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Late Pleistocene landscape changes and habitat specialization as promoters of population genomic divergence in Amazonian floodplain birds

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Abstract

Although vicariant processes are expected to leave similar genomic signatures among codistributed taxa, ecological traits such as habitat and stratum can influence genetic divergence within species. Here, we combined landscape history and habitat specialization to understand the historical and ecological factors responsible for current levels of genetic divergence in three species of birds specialized in seasonally flooded habitats in muddy rivers and which are widespread in the Amazon basin but have isolated populations in the Rio Branco. Populations of the white-bellied spinetail (*Mazaria propinqua*), lesser wagtail-tyrant (*Stigmatura napensis*) and bicolored conebill (*Conirostrum bicolor*) are currently isolated in the Rio Branco by the black-waters of the lower Rio Negro, offering a unique opportunity to test the effect of river colour as a barrier to gene flow. We used ultraconserved elements (UCEs) to test alternative hypotheses of population history in a comparative phylogeographical approach by modelling genetic structure, demographic history and testing for shared divergence time among codistributed taxa. Our analyses revealed that (i) all three populations from the Rio Branco floodplains are genetically distinct from other populations along the Amazon River floodplains; (ii) these divergences are the result of at least two distinct events, consistent with species habitat specialization; and (iii) the most likely model of population evolution includes lower population connectivity during the Late Pleistocene transition (~250,000 years ago), with gene flow being completely

disrupted after the Last Glacial Maximum (~21,000 years ago). Our findings highlight how landscape evolution modulates population connectivity in habitat specialist species and how organisms can have different responses to the same historical processes of environmental change, depending on their habitat affinity.

KEYWORDS
biogeographical barrier, comparative phylogeography, ecological traits, geogenomics, population structure, Rio Branco

1 | INTRODUCTION

Determining how interactions between habitat specialization and historical landscape processes shape genetic variation within a community is essential for understanding global patterns of diversification (Marske et al., 2020; Myers et al., 2019; Papadopoulou & Knowles, 2016). Studies on Amazonian diversification have traditionally focused on the role of vicariance and landscape geographical features (Haffer, 1969; Naka & Brumfield, 2018; Ribas et al., 2012). In the last decade, however, population genetic studies have shown the potential effect of ecological and habitat specialization on species differentiation (Barbosa et al., 2022; Burney & Brumfield, 2009; Harvey et al., 2017; Smith et al., 2014). For example, upland forest interior birds show greater genetic diversity and population structure than forest edge and floodplain species (Harvey et al., 2017). Even within single habitats, such as upland forests, understorey species show more genetic structure than canopy birds (Burney & Brumfield, 2009).

Patterns of genetic differentiation are less clear in the Amazonian floodplains, where species are specialized on a variety of river-created habitats, resulting in a highly heterogeneous and rich avifauna (Remsen & Parker, 1983; Rosenberg, 1990). Recent studies have shown that floodplain birds specialized in the understorey of river-edge forests tend to show greater population differentiation (Choueri et al., 2017; Luna et al., 2022; Thom et al., 2020) than species associated with open vegetation along the floodplains (Barbosa et al., 2022; Cadena et al., 2011). In this sense, different patterns of spatial genetic structure between codistributed species could be related to distinct habitat specialization (Barbosa et al., 2022; Choueri et al., 2017). However, the underlying effects of habitat specialization on genetic differentiation within Amazonian floodplain species in response to historical landscape change is still poorly studied (but see Barbosa et al., 2022). Understanding these associations offers insights into complex evolutionary and ecological processes, which include adaptation, speciation and the formation of biological communities (Papadopoulou & Knowles, 2016; Provost et al., 2021).

Recognizing the landscape features that shape biodiversity is a major challenge in studies that explore historical changes in the Amazonian floodplains (Prado et al., 2022; Pupim et al., 2019; Sawakuchi et al., 2022). One important aspect of Amazonian rivers is their water colour, often considered a proxy for the amount of suspended sediments, which build the Amazonian floodplains

(Junk et al., 2011). White-, clear- and black-water rivers not only differ in the concentration of nutrients, but their suspended sediment loads also influence riverine habitats within river valleys (Junk et al., 2011). White-water rivers, for example, have a high concentration of suspended sediments and maintain highly productive tall and extensive *várzea* forests, whereas black- and clear-water (low turbidity and nutrient-depleted) rivers produce less productive and narrower *igapó* forests (Junk et al., 2011, 2015). These distinct types of vegetation influence bird species abundance and composition (Laranjeiras et al., 2019). For instance, several species commonly found along white-water *várzeas* in central and western Amazonia are absent from black-water-associated *igapós* (Laranjeiras et al., 2019).

One particularly interesting case is that of the Branco and Negro rivers, located in the Guianan Shield, northern Amazonia (Figure 1). The Rio Branco is the main tributary of the Rio Negro, which is considered the world's largest black-water river (Goulding et al., 2003). As their Portuguese names imply, these two rivers have contrasting colours and very different biotas associated with their floodplains. The Rio Branco floodplains sustain vegetation floristically similar to other white-water *várzeas* found elsewhere in the Amazon basin, but quite different from black-water *igapós* present along the Negro (Junk et al., 2015). Therefore, the floodplains of the Branco are currently isolated from the Amazon River *várzeas* by the black waters of the Negro (Figure 1). This pattern of habitat fragmentation associated with habitat specialization results in the presence of white-water-associated fish and bird species populations isolated on the Rio Branco (Hubert & Renno, 2006; Naka et al., 2007, 2020), providing a unique example of potential vicariance caused by the influence of river water chemistry and sediment load on habitat characteristics (Laranjeiras et al., 2020; Naka et al., 2007, 2020; Thom et al., 2020). Also, disjunct distributions offer an opportunity to investigate the processes through which past environmental changes generated current biota assembly and diversity in Amazonian floodplains (Ribas & Aleixo, 2019; Thom et al., 2020).

