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Combining phylogeography and landscape genetics of *Xenopipo atronitens* (Aves: Pipridae), a white sand *campina* specialist, to understand Pleistocene landscape evolution in Amazonia

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Open vegetation (*campinas* and *campinaranas*) associated with white sand patches occurs in the form of islands in a forested matrix throughout the Amazon basin. Bird species restricted to these habitats have patchy distributions, although connectivity may have been influenced by past glacial cycles as a result of the substitution of forest by savanna. Because these landscape changes are a matter of debate in the history of Amazonia, we studied the diversification of *Xenopipo atronitens*, a white sand specialist, aiming to infer the effects of past climate changes. The split of *Xenopipo atronitens* from its sister species, *Xenopipo uniformis*, may be related to Tepuis erosion and retreat of escarpments during the Miocene, or to a dispersal event. Compared with birds from *terra firme* forest, *X. atronitens* has low genetic structure. Low levels of unidirectional gene flow were found from the Guyana Shield to adjacent areas. Demographic expansion starting approximately 25 kyr BP was detected for some populations and is probably related to the Last Glacial Maximum and subsequent climate improvement. Landscape genetic analyses indicate that the forested (*terra firme*) matrix acts as a barrier for the dispersal of *X. atronitens*. The results of the present study indicate that glacial cycles have deeply influenced Amazonian biogeographical history, demonstrating a complex interaction between forest and nonforest habitats during the Pleistocene.

ADDITIONAL KEYWORDS: birds – black manakin – demographic expansion – gene flow – Last Glacial Maximum – mtDNA – Tepuis.

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INTRODUCTION

The debate about which historical processes have led to the high species richness and patterns of endemism in Amazonia has been influenced heavily by the refuge hypothesis of Haffer (1969). This hypothesis proposed that Pleistocene glaciations led to a drier climate in the Amazon basin, resulting in the expansion of open habitats (savannas) and consequent fragmentation of the forests. Thus, the Pleistocene glacial cycles could drive diversification in Amazonia through cyclic expansion and retraction of the rainforests, through periodic vicariance.

After many years of debate over the refuge hypothesis (Bush, 1994; Colinvaux *et al*., 1996; Bush & Oliveira, 2006), recent studies have shown that diversification rates in Amazonia have been almost constant during the Neogene and Quaternary (Rull, 2011). Nevertheless, many studies still seek to understand the effects of Pleistocene climate change on the Amazonian landscape (Mayle *et al*., 2004; Anhuf *et al*., 2006; Beerling & Mayle, 2006) and on current species distributions (Bonaccorso, Koch & Peterson, 2006). Based mainly on paleovegetation modelling, these studies have achieved varied results, ranging from fragmentation and reduction of forested areas and forest species distributions (Anhuf *et al*., 2006; Bonaccorso *et al*., 2006), to less pronounced expansion of savanna habitats and no forest fragmentation (Mayle *et al*., 2004; Beerling & Mayle, 2006). Furthermore, recent phylogeographical and population genetics studies have found evidence of open habitats and/or dry forests expansion (Wüster *et al*., 2005; Quijada-Mascareñas *et al*., 2007; Bonvicino *et al*., 2009; Vargas-Ramírez, Maran & Fritz, 2010) and of refugia areas for forest animals (Fouquet *et al*., 2012) during the Pleistocene. Still, the degree to which glacial cycles have influenced the origin of current patterns of diversity and distribution of Amazonian taxa remains largely undetermined.

Throughout the Amazon basin, two main types of nonforested habitats are currently found: savannas (*cerrado*) and white sand scrub and woodland (*campina* and *campinarana*, respectively) (Anderson, 1981). Savannas are predominantly non-Amazonian, distributed within the basin mainly at the northern and southern limits, with some relicts found scattered elsewhere (Werneck, 2011). The other open habitat type is associated with white sand soils that are patchily distributed within the Amazon (Anderson, 1981). These white sand areas support stunted vegetation, mainly of sclerophyllous species, in a landscape ranging from open grasslands and scrubby vegetation (*campinas*), to forested habitats with a 15–20-m high canopy (*campinaranas*), and are usually adjacent to and surrounded by nonflooded upland forests (*terra firme*) (Anderson, 1981; Vicentini, 2004). Thus, for the most part of Amazonia, white sand *campinas* are in the form of islands within a forested matrix.

If a species is restricted to a specific insular habitat, past climate changes may have altered the extent and degree of connectedness among these habitat islands over time (Toumisto, 2007). Therefore, during the glacial cycles, the currently isolated *campinas* could have been more connected if the rainforest was replaced by open vegetation habitats, such as savanna, which is similar to white sand vegetation in its phytophysiognomy (Anderson, 1981). Furthermore, a drier climate in portions of Amazonia would favour the expansion of white sand habitats through sand transportation by rivers, as a result of increased erosion, combined with river channel displacements, with white sand habitats developing on palaeochannels (Latrubesse, 2002). Glacial periods would thus favour both the origin of new white sand areas and gene flow among populations currently isolated in *campina* patches, affecting the history of white sand species.

Phylogeographical patterns, genetic diversity, and diversification of several Amazonian bird species that occur in upland forests (Marks, Hackett & Capparella, 2002; Aleixo, 2004; Patel *et al*., 2011; Ribas *et al*., 2012) or floodplains (Aleixo, 2006; Cohn-Haft, Naka & Fernandes, 2007; Cadena *et al*., 2011) have been studied recently. The insular distribution of *campinas* suggests that populations of its species may be more isolated than those of Amazonian forest species. On the other hand, inventories in *campina* habitats reveal a widespread bird community with little local endemism (Oren, 1981; Diaz, Stiles & Telleria, 1995; Stotz *et al*., 1996; Alonso & Whitney, 2003; Borges, 2004; Poletto & Aleixo, 2005; Guilherme & Borges, 2011) and a uniform species composition of white sand communities throughout Amazonia, suggesting a high dispersal capability. Furthermore, historical changes on the Amazonian landscape could leave genetic signatures on white sand bird populations through varying connectivity among *campinas*. Therefore, the genetic study of white sand specialist birds can complement the current knowledge about Amazonian landscape history, revealing patterns that have not been detected so far.

In the present study, we provide the first account of the phylogenetic history and diversification of a white sand specialist bird species, the black manakin (*Xenopipo atronitens* Cabanis, 1847). Using a mitochondrial (mt)DNA marker, we investigate the phylogenetic origin of this species, examine the effects of the last glacial maximum on its phylogeographical pattern, and analyze the current landscape features that influence its genetic diversity on a local scale, which, in turn, helps with the interpretation of regional patterns.

MATERIAL AND METHODS

Sampling for phylogeographical and landscape genetics analysis

Tissue and blood samples from 18 white sand areas within Amazonia were obtained from the Instituto Nacional de Pesquisas da Amazônia (INPA), Museu Paraense Emílio Goeldi (MPEG), and American Museum of Natural History (AMNH) collections (56 individuals), and from our field expeditions (162 individuals) to the localities of Aracá (0°28′7.76′N and 63°28′32.20′W), Jaú (2°17′9.19′S and 58°51′53.92′W), and Uatumã (1°48′57.61′S and 61°45′50.33′W), hereafter referred to as landscapes (Fig. 1A; see also Supporting information, Table S1). On these three landscapes (approximately 50×50 km), we used a specific sample design (detailed below) with a more intense sampling for landscape genetics analyses (Fig. 2; see also Supporting information, Table S1). Our field expeditions to these landscapes occurred in July/2010 (Aracá), November/2010 (Jaú), and May/July/2011 (Uatumã).

