JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.12123

Rivers, refuges and population divergence of fire-eye antbirds (*Pyriglena*) in the Amazon Basin

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Keywords:

Amazon;
diversification;
neotropics;
Pyriglena;
refuges;
rivers;
speciation.

Abstract

The identification of ecological and evolutionary mechanisms that might account for the elevated biotic diversity in tropical forests is a central theme in evolutionary biology. This issue is especially relevant in the Neotropical region, where biological diversity is the highest in the world, but where few studies have been conducted to test factors causing population differentiation and speciation. We used mtDNA sequence data to examine the genetic structure within white-backed fire-eye (*Pyriglena leuconota*) populations along the Tocantins River valley in the south-eastern Amazon Basin, and we confront the predictions of the river and the Pleistocene refuge hypotheses with patterns of genetic variation observed in these populations. We also investigated whether these patterns reflect the recently detected shift in the course of the Tocantins River. We sampled a total of 32 individuals east of, and 52 individuals west of, the Tocantins River. Coalescent simulations and phylogeographical and population genetics analytical approaches revealed that mtDNA variation observed for fire-eye populations provides little support for the hypothesis that populations were isolated in glacial forest refuges. Instead, our data strongly support a key prediction of the river hypothesis. Our study shows that the Tocantins River has probably been the historical barrier promoting population divergence in fire-eye antbirds. Our results have important implications for a better understanding of the importance of large Amazonian rivers in vertebrate diversification in the Neotropics.

Introduction

Explaining the origins of Neotropical biodiversity has long been a major challenge. Despite several hypotheses being proposed during the last 150 years, many questions remain regarding the influence of ecological, geological and climatic processes on the origin of species in this region. Biological diversity is particularly high in the Amazonian lowlands (Myers *et al.*, 2000), and multiple hypotheses have been put forward to account for the origin (historical and biogeographical processes) and maintenance (ecological processes) of this diversity (Colinvaux *et al.*, 1996; Haffer, 1997a; Bates, 2001). To explain the origins of diversity, biologists have proposed the river (Wallace, 1852; Sick, 1967), refugium (Haffer, 1969), gradient (Endler, 1982), disturbance-vicariance (Colinvaux *et al.*, 1996) and palaeogeography hypotheses (mountain uplift and marine transgressions; Chapman, 1917; Bates, 2001).

Geographical 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

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rivers (hereafter the river hypothesis). Modern versions of this hypothesis hold that ancestral populations were continuous across Amazonia and became spatially subdivided by the formation of large Amazonian rivers, with subsequent reduction or interruption of gene flow, sometimes leading to subspecific or specific differentiation of populations on opposite interfluvia (Capparella, 1988, 1991; Haffer, 1992, 1997a). Several studies of Amazon Basin phylogeography have provided mixed support for this hypothesis. On the one hand, some studies have rejected the role of rivers as a significant barrier to gene flow among vertebrate populations in western Amazonia (Patton *et al.*, 1994; Lougheed *et al.*, 1999; Aleixo, 2004; Funk *et al.*, 2007). On the other hand, others have shown genetic differentiation congruent with the position of rivers in this same region (Aleixo, 2004; Armenta *et al.*, 2005; Cheviron *et al.*, 2005; Patel *et al.*, 2011; Ribas *et al.*, 2012) and phylogeographical breaks abutting large south-eastern Amazonian rivers (Aleixo, 2004; Ribas *et al.*, 2012), implying that rivers may be effective barriers to gene flow.

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, potentially, speciation. Subsequently, these populations would have experienced range expansions during the warmer and more humid interglacial periods (Haffer, 1969, 1997b). Many aspects of the refuge model have been challenged in the Amazon region; some of the criticisms derive from studies that show that forests may not have been as fragmented as hypothesized (Bush, 1994; Mayle *et al.*, 2004), that populations did not undergo predicted demographic changes (Lessa *et al.*, 2003; Aleixo, 2004), that estimates of the time since divergence among sister taxa pre-date the Pleistocene period (e.g. Hackett & Rosenberg, 1990; Mustrangi & Patton, 1997) and that a high degree of phylogeographical structure exhibited by some organisms over a relatively small geographical area (Patton *et al.*, 1994) is inconsistent with an expected population expansion from a reduced forest refuge area (Lessa *et al.*, 2003). New evidence does suggest, however, that forest cover might have been disrupted during the Pleistocene Epoch in the Amazon Basin, as indicated by some recent palynological (Siffedine *et al.*, 2001), phylogeographical (Wüster *et al.*, 2005; Quijada-Mascareñas *et al.*, 2007) and climate modelling (Bonaccorso *et al.*, 2006; Peterson & Nyári, 2007) studies. Despite the controversy surrounding the refuge model, it is surprising, given its importance for the development of Amazonian biogeography, that 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 study, we used mtDNA sequence data to examine the genetic structure within white-backed fire-eye [*Pyriglena leuconota* (Spix); 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 divergence of these populations reflect the palaeogeographical models proposed for the evolution of Amazonian drainage system or support the recently detected shift in the course of the Tocantins River? To address these questions we used two approaches: (i) phylogeographical and population genetic analyses to confront predictions from the river and the refuge hypotheses with the pattern of genetic variation in fire-eyes and (ii) an evaluation of more complex scenarios by using coalescent simulations (i.e. an explicit genealogical approach). Our analyses provide support only for the river hypothesis, a result that may have important implications for a better understanding of the importance of large Amazon rivers in vertebrate diversification in the Neotropics.

Study area, study system and geological and palaeoenvironmental history

The fire-eye genus *Pyriglena* (family Thamnophilidae), as currently recognized, includes three species: the fringed-backed fire-eye (*Pyriglena atra*), the white-backed fire-eye (*P. leuconota*) and the white-shouldered fire-eye (*Pyriglena leucoptera*) (Willis & Oniki, 1982; Zimmer & Isler, 2003). *P. leuconota* currently include 10 parapatrically or allopatrically distributed subspecies. One subspecies (*P. l. pernambucensis*) is isolated in the coastal areas of Northeast Brazil, north of the São Francisco River. Three other subspecies occur in the Amazonian region south of the Amazon and east of the Tapajós Rivers. Six subspecies occur from western lowlands of Brazil across central Bolivia and north along the eastern slopes of the Andes to central Colombia. In the western Andes, another isolated population occurs in the Tumbesian centre of endemism in north-western Peru and western Ecuador. Finally, the monotypic *P. leucoptera* occurs in parapatry with *P. atra* in the northern Brazilian Atlantic Forest. Fire-eyes are typical antbirds that inhabit *terra firme* forests across their range in the Amazon Basin and are regular ant-swarm followers (Willis & Oniki, 1978). Although they do not depend entirely on following ant swarms for their primary food sources, they do follow ant swarms for purposes of foraging (Willis, 1981).