To investigate the effect of floodplain habitat type on population connectivity along the Amazonian floodplains, we selected three passerine species from three different avian families which are specialized in distinct habitats within the *várzea* forest and have allopatric populations in the Branco and Amazon rivers, which are currently isolated by the black-water *igapó* forests of the lower Rio Negro (Naka et al., 2007; Figure 1). These species include two *Tessaria* sandbar scrub specialists, the white-bellied
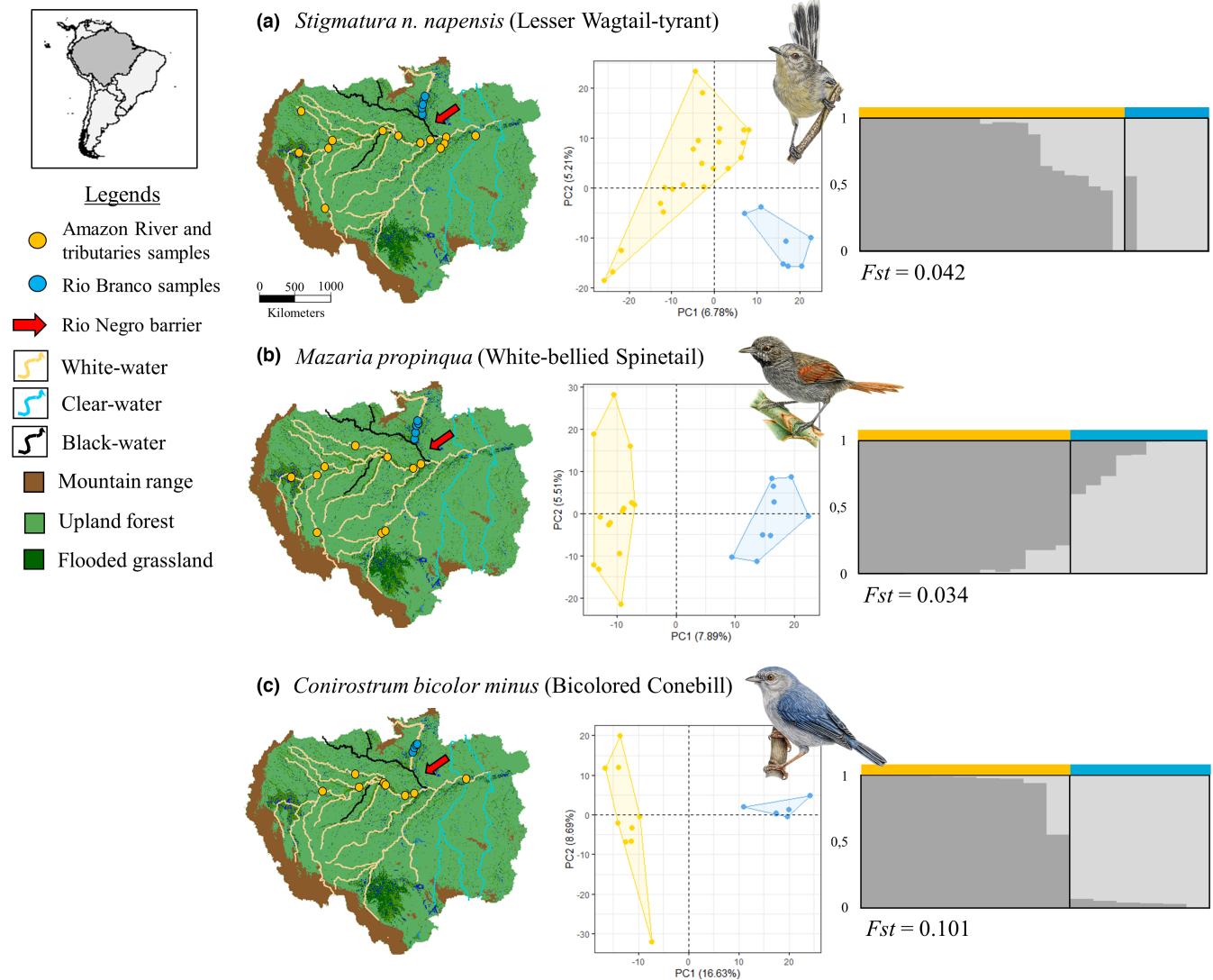
FIGURE 1 Sampling and genetic structure patterns between bird populations of the Rio Branco (blue) and the Amazon River and tributaries (yellow). The black-water *igapó* forests of the Rio Negro (red arrow) represent a putative ecological barrier for the three bird species associated with the *várzea* forests of the white-water rivers. Each panel shows the first and second axes of the PCA and boxplot of the *conStruct* analysis for (a) lesser wagtail-tyrant (*Stigmatura n. napensis*), (b) white-bellied spinetail (*Mazaria propinqua*) and (c) bicolored conebill (*Conirostrum bicolor minus*). Values of the genetic fixation indices (F_{ST}) are below the *conStruct* plot. Illustrations of the birds by Andreza Silva [Colour figure can be viewed at wileyonlinelibrary.com]

spinetail *Mazaria propinqua* (Furnariidae) and the lesser wagtail-tyrant *Stigmatura napensis* (Tyrannidae), and a *Cecropia* forest canopy specialist, the bicolor conebill *Conirostrum bicolor* (Thraupidae) (Rosenberg, 1990). There is a high degree of habitat specialization in the flooded forests, which suffer different levels of perturbation due to the seasonal flood pulse (Rosenberg, 1990). For instance, *Tessaria* scrub can be completely flooded during high-water periods, forcing specialists to search for other available areas, whereas *Cecropia*-dominated riverine forests may retain their canopies above the water allowing species to move to upper strata (Rosenberg, 1990; Rowedder et al., 2021). Therefore, different habitat specializations can potentially affect the patterns of gene flow (and thus levels of divergence) among populations across the landscape.

The lack of records of any of these three species along the lower Rio Negro suggests that they are indeed isolated on the Rio Branco (Cintra et al., 2007; Laranjeiras et al., 2019). Based on currently available evidence about Amazonian drainage evolution, at least four alternative scenarios could have generated these disjunct distributions, which would lead to distinct patterns of current genetic diversity: (i) a long history (~1 million years) of disconnection, consistent with the disjunction starting due to the establishment of the main channel of the Rio Negro in the Middle Pleistocene (Gautheron et al., 2022); (ii) a temporary connection of the Amazonas and the Branco rivers during the Late Pleistocene (250,000 years ago [ka]), when floodplains reportedly expanded throughout Amazonian lowlands (Pupim et al., 2019; Ruokolainen et al., 2019); (iii) a similar, yet later, connection initiated 250 ka,

interrupted by channel incision leading to abandonment of flood-plains (Pupim et al., 2019) and formation of a ria lake at the lower Rio Negro in the last 20,000 years (Latrubesse & Franzinelli, 2005); and (iv) a long historical connection of floodplains (>1 million years) disrupted by a relatively recent isolation during the Late Pleistocene due to the incision of the lower Rio Negro in the last 50,000 years (Prado et al., 2022; Pupim et al., 2019). Although the connectivity of floodplains within the Amazon River valleys remains poorly understood over time, especially for periods beyond the Late Pleistocene, the hypotheses above allow us to assess how population connectivity is affected by landscape changes operating over periods of a few million to thousands of years.

In this study, we use a comparative phylogeographical and population genetics approach to test those hypotheses. Our main questions include: (i) Is there genetic differentiation between the three allopatric populations selected in the Branco and Amazon rivers? (ii) Do differences in habitat specialization have a deterministic effect on the pattern of genetic differentiation and gene flow found for each species? (iii) To what extent can these divergences be related to the same historical process? We expect that bird populations from the Branco and Amazon rivers will show some degree of genetic differentiation (Naka et al., 2007, 2020). Furthermore, considering the underlying effect of habitat specialization on the level of genomic divergence, we expect that species associated with *Tessaria*-scrub (e.g., *S. napensis* and *M. propinqua*), which presumably disperse during the high-water season, will show less genetic differentiation than species associated with *Cecropia*-dominated riverine forests (e.g., *C. bicolor*).

