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Genomic variation in a widespread Neotropical bird (*Xenops minutus*) reveals divergence, population expansion, and gene flow

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ABSTRACT

The demographic and phylogeographic histories of species provide insight into the processes responsible for generating biological diversity, and genomic datasets are now permitting the estimation of species histories with unprecedented accuracy. We used a genomic single nucleotide polymorphism (SNP) dataset generated using a RAD-Seq method to investigate the historical demography and phylogeography of a widespread lowland Neotropical bird (*Xenops minutus*). As expected, we found that prominent landscape features that act as dispersal barriers, such as Amazonian rivers and the Andes Mountains, are associated with the deepest phylogeographic breaks, and also that isolation by distance is limited in areas between these barriers. In addition, we inferred positive population growth for most populations and detected evidence of historical gene flow between populations that are now physically isolated. Although we were able to reconstruct the history of *Xenops minutus* with unprecedented resolution, we had difficulty conclusively relating this history to the landscape events implicated in many Neotropical diversification hypotheses. We suggest that even if many traditional diversification hypotheses remain untestable, investigations using genomic datasets will provide greater resolution of species histories in the Neotropics and elsewhere.

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1. Introduction

Lowland humid forests in the Neotropics contain some of the highest biodiversity on the planet (Pearson, 1977). A number of hypotheses have been proposed to explain the origins of this diversity, most of which link biological diversification directly to tumultuous landscape changes that led to speciation via the geographic isolation of populations (Moritz et al., 2000; Antonelli et al., 2010). The hypotheses differ in the events and features implicated. These include the origins of major rivers in the Amazon basin (Sick, 1967; Capparella, 1987; Ribas et al., 2012), uplift of the Andes and other mountain ranges (Chapman, 1917, 1926), past fragmentation of humid forest due to expansion of arid habitats (Haffer, 1969) or marine transgressions (Nores, 1999; Aleixo, 2004), edaphic or climatic conditions associated with geologic arches (Lougheed et al., 1999; Wesselingh and Salo, 2006), and areas of displacement due to invasion by temperate taxa during colder periods (Erwin, 1979; Bush, 1994).

Studies evaluating these hypotheses have typically addressed them using gene genealogies to infer the timing of divergence and the geographic location of vicariance. Using the conceptual framework of vicariance biogeography, researchers have searched for shared phylogeographic (or phylogenetic) relationships among taxa that would suggest a common mechanism of biological diversification (e.g., Cracraft and Prum, 1988; Brumfield and Capparella, 1996; Hall and Harvey, 2002; Quijada-Mascareñas et al., 2007). In addition, molecular dating methods have been used to estimate the timing of population divergence events and to compare these dates to hypothesized events in the landscape evolution of the Neotropics (Patton et al., 2000; Weir, 2006; Santos et al., 2009; Ribas et al., 2012). Although some general patterns have emerged from these studies, such as the importance of landscape features in delimiting populations and the absence of an increase in diversification during the Pleistocene, no single dominant model relating historical diversification to landscape history has emerged from decades of genetic studies (reviewed in Haffer, 1997; Antonelli et al., 2010; Leite and Rogers, 2013).

Interrogating processes beyond divergence may prove to be more fruitful in informing species histories (Takahata et al., 1995, Kuhner, 2009). For example, signatures of population size changes found in studies of Neotropical organisms (Aleixo, 2004; Cheviron

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et al., 2005; Solomon et al., 2008; D'Horta et al., 2011) may evidence historical increases or decreases in habitat availability. Evidence of gene flow between populations, which may reveal instances of past connectivity between habitats or regions, has been uncovered in a few studies (Patton et al., 1994; Noonan and Gaucher, 2005; Maldonado-Coelho et al., 2013). In addition, a few studies have detected the effects of natural selection and sexual selection among populations (Mallet, 1993; Turner et al., 2004), which may be linked to past climatic changes or other events. Reconstructing how these diverse processes influenced modern phylogeographic patterns is challenging, but could provide new insight into the history of Neotropical diversification.

The availability of genome-scale datasets is improving inferences concerning the historical diversification of organisms (Li and Durbin, 2011; Frantz et al., 2013). Genomic data, when combined with methods that account for coalescent stochasticity, allow for the integration of information across many loci (Edwards and Beerli, 2000), and provide greater statistical power for testing models of population history (Pool et al., 2010). Analyses based on genomic data result in narrower confidence intervals in estimates of important parameters such as divergence times, effective population sizes, and migration rates between populations (Smith et al., 2014). Dense sampling across the genome increases the probability of obtaining data from migrant alleles or genomic regions that have been influenced by selection (Carlson et al., 2005). The application of genomic data to Neotropical systems (e.g., Nadeau et al., 2013) promises to allow further investigation of processes important in Neotropical species histories.

Here, we examine the utility of dense, genome-scale genotyping-by-sequencing (GBS) data for phylogeography and historical demography. We use a GBS dataset from a widespread lowland Neotropical bird species (*Xenops minutus*; Aves, Furnariidae) to (1) characterize the geographic structure of genetic variation in this species and (2) evaluate a series of predictions concerning its historical demography. *Xenops minutus* is relatively common in humid lowland forests west of the Andes from Mexico to northwestern South America and, east of the Andes, in the Amazon Basin and Atlantic Forest of eastern South America (Remsen, 2003). Eleven parapatrically or allopatrically distributed subspecies are currently recognized (Pinto, 1954; Dickinson, 2003; Remsen, 2003). Subspecies are cryptic, varying subtly in plumage or vocalizations, but this variation has not been studied quantitatively. Remsen (2003) suggested that the nominate subspecies of southeastern Brazil is distinct in plumage and in its smaller size and may merit species status. Although all subspecies inhabit forest, it is unclear whether there is geographic variation in microhabitat preference or other ecological traits. Previous phylogeographic studies (Burney, 2009; Smith et al., 2014) of *X. minutus* had limited genomic or geographic sampling, but found evidence for geographically isolated mitochondrial clades and deep genome-wide divergence between populations from either side of the Andes, respectively. Our goals were to determine how the population history of *X. minutus* influences modern patterns of genetic diversity, and to attempt to relate this history to the general landscape history of the Neotropics.

2. Materials and methods

2.1. Genetic data collection and processing

We sampled eight vouchered *X. minutus* from each of nine biogeographic areas for a total of 72 individuals (Fig. 1, Table S1). This sample included 7 of the 11 currently recognized subspecies (Dickinson, 2003; Remsen, 2003). The remaining four subspecies, distributed in Colombia, the northwestern Amazon Basin, and the northern Atlantic Forest of Brazil, were not included because we lacked sufficient genetic material. We extracted total DNA from frozen or alcohol-preserved pectoral muscle tissue using a DNeasy tissue extraction kit (Qiagen, Valencia, CA).