Fire-eyes are a suitable system to assess the effects of river barriers and geographical isolation into forest refuges on phylogeographical structure for two reasons. First, they have a widespread distribution in the understory of *terra firme* forests in south-eastern

Amazonia and results of this study may have strong implications for the diversification of other ecologically similar Amazonian birds. Second, an analysis based on a comprehensive geographical sampling of all populations of the entire genus revealed that fire-eye populations sampled west (the Xingu area of the endemism, Fig. 1) and east of the Tocantins River (the Belém area of endemism) and from Northeast Atlantic Forest (the Pernambuco area of endemism) form a well-supported clade with unclear phylogenetic relationships to other taxa/populations occurring in the Atlantic Forest south of the São Francisco River and forests flanking the Amazon Basin (Pantanal and Andes; Maldonado-Coelho, 2010;). Nonetheless, the sister relationship between the population (*P. l. similis*) occurring in the interfluvium Xingu-Tapajós (the Tapajós area of endemism) and all western South America *Pyriglena* populations is strongly supported (Maldonado-Coelho, 2010). Details of the diversification of fire-eyes at the continental scale will be published elsewhere. In this study, we focus on the clade composed by three subspecies of the white-backed fire-eyes sampled from the Xingu (subspecies *P. l. interposita*), Belém (subspecies *P. l. leuconota*) and Pernambuco (subspecies *P. l. pernambucensis*) areas of endemism (Fig. 1). Thus, preliminary information indicates that Amazonian fire-eyes undoubtedly diversified *in situ* and were subjected to the various historical and environmental changes that occurred there.

The Tocantins River is a long watercourse that spans a straight-line distance of over 2000 km from the central high Brazilian plateaus near the city of Brasília 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 phytogeographical 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 ecological barrier for the dispersal of several bird species (Haffer, 1992); (iii) forest refuges are hypothesized to have persisted on both sides of the Tocantins River during the last glacial maximum (LGM; Haffer, 1969; Fig. 1); (iv) the Tocantins River delimits Amazonian lowland areas of endemism to the west (Belém) and to the east (Xingu) (Silva *et al.*, 2005). Therefore, a framework to test alternative mechanisms of diversification will also provide insights into biogeographical processes underlying the origin of these lowland areas of vertebrate endemism. In addition, (v) the region in which the Tocantins River is located constitutes one of the few areas in the Amazon Basin that has been well documented from geological and sedimentological perspectives (Rossetti & Valeriano, 2007). Therefore, the Tocantins provides a unique spatial and temporal framework within which to test the role of Amazonian river dynamics on genetic structure of vertebrate populations. In addition, the study area is characterized by a

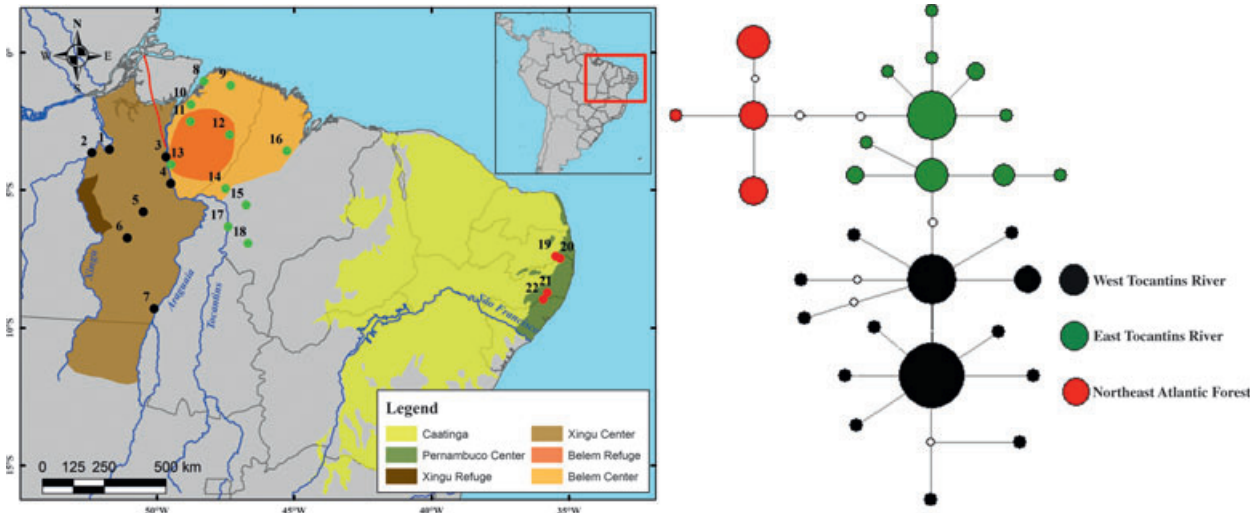

Fig. 1 Sampling localities and haplotype network of fire-eye (*Pyriglena leuconota*) populations along the Tocantins River valley and in Northeast Atlantic Forest, Brazil. Shaded areas west and east of the Tocantins River indicate the location of an unnamed (named Xingu here) and the Belém LGM forest refuges, following Haffer (1969). The red line depicts the palaeovalley of the Tocantins River during the Plio-Pleistocene/Mid-Pleistocene (Rosseti & Valeriano, 2007). Black, green and red circles represent sampling localities west and east of the Tocantins River and from Northeast Atlantic Forest respectively. The three areas of endemism are depicted. The haplotype network is based on ND2 sequences and each circle represents a different haplotype with size proportional to its relative frequency. See text for details.

complex geological and climatic history. Geomorphological data demonstrate that the Tocantins River shifted its course to the north-east in response to tectonic reorganizations, 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 (Sifeddine *et al.*, 2001).

A spatio-temporal framework to test the river and the LGM hypotheses

This study includes locales distributed both within (populations 11–13) and outside of the area of a proposed LGM refugium (populations 8–10 and 14–18) east of the Tocantins River (see Fig. 5 in Haffer, 1969 for refuge locations; Fig. 1 in this study). We also sampled west of the Tocantins River in areas outside a narrow and small, unnamed LGM refugium (populations 1–7, Fig. 1). Although different LGM refugia scenarios have been published for this region (e.g. Brown & Ab'Sáber, 1979; Prance, 1982; Brown, 1987), Haffer's hypothesis is discussed because it is the most popular one (see Bush & Oliveira, 2006) and it has been specifically proposed for birds.

Studies designed to test the river hypothesis have been criticized because of their failure to determine whether rivers had a main role in the diversification of organisms or if they acted only as points of secondary contact (Patton & da Silva, 1998). This is because the presence of phylogeographical breaks across a river might be the result of secondary contact, with the river having no part in diversification. In our study system, both the refuge and the river hypotheses predict that differentiation will be perpendicular to the river (Fig. 1). However, the river hypothesis assumes divergence in primary contact, whereas the LGM refuge hypothesis assumes that the river presents a barrier to secondary contact. Our sampling allows us to test a key prediction that distinguishes between primary and secondary contact. Specifically, one could reject the secondary contact hypothesis if populations bordering the river show no evidence of demographic expansion (Cheviron *et al.*, 2005; Maldonado-Coelho, 2012). A signature of expansion recovered in populations geographically close to the river could indicate that the river may be a point of secondary contact and that populations would have diverged under the influence of other processes (e.g. refuges); hence, such evidence can be used to reject the river hypothesis.