For landscape genetic analyses, we distributed a total of 16 sample sites in *campina* patches within the three studied landscapes (Fig. 2). The distribution and proportions of white sand vegetation and forested habitats are different among landscapes (Aracá 45.33%, Jaú 1.3%, and Uatumã 0.8% of white sand vegetation cover). Sampling sites were similarly distributed within landscapes, including sites at both margins of major rivers, and with between-sites distance ranging from approximately 4 to 43 km in each landscape (Fig. 2B, C, D). In the three landscapes, we mist-netted ten individuals at each sampling site (except for sites JA1 and UA10 from which 11 individuals were sampled; Fig. 2; see also Supporting information, Table S1), within an area of a radius of 800 m.

DNA extraction, amplification, and sequencing

DNA was extracted using the Promega Wizard Genomic DNA Purification Kit (A1125). Primers used for NADH dehydrogenase subunit 2 amplification (ND2) were L5204 and H6313 (Sorenson *et al*., 1999). Polymerase chain reaction cycling comprised: initial denaturation at 94 °C for 4 min, 30 amplification cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 2 min, finishing with 72 °C for 10 min. The sequencing reaction used the same primers and the BigDye Terminator Kit 3.1. Samples were run on a 3130x Genetic Analyzer from Applied Biosystems. Contiguous sequences were assembled and the alignment was made using CLUSTALW in BIOEDIT, version 7.1.3 (Hall, 1999).

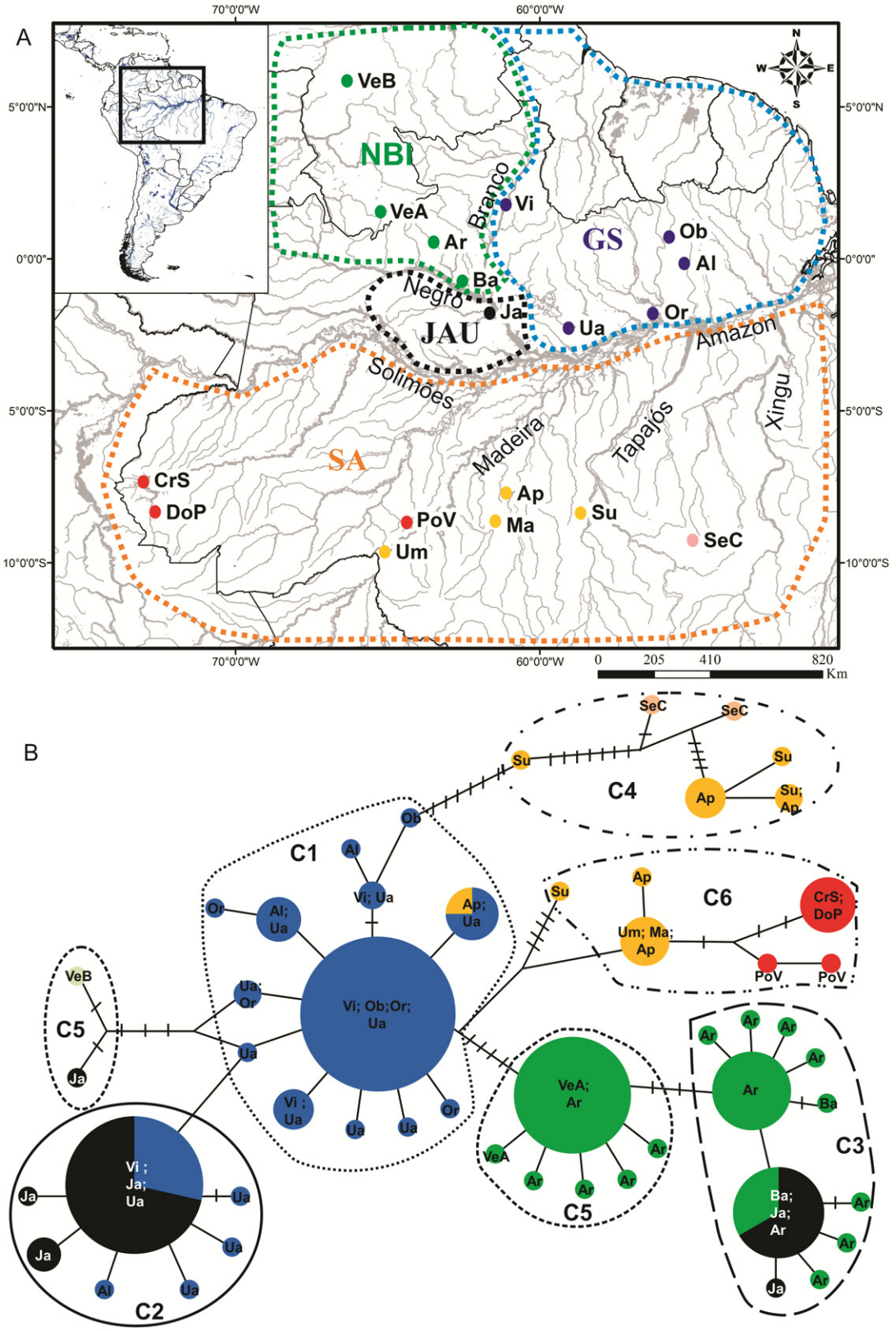
Interspecific relationships and the origin of *X. atronitens*

Only a few studies have tried to reconstruct the phylogenetic relationships between Pipridae species (Prum, 1990, 1992; Rêgo *et al*., 2007; Tello *et al*., 2009; McKay *et al*., 2010). *Xenopipo atronitens* grouped with *Xenopipo uniformis* (Salvin & Godman, 1884), endemic to the Tepuis, with high support (Prum, 1992; Tello *et al*., 2009), although the phylogenetic position of *Xenopipo holochlora* (Sclater, 1888) and *Xenopipo unicolor* (Taczanowski, 1884), both from the Andes, could not be resolved (Prum, 1992). Only one species from the genus, *Xenopipo flavicapilla* (Sclater, 1852), also from the Andes, has not been included in any phylogenetic analysis so far.

For the phylogenetic analyses, we used ND2 sequences from McKay *et al*. (2010), adding to this dataset sequences of four individuals of *X. atronitens* and four *X. uniformis* (for GenBank accession numbers, see the Supporting information, Table S1) obtained in the present study. Our final matrix included three *Xenopipo* species (*atronitens*, *uniformis*, and *unicolor*).