2 | METHODS

2.1 | Sampling

DNA was extracted from muscle tissue of multiple individuals that span most of the species' distributions within the Amazonian floodplains. The number of samples per population for each taxon varied from nine to 22 for the Amazon River and tributaries (hereafter Amazon River population), and six to nine for the Rio Branco (Figure 1; Table S1). We obtained genomic data from 23 individuals of *Mazaria propinqua* (data originally published in Barbosa et al., 2022; Bioproject ID: PRJNA736107), 29 *Stigmatura n. napensis* and 15 *Conirostrum bicolor minus*. To better understand the evolutionary history of these populations, we also included samples from non-Amazonian populations, including four samples of *C. b. bicolor* from the Brazilian coast and two samples of *S. napensis bahiae*, from the Caatinga dry forest in NE Brazil. Samples were obtained from several Brazilian and US institutions, including the National Institute for Amazonian Research (INPA), Museu Paraense Emílio Goeldi (MPEG), the Federal Universities of Pernambuco (UFPE) and Paraiba (UFPB), American Museum of Natural History (AMNH), and the Museum of Natural Sciences—Louisiana State University (LSUMNS) (see Table S1).

2.2 | Habitat specialization

We hypothesized that the distinct habitat specializations within the Amazonian floodplain are key factors in shaping the pattern of gene flow and genetic structure within species. The three bird species selected for our study occur mainly in river islands and are distinguished primarily by their habitat specialization (Rosenberg, 1990). Two of the taxa, *M. propinqua* and *S. n. napensis*, occur mostly at *Tessaria*-scrub-dominated habitats, whereas *C. bicolor minus* is restricted to *Cecropia*-dominated riverine forests (see Rosenberg, 1990). The type of habitat association is likely to affect patterns of occupancy and movement within the floodplains, as they are differently affected by the flooding regimes of Amazonian rivers (Wittmann et al., 2010). For instance, *Tessaria* scrub occurs in low-lying sandy beaches and may be completely flooded during the high-water period, when birds need to periodically move away from their territories in search of available habitat (Remsen & Parker, 1983; Rosenberg, 1990). On the other hand, *Cecropia*-dominated riverine forests are taller and do not flood completely, allowing species to use upper strata when flooded (Rowedder et al., 2021). Other ecological traits such as diet and mating system are not known to differ considerably among these three taxa (Hilty & Kirwan, 2020; Schulenberg & Rosenberg, 2020; Tobias et al., 2022) and therefore were not considered in our models of genomic differentiation.

2.3 | Genomic data processing

We obtained genomic data with a sequence capture approach using a probe set targeting 2321 ultraconserved elements (UCEs) and 96 exons commonly used in avian population genomic studies (Amaral et al., 2018; Luna et al., 2022; Thom et al., 2020). DNA extraction from tissue samples was performed with the Qiagen DNeasy kit, following the manufacturer's protocol. Genomic library preparation and sequencing were outsourced to RAPiD Genomics.

For each species, raw paired-end reads of 150 bp were obtained through automatic sequencing in the Illumina Hiseq 2500. Sequence quality control and reads count were evaluated in FASTQC version 0.11.5 (Andrews, 2014). Sequences were then demultiplexed and the reads from low-quality adapters and bases were removed (Phred quality <30, read length >150 bp) using ILLUMIPROCESSOR (Faircloth et al., 2012). The clean reads in contigs were assembled in TRINITY version 2.4 (Grabherr et al., 2011), using the default value of the nucleotide sequence size (kmer = 25). In total, 2417 contigs were mapped and identified using phyluce_assembly_match_contigs_to_probes implemented in PHYLUCE version 1.4 (Faircloth et al., 2012). Incomplete loci matrices were generated for each species (including missing loci for some samples), establishing a completeness value of 0.8. To avoid the inclusion of markers with different ploidy, we used BLAST version 2.7.7 (Camacho et al., 2008) to identify and remove loci linked to the

sexual chromosome Z, using the zebra finch genome as reference (Ensemble teGut3.2.4). Finally, all sequences were aligned in MAFFT (Katoh & Standley, 2013).

We constructed a matrix with all individuals for each species without filtering for missing data or trimming and selected the longest sequences as references for the single nucleotide polymorphism (SNPs) calling approach. Raw reads were aligned to the reference using BWA version 0.7.1 (Li & Durbin, 2009). The aligned reads with the references were mapped using a hard-masking low-quality criterion (<30), maintaining sites with the minimum read depth of >6, using the Unified Genotyper and Variant Annotator of GATK version 3.8.1 (McKenna et al., 2010). We removed the sequence regions not represented by the references, ordered the reads into groups, and excluded duplicate reads using Picard (Broad Institute; http://broad institute.github.iopicard/). The matrix assembly was performed by randomly calling one SNP per locus from a biallelic SNP database without missing data. The read-backed gametic phasing with GATK was performed on the variant call format (vcf) file containing the total of the called SNP variants assuming a threshold of 20. The set of variants per individual was then matched to the reference sequences using the seqcap_pop package (Harvey et al., 2016) to obtain the complete sequence for each locus, using a minor allele count (MAC) of 1.

2.4 | Phylogenetic analyses

To determine the relationships of sampled populations, we estimated a maximum-likelihood topology for all sampled individuals in each of the three species in IQ-TREE version 2.1.3 (Minh et al., 2020; Nguyen et al., 2015). This analysis was performed using a data set of concatenated sequences of UCEs and exons. The best evolutionary models of the sequences were obtained using MODELFINDER (Kalyaanamoorthy et al., 2017). Support value of the topology nodes were obtained through 1000 ultrafast bootstrap replicates (Hoang et al., 2018) and 1000 iterations of the approximate likelihood-ratio test (Guindon et al., 2010).

2.5 | Population genetic structure

We assessed the genetic structure within each of the three Amazonian floodplain bird taxa using multivariate and Bayesian approaches based on SNP matrices. First, we performed a visual inspection of the relationships between samples from the Rio Branco with samples from the remaining Amazonian floodplains (i.e., Amazon River and tributaries), using a principal component analysis (PCA), implemented in the R package *adegenet* version 2.1.5 (Jombart, 2008). Second, we tested whether population variation should be represented as discrete clusters or continuous clines using the R package *conStruct* version 1.0.3 (Bradburd et al., 2018). This Bayesian method models isolation by distance (IBD) within each genetic cluster, estimating the proportion of ancestry for each

sample from a set of two-dimensional population layers (K), considering that within each K the rate of genetic relatedness decreases as a function of distance (Bradburd et al., 2018). For each taxon, we estimate the presence of $K = 1–6$ (relative to the number of sub-basins sampled), comparing the spatial (accounting for the geographical distance between samples) and nonspatial models. For each K value, we used 90% of the data to train both models and performed 10 repetitions with 20,000 iterations per repetition. The spatial and nonspatial model comparison was performed by cross-validation, considering as the best model the result with the highest value of predictive accuracy (i.e., close to zero). However, additional K values may represent spurious covariations introduced by noise from the analyses (Bradburd et al., 2018). To avoid this issue, we compare the best model results selected in cross-validation with the total covariance contribution for each K within the same model, using as an arbitrary cut-off criterion the K with covariate contribution less than 0.02.