We also test two unambiguous predictions of the LGM refuge hypothesis. We suggest that the most critical predictions for testing the LGM hypothesis are historical and demographic. If the current population genetic structure of fire-eyes was affected by fragmentation of forests into LGM refuges as proposed by Haffer (1969), populations occurring east of the river should possess higher global levels of genetic diversity compared with populations west of the river, reflecting forest refuge size differences (Fig. 1). This prediction derives from (i) classical population genetics theory (Templeton, 2006), which predicts that a bottleneck effect of larger magnitude would result in a more pronounced loss of genetic diversity west of the river than east of it, and (ii) from theoretical (Nichols & Hewitt, 1994; Ibrahim *et al.*, 1996) and empirical studies (reviewed in Hewitt, 2004a,b), which show that episodes of range expansion have dramatic effects on the spatial pattern of genetic diversity. Thus, a larger geographical area to be colonized from LGM refuges in the western interfluvium would also contribute to a reduced genetic diversity relative to that east of the river. The second prediction is a signature of recent demographic expansion within a period corresponding to forest expansion after the LGM (i.e. during the last 20 000 years; Hewitt, 1996; Haffer, 1997a). Importantly, if the magnitude of population bottlenecks was proportional to the size of the two postulated refuges, the population size increment should be larger in the western interfluvium (Fig. 1). Accordingly, one could reject the LGM refuge hypothesis when biogeographical events and population processes fail to correspond. In other words, the hypothesized climatic (forest contraction and expansion) processes would be consistent with the historical demographic processes (changes in population size) if they have overlapping confidence intervals.

A genealogical framework to test the refuge hypothesis for Amazonia

We also used coalescent simulations to test historical scenarios that could be reflected in the branching patterns of the mtDNA gene tree and on the potential demographic processes affecting fire-eyes (reviewed in Richards *et al.*, 2007; Knowles, 2009; Hickerson *et al.*, 2010). Specifically, we simulated seven coalescent scenarios representing alternative hypotheses concerning the historical demographic causes of the mtDNA variation. We simulated the role of glacial extremes in temperature that occurred during the late Pleistocene as potential mechanisms driving the diversification of the three lineages recovered in the phylogenetic analyses presented below (east and west of the Tocantins River and Northeast Atlantic Forest). In addition, we simulated a null scenario of panmixia. A summary of the simulated scenarios is given in Fig. 2.

Materials and methods

Geographical sampling and laboratory molecular procedures

We sampled 32 individuals east and 52 individuals west of the Tocantins River (for details on samples, sampling

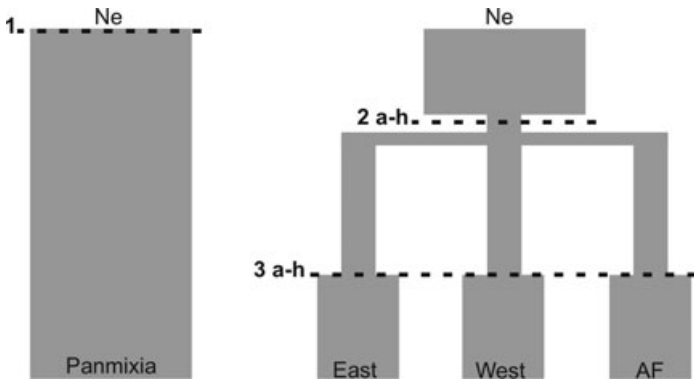

Fig. 2 Schematic view of historical demographic scenarios tested in the coalescent simulations. Numbers represent historical events as given in Table 2. U denotes a uniform prior distribution of the proportion of reduction of N_{ef} during the simulated bottleneck.

locations, population sample size and voucher information, see Fig. 1, Table 1 and Appendix S1).

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., Valencia, CA, USA). 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. We also always ran Polymerase chain reactions (PCRs) with blank samples to monitor potential contamination. Because the sampling source in our study was primarily museum skins with degraded DNA, we restricted our analysis for all individuals to one mitochondrial gene. For all the tissue and museum samples, we amplified all 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 amplifying 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′-GGTGG GTGAGTTGGGTAATG-3′) or HND2P1B (5′GCACCTT GGAGAACTTCTGG-3′); H5766 and LND2P2A (CATCG AGGCCACAACAAAAT) or LND2P2B (5′-AAAATCTCA CCACCCACGAG-3′); L5758 and HND2P3 (5′-GGCAAT GATTGTTGCTGTTG-3′); H6313 and LND2P4 (5′CTCC ATTAACGGGCTTTCTG-3′).

All fresh tissue samples available (23 individuals) were sequenced to generate a second data set of the mtDNA: ND2, NADH dehydrogenase subunit III (ND3;

Table 1 Sample size, number of haplotypes, haplotype diversity (h), nucleotide diversity (π) and historical demographic analyses (Ramos-Onsins and Rozas' R_2 test) for the 18 populations of fire-eyes (*Pyriglena leuconota*) west and east of the Tocantins River.

No.		Sample size	Number of haplotypes	Haplotype diversity	Nucleotide diversity ($\times 10^{-3}$)	R_2
	Western side	52	17	0.83 ± 0.04	7.0 ± 4.1	
1	Senador José Porfírio	5	3	0.70 ± 0.21	4.2 ± 3.7	0.29
2	Altamira region	7	2	0.48 ± 0.17	1.3 ± 1.4	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	0.23
5	Serra dos Carajás	16	8	0.83 ± 0.07	5.0 ± 3.5	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	0.18
	Eastern side	33	14	0.88 ± 0.04	5.3 ± 3.2	
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	0.20
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	0.21
15	Amarante	2	2	1.0 ± 0.5	2.8 ± 3.9	–
16	Grajaú	6	2	0.33 ± 0.21	0.9 ± 1.2	0.31
17	Porto Franco	1	1	–	–	–
18	Feira Nova	2	2	1.0 ± 0.50	8.8 ± 10.1	–

351 bp), cytochrome *b* (cyt *b*; 1045 bp) and the genes ATP-synthase 6 and 8 (ATPase; 776 bp). For amplification and sequencing, we used the following primers: ND3, primers L10755 and H11151 (Chesser, 1999); cyt *b* L14990, H16065, cytb.intf and cytb.intr (Brumfield & Edwards, 2007). We also designed external primers for ATPase6 and ATPase8: ATPasepyrL (CTCCATTAACGGG CTTTCTG) and ATPasepyrL (CATAGGCTTGAATTATGG CGAC).

Polymerase chain reaction profiles included an initial 2-min denaturation cycle at 95 °C, followed by 40 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, and completed with an additional extension at 72 °C for 10 min. 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 using BigDye v 3.0 Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Carlsbad, CA, USA), with 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 II (DNAstar) and visually aligned. The following measures were taken to ensure that the amplified DNA fragments did not include pseudogenes of nuclear origin: (i) inspecting for deletions, insertions and stop codons that would result in a nonfunctional protein, (ii) confirming a high transition rate at third codon positions and (iii) confirming a high transition-to-transversion substitution ratio characteristic of mitochondrial DNA (Arctander, 1995; Sorenson & Quinn, 1998).

We acknowledge that increasing the number of independent loci in our analysis would allow a better assessment of evolutionary relationships among the populations and of population demographics (Edwards & Beerli, 2000; Carstens & Knowles, 2007), which may decrease uncertainty in the estimates and allow the rejection of alternative biogeographical scenarios with more confidence. The degraded nature of most samples used in this work hindered the proper amplification of nuclear loci. However, mtDNA data often corroborate data from nuclear markers on patterns of population history in birds (see Zink & Barrowclough, 2008 for a review).

Phylogenetic analyses

Phylogenetic relationships among the 23 individuals sequenced for the four mtDNA genes (3213 bp total) were assessed with maximum parsimony (MP), maximum likelihood (ML) using PAUP v4.0 (Swofford, 2002) and Bayesian inference in MrBayes v3.2 (Ronquist *et al.*, 2011: http://mrbayes.sourceforge.net). Details of phylogenetic methods are presented in supporting information.