We sent 0.3–3.0 mg of each sample to the Cornell Institute of Genomic Diversity for genotyping-by-sequencing (GBS). GBS is a streamlined workflow for generating reduced representation libraries for Illumina sequencing, similar to other forms of RAD-Seq (Baird et al., 2008; Hohenlohe et al., 2010). Details of the laboratory methods can be found in Elshire et al. (2011). In brief, DNA from each sample was digested using the restriction enzyme PstI (CTGCAG), and both a sample-specific barcoded adapter and a common adapter were ligated to the sticky ends of fragments. Samples were pooled and fragment libraries cleaned using a QIAquick PCR purification kit (Qiagen). Libraries were amplified using an 18-cycle PCR with long primers complementary to the barcoded and common adapters, purified again using QIAquick, and quantified using a PicoGreen assay (Molecular Probes, Carlsbad, CA, USA). Samples were run on a partial lane (72 out of 96 samples) of a 100-bp single-end Illumina HiSeq 2000 run at the Cornell Core Laboratories Center.

The Cornell Institute of Genomic Diversity processed raw sequence reads using the UNEAK pipeline, an extension to TASSEL 3.0 (Bradbury et al., 2007). Briefly, UNEAK retains all reads with a barcode, cut site, and no missing data in the first 64 bp after the barcode. Reads are clustered into tags by 100% identity, tags are aligned pairwise, and any tag pairs differing by one bp are called as potential SNPs. To remove sequencing errors, any alleles represented by fewer than five reads or a frequency of less than 5% are filtered out (Table S2). Following processing with the UNEAK pipeline, we collapsed reverse complement tag-pairs and re-called genotypes using the method of Lynch (2009) as implemented in custom perl scripts obtained from T. A. White (White et al., 2013) and available at https://github.com/mgharvey/GBS_process_Tom_White/v1. We removed potential paralogs by filtering out SNPs with heterozygosity greater than 0.75, and we removed SNPs for which genotype calls were missing from more than 20% of the individuals. The hypothetical genomic distribution of the remaining SNP loci was investigated by aligning their tag-pair consensus sequences (with "N" inserted at the SNP site) to the Zebra Finch (*Taeniopygia guttata*) genome (Warren et al., 2010) using blastn (Altschul et al., 1990). *Taeniopygia guttata* is the most closely related species to *X. minutus* with a publicly available genome assembly, although the evolutionary distance between the two is considerable (Hackett et al., 2008). We used custom python scripts (available at http://github.com/mgharvey/misc_Python) to generate input files for further analysis.

2.2. Data analysis: Effects of distance and barriers

Isolation by distance and dispersal barriers are known to geographically structure genetic variation in Neotropical birds (Brawn et al., 1996; Cheviron et al., 2005; Cabanne et al., 2007). We evaluated the importance of these isolating forces using Mantel and partial Mantel tests, as well as a Bayesian model-based method. We used the kinship coefficient (Loiselle et al., 1995) calculated in the program SPAGeDi (Hardy and Vekemans, 2002) as an index of pairwise genetic relatedness between individuals. The kinship coefficient F_{ij} is the probability that two homologous genes are identical by descent, and is calculated as $F_{ij} = (Q_{ij} - Q_m)/(1 - Q_m)$ where Q_{ij} is the probability of identity by state between two individuals of interest for random genes and Q_m is the average probability of identity by state for genes coming from random individuals in the population. F_{ij} is a relatively unbiased estimator with low sampling variance (Hardy and Vekemans, 2002).

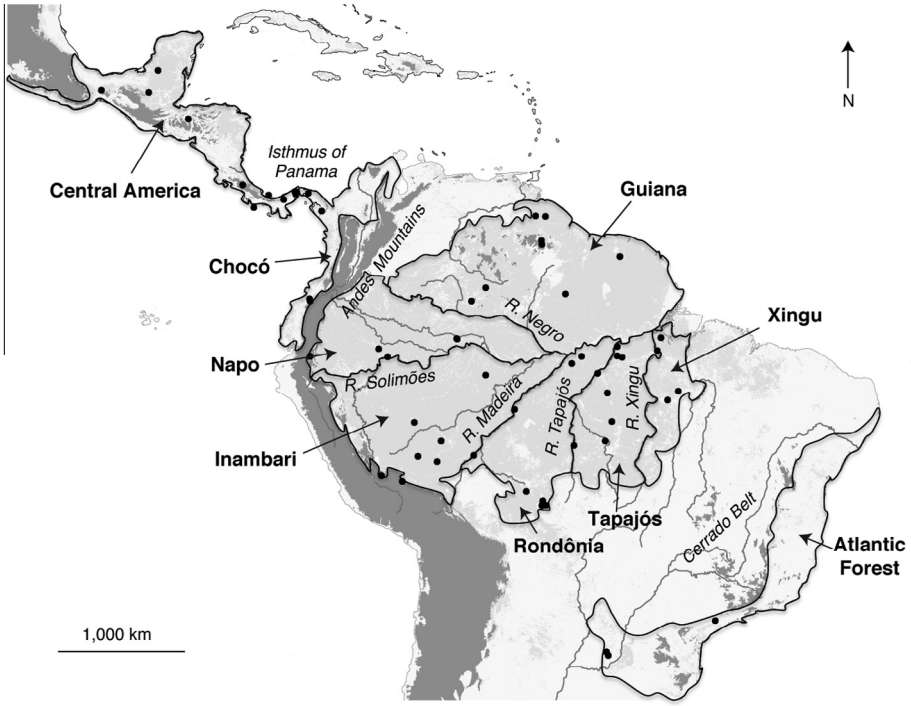

Fig. 1. Map showing sampling locations (circles), biogeographic areas (bold type) and dispersal barriers (italics) examined in this study.

We tested for isolation by distance across all individuals using a Mantel test comparing F_{ij} and geographic distance between individuals. Geographic distances were calculated as the Euclidean distances between sampling localities in SPAGeDi. To distinguish isolation by distance from discrete genetic breaks we conducted separate Mantel tests within each biogeographic area bounded by a major dispersal barrier, including the Isthmus of Panama, the Andes Mountains, major Amazonian rivers, and the cerrado belt of eastern Brazil that isolates Amazonia from the Atlantic Forest (based on Cracraft, 1985, Fig. 1). To investigate isolation due to the dispersal barriers, we used a partial Mantel test that controlled for geographic distance in testing the correlation between F_{ij} and whether individuals were on the same or different sides of putative barriers. We conducted separate analyses including all barriers and for each barrier individually. Only those individuals in the areas adjoining each barrier were used for the barrier-specific tests to remove confounding influences from other barriers. All Mantel and partial Mantel tests were carried out in the R package ecodist (Goslee and Urban, 2007) using 10,000 permutations of geographic locations with individuals to determine significance and a jackknifing procedure to estimate standard errors.

Because Mantel and partial Mantel tests assume linear relationships between variables (Legendre and Fortin, 2010), are confounded by spatial autocorrelation (Guillot and Rousset, 2013), and are unable to directly quantify the relative importance of predictor variables (Bradburd et al., 2013), we also used a new method, BEDASSLE (Bradburd et al., 2013). BEDASSLE overcomes these issues by modeling the covariance in allele frequencies between populations as a function of the predictor variables, and estimating model parameters in a Bayesian framework using a Markov chain Monte Carlo algorithm. We used BEDASSLE to estimate the relative importance of geographic distance and barriers across the entire distribution of *X. minutus*, as well as between each pair of adjacent populations separated by a specific dispersal barrier. We ran BEDASSLE using the beta-binomial model to account for over-dispersion due to variation in demographic histories across populations. All analyses were run for 10 million generations, sampling every 100. We examined traces, marginal and joint marginal parameter distributions, and MCMC acceptance rates every one to five million generations and adjusted tuning parameters according to the suggestions of Bradburd et al. (2013).