2.6 | Testing the barrier effect of the Rio Negro on genomic differentiation

For each species, we examined the effect of the Rio Negro barrier on genomic differentiation, while accounting for geographical distance by using the Multiple Matrix Regression with Randomization analysis (MMRR; Wang, 2013) in the R package *PopGenReport* version 3.0.7 (Adamack & Gruber, 2014). For this test, we calculated Rogers' genetic distance based on SNP matrices (DR; Rogers, 1972) between individuals using the function "dist.genpop(method = 4)" in the R package *adegenet* version 2.1.5 (Jombart, 2008), whereas the linear river distance (in kilometres) between samples was calculated using the function "riverdistancemat()" in the R package *riverdist* version 0.15.3 (Tyers, 2020). We performed a partial correlation between the genetic distance matrix and the barrier matrix while conditioning a third geographical distance matrix. The Rio Negro barrier matrix was represented by assigning a value of "0" between pairs of individuals on the same side of the barrier (e.g., within the Rio Branco and within the Amazon River) and "1" assigned to comparisons on opposite sides (e.g., Rio Branco vs. Amazon River). We also tested the correlation between genetic distance and linear river distance, without considering the barrier effect of the Rio Negro. Statistical significance (p) was tested by 99,999 random permutations of the genetic distance matrix, and correlation values (r^2) were interpreted as the level of the barrier effect on genetic differentiation beyond geographical distance.

2.7 | Modelling divergence scenarios

We tested alternative models of population divergence for each species using a machine-learning approach implemented in the R package *PipeMaster* version 0.0.9 (Gehara et al., 2017). For each divergence model, we used the MSABC coalescent simulator

(Hudson, 2002; Pavlidis et al., 2010) to simulate the genetic data and summary statistics. The models tested correspond to the four scenarios described above, which could have generated current disjunct distributions of bird populations in the Branco and Amazon rivers floodplains (Figure 2), and include: (i) deep divergence with gene flow starting at 1 million years ago (Ma); (ii) divergence with gene flow starting 250 ka; (iii) divergence with gene flow starting 250 ka, with subsequent gene flow cessation (i.e., complete isolation) at 20 ka; and (iv) divergence with gene flow starting 50 ka. We defined broad uniform prior distributions for current effective population size (min: 10,000; max: 500,000 individuals) and migration (min: 0.1; max: 2.0 migrants per generation). For the divergence times between populations, we set absolute time values in generations, considering one generation to be equal to 1 year for all three species.

For each model we conducted 50,000 simulations, calculating the following summary statistics from the UCEs and exon sequences: number of segregating sites, the proportion of shared alleles among populations, the proportion of private alleles in each population, the proportion of fixed alleles in each population, nucleotide diversity (π), Watterson's Θ (Watterson, 1975), Tajima's D (Tajima, 1989) and pairwise F_{ST} (Wright, 1950). We verified the adequacy of the simulated data with the observed data using PCAs for each species. The accuracy of the selected models was evaluated using a nonlinear neural network algorithm in the R package *caret* version 6.0–84 (Kuhn et al., 2017). To calculate the posterior distributions of the parameters for the best model, we used the "abc" function in the R package *ABC* version 2.1 (Csilléry et al., 2012), and the "cv4abc" function to assess the accuracy of the estimated parameters by performing 100 cross-validations and Pearson's correlation between the simulated and pseudo-observed data.

2.8 | Testing simultaneous population divergence

We used ECOEVOLITY version 0.3.2 (Oaks, 2019) to test if the genetic divergences between the Rio Branco and the Amazon River populations were temporally clustered or independent. This method uses a full-likelihood Bayesian approach to model divergence times between population pairs by calculating genealogy relationships and sampling posterior probability from models using a Markov chain Monte Carlo (MCMC) approach. These approaches assume (i) no migration after population divergence and (ii) the mutation rate is the same across populations and constant over time. While these assumptions are different from expectations for most divergence models simulated in *PipeMaster* (Figure 2), this inference is useful for modelling the last time that population pairs experienced significant rates of gene flow (Oaks et al., 2019). To perform this analysis, we used nucleotide sequences (nexus format) recoded in biallelic states. Then, we selected the concentration parameter of the Dirichlet process to correspond to the average of the putative divergence events between population pairs ($\alpha = 1.5$). The estimated divergence times between population pairs were based on the average mutation rate across species (mean μ rate $= 4.5 \times 10^{-10}$ substitutions per site per generation; see Table S2) estimated from the best demographic model (Model 3; Table 2) simulated for the three species in *PipeMaster*. We verified the sensitivity of the results by testing alternative concentration parameters of $\alpha = 2$ (sum of the divergence events divided by two). Five runs with 50,000 iterations chains per run were performed with a 10% burn-in and sampling every 50 iterations. The MCMC convergence parameters and the effective sample size (ESS) were accessed through pycosumchains, implemented in ECOEVOLITY (Oaks, 2019) and TRACER version 1.6 (Rambaut & Drummond, 2015).

FIGURE 2 Alternative divergence scenarios (Models) between floodplain bird populations of Rio Branco and Amazon River and tributaries. Model 1: deep divergence time since 1 Ma, with reduced gene flow between populations; Model 2: divergence with reduced gene flow since 250 ka; Model 3: divergence with reduced gene flow starting 250 ka, with subsequent lack of migration 20 ka; Model 4: divergence with reduced gene flow since 50 ka. Red arrows symbolize gene flow [Colour figure can be viewed at wileyonlinelibrary.com]
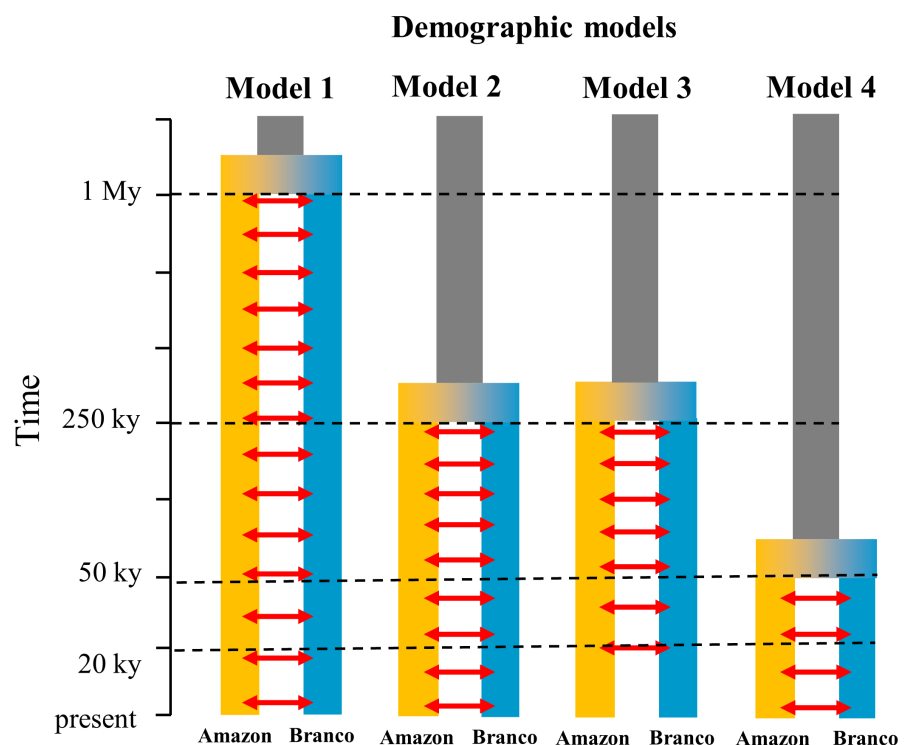
3 | RESULTS