For phylogenetic reconstruction, we used maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) methods. To select the best model of evolution, we used MODELTEST, version 3.7 (Posada & Crandall, 1998), based on the Akaike information criterion (AIC). The model selected was GTR+I+G and was used on the ML and BI analysis. The MP and ML analyses were performed in PAUP, version 4.0 (Swofford, 2002). Node supports were estimated by nonparametric bootstrapping with 1000 and 100 replicates for MP and ML, respectively. The BI analysis was run on MrBayes, version 3.2.1 (Ronquist

Figure 1. A, map showing the *Xenopipo atronitens* sampling localities used in the present study and the geographical areas used in the analysis (for more details, see text): Negro–Branco interfluvium (NBI), Guyana Shield (GS), Jaú (JAU), and South of Solimões/Amazon Rivers (SA). B, haplotype network; the colours of the circles and abbreviations are respective to the localities on the map (A); the size of the circles are relative to the number of individual with a certain haplotype, the smaller circle represents one individual; the shapes encompassing haplotypes (B) are the clusters identified by BAPS5 analysis (C1–C6). VeB, Venezuela (Bolívar); VeA, Venezuela (Amazonas); Ar, Aracá; Ba, Barcelos; Ja, Jaú; Vi, Viruá; Ua, Uatumã; Or, Oriximiná; Al, Alenquer; Ob, Óbidos; CrS, Cruzeiro do Sul; DoP, Dois Portos; PoV, Porto Velho; Um, Umirizal; Ma, Manicoré; Ap, Apuí; Su, Sucunduri; SeC, Serra do Cachimbo.



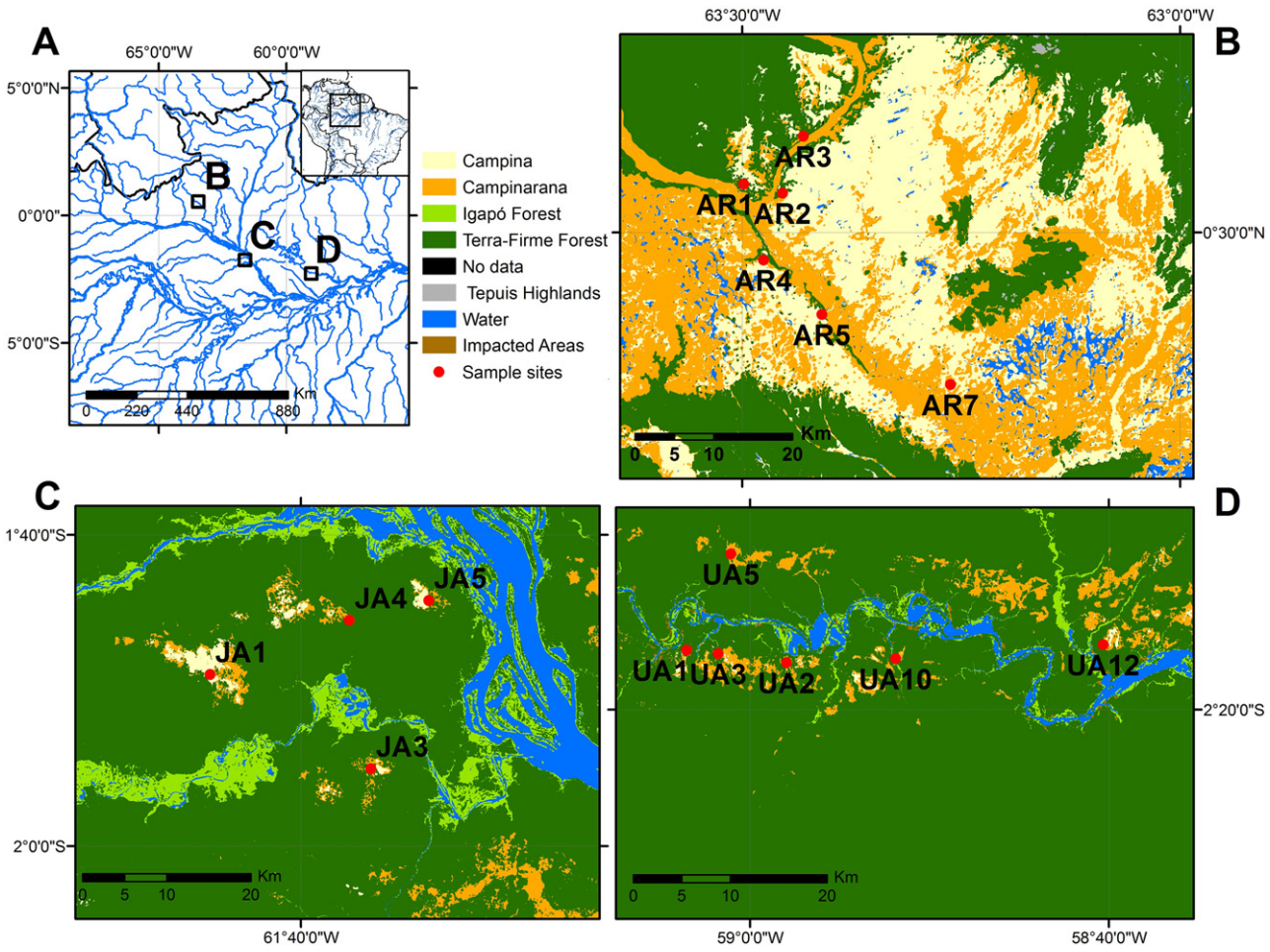


Figure 2. Distribution of the three studied landscapes on central and north Amazonia (A) and the classified maps for the Aracá (B), Jaú (C), and Uatumã (D) landscapes with respective sample sites. The abbreviations AR, JA, and UA followed by numbers correspond to sample site names in the Aracá, Jaú, and Uatumã landscapes, respectively.

et al., 2012), with two simultaneous analyses with four heated chains ran for 10×10^6 generations. The results were checked for effective sample sizes and good mixing of the chains with TRACER, version 1.5 (Rambaut & Drummond, 2007).

We used BEAST, version 1.6.2 (Drummond & Rambaut, 2007) to estimate the time to the most recent common ancestor of *X. atronitens* and *X. uniformis*. Only one individual per species was used in this analysis. We employed the Yule speciation process, the same evolutionary model selected above, a relaxed molecular clock with uncorrelated log normal rates, and a rate of 2.1% divergence per Myr among lineages (Weir & Schluter, 2008). Even though this rate is based only on cytochrome *b* sequences, it has been extrapolated to other mtDNA protein coding markers (Lovette, 2004), such as ND2, used in the present study. Furthermore, more recent studies have estimated a rate of evolution for ND2 of approximately 2.1% based mainly on model corrected genetic distances between markers, cross-validation methods, and paleogeographical time constraints (Miller *et al.*, 2008; Weir & Price, 2011).

Haplotype network and population structure in *X. atronitens*

The haplotype network was reconstructed using TCS, version 1.21 (Clement, Posada & Crandall, 2000) with a 95% connection limit. The software BAPS5 (Bayesian Analysis of Population Structure) (Corander & Tang, 2007; Corander *et al.*, 2008) was used to infer the number of clusters (k) based on our observed data. Initially, we ran the mixture model to access the most probable number of clusters using a range for the maximum number of clusters k from 1 to 20 (we ran the analysis four times to confirm the results). Then, we used the mixture result to perform an admixture

analysis using 200 iterations, 400 reference individuals, and 20 iterations per individual.

To test for isolation-by-distance (IBD) among all localities, we conducted a Mantel test using a matrix of linear geographical distance among localities (km) computed using GENALEX, version 6.5 (Peakall & Smouse, 2012) and an uncorrected *p*-distance matrix between all individuals computed with MEGA, version 5 (Tamura *et al*., 2011). The two localities from Venezuela were not included because we did not have the exact location of the samples. We also performed a partial Mantel test to evaluate the influence of riverine barriers on the genetic distances between pairs of individuals correcting for the geographical distance among them. We tested for the major rivers (Negro, Branco, Solimões/Amazonas, and Madeira) and for the black (Negro) and white water (Branco, Solimões/Amazonas, Madeira) rivers separately as a result of differences with respect to sediment load, which result in different types of flooded forest along the rivers. The same methodology was carried out for the individuals from Aracá, Jaú, and Uatumã at the landscape scale, testing for IBD and the riverine barriers, considering only the major river of each landscape, which were all black water rivers. We used ZT software (Van der Peer, 2002) to perform the calculations, and significance levels were assessed with 100 000 and 10 000 randomizations for phylogeographical and landscape scales, respectively.

