THE ROLE OF RIVERS IN THE ORIGINS, EVOLUTION, ADAPTATION, AND DISTRIBUTION OF BIODIVERSITY

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Editorial: The role of rivers in the origins, evolution, adaptation, and distribution of biodiversity

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Editorial on the Research Topic
The role of rivers in the origins, evolution, adaptation, and distribution of biodiversity

Rivers represent ubiquitous landscape features and affect biodiversity in fundamental ways. Not only do they provide the medium necessary to sustain aquatic life, but they also influence the structure and biodiversity of both riparian and non-riparian habitats. Therefore, rivers can potentially affect the origins, evolution, adaptation, and distribution of both aquatic and terrestrial biota. The goal of this Research Topic was to provide a forum to discuss recent advances in the study of the role of rivers in the ecology and evolution of biodiversity. Specifically, we aimed to highlight the current and historical role of rivers in the evolutionary process and reveal different ways by which rivers affect biodiversity. In this editorial, we will review (i) the role of rivers in the origin and evolution of species; (ii) how river reorganization can affect species diversity; (iii) the effect of riverine habitats as environmental filters; and (iv) the importance of community-based management for biodiversity conservation.

Rivers and the origin and evolution of terrestrial species

The association of rivers with the speciation process in non-aquatic species can be traced back to Wallace's explorations in the Amazon, where he documented that several species of primates had closely related, yet morphologically different, populations across some major rivers (Wallace, 1852). These observations were subsequently interpreted as evidence of the vicariant force of rivers in the speciation process, a model that became known as the *Riverine Barrier Hypothesis* (Sick, 1967; Capparella, 1988, 1991). Although there are many examples of rivers acting as biogeographical barriers throughout the world, it is in the Amazon that this phenomenon is more widespread and best documented. Nonetheless, it took over a century to fully appreciate the role of Amazonian rivers in defining species' distributions (Haffer, 1969, 1974; Hershkovitz, 1977; Cracraft, 1985). Increasingly detailed range maps have allowed biologists to investigate the role of rivers as biogeographical barriers for entire communities (Ayres and Clutton-Brock, 1992; Gascon et al., 2000; Hayes and Sewlal, 2004) and to formulate evolutionary hypotheses to account for congruent river-bounded distributions (Cracraft and Prum, 1988; Silva and Oren, 1996; Bates et al., 1998).

By the end of the 20th century, molecular studies started to link phenotypic and genotypic variation across riverine barriers (Capparella, 1988, 1991), inspiring legions of scholars who investigated the role of rivers in dividing evolutionary lineages (Marks et al., 2002; Aleixo, 2004; Ribas et al., 2012). These were followed by multi-taxon studies that began to reveal commonalities, but also some unique taxon-specific distribution patterns, including barrier effects of relatively minor Amazonian rivers (Naka et al., 2012; Boubli et al., 2015) and disparate times of divergence among co-distributed taxa (Naka and Brumfield, 2018). With the advent of next generation sequencing, multi-locus studies are shedding light into patterns of gene flow and introgression across rivers, particularly around river headwaters, where rivers are much narrower and potentially cease to represent meaningful biogeographic barriers (Pulido-Santacruz et al., 2018).

Despite major advances in the field, most of these studies were restricted to the Amazon basin and to either birds or primates. Recent studies, however, show that the dissecting power of rivers are not restricted to this region (Harcourt and Wood, 2011). Molecular studies have shown the role of rivers such as the Congo (Anthony et al., 2007), the Mississippi (Jackson and Austin, 2010), and the Paraná (Kopuchian et al., 2020) as potential current and historic biogeographic barriers. Similarly, in the last decade, studies evaluating the role of rivers as biogeographical barriers in non-avian and non-primate groups started to appear in the literature, including studies on lizards (Avila-Pires et al., 2012), frogs (Fouquet et al., 2012, 2015; Godinho and da Silva, 2018), invertebrates (Guilherme et al., 2022), and plants (Nazareno et al., 2017, 2019).

In this Research Topic, five articles include new data that broaden our understanding on the role of rivers in the speciation process, filling important taxonomic, geographical, and theoretical gaps. Three studies provide contrasting views on the role of Amazonian rivers in structuring different mammal and avian lineages. Whereas Silva et al. found that the distribution of Amazonian Phyllostomidae bats was not defined by rivers, Mourthé et al. found that rivers were key in structuring Amazonian primate diversity, finding a significant effect of annual discharge and river sinuosity on primate beta-diversity. Working on birds, Dornas et al. investigated the role of two eastern Amazonian rivers as barriers, in a region with few prior biogeographical studies. Using a comparative approach, these authors found that 14 avian lineages responded differently to these riverine barriers. These contrasting results suggest that (i) ecological traits and dispersal ability may predict the importance of rivers as biogeographical barriers, and (ii) different lineages may have different histories and be affected by rivers in different ways along their evolutionary history.

Using molecular data, two studies explored the role of rivers in the evolutionary history of the herpetofauna, including an Amazonian heliothermic lizard (*Kentropyx calcarata*) and a treefrog (*Dendropsophus elegans*) in the Atlantic Forest of Brazil. Cronemberger et al. evaluated the genetic structure of *K. calcarata* in the light of different evolutionary scenarios and found that although Amazonian rivers likely acted as barriers to dispersal, they were not the sole drivers of diversification. Pirani et al., on the other hand, describe the genomic divergence and phenotypic admixture of *D. elegans*, showing the effect of the Rio Doce as a biogeographical barrier. These results add to the growing body of information pointing this river in the Atlantic Forest as a major barrier, as shown in the past for small non-volant mammals (Costa, 2003), a species of gecko (Pellegrino et al., 2005) and a species of bird (Cabanne et al., 2008).

Quite surprisingly, until now we lacked basic knowledge on how riverine barriers affect species dispersal. Conducting a series of dispersal experiments in real-life conditions, Naka et al. evaluated how hundreds of individuals of dozens of bird species cope with the challenge of crossing a river gap in the Amazon basin. Using a methodology previously used in Panama by Moore et al. (2008), this study showed that nearly a third of the individuals tested failed at crossing even 100 m of open water. Their results revealed that ultimately, dispersal limitations are directly related to the flying apparatus of birds. Species with more rounded wings performed worse in the experiments than those species with more elongated ones. Surprisingly, ecological traits, such as habitat preference and river island specialization had little predictive power in the outcome of the experiments. These results open new perspectives on experimental studies to evaluate the dissecting power of rivers on biodiversity.

Riverine landscape evolution and diversification

Until very recently, most riverine studies viewed rivers as fixed vicariant forces. However, rivers do change through time. Drainage network reorganization can have pervasive effects on species distributions. One specific way by which rivers reorganize, is by a process known as river capture, where topographic changes may alter river networks (Bishop, 1995). The effect of these changes on biodiversity became known as the *River Capture Hypothesis* (Albert et al., 2018) and has shown great potential in the understanding of species distributions, particularly in fish. Recent studies have shown that river network rearrangements can also promote speciation in lowland Amazonian birds (Musher et al., 2022).

In this Research Topic, two studies investigate this phenomenon at two different scales. Val et al. conducted a comprehensive meta-analysis to test the *River Capture Hypothesis* using nearly 15,000 species of obligate freshwater fishes in more than 3,000 river basins. Their results indicate that fish species richness can be explained by landscape evolution models, including the *River Capture*, *Mega Capture*, and the *Intermediate Capture Rate Hypotheses*, supporting the conclusion that landscape changes represent a meaningful mechanistic driver of net diversification in riverine and riparian organisms. At a smaller scale, Sá Leitão et al. used genomic data to investigate if river reorganization could explain the genetic differentiation and structure of two Amazonian dwarf cichlids. Their results are consistent with the *River Capture Hypothesis* and offer a mechanistic link between the isolation and differentiation of fish populations and the drainage evolution of the basin, suggesting that the geological history of the region may be responsible for promoting species diversification.

Rivers as environmental filters

The ecological characteristics of rivers also affect the distribution of species, not only by restricting their movements, but also by providing differential habitats along its margins. Recent studies from northern Amazonia, have shown that water sediments are key to explaining bird species composition (Laranjeiras et al., 2019, 2021) and that avian communities respond promptly to changes in riverine habitats and climatic variables along ecological gradients (Naka et al., 2020). In this Research Topic, two articles show how habitat heterogeneity can drive compositional differences among both avian and butterfly species assemblages. Sinha et al. showed the influence of both biotic and abiotic factors in defining compositional differences among avian local species assemblages in the Himalayas. Using standardized avian surveys, they found that riparian bird communities in the drier and more seasonal Western Himalayas were poorer and more clustered phylogenetically and functionally than those communities in the Eastern Himalayas, pointing out the influence of habitat and climatic factors on patterns of avian beta diversity. Back in the Amazon, Rabelo et al. show that seasonal flooding of Amazonian forests strongly determines the composition of butterfly assemblages. In this case, small topographic variation can create distinct flooding gradients that directly affect species abundance and community composition. These results add to the growing body of work demonstrating that environmental filtering plays a crucial role in structuring biotic communities. Together, these results suggest that habitat heterogeneity can create the conditions that maintain distinct communities and even provide ecological gradients along which populations can diverge and possibly speciate.

This is, in fact, what Hay et al. found in their genomic study of the adaptive evolution of an Amazonian Characin fish. These authors found that variation in water characteristics was a key factor contributing to adaptive divergence. Specifically, variation in genes involved in acid-sensitive ion transport and light-sensitive photoreceptor pathways were strongly associated with water pH and turbidity variability. These results offer a hint at how river characteristics can drive genomic changes through natural selection, impacting the distribution of biodiversity in riverine habitats.

Conservation of riverine systems

Overfishing and overhunting represent significant threats to riverine biodiversity. While natural reserves are key for protecting riverine environments and their biodiversity, governments often fail in providing secure conservation. In fact, many protected areas in the tropics are themselves vulnerable to human activities (Laurance et al., 2012). Recent studies have shown that community-based conservation management can integrate both socio-economic needs with conservation goals in tropical ecosystems (Campos-Silva et al., 2018), providing benefits to entire biotas (Campos-Silva et al., 2021).

In this Research Topic, Andrade et al. analyze historical time series of protection of four different species of turtles in the Brazilian Amazon. Using data from 1974 to 2019, they estimate that over a million nests and more than 30,000,000 hatchings were protected by both government and community-based protection initiatives. They compare the effect of both kinds of protection, and showed that in some cases, government-based protection resulted in higher support capacity in the production of nests and hatchlings, but in other cases, communities were more efficient in protecting both nests and hatchlings. As such, they conclude that community-based protection and monitoring programs are an important component of conservation and should be incorporated by the government's environmental agencies for turtle management in the Amazon.

Final considerations

Despite the importance of riverine systems to both human wellbeing and biodiversity conservation, tropical rivers, which harbor an exceptional and disproportionate high number of species, are under assault (Latrubesse et al., 2017). Main threats include their use for energy production (i.e., hydroelectrical dams) and canalization to control their courses and allow navigation (Anderson et al., 2018). At the same time, climate change is disrupting natural patterns of rainfall and flooding worldwide (Barichivich et al., 2018), further modifying natural riverine ecosystems.

Although we have come a long way since Wallace's visit to the Amazon in the 19th century, our discovery of biological patterns is often outpaced by habitat destruction. Therefore, it is key to both increase protection of tropical rivers and accelerate and expand the kind of studies that are presented in this Research Topic. Understanding the complexity of riverine systems often requires great amounts of human and financial resources and we urge scientists to both deepen their Research Topics and use novel strategies to engage both local communities and the general public in the conservation of tropical rivers.

The increasing number of whole genome sequences available for an ever-growing number of taxa, allows us to better understand the past and present role of rivers as vicariate agents, as well as to understand current and past patterns of gene flow across barriers. On the other hand, ecological studies are broadening our understanding of rivers as environmental filters. Such advances can now be better contextualized by the outstanding advances in the understanding of the geologic, climatic, and geomorphological changes in riverine landscapes (Sawakuchi et al., 2022).

Unfortunately, the rate of destruction of many of these pristine systems is greater than the rate of new scientific discoveries. Particular attention should be given to rivers and their potential for evolutionary change in organisms when designing new protected area networks. We hope this Research Topic not only adds to the science of riverine biology, but also highlights the many opportunities that lay down the road, and at the same time call the attention to the urgent need of conserving the world's rivers, both for human wellbeing and biodiversity conservation.

Author contributions

LN did the leading writing. All authors contributed with ideas and reviews.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Role of River Flooding as an Environmental Filter for Amazonian Butterfly Assemblages

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Amazonian flooded (*várzea*) and upland (*terra firme*) forests harbor distinct assemblages of most taxonomic groups. These differences are mainly attributed to flooding, which may affect directly or indirectly the persistence of species. Here, we compare the abundance, richness and composition of butterfly assemblages in *várzea* and *terra firme* forests, and evaluate whether environmental gradients between and within these forest types can be used to predict patterns of assemblage structure. We found that both total abundance and number of species per plot are higher in *várzea* than in *terra firme* forests. *Várzea* assemblages had a higher dominance of abundant species than *terra firme* assemblages, in which butterfly abundances were more equitable. Rarefied species richness for *várzea* and *terra firme* forests was similar. There was a strong turnover in species composition from *várzea* to *terra firme* forests associated with environmental change between these forest types, but with little evidence for an effect of the environmental gradients within forest types. Despite a smaller total area in the Amazon basin, less defined vegetation strata and the shorter existence over geological time of floodplain forests, Nymphalid-butterfly assemblages were not more species-poor in *várzea* forests than in unflooded forests. We highlight the role of flooding as a primary environmental filter in Amazonian floodplain forests, which strongly determines the composition of butterfly assemblages.

Keywords: Amazonian floodplains, Lepidoptera, rarefaction, species composition, species density, species richness, *terra firme*, *várzea*

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INTRODUCTION

The number and composition of species at a given site is always a small subset of the regional species pool because environmental and biotic factors act together or separately to filter species from the regional pool and select the species composition at local scales (Hubbell, 2005). Vegetation type is the biotic feature most often used to represent the spatial distribution of forest-dwelling species, and several forest types occur in Amazonian landscapes.

Upland (*terra firme*) forests account for approximately 83% of the Amazon basin (Melack and Hess, 2010) and are located above the maximum seasonal flood levels of rivers, lakes, and large streams. *Várzea* forests are seasonally flooded by nutrient-rich white-water rivers for 6–8 months, and water-level fluctuations can reach up to 14 m (Junk et al., 2012). *Várzea* forests cover ∼7% of the Amazon basin (Melack and Hess, 2010).

Várzea and *terra firme* forests harbor distinct assemblages of trees (Wittmann et al., 2004), terrestrial mammals (Alvarenga et al., 2018), bats (Bobrowiec et al., 2014), birds (Beja et al., 2010), litter frogs (Gascon, 1996), and ants (Pringle et al., 2019). Poorer assemblages of several animal groups have been consistently documented in *várzea* forests (Haugaasen and Peres, 2005b; Bobrowiec et al., 2014; Alvarenga et al., 2018; Pringle et al., 2019), suggesting that seasonal inundation explains the lower number of terrestrial and understorey species. In contrast, *terra firme* should have higher species richness than *várzea* forest because it offers more niches associated with the understorey vegetation (Pereira et al., 2009).

It is expected that *terra firme* forests should contain more speciose assemblages of those species groups that can persist in both *várzea* and *terra firme* forests. *Terra firme* forests should have more species than flooded forests since they cover a much larger area (MacArthur and Wilson, 1967), have more stratified vegetation (MacArthur and MacArthur, 1961), and have existed over a longer period of geological time (Ruokolainen et al., 2018). With more species, it is also expected that species abundances in *terra firme* assemblages would be more equitable (MacArthur, 1969). On the other hand, *várzea* forests tend to have higher species abundance/biomass (Haugaasen and Peres, 2005b; Pereira et al., 2009; Pringle et al., 2019) due to the high forest primary productivity, as the white-water seasonal flooding fertilizes *várzea* soils (Haugaasen and Peres, 2006). Higher abundance/biomass in *várzea* forests due to the higher primary productivity has been documented mainly for mammals, but also for arboreal ant species (Pringle et al., 2019).

Butterflies are strongly associated with specific habitats at all life stages (Freitas et al., 2006) and are relatively sedentary in the larval stage, but are highly vagile in the adult phase and can have seasonal adaptations (phenological or migratory) to environmental changes (Diamond et al., 2011; Chowdhury et al., 2021). Vegetation gradients represent changes in the availability of food resources and physical conditions of the environment, which directly affect the spatial distribution of Amazonian fruit-feeding butterflies (Ribeiro and Freitas, 2012; Graça et al., 2015, 2017a). Therefore, environmental changes, such as seasonal flooding, can also filter species from the regional pool, affecting local species richness and composition.

This study compares the butterfly assemblages of *várzea* and *terra firme* forests in central Amazonia. Specifically, we aim (i) to test whether the density, richness and composition of butterflies differs between *várzea* and *terra firme* forests; (ii) to compare the species-abundance distribution between the two forest types; and (iii) to evaluate how the assemblage structure is associated with environmental (topography and vegetation) gradients between and within forest types. We expected to find a higher butterfly density in *várzea* forests because they have higher forest primary productivity, which represents higher availability of food resources than in *terra firme* forests. On the other hand, given that *terra firme* forests represent a more stable environment and cover a larger area, we expected higher species richness in this forest type. We also predicted that the species-abundance distribution would be evener in *terra firme* forests due to its higher species richness, in comparison with *várzea* forests, in which we should find a higher dominance of abundant species. We also expected to find strong turnover in species composition associated with forest type and environmental gradients.

MATERIALS AND METHODS

Study Area

Sampling was undertaken in three Amazonian protected areas: Amanã Sustainable Development Reserve, Mamirauá Sustainable Development Reserve and Baixo Juruá Extractive Reserve, in the Middle-Solimões (upper Amazon) River region, in Central Amazonia (**Figure 1**). These protected areas contain floodplains covered largely by *várzea* forests, which are adjacent to *terra firme* forests (ICMBio, 2009; IDSM, 2010). During the high-water season, *várzea* forests are flooded by nutrient-rich white-water rivers, with an average annual water-level range of 15 m. Highest river levels occur around May-June and minima in October-November (ICMBio, 2009; IDSM, 2010). Mean annual temperature and precipitation were around 26–31°C and 2,200–2,400 mm, respectively, with mean precipitation around 60–80 mm during the dry season (ICMBio, 2009;IDSM, 2010).

Sampling Design and Data Collection

Sampling was done in 15 plots located in *várzea* and 21 in *terra firme* forests (**Figure 1**) during the low-water season in all study areas (RDS Amanã in November–December 2017, RESEX Baixo Juruá in July 2018, RDS Mamirauá in August 2019). We were not able to conduct sampling in both high- and low-water seasons due to logistical constraints. The sampling design followed the RAPELD method as part of a long-term ecological project that aims to compare the distributions of multiple taxa (Magnusson et al., 2005). Plots (sample units) consisted of a 250-m long center line, separated by at least 500 m from one another (**Figure 1**).

Butterfly surveys were conducted via active and passive sampling. We placed six equally spaced baited butterfly traps along the center line of each plot. Traps were hung from tree branches in the forest understorey (∼1.5 m high). We baited the traps with a mixture of sugar-cane juice and bananas fermented for 48 h (Freitas et al., 2014) and visited them every 24 h to check for captures and replace the bait. We left the traps active for six consecutive days in each plot. This sampling effort is based on Graça et al. (2017b), who suggested that it is sufficient to identify ecological responses of understorey fruit-feeding butterfly assemblages.

We also used insect nets to sample low-flying Haeterini species and other Nymphalid species that usually are not caught with baited traps. On each visit to the plots, two researchers with standard 37-cm diameter insect nets actively searched for butterflies during 30 min. All captured individuals were collected for posterior species identification. Butterflies were identified to species level using on line resources[1] and the taxonomic literature. All identifications were verified by an

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[1] www.butterfliesofamerica.com

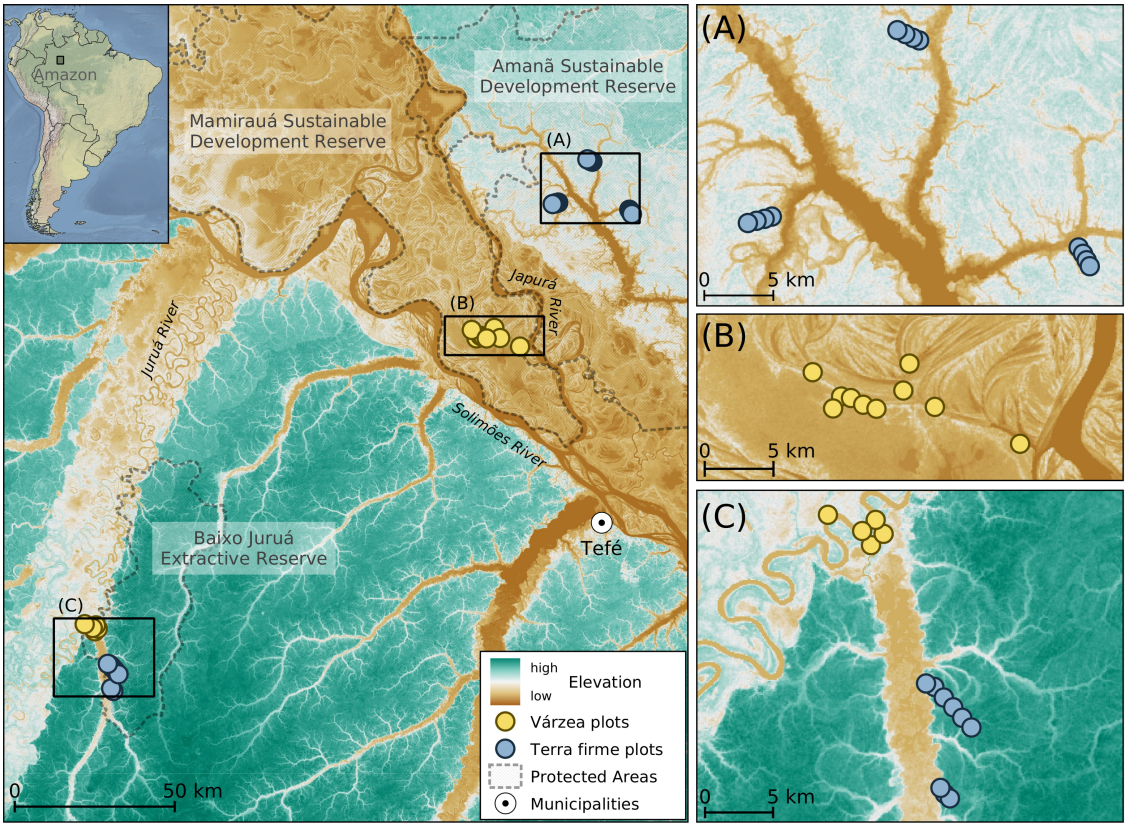

FIGURE 1 | Distribution of sample plots in várzea and terra firme forests. Maps show the distribution of sampling plots overlapped with an elevation Radar image from the Shuttle Radar Topography Mission (SRTM). Black rectangles in the left map are enlarged in right panels **(A–C)**.

expert taxonomist (T. Zacca). To avoid taxonomic uncertainty, especially because our study area is located in a region with many biodiversity-knowledge shortfalls (Hortal et al., 2015), where butterflies have been poorly inventoried (Santos et al., 2008), some specimens could not be identified to species level, so we identified them as morphospecies. Although not ideal, the use of morphospecies is a way to deal with taxonomic constraints in ecological studies, and they appear to provide a reliable alternative to taxonomic species in Lepidoptera (91% of matching accuracy; Derraik et al., 2002). All specimens were deposited in the Entomological Collection of the Mamirauá Institute for Sustainable Development, Tefé, Brazil.

We gathered topographic and vegetation data to characterize the environmental gradients across *várzea* and *terra firme* plots. Topography data consisted of elevation, height above nearest drainage (HAND) and flooded terrain during the high-water season, which were extracted from an image provided by the Synthetic Aperture Radar of the Japonese Earth Resources Satellite—JERS-1 SAR.[2] In the Amazon, JERS-1/SAR images indicate flooded-forest areas by brighter pixels, closed-canopy forests by median brightness, and open water as darker pixels. Vegetation data consisted of estimates of % of tree cover, canopy height, enhanced vegetation index (EVI) and net primary productivity (NPP) obtained from GIS databases (**Supplementary Table 1**). EVI is a vegetation index that is correlated with forest primary productivity and vegetation structure (Huete et al., 2002).

Data Analysis

We report two indices of butterfly diversity: species density and rarefied species richness. These measures emphasize different components of diversity while controlling for potential sampling bias. Species density records the number of species per sample unit. The rarefied species richness (hereafter "species richness"), is used to estimate expected species richness at constant total abundance, since increased number of species is expected as a random consequence of larger pools of individuals (Gotelli and Colwell, 2001).

We compared the total abundance and observed number of species per plot between *várzea* and *terra firme* forests with Kruskal-Wallis tests, as the data had non-normal distributions. We used rarefaction and extrapolation of standardized number of species to compare species richness in both forest types. We standardized the number of species by both number of sampled individuals and sampling coverage, following the recommendations of Chao et al. (2014). Rarefaction and extrapolation were based on sampling coverage, in

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[2] http://earth.esa.int

addition to sample size, because standardizing samples by number of individuals usually underestimates species richness of assemblages with more species (Chao and Jost, 2012). We also used Kolmogorov-Smirnov tests to compare the species-abundance curves from the two forest types and sampling methods.

We built a species by site matrix, recording each species abundance (columns) per plot (rows). Then we standardized the abundances by dividing the number in each matrix cell by the total abundance in the matrix row (plots) to reduce the discrepancy between sites with different number of samples. We summarized butterfly species composition by non-metric multidimensional scaling (NMDS) ordination with two axes, based on the Bray-Curtis dissimilarity index. Then, we used a permutational multivariate analysis of variance (PERMANOVA) to evaluate whether the species composition differed between the two forest types. We reran this analysis excluding singletons and doubletons, since rare species can introduce variation in the assemblage structure that may not be related to habitat (Beja et al., 2010). We used a principal component analysis (PCA) to summarize the environmental data from plots and used the first axis of this ordination to represent the environmental gradient across plots. We then used an NMDS with one dimension to reduce the dimensionality of data into only one axis, using the scores derived from this ordination to represent the butterfly species composition in each plot. We used this second NMDS ordination with only one axis because NMDS is not an eigenvalue technique, and it does not maximize the variability associated with individual axes of the ordination, so the axes are not orthogonal to each other (Legendre and Legendre, 1998). We then used the single NMDS axis, which represented the ordering of sites according to their similarity in species composition (i.e., the assemblage structure), as the response variable in a generalized linear model (GLM) to evaluate whether it changes with environmental gradients (PCA 1) and across forest types (*várzea* or *terra firme*). We included latitude and longitude as predictors in the GLM to account for potential effect of spatial gradients and tested for spatial autocorrelation in model residuals with Moran's I. All analyses were undertaken in the vegan 2.4-4 (Oksanen et al., 2013) and iNEXT (Hsieh et al., 2016) packages of the R 3.4.4 statistical software (R Development Core Team, 2018).

RESULTS

We captured 913 individuals belonging to 99 butterfly species (**Supplementary Table 2**), in a total sampling effort of 1,296 trap*days (540 in várzea and 756 in terra firme) and 144 h (60 in *várzea* and 84 in *terra firme*). The most frequently captured species in *várzea* forests was *Magneuptychia* aff. *ocnus,* whereas *Bia actorion* was the most frequently captured species in *terra firme* (**Supplementary Figure 1**). Singletons and doubletons were represented by 36 species (∼51%) in *várzea* forests and 23 species (∼51%) in *terra firme*. The number of species exclusive to *várzea* and to *terra firme* was 56 and 29, respectively, and 16 species were shared between the two forest types.

The median number of butterfly individuals counted per plot in *várzea* forests was 28 (first quartile (Q1) and third quartile (Q3) = 24 and 58, respectively), and it was significantly higher than the number of individuals counted in *terra firme* plots (Q1 = 5; median = 9; Q3 = 10; Kruskal-Wallis, $H = 22.64$, $p < 0.001$; **Figure 2A**). The abundance distribution of species also differed between the two forest types (Kolmogorov-Smirnov, baited traps: $D = 0.85$, $p < 0.001$; insect nets: $D = 0.71$, $p < 0.001$; both methods: $D = 0.66$, $p < 0.001$; **Figure 2C** and **Supplementary Figure 2**). The *várzea* assemblage had higher dominance of abundant species [three (4%) species made up 50% of all individuals, **Supplementary Figure 1**] than the *terra firme* assemblage, which had an evener distribution of species abundance [eight (18%) species accounted for 50% of individuals, **Supplementary Figure 1**].

The observed number of species per plot was also higher in *várzea* than in *terra firme* forests (Kruskal-Wallis, $H = 15.26$, $p < 0.001$; **Figure 2B**), with a median number of 9 species per plot in *várzea* forests (Q1 = 9; Q3 = 14) and 6 (Q1 = 5; Q3 = 7) species per plot in *terra firme* forests. However, when the species richness estimate was standardized by sample size and coverage, *várzea* and *terra firme* forests showed similar species-richness estimates (**Figure 3**). *Terra firme* assemblages had a lower estimated sampling completeness (84%) than *várzea* (94%; **Supplementary Figure 3**), despite the larger survey effort (21 surveyed plots in *terra firme* against 15 in *várzea*). Even with *terra firme* having a lower sampling completeness, the rarefaction and extrapolation of species-richness to the same number of individuals or coverage as the *várzea* samples showed similar curves (**Figure 3**), indicating that they have similar overall richness.

The NMDS ordination of plots along the two axes explained 53% of the variation in differences in species composition, whereas the NMDS with a single axis explained 33%. The PCA ordination of plots along the first two axes explained 66% of the variation in the environmental features of plots and the first axis (PCA 1) captured the environmental differences between *várzea* and *terra firme* plots (**Supplementary Figure 4**). Negative values of the first PCA axis were associated with *várzea* plots, whereas positive values were associated with *terra firme* plots (**Supplementary Figure 4**). *Várzea* plots had lower terrain elevation, were vertically nearer to drainage, subjected to flooding during the high-water season, and also had lower percentage tree cover and lower canopy height than *terra firme* plots (**Supplementary Figure 4**).

There was a marked difference between butterfly composition of *várzea* and *terra firme* forests (PERMANOVA, $F = 7.82$, $p < 0.001$), captured mainly by the first axis (**Figure 4A**) due to the strong turnover of species composition between forest types (**Figure 4B**). The exclusion of rare species (singletons and doubletons) did not change the pattern found (**Supplementary Figure 5**). The change in species composition was associated with forest types ($t = -4.59$; $p < 0.001$), but with little evidence for effects of environmental gradients within each forest type (*várzea*: $t = 0.09$; $p = 0.93$; *terra firme*: $t = -0.07$; $p = 0.94$; **Figure 4C**), after controlling for spatial effects of latitude and longitude.

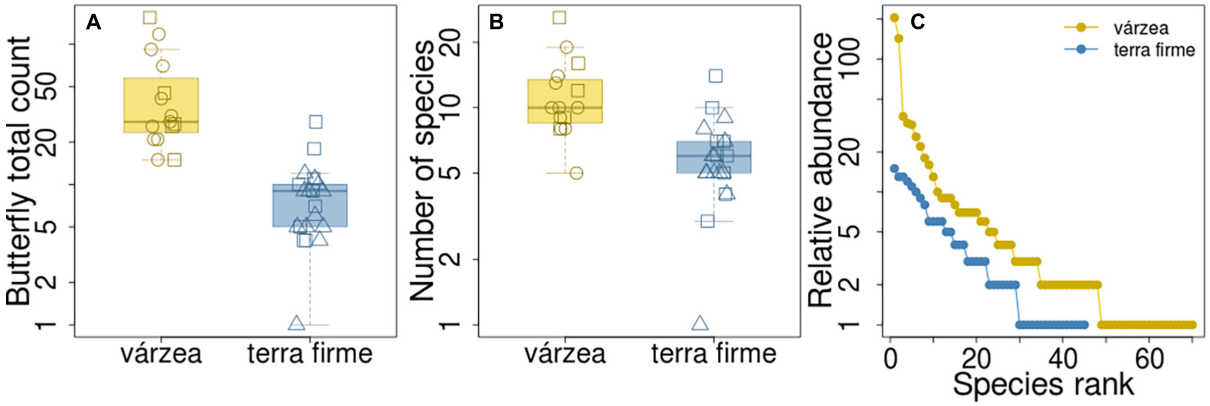

FIGURE 2 | Butterfly counts and number of species in *várzea* and *terra firme* forest plots. Difference in butterfly counts **(A)** and number of species **(B)** per plot between the two forest types. Square, circle and triangle symbols represent plots in Baixo Juruá, Mamirauá and Amanã reserves, respectively. **(C)** Assemblage rank-abundance distribution from the two forest types.

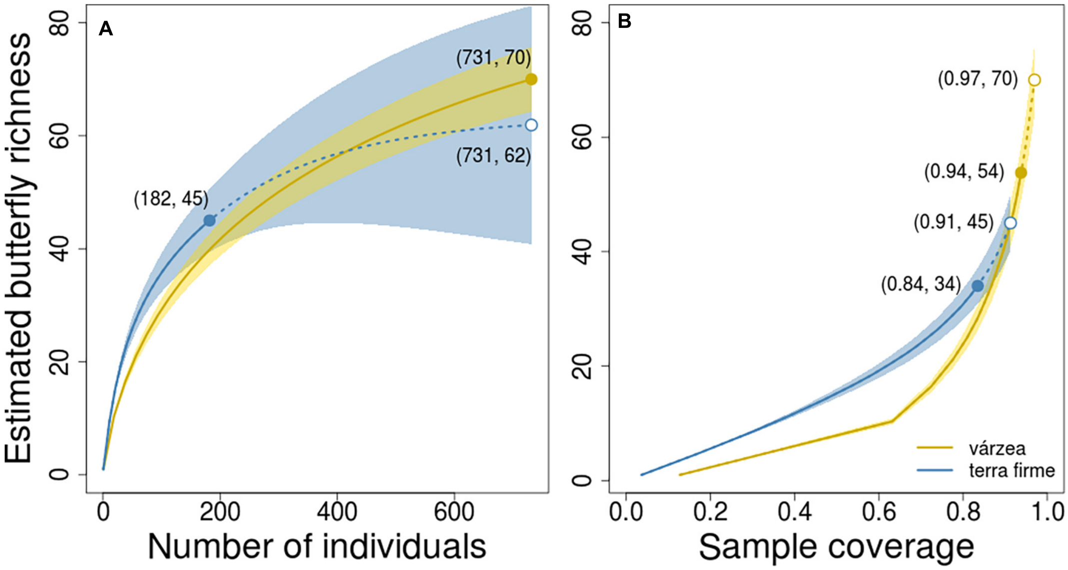

FIGURE 3 | Butterfly richness estimated by rarefaction (solid curves) and extrapolation (dashed curves) based on sample size **(A)** and completeness, **(B)** with corresponding 95% confidence intervals (shaded areas). Solid circles indicate the observed species richness and open circles indicate the extrapolated richness in *terra firme* assemblages based on number of individuals **(A)** or sample coverage. **(B)** Numbers within parentheses indicate the coordinates in both graphs. Although estimated richness in *várzea* seems to be higher than *terra firme* at its maximum sample size (731 individuals in "**A**") or completeness (0.97 of coverage in "**B**"), the confidence intervals overlap and indicate the there is no statistically significant difference in richness between the two forest types.

There was no spatial autocorrelation in model residuals (Moran's I: obs.: –0.01; exp.: –0.03; $p = 0.60$), even without including latitude and longitude among predictors (Moran's I: obs.: –0.07; exp.: –0.03; $p = 0.13$).

DISCUSSION

Bottom-Up Effects on Species Abundance and Richness

We found higher butterfly total abundance in *várzea* than in *terra firme* forests, which is the same pattern reported in studies of bats (Pereira et al., 2009) and primates (Haugaasen and Peres, 2005b). The higher abundance of herbivorous, frugivorous and nectarivorous species (such as butterflies, primates and frugivorous bats) in *várzea* is probably due to the higher availability of food resources for these species in these forests. Seasonal flooding by white-water rivers provides an extra input of nutrients in *várzea* soils, which increases forest primary productivity (Irion et al., 2010). Bobrowiec et al. (2014) found that the abundance of frugivorous bats in *várzea* forests is even higher during the high-water season. However, for Amazonian fruit-feeding butterflies, adults tend to be more abundant during the early and mid-dry seasons, and less abundant during the wet

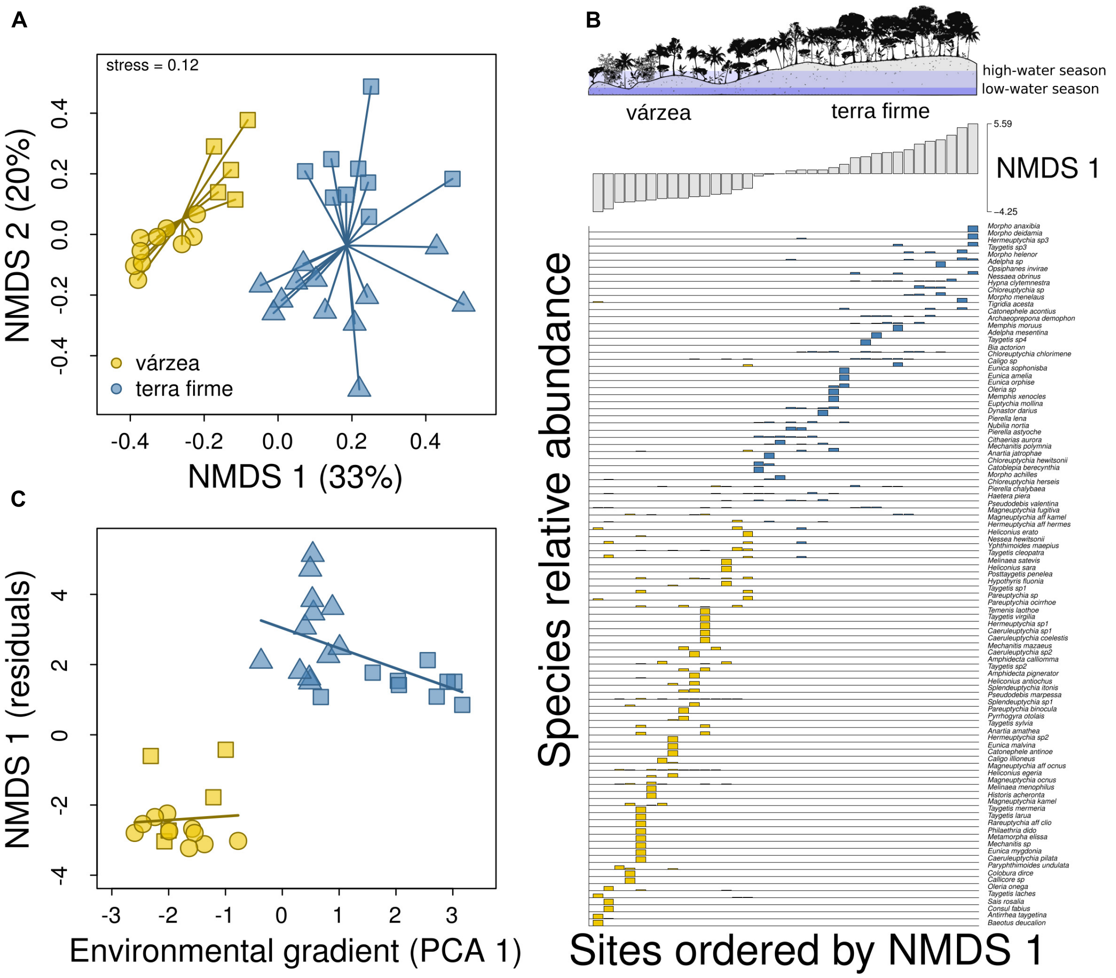

FIGURE 4 | Changes in species composition between *várzea* and *terra firme* forests. **(A)** Butterfly species composition in a bi-plot with the two axes derived from a NMDS ordination. Each point in the graph represents a plot located in *várzea* or *terra firme* forest and the distance between points represents the similarity of plots in terms of their species composition. Square, circle, and triangle symbols represent plots in Baixo Juruá, Mamirauá, and Amanã reserves, respectively. **(B)** Distribution of butterflies across sample sites. Sample sites are ordered by a single NMDS axis and bar heights show the relative abundance of butterfly species across *várzea* (gray) and *terra firme* (black) plots. **(C)** Change in species composition (NMDS 1) with environmental gradients (PCA 1) along and within each forest type, after controlling for the effects of latitude and longitude.

season (Barlow et al., 2007), when they probably occur in other life stages, such as herbivorous caterpillars.

We found that *várzea* forests also had higher species density (i.e., higher numbers of species per plot) than *terra firme*. This apparent difference in the number of butterfly species between the two forest types occurred because we sampled a much higher number of individuals per plot in *várzea* forest. When extrapolating the *terra firme* species richness to the same number of individuals/coverage as the *várzea* sample, the assemblages showed similar overall richness, even though *terra firme* covers a larger area, has more stratified forest structure and is much older than *várzea* forests. A similar species richness between flooded and unflooded forests was also found for arboreal ants (Pringle et al., 2019). Empirical experiments with arboreal arthropods demonstrated that bottom-up mechanisms in which long-term higher input of nutrients increases species density, but slightly decreases the richness of detritivores and herbivores due to increased dominance of common species (Haddad et al., 2000; Gruner and Taylor, 2006). This bottom-up process may explain why we found a higher species density, but not necessarily richness in *várzea* forests.

Previous studies have consistently found poorer assemblages in *várzea* forests for several animal groups (Haugaasen and Peres, 2005b; Bobrowiec et al., 2014; Alvarenga et al., 2018), including a recent study with butterflies (Oliveira et al., 2021). However, most of these studies reported only the species density (i.e., number of species per sampling unit) as a diversity index, and few attempted to estimate species richness by standardizing the number of species by sample size/coverage prior to undertaking such comparisons (but see Pereira et al., 2009; Oliveira et al., 2021). Therefore, the generalization of this pattern was likely based on species density (i.e., number of species per unit habitat), which is different from rarefied (or expected) species richness at constant total abundance (Gotelli and Colwell, 2001).

However, Oliveira et al. (2021) did estimate butterfly species richness as we did, but found higher species richness in *terra firme* forests than in *várzea*. Although our sampling design is different from theirs, which hampers comparisons between studies, there are possible reasons for this difference. One is that they sampled not only Nymphalidae species, but also five other families of butterflies, which increases the overall diversity evaluated. Also, as these authors acknowledge, their limited sample size may have affected their species-accumulation curves, which did not approach an asymptote, so comparisons between curves may have been compromised.

Environmental Filters and Adaptive Species Traits

Butterfly species composition changed with environmental gradients between forest types, but no effect of environmental gradients was detected within each forest type. It is likely that we did not detect the effects of environmental gradients within forest types because our measures of environmental variables were retrieved from GIS databases. Therefore, we caution that measuring micro-habitat characteristics in the field may be more appropriate to evaluate how assemblage structure responds to environmental gradients within each forest type.

On the other hand, species composition changed between *várzea* and *terra firme* forests. According to our PCA ordination, *várzea* forests are located at lower elevations nearer rivers, which causes inundation during the high-water season, and have lower tree cover and canopy height, whereas *terra firme* has a more complex forest structure and does not flood. The differences in butterfly species composition is probably mainly attributable to flooding, which is a direct barrier to the persistence of all ground-dwelling and understorey species during the high-water season (Haugaasen and Peres, 2005a), and even for flying species (birds, Beja et al., 2010; bats, Bobrowiec et al., 2014), such as butterflies.

We also found that *terra firme* assemblages from different protected areas had distinct butterfly composition (see separation of symbols captured by NMDS 2 in **Figure 4A**). We do not believe that these differences are due to an effect of the spatial distance between these assemblages, since we have controlled for potential effects of geographical distance and did not find spatial autocorrelation. While *terra firme* plots from Baixo Juruá Reserve are located in a region with very old soils from Tertiary sandstones, plots from Amanã Reserve are located on more recent soils from Late-Pleistocene, or *paleo-várzea* forests, as they have been called (Irion et al., 2010). Even so, *paleo-várzeas* are more similar to *terra firme* than to *várzea* in terms of topography and vegetation structure (taller and more stratified forests). As shown by the PCA ordination, these upland plots have distinct environmental conditions, depending on the geological formation in which they are located. Therefore, we believe that the distinct butterfly compositions among *terra firme* plots are probably due such environmental differences among study sites.

Habitat conditions may select for the evolution of adaptive traits and behavior, which in turn may affect the ability of species to disperse to and persist at local sites. For example, the evener rank-abundance distribution in *terra firme* forests had a considerable contribution from Haeterini butterflies, which tended to be more abundant in this forest type. Haeterini butterflies are low-flying ground-dwelling species that feed mainly on rotting fruits and other decaying material on the forest floor (Alexander and DeVries, 2012), and adults can be abundant throughout the year (Devries et al., 2012). Wing morphology in Haeterini butterflies has evolved as a response to their habitat-specific flight behavior, i.e., gliding in-ground along the forest floor (Cespedes et al., 2015). Therefore, it is possible that the adaptive wing shape traits of these species play an important role in constraining their dispersal through flooded forests.

Similarly, adaptive behavior may help to explain the larger abundance and richness of Ithomiini species in *várzea* forests. Ithomines commonly form large aggregations, also known as ithomine "pockets" (DeVries, 1987). This gregarious behavior normally occurs during the dry season, when temperatures are higher and air humidity is lower, so the pockets are located in shady forest sites close to water courses (Pinheiro et al., 2008). This adaptive behavior as a response to cope with adverse climate conditions was suggested to be the main factor explaining the formation of the pockets, rather than the occurrence of large concentrations of adult food resources (Pinheiro et al., 2008). Therefore, since most of the ithomine individuals were found in a few *várzea* plots located near the river banks (∼70 m) and very close to small streams, it is likely that the higher abundance of ithomines in *várzea* forests is an adaptation of these butterflies to seek suitable local climatic conditions.

The Role of Biotic Filters

The differences in species composition between the two forest types may also be explained by species interactions, especially with their host plants. For instance, the most frequently captured species in *várzea* assemblages were *Magneuptychia* aff. *ocnus*, *Pseudodebis marpessa,* and *P. valentina*. Larvae of *Magneuptychia* species feed mainly on grasses (Beccaloni et al., 2008), which have high growth rates and rapidly occupy available substratum during the low-water season in *várzea* forests (Silva et al., 2013). *Pseudodebis* species feed on the bamboo *Guadua angustifolia* (Murray, 2001), which was highly abundant in the *várzea* plots where we surveyed most *Pseudodebis* butterflies (Rabelo, person. obs.). On the other hand, *Bia actorion* and *Euptychia molina* were the most frequently captured species in the *terra firme* assemblages. *Bia actorion* feeds mainly on *Geonoma* palms (Freitas et al., 2002), which are considered *terra firme* specialists and rarely occur in *várzea* forests (Muscarella et al., 2019). Similarly, *Euptychia* butterflies are known for their strong relationship with their host plants, Selaginellaceae and Neckeraceae (DeVries, 1985; Hamm and Fordyce, 2016), which are often obligate terrestrial (*Selaginella*) and do not occur in floodplain forests (Poulsen and Balslev, 1991; Junk and Piedade, 1993). As most tropical caterpillars are host specialists and floristic diversity is closely associated with butterfly richness (Morais et al., 2011) and composition (Graça et al., 2015), the distribution of host plants is the primary biotic limitation affecting butterfly composition at local scales.

Another interesting example of how biotic interactions may explain our results can be seen in the Onega clearwing (*Oleria onega*). This species was the fourth most common species in *várzea* forests. *Oleria* are Ithomiini butterflies that are known to feed on alkaloid-rich host plants, which make the adults unpalatable to predators and all species are engaged in mimicry (Brown, 1987; Beccaloni, 1997). Although adults are unpalatable, it has been suggested that their eggs may be subject to predation or removed from leaves by *Ectatomma* ants, which are often found in *Solanum* species (Gallusser, 2002). As *Ectatomma* ants are weak swimmers (Yanoviak and Frederick, 2014) and do not normally occur in Amazonian seasonally flooded forests (Wilson, 1987), we hypothesize that their absence may favor the high abundance of *Oleria* in *várzea* forests.

CONCLUSION

We found that both *várzea* and *terra firme* forests have similar species richness, although the former forest type had higher species density likely due to its higher primary productivity. We also found a pronounced difference in butterfly species composition between *várzea* and *terra firme* forests. The strong turnover of butterfly species was associated with environmental differences between *várzea* and *terra firme*, but not with the environmental change within each forest type. Environmental conditions may select for the evolution of adaptive traits and behavior, which in turn may affect the ability of species to disperse to and persist at local sites. Therefore, our findings reinforce flooding as a primary environmental filter in Amazonian floodplain forests, which strongly determines the composition of butterfly assemblages, as well as the distribution of their interacting biota. The results of this study suggest that environmental and biotic filters override the effects of vegetation stratification and effects of source area on differences in the composition of butterfly assemblages in flooded and unflooded Amazonian sites at local scales.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the KNB Ecoinformatics repository (https://knb.ecoinformatics.org/view/doi:10.5063/B27SQT).

ETHICS STATEMENT

The study and collection of butterfly specimens was reviewed and approved by the Sistema de Autorização e Informação em Biodiversidade (SISBIO), Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio; permission #57444).

AUTHOR CONTRIBUTIONS

RR conceived and designed the study, collected and analyzed the data, and prepared the first draft of the manuscript. GP contributed to the study design, collected the data, and contributed to the manuscript preparation. JV contributed to the idea conception, study design, and manuscript preparation. WM contributed to study design, data analyses, and the manuscript preparation. All authors contributed to the article preparation and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.693178/full#supplementary-material

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River Reorganization Affects Populations of Dwarf Cichlid Species (*Apistogramma* Genus) in the Lower Negro River, Brazil

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Alterations, such as drainage network reorganization, in the landscape in the Amazon basin influence the distribution range and connectivity of aquatic biota and, therefore, their evolution. River capture is a geomorphic mechanism of network reorganization by which a basin captures large portions of the network of a neighboring basin, thus creating a barrier against species dispersal. In this study, the influence of river capture on the genetic differentiation and structuring of two dwarf cichlids species (*Apistogramma pertensis* and *Apistogramma gephyra*) is investigated in two tributaries of the lower Negro River. The analysis of 11 loci microsatellite and three mitochondrial DNA genes (*Cytochrome b*, *Citochrome c Oxidase subunit I* and *16S ribosomal RNA*) confirmed the populational isolation of two dwarf cichlids species, suggesting that they represent evolutionary significant units (ESU) that have been isolated—probably due to the river capture event. The paleovalley that resulted from the river capture is therefore an important physical barrier that separates the populations of the Cuieiras and Tarumã-Mirim Rivers. The findings herein provide evidence of a mechanistic link between the isolation and differentiation of fish populations and the drainage evolution of the Amazon basin, and indicate that the dynamic geological history of the region has promoted species diversification. The process described here partially explains the high diversity in the genus *Apistogramma* and the information obtained is beneficial to conservation programs.

Keywords: *Apistogramma*, microsatellite, mtDNA genes, fish, Amazon, paleovalley, river formation

INTRODUCTION

The Amazon region has gone through many landscape changes, such as the Andes uplift, marine incursions, and river shift (Rossetti and Toledo, 2007; Hoorn et al., 2010), since the Palaeogene period. These large-scale climatic and geologic events share important roles in the making of today's Amazonian biogeography and biodiversity (Hoorn et al., 2010; Rull, 2011). However, the biotic consequences of local landscape changes in the Central and Eastern Amazon that ensued in response to regional landscape changes, such as the formation of the transcontinental Amazon River, remain poorly understood.

Large rivers, such as the Negro River, constitute local barriers that prevent gene flow within many terrestrial species (Naka et al., 2012; Ribas et al., 2012; Boubli et al., 2015). As the position of large rivers changed during the geologic past, new barriers emerged, resulting in the isolation of populations (i.e., vicariant events) and, consequently, in speciation (Ribas et al., 2012; Alfaro et al., 2015; Boubli et al., 2015; Fernandes et al., 2015). Therefore, spatial patterns of aquatic biodiversity in the Amazon region are particularly promising for the investigation of the barrier-induced interruption of gene flow. These geological processes continue to reconfigure the drainage network over many areas of the Amazon River basin possibly due to its paleogeographic evolution (e.g., Hoorn et al., 2010; Stokes et al., 2018).

Endemic fish populations may have become isolated from widely distributed ones through landscape changes (Wise, 2011a,b; Albert et al., 2017), which makes them an ideal subject with which to study speciation related to geologic drivers of landscape change. The dwarf cichlids from the genus *Apistogramma* have over a hundred described species, which are well known to have either allopatric or sympatric geographic distributions (Kullander and Ferreira, 2005; Römer, 2006; Costa et al., 2019). It is estimated that many *Apistogramma* species still need to be described and thus species richness is probably underestimated (Estivals et al., 2020). The dwarf cichlids occur in all types of river waters (clear, black, and white) found in the Brazilian Amazon, though are rarely found in the main channels of the major rivers, which is a reflection of their ecology and restricted habitat requirements (Kullander, 2003). Nonetheless, most of them present a high endemic level (Estivals et al., 2020). The life history of cichlids includes parental care and territorialism, which explains their high rates of endemism. Their color patterns and morphological diversity are associated to the sexual dimorphism between males and females (López-Fernández et al., 2005; Maruska, 2014). Although little is known about the conservation status of this group of cichlids, many authors affirm that some species are sensitive to sudden changes in the environment. As such, these dwarf cichlids from the *Apistogramma* genus are an excellent model group for evolutionary studies related to landscape changes (Quérouil et al., 2015).

Herein, we studied the species *Apistogramma gephyra* and *Apistogramma pertensis*, which present insufficient data regarding their population status and conservation. These two species have been separated by a river capture event involving the Cuieiras and Tarumã-Mirim rivers (former lower reach of the pre-capture Cuieiras basin). Erosive processes across an active fault scarp (i.e., topographic step) led to stream capture and separation of what was once an integrated drainage basin (proto-Cuieiras River basin). The Cuieiras and Tarumã-Mirim Rivers formed a single south-flowing channel as evidenced by the continuous paleovalley that is now the drainage divide between these two basins (i.e., physical barrier) (**Figure 1**; Val et al., 2014). The proto-Cuieiras River basin was captured by a west-flowing tributary of the Rio Negro which had its headwaters at the topographic escarpment that still forms a drainage divide in other locations along-strike. The basins now form two separate drainage basins with a paleovalley containing paleochannel deposits in between. At this time, there is no geochronological constraint on the age of these paleochannel deposits which would provide a direct dating of the river capture event. Numerical modeling of knickpoint migration suggests a Pleistocene age (Val et al., 2014), however, we emphasize that such model uses underconstrained calibration parameters. Genetic ages of ground-birds in the lower Negro River valley suggest a Plio-Pleistocene age of landscape change in the region, also a plausible timeframe for river capture to have occurred (Ribas et al., 2012).

The Tarumã-Mirim River that was left behind retains the original populations that are probably under more pressure than the populations of the Cuieiras River. Both are threatened species affected by habitat degradation and anthropic presence, as well as overfishing due to their ornamental value (Römer, 2006; Junk et al., 2007; Ramos et al., 2015; Tougard et al., 2017). This study links a specific geologic process with biological diversification in the Amazon, and has implications for understanding the mechanisms that have generated the current high diversity patterns and species endemism (Albert et al., 2017), which may help to support management and government conservation policies.