2.3. Data analysis: Population assignment and admixture

We estimated the number of populations and conducted population assignment of individuals from all SNPs using methods implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000) and Structurama (Huelsenbeck et al., 2011). Given a fixed number of populations (K), STRUCTURE assigns individuals to populations probabilistically such that Hardy–Weinberg equilibrium and linkage equilibrium within populations are maximized. In addition to population assignment, STRUCTURE can be used to identify admixed individuals. We used STRUCTURE without specifying prior information on population membership, and used options for correlated allele frequencies and genetic admixture across populations (Falush et al., 2003). We conducted runs of 1,000,000 generations (after a 10,000-generation burnin) for each value between $K = 1$ and $K = 15$ and calculated Pr(X|K) to assess the results (Pritchard et al., 2000).

Structurama offers the option of jointly estimating the number of populations (K) and the assignment of individuals to populations using a Dirichlet process prior. We treated K as a random variable and provided an exponential distribution with a mean of nine as a prior for K, consistent with the number of biogeographic regions from which individuals were sampled. We also treated both K and the clustering variable α as random variables and examined the influence of three different gamma priors for α: (1,1), (5,1), and (10,1). For each analysis, we ran MCMC chains for 100 million generations, sampling every 25,000, and discarded 25% of the samples as burnin.

To uncover finer scale population structure we used ChromoPainter and fineSTRUCTURE (Lawson et al., 2012) with the subset of SNPs having no missing data across all 72 individuals. ChromoPainter considers each individual a possible recipient of "chunks" of

DNA from a panel of donor individuals. It assembles a "coancestry matrix" recording the number of recombination events between each donor and recipient. In our case, we considered all individuals as potential recipients and donors. Although using linked sites provides more power for population inference using this method, we lacked linkage information for our SNPs, so we treated them as unlinked. fineSTRUCTURE then performs model-based clustering using the information in the coancestry matrix. The normalization parameter c, or the effective number of "chunks", is used to rescale the elements of the coancestry matrix before calculating the likelihood, and can influence the amount of inferred population structure. We used a c value of $1/(n-1)$ where n is the sample size, following the recommendation in Lawson et al. (2012) for unlinked data, but also examined the effects of higher and lower c values.

Population structure is sometimes inferred incorrectly due to the presence of isolation by distance (Meirmans, 2012). We examined this possibility by conducting partial Mantel tests of the association between F_{ij} and both the set of populations estimated in fineSTRUCTURE and the set of populations estimated from STRUCTURE with $K = 5$ and Structurama with the gamma prior for alpha equal to (1,5), while controlling for geographic distance. Hereafter we refer to these as the fineSTRUCTURE populations and the STRUCTURE/Structurama populations, respectively.

2.4. Data analysis: Population expansion and migration

We estimated expansion within and migration between both the fineSTRUCTURE and STRUCTURE/Structurama populations using coalescent modeling in the program LAMARC (Kuhner, 2006, 2009). LAMARC has the advantage of being able to jointly estimate population growth and migration, both of which may be important processes influencing genetic variation in populations of tropical taxa (Moritz et al., 2000). We estimated the standardized population mutation rate ($\theta = 4N_e\mu$) and population growth rate (g, where $\theta_t = \theta_{present}^{-gt}$) for each population as well as the migration rate ($M = m/m\mu$, where m is the immigration rate per generation and $m\mu$ is the neutral mutation rate per site per generation) between adjacent populations separated by the dispersal barriers described above. We used the parameter-poor F84 model of sequence evolution because it is much faster than the alternative GTR model in LAMARC and because a simple model should be sufficient given that mutations are infrequent at the loci examined (SNPs represent a single variable site within an ~64 bp alignment). We set the transition/transversion ratio to 2. We used a Bayesian MCMC approach, and placed uniform priors on θ ($\log(1 \times 10^{-6}, 10)$), M ($\log(1 \times 10^{-10}, 100)$), and g (linear(−500, 1000)). We conducted 10 initial chains with 1000 iterations of burnin followed by 10,000 iterations, followed by 2 independent final chains of 5000 iterations of burnin followed by 10,000,000 iterations. We checked for convergence within and between chains using Tracer v.1.5 (Rambaut and Drummond, 2007), and we report estimates from the second final chain.

2.5. Data analysis: Natural selection

We conducted a preliminary examination of selection in *X. minutus* using a multi-population outlier scanning approach implemented in BayeScan 2.01 (Foll and Gaggiotti, 2008). BayeScan examines F_{st} values between each population and a common migrant gene pool for each locus. F_{st} coefficients are decomposed into a component shared by all loci (β) and a locus-specific component (α) that reflects selection. BayeScan then compares models in which selection (α) is and is not incorporated, and estimates the posterior probability for each model at each locus using a reversible-jump Markov chain Monte Carlo (RJ-MCMC) method. The posterior odds, or ratio of posterior probabilities, are used to decide on the best model and to define thresholds to determine sets of outlier markers. BayeScan is robust to complex demographic scenarios that might influence neutral differentiation (Foll and Gaggiotti, 2008). We examined the influence of selection based on analyses using both the STRUCTURE/Structurama and fineSTRUCTURE populations. We ran analyses using 20 pilot runs of 5000 iterations, a burn-in of 50,000 iterations, and a final run of 50,000 iterations. Prior odds for the neutral model were set to 10.

2.6. Data analysis: Species tree

We estimated the branching structure of populations using a species tree approach for both the fineSTRUCTURE and STRUCTURE/Structurama populations. Species trees were estimated using the coalescent method implemented in SNAPP (Bryant et al., 2012). SNAPP computes the likelihood of a species tree from unlinked biallelic markers rather than explicitly sampling gene trees. Any SNPs missing genotypes from all individuals in any of the populations were removed from the dataset. Also, due to the computational demands of analyzing the full dataset, we reduced each population to two randomly selected individuals (four haplotypes). We used a diffuse gamma prior for θ ($\alpha = 10$, $\beta = 100$) and a pure birth (Yule) prior for the species tree, with birth rate (λ) equal to 0.00765. For each population set, we conducted two runs of 5 million generations, sampling every 1000 generations. We determined the burnin and assessed MCMC convergence by examining ESS values and likelihood plots in Tracer v.1.5 (Rambaut and Drummond, 2007). We combined runs and used TreeAnnotator (Rambaut and Drummand, 2008) to determine the Maximum Clade Credibility tree and posterior probability values.

3. Results

3.1. Sequencing and datasets

GBS produced a total of 106,784 biallelic SNPs (Table S3). After collapsing reverse complements and filtering for observed heterozygosity and amount of missing data, the final data matrix contained 3379 SNPs and was 91.1% complete. Data have been deposited in Dryad (submission pending). We recovered hits to the *T. guttata* genome using blastn for 3247 of these SNPs. Hits were distributed across 31 of the 36 chromosomes, including the Z chromosome (Table S4). The chromosomes without hits were microchromosomes 16, LGE22, LG2, LG5, and MT. The number of hits per chromosome was positively associated with chromosome size ($r^2 = 0.836$, $p < 0.001$). We note, however, that the short length of GBS loci may result in low mapping accuracy and that *T. guttata* and *X. minutus* are distant relatives and synteny between the two genomes may be low.