Gene flow among geographical areas

We used the isolation with migration model (Hey & Nielsen, 2004) implemented in IMA software (Hey & Nielsen, 2007) to estimate the timing of population divergences and to determine whether gene flow has occurred subsequently. IMA simultaneously estimates for a pair of populations the parameters $\theta = 4Nu$ (where u is the mutation rate for the marker per year), such that three values of θ are estimated (θ_1, θ_2, and θ_A, for population 1, population 2, and the ancestral population, respectively); $m = m/u$, for m_1 and m_2 given in the coalescent; and, $t = tu$, the time of population splitting at t generations in the past.

We structured the analysis by separating the samples by geographical regions: Guyana Shield (GS, east of the Negro and Branco rivers, north of the Amazon river); Negro and Branco interfluvium (NBI); Jaú (JAU, west of the Negro river, north of the Solimões river); and south of the Solimões/Amazon Rivers (SA) (Fig. 1A). These geographical regions were defined based on the distribution patterns of Amazonian bird species and the hypothesized geographical barriers (major rivers) that isolate them (Silva, Rylands & Fonseca, 2005). We tested for the occurrence of gene flow among these geographical areas using pairwise analysis implemented on IMA. For each pairwise analysis, we made initial runs to define the priors for each parameter. Then, two longer runs were made with different seed numbers to verify the convergence of parameter values. For each of these runs, we used a 500 000 steps burn-in followed by at least 2.5×10^6 iterations sampling values every 50 steps, using 14 heated and one cold chain with geometric heating. For pairwise analyses returning migration values in both directions that were not different from zero, a final chain with 2×10^7 steps after burn-in and with migration set to zero was run. In these cases, because migration was not detected, we assumed the occurrence of incomplete lineage sorting as a result of recent population splitting. For those pairs that returned migration values different from zero, we tested whether a model that includes migration was better (or not) for explaining the data set based on the likelihood ratio test. Accordingly, we ran IMA on L mode sampling from 100 000 genealogies. Depending on the result from the likelihood ratio tests, a final chain with 2×10^7 steps was run considering (or not) zero migration. If the model with migration had a better fit to the data, we assumed that the phylogeographical pattern was influenced by gene flow.

Historical demography

Neutrality of ND2 sequence evolution was tested using the MacDonald and Kreitman test in DNASP, version 5.0 (Librado & Rozas, 2009). We tested for past demographic instability using Tajima's D (Tajima, 1989), Fu's F_s (Fu, 1997), and R_2 (Ramos-Onsins & Rozas, 2002) tests. Significance was determined by 10 000 coalescent simulations using DNASP, version 5.0 (Librado & Rozas, 2009). Three different analyses were performed: (1) including all sequences for *X. atronitens*; (2) analyzing each landscape independently; and (3) analyzing each cluster identified in BAPS5 independently.

We used BEAST, version 1.6.2 (Drummond & Rambaut, 2007) to construct a Bayesian skyline plot with the whole dataset for *X. atronitens*. This method uses a Markov chain Monte Carlo procedure to estimate the effective population size through time (Drummond *et al*., 2005). We ran 80 000 000 chains and the initial 10% were excluded as burn-in. The 2.1% rate of evolution for mitochondrial protein coding genes was used (Weir & Schluter, 2008). Using JMODELTEST, version 0.1.1 (Posada, 2008), we selected the best-fit model of nucleotide substitution based on the AIC (TrN + invariable sites). We verified the effective sample sizes and convergence of the chains with TRACER, version 1.5 (Rambaut &

Drummond, 2007). The same analysis was performed independently for individuals from north and south of the Amazon River, based on the results of the neutrality tests (see Results).

Landscape metrics and analysis

We used recent satellite images with a resolution of 30 m (LANDSAT TM5; US Geological Survey and NASA) with low cloud cover from years 2000, 2007, and 2011 for Aracá, Jaú, and Uatumã, respectively. We assumed that images within a 10 year-period represent current *campina* cover because geological processes that affect *campina* cover operate at a much larger temporal scale (Horbe, Horbe & Suguio, 2004). Moreover, human activity in these areas is negligible. To obtain categorical maps for each landscape, we used ENVI version 4.6 (ITT, Boulder, USA; http://www.exelisvis.com/ProductsServices/ENVI/ENVI.aspx) and performed a ML supervised classification with six pre-defined classes: *terra firme* forest, *campina*, *campinarana*, flooded forest, water, and anthropogenic areas. Because our model species is mostly restricted to *campina* but may also use *campinarana* forest (Oren, 1981; Ridgely & Tudor, 2009), we used two types of categorical maps for landscape analyses. Binary maps considering *campina* and other-cover types and maps with three classes: *campina*, *campinarana*, and other-cover types. To check the accuracy of our classification we used approximately 200 known field points per landscape and created a confusion matrix. The maps used in our analyses had an overall accuracy of > 90%.

To determine the effect of current landscape structure on the genetic variability of *X. atronitens*, we calculated six landscape metrics at each sampling point that considered area and isolation of *campina* patches in different ways (Table 1). We used three area-based metrics that represent different ways of quantifying the amount of habitat available for *X. atronitens* (area_c, area_cc, and area_x km). Because there are no data on dispersal distances for *X. atronitens*, we used three possible distances (5, 10, and 15 km) for the cumulative area (area_x km) metric. These values represent known dispersal distances for other Pipridae species in the Amazon region (Van Houtan *et al.*, 2007). We also used a fourth habitat availability measure, the perimeter of each *campina* patch (perim). Individuals of *X. atronitens* are associated with scrub vegetation typically found at the transition between open grassland *campina* and the more forested *campinarana* (Diaz *et al.*, 1995). Thus, the perimeter of a *campina* patch may be a better predictor of habitat availability than its actual area. We also calculated an isolation metric (enn) and a proximity metric that includes both area and isolation of *campina* patches (prox_x km). The proximity index was calculated based on the sum of neighbouring patches area within a given searching radius (again 5, 10, and 15 km from the focal patch), weighted by the distance to neighbouring patches (Gustafson & Parker, 1994). All landscape metrics were calculated using our categorical maps and ARCGIS, version 10.1 (ESRI, 2011) and FRAGSTATS, version 3.4 (McGarigal *et al.*, 2002).

We modelled nucleotide diversity (π) and haplotype diversity (H_D) as a function of landscape metrics. Models were contrasted using an information-theoretic approach based on AIC (Akaike, 1973), and using AIC corrected (AIC_c) for small sample sizes (Burnham & Anderson, 2002). Model selection based on information theory differs from usual methods based on null hypothesis testing and does not use the concept of significance. Instead, AIC model selection focuses on the strength of evidence. Model sets are simultaneously contrasted and the model with the lowest relative AIC_c value (i.e. $\Delta AIC_c = 0$) is considered best, whereas models with a $\Delta AIC_c \leq 2$ are considered equally plausible (Burnham & Anderson, 2002). We used normalized model weight (w_i) and the model evidence ratio (ER) to estimate how much likely is the best model compared to the constant (no-effect) model.