MATERIALS AND METHODS

Sampling

We collected 74 individuals of *A. gephyra* (30 from the Cuieiras River and 44 from the Tarumã-Mirim River), and 39 individuals of *A. pertensis* (27 from the Cuieiras River and 12 from Tarumã-Mirim River) (**Table 1**), thus a total of 113 specimens. The fishing efforts were similar in both rivers (**Figure 1**). The taxonomic identification of both species was carefully performed by two taxonomists from INPA's ichthyology group. The spinal cord of all animals was severed and the whole animal was placed in microtubes with 70% alcohol and transported to the Laboratory of Ecophysiology and Molecular Evolution (LEEM/INPA), where they were stored in a freezer at −80°C for posterior DNA extraction. The collection permits were provided by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio permit #34130-1). Sampling was carried out according to the Brazilian guidelines for animal care and authorized by the Committee on the Ethics of Animal Use and Care—CEUA of the Brazilian National Institute for Amazonian Research—INPA (approval number 048/2012).

Microsatellite Genotyping and Mitochondrial DNA (mtDNA) Sequencing

The total DNA was extracted of muscle tissue using the phenol-chloroform-isoamyl alcohol protocol described by Sambrook et al. (1989). For amplification, we used 11 microsatellite primers for *A. gephyra* and *A. pertensis* (Leitão et al., 2017). To sequence the partial mitochondrial genes, we developed two sets of primers for the genes 16S rRNA and cytochrome oxidase subunit I (*COI*), based on the partial sequences of these mitochondrial

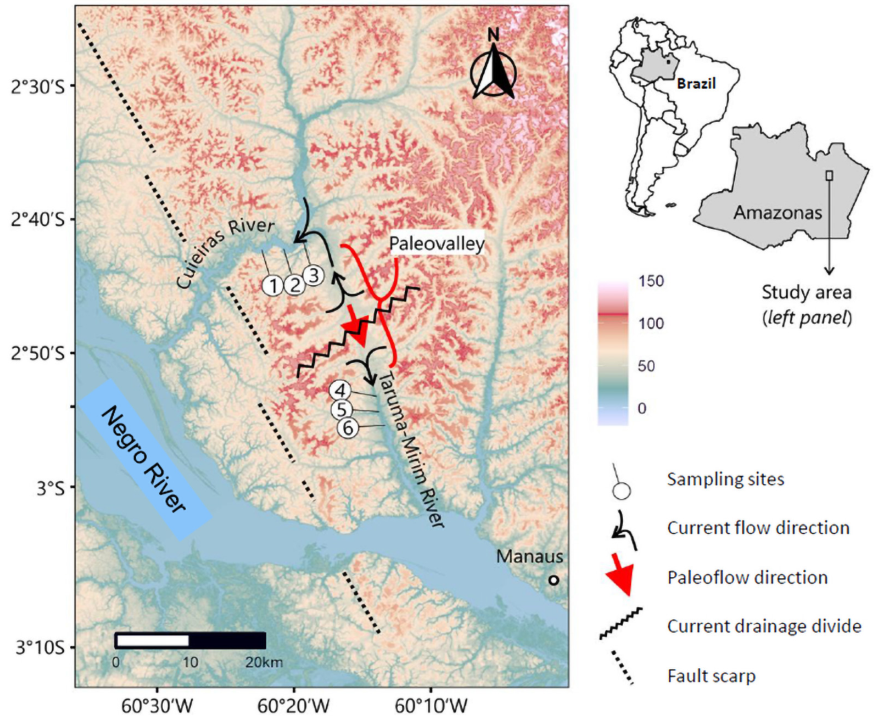

FIGURE 1 | Location and topography of the study site. Color scheme denotes elevations above mean sea level and was obtained from a digital elevation model (*Shuttle Radar Toporaphic Misson*—SRTM, http://earthexplorer.usgs.gov). Map produced by Rodrigo Nahum, 2020. Locations of sampling sites, rivers, and other features of the landscape are labeled. Current and past directions of river flow are highlighted by arrows (see key).

TABLE 1 | Allelic and genetic diversity within populations of *Apistogramma gephyra* and *Apistogramma pertensis* from the Cuieira River (CR) and the Tarumã-Mirim River (TMR), based on microsatellite data.

		Allelic diversity					Genetic diversity			
Species	**River**	**N**	**TNA**	**MNA**	**ENA**	**NPA**	**AR**	***He***	***Ho***	***Fis***
A. gephyra	CR	30	69	6.273	2.725	9	4.803	0.633	0.662	–0.047
	TMR	44	79	7.182	3.021	25	5.101	0.669	0.631	0.058
A. pertensis	CR	27	62	5.636	2.967	5	4.601	0.663	0.742	–0.123
	TMR	12	53	4.818	2.577	13	4.545	0.612	0.664	–0.089

N, sample size; TNA, total number of alleles; MNA, mean number of alleles; ENA, effective number of alleles; NPA, number of private alleles; AR, allelic richness; H_E, expected heterozygosity; Ho, observed heterozygosity: F_{IS}, fixation index; CR, Cuieiras River; TMR, Tarumã-Mirim River.

genes for the *Apistogramma* species available at GenBank—National Center for Biotechnology Information (NCBI). For the cytochrome b gene (*Cyt-b*), We used the primers developed by Taberlet et al. (1992) and Lydeard and Roe (1997), as suggested by Farias et al., 2001 (**Supplementary Table 1**). The polymerase chain reactions (PCR) conditions were as follows: for a 25 μL total volume, we used 4.0 μL of total DNA (50 ng μL^{-1}), 1.5 μL of forward primer (5 μM), 1.5 μL of reverse primer (5 μM), 5.5 μL of H_2O, and 12.5 μL PCR Master Mix 2X [Taq DNA polymerase (0.05 U), MgCl2 (1.5 mM), and dNTPs (0.4 mM)] (Fermentas, Waltham, MA, United States). Amplifications were performed with the following PCR profile: initial denaturation at 94°C for 2 min, followed by 35 cycles with denaturation at 94°C for 1 min, annealing at 50–51°C for 1 min, polymerization at 72°C for 1 min, and a final extension at 72°C for 5 min. We confirmed the amplified PCR products by electrophoresis in a 1.5% agarose gel using GelRed® (Invitrogen, Waltham, MA, United States), and we visualized it using an L-PIX Molecular Image transilluminator (Loccus Biotecnologia, Cotia, Brazil).

The genotyping of the microsatellites and the sequencing of the mitochondrial genes were performed in a genetic analyzer (ABI 3130xl, Applied Biosystems, Waltham, MA, United States) using GeneScan Liz-500 (–250) size standard to determine the fragment length of the microsatellite, and the Big Dye Terminator v3.1 kit for sequencing (Applied Biosystems, Waltham, MA, United States. The microsatellite alleles were scored based on the consistent pattern of their stutter peaks and on the peak intensity corresponding to each individual at each locus using GeneMapper v4.0 (Applied Biosystems, Waltham, MA, United States). The quality of the sequencing was verified using the 3,130 series data collection software v4 and Sequencing Analysis software v6 (Applied Biosystems, Waltham, MA, United States), respectively.

Microsatellite DNA Analysis

Using the following allelic diversity parameters of *A. gephyra* and *A. pertensis* populations, we calculated the total number of alleles (TNA), mean number of alleles (MNA), number of private alleles (NPA) using GDA v1.1 software (Lewis and Zaykin, 2002). Allelic richness (AR) was acquired using FSTAT v2.9.3.2 software (Goudet, 2001), and the number of effective alleles (NEA) was calculated using the mathematical model: NEA = $1/(1–H_E)$. We estimated the genetic diversity through the observed and expected heterozygosity (H_O and H_E, respectively) in Hardy–Weinberg equilibrium, fixation index (F_{IS}) using GENETIX v4.05.2 software (Belkhir et al., 2009).

We applied the mixture model (Admixture) ancestor to analyze the structures of the populations, which correlates the gene frequency among the studied populations. This was performed using STRUCTURE v2.3.1 (Pritchard et al., 2000; Falush et al., 2003) with a burn-in of 50,000 followed by 200,000 steps using the Markov chain Monte Carlo method (MCMC). Each analysis was repeated 10,000 times from a different randomly selected starting point, and convergence between independent runs was assessed via examination of α values and profile of posterior probabilities. The Q values from each of the 10 independent runs for each K scenario were extracted using the program STRUCTURE HARVESTER 0.6.92 (Earl and von Holdt, 2012) and summarized in the program CLUMPP 1.1.2 (Jakobsson and Rosenberg, 2007). Results were visualized in the program DISTRUCT 1.1 (Rosenberg, 2003), and a run for each analysis was performed for $K = 1$ and K = number of population samples + 2, with ten replicates for each K. We applied the value of ΔK suggested by Evanno et al. (2005) to identify the highest level of genetic division hierarchy.

The levels of genetic differentiation were analyzed using Wright's F-statistics F_{IS} and F_{ST} (Weir and Cockerham, 1984) using ARLEQUIN v3.5 software (Excoffier and Lischer, 2010), and the genetic distance (GD) was calculated using GENETIX v4.05.2. We also calculated the number of migrants (Nm) between populations by applying the F_{ST} values and the molecular variance (AMOVA) using the ARLEQUIN v3.5 software at the significance level of 5%.

Mitochondrial DNA Analysis

The sequences were edited and aligned using Geneious R7.1.8 (Biomatters Ltd., Auckland, New Zealand) and the Clustal W algorithm (Larkin et al., 2007). The genetic diversity, the total number of haplotypes (NH), the haplotype diversity (h) and the nucleotide diversity (π) (Nei, 1987) was obtained using DnaSP v5.10.1 (Librado and Rozas, 2009). Tajima's D test (Tajima, 1989) and Fu's FS test (Fu, 1997) were used to verify the occurrence of recent population size changes.

An analysis of molecular variance (AMOVA) was performed in ARLEQUIN v3.5. Population structure analysis was performed using the Bayesian approach implemented by BAPS 4.14 (Corander et al., 2007), in which the number of clusters was defined using an algorithm that estimates the distribution of haplotype frequencies of all populations tested. For these analyses, we applied two approaches: (1) two groups according to the rivers, to verify the segregation of the two species populations; and (2) two groups according to the species identification, to identify whether their groups are distinct and disconnected, and whether they present differences as populations between the rivers.

RESULTS

Microsatellite DNA Results

The analysis of allelic and genetic diversity between the populations of *A. gephyra* and *A. pertensis* from the Cuieiras River (CR) and Tarumã-Mirim River (TMR) revealed a similar mean number of alleles (MNA) (**Table 1**). The number of private alleles (NPA) ranged from 5 to 9 for *A. pertensis* and *A. gephyra* from the Cuieiras River, and 13–25 for *A. pertensis* and *A. gephyra* from the Tarumã-Mirim River, respectively.

The results of the structural analyses for the microsatellite data clearly show the separation of the two species within each studied river ($K = 2$) and that ΔK = 430 (**Supplementary Figure 1**) yield a clear separation between the species analyzed. The same result was observed with DNA mitochondrial data using BAPS. For $K = 3$ and $K = 4$, we observed the formation of two distinct populations for each species between the Cuieiras and Tarumã-Mirim Rivers, respectively (**Figure 2**). For K = 4, a possible sign of hybridization is suggested between the species *A. gephyra* and *A. pertensis* in the Cuieiras River, but in order to verify the actual existence of such hybridization process, it would be necessary to increase the number of samples, as well as increase the number of microsatellite markers.

The genetic distance (GD) between populations is compatible with the expected result for the genetic differentiation index (F_{ST}), and higher values are described between the populations of *A. pertensis* from the two rivers (0.228 GD and 0.285 F_{ST}) when compared to *A. gephyra* populations from the two rivers (0.120 GD and 0.128 F_{ST}), respectively (**Table 2**). The number of migrants (Nm) was 0.85 between *A. pertensis* from the Tarumã-Mirim River and Cuieiras River and, interestingly, 1.83 between the populations of *A. gephyra* from the two rivers.

The AMOVA analysis (**Table 3**) indicates that there are significant differences among individuals of the same population and between different populations for both species. These results are consistent with the F_{ST} values (**Table 2**), which show structure among different populations, as corroborated by the significant genetic structure when all sampled individuals were analyzed using the STRUCTURE program (**Figure 2**).

Mitochondrial DNA Results

The number of sequences obtained for the three mitochondrial genes were unequal among populations: 69 sequences for *16S*, 62 for *COI*, and 112 for *Cyt-b* (see **Table 4**). Despite this, the size of the amplicons and the quality of the DNA were acceptable for our main goal, which was to verify the structure of the two populations from the two species supposedly separated by

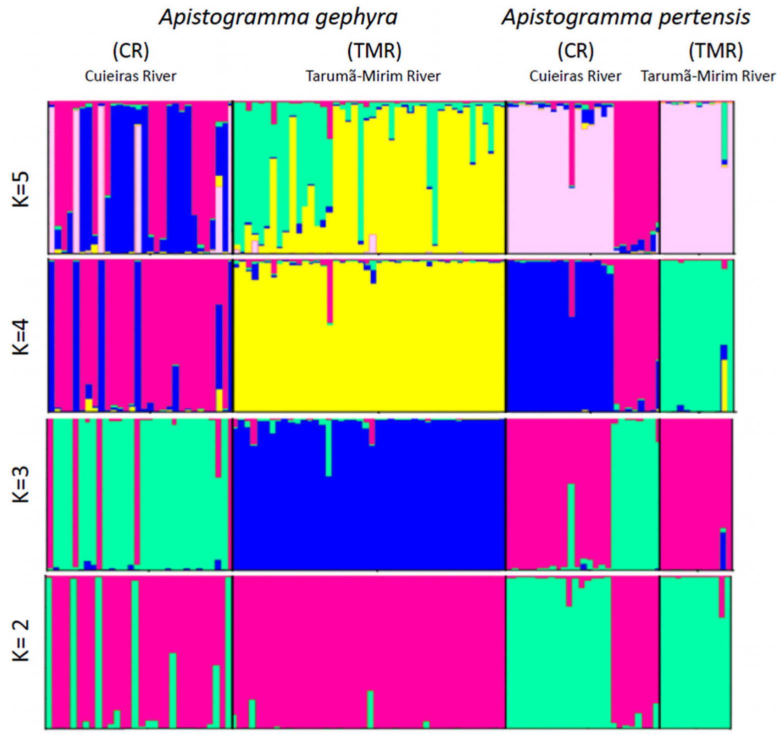

FIGURE 2 | Structure analysis. Graphs obtained using STRUCTURE software. Analysis of all the populations for both species (a) based on 11 microsatellite *loci* shows $K = 5$.

TABLE 2 | Upper diagonal: genetic distance; bottom diagonal of the bold: F_{ST} and Nm (in parentheses), based on microsatellite data.

		Apistogramma gephyra		***Apistogramma pertensis***	
		CR	**TMR**	**CR**	**TMR**
Apistogramma gephyra	CR	–	0.12005	0.06131	0.30251
	TMR	**0.1279* (1.83)**	–	0.17561	0.29286
Apistogramma pertensis	CR	**0.0633* (3.83)**	**0.1931* (1.17)**	–	0.22769
	TMR	**0.3603* (0.58)**	**0.3465* (0.60)**	**0.2584* (0.85)**	–

*F_{ST}, structuring index; Nm, number of migrants; CR, Cuieiras River; TMR, Tarumã-Mirim River. *Indicates a significant difference when $p < 0.05$.*

TABLE 3 | Analysis of molecular variance (AMOVA) of *Apistogramma gephyra* and *Apistogramma pertensis* based on the 11 microsatellite loci.

	A. gephyra* and *A. pertensis				
Source variation	**Sum of Squares**	**% Var**	**Component variance**	**Standard error**	***p*-value**
Between individual	387.500	85.3328	3.7176	0.1790	0.0002*
Within individual	336.488	–2.9846	–0.1300	–0.0155	0.7797
Within population	121.545	17.6518	0.7690	0.1916	0.0000*

**Indicates a significant difference when $p < 0.001$.*

the river capture. Due to this unequal sampling, we performed population analysis for each mitochondrial gene separately. The analysis of molecular variance (AMOVA, $p < 0.001$) for both species (*A. gephyra* and *A. pertensis*) indicates high variation within the species (**Table 5**), confirming that populations from both rivers are structured within each species for all genes.

Haplotype diversity (h) for *Cyt b* and *COI* genes was similar for *A. gephyra* and *A. pertensis* in the two rivers. For the *16S* gene, lower values (0.209 and zero) were observed for the populations of *A. gephyra* and *A. pertensis,* respectively, in the Tarumã-Mirim River when compared to the Cuieiras River. The genes *16S* and *COI* showed higher nucleotide diversity (π) in the Cuieiras River for both species when compared to the Tarumã-Mirim River. However, a low nucleotide diversity (π) was found in the *Cyt-b* gene in both *A. gephyra* and *A. pertensis* (**Table 4**).

The neutrality tests of Tajima (D) and Fu (Fs) showed negative values in the two populations of both rivers, and one positive value was observed in the Fu test for *A. pertensis* (*Cyt-b* gene). The neutrality tests were not significant for the *Cyt-b* gene in either species (**Table 4**). Therefore, the Tajima test indicates an excess of recent mutations in both populations of *Apistogramma*, while the Fu test did not suggest any recent population expansion in either of the *A. pertensis* populations based on the *Cyt-b* gene. The results obtained for the pairwise genetic differentiation index (F_{ST}) among the populations of dwarf cichlids showed significant statistical values ($P < 0.001$), with the only exception being detected for the *COI* gene in *A. pertensis* populations, which presented no difference in F_{ST}

TABLE 4 | DNA Polymorphism and neutrality tests for *Apistogramma gephyra* and *Apistogramma pertensis* populations from the Cuieiras River (CR) and Tarumã-Mirim River (TMR).

					Index of molecular diversity		**Neutrality tests**		
Species	**Gene**	**Population**	**NAS**	**NH**	**H**	**π**	***D***	**F_s**	***P***
A. gephyra	*16S*	CR	5	4	0.900	0.014	–2.376	–3.405	$p < 0.02$
		TMR	45	4	0.209	0.002			
	COI	CR	22	14	0.874	0.032	–1.592	–0.842	$p < 0.02$
		TMR	15	4	0.619	0.002			
	Cytb	CR	65	3	0.528	0.008	–0.461	–0.758	$p < 0.10$
		TMR	14	11	0.967	0.013			
A. pertensis	*16S*	CR	8	4	0.643	0.014	–1.193	–1.164	$p < 0.02$
		TMR	11	1	0	0			
	COI	CR	20	7	0.584	0.010	–2.441	–3.890	$p < 0.02$
		TMR	5	2	0.600	0.001			
	Cytb	CR	27	5	0.578	0.002	–0.673	0.415	$p < 0.10$
		TMR	6	5	0.933	0.006			

NAS, number of analyzed sequences; NH, number of haplotypes; S, polymorphic sites; h, haplotype diversity; π, nucleotide diversity; D, Tajima's index; F_S, Fu's index, and p, significance of the F_S value. Significance level ($p < 0.05$) – DNASp.

TABLE 5 | Analysis of molecular variance (AMOVA) of *Apistogramma gephyra* and *Apistogramma pertensis* based on mtDNA genes.

		Apistogramma gephyra			***Apistogramma pertensis***		
Gene	**Source variation**	**Component Var.**	**% Var.**	**F_{st}**	**Component Var.**	**% Var.**	**F_{st}**
16S	Between population	0.8925	55.64	0.721	1.8440	51.52	0.377
	Within population	0.7116	44.36		1.7353	48.48	
COI	Between population	2.4277	30.37	0.356	0.3035	7.39	0.280*
	Within the population	5.5651	69.63		3.8022	92.61	
Cytb	Between population	1.1678	35.27	0.352	0.3105	33.93	0.339
	Within population	2.1436	64.73		0.6047	66.07	

**Indicates a significant difference when $p < 0.001$.*

values ($P > 0.05$). The indexes for the genes *16S, COI,* and *Cyt-b* were, respectively, 0.721, 0.356, and 0.352, for *A. gephyra* from the Cuieiras River and the Tarumã-Mirim River; and 0.377, 0.280 and 0.339 for *A. pertensis* from the Cuieiras and Tarumã-Mirim Rivers (**Table 5**). This analysis also indicates high levels of population genetic structure among them.

Analysis of population structure in BAPS based on *16S rRNA* and *COI* genes clearly shows the separation of the two species between the Cuieiras and Tarumã-Mirim Rivers (**Figures 3A,C**). From the *Cyt-b* data, we observed a separation of all *A. pertensis* specimens, though we observed several clusters for *A. gephyra* (**Figure 3E**). When we analyzed the species separately, the results showed a clear differentiation between the rivers for both species. On the other hand, when we observed the *Apistogramma* populations for the Cuieiras River, we verified two or three clusters formed for the mitochondrial genes, and only one cluster in the Tarumã-Mirim River (*A. gephrya—16S rRNA*, *COI*, and *Cyt-b*; and *A. pertensis—16S rRNA* and *COI*, respectively) (**Figures 3B,D,F**). A similar result was observed for the microsatellite data after the analysis using the STRUCTURE program, by which we verified a higher gene flow between both species occurring in the Cuieiras River. In regards, the BAPS analysis corroborates the STRUCTURE analysis for microsatellite data (**Figure 2**).

DISCUSSION

Population Structure Is Affected by Landscape Changes

Geological events may have been important in the diversification of the genus *Apistogramma* (Römer, 2006), as well as the biology of this genus, and the habitat characteristics influence the genetic structure of their populations (see **Figures 4A–C**). Thus, understanding genetic structure is critical in evolutionary and conservation biology, and discriminating fish sub-populations is essential for detecting possible reproductive isolation (Santos et al., 2016).

The results from the microsatellite data indicate low levels of gene flow between populations of the same species from the different rivers (**Table 2**). These results suggest diversification among populations within each species and the possibility that a local adaptive process is occurring. On the other hand, we cannot discard the possibility that genetic drift caused allele fixation. In fact, the occurrence of local adaptation and random

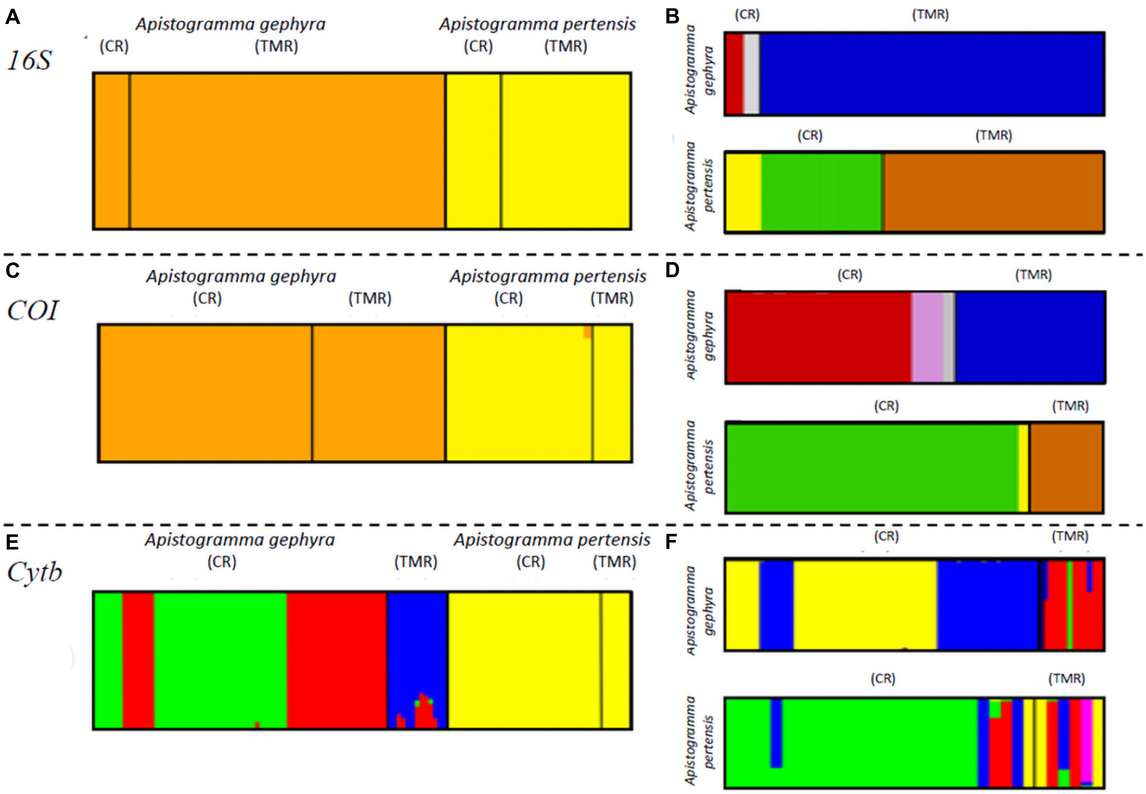

FIGURE 3 | Bayesian analysis of population structure (BAPS). **(A,C,E)** Refer to *Apistogramma gephyra* and *Apistogramma pertensis* from the Cuieiras River (CR) and Tarumã-mirim River (TMR) Rivers presenting, respectively, *16S, COI* and *Cytb* mitochondrial genes; **(B,D,F)** refer to both species analyzed separately between rivers using, respectively, the genes *16S, COI,* and *Cytb.*

allele fixation by genetic drift leads to a reduced number of genotypes (Blanquart et al., 2012). Moreover, local adaptation is also sensitive in small populations and to the balance between gene flow and local selection.

When gene flow is limited, specialized genotypes can be maintained in isolated populations and support local adaptation, thus providing important insight into evolutionary processes and adaptive divergence of populations (Blanquart et al., 2012, 2013). A key prerequisite for the emergence of local adaptation is the existence of a spatially heterogeneous environment, which generates a heterogeneous selective pressure (Blanquart et al., 2013). The region where the Cuieiras and Tarumã-Mirim Rivers are located is constantly changing, which involve this type of study. In addition, the drainage network of the Amazon is not static but is, in fact, constantly changing over time (Albert et al., 2018). All those factors induce allele fixation in these populations, genetic drift and local adaptation to habitat heterogeneity, thus making this a perfect ecosystem for evolutionary studies.

Interestingly, the nuclear data indicates genetic similarity between the two species of the Cuieiras River. However, this pattern is not seen in the mtDNA data and the two species form a well-supported high differentiation. We considered that these findings indicate that gene flow occurs in the Cuieiras River, which affects only the nuclear genome, though not the maternally inherited mtDNA. To have greater certainty regarding these processes, further studies must explore a higher and more variable number of markers (e.g., SNPs) and use more rapidly evolving mitochondrial genes (e.g., mtDNA control region).

Thus, the lack of support for intraspecific clades in mtDNA may be due to the slow evolutionary rate of the markers utilized in this study, while the nuclear microsatellites clearly show an intraspecific differentiation in the different drainage systems. Another explanation would be periodic connectivity or delayed disconnection between the Tarumã-Mirim and Cuieiras Rivers, as observed in ongoing drainage captures elsewhere in the Amazon (Stokes et al., 2018). This would allow eventual gene flow among sub-populations (or evolutionary significant units—ESU). Although the sample size was unequal for the populations of Tarumã-Mirim and Cuieiras Rivers, the level of polymorphism for both the microsatellites (Leitão et al., 2017) and the number of mitochondrial genes was substantial enough to reach these conclusions.

Tajima's D test results in negative values after a recent bottleneck, which indicates population expansion (Fu. 1997). The statistical values for Fs are also very sensitive to deviations from neutrality that are promoted by demographic changes. Herein, they resulted in negative values under most conditions and were positive only for *A. pertensis* (*Cyt b*).

The distribution of *Apistogramma* species is higher in Cuieiras River than the drainage basin of the Tarumã-Mirim River

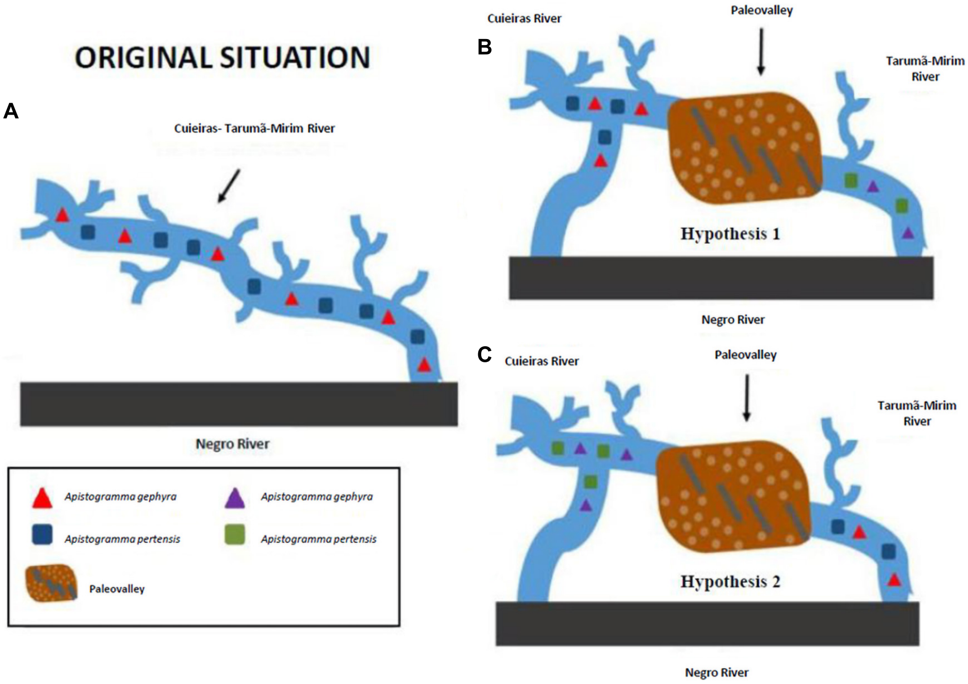

FIGURE 4 | Hypothetical scenarios. The hypothetical scenarios are delineated to propose an explanation for the strong genetic structure observed between the populations of *Apistogramma gephyra* and *Apistogramma pertensis* in the two rivers (Cuieiras and Tarumã-Mirim): **(A)** *A. gephyra* and *A. pertensis* populations from the Tarumã-Mirim River differentiated after the geographic isolation, with the lower Tarumã-Mirim River region being influenced by populations from the lower Negro River; and/or **(B,C)** the landscape changes in the course of the Cuieiras River and provided a wide area of distribution and allowed some gene flow between the species, thus adding more complexity to the differentiation between populations, with potential influences from individuals of the Anavilhanas populations just west of the mouth of the Cuieiras River.

(**Figure 1**). This is consistent with an increase in species range as the Cuieiras River has gained drainage area due to river capture. Thus, a larger gene flow has been detected between species of dwarf cichlids from the Cuieiras River, possibly due to mating occurring at higher levels, though with production of infertile offspring. We suggest that the mating observed between the two dwarf cichlids species in the Cuieiras River occur because these two species have not differed enough to prevent reproductive mating after the river capture event, which means they might still maintain a correlation with the original populations (Hypothesis 1, **Figure 4B**). In addition, both species from Tarumã-Mirim River have been genetically disconnected because they inhabit a fragmented and smaller area, and this may have caused higher environmental pressure on these populations. In addition, we must emphasize that these genetic studies are contemporary, and that the current landscapes may have a strong influence on our results. Thus, river captures may be considered a mechanism that promotes fish biodiversity, particularly in non-migratory fish species, and contributes to the genetic diversity at the population level, as in the present case (Albert et al., 2017).

An alternative explanation for the gene flow in the Cuieiras River is presented in Hypothesis 2 (**Figure 4C**): for the same reason as above, i.e., the populations in the Tarumã-Mirim River after river capture are the original populations prior to river capture (**Figure 4C**). Thus, our preferred explanation, prior to capture, is that these two species remained there, though with no gene flow.

The current pressure that affects the Tarumã-Mirim River is due to the urban expansion of Manaus, which probably affect these populations. The conservation approach over these populations must be taken to avoid disappearance in the future. In addition, the population of the Cuieiras River has evolved in a larger population with more landscape changes over time, which means that the river capture induced all the changes in this river and left the Tarumã-Mirim River behind. The latter remained a short (**Figure 1**) river and is the final part of the original river.

Based on the results, we are able to affirm that, based on the structured populations of both species in both rivers, these two species have given rise to four populations, and that these four populations will most probably generate new species through interruption of gene flow.

The current literature is of the consensus that a mosaic of events must have occurred at different times and spatial scales and that this created a complex scenario of spatial and temporal landscape changes that have influenced the biodiversity in the Amazon we see today (Hoorn et al., 2010, 2017; Rull, 2011, 2013; Gorini et al., 2014; van Soelen et al., 2017). Our study suggests that the populations of *Apistogramma* were affected by landscape changes that contributed to speciation and, eventually, species enrichment. Thus, even at smaller spatial scales, local landscape changes are important in creating biodiversity.

Conservation Implications

The cichlids are extremely interesting fish not only for adaptive radiation studies, but also for studies of behavior and reproductive characteristics (Turner, 2007). About 112 dwarf cichlids species are described as being part of the *Apistogramma* genus, and wide distribution throughout South America (Costa et al., 2019), particularly in the Amazon basin. This extraordinary diversity and high endemism rate found for dwarf cichlids has been explained by the geological, paleoclimatic, and water-level history that has played an important role in speciation events by isolating populations and favoring the establishment of reproductive barriers (Römer, 2006). Thus, new conservation strategies for these species and ecosystems should be rethought; mainly in relation to the vulnerability of the population structure of *Apistogramma* species that live in small basins and tributaries of large rivers to extreme anthropic changes.

Thus, for conservation measures to be effective, they must be based on the information between environment and organism, in order to avoid local extinction of threatened species. The integration of some types of analyses, such as species distribution, phenotypic variation, landscape change, and population genetics, can contribute to management and conservation strategies (Santos et al., 2011, 2016; Escobar et al., 2015; Gravena et al., 2015). In addition, knowledge of the biology of the species is vital for defining a conservation strategy, particularly in areas under environmental pressure. Most species of the genus *Apistogramma* are restricted to a single river or adjacent floodplains and nearly never found in the main river channel (Kullander, 2003; Römer, 2006).

Genetic population studies have constantly revolutionized our theories regarding evolutionary processes (De la Ossa-Guerra et al., 2020), and genetic variability in this case is considered an important factor for understanding these evolutionary processes and for planning conservation programs (International Union for Conservation of Nature [IUCN], 2020). Thus, understanding population structure is crucial for management actions for evolutionary significant units (ESU); however, developing conservation strategies for establishing priority units has historically been difficult. The low migration rate and the high population structure imply that *A. gephyra* and *A. pertensis* populations from Cuieiras and Tarumã-Mirim Rivers should be managed separately at the moment, each as an independent ESU (Avise, 2000; Baker et al., 2002) and, therefore, taxonomic units have to be conserved considering these aspects (De la Ossa-Guerra et al., 2020). This operational concept is often used to guide short-term management strategies, as it refers to current population structures and allele frequencies (Moritz, 1994).

The two dwarf cichlid species analyzed in this study (*A. gephyra* and *A. pertensis*) belong to two drainage networks that were separated by a river capture event. Based on our results, the paleovalley that now separates the Cuieiras and Tarumã-Mirim River basins is seen to have contributed to the local interruption of gene flow, and led to the development of structured populations of both species. According to De la Ossa-Guerra et al. (2020), in order to preserve evolutionary processes, species management must preserve natural networks of genetic connections among populations, rather than just the isolated populations within that network. On the other hand, our results support that geological changes induce new river courses that may cause genetic diversification in aquatic fauna, thus increasing its biodiversity. Moreover, given that events resulting in landscape changes, such as the one studied here, have been common throughout the Amazon basin during and after the Miocene (Rossetti et al., 2005, 2016; Almeida-Filho and Miranda, 2007; Hayakawa et al., 2010; Hoorn et al., 2010; Hayakawa and Rossetti, 2015), we may theorize that small geological processes are capable of producing vicariant events and should be considered within the complex framework of biodiversity drivers in the Amazon Basin. Further studies will clarify the ongoing population structure of *A. gephyra* in the Cuieiras River to better explain the influence of other surrounding geological events, such as the formation of the Anavilhanas Archipelago and the influence of the main channel (Negro River), on the emergence of new genetic information in this species.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI (accession: OK625450–OK625526 and OL310106–OL310174).

ETHICS STATEMENT

The animal study was reviewed and approved by Committee on the Ethics of Animal Use and Care—CEUA of the Brazilian National Institute for Amazonian Research—INPA (approval number 048/2012).

AUTHOR CONTRIBUTIONS

CL, PV, AV, and VA-V conceived the study. CL, CS, and ÉS conducted analyses, collected samples, obtained permits, and conducted laboratory procedures. CL and ÉS wrote the manuscript with input from all authors. VA-V supervised all study steps. All authors read and approved the present version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.760287/full#supplementary-material

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Landscape Evolution as a Diversification Driver in Freshwater Fishes

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The exceptional concentration of vertebrate diversity in continental freshwaters has been termed the "freshwater fish paradox," with > 15,000 fish species representing more than 20% of all vertebrate species compressed into tiny fractions of the Earth's land surface area (<0.5%) or total aquatic habitat volume (<0.001%). This study asks if the fish species richness of the world's river basins is explainable in terms of river captures using topographic metrics as proxies. The River Capture Hypothesis posits that drainage-network rearrangements have accelerated biotic diversification through their combined effects on dispersal, speciation, and extinction. Yet rates of river capture are poorly constrained at the basin scale worldwide. Here we assess correlations between fish species density (data for 14,953 obligate freshwater fish species) and basin-wide metrics of landscape evolution (data for 3,119 river basins), including: topography (elevation, average relief, slope, drainage area) and climate (average rainfall and air temperature). We assess the results in the context of both static landscapes (e.g., species-area and habitat heterogeneity relationships) and transient landscapes (e.g., river capture, tectonic activity, landscape disequilibrium). We also relax assumptions of functional neutrality of basins (tropical vs. extratropical, tectonically stable vs. active terrains). We found a disproportionate number of freshwater species in large, lowland river basins of tropical South America, Africa, and Southeast Asia, under predictable conditions of large geographic area, tropical climate, low topographic relief, and high habitat volume (i.e., high rainfall rates). However, our results show that these conditions are only necessary, but not fully sufficient, to explain the basins with the highest diversity. Basins with highest diversity are all located on tectonically stable regions, places where river capture is predicted to be most conducive to the formation of high fish species richness over evolutionary timescales. Our results are consistent with predictions of several landscape evolution models, including the River Capture Hypothesis, Mega Capture Hypothesis, and Intermediate Capture Rate Hypothesis, and support conclusions of numerical modeling studies indicating landscape transience as a mechanistic driver of net diversification in riverine and riparian organisms with widespread continental distributions.

Keywords: landscape evolution, tropical biodiversity, river capture, macroecology and macroevolution, biogeography, geobiology

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INTRODUCTION

Freshwater or continental fishes constitute one of the most species rich—and species dense—vertebrate faunas on Earth (Tedesco et al., 2017a). With more than 18,167 species freshwater fishes constitute about 26% of all living vertebrate species (Fricke et al., 2021), yet occupy a tiny fraction (less than 0.01%) of Earth's total surface area, and an even smaller proportion (0.001%) of Earth's total aquatic habitat volume (Lundberg et al., 2000). Such a high concentration of biodiversity in continental freshwaters has been termed the "freshwater fish paradox" (Tedesco et al., 2017b; McDermott, 2021). Freshwater fishes also exhibit high values of other prominent biodiversity metrics, such as ecological and physiological diversity (Helfman et al., 2009), genetic diversity (Manel et al., 2020) and phylogenetic and taxonomic disparity (Li et al., 2020; Su et al., 2021).

Studies of freshwater fish diversity have focused on salient features of landscape heterogeneity and landscape evolution, focusing on rapid speciation in tectonic lakes (McGee et al., 2020), ancient diversification in the global Greenhouse world of the Paleogene and Upper Cretaceous (Miller and Román-Palacios, 2021), the dendritic habitat architecture of river drainage networks and riverine population structure (Fagan, 2002; Thomaz et al., 2016), and the role of river capture dynamics in fragmenting and merging riverine ecosystems through time and space (Burridge et al., 2006, 2007; Albert et al., 2018a, 2020). From a macroevolutionary perspective, the rate of net lineage diversification is a function of the constituent rates of speciation and extinction, clade age, and depending on conditions, the carrying capacity or maximum species-density of a geographic region (**Figure 1**).

All evolutionary processes involve complex interactions among intrinsic organismal traits and extrinsic environmental factors (Jablonski, 2017; Saupe and Myers, 2021). Organismal traits associated with diversification in freshwater fishes include body size, feeding and locomotory specializations, habitat utilization, reproductive modes, and dispersal capacity (e.g., Davis et al., 2016; Kolmann et al., 2020; Burns, 2021). These functional traits may also affect the ecological processes that govern local species coexistence (alpha diversity) and regional species richness (beta diversity) (Winemiller, 1991; Matthews, 2012; Salgueiro et al., 2021). Yet the combined effects of these biological processes depend critically on physical environmental settings related to climate and geography, which alter the connections, persistence, and quality and configuration of freshwater habitats (Pringle, 2003; Smith et al., 2010; Dias et al., 2014). By altering the geographic range and/or configuration of landscape surfaces geomorphic processes may influence the rates of macroevolutionary diversification (**Figure 1**; see also Badgley et al., 2017).

The evolution of river drainage networks depends on landscape evolution processes that can be classified into one of two groups based on how they affect landscape equilibrium (Moodie et al., 2018): (1) Internal or autogenic processes (i.e., fluvial incision, aggradation, landsliding) occur continuously on all ice-free continental surfaces, transforming landscape structures like the geometry of fluvial networks and valley spacing toward erosional equilibrium (e.g., Perron et al., 2009; Willett et al., 2014; Scheingross et al., 2020). Such autogenic processes include differential exhumation of rocks with contrasting erodibility (e.g., Gallen, 2018), formation of stepped bedrock morphologies (e.g., Scheingross et al., 2019), and alluvial dynamics (e.g., Hajek and Straub, 2017); (2) external or exogenic perturbations, such as tectonism, precipitation change, and sea level change, modify the boundary conditions of underlying erosional processes, pushing landscapes away from erosional equilibrium (Densmore and Hovius, 2000; Crosby and Whipple, 2006; Kirby and Whipple, 2012; Whittaker, 2012). Erosion rates over landscapes increase with tectonic activity, but the latter possibly lowers the probability of river capture events in high-relief regions (Portenga and Bierman, 2011; Kirby and Whipple, 2012; Lyons et al., 2020).

Both autogenic and exogenic landscape evolution processes can drive changes in river basin base-level, defined as the lowest elevation or mouth of a drainage basin (Goudie, 2004). Base-level changes can promote disequilibrium in erosion rates that propagate upstream as a wave of migrating knickpoints, defined as a change in the channel slope which can be locally convex-up such as waterfalls and rapids, or concave-up such as a lake (Pazzaglia and Brandon, 1996; Tinkler, 2004). For the physical landscape, this imbalance is reflected as transient changes in topography and local relief (e.g., Gallen et al., 2013; Whipple et al., 2017). If the imbalance affects neighboring basins differently, they may create spatially heterogeneous relief across drainage divides (e.g., Gilbert, 1877; Forte and Whipple, 2018) and trigger discrete or continuous river network changes, such as river captures and geometric network disequilibrium, respectively (e.g., Willett et al., 2014; Beeson et al., 2017; Stokes et al., 2018).

River capture is a landscape evolution process in which topographic change alters the river networks and drainage areas of adjacent basins (Bishop, 1995). During this process a river network portion is disconnected from its original larger network and joined to the river network of the adjacent basin, all of which impact fluvial connectivity and resident biotas (Burridge et al., 2006). Over time the spatial configuration of river networks and the drainage area of basins can change as erosion and surface uplift alter topographic structure. A change in local boundary conditions such as tectonic uplift and climatic regime prompts surface processes, potentially including river capture, to adjust topography toward a new equilibrium until boundary conditions change again (e.g., Willett et al., 2014). A change in the spatial configuration of surface uplift or climate regime may accompany drainage area change if persistent at timescales longer than the time it takes for the landscape to respond to the new conditions (e.g., Whipple et al., 2017). While the topographic disequilibrium may eventually trigger river captures (i.e., Beeson et al., 2017; Whipple et al., 2017), it is the river capture process itself and its temporally discrete nature and magnitude that most likely affects the diversity profile of inhabiting aquatic species (e.g., Albert et al., 2017).

By altering the position of drainage divides, the size of basins, and habitat structure, river captures strongly affect the diversity and distribution of freshwater aquatic organisms (Burridge et al., 2006; Albert and Crampton, 2010; Albert et al., 2017). On

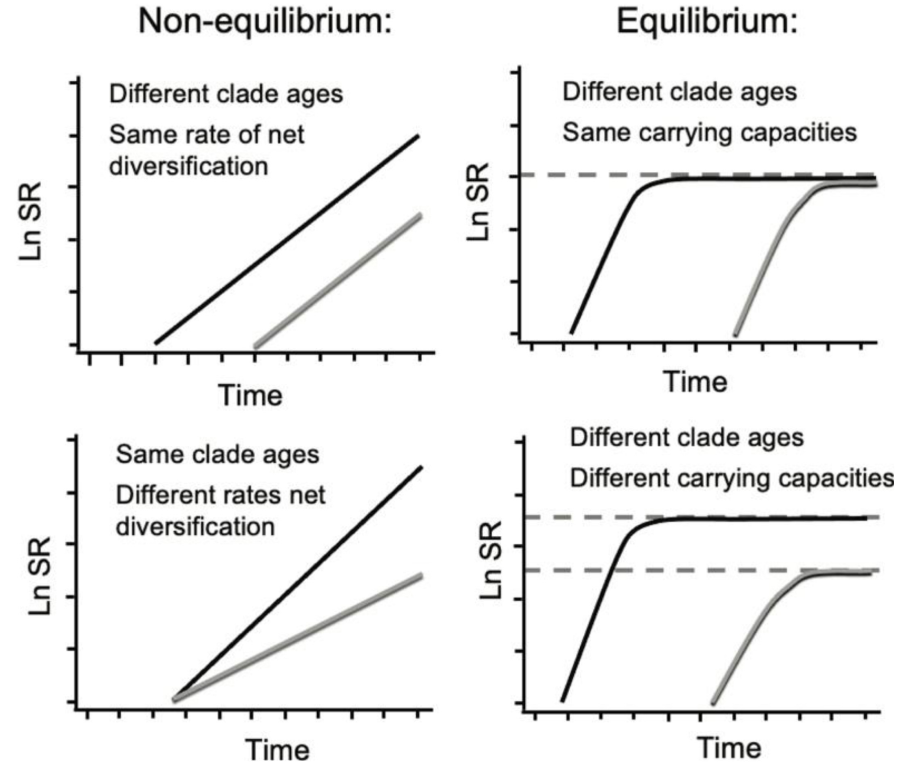

FIGURE 1 | Species richness (SR) through time profiles for two clades (black and gray curves) under the influence of different macroevolutionary processes. Modified from Rabosky (2010). **Left:** Under exponential diversification, SR is a function of clade age (or time *t*, x-intercept top right panel) or net rate of diversification (slope bottom right panel): $\delta = \lambda - \mu$, where δ is net diversification rate, λ is speciation rate and μ is extinction rate. **Right:** Under density-dependent diversification (where $\mu \rightarrow \lambda$ as $SR \rightarrow SR_{max}$ over time *t*), SR eventually becomes controlled by SR_{max} (depicted as dashed horizontal lines).

the other hand, landscape stability (i.e., equilibrium between erosion and uplift rates and spatially homogeneous erosion rates) or the slow migration of drainage divides impedes discrete river network rearrangements, thus allowing the accumulation of frequent and small-scale background erosional processes operating over extensive time periods (Sieben et al., 2018). Importantly, under an ever-changing geological substrate, a plausible and common scenario in continental interiors, equilibrium landscapes and therefore fixed drainage basin sizes may rarely be attained (e.g., Forte et al., 2016), especially when exogenic and autogenic processes interact to form feedback loops (Scheingross et al., 2020). Moreover, river captures accompany base-level fall, which may trigger other river captures both upstream and downstream of the capture point (e.g., Willett et al., 2014; Whipple et al., 2017; Giachetta and Willett, 2018). Therefore, landscape dynamics is understood to have multiple cascading and complex effects on the evolutionary diversification of continentally distributed groups of plants and animals (Ward et al., 2002; Coblentz and Riitters, 2004; Hoorn et al., 2010; Badgley et al., 2014; Rahbek et al., 2019; Igea and Tanentzap, 2021; Roell et al., 2021).

In this paper we ask if the fish species richness of the world's river basins is explainable in terms of river captures using topographic metrics as proxies. Specifically, we explore at what scale do landscape-species relationships emerge. Do the cumulative effects of smaller-scale river capture events ($<$10,000 km^2) that drive large scale watershed migration fully explain patterns of fish biodiversity, or do we need to understand the influence of rarer and larger-scale processes, tectonically driven or otherwise, like mega-river capture events ($>$ 10,000 km^2; Albert et al., 2018a, 2021)? We proceed from the expectation that basins with SR values close to that expected by regression against climatic or geographic variables (e.g., precipitation, topographic relief) are closer to species-equilibrium, reflecting feedback between rates of smaller-scale river capture events and rates of macroevolutionary processes (e.g., speciation and extinction). Contrariwise, basins with SR values far from these regressions are expected to be further from species-equilibrium, due to the historical effects of rare and large mega-river captures (Albert et al., 2018a).

We pursue this inquiry using correlations between species richness and topographic metrics of landscape structures known to be associated with landscape disequilibrium (e.g., Beeson et al., 2017; Sassolas-Serrayet et al., 2019), referred to in the field of biogeography as the River Capture Hypothesis (RCH; Albert et al., 2018b; Lyons et al., 2020). For this study, we employ a newly compiled dataset of fish species richness for most ($>$ 3,000) of the river basins located on ice-free continents (**Figures 2A,B**). We assess the quantitative influences of multiple landscape (e.g., latitude, elevation, topographic relief, tectonic

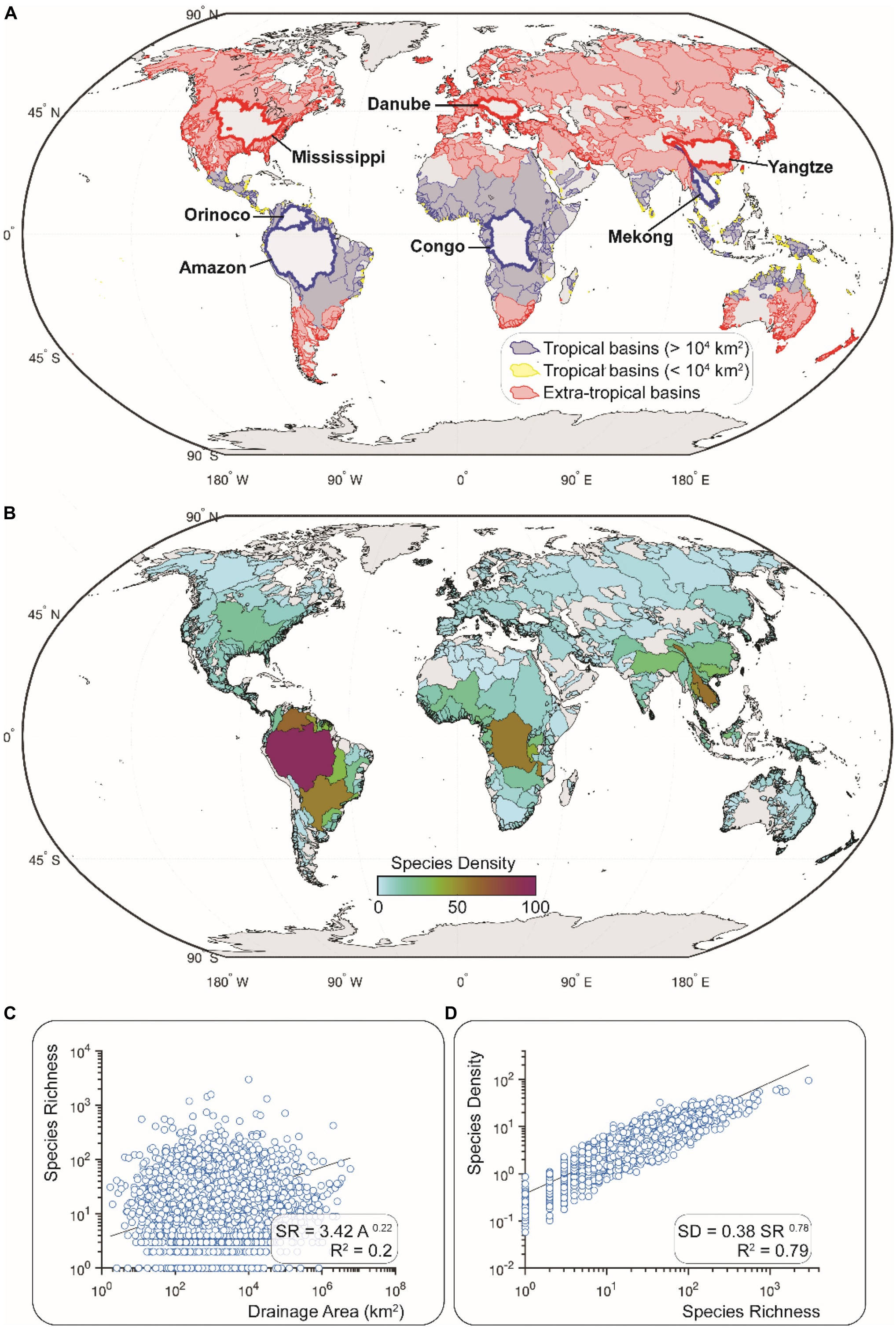

FIGURE 2 | River basins of the world (HydroSHEDS of Lehner et al., 2008; shapefiles provided by Tedesco et al., 2017a). **(A)** Basins grouped as either tropical (blues; *n* = 1,058) or extra-tropical (reds; *n* = 2,061) based on centroid latitude less than or greater than 23 degrees, respectively. Tropical basins with blue and yellow outlines have a surface area greater and lower than 10,000 km^2, respectively. Thicker outlines delineate large basins discussed in the text. **(B)** Species density (SD) values for all basins. SD calculated as SR/A^z, where SR is species richness, A is area (km^2), and *z* is the power-function exponent from a power-function regression of SR and A for the global dataset, not distinguishing between latitudinal, seismic, or elevation categories. **(C)** Species-Area Relationship for the global dataset used to produce the SD map in **(B)**. **(D)** Relationship between SD and SR values for all 3,119 basins in this dataset (see section "Calculation of Species Richness and Density" for a description of this calculation).

activity) and climatic (e.g., precipitation, temperature) variables on patterns of global freshwater fish diversity, adjusted for measures of habitat volume (e.g., basin area, river discharge). We find wide variation in biological responses among taxa and regions, presumably due to the effects of historically rare but geologically impactful events (e.g., tectonic uplifts) and other evolutionary contingencies (Losos et al., 1998). We also find that the spatial scale at which we look for the interaction between landscape evolution processes and biodiversity matters. For basins larger than 10,000 km^2, we find relationships with topographic metrics that are consistent with the RCH, albeit at smaller river capture scales. Based on our results, we propose that the biodiversity profiles of freshwater fishes bear predictable mechanistic relationships with the rates and scales of river captures under different ecological conditions and in different geological settings.

MATERIALS AND METHODS

Datasets

We used a global dataset of fish occurrence in discrete drainage basins to obtain species richness metrics (Leprieur et al., 2017; Tedesco et al., 2017a). These species richness values are point-estimates for fish "species inhabiting permanently or occasionally freshwater systems." This dataset is subject to expected errors associated with biodiversity sampling and taxonomic knowledge. Given the size of the dataset, we do not expect these potential errors to bias the results.