3.2. Effect of distance and barriers on genetic divergence

Plotting pairwise kinship coefficients between samples relative to geographic distance revealed wide variation in kinship across the distribution of *Xenops minutus* (Fig. 2). Mantel tests showed a signal of isolation by distance based on correlations between the kinship coefficient and geographic distance (Mantel r statistic = −0.4964, $p = 0.0001$). However, the signal for isolation by distance was less prevalent within areas; only the Napo, Rondônia, and Atlantic Forest areas showed significant ($p < 0.01$) evidence of isolation by distance and the slopes were generally shallow (Table S5). Partial Mantel tests across all areas and individuals revealed a relationship between kinship and whether individuals were on the same or opposite sides of barriers after controlling for isolation by distance ($r = -0.6467$, $p = 0.0001$). Examining each

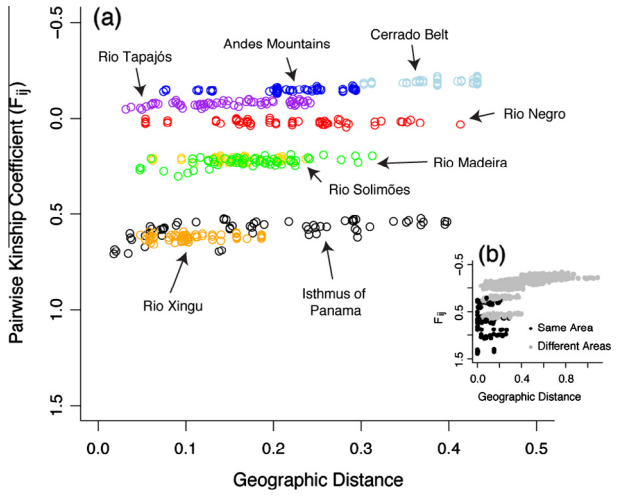

Fig. 2. Plots of pairwise kinship versus relative geographic distance (a) between individuals separated by a single putative barrier and (b) between all individuals including those within the same area (black points) or separated by one or multiple barriers (gray points). The *y*-axes are inverted so that points representing greater divergence appear toward the tops of the plots.

dispersal barrier separately, we found that all nine barriers showed a significant relationship ($p < 0.01$) with the kinship coefficient, and the slope of the Mantel correlation was generally steeper than in the within-area isolation by distance comparisons (Tables 1, S5). We observed the strongest correlations between dispersal barrier and kinship for the Isthmus of Panama, Andes Mountains, Rio Negro, and Rio Tapajós.

We discarded the first five million generations of all BEDASSLE MCMC chains and used the remaining posterior to estimate the ratio of the effect size of barriers versus the effect size of geographic distance (α_E/α_D). Across all barriers, the mean and median ratios were 0.413 and the 95% credible set was 0.322 to 0.464. The interpretation of this ratio is that the effect on genetic differentiation of separation by a barrier is equivalent to the effect of roughly 2000–2900 km of geographic distance. Examining each barrier separately, we found variation across barriers in the relative effect sizes of the barrier and geographic distance (Table 1). The Andes Mountains, Rio Negro, Rio Tapajós, and Cerrado Belt had the highest ratios, supporting the particular importance of these barriers in structuring genetic variation.

Table 1
Influence of barriers on genetic variation in *X. minutus*. Partial Mantel test *r*-statistics measure the relationship between pairwise kinship estimates and whether the two individuals are on the same or opposite sides of a barrier, controlling for geographic distance (lower *r*-statistics indicate a stronger relationship). The BEDASSLE α_E/α_D ratio measures the relative impact of barriers versus geographic distance on genetic similarity (higher values indicate a stronger relationship).

Dataset	Partial Mantel test *r*-statistic (SE)	BEDASSLE α_E/α_D ratio (credible interval)
Isolation by Barriers		
All barriers	−0.647 (−0.676, −0.612)*	0.416 (0.276, 0.588)
Isthmus of Panama	−0.716 (−0.809, −0.646)*	0.0773 (0.0619, 0.0975)
Andes Mountains	−0.737 (−0.798, −0.620)*	137 (22.3, 466)
Rio Negro	−0.797 (−0.843, −0.736)*	62.2 (21.5, 129)
Rio Solimões	−0.519 (−0.830, −0.359)*	0.125 (0.0781, 0.189)
Rio Madeira	−0.469 (−0.661, −0.357)*	0.0168 (0.00905, 0.0271)
Rio Tapajós	−0.844 (−0.924, −0.800)*	99.0 (35.3, 324)
Rio Xingu	−0.276 (−0.410, −0.180)*	0.0296 (0.0150, 0.0682)
Cerrado Belt	−0.531 (−0.712, −0.421)*	136 (10.8, 8,060)

* $P < 0.001$.

3.3. Population assignment and admixture

Analysis of P(*X*|*D*) from the STRUCTURE runs suggested $K = 5$ was the optimal value for number of populations (Table S6). The five clusters from the $K = 5$ analysis contained the individuals from (Central America + Chocó), Guiana, (Napo + Inambari + Rondônia), (Tapajós + Xingu), and Atlantic Forest (Figs. 3, S1). The four populations from the $K = 4$ analysis were similar, except the Guiana population was lumped with the (Napo + Inambari + Rondônia) population (Fig. S1).

The Structurama results were sensitive to the specification of the α prior. The (1,1) prior, with a small mean and narrow s.d. resulted in three populations; the (5,1) prior with an intermediate mean and s.d. resulted in five populations; the (10,1) prior with a large mean and s.d. resulted in four populations; and the prior based on an expected value of nine populations resulted in three populations (Fig. S1). The populations from all analyses included some combination of the same populations from the STRUCTURE analysis. The five populations from the Structurama analysis with an intermediate prior of (5,1) were identical to the five populations from the STRUCTURE analysis at $K = 5$ (Figs. 3, S1). These five populations were selected for use in subsequent analyses.

fineSTRUCTURE revealed more population structure than did STRUCTURE and Structurama. For $c = 1/(n-1)$, eight populations were detected (Figs. 3, S1, S2). These resembled the populations from the STRUCTURE analysis with K = 5 and the Structurama analysis with the (5,1) prior. fineSTRUCTURE, however, divided the (Central America + Chocó) population into two, with the break occurring west of the canal zone in Panama (an individual from Coclé just west of the canal is allied with the Chocó individuals), and identified a cluster within Central America comprising the two northwestern-most samples from foothill areas in Oaxaca and Chiapas, Mexico. In addition, fineSTRUCTURE separated seven of the eight individuals in the Napo region from those in the Inambari and Rondônia regions. The eighth sample from the Napo region allied with the Inambari and Rondônia samples. This sample was collected in the foothills of southern Ecuador not far from the Río Marañon, which is often considered the border between the Napo and Inambari regions. Varying the value of *c* within a narrow range did not strongly influence cluster assignment in fineSTRUCTURE, and did so in an intuitive manner (e.g. by combining two weakly divergent clusters). We selected the eight populations from the fineSTRUCTURE analysis with $c = 1/(n-1)$ for use in subsequent analyses.