For each dependent variable (π and H_D), we defined a model set based on possible hypotheses on the source of variation of genetic diversity, namely one model for each landscape metric (Table 1) in addition to models with additive and interaction terms to determine whether landscape context was also an

Table 1. Landscape metrics used as predictor variables for nucleotide diversity and haplotype diversity estimated at each sampling point in the Aracá, Jaú, and Uatumã landscapes, Amazonas, Brazil

Name	Landscape metric description
area_c	Size of the *campina* patch (ha) were the sampling point is found
area_cc	Size of the *campina* + *campinarana* patch (ha) were the sampling point is found
area_x km	cumulative area of *campina* (ha) in a 5-, 10- or 15-km radius from the sampling point
perim	Perimeter of the *campina* patch were the sampling point is found
enn	Distance to the nearest *campina* patch
prox_x km	Proximity index calculated with a 5-, 10- or 15-km radius from the focal *campina* patch (area based and distance weighted index)

important factor (i.e. to which landscape each group of sites belongs). We also included in the final model set a constant, intercept-only model. To avoid contrasting a large number of models simultaneously, we conducted a preliminary model selection to determine the best distance (5, 10 or 15 km) predicting the genetic diversity of *X. atronitens* for the cumulative area metric (area_x km) using both *campina* and *campina* + *campinarana* maps. For π, there was one single best model containing the 15-km radius cumulative area metric for *campina* habitat ($w_i = 0.8384$). For the proximity index, all plausible models contained metrics considering the three distances (see Supporting information, Table S2). Therefore, we selected a 15-km neighbourhood for the proximity index to match the value of the cumulative area metric. The final model set comprised 19 models. Similarly, for H_D, a preliminary model set was contrasted. In all cases, the constant (intercept-only model) was within the most plausible models (see Supporting information, Table S2). Therefore, the final model set for H_D comprised only 13 models. We used the generalized linear model (Crawley, 2007) with Gaussian error distribution after checking for the distribution of residuals. Landscape metrics were log transformed to reduce the range of values when necessary. Statistical analyses were performed using R, version 2.11.1 (R Development Core Team, 2009).

RESULTS

Interspecific relationships and the origin of *X. atronitens*

The result of the phylogenetic reconstruction for the three analyses (MP, ML, and BI) were very similar (see Supporting information, Fig. S1). *Xenopipo atronitens* and *X. uniformis* grouped together with high support in all analyses, confirming their sister relationship (Prum, 1992; Tello *et al*., 2009). However, the position of *X. unicolor* was not resolved, indicating that more analyses, including all species and more genetic markers, is needed to address the monophyly of the genus. In addition, the phylogenetic position of the *atronitens–uniformis* group is uncertain. The split between *X. atronitens* and *X. uniformis* is estimated to have occurred at the mid to late Miocene, at approximately 8.21 Mya [95% highest posterior density (HPD): 6.23–10.44 Myr].

Phylogeography and population genetic structure of *X. atronitens*

We obtained 214 ND2 sequences (905 bp) of *X. atronitens* (for GenBank accession numbers, see Supporting information, Table S1) from 18 localities (Fig. 1A). No missing data or gaps were present in the aligned matrix. The dataset contained a total of 71 polymorphic sites and 46 were parsimony informative. The overall nucleotide diversity (π) was 0.008 (±0.0004). The number of individuals and haplotypes; nucleotide and haplotype diversity per locality; and genetic distances among localities are provided in the Supporting information (Table S3).

A total of 51 haplotypes was recovered, which were grouped in six clusters (C1–C6) by BAPS5 analysis (Fig. 1B). The haplotypes from south of the Amazon River (SA) form two distinct groups (C4 and C6), both related to the Guyana Shield (GS) haplotypes (C1 group). The Jaú haplotypes (JAU) also formed two distinct groups (C2 and C3) with different phylogeographical affinities, one related to the GS haplotypes (C2) and the other (C3) related to haplotypes from the Negro and Branco interfluvium (NBI). GS and NBI did not share any haplotypes. Therefore, two main geographical breaks are observed, with none or little sharing of haplotypes, which corresponded to the Branco and Amazon Rivers (Fig. 1).

We found a positive correlation between genetic and geographical distances (Mantel test, $r = 0.55$, $P = 0.00001$) and a positive effect of rivers on genetic distances (partial Mantel test; $r = 0.26$, $P = 0.00001$). However, when differentiating between river types, we found that white water rivers had a significant effect on genetic distances ($r = 0.24$, $P = 0.00001$), whereas black water rivers did not affect genetic distances ($r = 0.02$, $P = 0.24$).

There was no IBD pattern among sites within landscapes (Jaú, $r = –0.002$, $P = 0.54$; Uatumã, $r = 0.01$, $P = 0.39$), except for sites in Aracá ($r = –0.08$, $P = 0.011$). However, geographical distance explained very little (8%) of the variation on genetic distance in this landscape. Also, there was no effect of rivers (all black water) acting as barriers within any of the three landscapes (partial Mantel test; Aracá, $r = 0.02$, $P = 0.21$; Jaú, $r = –0.05$, $P = 0.22$; Uatumã, $r = 0.001$, $P = 0.45$).

Gene flow among geographical areas

The general results of pairwise analysis on IMA identified low levels or absence of gene flow between geographical regions (Table 2). Low levels of unidirectional gene flow were identified only from Negro–Branco interfluvium (NBI) and Guyana Shield (GS) to Jaú (JAU) and from GS to South of Solimões/Amazon Rivers (SA) (Fig. 1; Table 2). All population splitting was centered in the Pleistocene during the last million years (Table 2).

Historical demography

The McDonald and Kreitman test did not reject the neutrality hypothesis for ND2 evolution ($P > 0.1$). The

Table 2. Summary of the IMA results among geographical areas identified as follows (for more details, see text)

Geographical pairs	m_1*	m_2*	t†	t (HPD90Lo)	t (HPD90Hi)
NBI/GS	0	0	468,823	216,258	718,232
NBI/JAU	0	0.84	415,153	36,306‡	3,155,485‡
NBI/SA	0	0	516,706	329,387	792,423
GS/JAU	0	0.90	552,48	28,939‡	5,248,619‡
GS/SA	0	3.25	881,347	286,766‡	5,259,142‡
JAU/SA	0	0	332,544	48,934	465,666

*Population migration rate ($q_1 \times m_1/2$) on the coalescent (*e.g.* m_1 = rate of migration from population 2 to population 1).
†Time of population splitting in years (t/u).
‡Nonreliable confidence intervals as a result of difficulties with parameters estimation (see text).
NBI, Negro–Branco interfluvium; GS, Guyana Shield; JAU, west of Negro River; SA, South of the Amazon River.