Using shapefiles in **Figure 2A** we obtained basin-wide average topographic and climatic metrics (see **Supplementary Dataset 1**). For topography, we used the 90 m resolution, Shuttle Radar Topographic Mission (SRTM) digital elevation model downloaded from OpenTopography (Farr et al., 2007) to compute basin-wide average topographic metrics (see section "Basin-Wide Topographic Metrics"). For climatic metrics, we used the monthly mean, 30-year reanalysis dataset for the period of 1961–1990 with 0.5° spatial resolution (New et al., 2002) and the 29-year monthly mean air temperature data (UDel_AirT_Precip v4.01 product) for the period of 1981–2010 with 0.5° spatial resolution (Willmott and Matsuura, 2001) provided by the Physical Sciences Laboratory (National Oceanic and Atmospheric Administration, NOAA).

Given that tectonic activity impacts biodiversity, we created two data partitions such as tectonically active or tectonically stable, which we identified based on seismic activity. Seismic activity was identified through Peak Ground Acceleration (PGA) data obtained from the Global Earthquake Model (GEM) (Pagani et al., 2018). GEM uses several methods to obtain PGA, including compilation of hazard maps and reduction of seismic data. In this dataset, PGA is the 10% probability of exceeding the 50-year reference shear wave velocities (see Pagani et al., 2018). We use a threshold of 3.2 to distinguish between tectonically active vs. stable regions based on the observation that the Amazon basin contains an average PGA of 3.2 (no-data grid cells are not averaged) and is a tectonically stable region.

Calculation of Species Richness and Density

Tedesco et al. (2017a) provides a list of fish species occurring in freshwater for 3,119 basins globally out of which we were able to acquire topographic data for 3,038. For every basin in the dataset, we calculated species richness (SR) as the total number of valid fish species (**Figures 2C,D**). Species density (SD) was then calculated *via* a regression of SR on drainage area. The Species-Area Relationship (SAR) assumes the form: $SR = Area^z$ (MacArthur and Wilson, 1966) and, therefore, SD was calculated as $SR/Area^z$, where the exponent z is obtained from the power-function regression of SR on Area (Rosenzweig, 2004; Albert et al., 2011). We then created data partitions based on seismic activity (active and stable) and latitude (tropics and extra-tropics) to assess differences in SAR based on geologic and climatic settings. Lastly, we focused on tropical regions as basins between 23.5 degrees latitude north and south, where most fish species live. We examined the effects of topography by separating basins into uplands and lowlands defined as median elevation above and below 500 m above sea level, respectively. We then assessed the importance of climatic and topographic predictor variables such as precipitation and relief, respectively, in individually predicting SD in the tropics. We create four sub-groups: (1) tectonically active highlands; (2) tectonically stable highlands; (3) tectonically active lowlands; (4) tectonically stable lowlands.

Basin-Wide Topographic Metrics

We used TopoToolbox to extract topographic metrics for each drainage basin (Schwanghart and Scherler, 2014). We obtained the average values for elevation above sea level as well as topographic relief. The latter was computed as the absolute range of topography over a 2,500 m moving window at every cell within a given basin.

Relief is a topographic metric that describes the local amplitude of topography. In tectonically active regions, relief scales directly with uplift rates and is often used as a proxy to identify relative differences in tectonic uplift (i.e., the rate of advective motion of rock) (e.g., Montgomery and Brandon, 2002; Kirby and Whipple, 2012). Relief also scales with a rock's resistance to erosion; harder (or softer) rocks promote steeper (or gentler) rivers (e.g., Hack, 1973; Duvall et al., 2004; Gallen, 2018). Thus, actively uplifting/eroding regions with highly variable lithology promote complex transient evolution and topography (e.g., Forte et al., 2016). Importantly, landscapes that are actively changing due to some past perturbation (i.e., base-level fall, river capture, or tectonic uplift) will have a positive correlation between relief, river steepness, and erosion rates within a given basin and, therefore, the amount of sediments actively fluxed through rivers (Portenga and Bierman, 2011; Kirby and Whipple, 2012; Gallen et al., 2013). Based on a previous modeling study, the ratio of the magnitude of the perturbation to the initial landscape relief dictates the degree of drainage reorganization, which in turn affects the placement or removal of dispersal barriers for aquatic organisms (Lyons et al., 2020; Stokes and Perron, 2020). Thus, average basin relief is a good metric for overall topographic steepness of a river basin and for linking topographic responses to autogenic and exogenic forcings. Also, it has been directly assessed with riverine species evolution in modeling studies (e.g., Lyons et al., 2020). Nonetheless, we emphasize relief is the time-integrated outcome of climate, tectonics, and surface and groundwater processes, and not strictly the outcome of landscape transience.

RESULTS

Distribution of Species Density Based on Tectonic Setting and Latitude

The SD values of freshwater fishes among river basins of the world vary systematically by seismic setting and latitude. We found a significant relationship (R^2 = 0.79; $p < 0.01$) between species richness (SR) and species density (SD) among all river basins worldwide. We found a relatively weak although significant relationship ($R^2 = 0.29$; $p < 0.01$) between SD and area among the largest basins (> 100,000 km^2; n = 133), but not for the medium (> 10,000–100,000 km^2; n = 504) or smaller basins (< 10,000 km^2, n = 2,482).

For both tropical and extra-tropical regions we observe that tectonically stable settings have a higher baseline species density value (i.e., y-intercepts of the SAR) but a lower slope (**Figures 3**, **4**). Moreover, the set of tropical basins exhibits a higher slope value than does the set of extra-tropical basins. Estimated this way, there is no significant relationship between fish species density and drainage area for all of the world's freshwater basins, although there is a modest relationship between these variables among the 133 basins larger than 10,000 km^2 ($R^2 = 0.12$; $p < 0.01$).

The most species-dense drainage basins are substantial outliers in each of the geographic sets of basins by tectonic activity and latitude (**Figures 3**, **4**). The statistical distributions of species density within each set reinforce the differences between tectonically stable and active regions (**Figure 5**). In all cases, the distributions are approximately log-normal, with the highest species densities with heavier tails in the tectonically stable group (**Figures 5A,B**). The average species density is higher in the tectonically stable regions compared to active areas irrespective of latitude (**Figures 5C,D**).

Species Richness in the Tropics—Upland vs. Lowland

Elevation (i.e., uplands and lowlands) is a well-known parameter controlling the distribution of aquatic species (Albert et al., 2018a). As expected, lowlands contain a 10-fold higher baseline species density (i.e., intercept of the SAR) but a lower slope. Basins affected by Neogene mega river captures (i.e., Amazon, Congo, Mekong) are exceptionally diverse and outliers in the SAR (**Figures 2B**, **6**).

Climatic and Topographic Drivers

In this study, we focus on river basins situated in the tropics from where a majority (66%) of freshwater fish species are known. Based on the observation that uplands and lowlands have differing relationships with topographic metrics depending on tectonic activity, we grouped basins based on drainage area, mean elevation, and tectonic activity and assessed four groups: tectonically active and stable lowlands and uplands.

Absent rainfall rates, we find no systematic relationships between species density and temperature, elevation, and relief when considering all basin sizes in each data subcategory. However, we find statistically significant correlations with these metrics for basins greater than 10,000 km^2 which is an approximate threshold at which landscape evolution processes might impact biodiversity (Albert et al., 2018a, 2021). Given this observation, we describe the following results for basins larger than this drainage area threshold.

Upland Basins

Of the four regressors used (rainfall rate, air temperature, average elevation, average relief), upland basins in tectonically active regions have statistically stronger relationships with topographic metrics than with climatic metrics (**Figure 7**). Tectonically active uplands have positive relationships with air temperature and negative relationships with elevation and relief (**Figure 7**). Of the climatic metrics, air temperature is a better predictor than rainfall rates and describes a positive relationship with species density (**Figures 7A,B**). Consistent with temperature gradients, basins at higher average elevations harbor lower species densities (**Figures 7B,C**). Similarly, basins with lower average relief are also more species dense (**Figure 7D**). Upland basins in tectonically stable regions do not have the same relationships as those in the tectonically active regions (**Figure 8**). Here, the most significant regressor for species density is the rainfall rate (R^2 = 0.57, $p < 0.01$). We observe no relationship with air temperature, elevation, or relief (**Figure 8**).

Lowland Basins

We observe no relationship between the metrics assessed here and species density (**Figure 9**). There are very few basins in lowland regions that are also tectonically active and even fewer basins in this subgroup that are larger than 10,000 km^2. Conversely, considering tectonically stable regions, basins in low elevations describe statistically significant relationships (**Figure 10**). Rainfall rate is the strongest predictor in this case ($R^2 = 0.52$, $p < 0.01$; **Figure 10A**) while relief is half as significant ($R^2 = 0.26$, $p < 0.01$; **Figure 10D**). Air temperature and elevation are insignificant in this subgroup (**Figures 10B,C**), which likely arise from the low range of average temperatures and elevations among tropical lowland basins.

In summary, total precipitation (as a proxy for total aquatic habitat volume) is a strong predictor of fish SD in tectonically stable areas, irrespective of upland or lowland areas, but not in tectonically active regions. Neither temperature nor elevation are strong predictors of SD among large lowland tropical basins. Topographic relief is a strong predictor of SD among large lowland but not large upland topical basins. We suspect this is because the absolute relief values represent a much larger proportion of elevational differences in flat (low relief) lowlands.

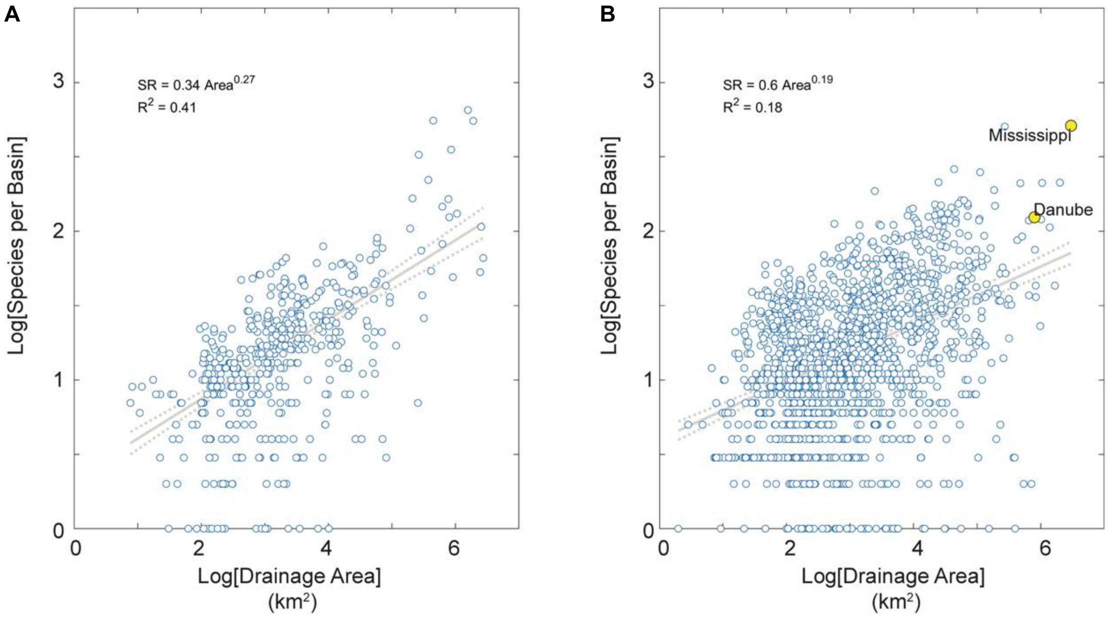

FIGURE 3 | SAR for **extra-tropica**l basins partitioned by **tectonic activity**. **(A)** Tectonically active basins in the dataset. **(B)** Tectonically stable basins in the dataset. Note these SARs extend over about six orders of magnitude in area and are all highly significant ($p < 0.01$). Note also these SARs have relatively low exponent values ($z < 0.30$), indicating relatively high shared species (and dispersal) among basins (Rosenzweig, 2004). Note further the lower exponent value in tectonically stable than active regions ($z = 0.19$ vs. 0.27, respectively) indicating on average more dispersal among basins of low-relief tectonically passive margins. Note finally in **(B)** the large and species-rich Mississippi and Danube river basins highlighted in yellow for reference.

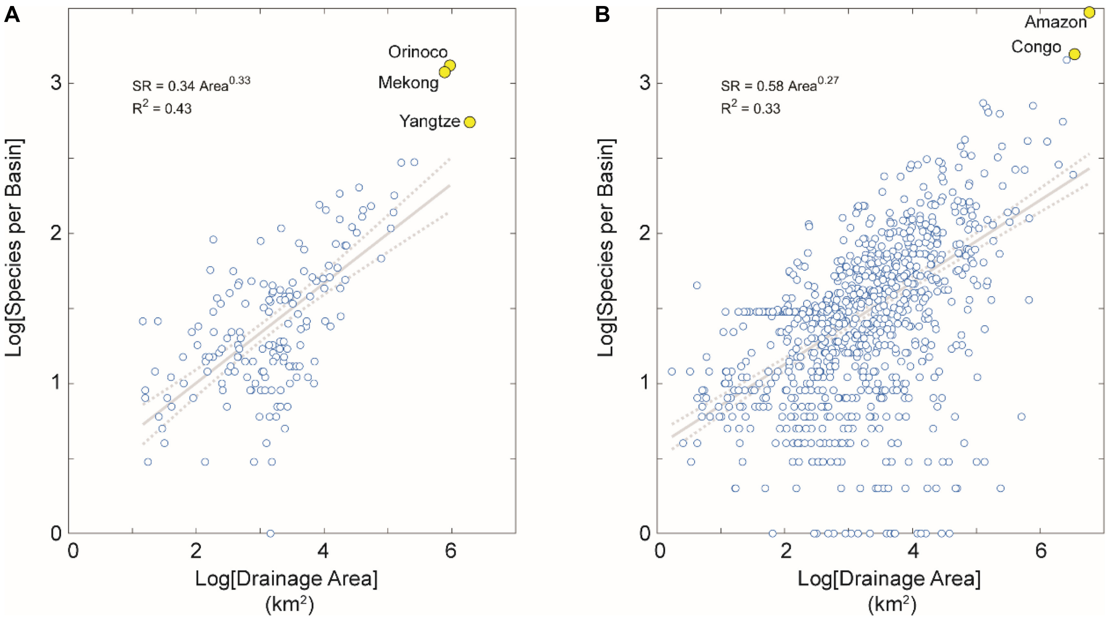

FIGURE 4 | SAR for **tropical basins** partitioned by **tectonic activity**. **(A)** Tectonically active basins in the dataset. **(B)** Tectonically stable basins in the dataset. Note the higher correlation between areal extent and SR for tectonically stable basins in the tropics than in the extra-tropics, but no such difference in these correlations between tectonically active basins in the tropics than the extra-tropics. We interpret this result as due to the more heterogeneous geographical conditions and geological history of the many river basins in extra-tropical and tectonically stable regions contained in this dataset (n = 1,478 basins, or 47% of the total). Note also several large-area and species-rich river basins highlighted in yellow for reference, including the Amazon and Congo basins with highest fish SR on Earth.

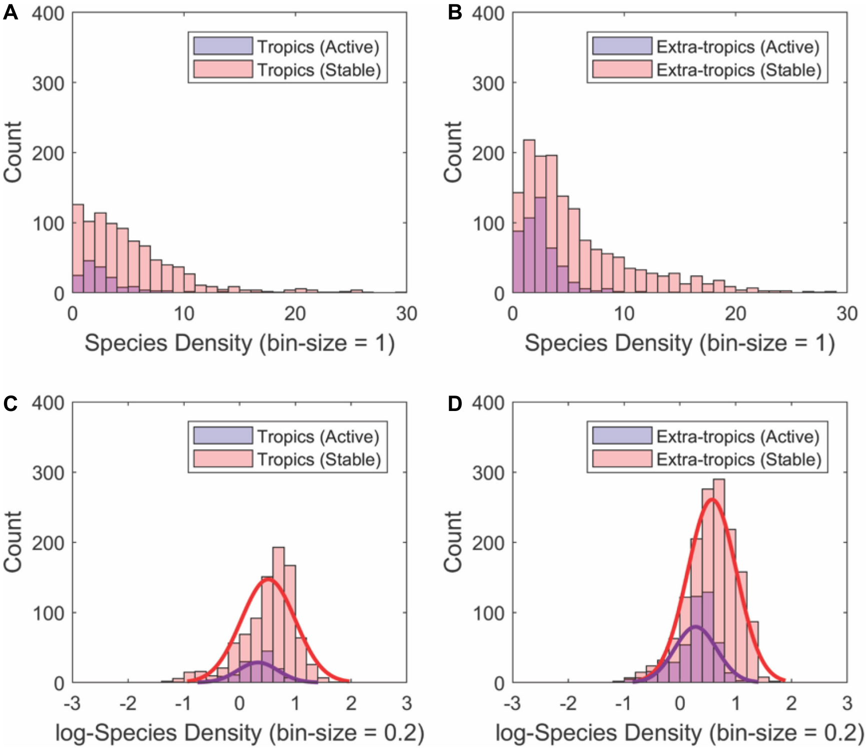

FIGURE 5 | Histograms showing the approximately log-normal distributions of fish SD values for river basins (*n* = 3,038) partitioned by geographic region [tropical **(A,C)** and extra-tropical latitudes **(B,D)**] and tectonic activity (active vs. stable). Species density for each subgroup was calculated using the regressions shown in **Figures 4**, **5**. In all cases, tectonically stable regions contain the higher mean species density as well as a greater amount of extremely high species density. Note the log-transforming these data distributions closely approximate normal distributions (see fits to bottom panels).

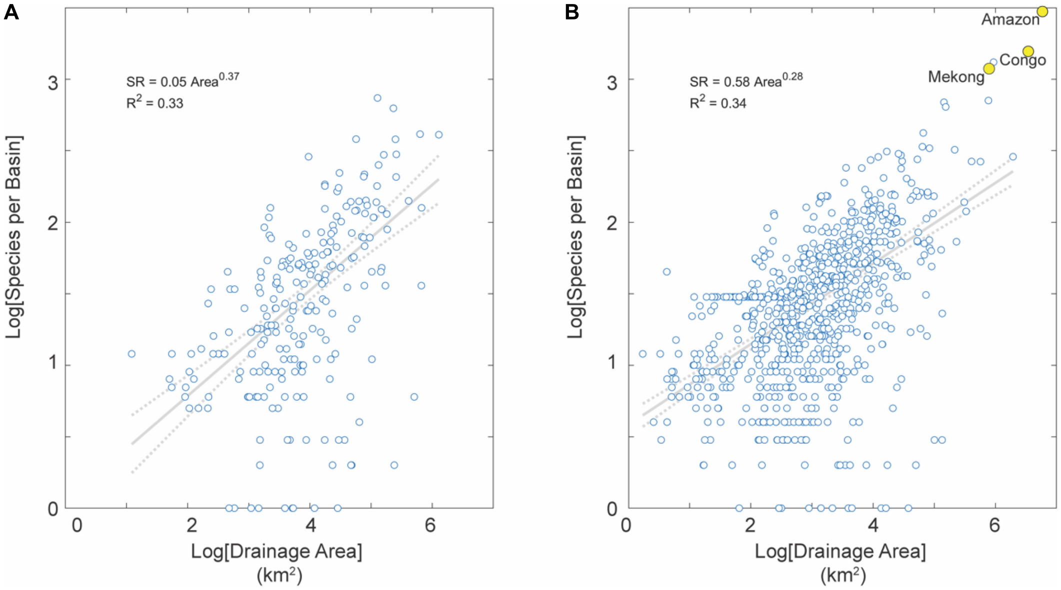

FIGURE 6 | SAR for basins in **uplands (A)** and **lowlands (B)** in the **tropics** (not distinguished by tectonic activity). Note the much larger number of basins in lowlands than uplands, reflecting the fact that the Earth is (mostly) flat with most basins in this dataset (78%) characterized by low average topographic relief (Willenbring et al., 2013). Note also several large-area and species-rich river basins highlighted in yellow for reference, including the Amazon and Congo basins with highest fish SR on Earth.

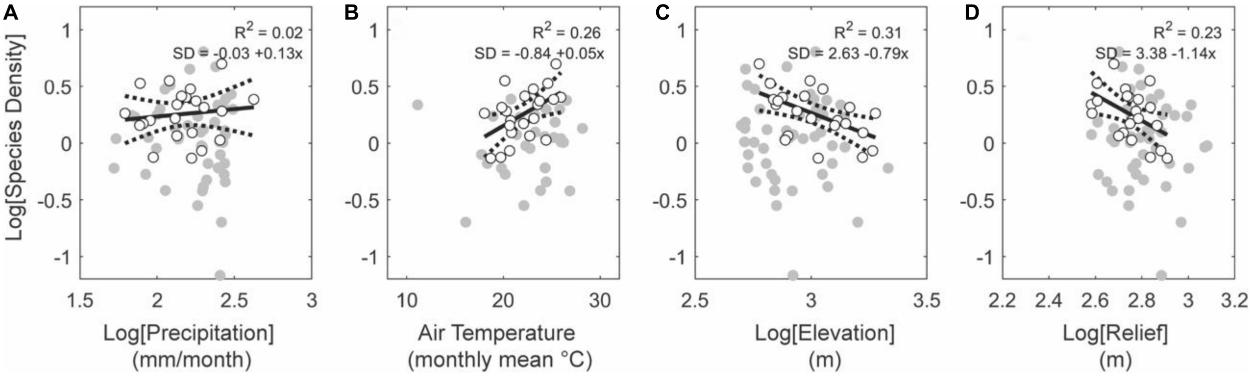

FIGURE 7 | Effects of climate and topography on fish species density for **upland** basins (average elevation above 500 m) in **active** tectonic settings. Note monotonic positive relationships between precipitation **(A)** and temperature **(B)**, and monotonic negative relationships with elevation **(C)** and relief **(D)**. The relationships reported in **(C,D)** contrasts with biodiversity patterns observed in mammals, birds, and vascular plants, which exhibit a mid-elevation richness peak in most regions of the world (McCain and Grytnes, 2010). Gray circles show all basins falling in this subset (i.e., upland, tectonically active regions) from which basins larger than 10,000 km^2 are shown with white circles.

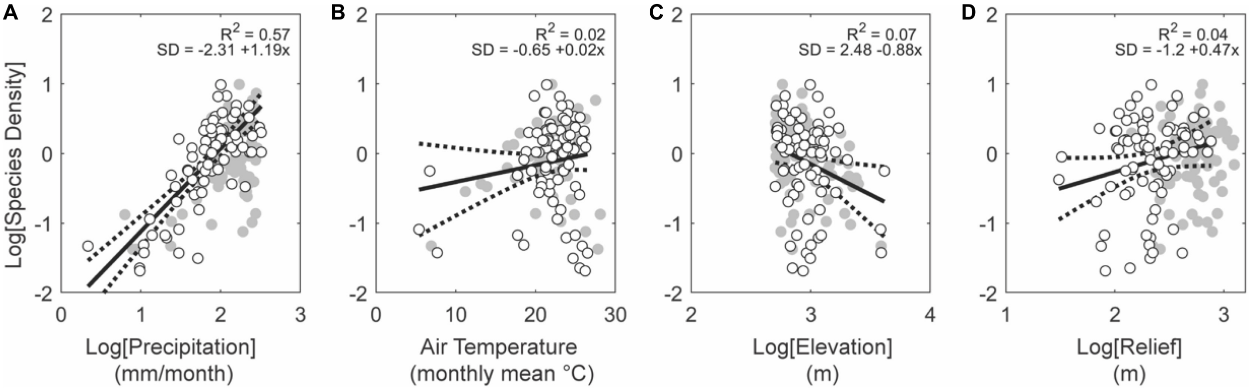

FIGURE 8 | Effects of climate and topography on fish species density for **upland** basins (average elevation above 500 m) in **stable** tectonic settings. Note monotonic positive relationships between precipitation **(A)** and temperature **(B)**, and relief **(D)**, and monotonic negative relationships with elevation **(C)**. Gray and white circles show all basins and white circles the basins larger than 10,000 km^2, both within this subset of data.

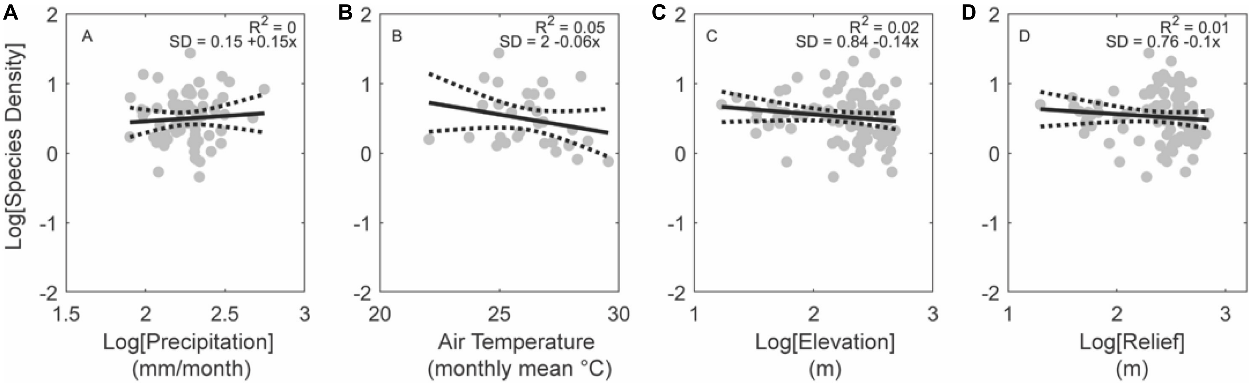

FIGURE 9 | Effects of climate and topography on fish species density for **low-elevation** basins in **active** tectonic settings. Note the lack of a significant relationship with precipitation **(A)**, temperature **(B)**, elevation **(C)**, or relief **(D)**. Regressions plotted for basins larger than 10,000 km^2 (gray circles; *n* = 92). Gray circles show all basins within this subset of data. No white circles are shown as very few basins in this subset are larger than 10,000 km^2.

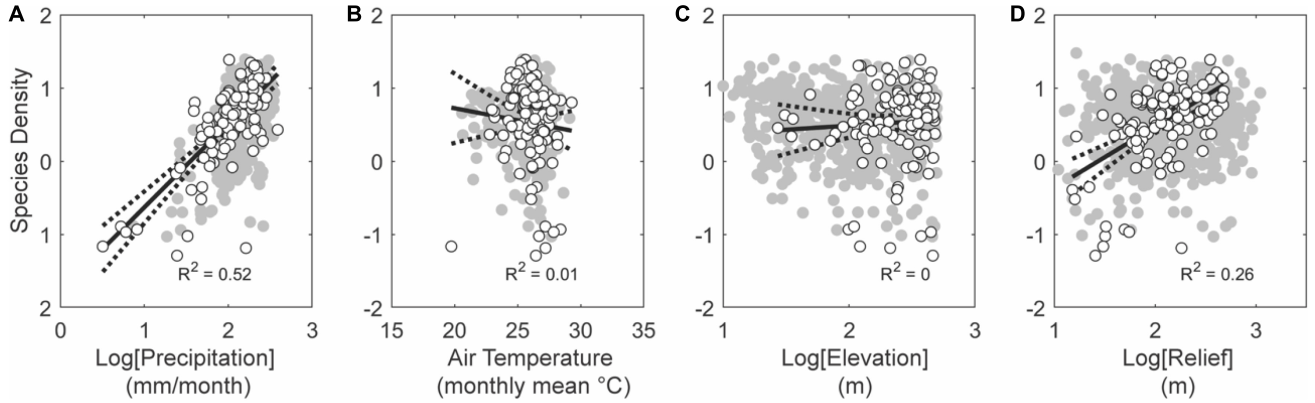

FIGURE 10 | Effects of climate and topography on fish species density for basins **low elevation** in **stable** tectonic settings. Note significant positive relationships between fish species density with precipitation **(A)** and topographic relief **(D)**, consistent with the hypotheses that total aquatic habitat volume (McGarvey and Terra, 2016), static riverscape heterogeneity (Thomaz et al., 2016), and dynamic riverscape landscape transience (Lyons et al., 2020; Stokes and Perron, 2020), promote net lineage diversification under these conditions. Gray and white circles show all basins and white circles the basins larger than 10,000 km^2, both within this subset of data.

DISCUSSION

General Macroecological Expectations

The results of regressions of freshwater fish SD values against two climatic (i.e., precipitation and temperature) metrics among river basins of the world are consistent with general macroecological expectations of highest diversity in wetter, warmer regions (Worm and Tittensor, 2018). Among freshwater basins globally, a disproportionate number of fish species inhabit the lowlands of large tropical river basins in South America, Africa, and Southeast Asia, under predictable ecological conditions of large geographic area, warm, humid tropical climate, relatively flat topography (i.e., low average relief), and high habitat volume (i.e., high precipitation and run-off) (Lundberg et al., 2000; Oberdorff et al., 2011; McGarvey and Terra, 2016; Leprieur et al., 2017; Antonelli et al., 2018b). The six river basins with greatest fish SR values (i.e., Amazon, Congo, La Plata, Orinoco, Mekong, and Tocantins basins) have a combined total of more than 9,200 species in an area of 13.7 million km^2, thus accounting for 51% of all freshwater fishes globally, in an area of just 14% of the 98.7 million km^2 of all river basins on Earth combined.

Among both tropical and extra-tropical basins, the species-area exponent is higher for tectonically (seismically) active than stable regions (**Figures 3**, **4**). This result suggests higher rates of dispersal among basins of low-relief tectonically passive margins, as indicated by previous studies showing higher SR in tectonically stable settings (Badgley et al., 2017; Griffiths, 2018). The higher correlation between area and SD for stable basins in tropics than extra-tropics indicates more heterogeneous geographical conditions and geological history of the many (n = 1,478 or 47% of the total) river basins in extra-tropical and tectonically stable regions (**Figure 4**).

The observed negative relationship between SD and average basin elevation among tectonically active uplands basins is consistent with previous observations of species occurrence along elevation gradients in the Amazon (Lujan et al., 2013), and across the South and North American continents (Smith et al., 2010 Griffiths, 2018). Relationships of SD with air temperature and precipitation in these regions (**Figures 7A,B**) are consistent with contributing roles of both contemporary ecological and historical (time-integrated) macroevolutionary effects driving down aquatic species diversity at higher elevations (e.g., Lujan et al., 2013; Hazzi et al., 2018). Similarly, the history of topographic growth, as opposed to present-day topographic or climatic conditions, is thought to be more important to the evolution and enrichment of many terrestrial (Castroviejo-Fisher et al., 2014; Antonelli et al., 2018a; Azevedo et al., 2020; Réjaud et al., 2020; Igea and Tanentzap, 2021) and aquatic (Smith et al., 2010; Badgley et al., 2017) vertebrate faunas.

General Macroevolutionary Expectations

Macroevolutionary theory predicts that regional SR values arise from interactions among three fundamental parameters: rate of net diversification (δ), time of net diversification (t), and regional carrying capacity (S_{max}) (Rabosky, 2010). The per-species net diversification rate (δ) is: $\delta = \lambda + d—\mu$, where per-species speciation (λ) and dispersal (d) rates add species, and per-species extinction rate (μ) removes species from a region. When diversity is unbounded (i.e., δ is independent of S_{max}), δ and t provide limits to diversity; i.e., δ = δ0, where δ0 is the intrinsic diversification rate. Under these non-equilibrium conditions, the number of species at time t is: $SRt = e^{\delta 0 \cdot t}$ (Cornell, 2013). Such a model applies when speciation and dispersal rates are low relative to total available niche space and/or geological age of a region, or when $\mu > \lambda + d$ for sufficient time that a diversity limit (i.e., SR_{max}) is not approached. When diversity is bounded, SRt depends on SR_{max} and t; i.e., $\delta = \delta 0—a\mathrm{SR}$, where a is the strength of diversity-dependent feedback on δ, the carrying capacity ($S_{max} = \delta 0/a$) depends on the time-integrated δ or $\delta t = \int [\lambda(t) + d(t) - \mu(t)]\, dt$, and expected equilibrium species richness is: $SRt = e^{\delta\ t}$ (Rabosky, 2013; Cornell and Harrison, 2014; Harmon and Harrison, 2015; Rabosky and Hurlbert, 2015).

Under this theory, the smaller difference in correlation values between tectonically active basins (5%) among tropical vs. extra-tropical (9%) basins is unexpected (Stanley, 2014; Albert et al., 2017), because there are many more tectonically stable basins (**Figure 4**). This result is partly due to the much wider range of SD values among large basins on stable terrains, in particular from the many large basins from cold boreal regions (e.g., northern Canada and Russia) and arid tropical regions (e.g., northern Africa, central Australia) with low SD values (**Figure 1** lower panels). There are fewer counterparts of these large low SD basins on active terrains.

Both macroecological and macroevolutionary models predict higher SD values in the dendritic geometry of river drainage networks (Fagan, 2002; Thomaz et al., 2016). River networks are a more spatially fragmented substrate than an equivalent Euclidean landscape with the same surface area (Rodriguez-Iturbe and Rinaldo, 2001; Dias et al., 2013). The hierarchical-branching of drainage networks is a more effective geometry for breaking up a geographically widespread species into daughter species (Wiens, 2002; Muneepeerakul et al., 2007; Tonkin et al., 2018). However, even more effective than a 3D dendritic surface for fragmenting and merging populations is a 4D dendritic surface changing in time; i.e., river capture (Albert and Crampton, 2010; Albert et al., 2017, 2018a).

The River Capture Hypothesis

The results of this study are consistent with several predictions particular of the RCH (**Figure 11**; Albert et al., 2018a). By merging geographic areas (geodispersal), river capture facilitates organismal dispersal and gene flow, and therefore acts to slow rates of speciation and extinction, i.e., lower species turnover (Albert and Crampton, 2010). However, by subdividing areas (vicariance) river capture also acts to increase rates of speciation and extinction. The results of this study suggest that genial ecological conditions are necessary, but not fully sufficient, to explain the basins with the highest diversity, consistent with the prediction of the RCH that dispersal across the watershed margins of adjacent lowland basins increases basin-wide SR and SD values. These increases occur both by adding individuals of different species, and adding individuals of existing species, thereby lowering the within-basin extinction rate (i.e., rescue effect; Brown and Kodric-Brown, 1977).

Dispersal of freshwater fishes among adjacent river basins may occur by multiple abiotic and biotic mechanisms (e.g., Tagliacollo et al., 2017). In some cases, the effects of geodispersal by river capture and biotic dispersal by organismal movements can be difficult to separate, for examples in seasonally flooded wetlands that straddle low-elevation drainage divides; e.g., Rupununi and Izozog swamps at the margins of the Amazon and adjacent basins which are sites of longer-term river captures and seasonal dispersal (Albert et al., 2011). In other cases, riverine corridors serve as ecological filters in which organismal trait values (adult body size, habitat utilization, tropic specializations) influence the species richness and composition of the biotic interchanges due to river capture; e.g., Casiquiare River.

The Mega Capture Hypothesis

The *Mega Capture Hypothesis* (MCH) predicts that large river captures (> 10,000 km^2) leave a disproportionately enhanced signature on the accumulation of basin-wide SR values in riverine and riparian taxa (Tagliacollo et al., 2015; Albert et al., 2018a, 2021). The basins with highest fish SD values (i.e., Amazon, Orinoco, La Plata, Congo, Mekong) have all been exposed to the effects of mega river captures within the past 20 Ma, which merged portions of the whole biotas of riverine and riparian taxa among adjacent basins (e.g., Bragança and Costa, 2019;

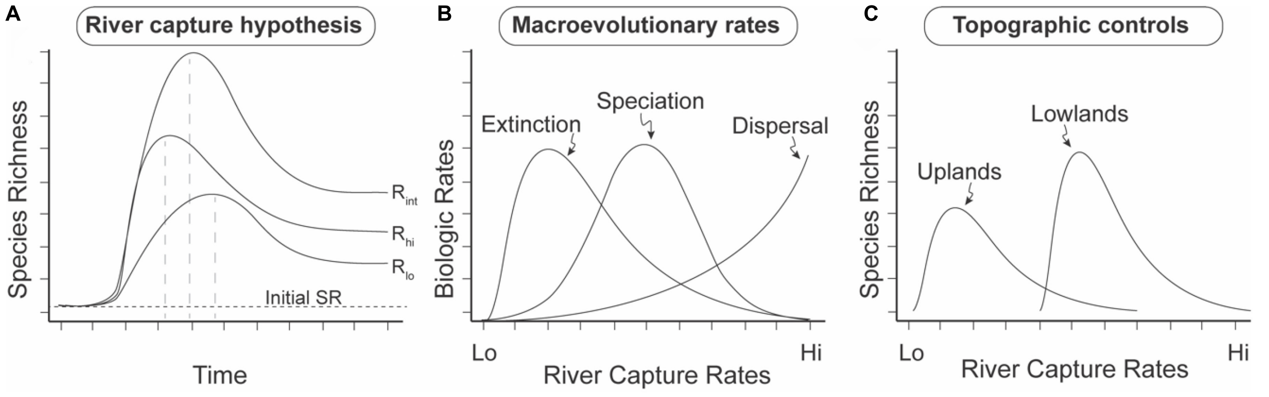

FIGURE 11 | Salient predictions of the River Capture Hypothesis (RCH) on macroevolutionary diversification in riverine and riparian taxa. Predictions from empirical (Albert et al., 2018a) and landscape modeling studies (Lyons et al., 2020; Stokes and Perron, 2020). SR = Species Richness. River capture rate is measured as the number of river capture events per unit time interval per unit area. **(A)** ***Intermediate river-capture rate hypothesis (IRH)***. Prediction 1: Transient SR_{max} values (dashed vertical lines), with subsequent decay to a higher post-capture than initial SR baseline. Prediction 2: Highest SR values at intermediate rates of river capture. **(B)** ***Macroevolutionary rates***. Prediction 3: Relative order of modal rate values in lowland, stable regions: extinction < speciation < dispersal. Rates integrated over the whole time interval of **(A)**. **(C)** ***Topographic controls.*** Prediction 4. Higher modal rates of river capture on low-relief, lowland landscapes, than on high-relief, upland landscapes based on landscape evolution modeling (Lyons et al., 2020). Prediction 5. Higher equilibrium SR on low-relief lowland landscapes. Log-normal river capture curves follow data from Goldberg et al. (2021).

Musher et al., 2019; Van Steenberge et al., 2020; Albert et al., 2021; Chen et al., 2021; Sun et al., 2021; van der Merwe et al., 2021).

Although SR and SD are significantly correlated among basins worldwide ($R^2 = 0.8$, $n = 3{,}038$, $p < 0.01$), basins with highest SD values drain primarily tectonically stable regions and these basins were assembled during the Neogene (c. 23–2.6 Ma) and Quaternary (2.6–0 Ma) through the action of mega river capture events (Albert et al., 2018a). Among the largest basins worldwide (i.e., those > 100,000 km^2), basins with the top 10 SD values are (in descending order): Amazon, Orinoco, Chao Phraya (Thailand), Mekong, Essequibo, Paraná-Paraguay, Congo, Tocantins, Uruguay, and Zhujiang (Pearl) basins. Under the RCH, the unexpectedly high SD values of these river basins, as assessed by their positive deviations from the regression in **Figures 3–6**, arose from the merging of multiple older and smaller basins through mega river capture events (Albert et al., 2018b, 2021; Sun et al., 2021). All these basins have undergone substantial changes to their watershed margins over the last 20 million years, with significant portions, sometimes>50% (e.g., Hoorn et al., 2010) of their modern areas having been affected by river capture during this time (e.g., Clark et al., 2004; Goudie, 2005; Hoorn et al., 2010).

The Intermediate Capture Rate Hypothesis

The *Intermediate Capture Rate Hypothesis* (ICH) predicts highest SD values on landscape with "just right" rates of river captures through time and at appropriate spatial scales (Albert et al., 2018a). The results of SD regressions against two landscape metrics (i.e., elevation and relief) are consistent with several predictions of the ICH. Maximum SD values are obtained in tectonically stable lowland basins (**Figure 10**), where river capture dynamics are expected to drive an excess of speciation and dispersal events as compared with extinction events per unit time, and therefore a net accumulation of SR through time. By contrast, SD values are lower, and not correlated with relief, among seismically active lowland basins (**Figure 9**) and stable upland basins (**Figure 8**). Finally, SD values are lowest, and are negatively correlated with relief, among the set of active uplands basins (**Figure 7**).

Basins with highest fish SD values are located within the tropics and on stable terrains, although not all basins in the tropics or on stable terrains have high fish species density (**Figure 2**). This is similar to the results of Albert et al. (2018a) who show differing river capture rates on stable vs. active tectonic platforms. Rates of river capture are poorly constrained on most landscapes worldwide, but preliminary evidence from South America indicates they may be several orders of magnitude faster on alluvial lowland sedimentary basins of continental interior than on upland cratonic regions (Ruokolainen et al., 2019; Goldberg et al., 2021).

Landscape evolution modeling results arrive at similar conclusions and suggest that river captures are likely more frequent and larger in low-relief landscapes (Lyons et al., 2020), which exhibit highest SD values worldwide (**Figure 2B**). Conversely, high relief in tectonically active landscapes acts to fragment the species range and increase extinction rates at smaller spatial scales as compared to low-relief landscapes (e.g., Smith et al., 2002; Albert et al., 2006, 2018a; Borregaard et al., 2012; Griffiths, 2018). Binned by relief, our dataset supports these directional relationships and reveals that SD values peak at an intermediate relief value when comparing tectonically stable lowlands with tectonically active uplands (**Figure 12**). The probability of river captures increases where neighboring basins erode laterally at different rates, which especially true near topographic escarpments with uniform rock types (Salgado et al., 2014; Willett et al., 2018; Calegari et al., 2021; Wang and Willett, 2021). The across-divide differences in mean relief, elevation, slope, and other topographic metrics that measure steepness, dictate the direction and rate at which divides migrate (Whipple et al., 2017). Importantly, in lower relief settings, landscape perturbations by long-wavelength (100 s of km) and low-amplitude (<1 km) uplift (i.e., dynamic topography; e.g., Bicudo et al., 2019), local uplift (i.e., faults), and base-level fall are likely to reach or surpass the observed ranges of relief, which more easily prompts drainage reorganization (Lyons et al., 2020). To the extent that the value of topographic relief affects the frequency of river captures across these landscape settings (e.g., Lyons et al., 2020), our findings are consistent with the expected effects of barrier displacement caused by landscape transience and support the ICH (Albert et al., 2017).

According to the macroevolutionary model outlined above, we may expect to see SR$_{max}$ values at intermediate rates of river capture under non-equilibrium conditions, when basin-wide species richness (SR) values are growing because the rate of speciation (λ) exceeds extinction (μ). However, under more equilibrium conditions when the rates of speciation (λ) and extinction (μ) are similar, SR may be more strongly controlled by the regional carrying capacity (SR$_{max}$; Rosenzweig, 2004; Albert et al., 2017). Results of this study indicate that rates of river capture on stable lowland continental platforms are sufficiently slow enough to allow speciation to occur among isolated portions of river networks through time (*t sensu* **Figure 1**), while also being sufficiently fast enough to allow dispersal to populate adjacent

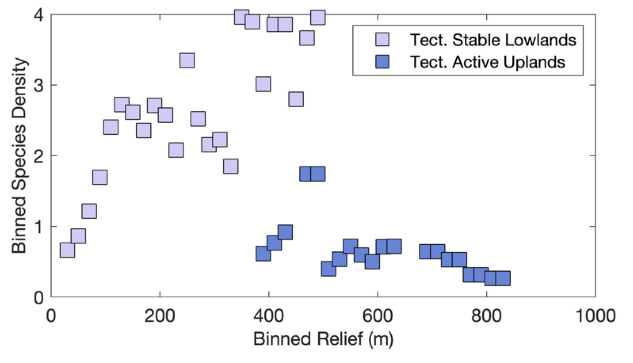

FIGURE 12 | Empirical evidence consistent with Intermediate Capture Rate Hypothesis. SD and relief data were binned in 20-m relief intervals for both sets of tectonically stable lowlands and tectonically active highlands as shown in **Figures 7**, **10**. Note highest SD at intermediate values of relief.

basins. This combination of parameter values allows SR values to increase in lowland sedimentary basins which experience higher rates of river capture and reduce the extinction risk of already resident species (Fagan, 2002; Tedesco et al., 2012; Douglas et al., 2013).

By contrast, slower river capture rates on tectonically stable and more erosion resistant upland regions (e.g., continental cratons) are expected to inhibit dispersal among adjacent basins, and thereby lower the rate of increase of diversity through time (lower δ *sensu* **Figure 1**). By the same logic, faster rates of river capture within alluvial lowland sedimentary basins of continental interior or coastal plains retard fish diversification, because populations do not become isolated for long enough to allow genetic divergence. In positing that lineage diversification dynamics arises from the multiple effects of dispersal and gene flow on speciation and extinction, the ICH resembles the Shifting Balance Theory (Wright, 1982) and Effect Hypothesis (Vrba, 1983), with the notable differences that, under the ICH, speciation may occur due to genetic drift alone rather than requiring natural selection, and species may therefore not necessarily be adapted to different ecological niches (Harvey et al., 2019). The hypothesis that highest SR values are obtained at intermediate capture rates reflects a larger perspective that all possible evolutionary drivers impose trade-offs on organismal diversification, achieving maximal effectiveness over a limited domain of parameter values; e.g., the intermediate disturbance and productivity hypotheses (Huston, 1994; Fraser et al., 2015).

The log-normal SD frequency distributions observed in this study often characterize biodiversity profiles and other biological systems that grow over time from the multiplicative interactions of many independent random variables (Crow and Shimizu, 1987; Rozenfeld et al., 2008; Magurran, 2013). As numerous studies suggest that landscape transience, river captures, and escarpment migration are common characteristics of intracontinental lowland regions (e.g., Harbor et al., 2005; Gallen et al., 2013; Val et al., 2014; Beeson et al., 2017; Gallen, 2018; Willett et al., 2018; Wang and Willett, 2021), landscape evolution processes might be a common underlying mechanism of diversification in continental regions.

Limitations of This Study

This study examines relationships of fish species richness with possible drivers among basins assigned to broad latitudinal categories (i.e., tropical and extratropical), but does not examine possible effects of latitude on habitat heterogeneity within river basins. Such an analysis would be complicated by many additional factors, with possible expectations for greater habitat heterogeneity in tropical than extra-tropical basins, for basins with N-S than W-E main-stem axis orientations, for upland than lowland basins, and for stable than active terrains. Many other potentially important factors could also be examined, including especially distance from continental geographical centroid or center of connectivity (Smith et al., 2010), and mean or maximum phylogenetic clade age (Miller and Román-Palacios, 2021).

This study also uses topographic metrics such as relief as proxy for landscape transience. Spatial variability in relief is not a unique outcome of exclusively landscape transience. The erosive susceptibility inherent to lithologic types, for example, also influences relief. The primary control of relief and its strong correlation with erosion has been recognized since the early days of geomorphology (Gilbert, 1877), and this correlation continues to be identified using state of the science techniques to measure erosion rates (e.g., von Blanckenburg, 2005). The cross-divide difference in erosion rates can be especially indicative of transient river network reorganization (e.g., Willett et al., 2014; Whipple et al., 2017). Future studies can incorporate cross-divide relief and erosion differences with computational tools such as Forte and Whipple (2018) along with our approach to further investigate links among river captures and species richness.

CONCLUSION

Results of this study are consistent with the predictions of several widely known macroecological and macroevolutionary models regarding the effects of landscape evolution on freshwater biodiversity; e.g., that the most diverse river basins are all located within tropical latitudes, on stable geological platforms, at lowland elevations, and in areas with high regional precipitation. However not all basins with these features exhibit high SR, due to local historical and geographic conditions, especially proximity and connectedness to species-rich basins that lie near the continental cores, e.g., Amazon, Congo, Danube, Mississippi and Yangtze basins. These discrepancies can be explained, and are predicted, by several models of species and landscape evolution, described here as the following non-mutually exclusive mechanistic hypotheses: the River Capture Hypothesis, Mega Capture Hypothesis, and the Intermediate Capture Hypothesis.

All the most diverse river basins are outliers in SARs (**Figures 4**, **6**) with SD values falling well above that predicted from smaller basins with similar properties. This result means that the predictors of SR based on analysis of many small rivers do not predict the SR of the most diverse basins. The largest basins are different from the others; they are evolutionary arenas with high rates of speciation (i.e., evolutionary cradles) and low rates of extinction (i.e., evolutionary museums) where lineage diversity has accumulated over many tens of millions of years. These results indicate contrasting effects of discrete (higher stream order) river capture events as compared with continuous (headwater or first-level stream order) watershed migration on fish diversity. Moreover, this study also suggests that mega-river captures at the lower and intermediate spatial scales are important drivers of tropical biodiversity. This study is the first to provide empirical support from freshwater fishes worldwide for the conclusions of numerical modeling and empirical studies indicating river capture and landscape transience as mechanistic drivers of net diversification in riverine and riparian organisms that have widespread continental distributions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

PV compiled existing data and collected topographic data for all basins analyzed and produced figures. PV and JA wrote the manuscript, which was revised by NL, NG, and JW. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.788328/full#supplementary-material

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Drivers of Taxonomic, Phylogenetic, and Functional Beta Diversity of Himalayan Riverine Birds

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Abiotic and biotic factors drive compositional differences among local species assemblages. Determining the influence of different drivers on beta diversity patterns can provide insights into processes governing community organization. Examining beta diversity patterns along taxonomic, phylogenetic and functional dimensions enables a nuanced understanding of underlying processes that govern community assembly and dynamics. The dynamic and complex riparian habitats in the Himalaya, and the hyper-diverse riverine bird community offer a fascinating setup to examine the role of environmental factors in influencing community structuring. Using a large dataset on river bird communities from field census across multiple drainages in the Indian Himalaya, we aimed at discerning processes that structure these communities through an understanding of pair-wise dissimilarities in species composition across sites. We determined the relative contributions of turnover and nestedness in taxonomic, phylogenetic, and functional beta diversity patterns in the Eastern and Western Himalaya that differ in species richness. Generalized Dissimilarity Modeling was used to examine the relative contributions of climatic, geographic, and anthropogenic factors toward explaining different metrics of beta diversity. The riverine bird communities in the drier and seasonal Western Himalaya were poorer in species richness, more phylogenetically and functionally clustered than that in the Eastern Himalaya. The contribution of the turnover component to the overall beta diversity was higher than the nestedness component in river bird communities, particularly in the Eastern Himalaya. Habitat and climatic factors differentially influenced the beta diversity patterns in both Eastern and Western Himalaya, with river width consistently explaining a large variation in beta diversity in the east and the west. The results show that environmental filtering plays a crucial role in structuring riverine bird communities in the Himalayan headwaters, highlighting the need to ameliorate the threats posed by the slew of hydroelectric projects and forest loss in the region.

Keywords: beta diversity (β), Brahmaputra, forest cover, Ganges, generalized dissimilarity modeling (GDM), nestedness, river width, species turnover

INTRODUCTION

Understanding patterns of community assembly and identifying underlying ecological processes across geographic gradients is a predominant theme in theoretical and applied ecology (Diamond, 1975; Vellend, 2010). Species diversity is shaped by current and historical conditions that include a range of environmental and geographical factors (Mittelbach, 2012). Traditionally, variation in species diversity has been examined at local (α-diversity) and regional (γ-diversity) scales; however, in the last three decades, variation in species diversity among sites (β-diversity) has also gained considerable attention (Harrison et al., 1992; Lennon et al., 2001; Soininen et al., 2007; Baselga, 2010; Legendre, 2014). Beta diversity is a measure of turnover in species diversity among sites, habitats, or along gradients (Whittaker, 1960). Beta diversity measures can be classified into variance-based and diversity partitioning-based metrics (Koleff et al., 2003; Legendre, 2014; Matthews et al., 2019). Availability of species-site matrix (as in the case of our study) enables the estimation of variance-based beta diversity metric (Matthews et al., 2019), which further allows us to determine the relative influence of "nestedness" and "turnover" components on compositional differences (Baselga, 2010).

Determining the processes that drive the variation in community compositional across sites is a fundamental challenge (Ricklefs, 2004). Past (e.g., historical climate and geographic barriers) and contemporary niche-based factors (e.g., climate, habitat complexity, and biotic interactions) and other factors (e.g., geographic distance, geometric constraints, and land-use change) may influence beta diversity patterns (Nekola and White, 1999; Ricklefs, 2004; Trøjelsgaard et al., 2015; Newbold et al., 2016; Barnagaud et al., 2017). The predictors that influence beta diversity patterns are categorized under niche-based processes or neutral processes (Hubbell, 2001; Chase and Myers, 2011). Deterministic niche-based processes emphasize variation in environmental gradients, where species are assembled through environmental filtering or biotic interactions, while neutral processes highlight the roles of dispersal limitation and genetic drift (Hubbell, 2001; Chase and Myers, 2011). For example, compositional differences in communities could be caused by geographic barriers that may prevent the dispersal of species, the absence of specific environmental factors (e.g., microhabitats) that are preferred by certain species, or the presence of superior competitors/predators. For instance, human induced land-use changes may filter species, especially habitat specialists, resulting in compositional changes across natural and human-modified landscapes (McKinney, 2006; Newbold et al., 2016; Barnagaud et al., 2017). Hence, identifying the drivers of beta diversity patterns can help explain the roles of historical processes (Fitzpatrick et al., 2013) and current environmental and anthropogenic activities (Capinha et al., 2015).

Studies on beta diversity often focus on variation in taxonomic composition across communities. These assessments assume that all species in an assemblage are similar in terms of the role they play in ecosystems. When they do so, they neglect the role of functionality in assemblage structure and ecosystem functioning (Villéger et al., 2008). Furthermore, the importance of examining functional and phylogenetic beta diversity is increasingly being acknowledged (McGill et al., 2006; Anderson et al., 2011; Branco et al., 2020; Qian et al., 2020). Functional traits may be responsible for the species' ecological role, interactions among species, and the interaction among species and its environment (Petchey and Gaston, 2006; Davies et al., 2007). Functional beta diversity measures are based on quantifying differences between species with respect to morphological, physiological, and behavioral traits that drive functional diversity. Phylogenetic measures explicitly integrate information on the evolutionary history of the species in a community and allow for incorporating information on trait conservatism (Webb et al., 2002; Cadotte et al., 2009). Complementary information provided by these measures enable a more comprehensive understanding of the ecological and evolutionary processes driving diversity patterns (Graham and Fine, 2008; Cavender-Bares et al., 2009).

The global plight of rivers has generated significant interest in documenting biodiversity patterns and determining the effects of environmental change on riverine biodiversity (Dudgeon et al., 2006). High-energy river systems in mountainous landscapes, with wide altitudinal ranges, complex topography, and geo-morphological dynamism provide diverse habitats into which species have proliferated (Ormerod et al., 1994; Townsend and Hildrew, 1994; Villéger et al., 2013; Branco et al., 2020). However, our understanding of beta diversity patterns in these riverine systems is relatively poor (Ormerod et al., 1994; Jacobsen et al., 1997). Although riverine habitats comprise a relatively small proportion of mountainous landscapes, they provide unique niches for avian species from surrounding forested areas (Palmer and Bennett, 2006; Sullivan et al., 2007; Sinha et al., 2019b). Many riverine birds specialize in aquatic and riparian resources and depend on these linear habitats for their survival (Buckton and Ormerod, 2002).

The Himalayan mountain range has among the highest diversity of birds in the world with species richness peaking in the Eastern Himalaya (henceforth, E Himalaya). Past climatic factors (such as extensive snow cover) and increasingly drier and seasonal environment in the Western Himalaya (henceforth, W Himalaya) are thought to be among the important factors driving differences in bird diversity between W and E Himalaya. Among riverine birds too, E Himalaya has the highest diversity of river birds globally (Buckton and Ormerod, 2002; Srinivasan et al., 2014). The W Himalaya is comparatively species poor (Sinha, 2021). The differences in species richness and environment offer an excellent opportunity to determine the role of environmental filtering (vis-à-vis other factors) in driving beta diversity patterns. While studies in the W and Central Himalaya have examined species-habitat relationships (Manel et al., 1999; Buckton and Ormerod, 2008; Sinha et al., 2019a,b), studies from the E Himalaya remain restricted to few studies providing species-specific information (Menzies et al., 2021). Hence, our understanding of the drivers of regional community composition and beta diversity patterns of river bird assemblages remains limited (Manel et al., 2000; Buckton and Ormerod, 2008).