Our phylogenetic analyses indicated that relationships among the three recovered clades were ambiguous (see below). We thus assessed the robustness of relationships recovered in our maximum-likelihood tree with an alternate topological placement of clades. To do this, we conducted a constrained ML search in PAUP using the four mitochondrial genes data set and contrasted their likelihood scores with the one obtained from the unconstrained ML search employing the Shimodaira–Hasegawa (S-H) test (Shimodaira & Hasegawa, 1999), with full optimization and 1000 bootstrap replicates.

Population genetic analyses

Haplotype network and patterns of genetic diversity

Given the problems associated with reconstructing relationships among recently diverged haplotypes (Posada & Crandall, 2001), we also inferred their relationships by constructing a median-joining network (Bandelt *et al.*, 1999) using NETWORK v. 4.610 (http://www.fluxus-engineering.com). Two haplotype networks were constructed: one with the ND2 gene (101 individuals) and another with the concatenated data set of four mitochondrial genes (23 individuals). Haplotype (h) and nucleotide (π) diversity were calculated for west and east of the Tocantins River as described in Nei & Kumar (2000), using ARLEQUIN (Schneider *et al.*, 2000). Uncorrected pairwise distances among haplotypes are presented as mean ± SD.

We also performed two exploratory analyses using the ND2 data set to test for the effect of the Tocantins River on the genetic structure of fire-eye populations. First, we used the analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) implemented in ARLEQUIN (version 3.1, Schneider *et al.*, 2000) for populations that contained more than one individual. Apportionment of the genetic variation was broken down among three hierarchical levels: among populations from opposite sides of the river, among populations on the same side of the river and among individuals within populations. Significance tests were based on 10 000 random permutations of the data set.

Second, if the Tocantins River was a long-term historical barrier to gene flow, genetic distances between populations on opposite sides of the river 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 (Manly, 2007; Legendre & Legendre, 2000) to

decompose the relative contributions of the long-term historical effect of the Tocantins River vs. IBD in explaining the genetic structure of fire-eye populations. In addition, we 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: (i) a matrix of pairwise corrected genetic distances, (ii) a matrix of pairwise geographical distances as a surrogate for IBD and (iii) 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). Geographical distances were measured as straight-line distances between populations.

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).

Historical demography

We use the ND2 data set to construct Bayesian Skyline plots in BEAST v1.4.6 (Drummond & Rambaut, 2007) for the populations west and east of the Tocantins River to estimate historical changes in population size. 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 using MODELTEST v3.7 (Posada & Crandall, 1998). This analysis was run for 2×10^8 generations with model parameters and genealogies sampled every 1000 generations under a strict molecular clock, of which the first 10% were discarded as a burn-in. The number of discrete intervals m was set to 10. Skyline plots were constructed using Tracer v1.4 (Rambaut & Drummond, 2007).

Here, we used two mutation rates: the widely employed interspecific rate of 2.1% (1.0×10^{-8} substitutions per site per lineage per year; reviewed in Weir & Schluter, 2008) and the intraspecific mutation rate estimate of 4.0% per site per million years of mitochondrial ND2 from the Galapagos mockingbirds (2.0×10^{-8} substitutions per site per lineage per year; Arbogast *et al.*, 2006).

We used a summary statistic to test the key prediction of the river hypothesis. Because simulation studies have shown that the R_2 test is statistically more powerful than other tests (Ramos-Onsins & Rozas, 2002), we use it to assess whether the populations would fit a population-stationary or a population-expansion scenario using DnaSP (Rozas *et al.*, 2003). Estimated values for this test 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 favour of an alternative scenario of demographic expansion.

The coalescent methods used to estimate historical demography are theoretically valid only when sequences are sampled from a single panmictic population. We thus performed the Bayesian demographic analyses separately for each of the two phylogeographical groups recovered on opposite sides of the Tocantins River, as we did not find strong within-basin population structure (see Results). Also, presence of population structure, number of loci sequenced or sampling scheme may lead to incorrect demographic inferences (Chikhi *et al.*, 2010). We tried to provide as dense a sampling as possible given the available individuals (museum specimens and fresh tissues). However, a full evaluation of the demographic history of fire-eyes will be possible only when a multilocus study is performed. Meanwhile, our results should be interpreted with caution as demographic analyses were based on a single marker.

Divergence times

We estimated divergence time among populations of fire-eyes for the ND2 data set using three approaches. First, given the shallow genetic divergences in our study, methods that account for gene divergence within the ancestral species seem to be a better and unbiased estimate of population divergence times as they may not overestimate times of population divergence and speciation events (Edwards & Beerli, 2000; Hey & Nielsen, 2007; McCormack *et al.*, 2010). For this reason, we first employed the Bayesian coalescent method developed by Hey & 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. Because likelihood ratio tests applied in initial runs to evaluate the fit of nested models within

the full IMa model could not reject models assuming no gene flow across the Tocantins River (results not shown), the subsequent runs were performed with migration rates set to zero values. Four 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 continued 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 trend-line plots and that effective sample sizes (ESS) among parameter values were at least 50 throughout the run (Hey & Nielsen, 2007; Hey, 2007). The peaks of the posterior distributions were taken as the estimates of the parameter values. Overestimation of divergence might 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% and the intraspecific mutation rate estimate of 4.0% divergence per Mya to estimate time of divergence. We assumed a generation time of 2.33 years, as determined in Maldonado-Coelho (2012).

We also estimated the dates of origin of all fire-eye clades and subclades using a Bayesian approach incorporated in BEAST. The time-to-most-recent-common-ancestor (TMRCA) was estimated across the Tocantins River assuming both Yule and coalescent tree priors (Drummond & Rambaut, 2007). These analyses sample the TMRCA values from the posterior density distribution generated by MCMC simulations. Only the concatenated data set of four mtDNA genes was used in these analyses. These approaches were based on the topology of the *Pyriglena* Bayesian tree for the concatenated data set including all lineages found in *Pyriglena* throughout South America and other antbird genera as outgroups (Maldonado-Coelho, 2010). The pattern of diversification of the genus *Pyriglena* in South America is beyond the scope of this study and we report only divergence time for populations across the Tocantins River. Here, we estimated divergence times assuming a Yule tree prior for lineages that have already been pruned, that is, for the clades and subclades identified in the phylogenetic analyses. We performed two analyses with the Yule prior: (i) using a reduced data set, which included one individual from each phylogeographical lineage of *Pyriglena* and (ii) with all individuals that have been sequenced for the four mtDNA genes (including nine and seven individuals west and east of the Tocantins River and seven individuals from Northeast Atlantic Forest). The analysis employing the coalescent prior also included all individuals that have been sequenced for all mtDNA genes.