Both the set of populations inferred from fineSTRUCTURE ($r = -0.6709$, $p = 0.0001$) and STRUCTURE/Structurama ($r = -0.7611$, $p = 0.0001$) explained kinship between individuals significantly, even after controlling for isolation by distance in partial Mantel tests (Table 1). An examination of the admixture estimates from the STRUCTURE analysis with $K = 5$ revealed relatively low admixture between populations (Fig. S3). A small amount of admixture was observed between Guiana and (Napo + Inambari + Rondônia) and between (Napo + Inambari + Rondônia) and (Tapajós + Xingu).

3.4. Population expansion and migration

LAMARC MCMC chains converged after 2–3 million generations, but were run to 20 million. In both the analyses of fineSTRUCTURE and STRUCTURE/Structurama populations, θ was smaller in the Atlantic Forest population than in all other populations except the Napo population in the fineSTRUCTURE analysis (Table 2). We detected significant population growth (confidence intervals not overlapping zero) in seven of the eight fineSTRUCTURE populations and all five of the STRUCTURE/Structurama populations (Table 2). Growth rates were higher in the (Tapajós + Xingu) and

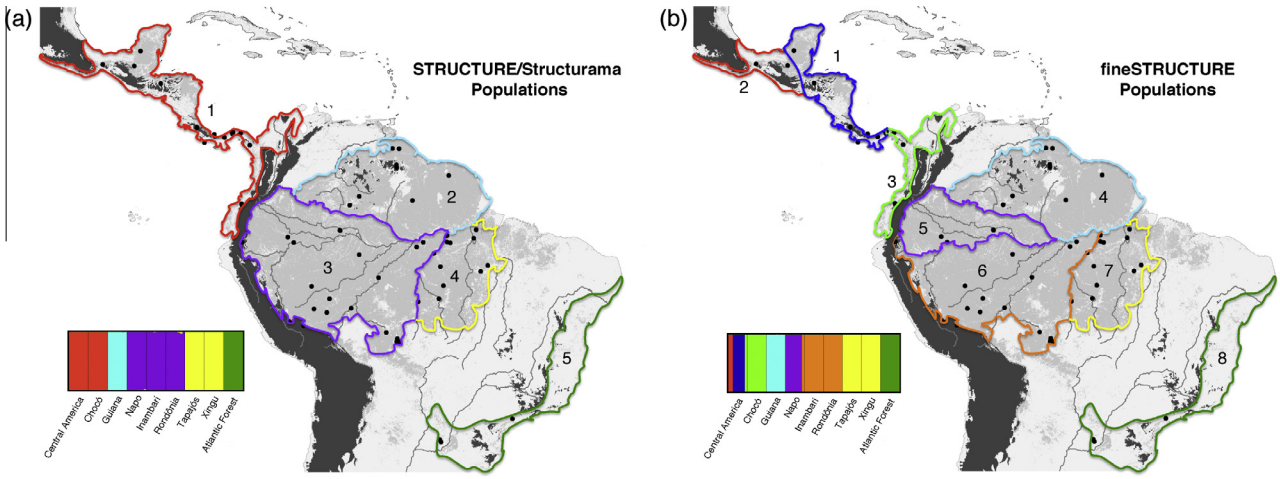

Fig. 3. Maps of the distributions of populations from (a) the STRUCTURE/Structurama analysis and (b) the fineSTRUCTURE analysis. Populations are numbered and numbers are consistent with subsequent tables and figures. The adjacent structure plots show population membership for all individuals from (a) the STRUCTURE analysis with $K = 5$ and (b) the fineSTRUCTURE analysis. Admixed individuals are shown in the structure plot for the STRUCTURE analysis, but fineSTRUCTURE does not estimate admixture.

Table 2
Theta (θ) and population growth rate (g) estimates from LAMARC for each STRUCTURE/Structurama and fineSTRUCTURE population (see Fig. 3).

Population	θ (95% CI)	g (95% CI)
STRUCTURE/Structurama		
1	5.2 (2.9, 9.2)	64.4 (48.8, 75.3)
2	8.4 (2.2, 9.8)	70.6 (52.7, 94.3)
3	9.9 (6.9, 10.0)	55.7 (47.5, 63.1)
4	8.1 (3.7, 9.8)	120.6 (94.8, 133.8)
5	1.0 (0.4, 5.2)	174.3 (112.0, 241.3)
fineSTRUCTURE		
1	8.7 (0.4, 9.8)	91.9 (−170.2, 208.4)
2	5.7 (0.5, 9.5)	87.5 (57.7, 212.1)
3	5.2 (1.9, 9.5)	80.4 (54.5, 100.0)
4	9.5 (2.9, 9.9)	96.7 (68.2, 107.5)
5	2.6 (1.1, 5.7)	42.0 (32.7, 57.4)
6	9.9 (6.8, 10.0)	66.5 (57.0, 76.9)
7	8.1 (3.3, 9.8)	119.9 (90.7, 134.3)
8	1.1 (0.4, 3.9)	204.3 (120.6, 258.9)

Atlantic Forest populations than in other populations, except for the Central American and Guianan populations in the analysis of fineSTRUCTURE populations.

We recovered significant non-zero migration rates (confidence intervals not overlapping zero) in six of the 14 pairwise estimates for the fineSTRUCTURE populations and three of the eight pairwise estimates for the STRUCTURE/Structurama populations (Table 3). Migration between Central American and Mexican populations in the analysis of fineSTRUCTURE populations was higher than between most other populations. Migration was also detected from the Chocó region to Central America (fineSTRUCTURE), from the (Napo + Inambari + Rondônia) population to the trans-Andean populations (STRUCTURE/Structurama), and from the (Tapajós + Xingu) population to the Atlantic Forest (both analyses). Within the Amazon Basin, analysis of the STRUCTURE/Structurama populations detected migration in both directions across the Negro River, and analysis of the fineSTRUCTURE populations detected migration from the Napo to the Guianan and (Inambari + Rondônia) populations and from the (Inambari + Rondônia) population to the (Tapajós + Xingu) population.

3.5. Natural selection

We detected no loci putatively under diversifying selection using BayeScan with the STRUCTURE/Structurama populations and the false discovery rate (FDR) set to 0.05 (Fig. S3). We did, however, detect 20 loci that were putatively under purifying or balancing selection (FDR = 0.05). In the analysis of the fineSTRUCTURE populations we detected 32 loci putatively under diversifying selection and 41 loci putatively under purifying or balancing selection (FDR = 0.05). Of the 20 loci putatively under purifying/balancing selection in the analysis of STRUCTURE/Structurama populations, 17 were also outliers putatively under purifying/balancing selection in the analysis of fineSTRUCTURE populations.

3.6. Species tree

We recovered well-supported topologies from the SNAPP species tree analyses of both the STRUCTURE/Structurama population set and the fineSTRUCTURE population set (PP of all nodes = 1.0). Runs converged after two to three million generations, so we used

Table 3
LAMARC estimates of migration rate (M) between populations for both the STRUCTURE/Structurama populations and fineSTRUCTURE populations (see Fig. 3).