Previous studies on avian assemblages in the Himalaya have emphasized the roles of temperature seasonality in shaping bird communities in the E and W Himalaya (Srinivasan et al., 2019). Birds are susceptible to changes in habitat structure due to human land use at both local and landscape scales (Tscharntke et al., 2005; Larsen et al., 2010). Variation in river topography related variables like river width, depth and flow influence community assembly processes through different pathways (Royan, 2015). Regulation of river flow is the most damaging impact on riverine systems resulting from anthropogenic activities (Dynesius and Nilsson, 1994), which is particularly evident in the developing countries of the Indian subcontinent (Grumbine and Pandit, 2013). With approximately 300 dams across 90% of the Indian Himalayan valleys, severe impacts on biodiversity, including species extinctions, are to be expected (Pandit and Grumbine, 2012).

In this study, we present data on river bird communities from field censuses across multiple drainages in the Himalaya. We assessed different aspects of avian diversity in the E and W Himalaya to (1) compare patterns in taxonomic, functional, and phylogenetic beta diversity; (2) determine the relative contributions of turnover and nestedness to beta diversity; and (3) examine the relative contributions of climatic, geographic, and anthropogenic factors on beta diversity. Given that W Himalaya has a distinctly drier climate, we expect its riverine bird communities to be more clustered than those in the E Himalaya, owing to the more substantial role of environmental filtering. Given the higher diversity of riverine birds in the E Himalaya when compared to the W Himalaya, we expect a more significant role of turnover (compared to nestedness) in the E Himalaya indicating the role of biotic processes in structuring riverine bird communities. If climate-, habitat-, and human-related factors influence beta diversity patterns, it would indicate the role of environmental filtering in structuring riverine bird communities. Whereas if geographic distance influences beta diversity patterns, it would indicate the role of dispersal limitation in structuring river bird communities.

MATERIALS AND METHODS

Study Area

The Himalaya spread over 3,200 km across seven countries, representing the tallest and youngest mountain range in the world. Western Himalaya extends from Pakistan to north-west India (west of Nepal), while E Himalaya extends from eastern Nepal to northern Myanmar (Rodgers et al., 2000). It has high biodiversity value as recognized by its four biodiversity hotspots, 60 ecoregions and 330 important bird areas (Myers et al., 2000). Riverine areas in the Himalaya harbor the highest diversity of specialist riverine birds in the world, among which several species are of conservation concern (Buckton, 1998; Sinha et al., 2019a; Menzies et al., 2021).

We conducted field surveys in the states of Arunachal Pradesh and Uttarakhand in the Indian part of the E and W Himalaya, respectively (**Figure 1**). Arunachal Pradesh is part of the Eastern Himalaya Biodiversity Hotspot, sharing its borders with Myanmar, China, and Bhutan (Mittermeier et al., 2011). It is drained by many rivers, such as the Noa Dehing, Kamlang, Lohit, Dibang, Siang, Subansiri, and Kameng which are important headwaters to the river Brahmaputra. In the west, Uttarakhand shares international borders with Nepal and China and is drained by torrential snow-melt rivers such as the Bhagirathi, Alaknanda, Mandakini, Pindar, Kosi, and Kali, several of which are headwaters to the Ganges. In the areas sampled in the E Himalaya, the vegetation is dominated by tropical and subtropical broadleaved evergreen forests. In the W Himalaya, the vegetation in riparian areas consists of conifers at higher elevations and subtropical vegetation at the foothills (Gaur et al., 2019).

Riverine Bird Surveys

Following Buckton (1998), we sampled river-dependent birds by walking along river banks and recording the number of individuals of different bird species detected. In the E Himalaya, we sampled 81 sites of variable lengths (average length = 1,285 m; range = 300 m–2 km; total effort = 101.3 km) across seven river drainages encompassing a wide elevational gradient of 60–2,000 m asl (**Figure 1**). In the W Himalaya, we sampled 53 river reaches of equal length (500 m; total effort = 26.5 km) in the Bhagirathi (main river and six first-order streams) and Amrut Ganga basins, important headstreams of the Upper Ganges, between an elevation gradient of 330–3,100 m asl. Surveys were carried out post-monsoon (mainly covering winter months) when several of the river bird species migrate to lower altitudes. Conducting surveys on foot during the monsoons is not feasible or safe since the rivers swell and prevent crossing for long stretches. Sampling was conducted between September to late January (2016–2018) in the W Himalaya and between late August to early March (2017–2019) in the E Himalaya. All species' nomenclature follows the Clements Checklist by the Cornell Lab of Ornithology (Clements, 2007). For more details on the field survey design, please see Menzies et al. (2021) for the E Himalaya and Sinha et al. (2019a) for the W Himalaya.

Phylogenetic Data

We built a phylogenetic tree of 39 bird species recorded in our field surveys by pruning the global bird phylogenetic tree obtained from www.birdtree.org (Jetz et al., 2012). The original tree in Jetz et al. (2012) was assimilated from genetic data of 6,693 species of extant birds. The backbone of the tree was constructed using 15 genes (19 loci) of 151 key species and time-calibrated with 10 well-known fossils (Jetz et al., 2012). A total of 10,000 trees were sub-sampled for our target species using a pseudo-posterior distribution[1] to obtain 100 trees, which were used to prepare a consensus tree used in further analysis. Packages "ape" (Paradis et al., 2004) and "phytools" (Revell, 2012) were used to construct a phylogenetic tree of the species recorded in the field.

Functional Trait Data

We obtained data on the trophic level, foraging niche, and morphometry from Supplementary Dataset 1 in Pigot et al. (2020). Birds were classified into four trophic guilds (herbivore, carnivore, omnivore, and scavenger) and 10 foraging niches

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[1] https://birdtree.org/subsets/

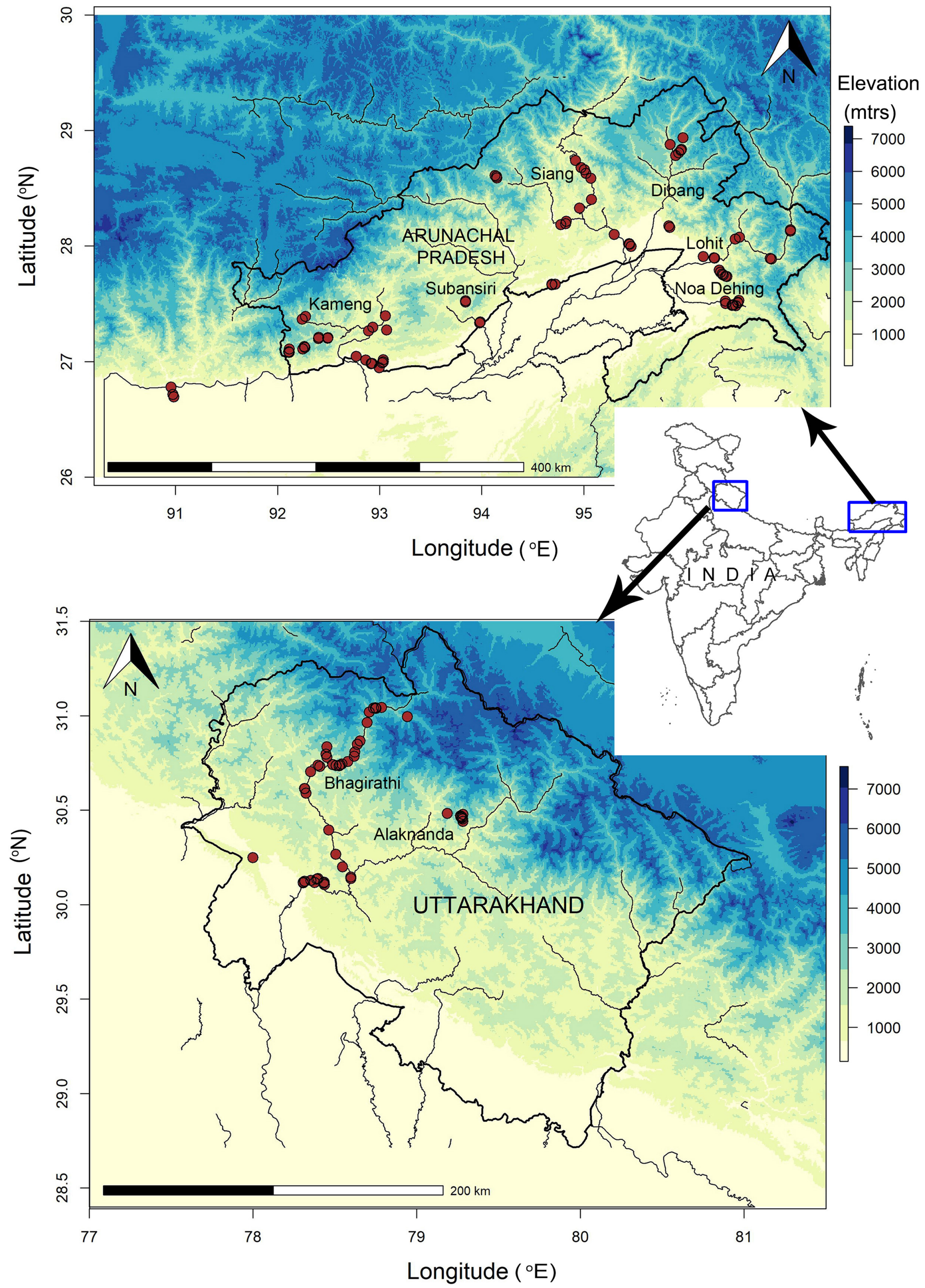

FIGURE 1 | Map showing the study area in the Indian Himalaya. Blue rectangles show the location of political states in the east and west where fieldwork was conducted and circles in red show locations of river stretches sampled. Eighty-one river reaches were sampled in the E Himalaya (Arunachal Pradesh) and 53 in the W Himalaya (Uttarakhand).

(e.g., aquatic dive, aquatic ground, aquatic perch, ground) (Pigot et al., 2020). For morphological trait data, we used the principal component scores reported for the overall body traits (comprising data on body mass, beak, wing, tail, and tarsus). For overall body traits, we used principal component scores of the first eight axes, which explained 99.9% of the variation among 9,963 bird species. Please see Pigot et al. (2020) for additional details on the functional trait data.

Environmental Data

Global patterns in species richness of river specialist birds highlight the role of net primary productivity (NPP) and temperature in shaping species richness patterns (Buckton and Ormerod, 2002). We downloaded net primary productivity data from the Terra Net primary productivity database from MODIS.[2] We obtained temperature seasonality (bio4) and annual precipitation (bio12) data from WorldClim (Fick and Hijmans, 2017). The recently developed human modification raster layer from Kennedy et al. (2019) and the forest cover data from Global Forest Watch[3] and its extracted values using a 500 m buffer along the surveyed river reaches were used in this study. We kept the buffer radius short as the life history parameters of the focal bird species are strictly tied to the riparian habitat (Buckton and Ormerod, 2008). We recorded elevation and river width at survey locations during fieldwork.

Analyses

Estimating Taxonomic, Functional, and Phylogenetic Diversity

To understand beta diversity patterns in river bird communities, we calculated taxonomic, phylogenetic, and functional diversity values between each pair of sites (81 sites in the east and 53 sites in the west). For this analysis, we did not use abundance-weighted diversity measures to control for variable sampling length of river reaches in the field. We used species richness values of each site to calculate taxonomic diversity and used Sorensen's dissimilarity matrix as implemented by the function "vegdist" in package "vegan" to calculate taxonomic beta diversity (Oksanen et al., 2019).

For phylogenetic and functional diversity, we used two dispersion metrics—mean pair-wise distance (MPD) and mean nearest taxon distance (MNTD)—which are calculated using the variation among traits or tips in a phylogeny, thus aiding in understanding the role of deterministic processes. MPD calculates the pair-wise distance between each species (including conspecifics) in the community and is independent of the species richness (Webb et al., 2002). MNTD calculates the distance to the closest non-conspecific relative and is hence dependent on the number of species in a community (Webb et al., 2002). While MPD is based on the overall structure of the tree, MNTD is based on the architecture of the tree near its terminals. Different drivers may influence basal and terminal architecture of the trees differentially, thus necessitating the use of the two measures. For functional diversity, we created a tree using the principal component scores for the different species associated with the overall phenotype (described above) from the global bird-trait dataset (Pigot et al., 2020); we did this using the UPGMA clustering method in package "phangorn" (Schliep, 2011).

MPD and MNTD were calculated for the phylogenetic and functional diversity of species assemblages in the E and W Himalaya separately. To examine the changes in phylogenetic and functional MPD and MNTD, we calculated the cophenetic distance using the function "cophenetic" in package "vegan" (Oksanen et al., 2019) and created a site-wise dissimilarity measure using phylogenetic and functional tree. MPD and MNTD were calculated as Bray-Curtis distance using functions "comdist" and "comdistnt," respectively, in package "picante" (Kembel et al., 2010). Additionally, we calculated the standardized effect sizes (SES) of MPD and MNTD for both the phylogenetic and functional diversity using established methods (Webb et al., 2002). We compared the observed estimates with the null distribution (n = 1,000 randomizations) while controlling for species richness. SES are informative as they make measures such as MNTD, which are sensitive to species richness, comparable. Negative values of SES indicate clustering (species are more closely related) in the community while positive values indicate overdispersion (species are more distantly related) (Webb et al., 2002; Kembel et al., 2010). We used a Mann-Whitney U-test to compare the different diversity measures across the E and the W Himalaya.

Total beta diversity was partitioned into its two constituent components—dissimilarity due to turnover (β_{turn}) and nestedness (β_{nest}) using the function "beta.pair" from the package "betapart" (Baselga and Orme, 2012). While turnover is the proportion of dissimilarity between a pair of sites due to the replacement of species, nestedness is the proportion of dissimilarity owing to species in a site being a nested subset of the other due to the addition or removal of species.

Determining Drivers of Beta Diversity

We used a non-linear regression-based method of Generalized Dissimilarity Modeling (GDM) (Ferrier et al., 2007) to model the taxonomic, phylogenetic, and functional beta diversity. Pair-wise dissimilarity in community composition across sites was modeled as a function of environmental covariates and geographic distance; this was done separately for the E and the W Himalaya to identify local and regional drivers of beta diversity for the three components. GDM is an extension of matrix regression where different covariates are fitted using splines. GDM explicitly accounts for the curvilinear relationship, depicting the dissimilarity of species between sites as ecological distance and identifying a subset of significant environmental predictors. This approach enables plotting predicted community turnover rates as a function of each variable (Ferrier et al., 2007). We used a combination of environmental (precipitation, temperature seasonality, primary productivity, forest cover), geographic (elevation, geographic distance, river width), and human disturbance variables to model beta diversity measures. Since the length of the sampling unit varied across different sites in the E Himalaya, we used sampling length as a predictor for the E Himalayan dataset. All spatial analyses were carried out in package "raster" (Hijmans, 2015). We calculated the variance explained by each predictor variable using the function "gdm.varImp" in "gdm" (Ferrier et al., 2007) package. All statistical analyses was carried out in software R ver. 3.6 (R Core Team, 2019).

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[2] https://ladsweb.modaps.eosdis.nasa.gov/missions-andmeasurements/products/MOD17A3/

[3] https://glad.umd.edu/projects/global-forest-watch

RESULTS

Riverine Bird Community

We recorded 3,897 individuals of 39 species of birds belonging to 15 families during combined field surveys. We encountered 2,996 individual birds consisting of 36 bird species in the E Himalaya and 901 individual birds from 17 species in the west (**Table 1**). Muscicapidae (old world flycatchers) and Alcedinidae (kingfishers) were the most represented families in both the east and the west. Plumbeous Redstart (*Phoenicurus fuliginosus*) was the most encountered species in W Himalaya and the second most encountered species in E Himalaya. Ibisbill (*Ibidoryncha struthersii*) was the least encountered bird in W Himalaya, whereas Black-backed Forktail (*Enicurus immaculatus*) and White-throated Dipper (*Cinclus cinclus*) were the least encountered species in E Himalaya (for species list see **Table 1**). We recorded four species of conservation concern, including the Critically Endangered White-bellied Heron (*Ardea insignis*), Endangered Black-bellied Tern (*Sterna acuticauda*), and the Near Threatened River Lapwing (*Vanellus duvaucelii*) and Great Thick-knee (*Esacus recurvirostris*). There was some evidence phylogenetic clustering for the W Himalaya sites as their median values were beyond −1.96 units from the null expectation (**Figure 2**).

Beta Diversity Patterns in the River Bird Community

We did not find differences in taxonomic and MNTD metrics for the phylogenetic and functional components ($p > 0.05$). However, we found greater clustering in phylogenetic ($W = 2{,}229{,}371$; $P < 0.001$) and functional ($W = 2{,}230{,}439$; $P < 0.001$) MPD for the W Himalaya than that for the E Himalaya (**Figure 2**). Beta diversity was highest for the taxonomic component compared to that for phylogenetic and functional components (**Figure 3**). MPD was higher than MNTD for both the phylogenetic and functional components of beta diversity (**Figure 3**).

The relative contribution of turnover and nestedness to the overall beta diversity varied across the different components and also between the E and W Himalaya. In the E Himalaya, there was a greater contribution of turnover to overall beta diversity as compared to that by nestedness for the taxonomic ($W = 10{,}556{,}567$, $P < 0.001$), phylogenetic ($W = 7{,}699{,}831$, $P < 0.001$), and functional ($W = 8{,}994{,}152$, $P < 0.001$) components (**Figure 3**). In the W Himalaya, there was greater contribution of turnover to overall beta diversity only for the taxonomic component ($W = 581{,}821$, $P < 0.001$). The overall turnover (β-total) was similar across E and W Himalaya for the taxonomic component ($P > 0.05$). However, the overall turnover was higher for the E Himalaya than that for W Himalaya for phylogenetic ($W = 1{,}698{,}385$; $P = 0.023$) and functional ($W = 1{,}756{,}820$; $P < 0.001$) components (**Figure 4**).

Drivers of Beta Diversity

The GDM explained 64–90% of the variation in different beta diversity measures, thus explaining beta diversity patterns in river bird communities in the Indian Himalaya (**Figure 5**). Overall, GDM explained maximum variance for the functional components of beta diversity followed by the taxonomic and phylogenetic components; comparatively, a higher proportion of variance was explained for the W Himalayan communities than that for the east (**Figure 5**). Maximum variance was explained for $\beta\text{-FD}_{MPD}$ (93%) followed by $\beta\text{-FD}_{MNTD}$ (82%) and β-TD (81.8%) in the west.

Results of spatial GDM showed that the habitat and environmental variables, rather than geographical distance, influenced the observed beta diversity patterns in river bird communities in both the east and the west (**Figure 5**). River width explained a bulk of the variation in beta diversity for most measures across all three components (**Figure 5**). The relationship between river width was non-linear and beta diversity increased exponentially with increasing river width, particularly in the E Himalaya and reached an asymptote in the W Himalaya (**Supplementary Figures 1–6**). While forest cover and elevation explained variation in beta diversity patterns in the east, rainfall was an important variable influencing beta diversity patterns in the west (**Figure 5**). Temperature seasonality, net primary productivity, and human modification showed limited roles in driving the beta diversity patterns (**Figure 5**). The relationships between the different predictors and beta diversity measures were non-linear and varied across the different measures and the E and W Himalaya regions (**Supplementary Figures 1–6**).

DISCUSSION

In this study, we found that river bird communities exhibited phylogenetic and functional clustering with a greater signal of clustering in the W Himalaya. The turnover component had a larger contribution in explaining the variation in beta diversity patterns than did the nestedness component, especially in the E Himalaya. Predictors that explained the variation of beta diversity varied across the different components (taxonomic, phylogenetic, and functional) and between the E and the W Himalaya. Climatic and habitat factors, rather than geographic distance explained the variation in overall compositional (taxonomic, phylogenetic, and functional) dissimilarity in riverine bird communities in the Indian Himalaya. Our results demonstrate the key role of environmental filtering in structuring riverine bird communities in the Himalaya. Given the strong influence of habitat features in influencing diversity patterns, ongoing threats to riverine habitats posed by hydroelectric projects and forest loss in the Himalaya can impact the riverine bird communities in this hotspot negatively.

Role of Environmental Filtering

A significant proportion of the variation in beta diversity patterns in riverine bird communities was best explained by habitat features such as river width, elevation, forest cover, and climatic factors such as precipitation. River width was a consistent driver of all measures of beta diversity (**Figure 5**). In the E Himalaya, beta diversity for all three measures increased

TABLE 1 | List of bird species recorded during field surveys in E and W Himalaya with their family, IUCN conservation status and trophic niche as classified by Pigot et al. (2020).

Bird species	Family	IUCN status	Trophic niche
Common merganser (*Mergus merganser*)[†]	Anatidae	Least concern	Aquatic predator
Ruddy shelduck (*Tadorna ferruginea)**	Anatidae	Least concern	Aquatic predator
Gray heron (*Ardea cinerea*)*	Ardeidae	Least concern	Aquatic predator
White-bellied heron (*Ardea insignis*)[†]	Ardeidae	Critically endangered	Aquatic predator
Great cormorant (*Phalacrocorax carbo*)*	Phalacrocoracidae	Least concern	Aquatic predator
Little cormorant (*Phalacrocorax fuscicollis*)*	Phalacrocoracidae	Least concern	Aquatic predator
Great crested grebe (*Podiceps cristatus*)[†]	Podicipedidae	Least concern	Aquatic predator
Great thick-knee (*Esacus recurvirostris*)*	Ardeidae	Near threatened	Invertivore
Ibisbill (*Ibidoryncha struthersii*)*	Ibidorynchidae	Least concern	Aquatic predator
River lapwing (*Vanellus duvaucelii*)*	Charadriidae	Near threatened	Aquatic predator
Long-billed plover (*Charadrius placidus*)[†]	Charadriidae	Least concern	Aquatic predator
Little-ringed plover (*Charadrius_dubius*)[†]	Charadriidae	Least concern	Aquatic predator
Small pratincole (*Glareola lactea*)[†]	Glareolidae	Least concern	Invertivore
Black-bellied tern (*Sterna auticauda*)[†]	Laridae	Endangered	Aquatic predator
Pallas's gull (*Ichthyaetus ichthyaetus*)[#]	Laridae	Least concern	Omnivore
Little stint (*Calidris minuta*)[†]	Scolopacidae	Least concern	Aquatic predator
Common sandpiper (*Actitis hypoleucos*)*	Scolopacidae	Least concern	Aquatic predator
Green sandpiper (*Tringa ochropus*)*	Scolopacidae	Least concern	Aquatic predator
Marsh sandpiper (*Tringa stagnatilis*)[†]	Scolopacidae	Least concern	Aquatic predator
Common greenshank (*Tringa nebularia*)[†]	Scolopacidae	Least concern	Aquatic predator
Blue-eared kingfisher (*Alcedo mininting*)[†]	Alcedinidae	Least concern	Aquatic predator
Common kingfisher (*Alcedo atthis*)*	Alcedinidae	Least concern	Aquatic predator
White-throated kingfisher (*Halcyon smyrnensis*)*	Alcedinidae	Least concern	Omnivore
Crested kingfisher (*Megaceryle lugubris*)*	Alcedinidae	Least concern	Aquatic predator
Pied kingfisher (*Ceryle rudis*)*	Alcedinidae	Least concern	Aquatic predator
White wagtail (*Motacilla alba*)*	Motacillidae	Least concern	Invertivore
Gray wagtail (*Motacilla cinerea*)*	Motacillidae	Least concern	Invertivore
White-browed wagtail (*Motacilla maderaspatensis*)[#]	Motacillidae	Least concern	Invertivore
Wallcreeper (*Trichodroma muraria*)*	Trichodromidae	Least concern	Invertivore
Brown dipper (*Cinclus pallassi*)*	Cinclidae	Least concern	Aquatic predator
Hodgson's redstart (*Phoenicurus hodgsoni*)[†]	Muscicapidae	Least concern	Aquatic predator
Plumbeous water redstart (*Phoenicurus fuliginosus*)*	Muscicapidae	Least concern	Aquatic predator
White-capped redstart (*Phoenicurus leucocephalus*)*	Muscicapidae	Least concern	Aquatic predator
Blue whistling thrush (*Myophonus caelereus*)*	Muscicapidae	Least concern	Omnivore
Black-backed forktail (*Enicurus immaculatus*)[†]	Muscicapidae	Least concern	Aquatic predator
Slaty-backed forktail (*Enicurus schistaceus*)[†]	Muscicapidae	Least concern	Aquatic predator
Little forktail (*Enicurus scouleri*)*	Muscicapidae	Least concern	Aquatic predator
White-crowned forktail (*Enicurus leschenaultia*)[†]	Muscicapidae	Least concern	Aquatic predator
Spotted forktail (*Enicurus macultus*)*	Muscicapidae	Least concern	Aquatic predator

Recorded both in the E and W Himalaya; [†]Recorded only in the E Himalaya; [#]Recorded only in the W Himalaya.

with river width, with a sharp rise in dissimilarity across river stretches wider than 150 m (**Supplementary Figures 1–3**). Larger river width is associated with river stretches at lower elevations with a higher discharge; they are also characterized by variable flow rates offered lotic to lentic habitats, which provide niches to a diverse array of riverine birds preferring slow- or medium-flowing river sections. The bank substrates are also variable (rocky or sandy), particularly in the lower sections of the rivers. Bouldery and pebbly bank substrates offer important microhabitats for birds such as dippers and redstarts, while the lower elevation sandy banks are preferred by wagtails, pratincoles, and lapwings (Sinha et al., 2019a). Since several riverine bird species show a preference for specific micro-habitats, there is likely to be higher species turnover as rivers progress from narrow headstreams to wider river stretches. Narrower river stretches support prey-base for a small group of specialist birds (e.g., redstarts and forktails) that predominantly have an insectivorous diet (Buckton and Ormerod, 2008; Sinha et al., 2019a). In comparison, wider river stretches offer greater river depths associated with larger prey such as fishes, amphibians, and mollusks, thus attracting other waterbirds such as kingfishers, cormorants, mergansers, gulls, and sandpipers to visit these river stretches opportunistically (for species list see **Table 1**).

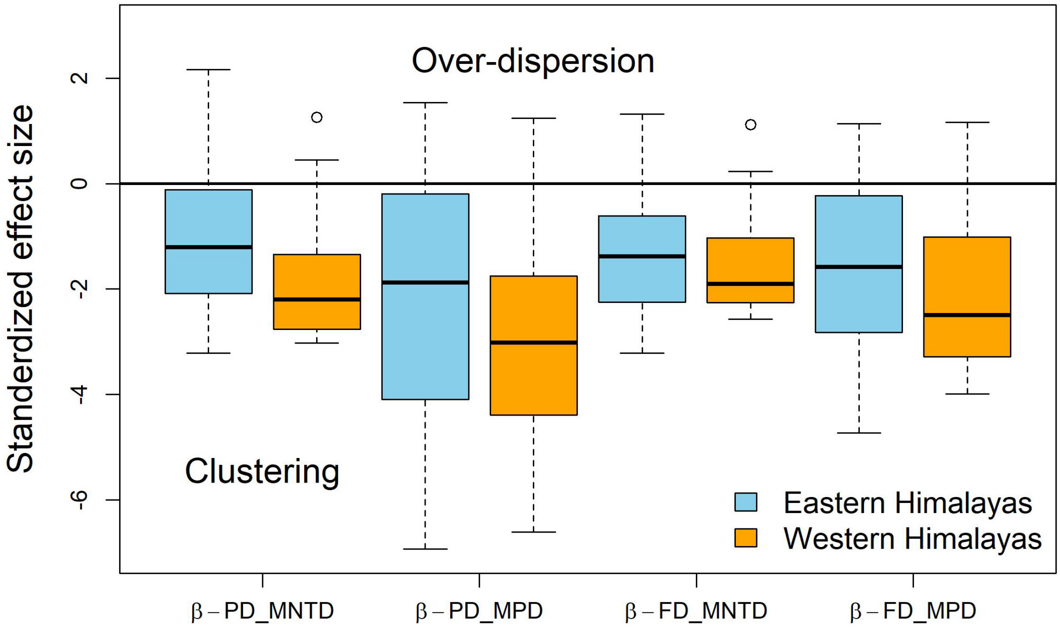

FIGURE 2 | Standardized effect size (SES) for mean pair-wise phylogenetic distance (MPD) and mean nearest taxon distance (MNTD) for phylogenetic and functional beta diversity in the W and E Himalaya.

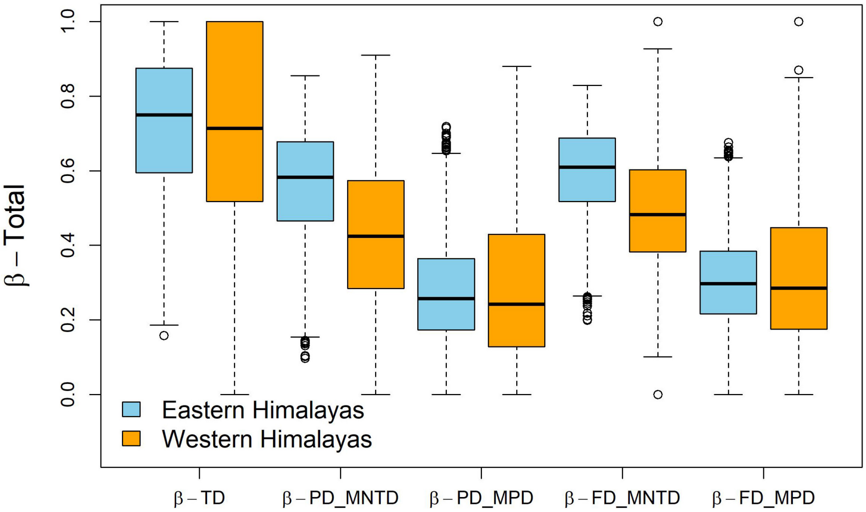

FIGURE 3 | Box-whisker plots showing pair-wise taxonomic, phylogenetic and functional β-total (Sørensen's pairwise dissimilarity) in river bird communities in the E and the W Himalaya based on field surveys in winter. TD, Taxonomic Diversity; FD, Functional Diversity; PD, Phylogenetic Diversity; MPD, Mean Pair-wise Distance; MNTD, Mean nearest taxon distance.

Interestingly, the relative influence of other drivers varied across the E and W Himalaya. Forest cover and elevation explained significant variation for the E Himalayan birds. Along with the river channel and flood plain, the riparian forests are also recognized as an integral part of the riverscape (Weins, 2002). Distributions of many riverine bird species are positively or negatively associated with tree cover (Sullivan et al., 2007; Vaughan et al., 2007; Sullivan and Vierling, 2012; Sinha et al., 2019a). Here, beta diversity increased with increasing forest cover, indicating a higher homogenization in bird composition in river stretches with lower forest cover (**Supplementary Figures 1–3**). Forests in the Himalaya are being lost at an alarming rate (Srivastava et al., 2002; Gaur et al., 2019; Sheth et al., 2020), which could potentially result in lower species turnover in riverine bird communities with cascading effects on overall diversity patterns in the long-term.

Elevation is an important driver of riverine bird diversity (Manel et al., 2000). Interestingly, at elevations above 300 m in

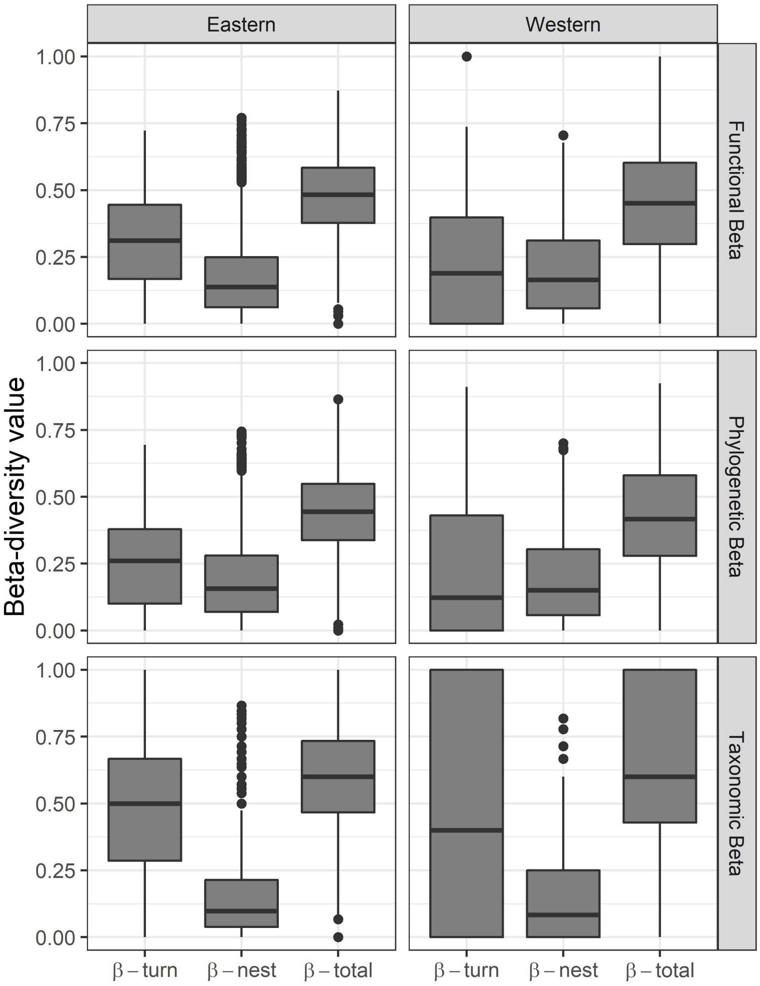

FIGURE 4 | Box-whisker plots showing beta diversity components (β-turnover, β-nestedness, β-total) of the river bird community in the E and W Himalaya.

the E Himalaya, the beta diversity patterns were consistent and showed a relatively lower species turnover at elevations below 300 m. In the E Himalaya, the lowermost elevations are characterized by higher anthropogenic disturbances, resulting threats such as lower forest cover, and boulder and sand mining (Srivastava et al., 2002; Menzies et al., 2021), which likely contribute to the lower species turnover in the lowland elevations. Anthropogenic activities negatively impact specialist bird species resulting in the persistence of generalist species that are able to withstand habitat changes (Sinha et al., 2019a) likely resulting in lower turnover in the more modified, low elevation riparian habitats. Additionally, lower elevation avian communities were dominated by large-body sized species (e.g., cormorants, mergansers, pratincoles, waders) which prefer wider river stretches and habitat features, like sand bars and river islands. These could be uniform across sites in the lower elevation which may have resulted in lower turnover. These aspects need to be explored in greater detail in future.

Climatic factors such as precipitation and temperature are related to the variation in beta diversity patterns (Naka et al., 2020; Wayman et al., 2021). We found that precipitation was an important factor in explaining the variation in beta diversity patterns in the W Himalaya. The W Himalaya is more seasonal and drier than the E Himalaya (Price et al., 2011). There was a lower species turnover in drier habitats as compared to wetter habitats (precipitation lower than ∼1,400 mm). E Himalaya is consistently wetter across the entire elevation gradient; therefore, precipitation does not play a significant role in influencing the beta diversity patterns (**Supplementary Figures 1–3**). Similar to forest birds, the W Himalayan riverine bird assemblage is a nested subset of the E Himalaya assemblage, which is more species-rich (Buckton and Ormerod, 2002; Price et al., 2011; Srinivasan et al., 2014). This indicates that species that were able to pass through the environmental filter imposed by a drier and variable climate in the W Himalaya were able to successfully colonize, despite the harsh climate. However, this pattern is also likely to be a consequence of past climatic influences. During the Pleistocene glaciation, the W Himalayan region was under ice, while evergreen forests persisted in the E Himalayan region (Owen et al., 2002; Srinivasan et al., 2014). Thus, the past and

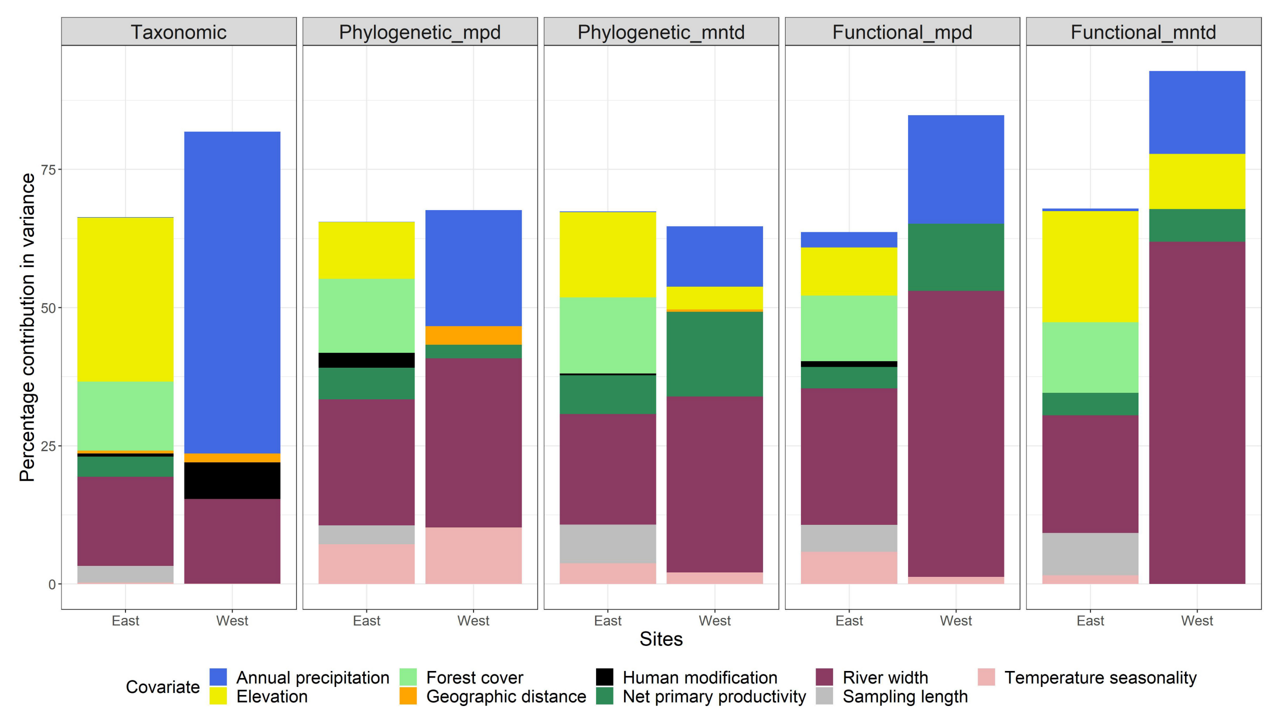

FIGURE 5 | Variance partitioning of different predictor variables across beta diversity measures (taxonomic, phylogenetic, and functional) for river birds at different scales.

present seasonality and variability in climate may have led to a greater clustering in riverine communities in the W Himalaya than that in the E Himalaya.

Contribution of Turnover and Nestedness

We found a greater contribution of the turnover component in taxonomic and functional beta diversity, especially in the E Himalaya. Higher turnover is a consequence of biotic interactions (e.g., competition), and greater nestedness is a consequence of local or regional extinctions, particularly in unfavorable and variable environments (Schemske et al., 2009; Soininen et al., 2018). As discussed earlier, the E Himalaya is more species-rich than the W Himalaya (Buckton and Ormerod, 2002); therefore, it is more likely that biotic interactions have an influence on turnover of species communities in the E Himalaya. Moreover, past (Pleistocene glaciation) and present (variable precipitation) climatic conditions likely cause similar influence of nestedness and turnover in the W Himalaya.

Differences Between Beta Diversity Components

There was a relatively higher turnover in taxonomic diversity than that in phylogenetic and functional diversity, which indicates that species are replaced by their close relatives with similar traits across sites. Himalayan rivers are characterized by high species richness consisting of multiple species within a lineage of riverine birds (e.g., three species of redstarts, five species of forktails, five species of kingfishers) that differ in their microhabitat requirements (Buckton and Ormerod, 2008; Sinha et al., 2019a). Riverine ecosystems are extremely dynamic because of high natural disturbances offering diverse microhabitats (Ward et al., 2002). Changing microhabitats and the associated replacement of species by their close relatives play a functionally similar role that likely results in the pattern observed in this study.

We found that the different beta diversity measures were related to similar predictors within a region (i.e., E or W Himalaya). However, the relative contribution of the different predictors in explaining the variation in beta diversity differed across the different measures (**Figure 5**), which has been reported elsewhere (Wayman et al., 2021). Interestingly, precipitation explained a large proportion of variation in taxonomic component of beta diversity in the W Himalaya (**Figure 5**), but a lower proportion of variation in phylogenetic and functional component. This result could be a likely outcome of closely related species (which are likely to be functionally similar) replacing each other along a gradient of precipitation. Moreover, the proportion of variation explained by forest cover across the different components was similar; this is a likely outcome of certain groups disappearing along the forest cover gradient and needs to be examined in greater detail in the future.

Limited Role of Distance and Habitat Modification

Geographical distance is an important driver of beta diversity patterns in birds (Wayman et al., 2021). However, we did not find a significant influence of geographical distance on beta diversity patterns in the E or the W Himalaya. The influence of distance on beta diversity patterns is explained by dispersal limitation (Myers et al., 2013; Weinstein et al., 2014). Birds are unlikely to be dispersal limited at the scale at which the analysis has been carried out in the E and the W Himalaya. Although we found limited evidence of the influence of habitat modification on beta diversity patterns in the W but not in the E Himalaya, past studies have documented birds being sensitive to anthropogenic influences (Sinha et al., 2019a; Abreu et al., 2020; Menzies et al., 2021). Fine-scale information on human disturbance may be able to provide additional insights on human impacts.

Limitations of the Study

This study was conducted primarily in the non-breeding season. Given that some riverine species exhibit altitudinal migration, beta diversity patterns and the influence of underlying drivers may vary. It will be important to conduct a similar study during the breeding season. However, conducting studies during the breeding season might be difficult due to inclement weather and associated flooding of the river. Nevertheless, the non-breeding season is also associated with a greater diversity of birds in the region as riverine stretches are being used by several other bird species that are dependent on riparian resources; hence, the non-breeding season is an important time for evaluating community organization. Given the high rates of human-driven modification of the Himalayan rivers, a systematic Himalaya wide survey across all drainages is required to determine the role of anthropogenic impacts on riverine systems.

Another potential shortcoming of the study could be variable sampling effort, particularly in the Eastern Himalaya. However, we used sampling length as a predictor in the variance partitioning analysis and demonstrate that very little variation in the beta diversity in E Himalaya is explained by the sampling length. Additionally, analysis of turnover in taxonomic beta diversity using a novel method (Zou and Axmacher, 2020) that helps control for variable sampling effort did not reveal any differences in beta diversity patterns (**Supplementary Figure 7**). Given this it is likely that variable sampling length will unlikely influence the outcome of this study.

CONCLUSION

This study provides evidence for the role of environmental filtering in structuring animal communities. This is the first study to examine turnover in riverine bird diversity in the Himalaya, the most species rich region for riverine birds and among the most threatened riverine habitats in the world. Globally, there are few studies that have examined the relative roles of different drivers on the different beta diversity measures of riverine birds and this study fills that knowledge gap. This study highlights the strong role of environment in driving beta diversity patterns. The wide climatic (precipitation), geographic (elevation), and habitat (river width, forest cover) gradient of the Himalayan mountain range contributes to the riverine bird diversity in the region. More than 160 dams have been proposed in the E Himalaya (Vagholikar and Das, 2010), which are likely to permanently alter the riverine systems with significant negative impacts on riverine bird communities as reported elsewhere (Abreu et al., 2020). In the Bhagirathi basin, the construction of the Tehri dam has converted a 75 km stretch of lotic habitat into a lentic habitat (Gaur et al., 2019). The riverine forests in the region also experience threats from severe habitat loss, destructive fishing methods, and sand and boulder mining, which can also be expected to impact riverine bird communities (Manel et al., 2000; Srivastava et al., 2002; Menzies et al., 2021). There is an urgent need to systematically determine the relative impacts of the different threats to riverine bird communities. Additionally, greater variability in precipitation patterns and river flows due to climate change (Prudhomme et al., 2014) will negatively impact the riverine bird communities (Royan et al., 2015), particularly in the drier and more variable W Himalaya.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because this was a purely observational study that did not involve the handling or collection of animals. The data collected in one region was approved by the Ethics Committee of the Nature Conservation Foundation.

AUTHOR CONTRIBUTIONS

RN and AS conceived the study. RM, MR, AS, and RN collected field data. NC ran the analysis with inputs from AS, RN, RM, and MR. AS, RN, NC, and RM wrote the manuscript with inputs from MR. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.788184/full#supplementary-material

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Riverscape Genomics Clarifies Neutral and Adaptive Evolution in an Amazonian Characin Fish (*Triportheus albus*)

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Understanding the role of natural selection in the evolution of wild populations is challenging due to the spatial complexity of natural systems. The richest diversity of freshwater fishes in the world is found in the Amazon Basin, a system where marked hydrochemical differences exist at the interface of major rivers with distinct "water colors" (i.e., black, white, and clear water). We hypothesize that divergent natural selection associated with these "aquatic ecotones" influences population-level adaptive divergence in the non-migratory Amazonian fish fauna. This hypothesis was tested using a landscape genomics framework to compare the relative contribution of environmental and spatial factors to the evolutionary divergence of the Amazonian characin fish *Triportheus albus*. The framework was based on spatial data, *in situ* hydrochemical measurements, and 15,251 filtered SNPs (single nucleotide polymorphisms) for *T. albus* sampled from three major Amazonian rivers. Gradient Forest, redundancy analysis (RDA) and BayPass analyses were used to test for signals of natural selection, and model-based and model-free approaches were used to evaluate neutral population differentiation. After controlling for a signal of neutral hierarchical structure which was consistent with the expectations for a dendritic system, variation in turbidity and pH were key factors contributing to adaptive divergence. Variation in genes involved in acid-sensitive ion transport pathways and light-sensitive photoreceptor pathways was strongly associated with pH and turbidity variability. This study improves our understanding of how natural selection and neutral evolution impact on the distribution of aquatic biodiversity from the understudied and ecologically complex Amazonia.

Keywords: evolutionary ecology, adaptation, ecological genomics, Amazonia, tropical diversification, landscape genomics, ddRAD, teleost

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INTRODUCTION

Natural selection is a ground-breaking conceptual framework to our understanding of evolution (Darwin, 1859). However, challenges with empirically studying adaptive divergence in nature has ultimately led to a bias toward using spatial factors to explain evolutionary patterns (Wang and Bradburd, 2014). Selectively neutral models of population divergence include Isolation by

Distance (IBD), where gene flow aligns with gradients of distance between populations (Wright, 1943; Sexton et al., 2014), and neutral hierarchical structure, where punctuated barriers restrict gene flow to particular geographic zones (Meirmans, 2012). When gene flow and differentiation better align with similar environments, an adaptive Isolation by Environment (IBE) model, where divergent natural selection acts on evolutionary advantageous phenotypes, might provide a more superior explanation for inferred patterns of population structure (Sexton et al., 2014; Wang and Bradburd, 2014).

The empirical challenge of clarifying patterns of adaptive divergence in wild populations can be addressed using "landscape genetics," a multi-disciplinary study that relies on explicit statistical quantification of environmental and geographic variables to test for the relative influence of each on genetic variation across the landscape (Storfer et al., 2007). More recently, the arrival of "landscape genomics," which utilizes genome-wide information to identify environmental features influencing genetic variation, has considerably increased the power to study local adaptation and adaptive divergence in wild populations—the initial outcomes of the process of divergent natural selection (Wang and Bradburd, 2014; Rellstab et al., 2015; Grummer et al., 2019). This includes assessment of a population's ability to evolve and maintain fitness in response to environmental changes, or their "adaptive capacity," an important issue in conservation management and applied evolution (Grummer et al., 2019). This is particularly timely as habitats across the globe are rapidly shifting in environmental properties, with many niches under notable threat (Thurman et al., 2020).

Limited research has explained how natural selection acts in aquatic environments (Kelley et al., 2016; Grummer et al., 2019). In rivers and streams, a constrained, unidirectional spatial flow of genes along well-defined migratory paths provides an ideal environment for the study of natural selection, as the impacts of geographic separation on genetic divergence can be controlled for (Hughes et al., 2009; Brauer et al., 2018; Attard et al., 2022). The Amazon, also known as Amazonia, holds the highest diversity of freshwater fishes in the world, yet little is understood about their evolution (Corlett and Primack, 2010; Beheregaray et al., 2014). Patterns of diversification in the Amazon have been mostly studied in the context of geographic isolation based on neutral genetic markers (Albert et al., 2011, 2020). Very few studies have assessed the role of natural selection in population divergence and evolution in the Amazon (reviewed in Beheregaray et al., 2014), an endeavor that requires information about adaptive genetic variation (Grummer et al., 2019).

The Amazon River network is composed of three distinctive river types with characteristic attributes resulting from the geological origin of water they contain, groundwater level, soil types, and vegetation (Val, 1995). Each water color shows a unique composition of pH, turbidity, water temperature and dissolved oxygen; thus "water color" can be used as an umbrella term to summarize differences in hydrochemical properties of water bodies. Variances in these hydrochemical parameters can drive constraints on the ecological communities inhabiting water bodies, and as such water color can be considered a major "aquatic ecotone" (Beheregaray et al., 2014). Water colors and their boundaries in the Amazon have been demonstrated to influence the distribution of forest floodplain avifauna (Laranjeiras et al., 2021) and the migratory paths of important fish species (McClain and Naiman, 2008).

From a landscape genetics perspective, several studies have investigated how genetic divergence in Amazonian fish populations might be influenced by water color (Cooke et al., 2012a,b,c, 2014; Beheregaray et al., 2014). This included the proposal of a framework capable of distinguishing the relative contributions of natural selection associated with aquatic ecotones vs. biogeographic history in the origin of fish population diversity (Beheregaray et al., 2014). These studies generally found support for water color as a selecting agent in Amazonian fish divergence. However, the limited number and the anonymous nature of the DNA markers used (amplified fragment length polymorphisms, AFLPs) offer little power to disentangle adaptive from neutral genetic variation (Luikart et al., 2003) and to gain insights about the functional relevance of putative adaptations (Grummer et al., 2019). Advancements in genome-wide screening technologies using single nucleotide polymorphisms (SNPs) has enabled a higher genotyping efficiency, data quality, analytical simplicity, and coverage across the genome (Luikart et al., 2003; Morin et al., 2004, 2009). This has allowed for greater power to differentiate adaptive from neutral genetic variation, enabling the identification of candidate loci influenced by natural selection (Vitalis et al., 2001; Morin et al., 2004, 2009; Seehausen et al., 2014).

This study focuses on the Amazonian benthopelagic fish *Triportheus albus* (Characiformes: Triporthidae) (Cope, 1871). *Triportheus albus* is a non-migratory species that has been recorded to grow up to 26 cm with a weight of up to 200 g (Giarrizzo et al., 2015). Little is known about the population dynamics and ecology of *T. albus*, a deficiency that applies to most other fish species in the Amazon (Prestes et al., 2010). This work aims to assess the relative contribution of spatial factors and environmental variation driving evolutionary divergence of *T. albus,* within the context of riverine "water color." We hypothesize that, after controlling for spatial covariables, evolutionary divergence due to divergent selection should be stronger between populations of *T. albus* found in different ecotones than between populations from the same ecotone. This possibility derives from the strong physiological constraints that major hydrochemical and environmental gradients impose upon aquatic communities in Amazonia (Junk et al., 1983; Saint-Paul et al., 2000). To test this hypothesis, we generated a genome-wide dataset for *T. albus* populations that was integrated analytically with environmental and spatial datasets within a landscape genomics framework. We employed model-based and model-free approaches to assess neutral population differentiation and several Genotype-Environment Association (GEA) analyses to test how genetic variation might be associated with environmental predictors embodying different water colors. We also identify gene regions involved with adaptation to different water habitats and predict their role in driving natural selection. To the best of our knowledge, this is the first landscape genomics study for an Amazonian fish, providing valuable insights into how spatial and environmental

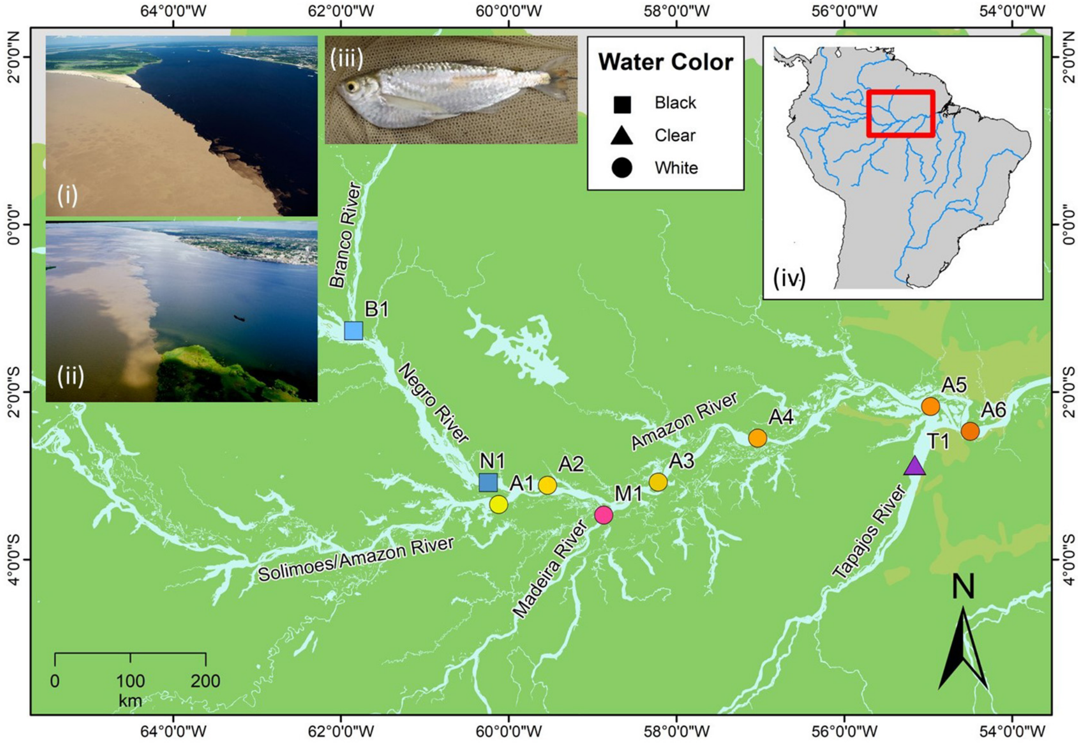

FIGURE 1 | Sampling localities of *Triportheus albus* in the Amazon Basin. Shape of site points correspond to water color of the locality (square = black water, triangle = clear water, circle = white water). Black water sites are labeled in blue points (B1 = Branco, N1 = Negro), clear water in purple (T1 = Tapajós), the white water Amazon in a gradient of yellow to orange (A1–A6), and the white water Madeira in pink (M1). Inset **(i)** shows the confluence of the Amazon and Negro rivers, and **(ii)** shows the confluence of the Amazon and Tapajós rivers. Inset **(iii)** depicts the study species *T. albus.* Inset **(iv)** displays the study area in northern South America.

factors simultaneously influence adaptation and population-level divergence within the ecologically rich Amazon Basin.