We performed partitioned analyses in BEAST after having inferred the appropriate nucleotide substitution model for each codon position in MODELTEST. We ran the MCMC under partitioned models of nucleotide substitution (one model for each mtDNA gene) and assuming a constant population size. Because the substitution rate variation among lineages can be substantial, independent of the divergence time frame under consideration (Arbogast *et al.*, 2002), we first tested if *Pyriglena* sequences were evolving in a clock-like fashion. Our preliminary runs estimated that the coefficient of variation of the mean branch rate variation was larger than one, indicating rate variation among branches (Ho *et al.*, 2005; Drummond *et al.*, 2006). Thus, we estimated divergence times between fire-eye populations of western and eastern riverbanks assuming 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 (Drummond *et al.*, 2006). To validate the application of the 2.1% corrected sequence divergence per Ma for cyt *b* in birds (Weir & Schluter, 2008), we compared pairwise model corrected genetic distances of the cyt *b* mtDNA gene alone with the genetic distances of the three remaining mtDNA genes. In this comparison, we employed the GTR-I model for corrected distances, and the corrected genetic distances between cyt *b* and the other mtDNA samples were strongly correlated ($F = 25475.330$; *regression* d.f. = 1; *total* d.f. = 1324; $r^2 = 0.95$). We defined the rate prior according to the divergence rate. For example, we assumed a rate prior to have a normal distribution with a mean of 0.0105 and standard deviation of 0.0034, corresponding to the sequence divergence rate of 2.1% ($\sigma = 0.68\%$, Weir & Schluter, 2008). Six analyses were performed and each analysis consisted of one model type (clock unconstrained) and one mutation rate (2% or 4%). For each analysis, two independent MCMC analyses were run for 60 000 000 generations, discarding the first 6000 000 as burn-in, and sampling parameter values every 1200 generations. In each independent run, we inspected for convergence of the chain to the stationary distribution using the program Tracer. The two independent runs were combined to obtain an estimate of the posterior distribution. This strategy ensured that the TMRCAs were well sampled (ESS values > 200).

Coalescent simulations

To test the effects of climatic extremes of the Pleistocene on branching and demographic patterns of fire-eyes, we generated coalescent simulations of distinct historical scenarios in BAYESSC (Anderson *et al.*, 2005). The statistical framework to evaluate the simulated scenarios followed Voight *et al.* (2005), which considers several summary statistics and results on a

single probability for each scenario. For each of the seven major glacial periods that occurred during the last million years, we simulated one ancestral population that had undergone a bottleneck and produced three descendant populations (refuges) that later expanded with the onset of more humid conditions after each glacial age (Fig. 2). The duration of each major glacial event was derived directly from raw palaeoenvironmental data (Lisiecki & Raymo, 2005a,b; Table 2). Here, we included all 101 individuals of the ND2 mtDNA data set. We simulated bottlenecks that reduced the female population effective size (N_{ef}) of each one of the three populations (east and west of the Tocantins River and Northeast Atlantic Forest) to 5%–20% (under a uniform prior) of the current size. In the scenario of panmixia, we assumed the temporal origin of all individuals as the TMRCA (given by BEAST) of all sampled individuals along the Tocantins River valley and in Northeast Atlantic Forest (Table 2). The N_{ef} of each population was obtained from theta values ($\Theta = 2\mu N_{ef}$) that were estimated in the IMa program. The 95% HPD of Θ for each population/lineage was: (1.47–2.17) west of the Tocantins River, (1.03–2.17) east of the Tocantins River and (0.14–1.45) for Northeast Atlantic Forest. We then calculated the effective number of females (N_{ef}) by solving the equation $\Theta = 2\mu N_{ef}$. Effective number of genes was introduced in the BAYESSC infiles as presenting a uniform distribution equal to the 95% HPD of the estimation of Θ. We ran the simulations using the mutation rates of 2.1% and 4%. The transition bias was 3.677 as estimated by MEGA5 (Tamura *et al.*, 2011). We incorporated a Kimura 2-Parameter model (gamma distribution = 0.4 and the number of mutation categories = 10) to allow for heterogeneous mutation rate. We assumed a generation time of 2.33 years (Maldonado-Coelho, 2012).

We performed 1000 simulations for each of the eight scenarios (seven refugia and one panmixia). Then, for each set of simulated data, we estimated five summary statistics (nucleotide diversity – π, Tajima's *D*, number of haplotypes – *h*, number of segregating sites – *S* and number of pairwise differences – pd) to obtain null distributions against which we tested the observed data (Hickerson *et al.*, 2006). The summary statistics for the observed data were estimated in DnaSP. To evaluate the goodness of fit of the simulated data to the observed data, we followed Voight *et al.* (2005) using the two-tailed empirical probability (*P*-value). We first computed a *P*-value for each of the summary statistics considered for each simulated scenario. Then, we combined these independent *P*-values using the *C* statistics and obtained its significance (global *P*-value) by comparing C_{obs} against a null two-tailed distribution of C_{sim} obtained from the simulated data. Then, we estimated the proportion *P* of observed values equal to or higher than the simulated distribution. The scenarios were rejected when $P < 0.05$.

Table 2 Timing of historical events used in the coalescent simulations by BAYESSC.

Historical event	Time in million years
1	μ = 2.1%: TMRCA 0.3098–2.7926; μ = 4%: TMRCA 0.1702–1.4937
2a	0.02 (± 10% as a uniform prior)
2b	0.11 (± 10% as a uniform prior)
2c	0.22 (± 10% as a uniform prior)
2d	0.32 (± 10% as a uniform prior)
2e	0.61 (± 10% as a uniform prior)
2f	0.7 (± 10% as a uniform prior)
2g	0.86 (± 10% as a uniform prior)
3a	0.07 (± 10% as a uniform prior)
3b	0.18 (± 10% as a uniform prior)
3c	0.27 (± 10% as a uniform prior)
3d	0.39 (± 10% as a uniform prior)
3e	0.68 (± 10% as a uniform prior)
3f	0.78 (± 10% as a uniform prior)
3g	0.94 (± 10% as a uniform prior)

TMRCA, time-to-most-recent-common-ancestor. Numbers represent historical events following Fig. 2. See text for details.

Results

DNA sequence variation and evolutionary relationships among populations

Fire-eye mtDNA base frequencies were similar to those found in other bird species, with an overall deficit of guanines (Johnson & Sorenson, 1998): $A = 0.318$, $C = 0.309$; $G = 0.091$; $T = 0.282$. For the mtDNA ND2 data set, a total of 14 and 11 unique haplotypes were recovered from the western and eastern sides of the Tocantins River, respectively; none of the haplotypes were shared across this river.

Phylogenetic relationships and genetic structure in fire-eyes

Individuals from populations of Northeast Atlantic Forest and populations on opposite sides of the Tocantins River each formed well-supported monophyletic groups (Fig. 3). Populations on opposite sides of the Tocantins River were reciprocally monophyletic in all analyses, but this relationship was not well supported by the Bayesian search (0.78 posterior probability) and by ML and MP (52% bootstrap for both). As revealed by the S–H test, enforcing reciprocal monophyly between the clade east of the Tocantins River and the clade of Northeast Atlantic Forest did not produce a significantly worse explanation of the data than produced by the optimal tree we recovered, in which clades west and east of the Tocantins River were sisters ($-\ln L_{unconstrained}$ = 14906.443 and $-\ln L_{constrained}$ = 14906.863, $P = 0.420$).