Table 3. R_2 (Ramos-Onsins & Rozas, 2002), F_s (Fu, 1997), and D (Tajima, 1989) values for all the sampled individuals of *Xenopipo atronitens*, for each landscape (Aracá, Jaú, and Uatumã) and for the identified clusters (shown as if from the north or south of the Amazon river)

Sample/cluster	R_2	F_S	D
All samples (214)	0.0501	**–16.893***	–1.17382
Aracá (60)	0.0607	–4.128	–1.20144
Jaú (41)	0.1493	5.504	1.01254
Uatumã (61)	**0.0487**	**–6.942**	**–1.62952**
C1 (North) (63)	**–1.52039**	**–6.362**	**0.0484**
C2 (North) (45)	**–2.05314**	**–6.695**	**0.0518**
C3 (North) (40)	**–1.81744**	**–6.555**	**0.0492**
C4 (South) (10)	–0.39123	0.471	0.1907
C5 (North) (37)	**–2.14442**	–2.747	**0.0461**
C6 (South) (19)	–0.34093	1.393	0.1388

*Values in bold were significant at 5%, except for Fu's F_S which is significant at 2% probability (Fu, 1997).
The number of individuals in each dataset or cluster is shown in parenthesis.

statistical tests showed signals of demographic expansion in populations of *X. atronitens* (Table 3). For the whole dataset (all 214 individuals), only Fu's F_S test was significant, although it is the most robust and recommended for analysis with a high number of samples (Ramos-Onsins & Rozas, 2002). When landscapes were analyzed separately, signs of demographic expansion were only found in Uatumã (Table 3). Testing for population expansion on clusters identified by BAPS5, we found population expansion only in the clusters from north of the Amazon River (Table 3).

The BSP also showed signs of population expansion despite the overlapping of confidence intervals (Fig. 3A). The expansion was dated to start approximately 25 000 years before present (BP). We also run BSP analyses grouping the clusters from the north

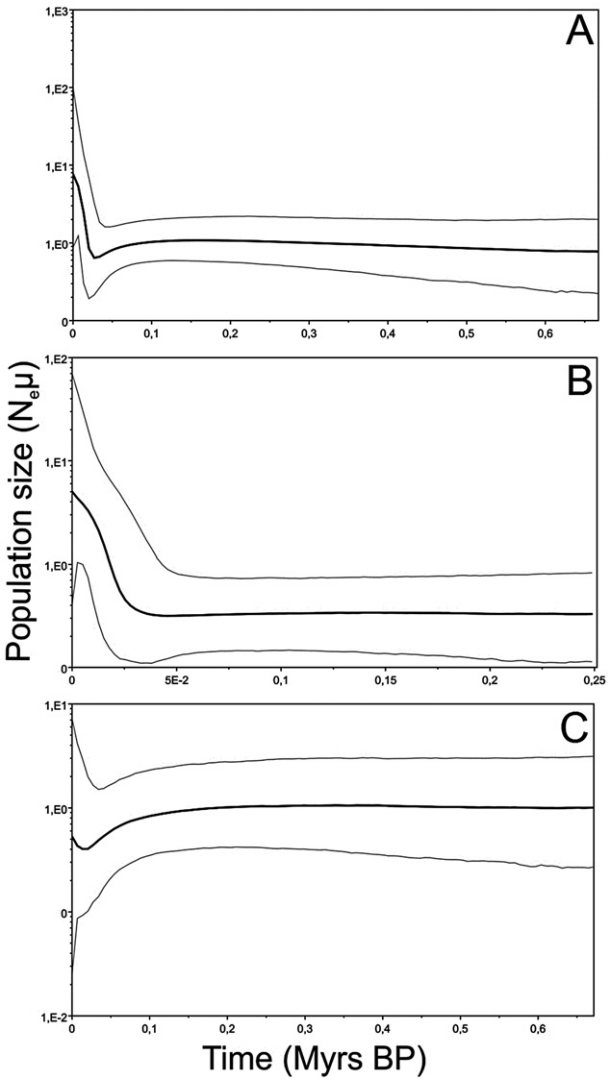


Figure 3. Bayesian skyline plots showing the demographic variation through time (million years before present, Myrs BP) for the whole dataset (A) and for the clusters from north (B) and south (C) of the Amazon River.

Table 4. Model selection results evaluating the effects of landscape metrics and landscape context on nucleotide diversity (π) and haplotype diversity (H_D) estimated at 16 sampling points in Aracá, Jaú, and Uatumã, Amazon, Brazil

Model	AIC_c	d.f.	ΔAIC_c	w_i	ER
Nucleotidic diversity					
π ~ area_15 km + landscape	**–182.4**	**5**	**0.0**	**0.9488**	**948.8**
π ~ area_cc × landscape	–176.1	6	6.4	0.0392	39.2
π ~ area_15 km × landscape	–171.3	7	11.1	0.0037	3.7
π ~ prox_15 km + landscape	–170.1	5	12.3	0.0020	2.0
π ~ area_c + landscape	–169.5	5	12.9	0.0015	1.5
π ~ perim + landscape	–169.5	5	13.0	0.0014	1.4
π ~ prox_15 km × landscape	–169.5	7	13.2	0.0013	1.3
Haplotype diversity					
H_D ~ area_cc × landscape	**–7.2**	**6**	**0.0**	**0.3435**	**1.58**
H_D ~ 1	**–6.3**	**2**	**0.9**	**0.2178**	**1.00**
H_D ~ area_c	**–5.6**	**3**	**1.6**	**0.1552**	**0.71**
H_D ~ perim	–5.5	3	1.7	0.1498	0.69
H_D ~ area_cc	–3.8	3	3.4	0.0628	0.29
H_D ~ enn	–3.2	3	4.0	0.0468	0.21
H_D ~ area_cc + landscape	–0.7	5	6.5	0.0134	0.06
H_D ~ area_c + landscape	1.6	5	8.8	0.0042	0.02
H_D ~ perim + landscape	1.8	5	9.0	0.0038	0.02
H_D ~ enn + landscape	2.6	5	9.8	0.0026	0.01

AIC_c, Akaike's information criterion adjusted for small samples; $\Delta AICc$, adjusted AICc relative to the top model; w_i, normalized model weight; ER, evidence ratio. Selected models (with $\Delta AICc < 2$) are highlighted in bold for each response variable. Only models with $w_i > 0.001$ are presented and ranked by ascending $\Delta AICc$.

and south of the Amazon River based on the results from the above statistical tests. The result for the northern clusters was very similar to the result obtained for the complete dataset, with expansion also dated approximately 25 000 years BP (Fig. 3B). By contrast, the southern clusters showed a signal of population retraction starting after the last interglacial (approximately 100 000 years BP) (Fig. 3C). However, the signal for demographic variation for the southern populations was weak and with large confidence intervals, preventing any reliable conclusions.

Current landscape structure and genetic variability

The best model selected for π indicated a positive relationship between the cumulative area of *campina* within 15 km of the sampling site and nucleotide diversity (π) ($R^2 = 0.9182$, $P < 0.001$). This model had high evidence ratio (Table 4) in relation to the other candidate models and was the only model within the $\Delta AIC_c < 2$ range. This model also included an additive term indicating a landscape effect with similar slopes among landscapes but different intercepts (Aracá: –0.0101, $P < 0.01$; Jaú: –0.0018, $P < 0.001$, Uatumã –0.0066, $P < 0.05$). Thus, the rate at which π declines with area is similar among landscapes, although the values of π are overall higher in the Jaú landscape (Fig. 4).

By contrast to nucleotide diversity, there was no evidence for an effect of current landscape structure on haplotype diversity (H_D). There were three equally plausible models (with $\Delta AIC_c < 2$) including the constant (no-effect) model, indicating that H_D variation across space is not related to current landscape patterns (Table 4).