3.1 | Genomic data summary

The data processing of UCEs and exon sequences, excluding the conserved probes, yielded a range variation from 2082 to 1761 loci across the three species (Table S3). Our SNP calling approaches yielded an average of 6889 SNPs per species with a mean read depth of 22.2 (Table S3). By selecting a single SNP per locus with no missing data, we obtained matrices ranging from 1071 to 1384 SNPs across species (Table S3).

3.2 | Phylogenetic relationships

Two of the species involved in this study (*Stigmatura napensis* and *Conirostrum bicolor*) have non-Amazonian populations. Our maximum-likelihood phylogenetic inferences recovered the monophyly of the Amazonian floodplain taxa, relative to the non-Amazonian ones in both cases (Figure S1). Phylogenetic relationships within Amazonian lineages revealed reciprocal monophyly for the Rio Branco populations, albeit with variable support. The Rio Branco phylogroup was well supported in *Co. bicolor* and *Mazaria propinqua*, with 100% and 94% bootstrap support, respectively (Figure S1). As for *S. n. napensis*, this node had low support, probably due to the recent nature of gene flow among these populations (Figure S1).

3.3 | Population genetic differentiation and structure

We found evidence of genetic differentiation between individuals from the Rio Branco and the other populations from the Amazon River and adjacent tributaries for all three species (Figure 1). The amount of variation on PC1 and PC2 were similar and lower in *S. n. napensis* (PC1: 6.73%; PC2: 5.21%) and *M. propinqua* (PC1: 5.51%; PC2: 5.25%), relative to *C. bicolor minus* (PC1: 16.63%; PC2: 8.69%), which could reflect the degree of divergence among population pairs between species. The third axis, PC3, explained 4.94% for *S. n. napensis*, 5.25% for *M. propinqua* and 7.87% for *C. bicolor minus*.

The cross-validation analyses using *conStruct* indicates that the genetic structure within each species can be better described as K = 2 (Figure S1). Genetic clusters depict the Rio Branco and the Amazon River with different admixture proportions between population pairs of each species (Figure 1). Models that include spatial information had a better performance in explaining the genetic structure in *S. n. napensis* and *C. bicolor minus*, indicating the presence of IBD within the genetic clusters. In contrast, for *M. propinqua*, the nonspatial model had a better performance, indicating a weak IBD effect (Figure S2). The contribution values of the K layers to the total covariance of the tested models can be found in Figure S3.
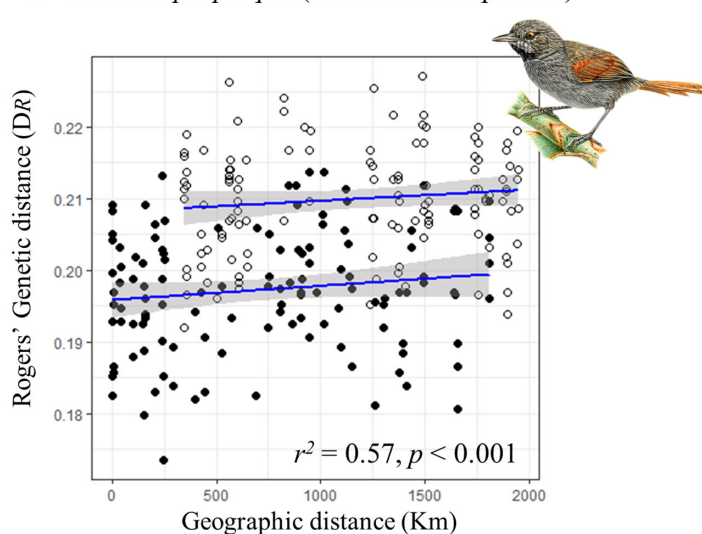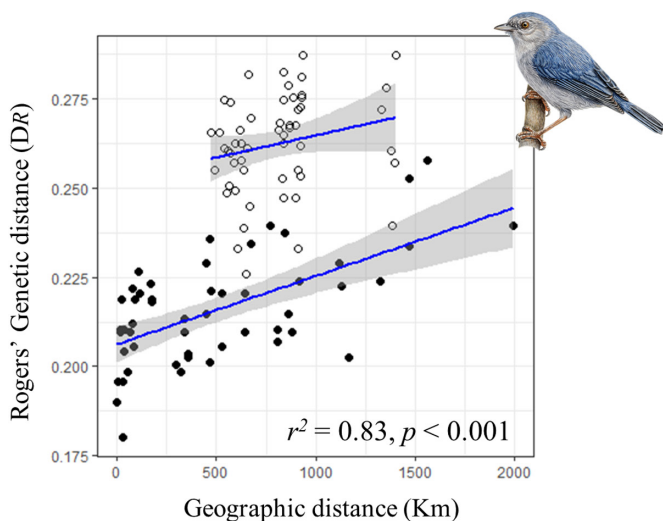
3.4 | Effectiveness of the Rio Negro as a barrier

Our results for the three species suggest that the Rio Negro has a significant effect as a barrier on genetic differentiation after controlling for geographical distance (Figure 3). However, the magnitude of the difference in the intercepts among regression slopes was respectively smaller in *S. napensis* and *M. propinqua* (Figure 3a,b) compared to *C. bicolor* (Figure 3c). When considering the Rio Negro barrier matrix, the correlation coefficient values varied considerably among species, with *C. bicolor* showing the highest coefficient ($r^2 = .83$, $p < .001$), followed by *M. propinqua* ($r^2 = .57$, $p < .001$) and *S. napensis* ($r^2 = .32$, $p < .001$). Conversely, considering only the effect of linear river distance on genetic distance, correlation values were lower for *C. bicolor* ($r^2 = .54$, $p < .001$) and *M. propinqua* ($r^2 = .31$, $p < .001$), but relatively higher for *S. napensis* ($r^2 = .41$, $p < .001$).