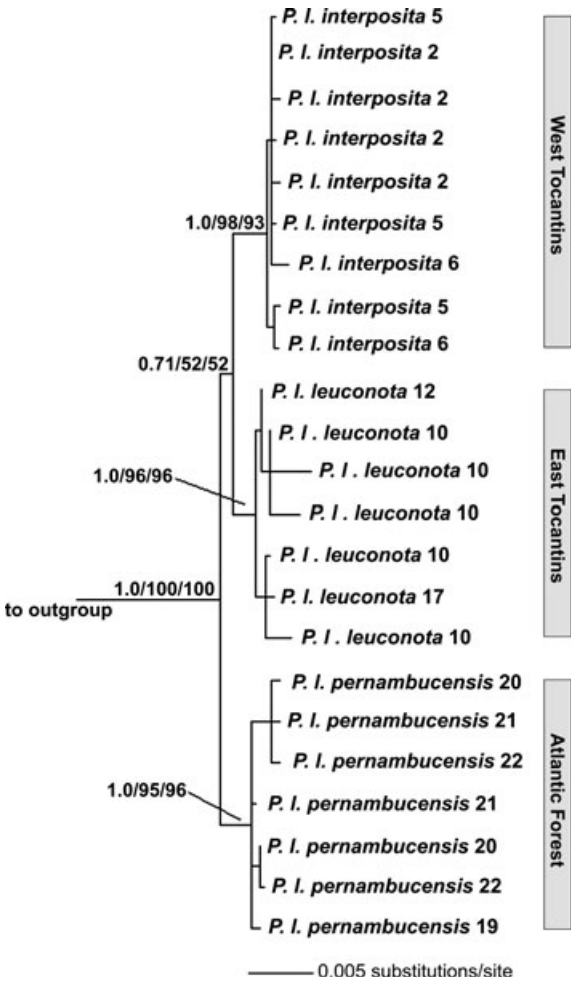

Fig. 3 Maximum-likelihood phylogram showing relationships among fire-eye haplotypes and populations based on combined analyses of 3213 aligned base pairs of four mitochondrial genes. Numbers following individuals represent localities as in Fig. 1. Recovered clades from the western and eastern sides of the Tocantins River and from Northeast Atlantic Forest are indicated by vertical bars. Numbers on nodes indicate Bayesian posterior probabilities and bootstrap values obtained under maximum likelihood and maximum parsimony respectively.

Results from the haplotype network show that haplotypes across the Tocantins River were sister (separated by two mutational steps) to the exclusion of the northeast Atlantic Forest haplotypes (separated from the Amazonian haplotype group by three mutational steps, Fig. 1). A second haplotype network recovered the same pattern of relationships when the four mtDNA genes (total of 3213 bp) were analysed. In this analysis, 13 mutational steps separated the groups of haplotypes across the river, whereas Amazonian and Northeast Atlantic Forest haplotypes were separated by 16 mutational steps (not shown). The mean pairwise uncorrected sequence divergence among unique haplotypes recovered on the western side of the river was 0.0033 ± 0.0014 (range 0.0096–0.0064) 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 across the river 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 sides of the river (Table 3). 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 east and west of the river and isolation by distance. 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, whereas 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 geographical distances (Fig. 4), as would be predicted by the IBD model. Rather, for similar geographical distances among populations, across-river comparisons always had a higher corrected genetic distance than comparisons from the same side of the river.

Testing the hypotheses

River

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 (Table 1). This pattern was further corroborated by the Bayesian analysis performed across the entire basin (see below), and it was not consistent with the expectation of the Tocantins River constituting an area of secondary contact. Thus, the key prediction of the river hypothesis cannot be rejected.

Table 3 AMOVA analysis for fire-eye (*Pyriglena leuconota*) populations grouped into west and east of the Tocantins River.

Source of variation	d.f.	Per cent of variation
Among population across the river	1	70.96**
Among populations from the same river side	16	3.96*
Within populations	65	25.08**
Total	82	

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

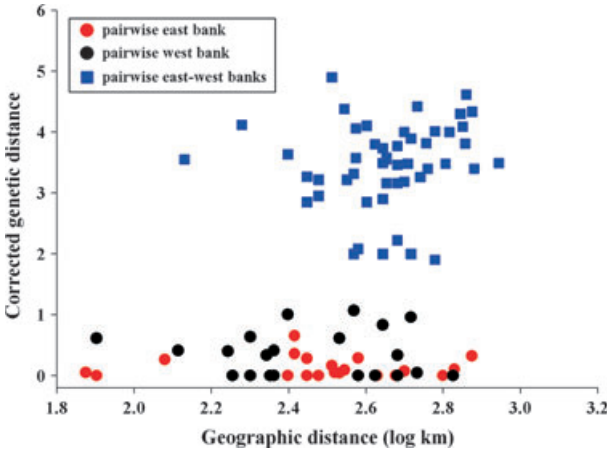

Fig. 4 Correlation of corrected genetic distances and geographical distances between fire-eye (*P. leuconota*) populations sampled within the same side (dots) and across (squares) the Tocantins River.

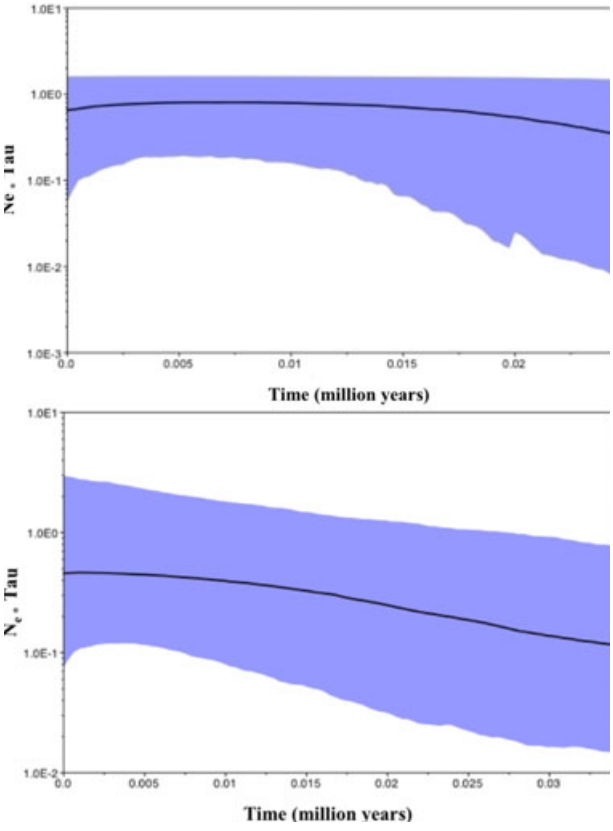

Fig. 5 Bayesian Skyline Plots depicting the demographic history of fire-eye populations west (top figure) and east (bottom figure) of the Tocantins River, with time axis scaled to the mutation rate of 2.1% per Mya. The solid line represents the median value for the log of the effective population size and the grey area represents the upper and lower 95% credible intervals. Time zero is the present, with values indicating time increasing towards the past.

LGM refuge

There were no significant differences in haplotype diversity for fire-eye populations sampled across the Tocantins River valley as predicted by the refuge hypothesis (Table 1; $h = 0.83 \pm 0.04$ and 0.88 ± 0.04 for, respectively, west and east of the Tocantins River; Mann–Whitney U-test = 15.5, $n = 15$ populations, $P = 0.15$). The index of nucleotide diversity also did not differ significantly between east ($\pi = 7.0 \pm 4.1$) and west ($\pi = 5.3 \pm 3.2$) of the Tocantins River (U-test = 22.0, $P = 0.54$).

The 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 size changes of fire-eye populations on both sides of the river. Although the credibility intervals of the Bayesian estimates were wide, the analyses showed trends that indicate stable population size on the western side and either growth or stable population size on the eastern side of the Tocantins River, respectively. These trends were not consistent with the expectation of a large population increase on the western interfluvium after the onset of humid periods as would be predicted by the LGM refuge hypothesis (Fig. 5).