Populations	M (95% CI)
STRUCTURE/Structurama	
1 → 3	0.0 (0.0, 0.2)
3 → 1	0.8 (0.0, 2.6)
2 → 3	3.3 (0.9, 7.2)
3 → 2	3.8 (0.4, 10.6)
3 → 4	0.9 (0.0, 3.5)
4 → 3	0.4 (0.0, 1.5)
4 → 5	2.0 (0.1, 8.7)
5 → 4	0.0 (0.0, 0.6)
fineSTRUCTURE	
1 → 2	31.6 (2.5, 92.9)
2 → 1	90.7 (12.5, 99.7)
1 → 3	2.6 (0.0, 9.6)
3 → 1	2.5 (0.1, 37.9)
3 → 5	0.0 (0.0, 0.6)
5 → 3	1.2 (0.0, 4.2)
4 → 5	0.0 (0.0, 0.6)
5 → 4	1.2 (0.0, 4.9)
5 → 6	4.3 (2.0, 8.6)
6 → 5	0.3 (0.0, 1.8)
6 → 7	1.9 (0.2, 5.2)
7 → 6	0.0 (0.0, 0.3)
7 → 8	4.3 (0.1, 12.3)
8 → 7	0.0 (0.0, 0.5)

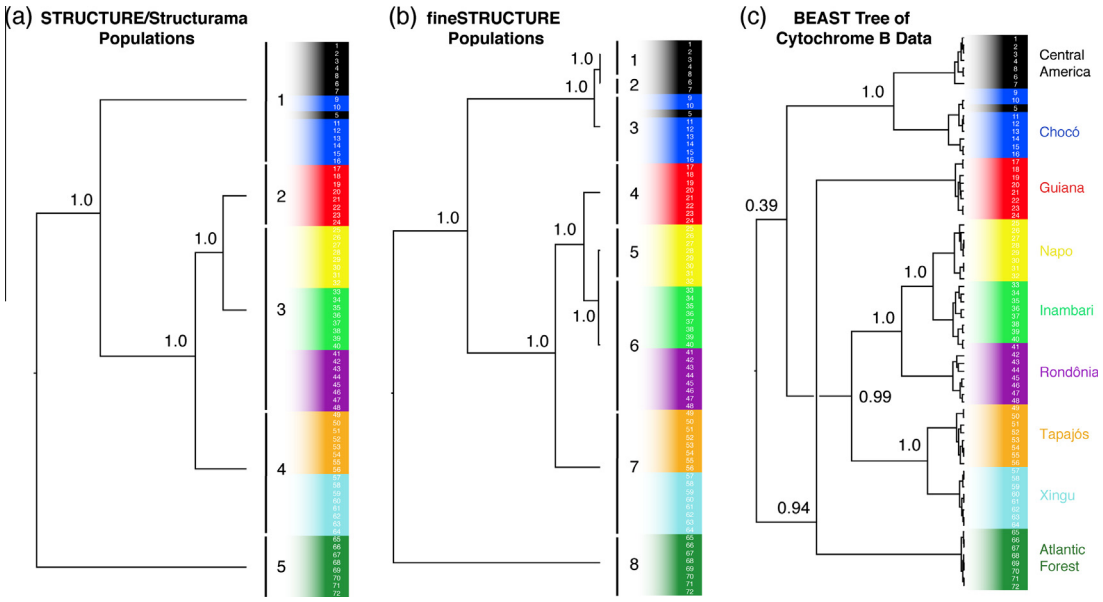

Fig. 4. SNAPP species trees of (a) STRUCTURE/Structurama populations and (b) fineSTRUCTURE populations based on the SNP data and a (c) BEAST gene tree of sequence data from the mitochondrial gene Cytochrome B showing discordance with respect to the species trees. Colors are used to differentiate the areas of endemism from which individuals were sampled, and do not correspond to the population assignments from Fig. 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

a burnin of three million generations. We ran both runs for each set of populations an additional four million generations and used the combined sample of 4000 trees to generate a Maximum Clade Credibility tree and posterior probability values for each node (Fig. 4). Topologies were consistent between the analysis of the STRUCTURE/Structurama populations and the analysis of the fineSTRUCTURE populations. Both estimated an initial divergence between the Atlantic Forest population and all other populations, followed by a divergence across the Andes. Within the Amazon Basin, both analyses estimated an earlier divergence across the Tapajós River followed by a subsequent divergence across the Negro River. Divergences between the two Central American populations, the Central American and Chocó populations, and the Napo and (Inambari + Rondônia) populations from the fineSTRUCTURE analysis were very shallow.

The SNP species tree was similar overall to a prior mitochondrial gene tree based on Cytochrome b data from the same samples used in this study (Smith et al., 2014; Fig. 4). It differed, however, in the placement of the Guianan population. In the SNP species trees, the Guianan population is sister to the (Napo + Inambari + Rondônia) clade with high support (PP = 1.0), and thus is nested within the clade containing the other Amazonian populations. In the mitochondrial gene tree, however, the Guianan population is sister, albeit with a very long intervening branch, to the Atlantic Forest population with high support (PP = 0.94).

4. Discussion

Prior studies of *Xenops minutus* based on mitochondrial sampling from many individuals (Burney, 2009) or genomic sampling from a few individuals (Smith et al., 2014) revealed deep phylogeographic structure associated with major landscape features, such as the Andes mountains and Amazonian rivers. Our GBS data identified the same phylogeographic breaks. Moreover, our results indicate the historical demography of *X. minutus* has been dynamic, with population size changes, migration and admixture between populations, and possibly natural selection.

We recovered positive population growth estimates for nearly all populations in the LAMARC analysis. Growth was greater in the (Tapajós + Xingu) and Atlantic Forest populations in the southeastern portion of the distribution than in most other populations. Signatures of population growth have been observed in some other Neotropical forest species (Aleixo, 2004; Cheviron et al., 2005; Solomon et al., 2008; D'Horta et al., 2011, but see Lessa et al., 2003). The significant migration rates and evidence of admixture confirm that connectivity between currently isolated populations has occurred over the history of *X. minutus*. We recovered significant non-zero estimates for 9 of 22 total migration parameters across two different analyses in LAMARC. Across the Andes Mountains and cerrado belt, we detected significant migration in only one direction – out of rather than into the Amazon Basin. The STRUCTURE analysis also suggested the presence of limited admixture in some populations. In addition, we directly identified an admixed individual: the individual from the Napo region that clustered with the Inambari SNP clade. Prior mitochondrial data from this individual (Burney, 2009) reveals a haplotype that clusters closely with other Napo individuals, rather than individuals from the Inambari region (Fig. 4). This admixed individual therefore has a Napo mitochondrial haplotype, but an Inambari nuclear SNP genotype. There are few previous estimates of migration rate between populations of Neotropical forest organisms isolated by barriers, and these mostly suggest that gene flow is low or absent (Patton et al., 1994; Noonan and Gaucher, 2005; Maldonado-Coelho et al., 2013). Hybridization and introgression between species and divergent forms have been uncovered in a few Neotropical taxa (Brumfield et al., 2001; Lovette, 2004; Dasmahapatra et al., 2010; Naka et al., 2012). We expect that increased genomic representation in datasets will reveal that migration, hybridization, and introgression are an important part of the diversification history of the Neotropics.