3.5 | Demographic model selection and co-divergence test

For the three bird species, the best divergence model selected was Model 3 (Figure 2), which describes decreased population connectivity starting at 250 ka, with gene flow being completely interrupted at 20 ka. Our neutral network analyses for model selection presented classification with >0.9 overall accuracy (Table 1). PCA results showed that simulated models fit the observed data, with considerable overlap between simulated and observed summary statistics (Figure S4). Model 3 had a higher probability for *C. bicolor* (posterior probability [PP] = 0.92) when comparing the probabilities obtained for *S. napensis* and *M. propinqua* (PP = 0.64 and 0.60, respectively; Table 1). For the latter two species, Model 2 (Table 1; Figure 2), which supports divergence with migration starting at 250 ka, had the second-highest probability value among the models tested (see Table 1). The confusion matrices indicated that even with some similarities between models (e.g., both Models 2 and 3 stipulate divergence time starting at 250 ka), it was possible to obtain high accuracy in their classifications (Figure S5).

Overall, comparisons of summary genetic statistics among population pairs across species showed higher genetic diversity for the Amazon basin population than for the Rio Branco populations (Table 2). Pairwise F_{ST} among populations were ~2.5× higher in *C. bicolor* ($F_{ST} = 0.101$), than for *S. napensis* ($F_{ST} = 0.042$) and *M. propinqua* ($F_{ST} = 0.034$). Despite the much smaller range of the Rio Branco population, the estimated parameters of the best-fit model (Model 3) supported a relatively similar effective population size (N_e) between the Amazon basin populations and the Rio Branco populations for the three species (Table S2). Estimates of the ancestral migration rate per generation had similar proportions among species, with a large overlap of the confidence intervals (Table S2). Pearson's correlation between pseudo-observed and simulated data indicated high accuracy of effective population size estimates >0.9, but not for gene flow parameters <0.6 (Figure S6). Despite the efficiency

(a) *Stigmatura n. napensis* (Lesser Wagtail-tyrant)
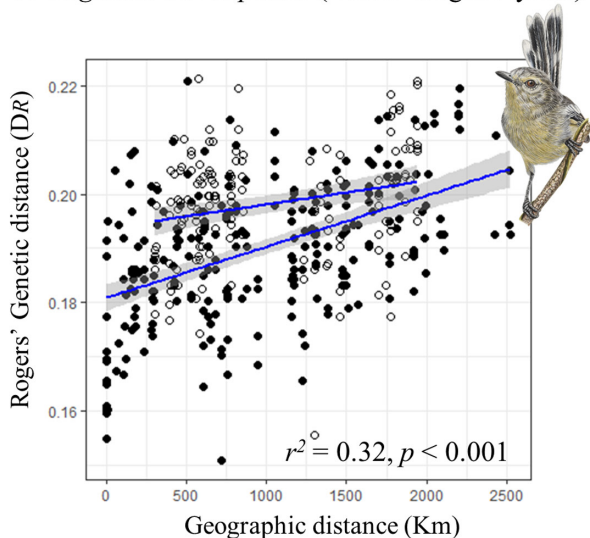
(b) *Mazaria propinqua* (White-belied Spinetail)

$r^2 = 0.32, p < 0.001$

$r^2 = 0.57, p < 0.001$

(c) *Conirostrum bicolor minus* (Bicolored Conebill)

Pairwise comparison between:

⬤ Individuals on the same side of the barrier.

◯ Individuals on opposite sides of the barrier.

$r^2 = 0.83, p < 0.001$

FIGURE 3 Testing the Rio Negro as a barrier to genetic differentiation by comparing individuals on the same side of the barrier (black dots) and opposite sides of the barrier (empty dots) while accounting for geographical distance in the bird species (a) *Stigmatura n. napensis*, (b) *Mazaria propinqua* and (c) *Conirostrum bicolor minus*. Illustrations of the birds by Andreza Silva [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Classification probabilities of alternative differentiation scenarios (Models), obtained with supervised machine learning using a neural network algorithm

Taxon	Model probability				Overall accuracy	Best model accuracy
	Model 1	Model 2	Model 3	Model 4		
Stigmatura n. napensis	0.05	0.30	0.64	0.00	0.93	0.92
Mazaria propinqua	0.02	0.35	0.60	0.03	0.93	0.92
Conirostrum bicolor minus	0.05	0.03	0.92	0.00	0.91	0.88

of this approach in classifying model probabilities that include different regimes of migration between populations, the low Pearson's correlation values between simulated and pseudo-observed data demonstrate the limitations in estimating this parameter.

Shared divergence results support two divergence events between pairs of populations (Figure 4a; PP >0.93, see Table S4), temporally clustering *M. propinqua* (mean Tdiv = 1173 years; 95% confidence interval [CI] = 593–1938) and *S. napensis* (mean

TABLE 2 Averages of the summary genetic statistics of the ultraconserved elements and exon sequences of the Branco and Amazonas bird populations.

Taxon	Population	Segregating site	π	Watterson's Θ	Tajima's D	F_{ST}	Private alleles	Fixed alleles
Stigmatura n. napensis	Branco	1.392	0.278	0.230	0.509	0.042	0.597	0.000
	Amazon	2.357	0.343	0.328	0.088			
Mazaria propinqua	Branco	1.211	0.411	0.354	0.370	0.034	0.639	0.000
	Amazon	2.571	0.516	0.658	−0.315			
Conirostrum b. minus	Branco	0.731	0.607	0.532	0.447	0.101	0.685	0.003
	Amazon	2.357	0.731	0.714	0.081			
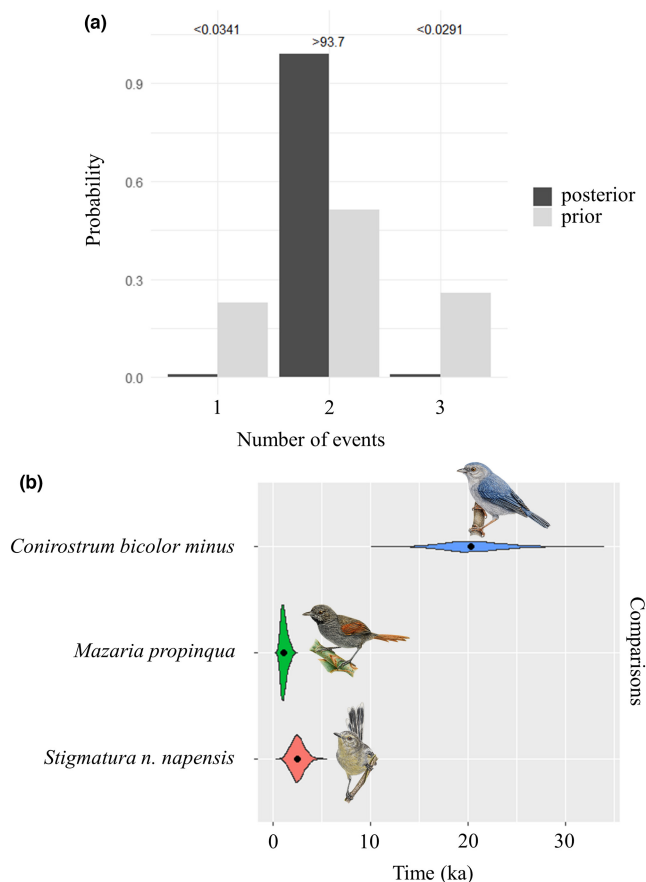
FIGURE 4 Results of the shared divergence test between pairs of bird populations in the Rio Branco and Amazon River and tributaries. (a) Probable distribution of the earlier (light bars) and later (dark bars) number of divergence events between pairs of populations. (b) Approximate posterior densities of divergence times (in thousands of years) for each pair of populations of *Stigmatura n. napensis*, *Mazaria propinqua* and *Conirostrum bicolor minus*. Illustrations of the birds by Andreza Silva [Colour figure can be viewed at wileyonlinelibrary.com]