MATERIALS AND METHODS

Sampling and Study Site

Our fieldwork explored over 1,100 km of total riverine distance in the Amazon Basin in February 2005 and in February 2008. Sampling was done at 10 sites representing five major river systems. These systems cover all three major hydrochemical environments of the Amazon Basin; the Amazon and the Madeira (white), the Negro (black), the Branco (seasonally black), and the Tapajós (clear) rivers (**Figure 1**). The white water Amazon River (known as Solimões upstream of Manaus) is central to the sampling transect and drains west to east, with the black water Negro, white water Madeira and clear water Tapajós rivers flowing into it. The black water portion of the Branco River that flows into the Negro was also included in the transect. Our sampling design consists of two ecological gradients, where black (Negro, **Figure 1i**) and clear (Tapajós, **Figure 1ii**) water meets white (Amazon) water, and two controls, where rivers of the same water color meet (Branco to Negro and Madeira to Amazon). Sampling was carried out in sandy and shallow beaches along the river banks and floodplains. This is the preferred habitat for *T. albus* (**Figure 1iii**), a species that has not been recorded in deeper river channels. Fish were caught with seine nets, euthanized, and muscle tissue preserved in 95% ethanol. Geographic coordinates of each site were obtained using a global positioning system (GPS). Measurements of pH, temperature (°C), turbidity (cm), dissolved oxygen (mg/L), and oxygen saturation (%) were collected *in situ* at each locality to assess environmental heterogeneity (**Table 1**).

Laboratory Procedures

A modified salting-out protocol (Sunnucks and Hales, 1996) was used to extract DNA from muscle tissue samples. Double digest restriction-site associated DNA (ddRAD) libraries were developed using a modified protocol by Peterson et al. (2012), as detailed in Brauer et al. (2016). Libraries were sequenced at Novogene across two lanes of Illumina HiSeq4000 at 150 base-pair paired-end reads. Details of the DNA extraction method and the ddRAD library protocol are in **Supplementary Appendix 1**.

Bioinformatics and Single Nucleotide Polymorphism Filtering

Illumina HiSeq4000 sequencing produced a total of 902,555,657 sequence reads, each of around 150 bp. Quality control tests were initially performed using FASTQC 0.11.8 (Andrews, 2010).

TABLE 1 | Sampling locations of *Triportheus albus* in different water "colors."

River color	River	Site	Coordinates	N	°C	pH	OD	O_2%	cm
Black	Branco	B1	1°15′59.00″S/61°50′55.00″W	15	29.8	6.9	6.9	90.4	83.4
	Negro	N1	3°4′44.00″S/60°14′44.00″W	14	29.7	5.2	6.4	82.3	76.0
White	Madeira	M1	3°28′14.00″S/58°52′5.00″W	13	29.7	7.1	5.7	82.4	5.5
	Amazon	A1	3°20′40.00″S/60°7′10.00″W	10	28.8	7.2	6.7	86.3	12.3
		A2	3°6′56.00″S/59°32′19.00″W	11	29.6	7.1	6.3	85.2	18.8
		A3	3°4′39.00″S/58°13′13.00″W	12	28.7	7.1	4.8	84.0	18.3
		A4	2°33′7.00″S/57°1′59.00″W	9	29.2	7.2	6.4	85.6	10.5
		A5	2°10′21.00″S/54°58′21.00″W	4	29.0	7.2	6.3	82.0	12.5
		A6	2°28′10.00″S/54°30′5.00″W	10	29.7	7.2	6.6	87.9	15
Clear	Tapajós	T1	2°52′17.00″S/55°9′38.00″W	16	29.5	6.7	7.1	97.5	118

Sampling locations, geographic coordinates, sample size (N) and average hydrochemical variables collected in situ; temperature (°C), pH, dissolved oxygen (OD; mg/L), oxygen saturation (O_2%) and turbidity (cm). All measurements, except turbidity, were averaged from riverbed and surface measurements.

Sequences were demultiplexed and barcodes and adapters were removed using process_ragtags 2.4 from STACKS (Catchen et al., 2013). Low quality bases ($Q < 20$) and reads (more than 5% Ns, less than 40 bp, or < 30 average Q) were removed using TRIMMOMATIC 0.39 (Bolger et al., 2014), SNP variants were characterized using the DDOCENT 2.7.8 pipeline (Puritz et al., 2014), as detailed in Sandoval-Castillo et al. (2018). The SNPs were filtered in a pipeline of steps from VCFTOOLS (Danecek et al., 2011). A detailed summary of the filtering process is found in **Supplementary Table 1**.

Genetic Diversity and Population Structure

Percentage polymorphic loci, expected heterozygosity (He) and observed heterozygosity (Ho) were calculated across the filtered SNP dataset for each sampling locality using ARLEQUIN 3.5.2.2 (Excoffier and Lischer, 2010).

Population structure was assessed using model-based and model-free methods. Pairwise FST among sampling localities was estimated with the Jukes and Cantor (1969) method using 1,000 permutations. *P*-values were adjusted for a false discovery rate (FDR) of 5%, using the false discovery script.[1] A principal component analysis (PCA) of the total SNP dataset was generated using the program ade4 (Dray and Dufour, 2007) in R. Population structure was also inferred using Admixture (Alexander et al., 2009). The number of ancestral populations (K) was assessed by comparing log-likelihood ratios for multiple independent runs of each K ($K = 1–7$) before using a cross-validation (CV) procedure with 10 replicates to identify the *K*-value that best explains the number of random mating populations (Alexander et al., 2009). Admixture coefficients were plotted using the ggplot2 package (Wickham, 2009) in R. Relationships among individual fish were then assessed based on the SNPs with a Neighbor-joining (NJ) tree in PAUP4 (Wilgenbusch and Swofford, 2003) and the GRT + G model (Tavaré, 1986), which best fitted our data according to the Bayesian information criterion implemented in ModelTest-NG (Darriba et al., 2020). The aim of this analysis was not to infer phylogenetic history in *T. albus*, but to instead obtain a visual representation of the relationships among individuals across localities that can be contrasted with results of other approaches used to infer population structure.

Controlling for Spatial Genetic Structure

Patterns of genetic structure are known to be influenced by the physical structure of river catchments, including the spatial separation between populations via IBD, and the hierarchical structure of the dendritic system (Excoffier et al., 2009; Fourcade et al., 2013; Brauer et al., 2018). Riverine distance between sampling localities was estimated using Google Earth Pro 7.3.3 (2020) to test if any inferred genetic differentiation is consistent with the IBD model. A global Mantel test based on 999 permutations was performed to assess correlation between riverine distance and linearized genetic distance between all localities (FST/1—FST) using the ade4 package in R (Dray and Dufour, 2007). A second Mantel test (999 permutations) exclusively assessed the relationship between riverine distance and linearized genetic distance of white water Amazon and Madeira River localities. Localities A5 and A6 were omitted from this analysis due to insufficient sample size. These relationships were visualized using regression plots. A pairwise matrix of riverine distances between localities was calculated and translated to a set of synthetic IBD coordinates for each locality in R using the isoMDS function in the MASS library (Venables and Ripley, 2002) for use as a control for IBD in subsequent GEA analyses.

Hierarchical structure can be identified through distinct breaks in allele frequencies between clusters of populations, which may coincide with geographical features such as river catchments (Meirmans, 2012). A matrix of population covariance across locality allele frequencies (Omega matrix) was calculated through the core model in the BayPass program [explained below under "Genotype-Environment Association (GEA) Analyses; Gautier, 2015]. This matrix infers gene flow resulting from elements of demographic history, including neutral models of hierarchical structure. This matrix was translated to a set of covariance variables using the MASS library (Venables and Ripley, 2002) in R. The Omega

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[1] https://github.com/carbocation/falsediscovery

coordinates were used as a control for hierarchical structure in successive GEA analyses.

Genotype-Environment Association Analyses

Five hydrochemical variables sampled *in situ* were considered in the GEA analyses; pH, temperature (°C), turbidity (cm), dissolved oxygen (mg/L), and oxygen saturation (%) (**Table 1**). These variables have been suggested as possible sources of ecological selection in fishes between Amazonian water colors (Beheregaray et al., 2014; Borghezan et al., 2021). The association between genetic and environmental heterogeneity was assessed using two multivariate approaches, redundancy analysis (RDA) (Legendre and Legendre, 1998) and Gradient Forest (Ellis et al., 2012), as well as by the univariate approach in BayPass (Gautier, 2015).

BayPass is a program for Bayesian population association analyses (Gautier, 2015). This Bayesian hierarchical model was proposed by Coop et al. (2010) as an FST-based approach to evaluate associations between variation in ecological variables and genetic markers, and to identify candidate loci for adaptive divergence while controlling for neutral covariance of alleles across populations. BayPass was used to investigate the signal of neutral and adaptive variation through three models; the core model, the standard covariate model, and the auxiliary model (Günther and Coop, 2013). Explanations of these three models are in **Supplementary Appendix 2**. The auxiliary model was used to identify candidate adaptive loci significantly correlated with each environmental variable over a log10(BF) threshold of 30.

The RDA is an asymmetric ordination method used to explore the relationship between multivariate response data and a set of explanatory variables using multiple linear regression (Legendre and Legendre, 1998). The RDA has been shown as a robust method to identify polygenic adaptive divergence through a strong ability to detect minor changes in allele frequency over many covarying loci (Grummer et al., 2019), while providing an optimal balance of low false-positive and high true-positive rates (Forester et al., 2018). An RDA was carried out using the vegan package in R studio (Dixon, 2003) comparing the response allele frequencies for each SNP with the five environmental variables (**Table 1**). To avoid collinearity, highly correlated variables were excluded using a variance inflation factor ≥10 (Dyer et al., 2010). First, we performed a standard RDA comparing variance in allele frequencies with variance in environmental variables. Subsequently, two partial RDAs were performed accounting for different aspects of spatial neutral population structure. These included synthetic IBD coordinates as a conditional variable and the synthetic Omega coordinates (see "Controlling for spatial genetic structure" section above) to control for other elements of demographic history, including hierarchical structure. A hierarchical analysis of molecular variance (AMOVA) and marginal AMOVA were performed to determine the significance of the partial and non-partial RDA models under a 0.05 threshold at 1,000 permutations in the R package vegan (Dixon, 2003). The SNPs exhibiting high contribution to environmental associations in the RDA (p = 0.05) were identified as candidate loci for adaptive divergence.

Random Forest (Breiman, 2001) is a machine-learning regression tree technique that can effectively handle thousands of DNA markers simultaneously to identify regions of the genome accounting for complex polygenic traits (Brieuc et al., 2018; Grummer et al., 2019). The original randomForest package was developed by Liaw and Wiener (2002). This was later modified by Ellis et al. (2012) into the gradientForest package, which extends the random forest method to the community level. Gradient Forest can pinpoint where compositional turnover occurs along an environmental gradient, which allows identification of important environmental thresholds that correlate to distinct changes in allele frequencies (Ellis et al., 2012). Gradient Forest was run to determine the correlation of allele frequencies with the predictor variables of environmental variation, synthetic IBD coordinates, and synthetic Omega coordinates (ntree = 750, mtry = number of variables/3, corr. threshold = 0.5). The cumulative importance of each predictor variable in shaping genetic populations was also assessed, with threshold values of each predictor in determining allele frequency breaks identified. Candidate adaptive loci were identified by assessing the R^2 weighted importance distribution of SNPs (**Supplementary Figure 7**). Loci above the upper elbow of this distribution curve (>0.86) were selected as candidates. As there is not a specific method implemented in random forest analyses to extract outlier SNPs (Goldstein et al., 2011; Laporte et al., 2016), the upper elbow approach used above has proven valuable in genome-wide association studies (Batley et al., 2019).

Functional Annotation

Candidate adaptive SNPs identified through RDA, Gradient Forest and BayPass methods were compared to select loci for functional analysis. The flanking sequence of each candidate SNP was aligned to the UniProt[2] Actinopterygii protein database using BLASTX 2.11.0 + (Altschul et al., 1997) with e-value threshold set to 1×10^{-6}. Gene ontology (GO) terms were assigned with a modified version of the python script GAWN.[3] The functional roles of annotated candidate loci were investigated further by exploring the relevant literature.

RESULTS

Sequencing Quality and Genetic Diversity

The SNP calling resulted in a total dataset of 743,123 raw SNP variants. After the filtering process (detailed in **Supplementary Table 1**), 15,251 high-quality SNPs were retained for analysis in 98 *T. albus* individuals (including four replicates). Genotyping error rates for the dataset were < 1%, with ∼0.05% missing data.

Adjacent Amazon River (Amazonas) localities with small sample sizes (A4, A5, and A6) were merged into a single sample to estimate genetic diversity. Genetic diversity was high and

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[2] https://www.uniprot.org/uniprot/

[3] https://github.com/enormandeau/gawn

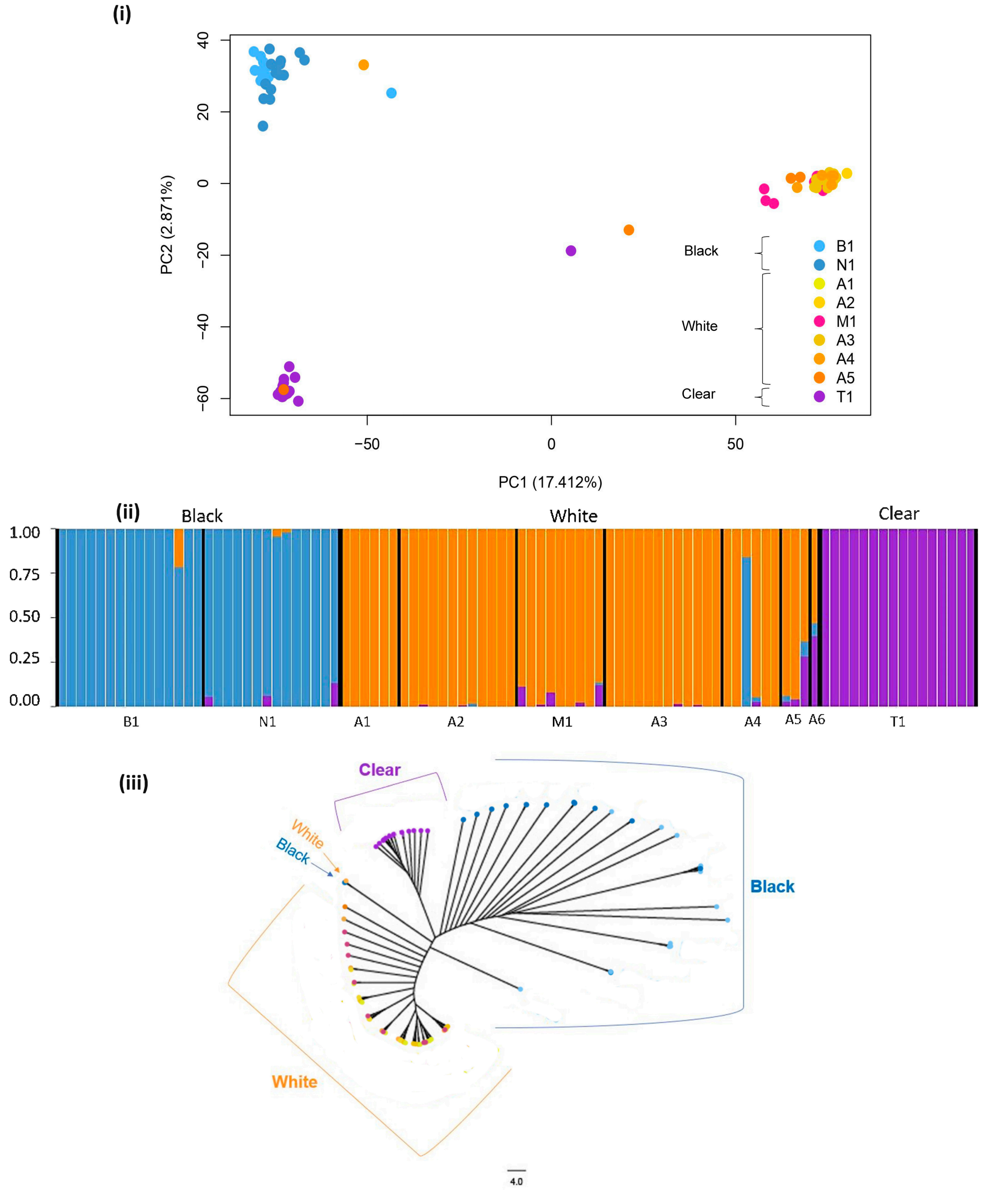

FIGURE 2 | Population genomic structure of *Triportheus albus* based on 15,251 SNPs. Site labels are described as B1, Rio Branco (black water); N1, Rio Negro (black water); A1–A6, Rio Amazonas (white water); M1, Rio Madeira (white water); T1, Rio Tapajós (clear water). **(i)** PCA plot with each point indicating an individual and color a sampled site (shades of blue = black water sites, shades of orange = white water sites, purple = clear water site); **(ii)** admixture plot with each vertical bar depicting an individual and color representing ancestry from each water color catchment (blue = black water, orange = white water, purple = clear water). **(iii)** Neighbor-joining tree generated with PAUP4 based on the GRT + G substitution model. Catchment water color is labeled.

similar among localities, with average expected heterozygosity of 0.263 (He; 0.252–0.300), average observed heterozygosity of 0.240 (Ho; 0.221–0.275), and average polymorphic loci of 64.9% (54.4–76.1%) (**Supplementary Table 2**).

Population Structure

Population structure was strong across the region sampled in the Amazon Basin. In general, genetic differentiation among localities was highest between white (A1–A6, M1) and black (B1, N1) localities, and between white and clear (T1) localities. Genetic differentiation was nil to low between localities within the white and black selective environment (**Supplementary Table 4**). Pairwise FST estimates were significant ($p < 0.05$) after 5% FDR correction between black water localities, between black and clear localities, and between black or clear and white water localities. Pairwise FST estimates between white water localities were not significant ($p < 0.05$) after 5% FDR correction (**Supplementary Table 4**).

For the PCA and subsequent GEA analyses, Amazon River localities A5 and A6 were merged to a single sample ($n = 4$), with

A4 ($n = 6$) kept as a separate sample. The PCA plot (**Figure 2i**) supported the pattern of population differentiation estimated via FST, showing distinct clusters of individuals among white, black, and clear water. A stronger level of separation was evident between white water *T. albus* and individuals from black and clear water localities, as explained by PC1 (17.412%). Clustering was also observable among black and clear water localities, explained by PC2 (2.871%)(**Figure 2i**). Two putative hybrids between white and clear water clusters were observed, originating from sites T1 and A5/A6. Possible migrants were also detected between black and white water, and between the clear water Tapajós and white water cluster (**Figure 2i**).

The Admixture analysis provided highest support for two genetic populations of *T. albus*, closely followed by three populations, as indicated by CV errors displayed in **Supplementary Figure 1**. The $K = 2$ plot combined the geographically distant black and the clear water samples into a single genetic population, with the second population represented by white water samples (**Supplementary Figure 2**). The $K = 3$ plot divided the black and clear catchments into two separate populations (**Figure 2ii**). An individual caught at A4 had ancestry to the black water population, supporting its migrant status suggested by the PCA. Two individuals from sites A5 and A6 shared ancestry between white and clear water populations, suggesting the possibility of an admixture zone (**Figure 2ii**).

The topology of the NJ tree was in agreement with results of population structure analyses (**Figure 2iii**). Individuals from the clear water Tapajós clustered as a single clade, whereas individuals from black and white water sites also comprised separate clades. Three individuals deviated from this pattern; one from white water A4, and one from each of the black water sites B1 and N1 (**Figure 2iii**). The outlier in site A4 corresponded to the potential migrant identified in the PCA and Admixture, supporting its suggested ancestry from the black water tributary. Potential hybrids from sites A5 and A6 identified in PCA and Admixture population structure analyses grouped closely to the clear water cluster in the NJ tree (**Figure 2iii**).

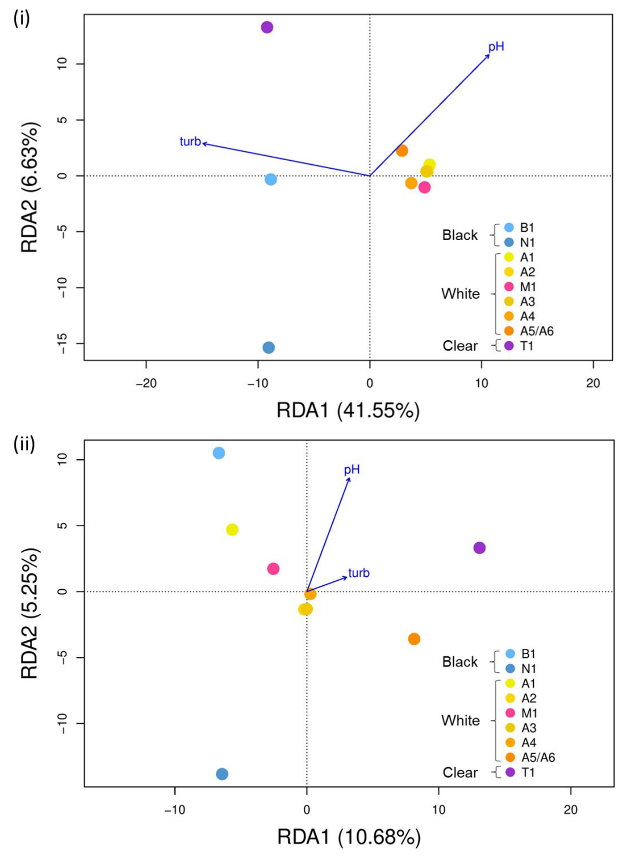

FIGURE 3 | Genotype-environment associations of *T. albus* in the Amazon Basin based on RDA. The RDA shows how genetic differentiation between localities is explained by non-correlated environmental variables pH and turbidity (turb) collected *in situ* at each site. **(i)** Unconstrained, without controlling for spatial structure, and **(ii)** constrained to population covariance calculated with BayPass (Gautier, 2015) to control for neutral hierarchical genetic structure. Each colored point represents a sampling locality. Black water Branco River (B1) and Negro River (N1) localities = shades of blue, white water Amazon River localities = gradient of yellow to orange (A1–A5), white water Madeira River (M1) = pink, clear water Tapajós River (T1) = purple. Based on 15,251 filtered SNP loci.

Genotype-Environment Associations and Controls for Models of Neutral Structure

The composition of environmental variables varied substantially between rivers of different water "color" (**Table 1**), and as such these are considered here as predictors of putative adaptive divergence within *T. albus*. The pH was considerably lower in the Negro River (5.2) than all other localities (6.7–7.2), including the seasonally black Branco River (6.9) (**Table 1**). Turbidity was substantially higher in the white water Amazon (14.5 cm) and Madeira (5.5 cm) sites compared to both black (79.7 cm) and clear (118 cm) water sites. Temperature remained relatively consistent across all systems. Dissolved oxygen was lowest at Amazon 3 (4.8 mg/L) and highest in the clear water Tapajós (7.1 mg/L). Oxygen saturation was greatest in the Tapajós (97.5%) and lowest at Amazon 5 (82.0%).

The Mantel test performed globally across white, black and clear water localities was not significant ($p = 0.14$), suggesting a relatively low effect of IBD at the broader regional scale. This was reflected in the global matrix regression plot (**Supplementary Figure 3i**) which shows no distinct pattern between riverine distance and FST among *T. albus* populations. The Mantel test among white water localities was also not significant ($p = 0.09$), although a trend of increasing genetic distance with geographic distance was observable in the regression plot (**Supplementary Figure 3ii**).

After controlling for highly correlated environmental predictors, pH and turbidity were included in the RDA model. The standard RDA separated individuals from white water localities from those in black and clear water sites (**Figure 3i**). This RDA model indicated that pH and turbidity accounted for 48.2% of total genetic variation and was globally significant ($p = 0.010$) (**Supplementary Table 6**). After controlling for IBD, which explained 37.7% of variation, pH and turbidity accounted for 32.0%, with the RDA model being globally significant

(p = 0.036) (**Supplementary Table 6** and **Supplementary Figure 4**). A clear pattern was less apparent in the RDA plot controlled by Omega (**Figure 3ii**). In that model, turbidity and pH explained 15.9% of the total genetic variation after accounting for demographic history (including hierarchical structure), which explained 53.9% of variation, with the RDA model not being significant (p = 0.384). From the RDA model conditional to Omega, we identified 1,749 candidate adaptive loci which associated highly with environmental predictors.

The Gradient Forest identified that Omega 2, pH, Omega 1, and turbidity all correlated significantly with allele frequency at a similar level to one another (**Figure 4i**). The synthetic IBD coordinates, dissolved oxygen, temperature, and oxygen saturation did not correlate highly with allele frequency (**Figure 4i**). Demographic history predictors Omega 1 and 2 were the strongest in influencing breaks in allele frequencies of *T. albus* across Amazonia (**Supplementary Figure 5**). This was closely followed by turbidity and pH, which both influenced compositional turnover of allele frequency to a high level (**Supplementary Figure 5**). Turbidity classified one distinct compositional turnover in allele frequency, defined by ∼42 cm (**Figure 4ii**), which separated white water localities from black and clear water localities (**Table 1**). The pH defined two allele frequency breaks occurring at threshold pH values of 6.2 and 7.0 (**Figure 4iii**), which generally corresponded to pH differences between each of the three water colors (**Table 1**). The Branco River (pH 6.9; **Table 1**) was an outlier to this pattern, grouping with the clear water population. Dissolved oxygen, oxygen saturation and temperature did not substantially define any turnover in allele frequency (**Supplementary Figure 6**). The Gradient Forest identified 53 candidate adaptive loci associated with environmental variables over threshold $R^2{}_C$ value 0.86.

The auxiliary model in BayPass (Gautier, 2015) identified 268 candidate adaptive loci, mostly associated with pH (67) (**Supplementary Figure 8**). Of the 1,749 candidate loci identified by RDA and 268 by BayPass, 53 loci were consistent between the two methods (**Supplementary Table 1**). The 53 candidate SNPs identified by Gradient Forest were not discovered by RDA or BayPass as outliers (**Supplementary Table 1**).

Functional Annotation

For the 53 unique candidate loci identified by both BayPass and RDA, and the 53 loci identified by Gradient Forest, 29 were annotated and assigned to 58 GO terms. Many genes annotated were components of cellular membranes, with the most common GO terms "integral component of the membrane" and "plasma membrane" (10 and four of 29 annotated genes, respectively). Twelve of these 14 genes had molecular functions associated with the binding of ions, nucleic acids, or ATP. The most common biological processes identified were related to signaling pathways (four of 29 annotated genes) (**Supplementary Table 7**). Most candidates were highly associated with pH (14 of 23) or turbidity (7 of 23) (**Supplementary Table 7**). Further information about GO Terms assigned to genes, environmental variables that genes were associated to, and regression coefficients of the correlation between genetic variation and variation in the associated environmental variables are reported in **Supplementary Table 7**.

DISCUSSION

Disentangling adaptive from neutral genetic diversity in wild populations is a difficult task, but the ease and power to do so has improved substantially with the development of genome-wide screening techniques and approaches in landscape genomics (Yeaman et al., 2016; Grummer et al., 2019). Here, we were able to identify the contributions of spatial and environmental factors in shaping genomic divergence in populations of the characin tetra *T. albus* from central Amazonia. Using a robust approach that integrated multivariate RDA and Gradient Forest, and univariate BayPass approaches, we have demonstrated that in addition to strong neutral hierarchical structure, adaptive divergence is likely to be occurring in *T. albus* populations across black, white and clear water selective environments.

Evolution and Population Genomic Structure

Our study is among the first aimed at assessing population genomic structure of an Amazonian aquatic species using genome-wide approaches. To the best of our knowledge, a study by Torati et al. (2019) examining stock structure in the teleost *Arapaima gigas* between South American basins using 393 SNPs represents the only prior attempt to this. Landscape genetic methods have been used to identify population structure among Amazonian catchments for the characin *T. albus* (Cooke et al., 2012a), the silver croaker *Plagioscion squamosissimus* (Cooke et al., 2012b), the riverine puffer *Colomesus asellus* (Cooke et al., 2012c), and the barred knife fish *Steatogenys elegans* (Cooke et al., 2014). Genetic structure between Amazon and Madeira River populations has also been identified for the catfish *Brachyplatystoma platynemum* (Ochoa et al., 2015). None of these studies have employed datasets and statistical approaches capable of inferring genome-wide neutral and adaptive signal (Luikart et al., 2003; Grummer et al., 2019). As such, comparisons of genome-wide diversity with our *T. albus* dataset are hampered by the remarkably small number of aquatic studies (including for characiform tetras from other basins). For instance, for the least-concern (IUCN) characiform *Astyanax mexicanus* (blind cavefish) in Northeast Mexico (Bradic et al., 2013), population-level observed heterozygosity ranged from 0.05 to 0.19. For the abundant atheriniform *Melanotaenia fluviatilis* (Murray River rainbowfish) in Australia, heterozygosity values ranged from 0.111 to 0.317 (Brauer et al., 2018). For our study, genetic diversity was not only moderate to high but was markedly similar across all sampled sites (observed heterozygosity ranged from 0.221 to 0.275), suggesting high adaptive potential for the inferred populations in our study region.

When assessing evolutionary patterns of freshwater biodiversity it is important to consider the history and timing of geological formation of river basins. The Characiformes originated over 100 million years ago (Ma) in Gondwana (Ortí and Meyer, 1997), and as such deep evolutionary divergence in the Neotropics should be assessed in relation to the uplift of the Andes and the initial development of the Amazon drainage basin (Rull, 2008; Hoorn et al., 2010a). Prior to the influence

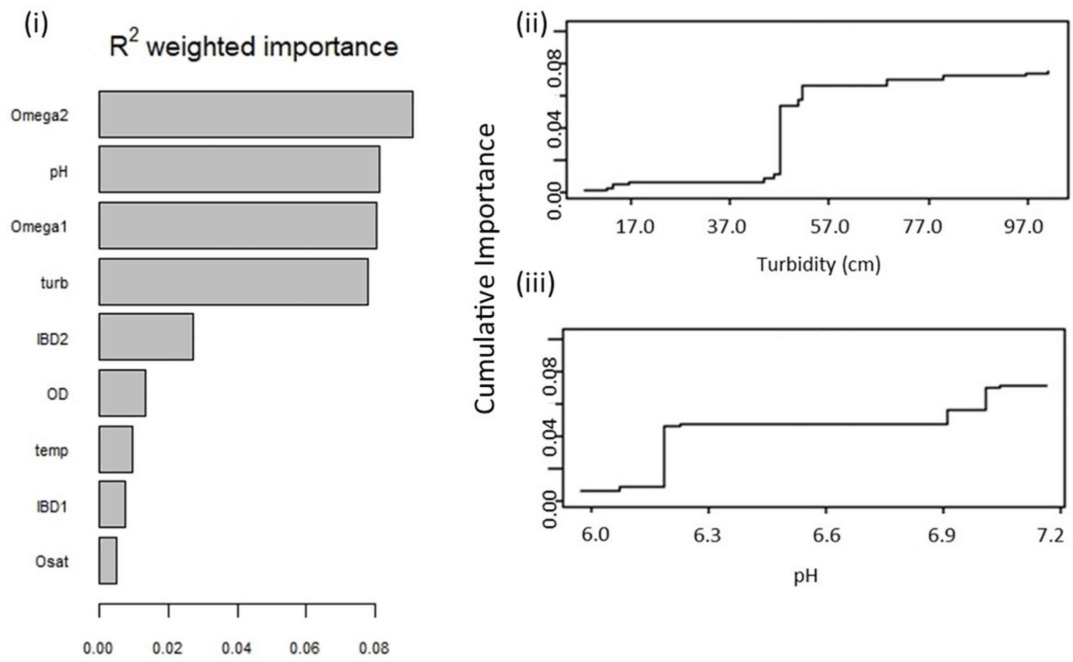

FIGURE 4 | Gradient Forest (Ellis et al., 2012) results for associations between environmental variables and genetic diversity of *Triportheus albus* across the Amazon Basin. Based on 15,251 genome-wide SNPs. R^2 weighted importance **(i)** clarifies the level of correlation between each predictor variable and allele frequency variation. Omega 1 and Omega 2 denote control variables of neutral hierarchical structure, measured through a matrix of population covariance translated to two synthetic coordinates. The IBD1 and IBD2 are controls of isolation by distance, measured using a matrix of geographic distance between localities. Turbidity (turb), pH, dissolved oxygen (OD), oxygen saturation (Osat), and temperature (temp) are hydrochemical variables measured *in situ* at each sampling locality of *T. albus*. Cumulative importance displays the allele frequency compositional turnover functions for important environmental predictor variables of *Triportheus albus* divergence across the Amazon Basin, turbidity **(ii)** and pH **(iii)**.

of the Andes, the Amazon was an undivided sedimentary basin dominated by the fluvial system of the cratonic shields of which the Negro and Tapajós rivers are derived from Figueiredo et al. (2009) and Hoorn et al. (2010b). Increased sedimentation, rising sea levels, and overfilling from the late Miocene (∼6.4 Ma) led to the establishment of west to east transcontinental flow and formation of the modern Andean-dominated Amazon River network by the late Pliocene (∼2.5 Ma) (Val, 1995; Campbell et al., 2006; Figueiredo et al., 2009).

Geomorphological evolution is thought to have impacted the phylogeography of *T. albus*. The work by Cooke et al. (2012a) based on 360 AFLP loci supported two historically distinct populations of *T. albus* in central Amazon; a white water Amazonas/Madeira population, and a black water Negro and clear water Tapajós population. Phylogenetic and phylogeographic analyses indicated a recent divergence between white water and black/clear water ecotypes of *T. albus* followed by demographic expansions dated to the late Pleistocene (Cooke et al., 2012a). These findings, paired with geomorphological and paleoecological evidence for the basin, indicate that the black/clear water ecotype is older, with the west to east transcontinental formation of the Amazon River promoting the colonization and demographic expansion of the white water *T. albus* ecotype (Lundberg et al., 1998; Campbell et al., 2006; Cooke et al., 2012a). This scenario is supported by comparative phylogeographic studies of co-distributed fishes; *Colomesus asellus*, *Plagioscion squamosissimus,* and *Steatogenys elegans* (Cooke et al., 2012b,c, 2014). Although assessing phylogeographic history of *T. albus* is outside the scope of this study, our results support the strong divergence between white and black/clear ecotypes, as well as the similarity between black and clear water populations detected by Cooke et al. (2012a). The latter was found despite the large contemporary riverine distance between the black water Negro and the clear water Tapajós (up to 1,100 km) and their different geological settings (Latrubesse and Franzinelli, 2005; Hoorn et al., 2010b), pointing to the strong influence of phylogeographic history (Cooke et al., 2012a) on contemporary patterns of population structure in *T. albus.*

In addition, the greater power offered by our genome-wide dataset revealed weaker, but biologically meaningful, genomic divergence between black and clear water populations. Only a low level of admixture was noted between black and white, and between clear and white populations, suggesting downstream migration following the direction of river flow; from tributaries into the main Amazon channel (**Figure 2ii**). This is consistent with the isolation with migration model results of Cooke et al. (2012a), which inferred a higher probability of unidirectional gene flow in *T. albus* from black and clear water into white water than of any other alternative scenario. Overall, population structure analyses indicated three contemporary genetic clusters for *T. albus* in central Amazonia that correspond to rivers of distinct water color. As genetic structure was not observed between the white water Amazon and Madeira rivers, divergence due to the confluence of a tributary alone remains an unsupported explanation. Similarly, a simple model of IBD seems insufficient to explain divergence patterns across the vast study region. Instead, our study results suggest that population divergence is also associated with the boundaries of catchments that harbor divergent hydrochemical conditions.

Neutral and Adaptive Contributions to Genomic Divergence

Species that inhabit contrasting selective environments provide a valuable opportunity to study divergent natural selection (Schluter, 2000). The dendritic arrangement of the Amazon Basin encompasses distinct ecotones of varying environmental conditions characterizing different water "colors," which may theoretically promote divergent selection and IBE across populations of aquatic organisms. For instance, Pires et al. (2018) described divergence and reproductive isolation between Amazon and Negro River lineages of the sailfin tetra *Crenuchus spilurus*. A landscape genetics study by Cooke et al. (2012a) suggested a putative pattern of IBE for *T. albus* across Amazonia, as well as several co-inhabiting fishes (Cooke et al., 2012b,c, 2014; Beheregaray et al., 2014). In those studies, evidence for putative divergent selection lay in patterns of diversification that aligned best with catchments of similar water properties, irrespective of riverine distance. The genomic dataset used in this study not only provides comparatively higher resolution than the above genetic datasets to infer fine- and broad-scale population divergences (Luikart et al., 2003), but also contains sequence information around candidate adaptive regions that might prove useful to identify mechanisms influenced by divergent selection.

The IBD model is not uncommon in freshwater systems and has been observed for species with varying dispersal potential (e.g., Hrbek et al., 2005; Hubert et al., 2007; Zieritz et al., 2010; Crookes and Shaw, 2016). The GEA analyses strongly indicated that genetic divergence among *T. albus* populations cannot be explained by a simple IBD model. Broadly speaking, we found no clear relationship between genetic and riverine distance across the entire study area in central Amazon, which was also reflected in both the RDA and Gradient Forest results. A strong pattern of genetic structure was found between white water and black/clear water localities, but not between white water Amazon and Madeira Rivers. Similarly, very low differentiation and a low but non-significant signal of IBD was present along the Amazon River, despite the ∼750 km separating sites A1 and A6. Critically, the low levels of neutral differentiation and corresponding reduced genetic drift along white waters are not expected to impact on our ability to characterize putative adaptive divergence within that selective environment (Grummer et al., 2019).

The stream hierarchy model (Meffe and Vrijenhoek, 1988) should be routinely considered when assessing gene flow among populations in complex dendritic spatial environments (Brauer et al., 2018). Consistent with the broad inferences of population structure, the RDA analysis demonstrated hierarchical structure as the prevailing factor driving genetic divergence among populations (**Figure 3ii**). Although pH and turbidity explained a substantial proportion of the variance in all RDAs, their contributions were not statistically significant as model complexity increased following the addition of demographic and spatial factors, such as hierarchical structure (**Supplementary Table 6**). This is expected due to the complex links between spatial-demographic factors and environmental variables and the resulting difficulties in separating adaptive from neutral variation (Grummer et al., 2019). As such, analyses did not suitably disentangle whether divergence followed a neutral hierarchical pattern resulting exclusively from the arrangement of tributaries, or a combination of neutral and non-neutral influences due to the varying environmental conditions within each of the catchments.

The Gradient Forest analysis also revealed neutral hierarchical structure as the greatest driver of genetic differentiation, however, turbidity and pH were revealed to correlate with allele frequency at comparably high levels (**Figure 4i**). Heterogeneity in turbidity created two distinct genetic populations of *T. albus,* separating the white water localities from the black and clear sites (**Figure 4ii**). The white Amazonian headwaters are subject to copious erosion in the Andes, and are consequently rich in suspended sediments (Sioli, 1984; Val, 1995; McClain and Naiman, 2008). Associated with this heavy sediment load is an abundance of nutrients and organic matter which facilitate a higher productivity and diversity of species in white waters than adjoining black and clear tributaries (McClain and Naiman, 2008; Hoorn et al., 2010a). However, characiformes are generally visually-orientated, surface-dwelling diurnal fishes (Tejerina-Garro et al., 1998), and consequently may be offered an ecological advantage in the transparent, mineral-deficient black and clear water tributaries (Wallace, 1854; Furch, 1984; McClain and Naiman, 2008). A detailed proposal of how Amazonian water types may promote differentiation in the sensory mechanisms of fish has been outlined by Borghezan et al. (2021).

Variation in pH across the basin is associated with the split *T. albus* into three distinct genetic populations corresponding to each water color (**Figure 4iii** and **Table 1**). The dark color of the Negro River is attributed to staining from tannins and humic acids leached by decaying vegetation (Wallace, 1854; McClain and Naiman, 2008), and as a result exhibits lower pH than their pH-neutral white and clear counterparts. The acidic, nutrient-poor properties of black water systems forms a challenging environment for aquatic species (Val, 1995), however, research has suggested that phenotypic plasticity of mechanisms conferring acid-base regulation has enabled *T. albus* to survive in harsh Negro waters (Araújo et al., 2017). The Branco River has white headwaters of mountainous origin, however, sampling took place near its junction with the Negro, thus this site was heavily influenced by cratonic black water. Even so, geochemical analyses have revealed the Branco River to be chemically and sedimentologically intermediate between black and white water (Küchler et al., 2000; Evangelista and Tosi, 2015). The clear water Tapajós represents a second intermediate physiochemical condition to the Amazon and Negro rivers (Duncan and Fernandes, 2010), hence why the pH of the Branco and Tapajós tributaries are similar. However, the influence of environmental discontinuities on population divergence presented by the Tapajós should be cautiously interpreted given the lack of replication in this river system.

A key limitation of the study was that sample size was small in terms of individuals for some white water sites, and in terms of locality replication for the Tapajós tributary. However, simulations by Gaughran et al. (2018) demonstrated that accurate estimates of genetic differentiation in highly structured populations can be obtained using thousands of SNPs and only 2–5 individuals per locality. Thus, merging sites A5 and A6 for analyses, should be sufficient to provide a reasonable representation of their genetic patterns, and is justified by their genetic similarity identified in population structure analyses. A greater number of sampled sites in the Tapajós would provide an improved understanding of the structure of the clear water tributary as a whole. The study was also limited as a whole genome reference is not available for *T. albus;* the reference of a closely related species would help to better characterize regions under selection. In addition, the hypothesis would be better tested by performing a comparative analysis on multiple co-distributed species, as done by Beheregaray et al. (2014) using a landscape genetics framework.

The discovery of 106 candidate loci highly associated with variation in the environment provides further support for adaptive divergent selection of *T. albus* across the Amazon Basin. Although no Gradient Forest candidates were identified as outliers in the RDA or BayPass analyses, it is important to consider that methods of outlier detection all vary in assumptions and balance between low false positive and high true positive rates (Narum and Hess, 2011). The strong associations of pH and turbidity with allele frequency that correspond to environmental differences between each water color, and the discovery of candidate adaptive genes for environmental associations, are supportive that characteristics of water color can perhaps induce selection and promote adaptive divergence between populations.

Functions of Candidate Adaptive Genes

Establishing the function of candidate adaptive genes and their ecological importance should be done cautiously, as a large fraction of genes remains without annotation of ecological relevance (Pavey et al., 2012). The correlation of potentially adaptive genes with mechanisms linked to mediation of the impacts of environmental fluctuations do not necessarily imply causation, but nevertheless can be useful to understand how adaptive divergence may arise (Grummer et al., 2019). Most candidates identified here were annotated to genes associated with ion channel activity (**Supplementary Table 7**), which is consistent with up-regulated GO terms for *T. albus* reported by Araújo et al. (2017). As aquatic environments are composed of a wide range of salinities, ion compositions, and pH values, fish are required to cope with challenging osmotic and ionic gradients (Hwang and Lee, 2007). Ion channels can be sensitive to changes in extracellular pH (Holzer, 2003), and fluxes of ions play an important role in maintaining acid-base homeostasis in freshwater fish (Goss et al., 1992). The black water Rio Negro drains the nutrient-poor soils of the Amazon forest, and consequently contains low concentrations of nutrients such as Na^+, Cl^-, K^+, Ca^{2+}, and Mg^{2+} (Furch, 1984; Val, 1995). The gene coding for the K^+ channel protein KCNK1 was identified in *T. albus* to be associated with environmental fluctuations across the study region, specifically in relation to pH in the RDA (**Supplementary Table 7**). The KCNK1 protein was found to be sensitive to low pH in zebrafish (Christensen et al., 2016), alluding that its function in *T. albus* may be sensitive to the strong fluctuations in pH across Amazonia.

Diurnal fishes such as the Characiformes depend highly on vision as a source of sensory information, and as a result have large, well-developed eyes (Guthrie, 1986; Tejerina-Garro et al., 1998). There is ample research demonstrating the effects of turbidity and light intensity on the feeding ability (Gardner, 1981; Rowe and Dean, 1998; Leahy et al., 2011), and antipredator behavior (Higham et al., 2015; Kimbell and Morrell, 2015) of fishes. Calcium ions and stores play an important role in the detection and transfer of light stimuli in photoreceptors (Krizaj and Copenhagen, 2002; Križaj, 2012). The ryanodine receptors (RYRs) are a family of Ca2+ release channels (Sutko and Airey, 1996) expressed in the photoreceptors of vertebrates (Križaj et al., 2004; Križaj, 2012). Photoreceptor pathways are sensitive to light (Križaj et al., 2011), and the RYRs have been linked to visual stimuli response in fish (Frank et al., 2019). The genes RYR2 and RYR3 were highly associated with environmental heterogeneity in *T. albus,* with RYR3 most highly correlated with variation in turbidity in the Gradient Forest analysis. Considering this, differences in light intensity produced by varying turbidity across Amazonia may be impacting the expression of the RYRs in *T. albus,* supporting sensory systems research directions suggested by Borghezan et al. (2021).

Fluctuations in dissolved oxygen is an issue in many aquatic systems including the Amazon (Junk et al., 1983) and can influence species distributions (Mandic et al., 2009) and cause negative impacts on freshwater fishes (Landman et al., 2005). Hypoxia is a condition in which cells suffer from oxygen deficiency (Hughes, 1973). Hypoxia has been found to affect the RYR2 gene in the large yellow croaker *Larimichthys crocea* (Mu et al., 2020). This gene was statistically associated to variation in oxygen saturation in *T. albus* (**Supplementary Table 7**). The connections of candidate genes to functions sensitive to environmental heterogeneity support the role of adaptive divergence in the evolution of *T. albus* across catchments of different water conditions.

CONCLUSION

By disentangling signal of adaptive and neutral divergence in *T. albus* across Amazonia using a novel landscape genomics framework, we have identified genetically distinct populations in black, white and clear water catchments of the Amazon Basin. The dominant driver of population divergence across the basin was hierarchical structure, which is expected in dendritic river systems (Brauer et al., 2018). However, pH and turbidity were identified as having a significant secondary influence on genetic patterns across the system, which was supported by the discovery of candidate adaptive genes with functions linked to mediating the biological impacts of hydrochemical fluctuations. Thus, pH and turbidity, which are of distinctive compositions in contrasting water colors, may be important agents of divergent natural selection. In spite of the known limitations of genome scans of selection (reviewed in Grummer et al., 2019), this study has improved our understanding of the evolutionary processes operating in a complex tropical system, which is vastly understudied in proportion to its immense biodiversity. The common IBD model appears as not being sufficient to explain broad patterns of genetic variation in this system. Importantly, the notable impact of IBE in freshwater fishes suggests that changing hydrological conditions of the Amazon Basin may have implications to their distribution and persistence. Understanding genomic vulnerability of Neotropical fishes amidst accelerated environmental and climatic change may be a valuable avenue for future research and would further help to inform on conservation management strategies for this diverse group of vertebrates.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: Figshare (https://figshare.com/articles/dataset/_/17109146).

ETHICS STATEMENT

The animal study was reviewed and approved by the Macquarie University Animal Welfare Committee 2007/033.

AUTHOR CONTRIBUTIONS

AH carried out the molecular laboratory work, performed the data analyses, and wrote the first manuscript draft. JS-C provided technical and analytical advice, supervised the laboratory work and data analyses, and made suggestions for the manuscript. GC carried out fieldwork and laboratory work. NC carried out fieldwork and provided guidance about project design. LB conceptualized and designed the project, obtained funding, carried out fieldwork, provided technical and analytical advice, critically reviewed, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.825406/full#supplementary-material

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Deep Genomic Divergence and Phenotypic Admixture of the Treefrog *Dendropsophus elegans* (Hylidae: Amphibia) Coincide With Riverine Boundaries at the Brazilian Atlantic Forest

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The Atlantic Forest (AF) domain is one of the Earth's biodiversity hotspots, known for its high levels of species diversity and endemism. Factors related to palaeoenvironmental dynamics, such as the establishment of vegetational refugia and river basins, have different impacts on biological communities and biodiversity patterns in this domain. Here, we sample genome-wide RADseq data from a widespread treefrog (*Dendropsophus elegans*), inhabiting natural and human-impacted ecosystems at the Brazilian AF to test the impact of riverine boundaries and climatic refugia on population structure and diversification. We estimate divergence times and migration rate across identified genetic breaks related to the rivers Doce, Paraíba do Sul, Ribeira de Iguape, and Paraguaçu, known to represent barriers to gene flow for other AF endemic species, and test the role of climatic refugia. Finally, we investigate the impact of spatio-temporal population history on morphological variation in this species. We recovered a phylogeographic history supporting three distinct clades separated into two geographically structured populations, corresponding to the north and south of AF. In addition, we identified an admixture zone between north and south populations in the latitude close to the Doce River. Our findings support a pattern of isolation-by-distance and the existence of a secondary contact zone between populations, which might have been promoted by gene flow during population expansion. Further, we found support for models considering migration parameters for all the tested rivers with different population divergence times. Based on the species history and the AF palaeoenvironmental dynamics, we corroborate the role of forest refugia impacting population structure for this species through recent range expansion after the Last

Glacial Maximum (LGM). The Doce and Paraíba do Sul Rivers coincide with the main genetic breaks, suggesting they might also have played a role in the diversification processes. Finally, despite finding subtle correlations for phenotypic data among different populations, variation is not strongly detectable and does not seem associated with speciation-level processes that could warrant taxonomic changes. Such results can be explained by phenotypic plasticity of the evaluated traits and by recent divergence times, where there has been insufficient time and weak selective pressures to accumulate enough phenotypic differences.

Keywords: phylogeography, ddRADSeq, refuge hypothesis, population structure, phenotypes, Neotropic, riverine barriers

INTRODUCTION

Spatial patterns of biodiversity are arranged according to species' evolutionary history, such as its responses to environmental variation. Current patterns of spatial genetic structure and gene flow among populations have been affected by past climatic fluctuations and the establishment of biogeographic boundaries, all modulated by species' ecology (Antonelli et al., 2018; Pirani et al., 2019; Sheu et al., 2020). In the Neotropics, climatic oscillations during the Quaternary have impacted species distribution and genetic patterns – a major process accounting for several species' current distributions (Carnaval et al., 2009; Cheng et al., 2013; Rull and Carnaval, 2020). It is long proposed that, during interglacial periods, the extension of Neotropical forested areas has been larger relative to open ecosystems (Vanzolini and Willians, 1970; Leite and Rogers, 2013). In contrast, during the Pleistocene glacial periods, biota experienced increased aridification and concentrations of CO_2, which likely resulted in retraction of forests and expansion of open areas (e.g., Antonelli et al., 2010). Consequently, populations of plants and animals associated with forested habitats that were unable to disperse across open areas could have been isolated for thousands of years, accumulating genetic differences that could potentially result in allopatric speciation (e.g., Resende et al., 2010; Damasceno et al., 2021).

In particular, a great number of studies have increased the understanding of the palaeoenvironmental dynamics effects on biological communities and biodiversity patterns of the Brazilian Atlantic Forest (AF) (Rosauer et al., 2009; Carnaval et al., 2014; Peres et al., 2020). Located along the eastern coast of Brazil, the AF is known for its high levels of species diversity and endemism (Peres et al., 2020), and it is considered one of the Earth's biodiversity hotspots (e.g., Martins, 2011; Moura et al., 2017). Historical forest refugia models of the AF (Carnaval and Moritz, 2008) are predicted to comprise higher phylogenetic diversity, suggesting that forest persistence influenced the biodiversity patterns of several taxa (e.g., Carnaval et al., 2009; Thomé et al., 2010; Martins, 2011; Peres et al., 2020). Furthermore, the Pleistocene climatic dynamics and forest refugia affected biota differently along with the broad latitudinal extension of the AF. At the north of the Doce River, larger forested areas persisted due to stable climates (e.g., Thomé et al., 2010; Carnaval et al., 2014), whereas in the south, smaller patches of forest occurred due to unstable climates and were likely colonized after the Last Glacial Maximum (LGM) (Carnaval et al., 2009). The presence of these different refugia along the domain might be explained by AF topographic complexity, large latitudinal range, and strong seasonality. The north and central regions are warmer and more humid than southern latitudes, which are colder and drier (Ab'Saber, 1977). However, contrasting this classic refugia hypothesis view, Leite et al. (2016) detected an expansion of suitable climatic conditions onto the exposed continental shelf during the LGM, which would have allowed forest and associated taxa to expand and not contract as previously proposed. A signal of population expansion during the LGM and the Last Interglacial (LIG) for small mammals supports such scenario.

In addition to climatic and vegetational refugia, the biodiversity of the AF is structured by topography – the domain comprises mountain ranges up to 2,500 m.a.s.l. (above sea level), lowland areas at sea level, and several river basins (e.g., Doce, Paraíba do Sul, and Mucuri Rivers – Pellegrino et al., 2005; Resende et al., 2010). These topographic features coincide with species-pairs genetic breaks recovered for several taxa (e.g., Thomé et al., 2012, 2014; Rodríguez et al., 2015; Cazé et al., 2016; Sabbag et al., 2018; Menezes et al., 2020). Furthermore, the AF rivers also play an important role in promoting species diversification and structuring populations of dry adapted taxa (e.g., São Francisco River; Werneck et al., 2015). One of the AF rivers that seems to be a congruent dispersal barrier across several taxa is the Doce River (Costa, 2003; Pellegrino et al., 2005; Martins, 2011; review Peres et al., 2020), including our focal species, *Dendropsophus elegans*, even though it is role as a primary driver of divergence is still unclear (Tonini et al., 2013). In addition, the Doce basin is also a region of significant species turnover, highlighting that the climate differences between the north and south of AF could be responsible for this division, rather than the river itself (Fiaschi and Pirani, 2009; Saiter et al., 2016; Peres et al., 2020; Rezende et al., 2020).

For a complete association between geography and genetic divergence, it is important to consider the persistence of populations' isolation and the timing of isolation events. Likely, populations remained isolated long enough to accumulate genetic and phenotypic differences (e.g., the timing of divergence across margins of the Doce River varies among taxa; Cabanne et al., 2008). As a result, several phylogenetic studies in amphibians (e.g., Wynn and Heyer, 2001; Gehara et al., 2014) highlighted the existence of cryptic lineages into nominally widespread tropical species (e.g., Pellegrino et al., 2005; Cabanne et al., 2008).

Here we explore the impact of river basins and Quaternary climatic oscillations on the population structure of an AF endemic treefrog species. The focal species of our study, *D. elegans* (Hylidae, Amphibia), is a common treefrog that usually inhabits native herbaceous vegetation in wetlands near open areas but also human-impacted regions at the AF (Izecksohn and Carvalho-e-Silva, 2001). Its distribution ranges from the northern state of Pernambuco to the southern state of Rio Grande do Sul in Brazil, spanning most of the AF environmental gradient (see **Figure 1**). The species is part of the clown treefrog *Dendropsophus leucophyllatus* group, associated with South American rainforests (Amazonia and AF – Duellman et al., 2016). In this group, most of the species' diversity occurs in the Amazonia (11 lineages – Pirani et al., 2020) and only *D. elegans* and *Dendropsophus nekronastes* in the AF (Dias et al., 2017), with distinguishable morphological features that set them apart from close relatives (Orrico et al., 2021).