Although we detected a small proportion of loci under purifying or balancing selection, the detection and interpretation of loci under purifying or balancing selection (ie. lower divergence than expected) is challenging (Teacher et al., 2013) due to the diversity of processes that might underlie such a pattern. The detection of diversifying selection at a small proportion of loci in the BayeScan analysis of fineSTRUCTURE populations, but not in the analysis of STRUCTURE/Structurama populations, suggested that diversifying

selection has occurred between the most recently diverged populations. We found, however, that none of the outliers putatively under diversifying selection showed large allele frequency differences between populations that were only separated in the fineSTRUCTURE population set. Null F_{st} distributions may be overly narrow when some populations are recently diverged and have highly correlated allele frequencies, resulting in false positive outliers (Excoffier et al., 2009). Correlated allele frequencies between recently diverged populations in the fineSTRUCTURE analysis, rather than diversifying selection, are likely responsible for the positive outliers in that analysis.

Accurately mapping loci to an annotated genome assembly may permit further evaluation of putative outliers (Stapley et al., 2010), but is complicated in our study by the absence of a genome assembly for *X. minutus* or any close relative, as well as the short length (~64 bp) of the GBS loci. Because we lack an independent method of verifying outliers, our results are very preliminary with regards to the importance of selection in this system. In addition to the problems mentioned above, the total number of loci putatively under selection across both BayeScan analyses (76 loci, 2.2% of the total) is smaller than in many other studies (reviewed in Nosil et al., 2009), suggesting a relatively minor role for selection in the history of *X. minutus*.

4.1. Relating species history to landscape history is challenging

Although we recovered a detailed estimate of the history of *X. minutus*, relating this history to the landscape history of the Neotropics and to hypotheses of Neotropical diversification in general is challenging. Similar issues have been encountered in other studies, such that few general patterns have emerged that convincingly relate landscape history to diversification history within species (Antonelli et al., 2010; Brumfield, 2012). The difficulty stems in part from the incomplete knowledge of Neotropical landscape history on spatial and temporal scales relevant for species evolution (Bush, 1994; Bush and Flenley, 2007) and from the shortage of unique testable predictions under different hypotheses of Neotropical diversification (Brumfield and Capparella, 1996; Tuomisto and Ruokolainen, 1997). Another challenge is that species distributions appear to be dynamic on much shorter timescales than those on which landscape evolution occurs, potentially erasing the signal for important events and resulting in pseudo-congruence (Haydon et al., 1994; Sanmartín et al., 2008; Brumfield, 2012). Finally, different species are likely to have responded in different ways to the same history depending on their ecologies, such that few general patterns may exist (Aleixo, 2006; Rull, 2013).

We did find that major Neotropical landscape features, including the Andes, Amazonian rivers, and the cerrado belt isolating Amazonia from the Atlantic Forests, accounted for much of the genetic structure within *X. minutus*. The species tree topology for *X. minutus* contains similar area relationships to those found in other phylogenetic analyses (Weckstein and Fleischer, 2005; Aleixo and Rossetti, 2007). Divergence across barriers may be evidence of vicariance associated with barrier origin, dispersal across an existing barrier followed by differentiation (Mayr, 1963), or the role of the barrier in structuring variation that arose elsewhere due to unknown historical processes (Brumfield, 2012). The potential for pseudo-congruence between barriers and distributions combined with recent evidence that dispersal is more important than vicariance in the histories of some Neotropical groups (Fine et al., 2014; Smith et al., 2014) suggests that the null hypothesis of shared area relationships used in vicariance biogeography is inappropriate. In addition, existing hypotheses of Neotropical diversification include few explicit predictions about relationships between areas of endemism (Bates et al., 1998; Leite and Rogers, 2013), and replicate simulations illustrate a remarkable amount of phylogenetic discordance even under identical vicariance scenarios (Endler, 1983). Because of these issues, the divergence patterns in *X. minutus* tell us relatively little about the historical landscape or climatic events responsible for the modern genetic structuring in this species.

Dating the divergences between populations could allow determination of whether they were coincident with barrier formation, providing circumstantial support for particular vicariance hypotheses. Although dating the SNP divergences is problematic because we lack substitution rate estimates for GBS loci (see below), a previous dating analysis using mitochondrial DNA suggested that *X. minutus* populations diverged within the time span that the Andes Mountains and Amazonian Rivers are thought to have reached their modern conformations (Smith et al., 2014). *Xenops minutus* populations across the Andes diverged 4.58 (s.d. = 3.04–5.98) Mya and populations within the Amazon basin (aside from the Guianan population with a potential spurious placement in the mitochondrial tree, see below) began diverging 2.91 (s.d. = 1.89–4.00) Mya. Similar Pliocene divergence dates have been estimated for many other Neotropical taxa including fish (e.g., Lovejoy et al., 2010; Lundberg et al., 2010), plants (e.g., Pennington and Dick, 2010), amphibians (e.g., Santos et al., 2009), birds (e.g., Weir, 2006), and mammals (e.g., Costa, 2003). These dates coincide roughly with the final uplift of the Andes and the coincident formation of the contemporary fluvial system of the Amazon in the last 10 My (Mora et al., 2010). However, the concordance of divergence dates with the vast time span associated with the origin of these dispersal barriers provides only rough, circumstantial support. The crucial details of how dispersal barriers interdigitate with other factors, such as population size flux, changes in forest distribution (Bush and Flenley, 2007), changes in forest composition and niche availability (Jaramillo et al., 2010), changes in avian community composition (Ricardo Negri et al., 2010), and local extinctions and re-colonizations are not considered. This uncertainty suggests a nuanced understanding of how the Andes and Amazonian rivers influence speciation within lineages is not achievable using area relationships and divergence dates, and that our focus should be on other aspects of the speciation process.

The evidence we found for population expansions in *X. minutus* provides support for a prediction of the forest refugia hypothesis that humid lowland forests were once more restricted due to the expansion of savanna (Haffer, 1969). Some palynological analyses also support the idea that lowland Neotropical humid forest was once more restricted (Absy et al., 1991; Burnham and Graham, 1999). Recent isotopic evidence suggests that precipitation was lower in the eastern Amazon, but not the western Amazon, during the last glaciation (Cheng et al., 2013), consistent with our observation of greater population growth in that area. Unfortunately, knowledge of the recent history of forest cover in the Amazon is limited and contentious (Behling et al., 2010). The marine incursion hypotheses might also predict population growth following the recession of water levels, although growth is expected to be greatest in the western Amazon Basin (Aleixo, 2004), contrary to the pattern we observed. Other events such as disease (e.g., Daszak et al., 2003), changes in abiotic climate conditions (e.g., Sillett et al., 2000), or changes in competitive interactions (e.g., Koenig, 2003), predation (e.g., Wittmer et al., 2005), or resource availability (e.g., O'Donoghue et al., 1997) might also have driven population size changes. Although the population expansion we observed in *X. minutus* may be attributable to recent increases in forest habitat in the lowland Neotropics, we cannot exclude other equally likely causes.

Migration and admixture between populations supports the idea that populations have experienced periodic connections in the past. Habitat connectivity, however, might have occurred under any of various hypotheses of Neotropical diversification

and does not aid in discriminating among them. Future improvements in our understanding of past habitat distributions combined with improved methods of inferring and dating admixture events may allow us to correlate episodes of migration and gene flow with individual events of habitat connectivity (Gillespie and Roderick, 2014).