The models that we established according to the palaeoenvironmental data enabled us to reject the effect of the seven climatic extremes of the Pleistocene as a driving force shaping the genetic diversity and demographical patterns in fire-eyes. The results of coalescence simulations in BAYESSC indicated that all eight scenarios cannot be reconciled with the observed data, although the simulations for the panmixia scenario using the mutation rate of 2.1% presented a probability close to the threshold of acceptance (Table 4).

Divergence times

The IMa analysis indicated that the posterior distribution of population divergence time t across the Tocantins River peaked at 2.78 (95% HPD: 1.19–5.22), resulting in divergence estimates of 0.267 Mya (95% HPD: 0.114–0.501 Mya) and 0.134 Mya (95% HPD: 0.057–0.251 Mya), assuming the divergence rates of 2.1% and 4% per million years respectively. These estimates suggest that population divergence across the Tocantins River occurred during the late-Pleistocene. The TMRCA estimates for the concatenated data set using a Yule prior and mutation rates of 2.1% and 4% also place the initial divergence across the Tocantins River within the middle-late Pleistocene for both the pruned data set (one individual per lineage; 0.34 Mya, 95% HPD: 0.15–0.56 Mya for 2.1% and 0.16 Mya, 95% HPD: 0.07–0.26 Mya for 4%) and for the full data set (several individuals per lineage; 0.71 Mya, 95% HPD: 0.23–1.44 Mya for 2.1% and 0.34 Mya, 95% HPD:

Table 4 Probabilities (*P*) for nucleotide diversity (π), Tajima's D, number of haplotyes (*h*), number of segregating sites (*S*) and number of pairwise differences (pd). Values of (*P*) represent overall probabilities for models of demographic scenarios for fire-eyes. Simulations were performed employing 2.1% and 4% divergence rates.

Demographic scenario	μ	$P\pi$	*P*D	*Ph*	*PS*	*P*pd	PC_{obs}
a - Panmixia (null model)	2.1%	0.562	0.328	0.16	0.106	0.634	0.05
	4%	0.536	0.27	0.148	0.104	0.532	0.04
b - Refuge hypothesis bottleneck 1	2.1%	0.04	0.014	0.0001*	0.006	0.022	0.0001*
	4%	0.042	0.012	0.01	0.002	0.022	0.0001*
c - Refuge hypothesis bottleneck 2	2.1%	0.034	0.01	0.114	0.0001*	0.024	0.0001*
	4%	0.014	0.004	0.164	0.0001*	0.006	0.0001*
d - Refuge hypothesis bottleneck 3	2.1%	0.012	0.31	0.0001*	0.222	0.196	0.0001*
	4%	0.002	0.0001*	0.128	0.0001*	0.002	0.0001*
e - Refuge hypothesis bottleneck 4	2.1%	0.01	0.0001*	0.136	0.0001*	0.004	0.0001*
	4%	0.0001*	0.0001*	0.136	0.0001*	0.0001*	0.0001*
f - Refuge hypothesis bottleneck 5	2.1%	0.0001*	0.0001*	0.16	0.0001*	0.0001*	0.0001*
	4%	0.0001*	0.0001*	0.162	0.0001*	0.0001*	0.0001*
g - Refuge hypothesis bottleneck 6	2.1%	0.0001*	0.0001*	0.146	0.0001*	0.0001*	0.0001*
	4%	0.0001*	0.0001*	0.174	0.0001*	0.0001*	0.0001*
h - Refuge hypothesis bottleneck 7	2.1%	0.0001*	0.0001*	0.162	0.0001*	0.0001*	0.0001*
	4%	0.0001*	0.0001*	0.136	0.0001*	0.0001*	0.0001*

*Observed statistic that fell off the simulated distribution.

0.12–0.71 Mya for 2.1%). The TMRCA estimates based on a coalescent prior and all individuals for the concatenated data set estimated the divergence across the Tocantins River to have been in the middle-late Pleistocene, as divergence time and associated credibility interval values were 0.55 Mya (95% HPD: 0.23–1.02 Mya) and 0.27 Mya (95% HPD: 0.12–0.51 Mya), assuming divergence rates of 2.1% and 4% Mya respectively.

Discussion

Amazonian fire-eyes population divergence: rivers or refuges?

Rivers

We tested one key prediction of the river hypothesis. Our data fully support this prediction, as none of the historical demographic analyses showed any signature of significant changes in population effective sizes. The historical role of the Tocantins River on the phylogeographical structure of fire-eyes is also supported by the results of AMOVA, the partial Mantel test and the reconstructions of evolutionary relationships among the populations. The phylogenetic analyses and haplotype network indicate that the first separation could have been between Northeast Atlantic Forest and south-eastern Amazonia, with subsequent divergence across the Tocantins River. However, the sister relationship between the populations across the Tocantins River is not strongly supported by tree-building methods as they exhibit low statistical support. A sister relationship between the Northeast Atlantic Forest and populations on the eastern bank of the Tocantins River cannot be rejected under a maximum-likelihood framework for the four mtDNA genes data set. Likewise, the haplotype network cannot place the populations across the river unambiguously as sister with respect to the Northeast Atlantic Forest population. However, even in the event that populations east of the river are sister to Northeast Atlantic Forest populations, it is also conceivable that an 'ancient' barrier effect of the Tocantins River has been of primary importance in the origin of the clade west of the river, followed by further divergence between the clade east of the river and the clade of Northeast Atlantic Forest.

In fact, if the populations within the ancestral range of fire-eyes were isolated due to a process other than the Tocantins River (e.g. by forest refuges) with subsequent differentiation followed by secondary expansion towards the river, a significant population size increase should be recovered in either river basin. Although further sampling might recover a signature of expansion in some sections along the river, our data rule out the possibility of an extensive range expansion from LGM refuges as indicated by the R_2 test, and the Bayesian Skyline Plots and coalescent simulations. Moreover, the fact that populations west and east of the Tocantins River are monophyletic implies that this river has been a long-term historical barrier to Amazonian fire-eyes, as reciprocal monophyly can be attained only after relatively long periods of isolation (Neigel & Avise, 1986; Funk & Omland, 2003).

Dispersal across the river also cannot be reconciled with the pattern of divergence of fire-eyes, as neither a paraphyletic scenario nor an extensive signature of demographic expansion on either side of the river was recovered. It is also noteworthy that the pattern of

haplotype relationships observed in the network suggests that the postulated river channel shift could not 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).

The view that large rivers act as effective barriers to dispersal of vertebrates inhabiting opposite interfluvia 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 & da Silva, 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 Amazonia, for example, several phylogeographical studies have shown that some rivers are significant barriers to gene flow for terrestrial vertebrates (Aleixo, 2004; Cheviron *et al.*, 2005; Funk *et al.*, 2007), although exceptions to this pattern have been found (Patton *et al.*, 1994; Patton & da Silva, 1998; Aleixo, 2004). In south-eastern Amazonia, major lineage splits of a passerine bird coincide with both the Tapajós and the Xingu rivers, but not with the Tocantins River (Aleixo, 2004), and a study in the headwaters of the Tapajós River found a significant river effect for some but not all forest bird species considered (Bates *et al.*, 2004). However, our results agree with a recent study that suggests that the evolution of the modern Amazon drainage system (including the Tocantins River) underlies the diversification of an understory *terra firme* forest avian group (Ribas *et al.*, 2012).