DISCUSSION

Interspecific relationships and the origin of *X. atronitens*

The sister relationship between *X. atronitens* and *X. uniformis* has been corroborated by different datasets (Prum, 1992; Tello *et al.*, 2009; present study). The former is related to white sand vegetation areas of lowland Amazonia, whereas the latter occurs from mid to high elevation in the Tepuis region. Parts of the Tepuis are sandstone formations (Maguire, 1970; Briceño & Schubert, 1990), a kind of sediment similar to the one that forms the *campinas* and *campinaranas* of lowland Amazonia (Anderson, 1981), which could characterize similar habitat types. Also,

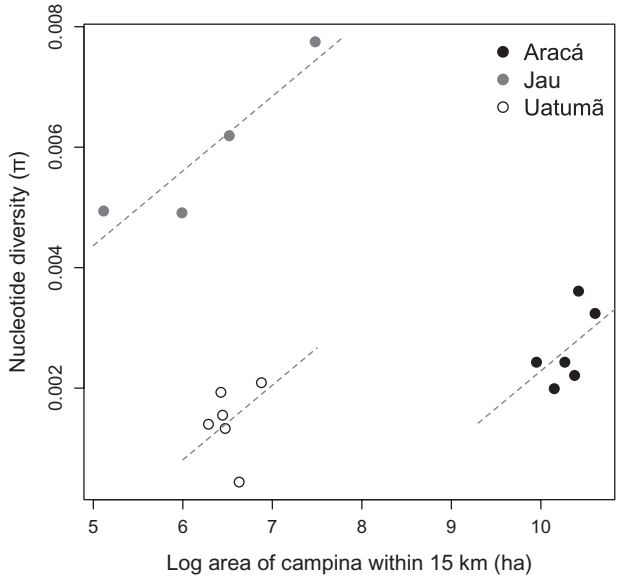


Figure 4. Relationship between area and genetic diversity (π) in the three landscapes in northern and central Amazonia, Brazil. Linear fit was taken from the best model selected for nucleotide diversity.

the largest areas of white sand soil in the middle and upper Negro River region may be fluvial deposits of sediments originating from the Tepuis sandstone formations (Coomes & Grubb, 1996). The isolation of ancestral populations of *X. atronitens* and *X. uniformis* on the lowlands and highlands, respectively, could have followed the isolation of the Tepuis highlands during the formation of escarpments and the retreat of the more recent slopes. The genesis of the Tepuis landscape through the erosive process lasted at least 70 Myr (Briceño & Schubert, 1990; Briceño, Schubert & Paolini, 1990), although there is evidence that an intensive erosive process occurred at the Miocene and Pliocene producing a great amount of sediments deposited along the riverine basins of the Guyana Shield (Gansser, 1954). The dating we obtained (8.21 Myr BP; 95% HPD: 6.23–10.44 Myr BP) for the split between *X. atronitens* and *X. uniformis* is coincident with this period of intense erosion and retraction of the Tepuis escarpments. Thus, the erosive process that shaped the more recent slopes of the Tepuis formation may have lead to the origin of these two species through the isolation of lowland and highland populations.

Oren (1981) observed the relationship among bird species from white sand vegetation areas and those from the Tepuis, and associated the origin of Tepuis species with dispersion from the white sand *campinas*. Huber (1988) considers the white sand *campinas* as an analogous ecosystem to the herbaceous vegetation on the Tepuis summits. Thus, it is plausible (as an alternative hypothesis) that the split between *X. atronitens* and *X. uniformis* could correspond to a dispersion event. However, it is interesting to note that, among the few groups studied so far including species from the Tepuis and the adjacent lowlands, all the highland/lowland splits date to the Miocene (Givnish *et al.*, 2000; Salerno *et al.*, 2012), indicating a relationship between Earth history and the origin of these taxa. These results indicate that Pleistocene climatic cycles apparently have not been the main drivers of highland/lowland speciation, even though they could still be related to diversification within the Tepuis highland lineages (Mayr & Phelps, 1967; Rull, 2005).

Phylogeographical patterns of *X. atronitens*

The ancestral distribution area of a species is often related to a central position on the haplotype network, a higher genetic diversity, and the most frequent haplotype (Posada & Crandall, 2001). It can also be related to the probable distribution of the ancestral species. Both phylogenetic and phylogeographical evidence suggest that the ancestral distribution of *X. atronitens* was centred on the Guyana Shield, adjacent to the Tepuis mountains and, from there, it expanded its distribution to the other white sand areas of Amazonia.

Alonso & Whitney (2003) had already suggested the Guyana Shield as the ancestral area of the white sand bird community. Based on studies on white sand vegetation areas of northern Peru, they concluded that the bird community of this region was of Guyanan ancestry, and accessed the region from the sand belts of north-western Amazonia. In the present study, we observed that the samples from south-western Amazonia (closest to Peru study sites) are related to the samples from south-eastern Amazonia. Poletto & Aleixo (2005) also related a bird community from a south-western *campina* (Guajará, south Amazonas, Brazil) as being biogeographically related to those from the south-eastern Amazon (but part of the community has also been related to the north-western Amazonian *campinas* from the upper Negro River). These results do not discard the Guyanan origin of the white sand bird community but, instead, add another hypothesis for its dispersion through Amazonia. Instead of expanding its distribution through sand belts of western Amazonia, the *campina* avifauna may have expanded through south-eastern Amazonia, crossing the Amazon River, as supported by our results showing gene flow between Guyana Shield and southern Amazonia (Fig. 1, Table 2).

Because this is the first phylogeographical study of a white sand vegetation specialist bird species, our

results can only be compared to patterns observed for Amazonian bird species from other habitats. The upland *terra firme* forest bird species usually have monophyletic lineages or species and subspecies separated by the major Amazonian rivers (Marks, Hackett & Capparella, 2002; Aleixo, 2004; Patel *et al*., 2011; Ribas *et al*., 2012). This occurs especially for understory species (Burney & Brumfield, 2009). Otherwise, typically savanna (cerrado) species appear to have low population genetic structure, even across large geographical distances and biogeographical barriers (Bates, Tello & Silva, 2003). The genetic structure of *X. atronitens*, on the other hand, shows an intermediate pattern between the observed for forest and savanna taxa. Although gene flow in *X. atronitens* appears to be more restricted by the white water rivers, surrounded by *várzea* flooded forest, than by the black water Negro River, surrounded by *igapó* flooded forest, both of these river types are equally important in isolating *terra firme* bird populations (Fig. 1).

Xenopipo atronitens has occasionally been observed in *igapó* forests (Oren, 1981; Borges, 2004) but was never registered in *várzea* forests. Furthermore, haplotype sharing occurs between localities on opposite margins of the Negro River, which is an important geographical barrier for *terra firme* forest species in Amazonia. The Negro River harbours two important archipelagos: one before and another after the connection with the Branco River (Latrubesse & Franzinelli, 2005). The origin of these archipelagos is very recent, estimated to occur between 14 000 and 1000 years ago (Latrubesse & Franzinelli, 2005). Thus, the capability of *X. atronitens* with respect to eventually using *igapó* habitats, plus the occurrence of the archipelagos diminishing the distance between margins of the Negro River, may favour gene flow across this river.