Based on the challenges associated with connecting the species history of *X. minutus* to landscape history, we suggest the common practice of relating single species histories to landscape events is unproductive. As an alternative, we suggest an initial focus on evaluating the importance of different historical processes (including divergence, but also population size changes, migration, and selection) using genomic datasets within individual species or species complexes. With many such datasets in hand, comparative methods may permit determination of the importance of each process along taxonomic, temporal, and spatial axes. This information, perhaps combined with more information on the combined effects of processes shaping landscape history, may ultimately permit evaluation of each hypothesis of Neotropical diversification across assemblages, timescales, and regions.

4.2. Limitations and prospects for GBS data in phylogeography

Genotyping-by-sequencing data allowed us to conduct a variety of population genetic, phylogeographic, and phylogenetic analyses. We did, however, encounter some potential shortcomings of GBS data for addressing phylogeographic questions in our non-model system. The large amount of missing data observed in our dataset prior to filtering suggests the need for further optimization of coverage relative to the number of targeted loci, but better coverage could be achieved by using different enzymes or multiple enzymes (Peterson et al., 2012). The locations to which we were able to map loci may be inaccurate, both because of the potential for spurious alignment due to the short length of the GBS reads, and because of the evolutionary distance between *X. minutus* and *T. guttata*. This issue may be reduced in the future if longer read lengths can be obtained, or if a genome from a species more closely related to the study species becomes available. Perhaps the greatest limitation of GBS is that no standard evolutionary rate exists for the targeted loci for the purpose of dating divergences or converting demographic parameters. As a result, we were largely limited to making relative comparisons of raw parameter estimates in this study. Furthermore, the processing pipeline for GBS and other RAD-Seq data complicates the future development of standard rates that could be used across groups of organisms. Because identity thresholds are applied to each dataset for assembly, mutational spectra may be biased to different degrees across datasets (Huang and Knowles 2014, Ilut et al., 2014). More informed assembly protocols or methods for correcting rates based on the level of truncation in a dataset may alleviate these issues in the future.

Despite some limitations, genomic data from GBS have provided a more complete picture of the history of *X. minutus* than would be possible with a few markers. The history inferred from genomic SNPs is likely to better reflect the true history of *X. minutus* populations than a single-locus dataset (Edwards and Beerli, 2000). In addition, genomic data have allowed us to investigate processes that are difficult to evaluate with a single marker, such as migration and selection. More efficient laboratory methods and new analytical tools will surely increase the utility of genomic datasets as they come into more widespread use.

Since divergence histories based on mitochondrial data have been the primary source of information for studies of Neotropical phylogeography (Haffer, 1997; Antonelli et al., 2010; Leite and Rogers, 2013), the discordance between the mitochondrial gene tree and genome-wide SNP species trees in this study is alarming. This discrepancy might occur if deep coalescence of the mitochondrial haplotypes from the Guianan and Atlantic Forest populations resulted in a mitochondrial genealogy that does not represent the species history. Alternatively, recent nuclear gene flow between Atlantic Forest and Guianan populations might produce a similar result, but we consider this less likely due to the geographic distance between these populations and because gene flow would have to have influenced a substantial portion of the genome to result in the relationship recovered from the GBS loci. Discordance between mitochondrial and nuclear SNP datasets is not surprising, given the number of prior studies reporting similar mito-nuclear discordance (Funk and Omland, 2003; Chan and Levin, 2005). The observed discordance deepens concerns about the utility of mitochondrial DNA as a record of population history and reaffirms the importance of shifting to genome-wide datasets for phylogeographic research.

4.3. Systematics of Xenops minutus

Our results support the presence of at least three deeply divergent clades experiencing little to no gene flow within *Xenops minutus*. The trans-Andean clade of Central and northwestern South America includes the subspecies *mexicanus* (Sclater, 1856), *ridgwayi* (Hartert and Goodson, 1917), and *littoralis* (Sclater, 1861). The trans-Andean subspecies *olivaceus* Aveledo and Pons, 1952 and *neglectus* (Todd, 1913) were not sampled in our study or previous studies but may also belong to this group. The Amazonian/Guianan clade includes the subspecies *genibarbis* (Illiger, 1811); *obsoletus* Zimmer, 1924; and *ruficaudus* (Vieillot, 1816). The northwestern Amazonian subspecies *remoratus* Zimmer, 1935, not sampled in our study, may also be in this group, although mitochondrial data suggest that this population is highly divergent (Burney, 2009). Populations from the northern Atlantic Forest are most similar to the Amazonian/Guianan clade based on mitochondrial data (Burney, 2009). These populations were described as a unique subspecies (*alagoanus* Pinto, 1954), but this taxon has been omitted or overlooked by most subsequent authors (Dickinson, 2003; Remsen, 2003) and was not sampled in our study. Finally, the nominate subspecies (Sparrman, 1788) of the Atlantic Forest represents the third deeply divergent clade, and is highly distinct genetically despite some amount of gene flow from Amazonian populations to the northwest.

All three clades are diagnosable vocally. The trans-Andean clade has a much more rapid, nearly trilled, song than other clades. The Amazonian/Guianan clade has a slower song with rising, "hill-shaped" (Isler et al., 1998) notes. The nominate subspecies also has a slow song, but the notes are upslurred giving them a distinct "twanging" quality. Interestingly, populations from the northern Atlantic Forest (*alagoanus*) have a song more similar to Amazonian birds, and thus may be part of the Amazonian/Guianan clade. Plumage is variable geographically, but much of the variation appears to be clinal (Remsen, 2003). Within the trans-Andean clade, plumage is highly variable with a rough trend from red and plain in the north to olive and streaked in the south. Plumage is also variable in the Amazonian/Guianan clade, although most populations are intermediate in color and show moderate to heavy streaking. Only the nominate subspecies is highly distinct in plumage (Remsen, 2003), with a white throat, reddish coloration, and plain underparts.

We suggest that the three deeply divergent clades described above represent phylogenetic species due to diagnosable vocal, genetic, and (in the third clade) plumage differences. They may merit biological species status based on the fact that they exhibit little to no detectable gene flow, although further research is required to determine whether they might currently interbreed. The northwestern Amazonian clade found by Burney (2009) may represent a fourth phylogenetic species, although it would be

desirable to confirm this result with additional independent genetic markers, vocal data, and field work to determine if populations come into contact in the northwestern Amazon Basin. The populations from Guiana and from the Tapajós/Xingu areas of endemism may also merit species status because they were recovered as distinct populations and show moderate divergence in the species tree. These two clades are less divergent, however, than the three mentioned above, and we were also unable to find obvious morphological or vocal characters distinguishing them. Further research involving improved geographic sampling and formal morphological and vocal analyses may clarify the status of these and other, un-sampled populations.

Data accessibility

SNPs and individual genotypes: (NCBI dbSNP ss# 1536954775–1536958153).

Genotype data: Dryad (http://dx.doi.org/10.5061/dryad.3j0b1).

Custom scripts: Github (https://github.com/mgharvey).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2014.10.023.

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