Previous molecular assessment for *D. elegans* based on mitochondrial DNA (mtDNA) revealed a clear phylogeographical structure with three main clusters along the latitudinal gradient of the AF (north, central, and south clades; see Tonini et al., 2013). These clusters intersected with inferred paleoclimatic models that generally agree with the Late Quaternary climatic stability scenario proposed for the AF by Carnaval and Moritz (2008) and Carnaval et al. (2009). Another study recovered the divergence time between *D. elegans* and its sister group in the *D. leucophyllatus* species group to 3-2 million years ago (Mya) on the Plio-Pleistocene (Pirani et al., 2020). Although the genetic structure and past geographic distribution of *D. elegans* fit well with the refuge hypothesis, geographic barriers, such as the Doce and Paraíba do Sul Rivers, also coincide with the main mtDNA phylogeographic breaks (Tonini et al., 2013) and their role in this species diversification history was never properly explored. In addition, it is important to emphasize that ecological patterns of advertisement call variation among individuals do not correlate with the species' proposed phylogeographic history (Forti et al., 2017). The apparent mismatch between genetic and phenotypic variation can help uncover processes involved in species evolutionary histories (Zamudio et al., 2016) and raises the question of whether other mechanisms are likely to be related to the observed diversification pattern. For example, geographical barriers and persistence of gene flow among populations could be responsible for ecological uniformity (Zamudio et al., 2016) at a given period (e.g., Leite et al., 2016).

Here we use genome-wide data and model-based phylogeographic analyses to untangle the history of population genomics and morphological differentiation in this treefrog species. We estimate the time of populations' diversification, demography, and gene flow between genetic breaks (which coincide with the Doce, Paraíba do Sul, Ribeira de Iguape, and Paraguaçu Rivers) to test whether the genetic structure is related to geographic barriers and/or climatic refugia. We also evaluate whether observed patterns of genetic diversity and phylogenetic structure are related to phenotypic variation for *D. elegans* populations.

MATERIALS AND METHODS

Sampling and RADseq Genomic Data Generation

Genomic data were collected from 44 samples (36 *D. elegans* samples, plus four samples of *Dendropsophus anceps* from AF, two samples from *Dendropsophus minutus*, and two from *D. leucophyllatus* from the Amazonian rainforest as outgroups; representing a total of 25 localities; see **Supplementary Table 1** in **Supplementary Material 1**).

The DNA was extracted from the muscle or liver of each individual using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer's protocol. Our sampling of *D. elegans* individuals is a subsampling of two double digest Restriction-site Associated DNA (ddRAD) libraries sequenced for a larger scale sampling of the *D. leucophyllatus* species complex (which included ten described species and four new lineages). For a complete description of the genomic library preparation and DNA sequencing process, see Pirani et al. (2020). A complete list of voucher information used in this study is provided in **Supplementary Table 1** in **Supplementary Material 1**.

Data Processing

To infer interspecific relationships of *D. elegans* and close relatives, raw data was processed using the ipyRAD v.0.7.17 pipeline[1] (Eaton, 2014; Eaton and Overcast, 2020) using *de novo* assemble from the fastQ files obtained from the Illumina sequencing run. All the 44 samples were processed together with the following parameter specifications. First, sequences were demultiplexed using the program step 1, where the restriction sites and barcodes were trimmed from approximately 544 million reads. Because the number of reads stabilizes above 500,000 per sample, we eliminated seven *D. elegans* samples below this threshold before conducting the next steps in the remaining 37 samples. The number of reads per individual ranged between 551,291 and 2,395,613 (**Supplementary Table 2** in **Supplementary Material 1**). After that, sequences with more than five base-calling errors were discarded. Heterozygosity and error-rate were estimated from the base counts at each site across all clusters, and the averages were used to establish consensus sequences. We excluded clusters with less coverage than a minimum depth of five to ensure accurate base calls. Consensus sequences from all samples were clustered by sequence similarity, with their input order randomized, using the same similarity threshold as the within-sample clustering (90%). Note that the parameter values were in line with other recent studies focusing on within-genus divergences (e.g., Huang, 2016). All the fragments were trimmed to a minimum length of 110 bp. Any cluster appearing heterozygous at the same site across more than four samples was discarded. A cluster with sequence success of

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[1] http://ipyrad.readthedocs.io/#

fewer than 15 individuals was also excluded from the output. The remaining clusters were treated as RAD loci, i.e., multiple alignments of putatively orthologous sequences assembled into phylogenetic data matrices. We achieved the final dataset after running all the ipyrad steps and excluding excessive variation arising from clustering errors. We also exported only one random SNP per cluster, so our data comprised only potentially unlinked SNPs. The complete dataset with *D. elegans* samples plus outgroups had a total of 29,579 potentially unlinked SNPs for 37 individuals (29 from *D. elegans* and eight outgroups). This dataset presented 63% of missing data, and it was only used to infer phylogenetic relationships. Because of the higher missing data frequency present among the outgroup samples, a secondary dataset including only the 29 individuals of *D. elegans* was generated. This dataset contained 19,151 potentially unlinked SNPs with only 5% missing data. It was used to calculate summary statistics and perform all the other analyses, including STRUCTURE and X-origin (see methods below for details). See **Supplementary Table 2** in **Supplementary Material 1** for the number of loci used for each downstream analysis. All the ipyRAD steps were run under parallel execution with eight threads in the University of Michigan flux computing cluster.

Inferring Species Tree/Population Tree

The final potentially unlinked SNP dataset resulting from ipyRAD for *D. elegans* species and outgroups was used to infer the species phylogeny and contrast patterns of divergence between lineages. To access the evolutionary relationships among individuals, we manually converted the output from ipyRAD to nexus format and performed a species tree reconstruction using the coalescent-based program SVDquartets (Chifman and Kubatko, 2014), implemented in PAUP* v. 4.0d147 (Swofford, 2002). We evaluated all possible quartets, selected trees using the QFM quartet assembly and performed bootstrapping with 100 replicates to calculate branches' support. The tree was rooted in *D. minutus* (Pirani et al., 2020). We also used RAxML v. 8.2.8 (Stamatakis, 2014) to infer branch lengths for the total concatenated dataset of 3,852,884 loci (all fragments including invariant sites), using the GTRCAT model and a bootstrapping of 100 replicates. All the analyses above ran under parallel execution with 16 threads in the University of Michigan flux computing cluster.

Characterizing Population Structure and Genetic Diversity Across Rivers

To evaluate the population structure within *D. elegans*, we used a Bayesian approach implemented in STRUCTURE version 2.3.4 (Pritchard et al., 2000) using 5,000 potentially unlinked SNPs randomly selected with no outgroups (total of 27 individuals). This subset allowed us to avoid the exceptionally time-consuming computations typical of large datasets while still covering our complete sampling range. We ran 10 replicated analyses over a range of *K* from 1 to 4, based on the results from a previous study that recovered three genetic clades based on a phylogenetic inference (Tonini et al., 2013). Further, a hierarchical genetic structure was performed within each initial cluster identified by STRUCTURE (i.e., Thomaz et al., 2017). The hierarchical subset of individuals contained within genetic clusters were run with *K*-values ranging from 1 to 4, where individuals were assigned probabilistically to each genetic cluster. Each STRUCTURE analysis was run for 500,000 generations following a burn-in of 200,000 generations, with the possibility of mixed ancestry. We compared the analyses using the ΔK of Evanno et al. (2005) implemented in STRUCTURE HARVESTER (Earl and vonHoldt, 2011) to identify the *K* number of genetic clusters that best fit the data and plotted results with individuals in the geographical order of their appearance using the CLUMPAK pipeline (Kopelman et al., 2015). In addition, to avoid any bias, we also plot the results using Structure Selector (Li and Liu, 2018), using Puechmaille's method (Puechmaille, 2016).

Genetic diversity was measured within populations as informed by results obtained from population structure analysis. A third clade was identified in the phylogenetic analyses corresponding to the region between Doce and Paraíba do Sul Rivers. The same was recovered from our population structure analysis as an admixture zone (see Results below). Thus, we separated this contact zone as a different "population" (see section "Results"; **Figure 2**) with the intention to test if this admixture event was established by primary or secondary contact demonstrated by genetic diversity levels (that is, higher diversity if the contact was established posteriorly). For that, we calculated the average nucleotide diversity (π) in Arlequin 3.5.2.2 (Excoffier and Lischer, 2010) based on polymorphic sites.

Genetic differentiation across populations (considering the contact zone as a different population; see **Figure 2**) was measured by pairwise F_{ST}-values, with their significances assessed from 10,000 bootstrap replicates with a Bonferroni correction for multiple comparisons, calculated in Arlequin 3.5.2.2 (Excoffier and Lischer, 2010). Additionally, the hypothesis of isolation-by-distance among localities was calculated in Arlequin 3.5.2.2 (Excoffier and Lischer, 2010) using the Mantel test to compare the genomic and geographic distance matrices. Geodesic distances between populations were estimated with the function gDistance in the R package shapefiles (Bivand, 2006).

We used the Estimated Effective Migration Surfaces (EEMS) method to evaluate geographic barriers and gene flow patterns (Petkova et al., 2016). This method estimates gene flow from geo-referenced genetic samples and identifies potential barriers separating areas where the decay of genomic differences across geographical distances is higher than expectations under a model of isolation-by-distance. For this approach, a triangular grid from −34° to −51° of longitude and −27° to −8° of latitude was built, spanning the entire geographic range of sampling in the Brazilian AF for *D. elegans* with 600 demes used as a parameter. In this analysis, the migration parameter *m* is estimated by Bayesian inference for every edge of the grid by Markov chain Monte Carlo (MCMC) sampling, with each individual being assigned to the nearest vertex of the grid based on observed genetic dissimilarities on the potentially unlinked SNPs dataset. When viewed graphically across the species distributions, areas of reduced gene flow can be visualized based on estimates of the posterior probabilities of *m*. We performed eight independent runs to assess convergence, with 10 million MCMC iterations, with 2 million burn-in and a thinning of 9,999 used for each run. Convergence among runs was accessed with the R package rEEMSplots, available with the EEMS pipeline.

Divergence History and Migration Across River Barriers

We estimated divergence models with and without migration between genetic breaks of *D. elegans* following the empirical results obtained from our genomic analyses, where the rivers Doce, Paraíba do Sul, Paraguaçu, and Ribeira de Iguape demonstrated to have an impact on population structure (see section "Results"). For this analysis, we used the method based on the joint site frequency spectrum (SFS) implemented in FASTSIMCOAL2.6 (Excoffier et al., 2013). We estimated the divergence times T_{DIV}, population size N_{POP}, ancestral size $N_{ANCSIZE}$, and migration M_{MIG} (for the models including migration) across each genetic break. We intended to test if these rivers act as barriers reducing gene flow between populations in different margins and to estimate diversification time across each genetic break. Thereby, we could correlate the results with an older diversification scenario; or if more recent events were more likely to affect populations diversification and consequently the formation of the contact zone.

For the input dataset, ipyRAD was run to generate a dataset with 0% of missing data (total of 2,797 SNPs) (following the program requirements; see Excoffier et al., 2013). The vcf file with potentially unlinked SNPs was converted to Arlequin format using PDGSpider (Lischer and Excoffier, 2012). We calculated the folded joint SFS (i.e., minor allele) using Arlequin 3.5.2.2 (Excoffier and Lischer, 2010). The effective population size of one side of each river (N_{POP0}) was fixed to improve the accuracy of parameter estimates from the SFS – following the recommendations of the program (Excoffier and Foll, 2011). The other parameters were estimated from the SFS using uniform priors (see **Table 2** for details). To calculate the N_{POP0} directly from the empirical dataset, we based our calculation on the nucleotide diversity (π) of variant and invariant sites, where $\pi = 4N\mu$, assuming a mutation rate of 3.46×10^{-8}. We used the regression formula for cellular organisms to estimate the mutation rate (Lynch, 2010), based on genome sizes estimated in related species (i.e., *Dendropsophus microcephalus* for *D. elegans*[2]), and considering one generation per year (Duellman, 1974). A total of 40 runs were conducted for each model, and we present the point estimate of the highest likelihood across runs, as well as 95% confidence intervals on the parameter estimates calculated using a parametric bootstrap of 100 simulated datasets. The analysis was based on 100,000 to 250,000 simulations for likelihood estimation with a stopping criterion of 0.001 and 10–40 expectation-conditional cycles (ECM). All the models were performed with eight threads in the Smithsonian Tropical Research Institute computing cluster.

Recent Range Expansion

To understand the historical demography of *D. elegans*, we also tested if north and south populations had signals of range expansion after the LGM using the X-ORIGIN pipeline (He et al., 2017). The program is designed to estimate origins by applying the pairwise spatial statistics Ψ that estimate expansion origin from derived SNPs data. It is best suited for estimating refugia for species that experienced distributional shifts during LGM (see He et al., 2017), which is our hypothesis for *D. elegans*.

For the input dataset, we used the vcf file with 0% missing dataset generated for FASTSIMCOAL2.6 (see methods above). We converted it to nexus format as input (program requirement format for the precheck.R script) at PDGSpider (Lischer and Excoffier, 2012). Further, to assign individuals to populations, we separated them by geographic localities using a buffer of 50 km. After applying the buffer, localities represented by only one sample, and the contact zone samples, were removed from this analysis. For details, see **Supplementary Table 3** in **Supplementary Material 1**.

Correlation Between Phenotypes and Genomic Divergence

Morphometric and morphological data were collected from 1,020 individuals of *D. elegans* (867 males, 126 females) from 109 localities (for details, see **Supplementary Tables 1, 2** in **Supplementary Material 2**). To test if differences in body shape are correlated with genetic divergence among *D. elegans* populations, we measured 18 morphometric distances for all the analyzed specimens: SVL (snout-vent length), HL (head length), HW (head width), ED (eye diameter), END (eye nostril distance), TD (tympanum diameter), UEW (upper eyelid width), IOD (interorbital distance), IND (internarial distance), NSD (tip of snout nostril distance), HU (humerus length), RUL (radius-ulna length), HAL (hand length), THL (thigh length), TL (tibia length), FL (foot length, including tarsus), 3FD (third finger disc diameter), and 4TD (fourth toe disc diameter). All measurements are in millimeters (*mm*) using a digital caliper under a stereo dissecting microscope; measurements follow Napoli (2005). To further characterize morphometric data clustering, we performed a principal component analysis (PCA) using the "prcomp" function in R (R Core Team, 2020) and coloring the samples according to the inferred genomic structure. The complete list of visited Herpetological Collections in Brazil is presented in **Supplementary Table 2** in **Supplementary Material 2**.

We did a series of standards on outline drawings for color patterns (general dorsal pattern, upper surface of shank, and upper surface of forearm) and for a dorsal outline of the snout (following Heyer et al., 1990) to assess phenotypic variation across the study area (**Figure 1**) and to test for correlation among dorsal coloration and outline of snout patterns against genetic divergence. The dorsal body surface was characterized by a rectangle or hourglass of brown color, framed by a yellowish-white band; D1 (full rectangle frame); D2 (incomplete rectangle frame close to the eye and/or sacral region); and D3 (fragmented rectangle frame) (see **Figure 5** for dorsal color variation drawings). The upper shank surface was characterized by a longitudinal white-yellowish longitudinal strip covering almost the entire dorsal surface of brown background color: T1 (single and extensive white-yellowish band); T2 (three rounded

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[2] www.genomesize.com

white-yellow spots); and T3 (two yellow-white spots). The upper forearm surface was characterized by yellowish-white crossbones on brown background color: UA1 (immaculate); UA2 (a yellowish-white spot on the elbow); and UA3 (two yellowish-white patches, one on the elbow and one in the anterior region of the forearm). Further, we also registered three muzzle formats in dorsal view for the *D. elegans* specimens: S1 (rounded); S2 (pointy); and S3 (truncated) (see **Supplementary Table 3** in **Supplementary Material 2**). For specimens in which the design was partially discolored or damaged were removed from the analyses – we only recorded pattern states that were clearly visible. This type of "by eye" phenotypic categorization has been shown to be highly supported by quantitative analyses of standardized color photographs (Dugas et al., 2015).

To determine whether color phenotypes (the dorsal surface, the upper surface of the shank, staining the upper surface of the forearm, and muzzle formats in dorsal view) correlate with geography, we performed a permutational multivariate analysis of variance (PERMANOVA) on each trait, with sampling site nested within populations. All analyses were performed on normal-quantile-transformed data. We performed all transformations and statistical tests in R (R Core Team, 2020).

RESULTS

Species Tree and Intraspecific Diversity

The SVDquartets analysis inferred a highly supported topology recovering *D. elegans* as a monophyletic species sister to *D. leucophyllatus* (100% bootstrap; see **Figure 2**), both from the same species group. *Dendropsophus anceps* appears as a sister to the *D. elegans* and *D. leucophyllatus* clade, but with low node support (50%, **Figure 2**) (see also Figure 2 in Pirani et al., 2020).

At the intraspecific-level relationships, *D. elegans* is represented by three main geographically distinct clades (**Figure 2**): the north clade (61% bootstrap) that is distributed northern of Doce River, at Minas Gerais state to the Alagoas state (see **Figure 1**); the south clade (100% bootstrap), with the northern limit of the distribution corresponding to the Paraíba do Sul River extending southern into Paraná state; and the central clade (87% bootstrap), with a narrow distribution between the Doce River to the north of the Paraíba do Sul River, including Espírito Santo and Minas Gerais states (**Figure 1**). All these results are also supported by the RAxML tree (see **Supplementary Figure 2** in **Supplementary Material 1**).

Population Structure and Genetic Diversity Across Rivers

Population structure analyses identified two main genetic clusters (K = 2; **Figure 2** and **Supplementary Figures 3, 4** in **Supplementary Material 1**) across the geographic range of *D. elegans*, being congruent with the north and south clades from the phylogenies, geographically separated by the Doce and Paraíba do Sul Rivers (**Figure 1**). Within each main cluster, probabilistic assignment of individuals to the respective genetic clusters revealed evidence of a substructure corresponding to the position of the rivers Paraguaçu, within the north cluster, and Ribeira de Iguape in the south cluster (K = 2 for both cases; **Figure 2**). Moreover, we found evidence of haplotype admixture between north and south clades in samples positioned in the central clade in the phylogenetic inferences, which we identified as a contact zone (see **Figures 1**, **2**).

Pairwise genetic differentiation (F_{ST}) was high across north and south clades (F_{ST} = 0.408, **Table 1**), and the samples from the contact zone demonstrated to be genetically closer to the north than to the south clade (F_{ST} = 0.246 and 0.325, respectively; **Table 1**), which differs from the genealogical results. Furthermore, *D. elegans* localities had overall genomic distances positively correlated with geographic distances (r = 0.68; P-value < 0.01; **Table 1**). For the genetic diversity analyses, the central clade that corresponds to the contact zone showed one order of magnitude higher values of genetic diversity ($\pi = 0.014$; **Table 1**), in comparison with the north ($\pi = 0.003$) and the south clades ($\pi = 0.006$, **Table 1**).

Population genetic structure was supported by the EEMS analysis, which detected reduced gene flow corresponding to the major AF rivers (**Figure 3**). For instance, the Doce River was inferred as a low migration zone (e.g., potential barrier) between the north and south populations. Reduced migration was inferred within the north population in areas that corresponded to the Paraguaçu and São Francisco Rivers. For the south population, we did not find any strong restriction of gene flow that corresponds to the Paraíba do Sul River. In contrast, a reduced migration was inferred between the samples in the area between Tietê and Ribeira de Iguape Rivers (**Figure 3**).

Divergence History and Migration Across River Barriers

FASTSIMCOAL26 results indicated divergence with migration as the best-fit models for all the tested rivers (Paraguaçu, Doce, Paraíba do Sul, and Ribeira de Iguape Rivers), even though migration estimates vary between them (see **Table 2**). The highest migration estimate was across the Paraíba do Sul River, which coincides with the oldest divergence time among populations (3 Mya ago). The migration estimated per generation over this river from the southern population to the contact zone is almost two migrants per generation ($\sim$1.91 from south 1 to central and 0.42 migrants per generation in the opposite direction). The lowest migration rates were found across the Ribeira de Iguape River, within the south population, with less than one migrant per generation (0.01 for south 1 to south 2 and 0.04 in the opposite direction). Although the divergence time between the Ribeira de Iguape River margins is recent (0.36 Mya) in comparison to the Paraíba do Sul, this estimate is closer to the divergence times between populations separated by the Doce and Paraguaçu Rivers (0.24 and 0.16 Mya, respectively). Migration rates across the Doce River, between the contact zone and the north population, indicates almost one migrant per generation (0.29 for north 2 to central and 0.99 in the opposite direction). Across the Paraguaçu River, within the north population, the results present less than one migrant per generation ($\sim$0.13 for north 1 to north 2 and 0.48 in the

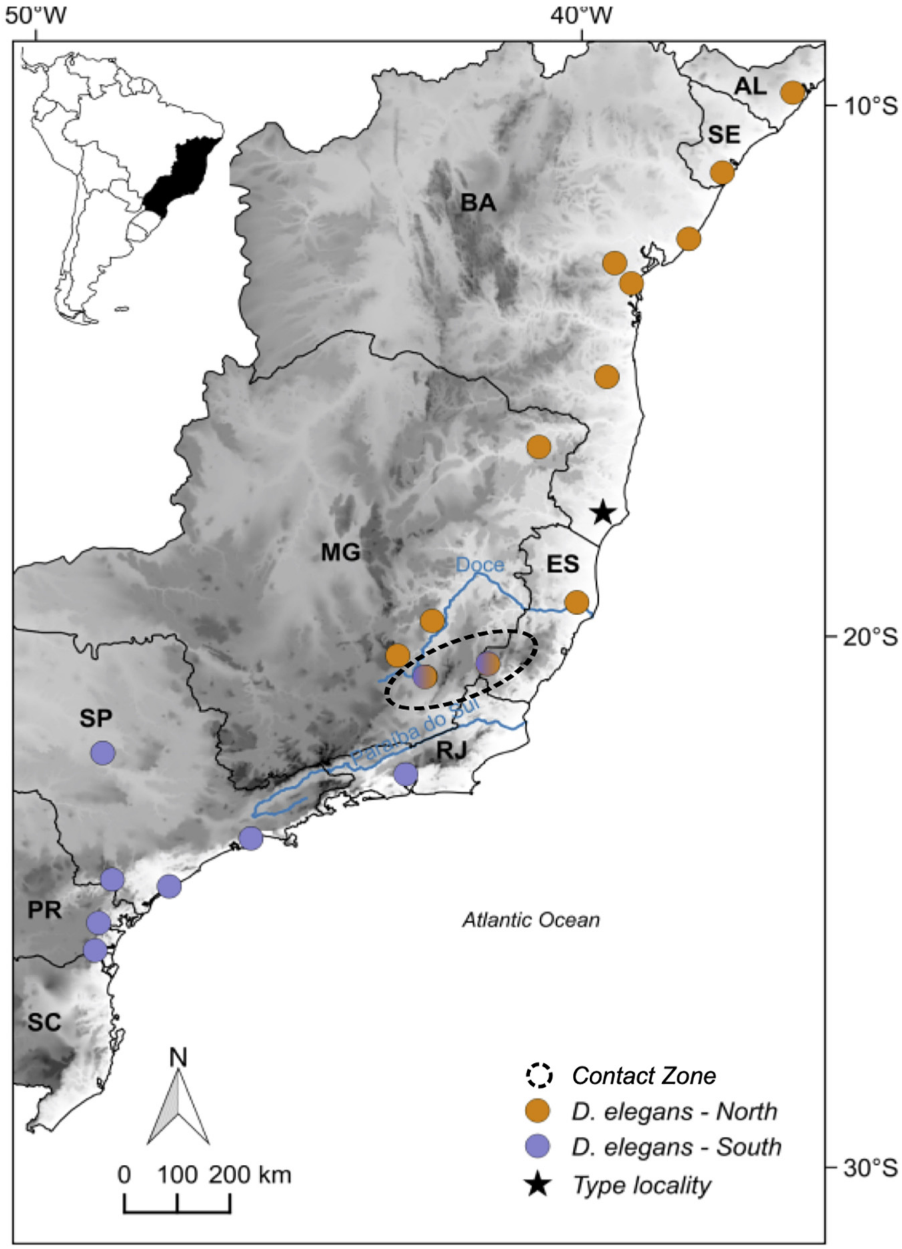

FIGURE 1 | Distribution of the genomic sampled localities of *Dendropsophus elegans* from the Atlantic Forest across each population with different colors: north (orange), contact zone (black dashed circle), and south (purple); also the type locality (start). Elevation is shown in gray scale ranging from white (0–50 m above the sea level) to black (3,900–3,950 m above sea level). Brazilian states: Alagoas (AL), Sergipe (SE), Bahia (BA), Espírito Santo (ES), Minas Gerais (MG), Rio de Janeiro (RJ), São Paulo (SP), Paraná (PR), and Santa Catarina (SC).

opposite direction). All the parameter estimates are represented in **Table 2**.

The X-ORIGIN analysis indicated a significant recent range expansion signature after the LGM for the north population in the southern direction (P-value = 0.00117). However, expansion was not detected for the south population (P-value = 0.72753). Further, samples located in southern Bahia and Minas Gerais state (included into the north population) presented higher pairwise Ψ statistics in comparison to the other localities (see **Supplementary Table 3** in **Supplementary Material 1**), meaning that there is an expansion signature left in the data.

Correlation Between Phenotypic and Genomic Divergence

Variation of morphometric measures among *D. elegans* specimens did not evidence any specific pattern between north and south populations and the contact zone (see

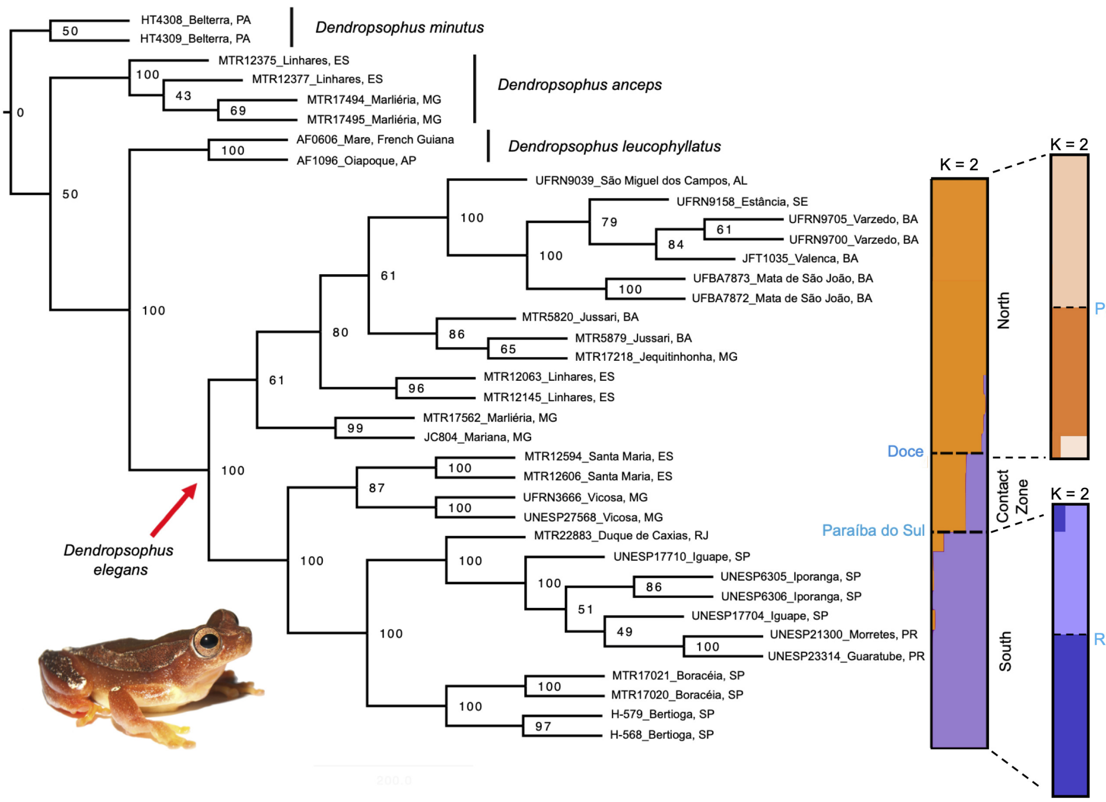

FIGURE 2 | Phylogeny of *Dendropsophus elegans* species recovered by SVDquartets on the potentially unlinked SNPs dataset. Bootstrap values for the nodes are presented for the complete tree. The right bars represent the population structure inferred by STRUCTURE analysis showing the most probable number of groups (*K* = 2) as different colors along a latitudinal scale, whereas populations are labeled and separated by the black dashed line (corresponded to Doce, Paraíba do Sul, Paraguaçu, and Ribeira do Iguape Rivers). North population (orange), south population (purple), and the contact zone. Photograph: Rodrigo Tinoco.

Supplementary Table 4 in **Supplementary Material 2**). The PCA visualizes the overlap of individual measurements between these two populations (PCA), where the first principal component (PC1) explained 63.83% of all morphometric variance, followed by PC2 and PC3 that explained 7.96% and 5.50%, respectively (**Figure 4**). The most important variable explaining the morphometric overlap between populations along PC1 is the SVL; in the PC2, the head length (HL) was the most relevant variable, followed by the HW in the PC3. In general, body sizes are similar among sampled localities, but there is emphasis on larger body size for individuals in a narrow coastal strip that extends from southern Bahia, south of Jequitinhonha River (18°S, 39°W), to the southwest of Espírito Santo state (19°S, 40°W).

For the dorsal pattern coloration results, no correlation was recovered for the upper surface of the forearm and the muzzle formats in dorsal view (*P*-value = 0.156 and 0.713, respectively). While we recognized spatial variation along with localities for the dorsal and upper shank surface patterns (see **Figure 5**), no correlation was found for the dorsal surface analysis (*P*-value = 0.06), and only the shank surface had a significant variation (*P*-value = 0.001) on the PERMANOVA analyses. The D1 body pattern was the most frequent (80% of the individuals) distributed along the species range. The D2 pattern occurred in almost all the localities but was infrequent (18.2% of the individuals) and tended to decrease or disappear on the extremes of the species distribution. The D3 pattern was the most uncommon (1.2% of the individuals), only occurring in the contact zone region, concentrated at Minas Gerais and Espírito Santo states, located in the region of the Doce River valley. The T1 upper shank surface pattern occurred in most localities (69.8% of the individuals) and was more frequent along the southern localities, decreasing considerably northwards. The opposite situation happened for the T2 pattern, which decreases in frequency southwards. The pattern T3 only appeared in the center of the species distribution, with a very low frequency (5.8% of the individuals) (for details, see **Figure 5** and **Supplementary Table 3** in **Supplementary Material 2**).

DISCUSSION

By analyzing genome-wide SNPs of *D. elegans* populations, our study revealed a strongly resolved phylogeny with three main clades distributed along a latitudinal gradient, consistent with the mitochondrial pattern previously recovered (Tonini et al., 2013). A recent genomic study at the species group level also demonstrated an initial separation of *D. elegans* into

TABLE 1 | Pairwise F_{ST}-values between population pairs and nucleotide diversity (π) for each population.

Population			
	South	**North**	**Contact zone**
South	0.000	–	–
North	0.408	0.000	–
Contact zone	0.325	0.246	0.000
π	0.006	0.002	0.013

Significant values are shown after correcting for multiple comparisons using a Bonferroni correction (i.e., $\alpha = 0.002$).

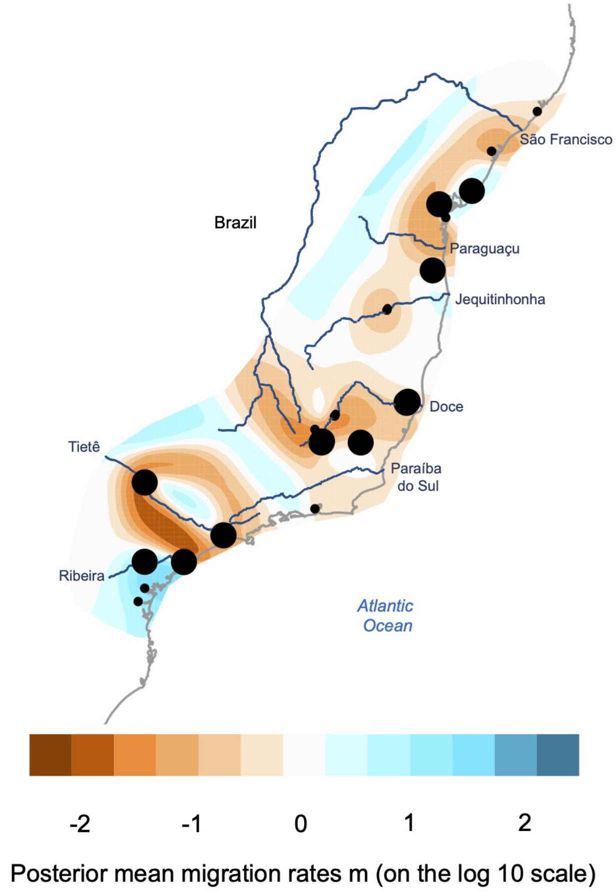

FIGURE 3 | Estimated effective migration surfaces (EEMS) plot showing the effective migration rates (m) on a log10 scale for *Dendropsophus elegans* averaged across eight independent runs. Geographic regions of low migration are presented in orange-brown, representing hypothesized migration barriers. Areas in blue represent geographic regions where samples are connected by migration rates higher than that expected under isolation by distance. Dots represent the sampled localities, with dots sizes proportional to sample sizes.

three lineages dated for the Plio-Pleistocene delimited by the Paraguaçu and Doce Rivers (3-2 Mya; see Pirani et al., 2020). Here we show that, following a relatively long period of low divergence (e.g., long-branches on Pirani et al., 2020), population history of *D. elegans* became very dynamic over short time intervals, probably associated with climatic fluctuations and riverine barriers in the AF. We found support for the riverine barrier hypothesis structuring population along a north–south gradient in different time periods but also allowing gene flow between rivers margins. It is also likely that a recent range expansion in the north population after the LGM (∼21 Kya) had caused an admixture event between the north and south populations by secondary contact. This result was also supported by Tonini et al. (2013) for the clades from southern Bahia, causing admixture at the central portion of the environmental gradient. During the LGM, cold temperatures supposedly promoted forest reduction into a large forest refugium to the north of the Doce River (Bahia refuge) and scattered refugia to the south. This colder and drier period was subsequently followed by warmer temperatures and expansion of the AF areas (Carnaval and Moritz, 2008), which could have facilitated secondary contact and gene flow between *D. elegans* in central AF (see also Peres et al., 2020).

We found support for three main intraspecific clades as a result of tree-building methods (see **Figure 2** and **Supplementary Figure 3** in **Supplementary Material 1**) with a high pairwise genetic differentiation between them (see **Table 1**). In contrast, the population structure analysis recovered two populations, north and south, with admixture between them along a contact zone – in which samples correspond to the central clade on the genealogical analyses (see **Figure 2**). The lack of evidence for the contact zone as a third unit in the population genetics analysis might be expected since the other analyses performed recovered a recent admixture event. Despite a known bias that introduces a problem of selecting the optimal number of clusters, where the ΔK method tends to frequently identify $K = 2$, even when more subpopulations are present (Janes et al., 2017), it is unlikely that our recovered $K = 2$ was an artifact of an analytical problem, but rather the pattern resultant from biological events, such as admixture. Another interesting fact resulting from our tree-building methods is that the phylogenetic splits do not follow the exact divergence times for each river (see **Table 2**). This result could be explained by gene flow among populations over time across river margins or changes in the river course, allowing gene flow in different time periods. The reconstruction of the phylogenetic method used for this study is exploratory and does not consider population parameters and migration among individuals and clades.

Our results have been consistent in terms of the relative impact of rivers in the species genetic structure and diversification, where all the major AF rivers coincide with species genetic breaks, though with different divergence times, and also allow varying degrees of migration between margins, acting as "soft" barriers. Although we find gene flow and secondary contact between populations, these events seem restricted to the Pleistocene (see **Table 2**). Our results make a compelling case for the association of Doce and Paraíba do Sul Rivers separating populations, and their relative role in the species diversification process (Tonini et al., 2013; review Peres et al., 2020). For instance, the Doce River has been consistently recovered as a geographic barrier by different analyses for *D. elegans*. At the same time, the Paraíba do Sul River was not detected as a barrier by the EEMS analysis (see **Figure 3**). Such results demonstrate the different

TABLE 2 | Parameter estimates for the *Dendropsophus elegans* divergence time along different rivers.

River	Parameter	Description	Estimate	Bootstrap	MaxEsthood/MaxObshood
Paraguaçu	N_{POP0}	North 1 N_e (7 samples)*	63,600	–	–15,293.24
	N_{POP}	North 2 N_e (7 samples)	16,911	(14,328–20,765)	–15,116.06
	$N_{ANCESIZE}$	Ancestral *Ne*	11,249	(8,979–13,013)	
	T_{DIV}	Divergence time	16,740	(14,083–21,569)	
	MIG_{12}	Migration north 1 → 2	0.000000752908	(0.000001–0.000001)	
	MIG_{21}	Migration north 2 → 1	0.0000028315	(0.000002–0.000003)	
Doce	N_{POP0}	North 2 N_e (7 samples)*	92,500	–	–14,159.21
	N_{POP}	Central N_e (4 samples)	27,401	(22,789–35,230)	–14,085.50
	$N_{ANCESIZE}$	Ancestral *Ne*	17,553	(13,726–19,474)	
	T_{DIV}	Divergence time	24,350	(20,002–32,540)	
	MIG_{12}	Migration north 2 → central	0.0000010654	(0.000001–0.000001)	
	MIG_{21}	Migration central → north 2	0.00000359644	(0.000003–0.000004)	
Paraíba do Sul	N_{POP}	Central N_e (4 samples)	474,951	(350,719–446,012	–9,977.03
	N_{POP0}	South 1 N_e (5 samples)*	105,000	–	–9,933.67
	$N_{ANCESIZE}$	Ancestral *Ne*	101,185	(65,633–186,571)	
	T_{DIV}	Divergence time	341,950	(168,697–354,464)	
	MIG_{12}	Migration south 1 → central	0.000000401461	(0.000000–0.000000)	
	MIG_{21}	Migration central → south 1	0.000000088753	(0.000000–0.000000)	
Ribeira de Iguape	N_{POP0}	South 1 N_e (5 samples)*	105,000	–	–6,080.81
	N_{POP}	South 2 N_e (6 samples)	15,965	(6,415–18,532)	–6,014.27
	$N_{ANCESIZE}$	Ancestral *Ne*	200,735	(299,026–190,050)	
	T_{DIV}	Divergence time	36,425	(34,016–218,935)	
	MIG_{12}	Migration south 1 → 2	0.000000042605	(0.000000–0.000000)	
	MIG_{21}	Migration south 2 → 1	0.000000280198	(0.000000–0.000002)	

Point estimates are those identified in the migration models run of the 40 model selection replicates and presented with 95% confidence intervals in parentheses.
**Fixed population size.*
N_e, effective population size; T_{DIV}, timing of divergence per generation; $N_{ANCESIZE}$, ancestral size; MIG, migration; Central, contact zone.

impacts of gene flow on species population structure, which could also be responsible for the ecological and phenotypic uniformity of *D. elegans* (e.g., Zamudio et al., 2016). Other geographic barriers recovered in this study seem to restrict gene flow across *D. elegans* populations and not only the Paraguaçu River (**Figures 2, 3**) as discussed in a large study for the species group, where *D. elegans* belongs to (see Pirani et al., 2020). Beyond rivers, the fact that an area of reduced gene flow inferred by EEMS does not precisely match the location of the Riberia de Iguape River raises the question that other environmental or geomorphologic barriers may be responsible for the *D. elegans* divergence process. This location presents a species turnover due to the different AF ecoregions, where the north geographic distribution of Araucaria mixed forest (north distribution between Tietê and Ribeira Rivers) could be responsible for the highest restriction of gene flow detected for the EEMS analysis (**Figure 3**; see also Thomé et al., 2014; Peres et al., 2020).

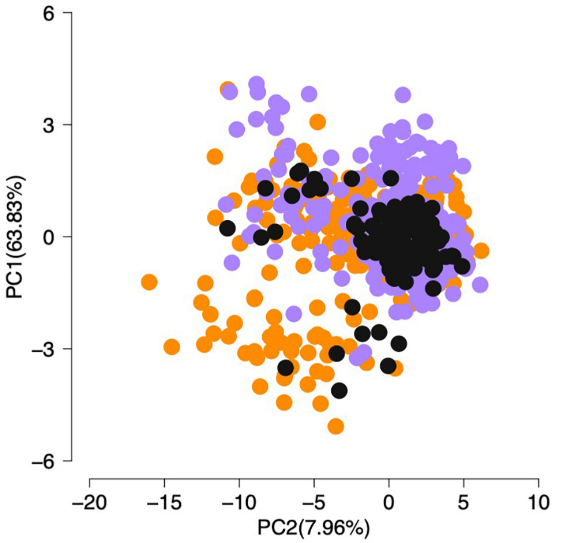

FIGURE 4 | Principal component analysis (PCA) from morphometric data of *Dendropsophus elegans* samples. Colors are following the genomic population structure: north (orange), contact zone (dark gray), and south (purple).

Despite physical barriers restricting species movements and affecting genetic variation (e.g., Pellegrino et al., 2005; Damasceno et al., 2021), organismal dispersal ability is also potentially correlated to species ecology and environmental variation. Yet, it is not surprising that the region between Doce and Paraíba do Sul was a contact region for *D. elegans* populations. This region is known as a turnover from the northeast and southeast of AF community composition (plants and animals – Fiaschi and Pirani, 2009; Peres et al., 2020), which have accumulated significant species differences to produce such pattern (see Brown et al., 2020), and it could also explain the oldest divergence time recovered for the Paraíba do Sul River (see **Table 2**; Rezende et al., 2020). Thomé et al. (2012) highlighted

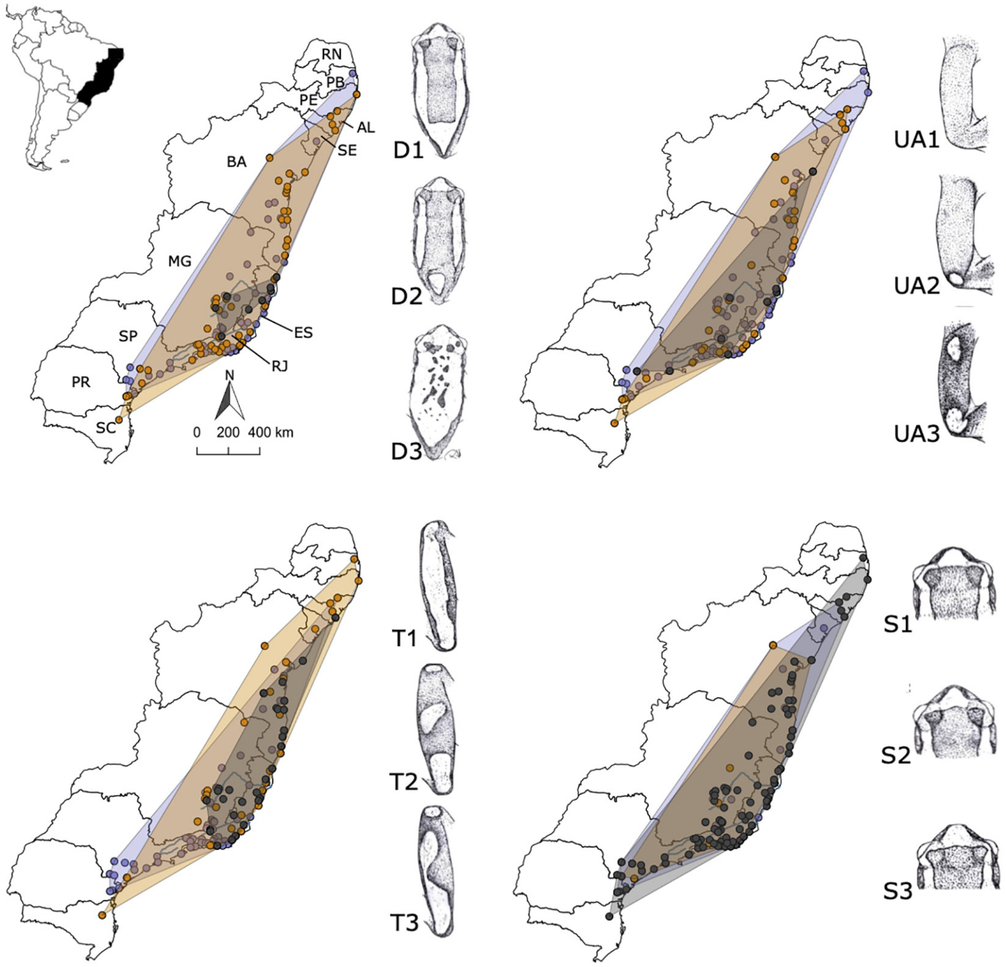

FIGURE 5 | Distribution maps and illustration of the dorsal coloration patterns found within sampled individuals of *Dendropsophus elegans*, with range shapes colored by genomic populations: south (purple: patterns D1, UA1, T1, and S1); north (orange: D2, UA2, T2, and S2); and contact zone (dark gray: D3, UA3, T3, and S3). Brazilian states: Rio Grande do Norte (RN), Paraíba (PB), Alagoas (AL), Sergipe (SE), Bahia (BA), Espírito Santo (ES), Minas Gerais (MG), Rio de Janeiro (RJ), São Paulo (SP), Paraná (PR), and Santa Catarina (SC).

this region as a contact zone between a north range species – *Rhinella crucifer* and a south range species – *Rhinella ornata*, resulting in the hybrid species – *Rhinella pombali*. The support for this congruence comes from the marked environmental differences accumulated between north and south of AF due to climatic and refugia dynamism (Carnaval et al., 2014), added to a complex topography, where past landscape changes driven by climatic fluctuations from old periods, not only Pleistocene, could have different effects on species diversification (Thomé et al., 2010, 2012, 2014; Leite et al., 2016). Even though *D. elegans* is a common species that occupies natural and human-impacted ecosystems, environmental differences along the AF and the cold temperatures during different periods could also have caused a reduction of gene flow between north and south populations, helping drive the genetic differences present in this study (see Paz et al., 2020).

We found a latitudinal variation on the species phenotypic pattern that contrasts with the genetic pattern. Individuals from the north and south extremes have more phenotypic similarities than individuals from the contact zone. Such result recognizes differences, even if not significant for most of the characteristics, in the frequency of species dorsal pattern (one of the diagnostic characters of the species group; **Figure 5**) related to geography, highlighting less phenotypic disparity between individuals from the north and south ranges than from the contact zone. In the north, individuals' dorsal pattern is mostly a continuous white line around the dorsal side, legs and arms. In contrast, individuals from the south have the same pattern but discontinued lines (**Figure 5**). Individuals in the contact zone show both dorsal patterns associated with north and south populations, in addition to the appearance of other phenotypes, the D3 pattern. As demonstrated by our results, these phenotypes are not associated with species differences, where the contact zone pattern is shared with the north and south clades. The increased diversity of *D. elegans* phenotypes in the contact zone could have resulted from the recent admixture events between north and south populations. This uncoupling between genetic and phenotypic variation can be caused by several evolutionary processes (Zamudio et al., 2016). For example, phylogeographic structure with little or non-detectable phenotypic variation or when variation patterns are not geographically cohesive, such as seem to be the case of *D. elegans*, it can be maintained through evolutionary processes such as retention of ancestral polymorphism, parallel adaptation to locally variable conditions or phenotypic plasticity (Zamudio et al., 2016). Understanding the adaptive value of the phenotypic variation along the AF environmental gradients is key to sorting out these processes, and our results can help target sampling to test them.

A hypothesis to explain why geographically distant populations of *D. elegans* would have less phenotypic disparity but higher genetic differences, is that since these populations are isolated by distance there are reduced opportunities for character displacement and reinforcement, and environment and species pressures in the ancestral range would be the same as in the derived range of genetic clusters (Pyron et al., 2015). Suppose past environmental conditions are similar and there is no selection on character displacement on the latitudinal extremes of *D. elegans* range due to isolation by distance. In that case, disjunct populations might tend to retain ancestral phenotypic conditions (Zamudio et al., 2016). In contrast, secondary contact with admixture between north and south populations along the contact zone would lead to higher rates of character displacement away from the phenotypes observed in north and south, resulting in higher phenotypic diversity (e.g., Barrera-Guzman et al., 2018). Thus, the unique existence of a dorsal color phenotype at the contact zone could indicate early stages of character displacement, but more in-depth phenotypic analyses are needed to confirm or refute this hypothesis.

Even though mitochondrial and genomic distance between north and south clades are high – 9.3% (Tonini et al., 2013) and $F_{ST} = 0.40$ (**Table 1**) indicating them as evolutionarily independent genomic lineages (see also Pirani et al., 2020), gene flow was recovered along genetic breaks (see section "Results"). Estimated divergence analysis demonstrated that different clades of *D. elegans* have not had enough time since isolation to accumulate phenotypic differences (see **Supplementary Table 4** in **Supplementary Material 2** and **Figure 5**), not confirming the taxonomic hypothesis of multiple species. For instance, the effort by Forti et al. (2017) analyzing acoustic traits of *D. elegans* for these three clades did not find consistent differences in advertisement calls among individuals correlated with the species phylogeography. Instead, males from distant sites were grouped together without any population congruence.

CONCLUSION

The phylogeographic history of *D. elegans* supports three main distinct clades separated into two geographically structured populations distributed at the AF northern and southern ranges, with a contact zone. Genomic breaks coincided with the main AF rivers (Ribeira de Iguape, Paraíba do Sul, Doce, and Paraguaçu), suggesting their strong role in the species diversification process even though allowing gene flow in certain periods. Moreover, based on the species' natural history and the AF palaeoenvironmental dynamic, we corroborate the role of forest refugia during the Pleistocene impacting population structure for this species by a recent range expansion in the north population after the LGM, allowing a secondary contact between north and south lineages (see **Table 1**). Our results also confirm the pattern of isolation by distance for *D. elegans*. Finally, the latitudinal variation on *D. elegans* phenotypic pattern contrasts with the genetic pattern, with north and south presenting more phenotypic similarities than the contact zone. Such a pattern can be explained by phenotypic plasticity of the evaluated traits and by the recent times of divergence, where there has been insufficient time to accumulate enough phenotypic differences to support the taxonomic hypothesis of multiple species.

DATA AVAILABILITY STATEMENT

Supplementary Material for the analyses performed here can be found online at the GitHub repository (https://github.com/renatapirani/Deep_genomic_divergence_and_phenotypic_admixture_of_the_treefrog_Dendropsophus_elegans). The raw data and accession number(s) can be found below: NCBI SRA BioProject, accession number PRJNA773141.

ETHICS STATEMENT

Ethical review and approval was not required for this study in accordance with the local legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

RP, FW, and JT designed the study. RP, FW, and LK contributed with funding and resources. MN and LE contributed with the phenotypic data. RP carried out the molecular lab work and data analysis, with help from AT and JT. RP, AT, FW, and JT drafted the manuscript. All authors reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.765977/full#supplementary-material

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Community-Based Conservation and Management of Chelonians in the Amazon

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Chelonians represent an important resource in the Amazon, either as a source of protein at the base of the food chain of aquatic and transition ecosystems, or in the dispersion of seeds of plants from floodplains and flooded forests. The consumption and predatory exploitation of their meat and eggs by local populations has been, and still is, one of the main threats to these animals. Community-based conservation projects allied to official protection programs have been restoring populations of chelonians of the genus *Podocnemis* throughout the Amazon since 1974. In this study, we analyzed the historical time series of protection data of *Podocnemis expansa*, *P. unifilis*, *P. sextuberculata* and *P. erythrocephala* in areas protected by the government and communities in the Amazonas state and northwest of Pará state. Between 1974 and 2019, 230,444 nests and 21,350,201 hatchlings of *P. expansa*, 170,076 nests and 3,229,821 hatchlings of *P. unifilis*, 647,715 nests and 6,410,092 hatchlings of *P. sextuberculata* and 24,617 nests and 168,856 hatchlings of *P. erythrocephala* were protected. Community protection schemes emerged in 1990, and covered 80.7% of the areas and produced 64.2% of *P. unifilis* hatchlings and 44.6% of *P. sextuberculata* hatchlings. The areas with the highest production of *P. expansa* remain under government protection (57.4%). Using the time series of production of nests and hatchlings per beach, logistic growth curves were estimated, and the values of r and K were compared between the two protection systems (government and community). Beaches controlled by the government showed higher support capacity in the production of nests (1,910.7 ± 1,035) and hatchlings (211,513 ± 93,031) of *P. expansa* and *P. sextuberculata* (81,160 ± 34,924 hatchlings). However, the communities were more efficient in protecting nests (r = 0.102 ± 0.2315) and hatchlings (r = 0.282 ± 0.166) of *P. unifilis*. Community-based protection and monitoring programs are an important component that should be incorporated by the government's environmental agencies for the management and conservation of turtles in the Amazon.

Keywords: freshwater turtles, participatory management, monitoring, population models, *Podocnemis*

INTRODUCTION

At approximately 240 million years old, chelonians are one of the oldest reptile groups. Of the 360 species currently recognized worldwide, 56–61% are threatened (Rhodin et al., 2018; Stanford et al., 2020). The Amazon is considered one of the regions with the greatest wealth of chelonians in the world, comprising 18 species, 11 of which are aquatic, 5 semi-aquatic and two terrestrial species (Rueda-Almonacid et al., 2007; Ferrara et al., 2017). However, this diversity may be much greater (Carvalho et al., 2016; Cunha et al., 2019; Vargas-Ramírez et al., 2020). Five species of Podocnemididae are found in the region: the giant South American river turtle, *Podocnemis expansa* (Schweigger, 1812); the yellow-spotted river turtle, *P. unifilis* (Troschel, 1848); six-tubercled river turtle, *P. sextuberculata* (Cornalia, 1849); red-headed river turtle, *P. erythrocephala* (Spix, 1824); and the big-headed turtle, *Peltocephalus dumerilianus* (Vogt, 2008).

Chelonians play an important role in the ecosystem as the basis of food chains in aquatic, transitional and terrestrial environments (Campos-Silva et al., 2018) and assist in seed dispersal, mineral cycling and carbon storage (Jerozolimski et al., 2009; Nascimento et al., 2015; Lovich et al., 2018). They are a food source for a wide diversity of predators ranging from invertebrates such as ants, fly larvae, northern mole cricket, to vertebrates, such as fish, alligators, birds, lizards and even jaguars (Salera et al., 2009; Andrade et al., 2016; Erickson and Baccaro, 2016). Beaches where a large number of turtle nests occur provide positive co-benefits for other aquatic and terrestrial animal species that are attracted there (Campos-Silva et al., 2018).

Amazon turtles synchronize their life cycle with the variations in the water level of the rivers and lakes (Alho and Pádua, 1982; Vogt, 2008). During the flood season of the Amazonian rivers, these flooded areas are used as places of shelter and for feeding (Garcez et al., 2012; IBAMA, 2019a). Chelonians have mostly fruit-based (17–30.8%) and seed-based (17–18.9%) diets (Fáchin-Téran and Vogt, 2014; Garcez et al., 2020; Oliveira et al., 2020) thereby acting as potential seed dispersers and play an important role in the dynamics of the regeneration of floodplains and flooded forests (Eisemberg et al., 2017).

The turtles have developed features such as a protective shell and a life history characterized by seasonal and mass egg laying, producing many hatchlings (r strategists), with delayed maturity, prolonged reproductive life and great longevity that has allowed for adaptation in many ecosystems. These same features have made them vulnerable to extinction in the face of threats caused by man (Stanford et al., 2020).

Chelonians have always been an important food resource for the riverine populations of the Amazon (Pezzuti et al., 2010; Andrade, 2017). The native Indians, especially those ethnicities that lived in the floodplain areas, exploited this resource by consuming the eggs and meat of turtles (Smith, 1979; Prestes-Carneiro, 2013; Meza and Ferreira, 2015), with evidence that some of these species had areas of their current distribution influenced by human translocations in pre-and post-Colombian periods (Guix, 2020).