Tdiv = 2518 years; 95% CI = 1274–3938) as the most recent divergence, and *C. bicolor* (mean Tdiv = 20,560 years; 95% CI = 15,166–26,692) with relatively old and independent divergence (Figure 4b). These results were consistent in alternative Dirichlet process concentration parameters α = 2 (Figures S7 and S8). The divergence time supported by ECOEVOLITY agreed with the best divergence

model estimated in *PipeMaster* (Model 3, Table 1) for *C. bicolor*, but was discordant for *S. napensis* and *M. propiqua*, which showed recent divergence times (Figure 4b).

4 | DISCUSSION

This study shows the potential vicariant role of the spatial variation in flooded forest habitat and sediment load along Amazonian floodplains. Specifically, we show that the black-water *igapós* of the Rio Negro have prevented gene flow between populations associated with two white-water *várzea* systems for tens of thousands of years, with isolation starting in the Late Pleistocene, possibly associated with the retraction of floodplains across the lowland Amazon basin due to channel incision (Pupim et al., 2019). Our analyses show that: (i) in all three bird species investigated, the Rio Branco populations are isolated and genetically distinct from those of *várzeas* in the Amazon River and tributaries; (ii) levels of genetic differentiation and tests of shared divergence indicate that there were at least two distinct divergence events, which can be related to habitat specialization and/or different dispersal abilities; and (iii) the most likely differentiation model includes decreased population connectivity since the Middle–Late Pleistocene transition (~250 ka), with gene flow being completely interrupted after the Last Glacial Maximum (LGM; ~20 ka), corresponding to the development of the current ria lake that occupies the lower Rio Negro.

4.1 | Population differentiation between Amazonian white-water rivers

Previous genetic studies have proposed that the low population differentiation along Amazonian floodplains (as well as the lower species diversity when compared to upland *terra-firme* forest) is a result of factors such as high environmental connectivity and species dispersal ability (Aleixo, 2006; Cadena et al., 2011; Harvey et al., 2017). However, recent studies have revealed contrasting patterns of genetic differentiation within Amazonian floodplain bird species, suggesting that the main driver of divergence among populations was Late Pleistocene fluvial dynamics leading to variations in

the flooded forest continuity (Choueri et al., 2017; Luna et al., 2022; Thom et al., 2020). Here, the levels of population differentiation observed among birds specialized in white-water *várzea* relate to spatial variation in riverine vegetation structure (i.e., *várzea* vs. *igapó*) generated by changes in drainage configuration and sedimentation patterns through time. These results add further evidence to the evolutionary complexity in the Amazonian floodplain system, suggesting that the interaction between species habitat affinity and spatial heterogeneity of the environment over time has driven the current distribution of species, the composition of communities (Bogotá-Gregory et al., 2020; Laranjeiras et al., 2019, 2020) and phylogeographical patterns (Barbosa et al., 2022; Luna et al., 2022; Thom et al., 2020).

4.2 | Habitat specialization and dispersal in Amazonian floodplain birds

To account for both historical and ecological drivers of population genetic differentiation within bird species associated with *várzea*, we compare the analysis of demographic model selection with shared divergence tests. Our demographic models support that the differences between pairs of populations were shaped by the same general historical process (Model 3), while the shared divergence test favours alternative explanations of at least two divergence events between the Branco and Amazon avian populations (Figure 4).

It is worth noting that these complementary analyses suggest that the current connection between the Rio Branco and the Amazon River populations is unlikely, reinforcing the hypothesis that the Rio Negro *igapó* forest is a biogeographical barrier to *várzea* forest specialists (Naka et al., 2007, 2020). The formation of this barrier could be related to the retraction of flooded environments (*várzea* and *igapó*) in the Amazonian lowlands since the Middle–Late Pleistocene transition (Pupim et al., 2019), including the formation of the ria lake at the lower Rio Negro after the LGM (Latrubesse & Franzinelli, 2005). Second, since ECOEVOLITY analyses do not consider gene flow after divergence, we can interpret that the mean divergence time estimate reflects the last time these populations experienced significant gene flow in the past (Leaché et al., 2020; Oaks et al., 2019). Recent gene flow mediated by habitat specialization could also explain the high probability of the alternative divergence models, such as Model 2, which assumes current gene flow between populations for both *Tessaria* sandbar scrub specialists *Stigmatura napensis* and *Mazaria propinqua* (Table 1). Variation in divergence time is often interpreted to be the result of distinct dispersal histories mediated by differences in ecological traits (Kopuchian et al., 2020; Nazareno et al., 2021; Provost et al., 2021; Thomaz & Knowles, 2020). Therefore, the distinct divergence times between pairs of floodplain bird populations studied here could be interpreted as differential migration histories across the lower Rio Negro *igapó*-dominated floodplains during a progressive isolation process.

For Amazonian birds, habitat association is a strong predictor of genetic differentiation across the landscape (Barbosa et al., 2022; Burney & Brumfield, 2009; Choueri et al., 2017; Harvey et al., 2017). In this sense, differences in habitat use could determine the pattern of genetic differentiation of floodplain organisms across geographical or ecological barriers. The correlation coefficient values derived from our MMRR analysis support this hypothesis, where the *Tessaria* shrub specialists *S. napensis* and *M. propinqua* were less affected by the Rio Negro as a barrier when compared to the *Cecropia*-dominated forest specialist *Conirostrum bicolor* (Figure 3). While the difference in the correlation coefficient between species is not a strict measure of phylogeographical concordance, it does scale the effectiveness of the Rio Negro black-water *igapó* in shaping the genetic differentiation of white-water *várzea* birds associated with *Tessaria* shrub habitats relative to species associated with *Cecropia* forest.

We acknowledge that the lack of replication of species specialized in *Cecropia*-dominated forest may limit our statistical inferences, but this approach offers us some insights into the underlying effects of habitat use on the genetic variability and connectivity through time of Amazonian floodplain birds. In an ecological context, fluvial processes of sedimentation and erosion constantly affect floodplain forests, generating different vegetation successional stages that can be divided into discrete categories (Junk et al., 2012). Vegetation such as *Tessaria*, *Salix*, and *Gynerium* are pioneers in occupying recent sedimentary substrates emerging during the low water season (Kalliola et al., 1991). These vegetation types are often submerged during high water periods due to their short stature, whereas early-secondary *Cecropia* forests need decades to become mature and, due to their height, are usually only partially submerged during high water season (Junk et al., 2011, 2012; Wittmann et al., 2010). To cope with this temporal and spatial variation, species specialized in pioneer *Tessaria* vegetation (such as *M. propinqua* and *S. n. napensis*) probably move horizontally across the landscape in search of their preferred habitats during the flooding season, while species specialized in early-secondary *Cecropia* forests (such as *C. bicolor minus*) move vertically in the forest stratum (Rosenberg, 1990; Rowedder et al., 2021), and probably do not disperse as much. These distinct dispersal strategies triggered by seasonal flooding could affect patterns of gene flow and population structure across the landscape.