Pleistocene refuges

We tested two predictions derived from the LGM refuge hypothesis, neither of which was supported: (i) variation in genetic diversity did not differ west and east of the Tocantins River, and (ii) the absence of a population expansion of the magnitude of the refuge area hypothesized for the western river side as indicated by the Bayesian Skyline Plot refutes the scenario proposed by Haffer (1969). In the Tocantins River valley, Haffer 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 LGM (Fig. 1). Our analyses reject the key prediction of population decline derived from Haffer's hypothesis. This decline in population size would be expected mainly west of the river, given the hypothesized small forest refuge area. Thus, the null hypothesis that populations have been stable or even growing cannot be rejected in favour 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 had occurred.

In the coalescent framework employed in our study, we did not examine in detail more complex demographic scenarios of Pleistocene climatic changes on fire-eye's genealogical structure. However, we did establish a general test of the idea that climatic extremes during the last million years are responsible for current fire-eye phylogeographical structure. We could reject all scenarios taking into account the influence of seven major glaciations derived from the palaeoenvironmental data.

Estimated divergence times

Population divergence times based on the IMa and the BEAST Yule and coalescent prior estimates indicate divergence across the Tocantins River to have occurred not earlier than the middle-late Pleistocene. We cannot distinguish between the river and the LGM Pleistocene refuge hypotheses regarding divergence time because under both hypotheses, population divergence could have occurred during the late Pleistocene. On one hand, one could 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 late Pleistocene or Holocene (Rosseti & Valeriano, 2007). On the other hand, according to the LGM Pleistocene refuge hypothesis, divergence time should be congruent with the duration of the last glacial period (i.e. not older than 80 000 years bp; Hooghiemstra *et al.*, 2000) and with shallow levels of genetic divergence representing isolation of populations in two refuge areas during the last glaciation (Hewitt, 2004a). We found shallow levels of genetic divergence, and credibility intervals of population divergence time and TMRCA between populations sampled across the Tocantins River for one IMa and one BEAST (pruned data set assuming a Yule prior) estimate are consistent with the temporal scenario posited by the refuge model. Assuming that glacial periods lasted approximately 60 000 years (Hooghiemstra *et al.*, 2000), divergence estimates based on the assumed mutation rate of 4% for both analyses could be reconciled with the timing predictions of the LGM refuge hypothesis.

Avian behaviour, ecology and rivers

One relevant issue when investigating geographical mechanisms of population divergence is the extent to which the ecology of the study species might play a role in determining the effectiveness of a geographical barrier to gene flow (e.g. Burney & Brumfield, 2009). In the context of our study, an important question is whether large Amazonian Rivers constitute stronger geographical barriers to obligate and regular ant-swarm followers than to occasional and nonfollower bird taxa.

Although many factors (e.g. effective population size at the time of gene flow cessation) may influence genetic differentiation across a river, the fact that antbirds seem to have one of the lowest dispersal capabilities among small Neotropical birds (Moore *et al.*, 2008) could account for the observed phylogeographical break detected in fire-eyes across the Tocantins River. We also suggest that the barrier effect of a river may well be tied to reliance of birds on army ants. Fire-eyes are regular ant-swarm followers (Willis & Oniki, 1978; Willis, 1981; pers. obs.). According to foraging theory, prey search time is a fundamental component in determining the value of a prey type (Charnov, 1976). Hence, an optimality approach to foraging predicts that birds that are obligate or regular ant-swarm followers should chose between continuing to follow a particular swarm or to leave it and cross a river in such a way as to maximize their foraging efficiency. Continuing to follow swarms on one side of the river should be less costly energetically than crossing a large Amazonian river to search for a different swarm because ant swarms are uncommon and birds are unlikely to know the position of multiple bivouacs (Swartz, 2001; Wilson, 2004; Chaves-Campos, 2011) or to encounter a new swarm by chance (Swartz, 2001; Wilson, 2004; Logan *et al.*, 2011). Although this is a simplistic view (Green, 1987), we hypothesize that large rivers would always constitute a more impressive barrier to both obligate and regular ant followers than to occasional followers and species that do not follow ants.

Amazonian fire-eyes do not occur in the upper reaches of the large south-eastern Amazonian rivers (Tapajós, Xingu and Tocantins), where streams that constitute the headwaters are either bordered by gallery forests situated within the Cerrado phytogeographical domain or are bordered by dry forests (Somenzari *et al.*, 2011; pers. obs.). It is conceivable that the rarity of ant swarms in more seasonal and harsh habitats (Willis & Oniki, 1982) and/or habitat selection could explain the absence of fire-eyes in the headwater forests of the Tocantins River. The absence of fire-eyes in the headwaters of most Amazonian rivers, which are less wide and hence constitute weaker barriers to dispersal in these regions, might enhance the river barrier effect. Future phenotypic and molecular studies as well as translocation experiments could be adequate to test these hypotheses.

Conclusions

Our dense sampling in combination with both traditional and model-based phylogeographical approaches allowed us to derive testable predictions from the river and the LGM refuge hypothesis and to discuss our findings concerning the nature of each process. Results of this study provide strong evidence that rivers are the mechanism underlying population divergence of Amazonian fire-eyes. They also agree with recent geological (Rossetti *et al.*, 2005; Campbell *et al.*, 2006; *contra* Hoorn *et al.*, 2010) and molecular studies (Patel *et al.*, 2011; Ribas *et al.*, 2012) that have suggested that the modern Amazonian drainage system originated during the Pleistocene. Our results also imply that the mechanism underlying the origin of the Xingu and Belém areas of endemism could well be the formation of the course of the Tocantins River. Amazonian rivers can be significant barriers to animal dispersal in the Amazon Basin (Wallace, 1852; Sick, 1967; Haffer, 1992, 1997a,b); our study adds another piece of information to this discussion by showing that, although a recent feature (i.e. late Pleistocene), the formation of the Tocantins River seems to be the mechanism responsible for population divergence in fire-eye antbirds.

Acknowledgments

P.M.Z. Coelho provided critical logistical support for MM-C's field work in Brazil. C. Miyaki kindly helped with collecting and exportation permits. We are very grateful to A. Aleixo (MPEG, Brazil) for his invaluable assistance and for providing field logistic support. Collecting and exporting permits were issued by the IBAMA. The following institutions generously provided tissue and skin samples: A. Aleixo; C. Miyaki (LGEMA, USP, Brazil); D. Ditmann and R. Brumfield (MNS, LSU); J. Bates and D. Willard (FMNH, Chicago); and M. Braun (Smithsonian Institution, Washington D.C.). MM-C is thankful to CNPq (The National Research Council of Brazil) for an overseas doctoral fellowship and to FAPESP (São Paulo Research Foundation) for a postdoctoral fellowship. We are thankful to D. Cadena and J. Bates for the valuable help with laboratory issues. J. Bates, B. Loiselle, M. Svensson-Coelho, F. Zapata, F. Martins, E. Miller, A. Aleixo, the editor Jacqui Shykoff and two anonymous reviewers provided valuable suggestions on the manuscript. This study was funded by CNPq-Brazil, NSF (Doctoral Dissertation Improvement Grant OISE-0555482 to MM-C and research grants to RER), Whitney R. Harris World Ecology Center (Parker-Gentry Fellowship) at UMSL, UMSL Department of Biology (Raven Fellowship), the AMNH (Frank Chapman Memorial Fund), St. Louis Audubon Society, Sigma Xi and Idea Wild. The authors have no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Collection locality, sample size, tissue or dry skin source, voucher number and Dryad accession number for specimens of White-back fire-eye (*Pyriglena leuconota*) sequenced in this study.

Received 23 September 2012; accepted 4 January 2013