With the arrival of the Portuguese colonizers in the sixteenth century, this exploitation became even more intense, with millions of turtles of the *Podocnemis* genus being slaughtered. Millions of eggs from these turtles were also used for the production of oil for public lighting, in the preparation of food and even mixed with pitch to caulk sailing vessels (Schneider et al., 2011; Fiori and Santos, 2013, 2015; Andrade, 2017). It was only in 1849 that the first prohibition appeared in the Amazon region, Brazil, to protect the nesting beaches in the Solimões, Amazonas and Negro Rivers, since these species, mainly *P. expansa*, had begun to disappear (Andrade, 2015). In 1967, the Brazilian Federal Government, through law N° 5,197/67 (the Fauna protection law), prohibited the capture and marketing of wildlife, thus making it illegal to exploit turtles—a practice that for centuries had been carried out by the peoples of the Amazon, and as a result caused an impact on the extractive economy of the region (Benchimol, 1999; Antunes et al., 2016, 2019).

The main threats to the chelonians in the Amazon are the rampant overexploitation of adults and eggs (Pantoja-Lima et al., 2014; Morcatty and Valsecchi, 2015; IBAMA, 2019a; Charity and Ferreira, 2020), the loss and degradation of aquatic and terrestrial habitats (feeding, dispersal, reproduction, nesting) due to deforestation, the expansion of urban areas (Conway-Gomez, 2007; Bowne et al., 2018) and the implementation of hydroelectric dams, highways, mining, and even uncontrolled tourism on nesting beaches (Fagundes et al., 2018; ICMBio, 2018; IBAMA, 2019a).

The commercial capture of chelonians is one of the factors that, even today, contributes most to the decline of aquatic and terrestrial turtle populations throughout the Amazon (Hernández and Espín, 2003; Fachín-Terán et al., 2004; Pezzuti et al., 2010; Schneider et al., 2011; Norris and Michalski, 2013; Penaloza et al., 2013; Pantoja-Lima et al., 2014; Harju et al., 2017; Morcatty et al., 2020). In general, these resources are exploited by local communities for subsistence consumption or sold to nearby cities or large regional centers such as the cities of Manaus, Santarém and Belém (Canto et al., 1999; Andrade, 2008; Nascimento, 2009).

It is estimated, based on the seizure data of the environmental agencies and the police, that between 1992 and 2011, 86,949 chelonians (13,289 *P. expansa*; 3,933 *P. unifilis*; 19,279 *P. sextuberculata*, 474 *P. erythrocephala*, 49,583 *Podocnemis* sp., 184 *Peltocephalus dumerilianus*, 195 *Chelonoidis* sp. and 9 *Chelus fimbriatus*) and 42,941 eggs were seized in the Amazonas state alone (Nascimento, 2009; Andrade, 2015), which represents from 52 to 57% of all animals seized (Canto et al., 1999; Nascimento, 2009). Between 2012 and 2019, 11,894 chelonians (29% *P. expansa*, 27% *P. unifilis*, 7% *P. sextuberculata*, 5% *P. erythrocephala*, 1% *P. dumerilianus* and 31% *Podocnemis* sp.) and 16,090 eggs were seized (Charity and Ferreira, 2020).

With an annual average of seizures of 4,347 chelonians/year and 2,147 eggs/year, between 1992 and 2011, and decreasing to 1,487 animals/year and 2,011 eggs/year, between 2012 and 2019, this may indicate a reduction of enforcement and control actions in the region, but could also be because of improved protection.

In 1979, the project "Chelonians of the Amazon" emerged, through which the government began to protect the breeding areas of *Podocnemididae* that still existed in the Amazon. In addition, in the 1990s, several conservation activities were developed by the Amazonian riverine communities, especially to organize fisheries agreements (Pinto and Pereira, 2004). This participation in the process of implementation and monitoring the management of aquatic resource has been called co-management, community management or participatory management (Berkes, 2009; Freitas et al., 2009; Campos-Silva et al., 2020).

In 1999, in the middle of this period of changes, the Federal University of Amazonas (UFAM) created, in partnership with IBAMA and riverines of Terra Santa, a community-based chelonian conservation program in the lower Amazon, called "Pé-de-pincha," has already returned 5,204,849 chelonian hatchlings back to nature (Andrade, 2017). Other actions of community conservation of chelonians have been recorded in the Brazilian Amazon (Miorando et al., 2013; Waldez et al., 2013) as well as in other countries of the Amazon Basin and Orinoco River (TCA, 1997; Soini, 1999; Towsend, 2008; Hernández et al., 2010; Harju et al., 2017).

Most of the protection programs for *P. expansa* and *P. unifilis* in the Amazon have sought to protect breeding females, nests and hatchlings, which seems to have contributed significantly to averting these species from the risk of extinction (Cantarelli et al., 2014; IBAMA, 2016; Pezzuti et al., 2018; Forero-Medina et al., 2019; Norris et al., 2019). However, turtles are long-living animals, and models that use demographic estimates of age, growth, fertility and survival are key to their management (Spencer, 2002; Zimmer-Shaffer et al., 2014). For *Podocnemis expansa*, there have been few studies aimed at estimating their population growth (Diniz and Santos, 1997; Norris et al., 2019; Rachmansah et al., 2020).

In order to adequately monitor conservation efforts, it is necessary to know if there is an increase in the populations of turtles through the work of protecting eggs and hatchlings in managed areas, both in areas protected by the State, and in areas protected by communities, and whether there are differences between these management systems. The systematization of the protection data of the nests and hatchlings of turtles in areas protected by the federal government or programs in community-based conservation in the state of Amazonas, would be one way. This, together with population attributes of the structure and the population dynamics of these stocks, will allow us to estimate population models for local turtles of the *Podocnemis* genus. Thus, we will able to evaluate the efficiency of these programs and predict the possible future impacts of these types of management systems on the conservation of these species.

The present study had the following objectives: (a) characterize and evaluate the different chelonian conservation and management systems for *Podocnemis expansa*; *P. unifilis*; *P. sextuberculata*; *P. erythrocephala* in the Amazonas state and in northwest Pará state, Brazil; (b) analyze the production data from the official federal environmental agency protection system of chelonian nesting beaches (1974–2019) and the conservation data from the community management system of chelonians (1999–2019) in these areas of the Amazon; (c) generate mathematical population models that simulate the evolution of the production of nests and hatchlings from the data of the management systems of chelonians analyzed; (d) estimate the intrinsic growth rate (r) and the support capacity (k) from the models generated.

MATERIALS AND METHODS

Area of Coverage and Geographical Location

This study was developed from the analysis of the historical data series (1974–2019) of conservation of chelonians of the Amazon Chelonian project (PQA) of the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) and the National Center for Research and Conservation of Reptiles and Amphibians (RAN) at the Chico Mendes Institute of biodiversity (ICMBio). Data was obtained from the locations that participate in the Pé-de-pincha Program of the Federal University of Amazonas (UFAM) in the physiogeographic zone of the middle-lower Amazon River and on the Trombetas, Nhamundá, Uaicurapá, Andirá, Marau, Uatumã, Madeira, Negro and Tefé Rivers, for the period from 1999 to 2019; and also in the areas of PROBUC (Biodiversity Monitoring Program and the Use of Natural Resources) on the Juruá and Purus Rivers, and from the Center for Preservation and Research of Aquatic Chelonians (CPPQA), on the Uatumã River, over the same period. The map of the sites used in the analyzes is presented in **Figure 1**.

Methodology

We analyzed 82 technical reports and field records of the areas protected by PQA—IBAMA in the Amazonas state, as well as the data provided by the Chelonian Integrated Data System (SISQUELONIOS) of the RAN—ICMBio, for the period from 1974 to 2019. In addition, we analyzed 138 beach protection permits that were granted in the period between 1964 and 2004 by the respective environmental agencies: Department of Hunting and Fishing of the Ministry of Agriculture (until 1966), 58% of the permits; Brazilian Institute of Forest Development (IBDF) from 1967 to 1989, 27%; IBAMA between 1989 and 2004, 14%; and permits from the respective municipalities, 1%.

The chelonian management systems adopted in this period in different areas and rivers were identified and

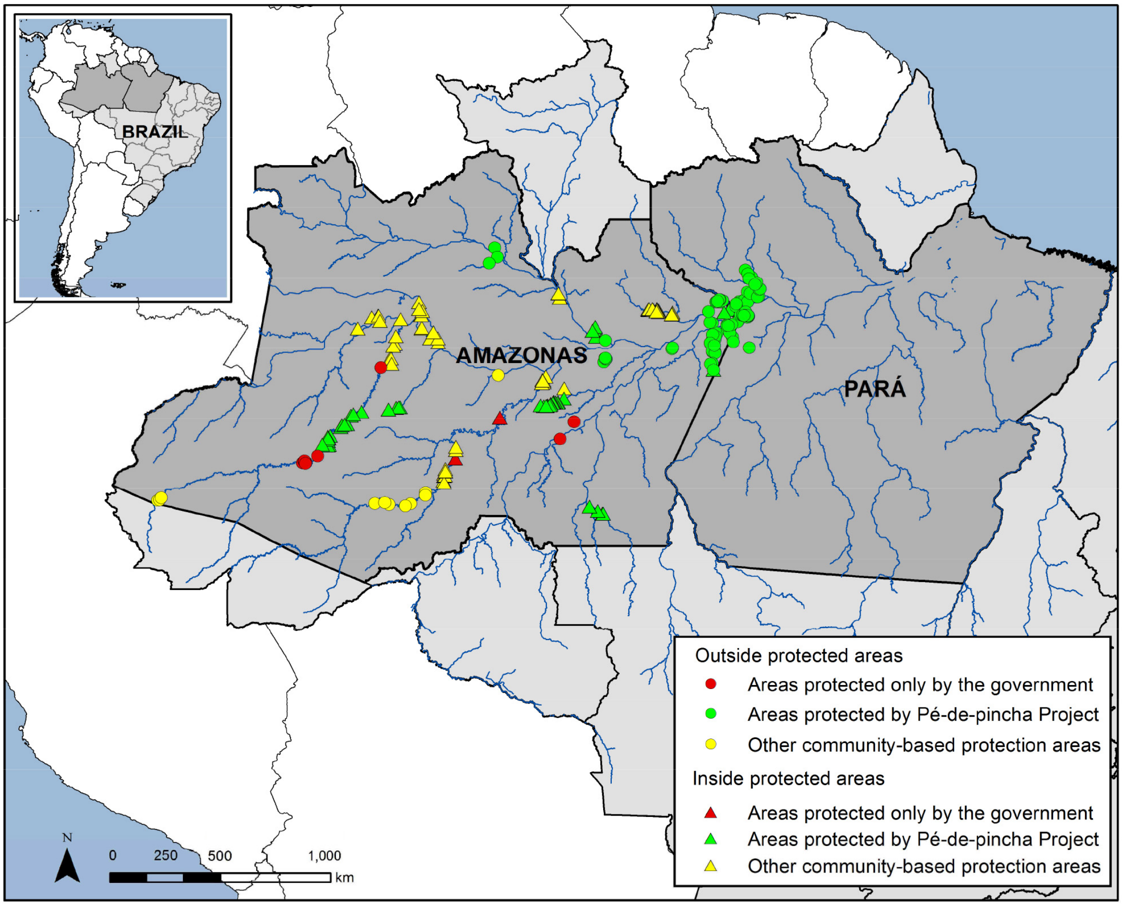

FIGURE 1 | Chelonian conservation areas in the Amazonas state and the northwestern section of the Pará state, Brazil.

characterized. The existing data on the number of nests, eggs and hatchlings of giant South American turtles (*Podocnemis expansa*), yellow-spotted river turtles (*P. unifilis*), six-tubercled river turtles (*P. sextuberculata*) and red-headed river turtles (*P. erythrocephala*) and the time and resources applied in the conservation work of the chelonian nesting beaches by the federal government were inserted in a database and tabulated in a spreadsheet (Excel 2013) and then analyzed.

Similarly, data on the number of nests, eggs and hatchlings of the studied *Podocnemis* species and the time and resources applied in the work of community conservation of chelonians through the Pé-de-pincha program and in the PROBUC, for the period from 1999 to 2019 were also tabulated and analyzed. These data were analyzed and used for the formulation and simulation of mathematical models.

Mathematical Modeling, Model Validation and Statistical Analysis

The data were tabulated and two sample groups were considered: (1) the production of chelonians in areas with exclusive protection by the government (federal or municipal), and (2) in areas subject to community management. The production data of nests and hatchlings by species on each beach were related to the length of protection time each area had received. After the Pearson's correlation analysis, regression analysis (linear, quadratic and polynomial) was performed, through which the existence of growth trends in the number of nests and hatchlings on each nesting beach was verified. These analyses were performed using the statistical programs MINITAB and STATISTICA v.7. The parameters of the regressions with the best fit were used in the population growth models: logistic and Gompertz (Barry, 1995; Gotelli, 2007). The curve estimates were made using the PAST 2.08 statistical program, and subsequently defined as a general model of analysis of the logistic curve:

- Logistic model:

$$Nt = \frac{K}{1 + [\frac{K-No}{No}] \times e^{-rt}}$$

where Nt = total number of nests/egg-laying females or hatchlings produced in time t; No = number of nests, egg-laying females or hatchlings produced in the first year of protection work; r = intrinsic population growth rate; E = Napier's logarithmic constant = 2.717; K = carrying capacity (Gotelli, 2007).

With the curve models established for each breeding site for each species, the coefficients r and K of the growth models of the number of nests and hatchlings were defined. K values were determined by the maximum population density. The values of r and K were obtained directly from the models estimated by the PAST program, or through the first derivation of the logistic curve of population growth. The support capacity, K, was also estimated by regression between N and r, with K being equal to the value at which the line cut the X axis. The value of r was estimated from K and the size (N) of the population (Krebs, 1986; Brower et al., 1989). When the rate of intrinsic growth (r) was greater than zero, it meant that the population was growing; if it was equal to zero, it meant that it had stabilized; and, if it was less than zero, it meant that it was declining (Krebs, 1986). For each site, species and population indicator (nests or hatchlings) analyzed, we estimated the values of the instantaneous growth rate (r), the carrying capacity (K) of the nesting site and the equation of the estimated logistic curve, as well as performing the AKAIKE adjustment for the model tested.

To validate the model, several simulations were performed with the estimated models, which generated annual data that were compared with the actual data obtained from the historical series (1974–2019) of production of the number of nests/egg-laying females and chelonian hatchlings produced in the areas under community management. The robustness of the models was also evaluated using data that were collected directly in the field by the authors (1999–2019).

To evaluate the efficiency of the existing chelonian protection systems in the Amazon, the nesting beaches were divided into three groups (areas in the Juruá River, areas in the Purus River and areas of the Pé-de-Pincha program, in the middle-lower Amazon river) and two treatments or protection systems (areas of exclusive management by the government and areas of community management). Each protected beach was considered an observation, and each year was considered a repetition. The variables analyzed were the instantaneous growth rate r and the carrying capacity K of the number of nests/egg-laying females and the total number of hatchlings released on each beach.

A two-way analysis of variance was applied with factor 1 being the river or physiographic zone and factor 2 being the system of management (Sokal and Rohlf, 1990; Ferreira, 1991) in order to compare the means of the rates of growth r and carrying capacity K in the production of the nest and hatchlings of the systems of conservation considered (government and community) for the three different species of turtles studied (*P. expansa*, *P. unifilis*, and *P. sextuberculata*), whereafter a *post hoc* Tukey test was applied (Sokal and Rohlf, 1990; Zar, 1999). Statistical analyses were performed using the MINITAB program.

RESULTS

Analysis of Historical Data Series on the Conservation of Turtles in the Amazon

A total of 2,318 records of annual production of chelonians, relating to 109 areas and 244 nesting beaches (207 in the Amazonas state and 37 in the northwest of Pará state) in 16 river channels of the western Amazon were analyzed in the survey of the historical data series (45 years of information). Of these areas, 52.9% are located in federal or state conservation units (CU), and 47.1% are outside of CUs. The Pé-de-pincha program was implemented in 76% of these areas (184 beaches), 56% of them were outside CUs (**Figure 1**).

Between 1974 and 2019, 230,444 *Podocnemis expansa* nests, 170,076 *P. unifilis* nests, 647,715 *P. sextuberculata* nests and 24,617 *P. erythrocephala* nests were protected, which produced 21,350,201 *P. expansa* hatchlings, 3,229,821 *P. unifilis* hatchlings, 6,410,092 *P. sextuberculata* hatchlings and 168,856 *P. erythrocephala* hatchlings (**Supplementary Table 1** presents all the annual protection data of the chelonian nests and hatchlings from the 1974–2019 historical series; and **Supplementary Table 2** shows a summary of the total number of nests, eggs and hatchlings protected, as well as the recorded number of defective hatchlings, stillbirths, unviable eggs, predated nests, nests damaged by rainfall or erosion and the seizures recorded in the Amazon state).

The increase in the number of protected *Podocnemis expansa* nests and hatchlings was related to the increase in number of protected areas (**Figure 2** and **Supplementary Figure B**).

From analysis only of the beach protection permits, it observed that were sent to 57 chelonian nesting beaches (locally known as “tabuleiros”) mainly in the Purus (36), Juruá (6) and Solimões (14) Rivers. Most of the permits were granted to the beaches managed by rubber plantation owners or their surviving heirs, mainly in the Purus (101). Before 1967, when the wildlife trade in Brazil was banned, the owners of beaches had an interest in this type of permit (34.5/year). Some of these authorized beach owners sold chelonians and their eggs to cover the expenses and earn income. After the ban, this number fell to 6.8/year.

For *P. expansa*, more consolidated information was available than for the other species, since this species is the main target of conservation efforts of environmental agencies. Not all reports presented data on nests and hatchlings of *P. unifilis* and *P. sextuberculata*, however, the data on *P. unifilis*, when recorded, were based on the marking of nests and counting of hatchlings, whereas the data on *P. sextuberculata* were based, for the most part, only on estimating the number of nests and hatchlings (**Figure 3** and **Supplementary Figure C**).

Data on *Podocnemis erythrocephala* only began to be systematically recorded from 1995 in communities in the Negro River and by the Pé-de-pincha program in the Nhamundá, Jamari, Andirá, Uaicurapá, Mamuru, Marau, Matupiri Rivers and Juruti Lake, and by CPPQA, on the Uatumã River (**Supplementary Figure D**). These groups protected 1,116 ± 942 nests and produced 7,675 ± 8,648 hatchlings per year.

With regard to the rivers where the protected beaches were located, initially, efforts were concentrated on the Purus, Juruá and Solimões Rivers. From 1999 onward, with the increase in the areas of community management, there was a diversification in the environments where the turtles were protected, and the expansion in the number of rivers covered, these being the middle-low Amazonas, Andirá, Sapucuá-Trombetas, Nhamundá, Negro, Madeira, Uatumã, Marau and Tefé Rivers.

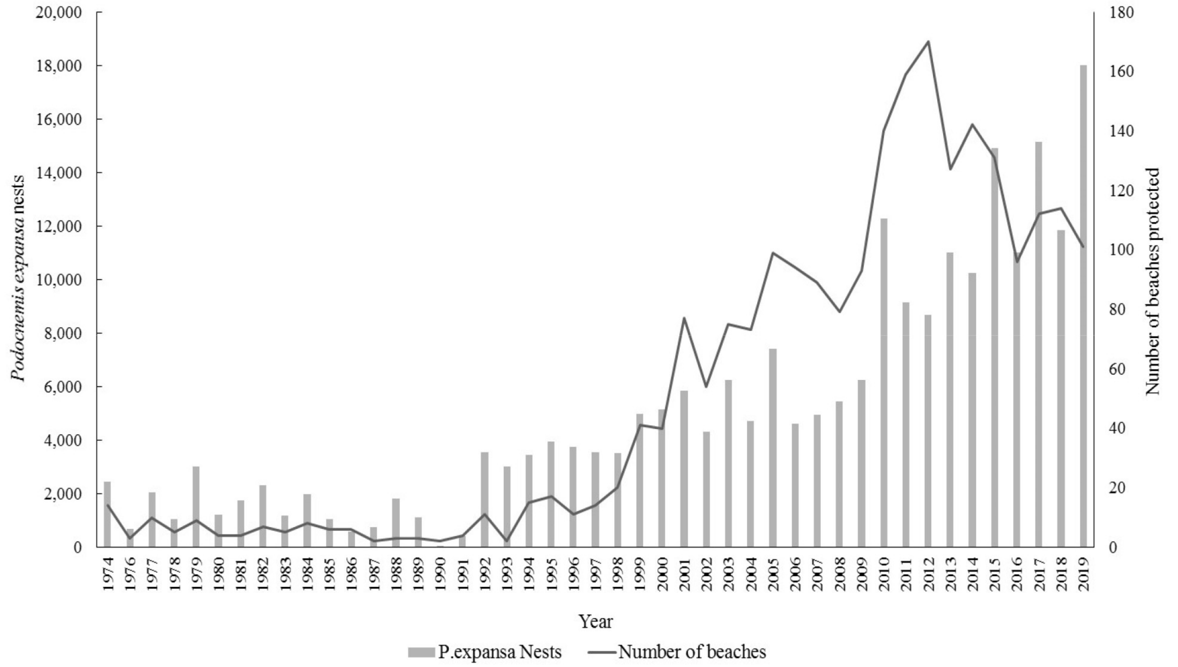

FIGURE 2 | Nests of Giant South American turtle (*P. expansa*) protected in the Amazonas state and the northwestern section of the Pará state, Brazil, between 1974 and 2019. Source: PQA/IBDF/IBAMA CPPQA and Pé-de-pincha program technical reports.

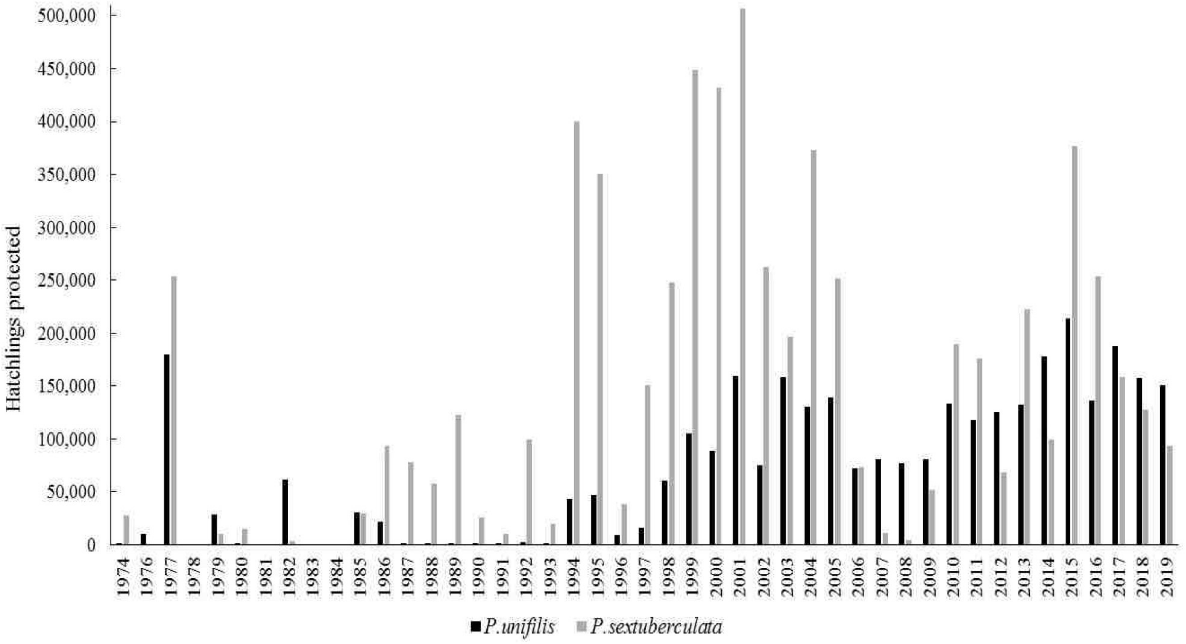

FIGURE 3 | Yellow-spotted river turtle (*P. unifilis*) and six-tubercled river turtle (*P. sextuberculata*) hatchlings protected between 1974 and 2019 in the Amazonas state and the northwestern section of the Pará state, Brazil. Source: PQA/IBDF/IBAMA CPPQA and Pé-de-pincha program technical reports.

The Purus River was responsible for 62.8% of the nests and 56.8% of the hatchlings of *P. expansa* protected over the 45 years in the Amazon, followed by the Juruá River (34.6% of the nests and 41.3% of the hatchlings). Across all other rivers, the production of nests and hatchlings reached 2.7 and 1.9% of the total, respectively (**Supplementary Figure E**).

Podocnemis unifilis hatchling production was 40.2% on the Juruá River, followed by the Purus River with 14.4% and the community protection areas of the Pé-de-pincha program for the middle-low Amazon River (14.2%), Sapucuá/Trombetas Lake (14.2%) and Andirá River (9.0%) (**Figure 4**).

For *Podocnemis sextuberculata*, 64.5% of the hatchlings were recorded in the Juruá River, 30.5% in the Purus, 1.6% in the middle Amazonas River and the rest in the other areas. In the case of this species, due to the large number of nests in the Purus and Juruá rivers, only estimates were performed. Only in community-based protection areas with few nests was it possible to check all nests and hatchlings.

In the Amazonas state, the following types of protection for the chelonian nesting beaches were identified:

(1) Owner of the beach or rubber plantation owner: The owner of the beach or "beach captain" (as he was locally known) that protected the nesting area by marking the beach with flags and preventing other people from removing eggs and adults from the area, and who had official authorization to do so. This system accounted for 100% of the protected areas from 1964 to 1976, when the federal government began to protect and monitor these areas. It ended in 2005. This system did not involve public funding and the protection work was carried out at the expense of the owner of the area (private initiative), but labor was provided by the local residents. Therefore, this work can be considered as a mixed form of participatory resource management and a precursor of community-based protection. It corresponded to 4% of all the analyzed protected areas.

(2) Government: These areas were considered as nesting areas protected by the federal government, and these involved protection carried out only by employees of the federal environmental agencies or by persons hired by them, without the participation of communities, and using federal resources. This system was used in the areas of large production of turtles in the Amazonas state (Purus and Juruá Rivers). It has accounted for 9.6% of all protected areas in the last 45 years.

(3) Community-based: The community-based chelonian protection system involved the communities of each region in all phases, from the perception and decision to protect these animals, the organization and labor for the execution of protection services and monitoring of the beach, recording of data (nests and hatchlings) and the holding of release events. The resources for these actions were obtained from the communities themselves or from partner institutions that supported these initiatives. This system may also involve the participation of environmental agencies providing logistical support, but the actions of protection are eminently community-based, and it is considered participatory management. Between 1974 and 2019, this system corresponded to 80.7% of the total protected areas.

(4) Municipal Authorities: In some municipalities, due to the absence of environmental agencies, the municipal authorities assumed the role of conservation of local turtles. Usually, they defined a municipal reserve area and made resources (financial, human and logistical) available for the execution of the monitoring and control of the nesting areas, but without the participation of the communities at any stage. This protection system corresponded to 2.9% of all evaluated protected areas.

(5) Specialized Centre: One specific case of protection by a specialized center was recorded. The Center for Preservation and Research of Aquatic Chelonians (CPPQA) belongs to a state company (Eletronorte/Eletrobrás) and began its chelonian conservation actions of as a form of environmental compensation, and then expanded them to the communities of the Uatumã River. The CPPQA worked in 2.8% of the areas analyzed from 1994 to 2019.

If we analyzed the number of beaches protected by the different systems of conservation of chelonians in the Amazonas state in the last four decades (**Figure 5**), it can be observed that from the 1990s there was an expansion of community-based protection systems. Which, in addition to replacing the system of the owner or captain of the beach, allowed for the exponential increase of the chelonian protection areas.

When analyzing the production of hatchlings in each protection system, it was found that although the beaches protected by the federal government correspond to only 10%, they account for the production of 13,732,700 hatchlings of *P. expansa*, or rather, 57.4% of the total protected turtles. The protected areas of the municipalities come in second place, with 23% of the total hatchlings produced. The community system accounted for 13.9% of protected turtle hatchlings (**Figure 6**). The increase in community-based protection of hatchlings of *P. expansa* between 1999 and 2019 was mainly due to the increase in the number of beaches involved in the Pé-de-pincha/UFAM program, which currently provides 93.9% of community protection of *P. expansa*.

When analyzing the production of *Podocnemis unifilis* hatchlings, it was found that the community conservation system is responsible for the protection of 2,112,070 hatchlings, or rather, 64.2% of the total production. Beaches protected by the federal government only come in second place with 19.4% of total production. Beach owners produced 6.3% and municipalities 7.6%.

The increase in the participation of the community-based conservation system from 1999 onward in the production of *P. unifilis* hatchlings, was mainly due to the increase in the areas of the Pé-de-pincha program, which has this species as its emblem. Initially, the program accounted for 15.9% of the community conservation of *P. unifilis* and, by 2019, it had reached 96.2% of the community production of hatchlings of this species.

For the production of hatchlings of *P. sextuberculata*, it was found that 44.6% of hatchlings were produced in areas of community management, 25% on beaches protected by federal environmental agencies, 20.1% by municipal authorities and 10.2% by beach owners (**Supplementary Figure F**). For *P. erythrocephala*, the first records of protection of the species only began in communities on the Negro River in 1995 and,

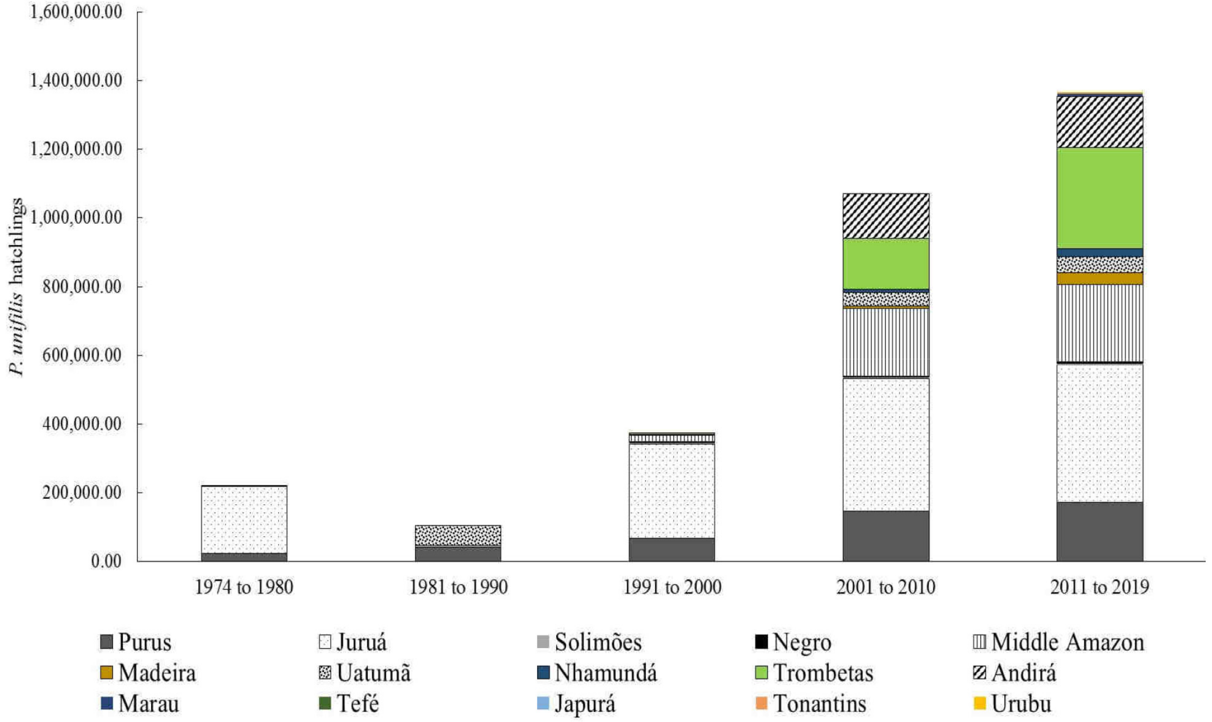

FIGURE 4 | Number of *P. unifilis* hatchlings protected between 1974 and 2019 for different rivers in the Amazonas state and the northwestern section of the Pará state, Brazil.

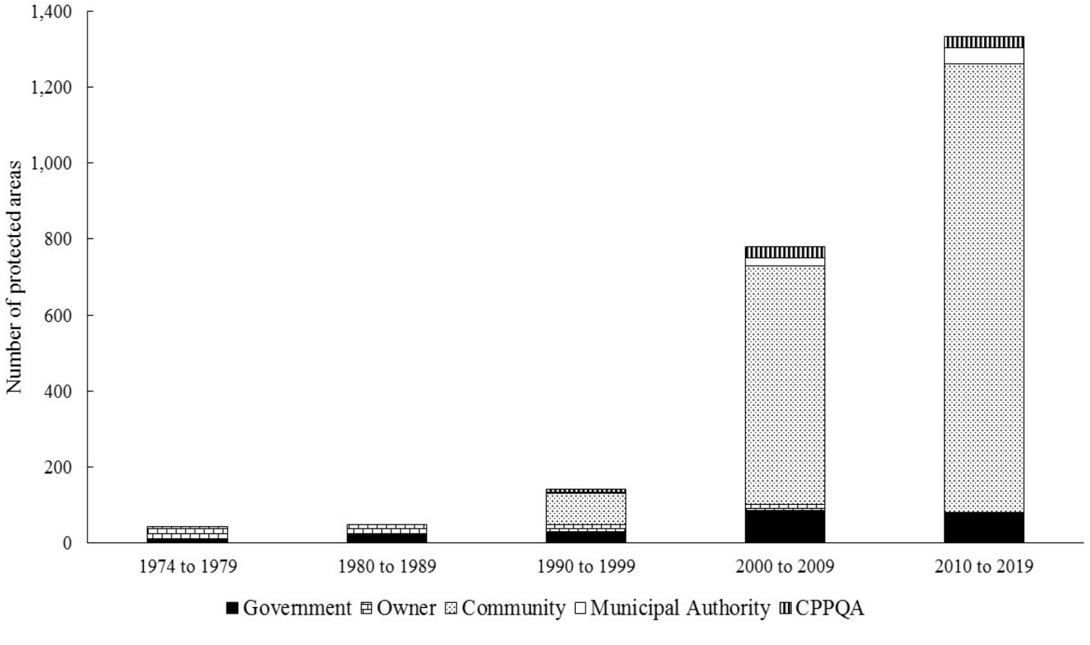

FIGURE 5 | Number of chelonian nesting beaches protected by different management systems in the Amazonas state and the northwestern section of the Pará state, Brazil, between 1974 and 2019.

from 1999, expanded to the Pé-de-pincha program areas. Of the hatchlings of *P. erythrocephala* protected, 58.8% were protected through the community conservation system, 40.3% by the government and 0.9% by the CPPQA. If we consider only the areas of community protection, the Pé-de-pincha program conserved 98.4% of the production of hatchlings of this species.

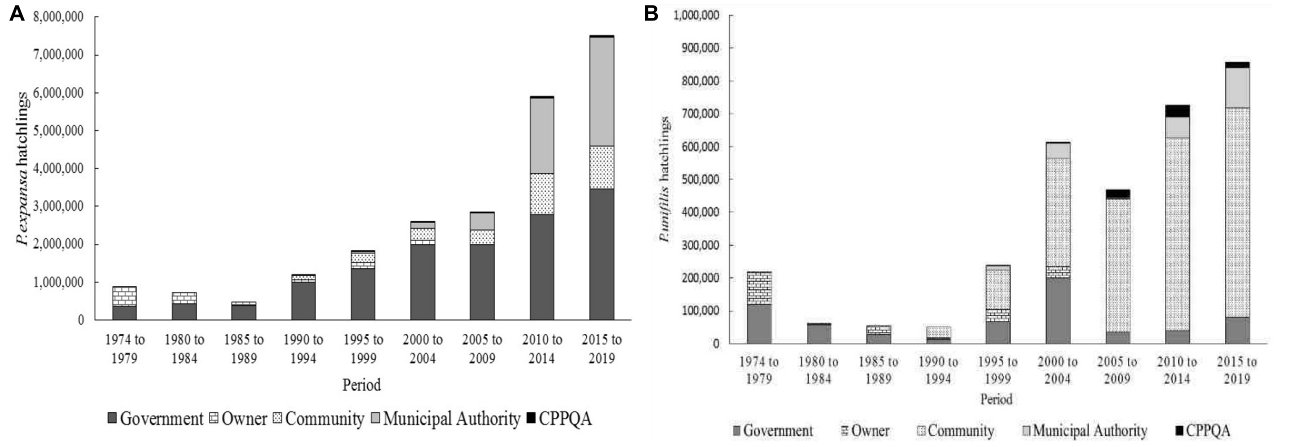

FIGURE 6 | Participation of the different systems of protecting the production of chelonian hatchlings in the Amazonas state and the northwestern section of the Pará state, Brazil, between 1974 and 2019: **(A)** *P. expansa*; **(B)** *P. unifilis*.

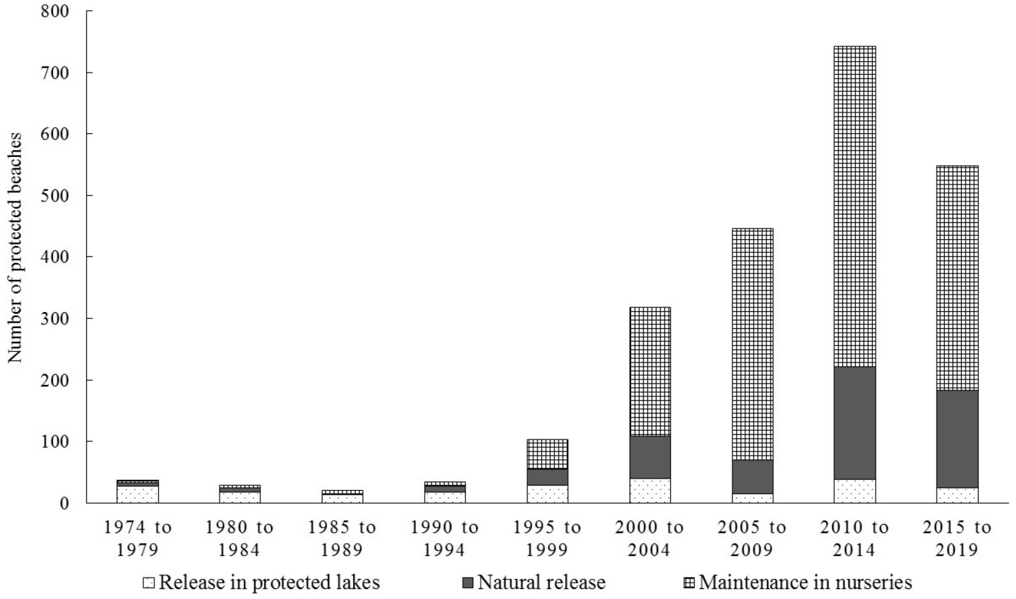

FIGURE 7 | Number of protected beaches with different types of hatchling management: natural release; transport of hatchlings to lakes protected; and maintenance in nurseries or headstarting, for the period from 1976 to 2019, in the Amazonas state and the northwestern section of the Pará state, Brazil.

The different ways of managing the hatchlings of the protected nests was also evaluated (**Figure 7**). We categorized these according to the level of human intervention in the natural process of hatching and birth of offspring. Several levels of intervention were found then, the management of hatchlings was classified into three levels of intervention:

(1) Natural: We considered natural management of hatchlings to be when there was only the counting of nests, and the hatchlings emerged from the nests and proceeded to the river without human intervention. Or in places where it was impossible count the nests and only the hatchlings were counted. We also included places where the nests were opened manually and the hatchlings counted and released immediately on the shores of the beach.

(2) Transport of hatchlings to lakes protected from natural predators: This system was widely used by beach owners, and was performed in almost 100% of the areas until 1976. It consisted of the manual removal of the hatchlings (especially *P. expansa*) from the marked nests. They were then transported to lakes, away from the beach and away from the main aquatic predators of the hatchlings (fish and caimans). These lakes usually had enough aquatic macrophytes in which the hatchlings could shelter, as well as being

places where adult turtles would go during the flood season of the river.

(3) Maintenance in nurseries or headstarting: this classification comprises the places that maintained the hatchlings in captivity for different periods before release (from 7 to 90 days providing supplementary feeding) and using different types of nursery tanks (fenced natural lakes, wooden cages, net-tanks, water tanks, etc.).

Of the *P. expansa* hatchlings protected, 51.4% were released immediately; 24.1% were transported to protected lakes and 24.4% were kept in a nursery for a period before being released. Among the protected *P. unifilis* hatchlings, 28.1% were released naturally, 10.9% were transported to lakes and 61% were kept in nurseries before release. In the case of *P. sextuberculata* hatchlings, 39.5% naturally emerged from their nests, 37.5% were transported to the lakes and 23.1% were kept in a nursery before being released. For hatchlings of *P. erythrocephala*, 36.4% were released naturally and 63.6% were kept in a nursery.

It was estimated that, in the 45 years analyzed, US$2,340,410 were directly invested in the protection of turtles in the Amazonas state and northwest of Pará state by IBAMA and the Pé-de-pincha program (equivalent to US$8,000 to US$52,000/year). The Pé-de-pincha program accounted for 87% of these expenditures between 2010 and 2015. The average cost per protected hatchling was estimated at US$ 0.25 ± 0.2. In government protected areas, the average cost was US$0.13 ± 0.12 per hatchling, and in community-based conservation areas, this cost was US$0.33 ± 0.19 per hatchling. Even though community-based protection was almost all voluntary, the costs per hatchling were slightly higher due to the need for training and the meeting of some socio-economic demands of the communities, such as donations of basic food supplies.

Turtle Population Growth Models in the Amazon

The initial analysis of the data was performed by constructing column graphs of the time series and evaluating the trend lines of the graphs for each species and beach analyzed (**Figure 8**). Only sites with a historical series of more than 10 years of data were analyzed.

It was analyzed the time series of the annual production of nests and hatchlings of *P. unifilis* and *P. sextuberculata* in different nesting sites (**Figures 9A–F**).

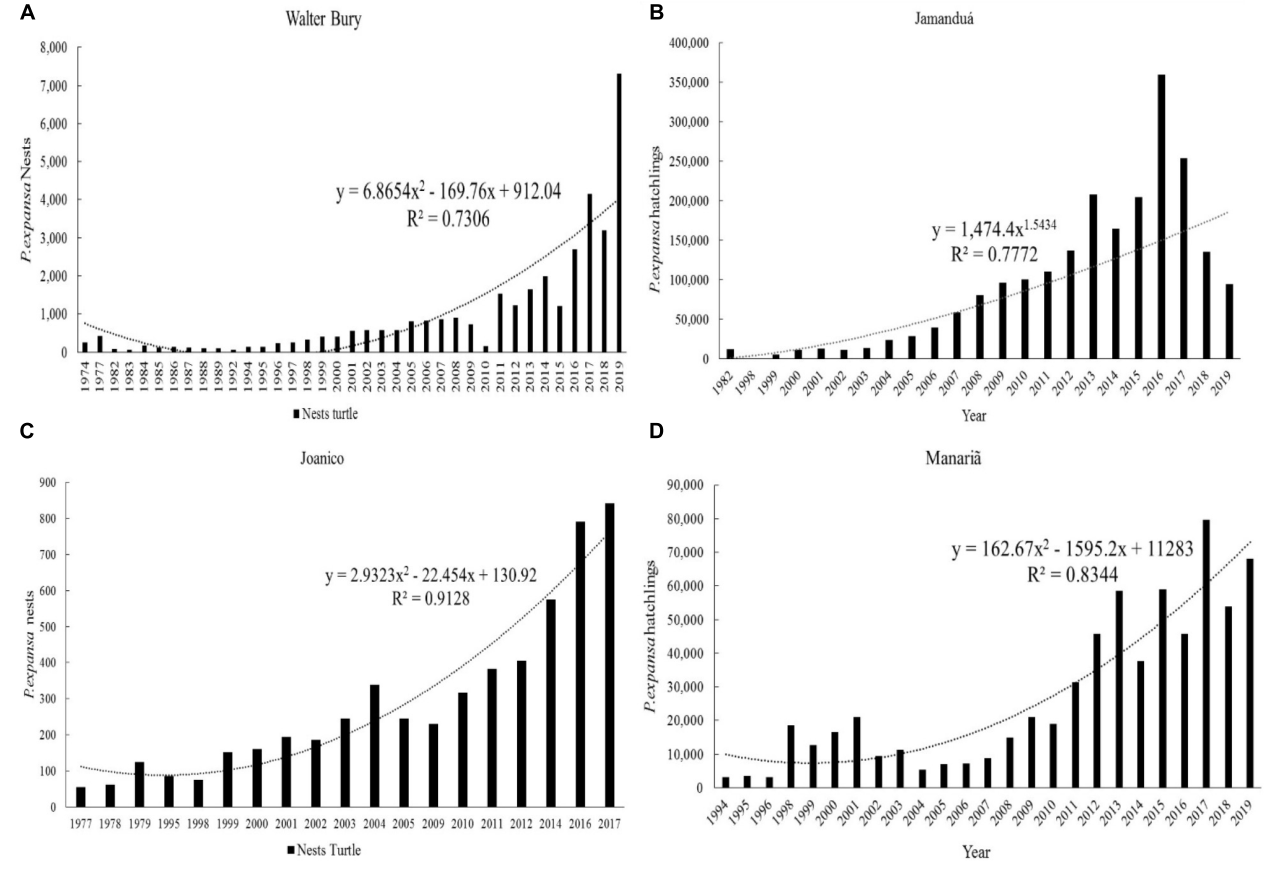

FIGURE 8 | Time series of the production of *P. expansa* nests and hatchlings at nesting sites of the Juruá and Purus Rivers: **(A)** Nests—Walter Bury; **(B)** Hatchlings—Jamanduá; **(C)** Nests—Joanico; **(D)** Hatchlings—Manariã.

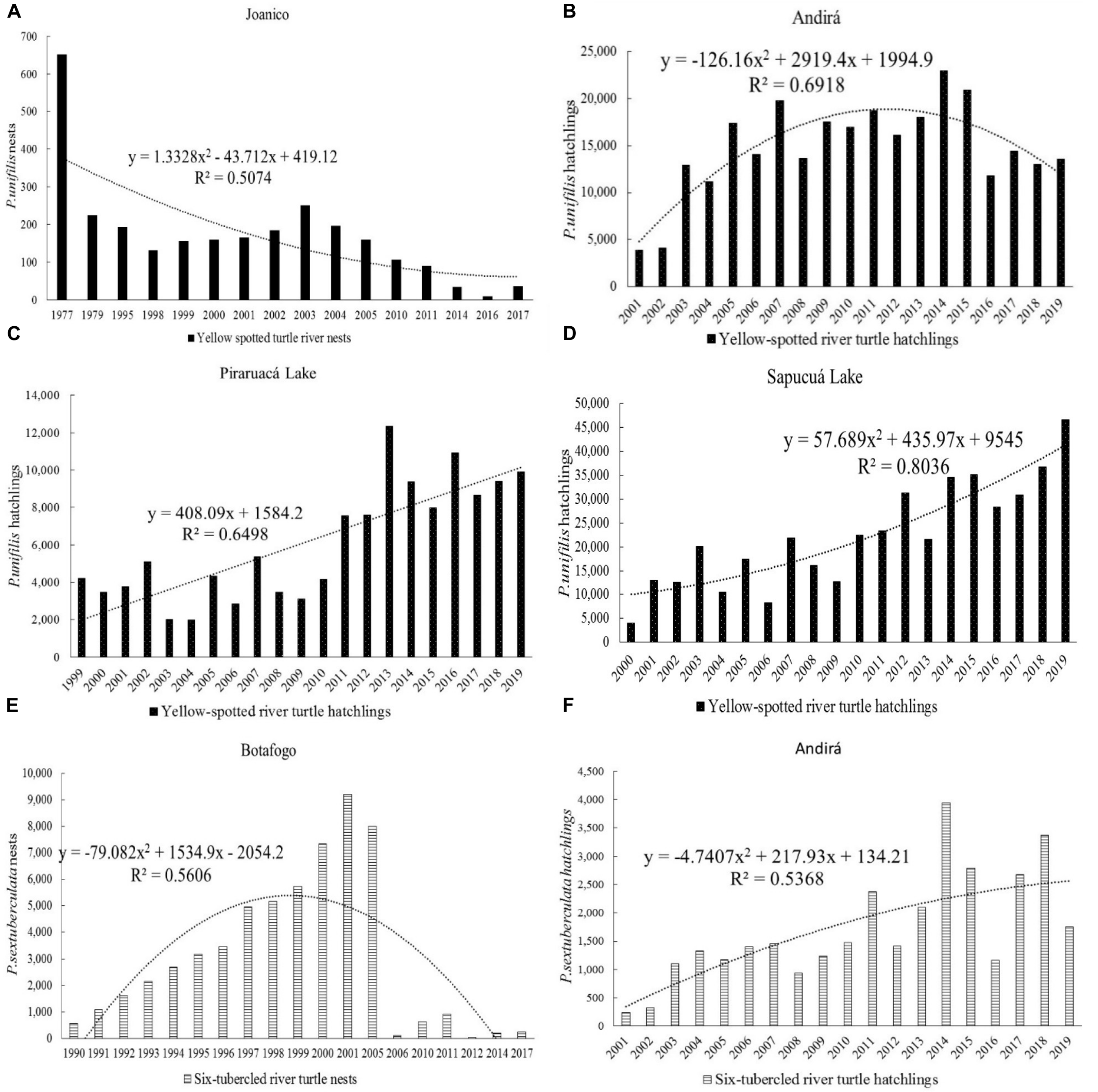

FIGURE 9 | Time series for the production of *P. unifilis* and *P. sextuberculata* nests and hatchlings at different nesting sites: *P. unifilis*: **(A)** Nests in the Joanico, Juruá River; **(B)** Hatchlings in the Andirá River; **(C)** Hatchlings in the Piraruacá Lake; **(D)** Hatchlings in the Sapucuá Lake; *P. sextuberculata*: **(E)** Hatchlings in the Botafogo, Juruá River; **(F)** Hatchlings in the Andirá River.

After the analysis of the time series of the production of nests and hatchlings of the *Podocnemis* species studied for each beach, only those with R^2 above 50% were selected to fit the logistic model. A total of 28 areas of the Juruá, Purus, middle-lower Amazon, Andirá, Nhamundá, Sapucuá/Trombetas and Negro Rivers were selected.

For each selected area, attempts were made to adjust the logistic growth to the curve model. For each area or nesting site analyzed, a curve relative to each species was generated (**Figures 10A–D**).

In total, 160 logistic growth curves for number of nests/reproductive females and hatchlings were estimated and adjusted for the 28 breeding areas analyzed; 48 for *P. expansa*, 50 for *P. unifilis*, 50 for *P. sextuberculata* and 12 for *P. erythrocephala*.

There was a significant difference ($F = 6.35$, gl = 22, $p = 0.02$) between the growth rate r of the number of hatchlings of *P. expansa* from government protected areas ($r = 0.172 \pm 0.055$) and those from communities ($r = 0.085 \pm 0.197$), and also between the growth rate r of the number of nests/reproductive females ($F = 4.97$, gl = 22, $p = 0.038$)—**Table 1**. There was also a significant difference ($F = 30.49$, gl = 22, $P < 0.0001$) between the carrying capacity of the growth curves of the number of *P. expansa* nests and hatchlings in government protected areas ($K_{nests} = 1{,}910.7 \pm 1{,}035$ nests; $K_{hatchlings} = 211{,}513 \pm 93{,}031$

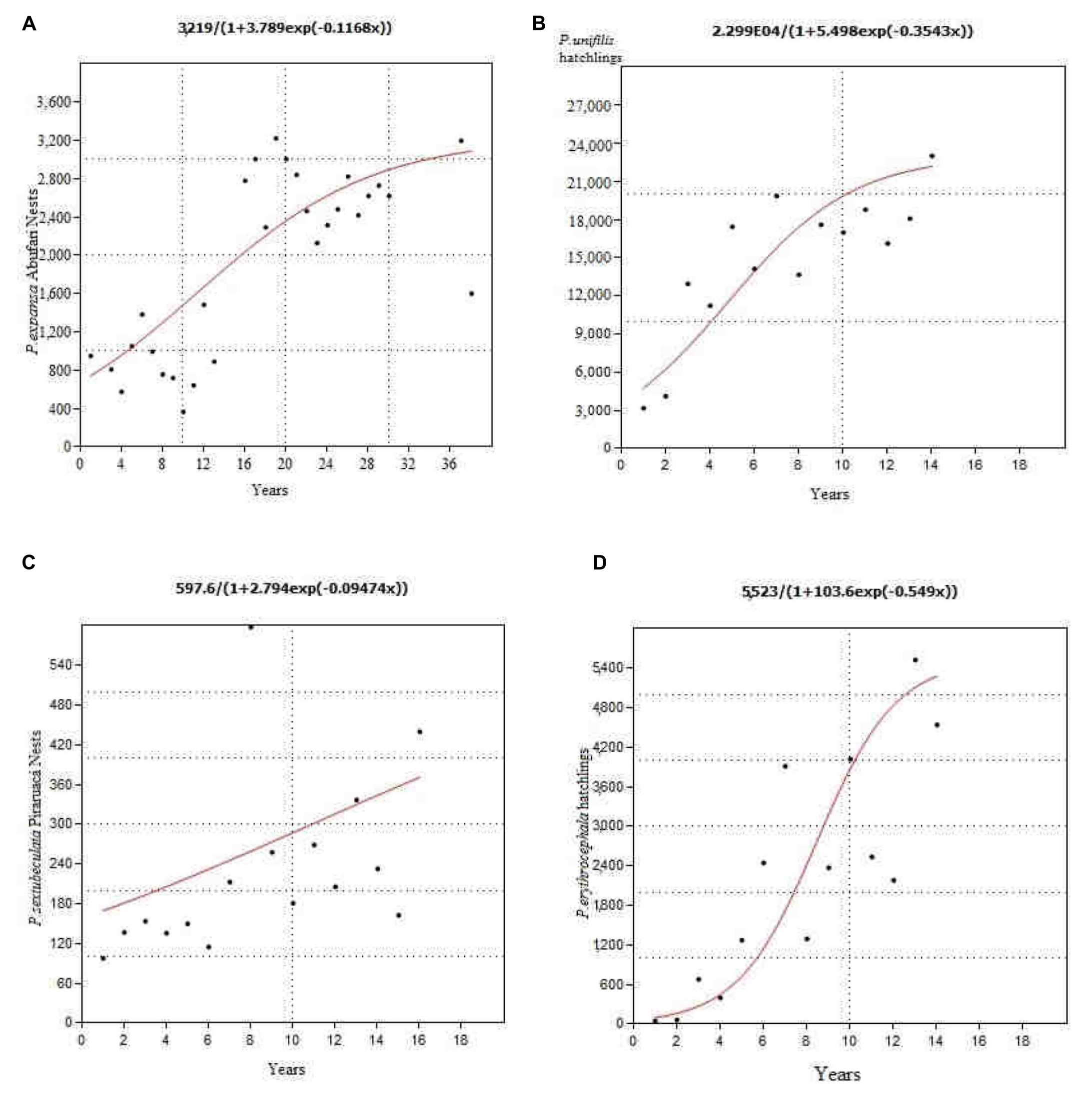

FIGURE 10 | Logistic growth curves of the number of nests and hatchlings **(A)** nests of *P. expansa*, Abufari, Purus River; **(B)** hatchlings of *P. unifilis*, Andirá River; **(C)** nests of *P. sextuberculata*, Piraruacá Lake, middle Amazon; **(D)** hatchlings of *P. erythrocephala*, Andirá River.

offspring), which was higher than those protected by the communities (K_{nests} = 268.3 ± 303.5 nests; $K_{hatchlings}$ = 29,789 ± 35,716 hatchlings).