Data on dispersal capacity, however, indicate that *C. bicolor* is a better disperser than both *M. propinqua* and *S. napensis* (Naka et al., 2022). In dispersal challenge experiments conducted on the Rio Branco, *C. bicolorcolour* managed to safely cross river gaps in all trials (10:0), whereas *S. napensis* failed in 25% and *M. propinqua* in more than 50% of the trials. Therefore, it is possible that habitat availability during the flooding season, rather than dispersal ability, is a key factor explaining the historical disruption of gene flow. However, further studies with a larger collection of species are needed to better determine the contribution of these ecological traits to the pattern of genetic structure and differentiation across Amazonian floodplain birds.

4.3 | Evolutionary responses to floodplain environmental dynamics in the Late Pleistocene

Our genomic data support the hypothesis that during the Late Pleistocene, the widespread deposition of fluvial sediments formed extensive white-water floodplains in northern and central Amazonia (Passos et al., 2020; Pupim et al., 2019), which may have allowed habitat connectivity (and subsequent gene flow) between the bird populations associated with the *várzeas* of the Branco and Amazon rivers. This connectivity was probably reduced after the LGM and throughout the Holocene due to river incision (Pupim et al., 2019). The drier climate in northern Amazonia during the LGM (Zular et al., 2019) also probably reduced sediment input from the Branco into the lower Rio Negro and the formation of a ria lake (Latrubesse & Franzinelli, 2005), possibly increasing the effectiveness of the lower Negro as a barrier for specialized white-water floodplain taxa.

In a historical context, climate-driven variations in sediment input from the Rio Branco to the lower Rio Negro may have affected the connectivity of *várzea* floodplain bird populations by gradually replacing their preferred habitat over time (Laranjeiras et al., 2020). For example, *Cecropia* forests and *Tessaria* scrub are largely associated with high sediment load rivers (*várzeas*) and are currently scarce in black-water rivers (*igapós*) with low sediment load, such as the Rio Negro (Akabane et al., 2020). The sedimentary input from the Branco to the eastern margin of the Negro causes a slight increase in soil fertility in this region, allowing the colonization of typical *várzea* vegetation (Junk et al., 2015). The sediment supply of the Rio Branco during the Late Pleistocene was possibly modulated by precipitation changes driven by precession (~23,000 years) insolation cycles (Cheng et al., 2013) and/or abrupt millennial (1000–3000 years) climate events (Häggi et al., 2017; Zular et al., 2019). Thus, precipitation changes in the northern Amazon could be an important driver of the connection between the *várzeas* of the Branco and Amazon rivers. However, precipitation and sediment discharge have varied at relatively high frequency (23,000-year cycles to 1000–3000-year events) compared to ten to a hundred thousand years needed to build or abandon extensive floodplains (Pupim et al., 2019). Changes in connectivity of floodplains forced by these ~23,000-year precipitation cycles or 1000–3000-year events would be unsuitable to promote isolation and restriction of gene flow in populations of specialists in *várzea* habitats.

The Rio Negro responded to changes in base level and sediment supply leading to widespread sediment deposition (floodplain expansion) and incision (floodplain fragmentation) phases during the Late Pleistocene (Latrubesse & Franzinelli, 2005). Specifically, during the Late Pleistocene and Middle Holocene (15–6 ka), a large ria lake developed in the lower Negro (Irion et al., 1997, 2010; Latrubesse & Franzinelli, 2005), although specific dates for the beginning of channel flooding and sediment filling of the Negro ria lake are not available. Sediment accumulation in the Negro ria lake promoted the development of the Anavilhanas archipelago during the Holocene (Latrubesse & Franzinelli, 2005). The Anavilhanas archipelago represents the main *igapó* area between the Branco mouth and the

Amazon *várzeas* and its development during the Holocene coincides with the most recent connections between the population pairs of *S. napensis* and *M. propinqua* (Figure 4b). The formation of sandbar scrub habitats in middle channel bars of the Anavilhanas archipelago and marginal beaches along the Negro ria lake could have served as a stepping-stone for migration and contact of populations from the Branco and Amazon rivers (Laranjeiras et al., 2020) until the ria became dominated by black-water *igapó* forests. However, while this is a possible explanation for the patterns observed in our genomic data, further studies are needed to characterize the development of *igapó* forest in the lower Negro during the Late Pleistocene and Holocene and test its impact on population connectivity (i.e., gene flow) across the Amazonian floodplains.

4.4 | Conclusions and future perspectives

In this study, we combine scenarios of the changes in the continuity of floodplains within river valleys of the northern and central Amazon basin during the Late Pleistocene and the effects of habitat specialization on dispersal to test alternative hypotheses of genomic differentiation among Amazonian floodplain birds. Our findings suggest that habitat specialization is a potential underlying factor in population connectivity, possibly driving different evolutionary responses to the same historical processes of environmental change. Habitat use could reflect general differences in population connectivity (i.e., historical changes in the composition of seasonally flooded forests), an effect linked to variation in sediment supply and accumulation in Amazonian drainage basins. Specific stages of floodplain development could provide temporary connections depending on the species' habitat specialization. This suggests that even though the three lineages of birds studied here are specialized in vegetation from floodplains of rivers with high sediment load, such as the Branco and Amazon, differences in habitat use within these floodplains may have shaped their current genetic variability. In addition, the different responses of population connectivity to historical environmental change also offer insights into current threats faced by Amazonian floodplains. For instance, the Santo Antonio and Jirau hydroelectric dams on the white-water Madeira River, one of the largest tributaries of the Amazon River, have rapidly changed habitats used by floodplain birds (Melo et al., 2021). This is an important aspect of conservation, since rapid anthropogenic changes can reduce connectivity among populations, increasing the risk of local extinction by isolating small populations. Future studies should include additional species with different ecological requirements to contrast patterns of genetic diversity and population structure from both macro- and microevolutionary perspectives, especially in the Rio Branco, which harbours a unique bird community with several endemic species.

CONFLICT OF INTEREST

The authors declare they have no conflicts of interests.

DATA AVAILABILITY STATEMENT

Illumina raw reads data have been deposited in the Sequence Read Archive for *Stigmatura napensis* and *Conirostrum bicolor* (Bioproject ID: PRJNA889938) and *Mazaria propinqua* (Bioproject ID: PRJNA736107). The variant call format inputs (vcf; both unfiltered SNPs and one SNPs per locus) used in the population genomic analyses are available in the Dryad digital repository (Luna et al., https://doi.org/10.5061/dryad.3tx95x6kb).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.