The estimated gene flow in the isolation with migration analyses was always unidirectional, with individuals coming from Guyana Shield (GS) to Jaú (JAU) and South of Solimões/Amazon Rivers (SA), and from Negro-Branco interfluvium (NBI) to JAU (Fig. 1, Table 2). The detected gene flow between opposite margins of the Negro River shows that this river is a more permeable barrier for populations of *X. atronitens* compared to white rivers in the region. However, a higher gene flow was identified from GS to SA, which are separated by the Amazon River, a wide white water river. These results probably indicate an instance of historical gene flow through the lower Amazon River because populations on opposite sides of this river represent the oldest split within the species (Table 2). Other comparisons identified the Solimões/Amazon River (JAU/SA) and the Branco River (NBI/GS) as representing important barriers interrupting gene flow between opposite margins (Table 2). The low genetic distance among haplotypes in different geographical regions (Fig. 1B) was a result of recent (mid-Pleistocene) population splitting (JA/SA; NBI/SA; NBI/GS) (Table 2), suggesting that these populations may have reached some regions of Amazonia only in the recent past. This process may have been facilitated by higher connectivity among *campinas* as a result of changes in the forest structure related to drier periods (Cowling, Maslin & Sykes, 2001).

Demographic expansion since the Late Pleistocene

The clusters located north of the Solimões/Amazon Rivers showed signs of demographic expansion starting approximately 25 000 years ago, which corresponds to the final portion of the last glacial period (110 to 11.7 kya), whereas southern clusters showed a slight signal of population retraction during the last interglacial (Fig. 3). Carneiro-Filho *et al*. (2002) observed a higher activity of sand dunes, which are covered by *campinas* or *campinaranas*, in the middle Negro River region during the late Pleistocene and Holocene. The activity of sand dunes was related to drier climate phases, which can account for increased wind velocity, reduced precipitation and/or longer dry season, and reduced vegetation cover (Carneiro-Filho *et al*., 2002; Teeuw & Rhodes, 2004). In southern Amazonia, Latrubesse (2002) found evidence for Late Pleistocene age (20 kyr) white sand regions, related to river channel migration as a result of a drier climate and more open vegetation cover. Furthermore, according to Latrubesse (2000, 2003), during the Middle Pleniglacial (65–24 kyr BP) aridity in Amazonia reached its maximum, a period even drier than during the Last Glacial Maximum (LGM) (approximately 18 kyr BP). However, Rossetti *et al*. (2012) suggested a recent (Late Pleistocene and Holocene) genesis of open habitats in north, south, and eastern Amazonia, related to fault reactivations.

According to the observations reported above, population demography in *X. atronitens* appears to be related both to increased habitat availability, with the genesis of new white sand habitats, and with the capacity of these white sand habitats to support bird populations. During very dry periods, white sand areas may suffer from drought because the sandy soil has low capacity of retaining water. This, however, appears to be different in southern campinas, that are characterized by soils with a higher proportion of clay (Adeney, 2009), allowing higher water retention. The recent population expansion in northern populations may be related both to more available white sand habitats and to more favourable climatic conditions

after the LGM, which allowed larger populations to occupy these white sand regions. On the other hand, white sand patches are quite smaller in southern Amazonia, and this (associated with their larger capacity of water retention) may have maintained viable population sizes throughout the LGM, without a significant expansion when the climate became more favourable.

Current landscape structure and genetic variability

The influence of current landscape structure on the distribution of genetic diversity of *X. atronitens* provides additional information that helps the interpretation of the demographic scenario described above. The observed reduction of genetic diversity with area, based on current landscape structure, indicates that individuals of *X. atronitens* belonging to sites with reduced habitat availability (i.e. to smaller populations) tend to be more related to each other than individuals belonging to sites with a larger amount of habitat availability (i.e. to a large population). Small populations tend to loose genetic diversity through the effect of reduced immigration and an increased effect of genetic drift (Templeton, 2006). Therefore, this result indicates that populations of *X. atronitens* are relatively isolated in areas with a low amount of *campina* and that the forested area (mostly *terra firme* forests) that surrounds *campina* patches can currently act as dispersal barriers.

Rivers within landscapes did not act as dispersal barriers, most likely because Jaú, Aracá, and Uatumã rivers are all black water rivers. The lack of any model improvement when the area of *campinarana* forest was added to the amount of available habitat suggests that this forest type is not as important as more open vegetation types with respect to determining habitat availability for *X. atronitens*. However, the degree to which *campinarana* and *igapó* forests are more permeable to dispersal than *terra firme* forest needs to be tested.

Despite the lower overall connectivity in Jaú and Uatumã landscapes, area had a similar effect on genetic diversity compared to its effect on the more connected Aracá landscape. Therefore, genetic diversity at focal points is mostly affected by local, small-scale processes independent of the overall landscape context. On the other hand, overall genetic diversity, which is much higher in Jaú, appears to be influenced by population history because the haplotype network and the isolation with migration analyses have shown that haplotypes in Jaú have originated by migration from two distinct regions: NBI and GS.

An integrated scenario for the evolution of white sand *X. atronitens* populations in Amazonia

Landscape genetic analyses indicate that *terra firme* forests are less permeable to dispersal, acting as a barrier to gene flow for *X. atronitens*. On the other hand, the results of isolation with migration analysis indicate unidirectional gene flow among some Amazonian regions, and isolation between these different regions during the Pleistocene. These results suggest that Amazonian landscapes have gone through important changes during glacial cycles, and that these changes probably allowed for higher gene flow among white sand populations in the past.

Studies on both northern (Carneiro-Filho *et al*., 2002; Horbe *et al*., 2004; Teeuw & Rhodes, 2004) and southern (Latrubesse, 2002) Amazonian *campinas* indicate that this habitat appears to respond to historical changes in climate. In northern *campinas*, increased sediment deposition, most of it coming from the Tepuis, and aeolian activity, appear to have caused the increase of the white sand patches during drier climatic periods. In southern regions, recent deposition of white sands appears to be a result of river channel migration and paleochannel formation in drier periods (Latrubesse, 2002; Rossetti *et al*., 2012). Additionally, demographic changes in northern populations of the studied white sand species indicate growing populations when the climate became more favourable, after the LGM.

Thus, Pleistocene glacial cycles appear to have influenced the population dynamics of *X. atronitens*, a white sand specialist, in several ways: by cyclically increasing the availability of white sand soils suitable for growth of *campinas* on subsequent wetter periods; by influencing the population sizes on isolated patches of white sand habitat, with conditions deteriorating during very dry periods; and by facilitating gene flow among currently isolated populations when forest structure is affected by drier periods.

The pattern of current and historical genetic diversity recovered in the present study for *X. atronitens* represents the first study of a white sand specialist bird species, and corroborates the idea that glacial cycles have deeply influenced Amazonian biogeographical history, suggesting a complex interaction between forest and nonforest habitats during the Pleistocene.

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ARCHIVED DATA

All DNA sequences used in the present study have been archived at GenBank.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Phylogenetic tree obtained by maximum likelihood and node supports from the maximum parsimony, maximum likelihood, and Bayesian inference analyses.
Table S1. General information of the samples used in the study and GenBank accession numbers.
Table S2. Model selection results evaluating the distance (5, 10 or 15 km) that predicts best the variation of nucleotide diversity (π) and haplotype diversity (H_D) for the cumulative area metric and the proximity index metric.
Table S3. Number of individuals, segregating sites, nucleotide diversity, and haplotype diversity with its respective standard errors, and number of haplotypes for all the samples and for each locality used in the present study.