The first two regions (Purus and Juruá) were older areas of protection of chelonians (30–45 years), which still had large populations of *P. expansa*, *P. unifilis* and *P. sextuberculata*. The last region covered the areas of action of the Pé-de-pincha community management program of chelonians, in the physiographic zone of the middle-lower Amazon, with an accumulated 20 years of protection.

It was found that the effect of rivers/areas was actually affecting the initial analysis. There is a significant difference between growth rates r in the number of hatchlings of *P. expansa* for river/nesting areas ($F = 9.41$, gl = 22, $P = 0.001$).

It was observed that, although there was a significant effect ($P < 0.04$ and $P < 0.02$) by the ANOVA, there is no significant difference between the means of nesting areas protected by the government and the communities for the instantaneous growth rate r of the number of nests and hatchlings of *P. expansa* when verified by the Tukey test ($P < 0.05$).

There was a significant difference ($P < 0.001$) between the rivers/protection areas for the r rate of the number of hatchlings of *P. expansa*. The rates (r) of the *P. expansa* hatchlings of the middle-lower Amazon/Pé-de-pincha project were higher ($r = 0.319 \pm 0.089$) than those of the Purus River ($r = 0.008 \pm 0.154$) and did not differ statistically from those of the Juruá River ($r = 0.08 \pm 0.153$) when verified by the Tukey test ($P < 0.05$).

When the analysis on the K carrying capacity of *P. expansa* nests and hatchlings was performed, it was observed that there was no difference between the different areas. There was only a difference between the support capacity of the beaches administered by the government, with much higher averages (Tukey, $P < 0.0001$) of nests (1,910 ± 1,035) and hatchlings (211,513 ± 93,031) than the beaches of the communities. This is related to the fact that the federal government has chosen the areas with the largest populations and prioritized the protection of *P. expansa*, in addition to having greater power of supervision and control than the community has to protect these large stocks of *P. expansa* from being targeted by the traffickers of turtles.

For *P. unifilis*, there was no significant difference in the carrying capacity (K) of nests and hatchlings in the reproductive sites analyzed, neither by the different protection systems ($F = 3.18$, gl = 25, $p = 0.089$), nor between different areas ($F = 1.12$, gl = 25, $p = 0.344$)—**Table 2**. We found that the stocks of reproductive females nesting in sites protected by communities are similar to the average amount managed by government agencies. Acting in a greater number and diversity of areas (80.7%), the community system of protection of chelonians is responsible for most of the production of hatchlings of *P. unifilis* (64.2%), unlike *P. expansa*, where most of the production is in areas protected by the government.

Significant differences for the rates of growth (r) of the number of nests and hatchlings ($F = 11.52$, gl = 25, $P < 0.0001$) were found between the rivers/areas evaluated. The areas of the middle-low Amazon where the Pé-de-pincha project operates showed growth rates of *P. unifilis* nests that were higher (0.33 ± 0.19) than the areas of the Juruá (−0.004 ± 0.12) and Purus (−0.044 ± 0.144) Rivers.

The negative r values found for most beaches of the Juruá and Purus Rivers (above 60%) could simply mean that the data for this species were underestimated by the lower number of records by beach monitors who would be favoring records of nests of *P. expansa*. However, when analyzing the historical production series of these areas, it was also found that, as the number of *P. expansa* females nesting on a beach increased, the number of nests of *P. unifilis* decreased in that area (**Figure 11**).

Some of these beaches have been systematically monitored by the authors for more than 20 years, ensuring the authenticity and the same effort to record data for both *P. unifilis* and *P. expansa*, and, as such, this negates the possibility of underreporting of data for *P. unifilis.* This phenomenon had already been reported by the beach monitors of the Juruá and Purus Rivers, and was confirmed with the field data.

In the nesting areas analyzed, the number of *P. expansa* females laying eggs exceeds that of *P. uniflis* by around 13.7 ± 5.2 years of beach protection.

For *P. sextuberculata*, no significant differences were found between growth rates (r) of the number of hatchlings ($F = 2.43$, gl = 27, $p = 0.132$)—**Table 3**. However, there was an effect ($F = 10.9$, gl = 27, $P = 0.004$) of the protection system on the growth rate (r) of the *P. sextuberculata* nests, which was higher in the areas protected by the government ($r = 0.219 \pm 0.228$). There was also an effect ($F = 4.09$, gl = 27, $P < 0.03$) of the protection system observed on the carrying capacity (K) of production of hatchlings, which was higher in the areas protected by the government ($K = 81{,}160 \pm 34{,}924$ hatchlings), although there was no difference in the capacity of nests between beaches protected by the government and the communities.

For carrying capacity (K) of nests and hatchlings of *P. sextuberculata,* there were differences between the rivers/areas analyzed ($P < 0.04$ and $P < 0.01$, respectively), and the beaches

TABLE 1 | Summary of the ANOVA of instantaneous growth rates r and carrying capacity K for number of nests/reproductive females and hatchlings of *Podocnemis expansa*.

Index	r hatchlings**	K hatchlings**	r nests	K nests**
River/area	($P < 0.001$)*	($P < 0.43$)	($P < 0.63$)	($P < 0.25$)
Juruá	0.08 ± 0.153^{AB}	84,684 ± 96,856	0.089 ± 0.139	679.2 ± 768.6
Purus	0.008 ± 0.154^{B}	109,986 ± 117,203	0.064 ± 0.182	1,275 ± 1,386.4
Middle Amazon/Pé-de-pincha	0.319 ± 0.089^{A}	3,674 ± 5,138	0.043 ± 0.167	35.9 ± 11.4
Protection system	($P < 0.02$)*	($P < 0.0001$)*	($P < 0.04$)*	($P < 0.0001$)*
Government	0.172 ± 0.05	$211{,}513 \pm 93{,}031^{A}$	0.177 ± 0.06	$1{,}910.7 \pm 1{,}035^{A}$
Community	0.085 ± 0.197	$29{,}789 \pm 35{,}716^{B}$	0.038 ± 0.151	268.3 ± 303.5^{B}

**Significant difference using ANOVA F-test (P < 0.05) for groups (river/area) and treatments (protection system). **Means followed by different letters in the columns show significant difference using Tukey test (P < 0.05).*

TABLE 2 | Summary of ANOVA of instantaneous growth rates r and carrying capacity K for number of nests/reproductive females and hatchling of *Podocnemis unifilis*.

Index	r hatchlings**	K hatchlings	r nests**	K nests
River/area	$P < 0.0001$*	$P < 0.344$	$P < 0.0001$*	$P < 0.079$
Juruá	-0.033 ± 0.129^{B}	19,746 ± 28,353	-0.004 ± 0.118^{B}	506.2 ± 425.3
Purus	-0.003 ± 0.134^{B}	11,816 ± 10,352	-0.044 ± 0.144^{B}	620.3 ± 385.8
Middle Amazon/Pé-de-pincha	0.282 ± 0.166^{A}	25,067 ± 28,109	0.330 ± 0.192^{A}	1080.9 ± 848.1
Protection system	$P < 0.807$	$P < 0.089$	$P < 0.203$	$P < 0.237$
Government	−0.024 ± 0.168	30,779 ± 37,273	0.049 ± 0.155	802.4 ± 421.4
Community	0.095 ± 0.2073	16,252 ± 20,223	0.102 ± 0.231	672.2 ± 662.7

**Significant difference using ANOVA F-test (P < 0.05) for groups (river/area) and treatments (protection system). **Means followed by different letters in the columns show significant difference using Tukey test (P < 0.01).*

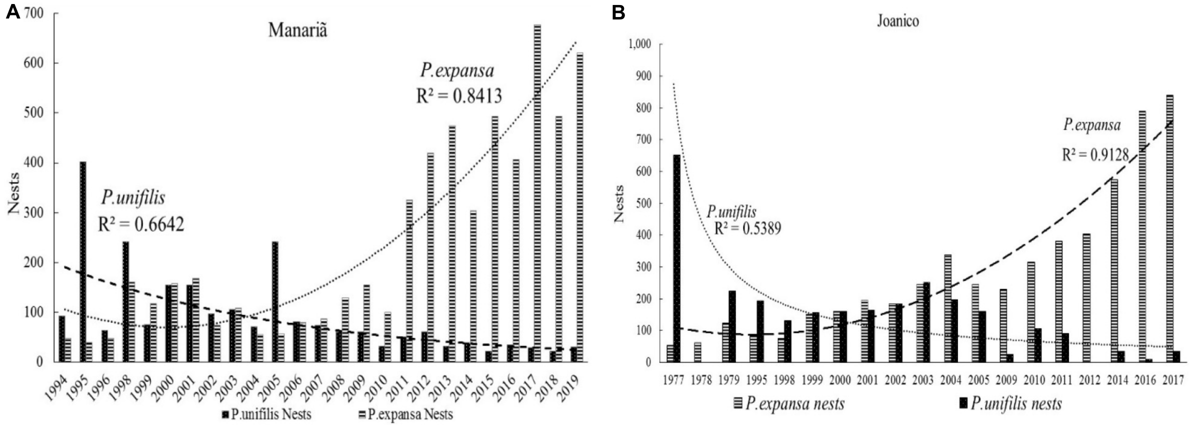

FIGURE 11 | Comparison between the trend lines of the production of nests of *P. expansa* and *P. unifilis*: **(A)** Manariã beach, middle Juruá; **(B)** Joanico beach, lower Juruá.

TABLE 3 | Summary of the ANOVA of instantaneous growth rates (r) and carrying capacity (K) for number of nests/reproductive females and hatchlings of *Podocnemis sextuberculata*.

Index	r hatchlings	K hatchlings**	r nests**	K nests**
River/area	$P < 0.05$	$P < 0.014$*	$P < 0.119$	$P < 0.036$*
Juruá	0.006 ± 0.354	48,687 ± 32,417^A	−0.087 ± 0.315	6,418 ± 4,017^A
Purus	0.042 ± 0.086	54,650 ± 38,024^{AB}	0.062 ± 0.102	5,028 ± 3,448^{AB}
Middle Amazon/Pé-de-pincha	0.330 ± 0.347	4,548 ± 4,153^B	0.066 ± 0.098	563 ± 253^B
Protection system	$P < 0.13$	$P < 0.03$*	$P < 0.004$*	$P < 0.297$
Government	0.207 ± 0,241	81,160 ± 34,924^A	0.219 ± 0.228^A	7,192 ± 3,184
Community	0.067 ± 0.367	29,231 ± 27,117^B	−0.097 ± 0.216^B	3,999 ± 4,126

**Significant difference using ANOVA F-test (P < 0.05) for groups (river/area) and treatments (protection system). **Means followed by different letters in the columns show significant difference using Tukey test (P < 0.01).*

of the Purus and Juruá Rivers presented higher production of hatchlings and a greater number of nests than the areas of the middle Amazon River. In addition to the Purus and Juruá Rivers being white water rivers and, therefore, having greater distribution and abundance of this species, there seems to be a positive association between the increase in populations of *P. sextuberculata* and *P. expansa*, which is contrary to what occurs with *P. unifilis*. The competition for space on the beach for egg-laying seems to be eased by the difference in nesting periods, as *P. sextuberculata* usually reach their peak egg-laying before the *P. expansa*.

It was observed that in the Juruá River, even with its greater support capacity for *P. sextuberculata* nests, the populations presented negative instantaneous growth rates (r) for the nests and very low rates for the hatchlings, which may indicate the reduction of these stocks.

This could be just an underestimation of the data due to the lack of records of the number of nests and hatchlings of *P. sextuberculata* on each beach. In fact, due to the huge amount of simultaneous egg-laying of *P. sextuberculata* (sometimes more than 1,000 nests per night), this would require great effort to count the nests. In addition, the communities that monitor the beaches of the middle Juruá River have stopped estimating the production of nests and hatchlings since 2007, when the PROBUC protocol was implemented in the state conservation units, which prioritized only the registration of data for *P. expansa* and *P. unifilis*.

However, it should be noted that this specie was the most caught for consumption and sale (377 nests and 32,756 animals) according to the seizure data and consumption data collected by PROBUC and Andrade (2008, 2017), and it is possible that these negative rates actually indicate the reduction in stocks of *P. sextuberculata* in the Juruá River.

It was not possible to perform the same ANOVA for *P. erythrocephala* because curves were estimated for only 12 sites, that form of community protection systems. This species showed growth rates (r) for number of nests and hatchlings ($r = 0.42 \pm 0.3$) that were higher than those for the other species analyzed.

To estimate a model of the general logistic curve for each species according to the protection system, 72 growth curves of nests/reproductive females on the beaches were selected, and the curves with growth rate (r) with negative values were eliminated. Simulations were performed with the selected curves up to 50 years of conservation, and the generated values were used to estimate a general growth curve of the number of nests/reproductive females of each species as a result of the protection system (**Figure 12**).

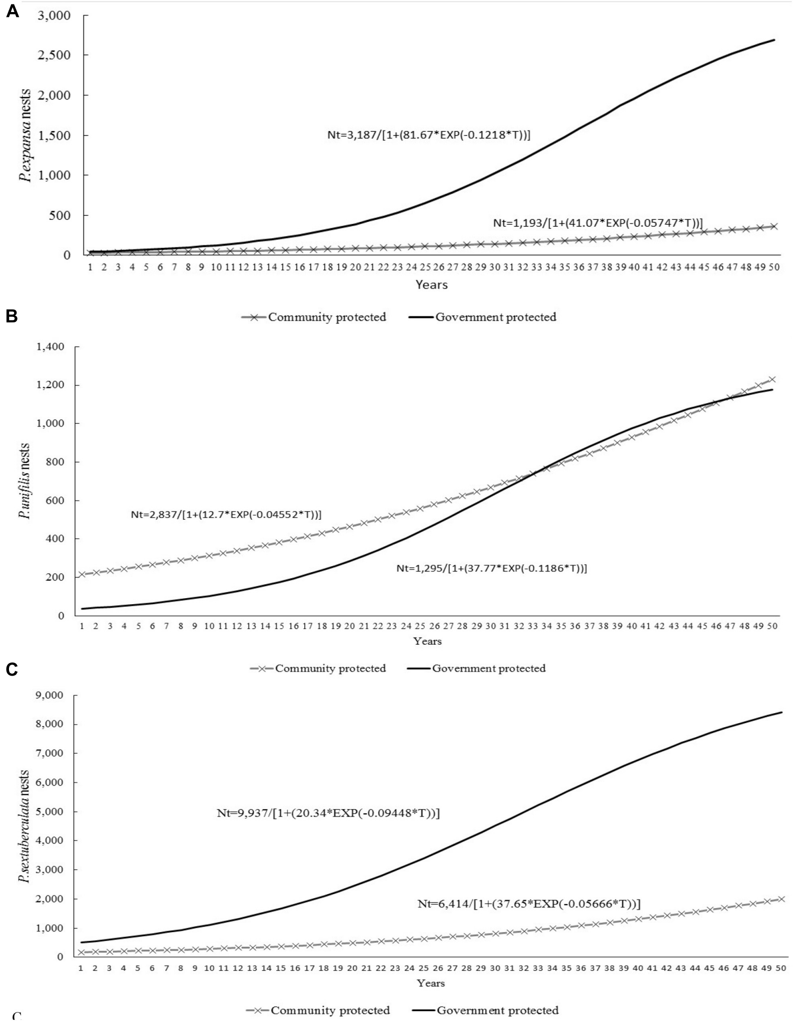

FIGURE 12 | Logistic growth curves of the number of nests/reproductive females in the Amazonas state and the northwestern section of the Pará state, Brazil: **(A)** *P. expansa*; **(B)** *P. unifilis*; **(C)** *P. sextuberculata*.

After estimating the logistic growth model curves by species and by protection system, the proposed models were validated by comparing the estimated values to the real values using the Spearman correlation, and for most of the curves, the correlation was significant.

DISCUSSION

Analysis of the Historical Data Series for Conservation of Chelonians in the Amazon

The analysis of the historical data series of chelonian protection in the Amazonas showed that an increase in the number of protected areas directly provided an increase in the number of protected nests and hatchlings. However, when comparing the 230,444 nests and 21,350,201 hatchlings of *P. expansa* protected for 45 years (5,121 ± 4,417 nests/year and 474,449 ± 411,488 hatchlings/year) with the estimates of 400–500,000 turtles females laying eggs in the Solimões and Madeira Rivers in the nineteenth century (Bates 1863 *apud* Smith, 1974 and 1979), it was noticed that these populations of *P. expansa* perhaps represent less than 1–2% of the original population in the Amazonas state.

However, it should be considered that in the surveys done in the 1970s by the IBDF in the 26 main beaches of the Solimões, Uatumã, Purus and Juruá Rivers, only between 1,750–2,767 egg-laying *P. expansa* females were recorded (Alfinito, 1978; Corrêa, 1978). In the first 10 years of protection, total production was estimated at 9,323 turtles nests in just two areas (Abufari and Walter Bury) in the Amazonas state (average 1,767 nests/year) (IBAMA, 1989). Cantarelli (2006) and Cantarelli et al. (2014) estimated that from 1975 to 2004, 6,163,521 hatchlings of *P. expansa* (2,732 nests/year) were protected on 12 beaches of the Purus and Juruá Rivers in the Amazonas state, with an increase of 25% per year, and the cost of protection per hatchlings was estimated at US$0.09.

In this study, when analyzing only the last 10 years (2009–2019), the average number of egg-laying *P. expansa* females in protected areas in the Amazonas state increased to 11,681 ± 3,321 animals/year, which represented an increase of 636% in 45 years (14% per year), with a cost of US$0.1 to 0.5 per protected hatchling.

Cantarelli et al. (2014) estimated that there were 30,000 *P. expansa* egg-laying females in the Brazilian Amazon. Forero-Medina et al. (2019) estimated that there are 147,000 *P. expansa* females in six Amazonian countries (Brazil, Colombia, Bolivia, Peru, Venezuela and Ecuador) of which 109,473 females were in Brazil alone in 2014. In this study, the estimated number of protected *P. expansa* females in the Amazonas state would represent only 10.6–16.4% of the total estimated turtle females for the whole of the Amazon. According to IBAMA (2019a) and Fagundes et al. (2021), only between 9.4 and 10.7% of priority areas for conservation of this species receive protection in the Amazonas state.

For *P. unifilis*, the analysis of the historical series (1974–2019) showed a production of 170,076 nests and 3,229,821 hatchlings (mean of 3,779 nests/year and 71,774 hatchlings/year) and, for *P. sextuberculata*, a production of 647,715 nests and 6,410,092 hatchlings (mean of 14,394 nests/year and 142,446 hatchlings/year) was observed. Cantarelli (2006) reported that 9,240,264 *P. unifilis* and *P. sextuberculata* hatchlings (318,629 hatchlings of the two species/year) were protected by the PQA (1975–2004).

With regards to the production of *P. expansa* hatchlings, it was found that the federal environmental agencies prioritized the protection of the most productive areas of turtles, while the communities usually conserved areas with smaller populations of turtles (a result of intense human predation) or in environments where there is a greater occurrence of *P. unifilis*, thus prioritizing this second species.

It was also found that, for the smaller species of *Podocnemididae* (*P. unifilis*, *P. sextuberculata* and *P. erytrocephala*), the community conservation system managed to cover a larger number of areas and that produced larger quantities of hatchlings for the restocking of the areas, while for *P. expansa*, the protection system of the federal environmental agencies managed to protect larger quantities of nests and hatchlings, in a few high production nesting areas.

The community conservation of chelonians began to gain strength between 1990 and 1999, by which time it protected 58.2% of the areas, and expanded to 80.6% of the areas between 2000–2009, until it became responsible for the protection of 88–94% of the chelonian breeding areas in the Amazonas state between 2010 and 2019. The conservation actions have been developed by the Amazonian riverine populations, including the adoption of conservation management systems of turtles with the management of resources guided by various forms of social organization, in which local residents and all stakeholders are key (Pinto and Pereira, 2004; Lima et al., 2017).

The mobilization and organization of communities to protect the fishery resources in the lowlands of Solimões/Amazon Rivers eventually influenced the emergence of community initiatives for conservation of chelonians, as was the case of the Pé-de-pincha Program in the middle Amazon River (Batista et al., 2004; Pinto and Pereira, 2004; Andrade, 2015) and community management areas in Santarém (Miorando et al., 2013).

The Pé-de-pincha Program is one of the largest community management and volunteer programs in the world. The name originates from the footprints that yellow-spotted river turtles leave on the beach, which are similar to bottle cap imprints (that the riverine community call "pincha"). Thus, an extension program and community management of chelonians was born in which the community members are trained and work voluntarily to protect nests and take care of the hatchlings until they return to their natural habitat (Andrade, 2012, 2015, 2017; Lima et al., 2017). The Pé-de-pincha Program has been implemented in 123 communities in 18 municipalities of the Amazonas and west Pará states, helping to protect 184 nesting sites (56% outside of conservation units) in an area equivalent to 2.7% of the Amazon (Andrade, 2017).

In order to stimulate and maintain the mobilized communities, in addition to protecting the turtles, the Pé-de-pincha Program has been active in training volunteers and seeking sustainable economic alternatives. It has trained 219 volunteer environmental agents and 148 environmental managers in techniques related to conservation of chelonians and raised awareness with lectures to over 86,507 participants, and trained 1,350 teachers of rural schools in environmental education (Andrade, 2017). To encourage income generation, it has held courses in fish technology, poultry breeding, medicinal plants, community gardens and breeding of turtles for 5,798 participants. The total number of people involved were 28,379 (directly) and 314,845 (indirectly) and, as such, it is one of the largest volunteer programs in the country (Andrade, 2017).

In the various conservation units (extractive reserves and sustainable development reserves) of the Juruá and Purus River, there are also community management systems that have helped protect more than 30 nesting areas of turtles, during more than 40 years of conservation work. Unlike the areas of the Pé-de-pincha Program, these are areas that still have large stocks of *P. expansa, P. unifilis* and *P. sextuberculata* (Andrade, 2008, 2015; Fonseca et al., 2011; Campos-Silva et al., 2018).

However, there are other areas in which there has been work on community management of turtles such as in the Sustainable Development Reserve (RDS) of Mamirauá (Oliveira, 2006); the RDS Uatumã, RDS Piagaçú-Purus (Waldez et al., 2013), in Juruti and in Aritapera/Santarém in the lower Amazon (Miorando et al., 2013). This has also occurred in the states of Amapá, Rondônia and Acre (Silva et al., 2019), as well as in other Latin American countries such as Venezuela (Hernández et al., 2010); Colombia (TCA, 1997); Peru (Soini, 1999; Harju et al., 2017); and Ecuador (Towsend, 2008).

In 1996, thanks to this conservation work by the federal government, together with numerous community protection initiatives, *P. expansa* was removed from the list of endangered animals in Brazil (Cantarelli et al., 2014; Forero-Medina et al., 2019). But, since 2003, the federal government has been reducing the volume of resources devoted to the protection of this specie in the region, which has caused a reduction of between 15.5 and 46.8% in the production of hatchlings on some beaches protected only by the environmental agency without community support (Andrade, 2008).

In the state of Amazonas, which covers 28.4% of the Amazon, by 2015, 212 chelonian breeding areas in 15 rivers had received some protection, mainly for nests and hatchlings, with an average production of 1,077,768 ± 115,466 hatchlings/year: 35.3 ± 26.1% of *P. expansa*; 19.3 ± 25.4% of *P. unifilis* and 40.9 ± 32.6% of *P. sextuberculata* (Andrade, 2017). Most of these areas were protected through the work of local communities interested in the maintenance of this natural resource, and these areas increased from 69 (58.2%) in 2001 to 193 (93.4%).

Andrade et al. (2004) analyzed the effective costs for the production of hatchlings protected with community-based management in the middle Amazon, and estimated an average value of US$0.6–0.8/hatchling and a profitability of 120.1%, when considering the estimate of the value of environmental services provided by the communities. Campos-Silva et al. (2018) estimated that the value for maintaining all areas of community-based management of chelonians would be around US$833,000 annually for the Brazilian Amazon.

Miorando et al. (2013) evaluated the effects of community management on the protection of *P. sextuberculata* in the lowlands of the Amazon, and noted that areas with community-based management have a higher number of catches of animals per units of effort, which shows the importance of this protection system.

Initiatives involving local communities and institutions carried out in the Amazon have resulted in increased protection of nesting habitats and recovery of *Podocnemididae* populations (Cantarelli et al., 2014; Andrade, 2017; Lima et al., 2017; Campos-Silva et al., 2018; Forero-Medina et al., 2019). Participatory community monitoring programs (PCM) have become an important methodological innovation for the management of biodiversity conservation in protected areas (Costa et al., 2018).

The idea of decentralization in the management of natural resources and the involvement of local populations has gained a lot of credit in the formulation of public policies and regional development projects, and, as such, avoids the classic options of privatization of resources or exclusive control by the State (IPEA, 2010). These changes have been occurring worldwide and have a strong emphasis on communities and local impacts of policies based on community management (Campos-Silva et al., 2020). Conservation actions and sustainable management of natural resources require initiatives also integrated by participatory monitoring and management, in which the information collected by resource users helps guide local decision makers on conservation management (Kennet et al., 2015). However, it is necessary to fully integrate the diverse interactions between individuals, communities and institutions and the complex Amazon ecosystem in this type of long-term environmental monitoring (Oliveira Júnior et al., 2020).

The chelonian conservation programs, carried out for a long time through the government environmental agencies (IBAMA, ICMBio) or through community management (Pé-de-pincha Program), have shown that by maintaining the protection effort, there is a tendency to increase the number of females laying eggs and hatchlings being produced on each beach (Andrade, 2015; IBAMA, 2016; Campos-Silva et al., 2018). With the regional strengthening of protected areas and community-based management, it is possible to anticipate a recovery scenario of freshwater turtle stocks in the Amazon in the coming decades (Norris et al., 2019).

In 2017, the Amazonas state officially recognized the efforts of the communities by creating 265 chelonian protection areas and regulating a community system of chelonian breeding, and allowing the communities to carry out breeding and fattening of a portion of the hatchlings of *P. expansa* (10%) and *P. unifilis* (20%) that they protect (CEMAAM resolutions N°25 and 26/17-DOE, DOE, 2017). This possibility of *ex situ* management of turtles to rear them in semi-natural conditions to be commercialized seems to be a solution to generate income and cover part of the expenses for the protection of the species (Campos-Silva et al., 2018).

The Amazonas state has the largest number of registered chelonian breeders in Brazil (27), with more than 150 thousand animals in captivity (*P. expansa* and *P. unifilis*) and an annual supply of 2,623 ± 561 animals/year (15 tons/year) (Andrade, 2008; Garcez, 2009; IBAMA, 2019b). This seems to be promising, not only from an economic point of view, but also for its relevance in food security and culture of the peoples of the region (Dantas-Filho et al., 2020).

Turtle Population Growth Models in the Amazon

The tabulation of the historical series of data of protection of *Podocnemis* species permitted us to analyze the variation in the number of females nesting on each beach and the number of hatchlings produced over the years. Although the number of nests do not necessarily indicate the number of reproductive females on the beach (especially in the case of species with multiple layings, such as *P. unifilis* and *P. sextuberculata*), and also do not constitute the total number of individuals in a population, as males and females that did not reproduce, young and hatchlings were not counted, it was considered that their variation over time could be the only consistent indicator of population variation in those protected breeding sites. The same can be considered with regard to the number of hatchlings produced, which is directly related to the number of nests, and which allows us to estimate the rate of hatching or reproductive success of each species on each beach, over the years.

It should be taken into account that, in very few (9.8%) of the sites analyzed were larger and long-term surveys carried out with animal capture, marking and biometrics, which would have allowed the recording of basic information about the population structure (sex ratio, size classes, abundance).

Considering that for *Podocnemis expansa* there are few studies aimed at estimating population growth models (Corrêa, 1978; Diniz and Santos, 1997; Norris et al., 2019; Rachmansah et al., 2020), it is understood that it would be more robust to use the real data of variation of nests/reproductive females and hatchlings as indicators of population variation.

Thus, we chose to use a simpler model of population growth to verify that the data of nests and hatchlings fit the models of growth curve tested [logistic, as suggested by Hailey and Lambert (2002)]. A model of continuous growth was used rather than a discrete model and with delay in density-dependent response, because when the intrinsic growth rates (r) are less than 1.0 (as shown to be the case of the analyzed species), the behavior for the discrete model is similar to the continuous one (Gotelli, 2007).

In this study, it was possible to use the regression coefficients of the non-linear curve model (r and K) as explanatory variables of the temporal trend of each nesting site and compare them with the coefficients of equations of other areas.

The growth rate of the number of nests/reproductive females per unit of time (r_t) is specific for each species, as well as for the nesting site analyzed, and for a given period of time of protection of the area. Therefore, it made no sense to obtain the mean values of r for each protection system used.

The same reasoning applies to the carrying capacity (K) of each nesting beach in relation to the number of nesting females. The competition for space for the nests in the sand of the beach can reach such a level that there will be overlapping of nests and the females will end up digging up and removing the eggs of the others. In addition, some females would end up looking for other places to nest. In other words, each breeding site has a support capacity (K) for nests and hatchlings produced that is limited, mainly, by the space factor. This constant is also specific for each species and laying site and, therefore, it would not make sense to use the average value of K to generate a unique model for the population growth curve.

However, the population parameters r and K are values that have units that allow comparison between one conservation area and another. Units of r signify individual by individual and unit of time (Gotelli, 2007). Thus, it was possible to compare the instantaneous growth rates between the different protection systems used over time in the breeding areas of chelonians in the Amazonas state.

Bence (1995) suggested the use of long time series to estimate prediction equations, from which the estimates of the mean, the regression intercept would be more reliable, as well as for when the explanatory variable had a temporal tendency. Barry (1995) reported that several studies consider the non-parametric estimation S functions of time variation through non-linear models as the most appropriate method for estimating growth curves.

We have followed all of the steps set out by Pereira and Arruda (1987) in order to carry out the practical adjustment of a time series of data to the various models of the growth curve: (a) first, we assessed the correlation and regression analysis between the data of the production of the population (number of nests, number of hatchlings) and years; (b) then we estimated regressions that best fitted the relationship between the variables; c) when the regressions were significant, the simulations were done in fit of the curve non-linear models (Logistic and Gompertz); d) and, finally, when the best models were identified, these were validated by comparison with the real distributions obtained from the data for each beach.

Podocnemis expansa lay eggs in synchrony with the flow of the Amazonian rivers (Alho and Pádua, 1982), and offspring births occur in the months from October to December. This would characterize, according to Gotelli (2007), a discrete population growth model that, moreover, would be subject to environmental stochasticity (variability associated with good and bad years of production of hatchlings, as for example, with the loss of nests in large floods) and with delays in time (turtles take a long time to become reproductively active). However, according to this same author, if the growth rate (r) is small ($r < 2.0$) the behavior of the discrete equation is similar to the continuous curve.

For growth models with delay, May (1976) defined that the response time of the population is inversely proportional to the growth rate (r) ($t = 1/r$), and therefore, slow-growing populations have long response time, but if this delay quotient (r.t) is also small ($0 < rt < 0.368$), the population will also grow smoothly until the support capacity is reached.

In the estimates of the r values for the logistic growth curves of nests and hatchlings analyzed in this study, the values were always below 1.0, and behaved as a continuous model with a low delay quotient, which may be explained by the fact that, although there is a very large initial pulse of births, compensatorily, there is also a large pulse of mortality in the initial phase of life of these turtles.

For *P. expansa*, the lower and often negative r rates of the Purus River nesting areas seem to be linked to the following two factors: (1) the vast majority of community nesting areas were initially managed in the system of beach owners, suffered exploitation of their resources and were subsequently abandoned and looted, which caused the drastic reduction in the number of nesting matrices when compared to the initial stocks. (2) The second factor may be that the population is close to reaching the carrying capacity of the beach.

In the middle Purus, only the nesting areas of *P. expansa* under government protection had positive r rates, even so, with low values, as in the case of Abufari, the largest nesting areas of *P. expansa* in the Amazonas state, which presented r rates equal to 0.134 for hatchlings and 0.117 for nests, which could indicate a dampening of the population growth rate due to the number of egg-laying females being close to the support capacity of the beach.

Apparently, the same situation did not occur in the Juruá River, especially in the middle Juruá, where the process of community organization seems to have been stronger. The communities of rubber tappers have replaced the old beach owners in the defense and protection of the nesting areas. In addition, the environmental groups joined forces with the rubber tappers, and together they sought to create extractivist reserves that eventually further protected *P. expansa* nesting areas of the Juruá River.

On the other hand, it was found that in the areas of the middle Amazon/Pé-de-pincha, the r rates were higher, which can be explained by the fact that in these areas the populations of *P. expansa* were drastically reduced, and therefore there is a significant amount of environmental resources available for just a few animals. In this region, the population growth curve would still be at the beginning, thus, with a much higher initial growth rate. This can be seen as a good indicator of the potential for recovery of chelonian populations through community work.

For *P. unifilis*, the negative or low instantaneous growth rates (r) on the protected beaches of the Purus and Juruá rivers could be related to underreporting of data. But, the most likely hypothesis that the increase in recruitment of females of *P. expansa* that reach reproductive age increases the competition with females of P. unifilis for space on the river banks and on the beach sand. Because they are smaller than the *P. expansa*, females of *P. unifilis* are probably forced to look for other spaces on the beach closer to vegetation or at the ends of the beach, or even migrate to other areas, such as ravines or other beaches. Since these other sites are not monitored, the records only show the main beaches and these end up indicating a reduction in the number of female *P. unifilis* nesting on that beach.

Another assumption would be that the actual reduction of female of *P. unifilis* on the Purus and Juruá beaches is a result of the capture of adults and the harvest of eggs for consumption and illegal sale. The analysis of data regarding predation of nests (558) of *P. unifilis*, specifically in the protected beach areas, revealed that this was in fact greater than that of the predation of nests (300) of *P. expansa*. But, the seizures of *P. expansa* (20,915) was greater than that of the *P. unifilis* (14,505).

The higher rates of growth r recorded in the areas of community management under the Pé-de-pincha Program are probably due to the fact that this program has prioritized *P. unifilis* as a key species (rather than *P. expansa*), and protects some areas where only *P. unifilis* occur.

Another aspect is the fact that these areas are also depredated areas, with reduced stocks of the species, which means that in these places the population growth curve of *P. unifilis* is still in its initial phase, for which the intrinsic growth rates r are usually higher.

In small chelonian populations, such as those in the middle Amazon, the Allee effect is believed to occur, with growth rates being reinforced by the increase in population size, probably due to the greater reproductive efficiency of the chelonian groups (Gotelli, 2007; Silva et al., 2009; Silva, 2020).

According to Crouse (1999), population models for chelonians should provide a tool to predict the likely response of a population over time and, above all, allow us to identify data gaps, which is the predictive capacity of the model.

We started with long time series (15–45 years) for the production of nests and hatchlings. Instead of using rates or estimated predictive values, we used real data regarding what occurred over time with the population of females nesting at each site. The proposed models are a simple tool, based on analyses of trends in the production of nests and hatchlings, but that do not lose their predictive character as a population model. This tool can be enriched by incorporating information on population structure and dynamics from long-term studies that are already being conducted with *P. expansa*, *P. unifilis* and *P. sextuberculata* in the Juruá, middle Amazon and Andirá Rivers (Andrade, 2012, 2015).

Models in matrices can be used in ecological models, but they represent a device where the reality of it is partly sacrificed for the benefit of using the special properties of the matrix in the formulation of the model. However, it should be considered that modeling and simulation basically demand five key elements that are actual data in the field, a systematic framework for observation and recording of data (the time series of the variables), a proposed basic model, a derived aggregate model, and simulations (Santos, 1986), all of these prerequisites were used in our study.

Ehrhart et al. (2014) performed a trend analysis of time series of nesting data and reproductive success of *Caretta caretta* in which they used simple methods of evaluation through descriptive statistics of the data (graphics of the number of nests produced annually and tables with the annual data of nests, hatchlings and hatching rates), as was done in this study with the production data for *Podocnemis* species.

Diniz and Santos (1997) were the first to propose a qualitative study of the population growth of *Podocnemis expansa* by using a mathematical model that employed the Leslie matrix, with some data from biotic parameters. However, based on the value obtained from the Kojima quota for the biotic parameters ($\beta \leq 0.745296820391 < 1$), he predicted that the populations of *Podocnemis expansa* would be extinct in Brazil, unless it was possible to increase the survival rate of offspring to 20% in the first year of life. Before him, Corrêa (1978) had already tried to propose a projection model for the offspring of a female of *P. expansa* for 30 years (evolution of the herd until the 4th generation). For this, he considered the average of 100 eggs/nest, sexual maturity of the mother at 8 years of age, useful life of 30 years, probability of the sexual ratio of the offspring of 50% males and 50% females.

It should be considered, however, that the models of Corrêa (1978) and Diniz and Santos (1997) are only models of reproduction projection, based mainly on the mean values of reproductive indices, such as number of eggs/nest and hatch rate of offspring, which when inserted deterministically in a matrix model estimate simulated values not based on comparisons with the actual data of field the over time.

The protection of eggs and nests alone was not enough to replenish depleted stocks of sea turtles. The models showed that small reductions in annual survival of juveniles and adults can have a profound effect on population dynamics by reducing population growth. Thus, the most complete models built for the sea turtle population, as well as models for the other species of turtles, point to the need to maintain high annual survival of all phases of life in order to recover declining populations (Crouse, 1999).

Norris et al. (2019) used a population projection model of the Lefkovitch matrix based on stages (eggs, hatchlings, juveniles and adults) to evaluate *P. unifilis* management scenarios, and estimated that large areas could be recovered if headstarting of hatchlings is practiced more widely.

Rachmansah et al. (2020) analyzed the biological viability of sustainable management in species of tropical turtles using life history data (number of eggs, age to sexual maturity, annual survival), latitude and climatic variables that were examined using generalized additive models (GAMs) and also the Lefkovich matrix population model structured in stages. Based on the tested modeling, these authors concluded that harvesting of adult tropical turtles would not be viable, but that sustainable egg harvesting would be.

A simpler population trend analysis protocol, such as the model proposed in this study, can help environmental managers and community leaders to better understand the dynamics and efficiency of their protective actions, and assist in decision-making.

Future Perspectives and Recommendations

Environmental agencies are giving more and more space to community-based management, investing in the implementation of participatory monitoring programs for natural resources. It is necessary that environmental managers work to have greater community involvement in turtle conservation programs, no matter how long this process may be.

Training in management and environmental awareness is gradually changing communities' perception of turtles. These changes in perception will not necessarily bring about changes in the use of these animals for subsistence consumption, but they will certainly influence the creation of rules and agreements for use.

With the advancement of technology, new ways for community members to collect data from nests and hatchlings of turtles on protected beaches should be adopted. To replace the old field sheets, applications on cell phones for recording monitoring data and sending them online to the database systems of environmental agencies have already begun to be tested. Also, the use of drones to estimate female turtles and their nests on a beach.

At the same time, new protocols for the monitoring of turtle populations, with the capture and recording of information about adult animals (and not just the nests and hatchlings), as well as the recording of data on the consumption of these species began to be implemented in protected areas in the Amazon. In addition, it is necessary that new applied ecology researches are carried out to obtain information about the structure and population dynamics of Podocnemidids in the different rivers of the region. This will help to build more robust population models that will help to simulate different scenarios for the conservation of these animals.

In areas with reduced turtle populations and where communities have an interest in trying to recover stocks, we suggest that other conservation tools can be adopted to ensure nest protection and improvement in hatching and hatch survival rates. Simple strategies historically used by beach monitors, such as the transfer of nests from threatened areas (by flooding or predation) to areas protected by the community, or the use of headstarting, to protect the hatchlings more by releasing them in protected natural lakes or keeping them in nurseries before release. Preferably, such strategies should be accompanied by research and used only until the population recovers.

Another important issue is that as local turtle stocks have increased, there has been, as a consequence, increased pressure from groups outside the community to illegally catch and sell these animals, and also increased community complaints about the lack of turtles. surveillance.

Faced with this situation of lack of control by environmental agencies and predatory outflow of resources, communities that protect turtles began to seek, with the environmental authorities, a form of remuneration for the environmental services they provide, or sustainable possibilities of income generation, through community creation of turtles (*ex situ* management) or quotas for extraction. Then, in 2017, in the state of Amazonas, the first authorized community agriculture units for turtles began operating. But other initiatives such as ecological tourism could be a good future option for these communities.

CONCLUSION

The riverside communities of the Amazon view the chelonians as an important food source, and it is one which has been managed and conserved by them with participation in different systems of use and protection throughout history.

The community system of protection of chelonians is responsible for most of the conservation areas of chelonians in the Amazonas state, and ensures that a great diversity of nesting environments are conserved in several rivers. In addition, the community management of chelonians has demonstrated that it is productive as a form of co-management, and is effective in increasing and protecting stocks of chelonians in the region, as well as being efficient, since it protects the species at a low operational cost.

Community-based chelonian conservation programs have their voluntary character and community mobilization as their strong point and, as such, have guaranteed the continuity of protection actions, even in the face of possible crises due to lack of support from environmental agencies. As tools for participatory management of natural resources, it is an important conservation strategy, complementary to the protection system created by the government.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical approval was not required for our noninvasive study since we did not collect any biological samples nor interfere with the behavior of the study species. Permission to collect observational data from river turtle nesting areas was granted by research permit number IBAMA/SISBIO No.19232/2011.

AUTHOR CONTRIBUTIONS

The present study was part of the doctoral thesis of PA, who carried out the compilation, tabulation and consolidation of all the data, as well as the statistical analyses, elaboration of graphs and discussion of the results. The other co-authors collaborated in collecting data in the field, tabulating annual data, recording and analyzing specific situations in different areas of chelonian protection, as well as helping to discuss the results obtained.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.769328/full#supplementary-material

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Flying Over Amazonian Waters: The Role of Rivers on the Distribution and Endemism Patterns of Neotropical Bats

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The Amazon comprises many of the largest rivers in the planet and also houses some of the richest bat communities in the world. Rivers are important geographic barriers for the dispersal and distribution of different taxa worldwide and, particularly in the Amazon region, they form the conceptual and empirical bases for the recognition of the so-called Areas of Endemism of terrestrial vertebrates. Despite the vast literature on the role of rivers on vertebrate community structure in the Amazon Forest, this process has never been investigated using a comprehensive dataset of Neotropical bat communities in the region. In this study, we aimed at: (1) evaluating the patterns of bat endemism across the Amazon Forest; (2) testing for the relationship between the distribution of bat species in the Amazon and the interfluve Areas of Endemism as currently recognized, and; (3) analyzing the importance of major Amazonian rivers in bat beta-diversity (turnover and nestedness) in the Amazon. Our results indicate that rivers are not major barriers for the current distribution of most bat species, and bat community composition breaks were divided into two clusters separating the east and west regions, and a third cluster in northern Amazon. In addition, there was no significant overlap among species distribution limits and the interfluve Areas of Endemism. Interestingly, the geographic patterns that we found for bat communities composition breaks highly resembles the one recovered using bird communities, suggesting that similar ecological and historical drivers might be acting to determine the distribution of flying vertebrates in the Amazon. Moreover, Amazonian bat distribution and endemism patterns were likely shaped by factors other than rivers, such as species interactions and the current environmental conditions. In conclusion, our results highlight the importance of modern analytical approaches to investigate large scale ecological patterns in the Neotropical region, and also challenge the widely recognized role of rivers on the determination of community structure and endemism patterns in the Amazon Forest, at least for bats.

Keywords: beta-diversity, biogeography, Chiroptera, South America, geographic barriers

INTRODUCTION

Rivers have long been hypothesized to be ecological and geographical barriers for the dispersal and colonization of new habitats for different taxa (Wallace, 1854; Napier, 1976; Hershkovitz, 1977). Not surprisingly, rivers represent one of the most important drivers of species distribution and endemism worldwide (Harcourt and Wood, 2011; Ramachandran et al., 2017; Mahulu et al., 2021). The Amazon region is not only one of the most biologically diverse regions on the planet, but also comprises the largest network of rivers (Junk, 1997). Four of the 10 largest rivers and 20 of the 34 largest tropical rivers are located in the Amazon region (Latrubesse et al., 2005). The role of the vast Amazonian hydric system on the diversification patterns and community assembly processes of Neotropical organisms has been investigated for many animal groups, such as primates (Ayres and Clutton-Brock, 1992; da Silva and Oren, 1996), amphibians and lizards (Moraes et al., 2016), butterflies (Hall and Harvey, 2002), comparatively among different taxonomic groups (Santorelli et al., 2018) and, in particular, using birds as model organisms (Ribas et al., 2005, 2012; Ferreira et al., 2017; Oliveira et al., 2017; Naka and Brumfield, 2018).

The Areas of Endemism (AoE) hypothesis, initially proposed by Cracraft in 1985 using birds as model organisms (Cracraft, 1985), gained extensive support in the scientific literature in the following decades, with the evidence mostly arising from historical biogeography studies (Fernandes et al., 2012; Ribas et al., 2012; d'Horta et al., 2013; Lutz et al., 2013). In this scenario, distinct AoEs would be located at the interfluves of major Amazonian rivers (**Figure 1**), which are believed to harbor unique species communities (Ayres and Clutton-Brock, 1992; Gascon et al., 2000; Silva et al., 2005). However, the role of Amazonian rivers on actual endemism patterns of birds was only quantitatively tested using a large distribution dataset and community ecology regionalization methods by Oliveira et al. (2017). These authors showed that the AoEs had no support from an actual test of biotic regionalization using a dataset that covered a broad geographic area in lowland Amazon. Moreover, many studies have failed to provide evidence that supports the role of major Amazonian rivers as geographic limits for terrestrial species (see Aleixo, 2004; Fernandes et al., 2013; Fecchio et al., 2018; Santorelli et al., 2018; Dambros et al., 2020), questioning the current knowledge and definitions of AoE in the Amazon. Nonetheless, the role of large Amazonian rivers acting as dispersal barriers is usually undisputed, and this has been demonstrated using both distribution and divergence patterns as inferred by genetic data (Maldonado-Coelho et al., 2013; Weir et al., 2015; Pirani et al., 2019).

The Amazon region (6.9 million km^2) covers more than a third of the total Neotropical region area, harboring more than 170 bat species, which represents over 10% of the world's bat diversity, and more than 100 species might be recorded in a single locality (Medlin et al., 2010; Burgin et al., 2018; Santos et al., 2019). Even though some bat species are able to make long-distance dispersals (Arnone et al., 2016; Esbérard et al., 2017), many Neotropical species have small home ranges and specific ecological requirements, which can particularly limit their distribution ranges and contribute to the origin and maintenance of local endemism patterns (Meyer et al., 2005; Henry and Kalko, 2007; Voss et al., 2016). Surprisingly, and considering that the AoE hypothesis was proposed using flying vertebrates as models, no large-scale study has ever tested if the Amazon rivers might be defining species distribution limits and, hence, influencing the maintenance of AoEs for bats in the Amazon Forest.

The few studies that analyzed the effect of Amazonian rivers on bat distribution showed that rivers were not an important barrier for the dispersal of bat species (Santorelli et al., 2018), with climate showing to be a more important variable to predict similarity in species composition than geographic distance (Dambros et al., 2020). In addition, endemism areas were responsible for explaining less than 10% of the observed difference in species composition among sites (Dambros et al., 2020). Nonetheless, three AoEs in the Amazon showed significantly different species compositions among them (Dambros et al., 2020). These are local-scale studies that sampled a relatively small geographic area within the Amazon, and only included a small number of bat species, i.e., ∼60 bat species (Dambros et al., 2020) and 76 species (Santorelli et al., 2018) from a total of more than 170 bat species known to occur in the biome (Santos et al., 2019). Noteworthy, the study of Tavares et al. (2017) analyzed 26 Amazon localities, with 161 sampled bat species, and proposed that the composition of Amazon bat species can be divided into three zones: eastern Brazilian Amazon, Guiana Shield, and western Amazon. However, this study did not use spatially explicit analyses, rather basing its geographic interpretations on the composition distance among bat assemblages. Thus, a more comprehensive evaluation on the impact of rivers as dispersal barriers for bats and the importance of AoEs to explain differences in species composition within the Amazon rainforest is still warranted.

Recently proposed endemism metrics that integrate information on geographic rarity and phylogenetic divergence among the biota of a particular region have a stronger explanatory power in the detection of endemism patterns, compared to traditional methods (Crisp et al., 2001; Rosauer et al., 2009). Moreover, advances in the identification and differences in the phylogenetic composition of AoEs can also help in exploring the evolutionary significance of such areas, as made possible by the Categorical Analysis of Neo and Paleo-endemism (CANAPE, Mishler et al., 2014). The CANAPE is capable of distinguishing areas containing significant concentrations of rare long branches (paleo-endemism), rare short branches (neo-endemism), or mixed endemism. Taken together, these metrics allow for the identification of complementary areas of biodiversity that have unique evolutionary histories and characteristics and, therefore, should be taken into account in studies investigating geographic regionalization patterns (Mishler et al., 2014; González-Orozco et al., 2015; Scherson et al., 2017; López-Aguirre et al., 2018; Veron et al., 2019; Azevedo et al., 2020).

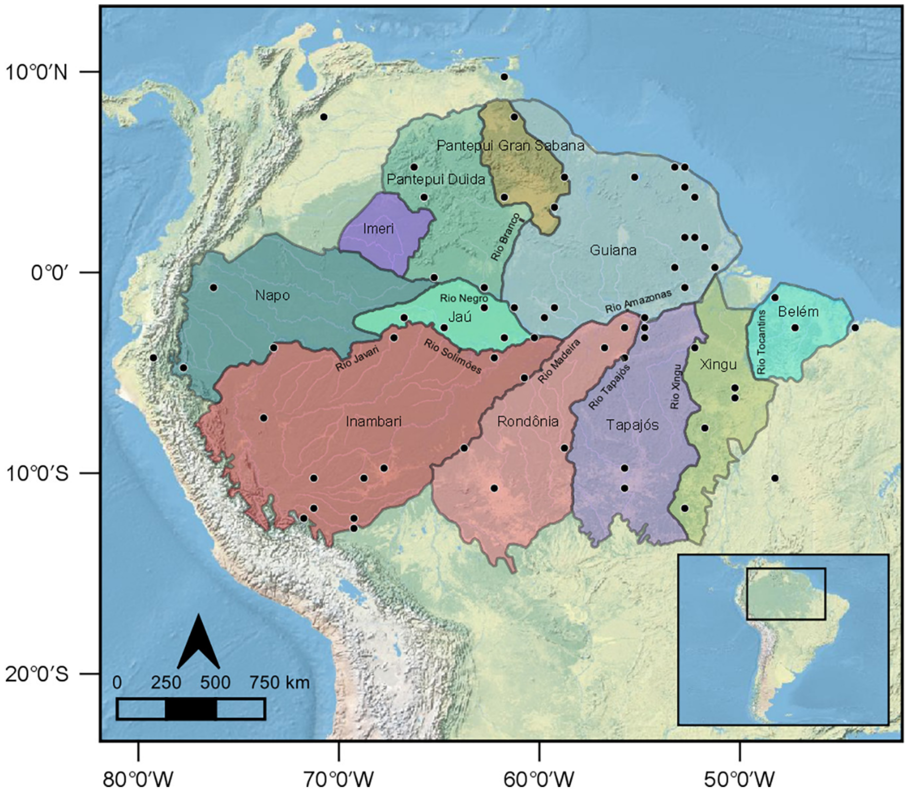

FIGURE 1 | Geographic location of the 64 bat communities used in this study (black circles). For reference, the figure depicts the interfluve hypothesis of lowland Amazonian vertebrates Areas of Endemism. This currently accepted Areas of Endemism classification was proposed by Cracraft (1985) and subsequently modified by da Silva and Bates (2002), Naka (2011), and Borges and da Silva (2012).

Considering the above-mentioned methodological advances, our main goal was to understand patterns of beta diversity and bat endemism in *cis*-Andean Amazonia and their relationships with the large river system of the Amazon. Specifically, we aimed at:

(1) Inferring patterns of bat endemism in *cis*-Andean Amazonia using species richness corrected endemism metrics and inferring the location of areas with evidence of neo-, paleo-, and mixed endemism; If the interfluve AoE hypothesis is indeed a strong predictor of terrestrial vertebrate endemism patterns in the Amazon, it is expected that rivers would play an important role on bat endemism patterns. However, as previously shown for birds (Oliveira et al., 2017), this hypothesis might not hold true for flying vertebrates. Hence, we predict that (i) endemism patterns will not be correlated with the geography of interfluve AoEs as currently proposed (Scherson et al., 2017).

(2) Investigating if there is a relationship between the distribution of bat species in the Amazon and areas of endemism as traditionally proposed (interfluves AoE). If the interfluves AoE hypothesis is true, each bat species should be mainly distributed within one or, at least, a few AoEs. Nevertheless, many bat species are able to fly long distances and over large rivers, meaning that rivers should not be strong geographic barriers and would not be a process influencing endemism patterns for these organisms. Thus, we predict that (ii) the limits of the distribution ranges of each bat species will not depict a high fit to the interfluve AoEs limits, as also shown for birds (Oliveira et al., 2017).

(3) Estimating patterns of bat beta diversity and its decomposed components (turnover and nestedness) in order to understand the relationship between species assemblage breaks and the geography of major Amazon rivers, and verifying the biotic similarity among communities using phylogenetic measures of beta-diversity. Again, if the interfluve AoE hypothesis holds true, rivers should play a clear role in the variation of bat species composition across the Amazonian landscape. However, evidence suggests that processes other than the effects of rivers in the Amazonian geography should be responsible for the variation in species composition (Silva et al., 2019; Dambros et al., 2020). Hence, we predict that, (iii) there will be no direct correlation among geographic breaks on species composition and the main Amazonian riverine system. Finally, if the interfluve AoEs define a general community structure pattern for bats, we would expect that communities within each AoE would be more similar to each other compared to communities in other

AoEs. Hence, and following the same reasoning of our previous expectations in relation to the lack of influence of the interfluve AoEs on bat biogeograhic patterns in the Amazon, we predict that, (iv) beta-diversity among communities within each AoE will not be smaller than beta-diversity among communities in different AoEs, and that (v) communities within each AoE will not be more similar to each other in terms of phylogenetic community structure (Scherson et al., 2017).

MATERIALS AND METHODS

Communities and Phylogenetic Dataset

We used a previous compilation by Santos et al. (2019) that assembled data for 44 bat communities in the Amazon as a starting point for our dataset. In addition, we included another eight communities available in the literature (Peracchi et al., 1984; Martins et al., 2006, 2011; Patterson et al., 2006; Peters et al., 2006; Rex et al., 2008; Carvalho et al., 2018; Carrasco-Rueda et al., 2021). Finally, we supplemented our database by extracting information on the distribution of individual bat species in the Amazon from Aguiar et al. (2020). Because of recent taxonomic changes, we removed all records of the species *Pteronotus parnellii* from the dataset and only included data for undisputed *P. rubiginosus* and *P. alitonus* records (Pavan et al., 2018). Because we are using community and individual species records, we defined bat communities and eliminated duplicate data by using a grid with 0.50° × 0.50° (∼50 km) cells, and created a binary matrix with species occurrence in each community (i.e., presence and absence data for each grid cell). To reduce biases related to differences in sampling effort and methodology from different sources, we applied a threshold of a minimum of 20 species for each cell to be included in the final communities dataset, which should represent a well-sampled community in the Amazon region (e.g., Tavares et al., 2017). All species identified only at the genus level were excluded from the dataset. We checked all records to verify the presence of misidentified species based on species ranges as provided by Gardner (2008) and Rojas et al. (2018), and used current species names as available in the Mammal Diversity database.[1]

As most of the data described above derives from studies that used mist nets, and this sampling method is not particularly efficient for non-Phyllostomidae bat species in the Neotropical region (Marques et al., 2016), the presence of some rarely sampled species might bias our general inferences. In order to evaluate the extent of such possible issue, we used two different datasets to run the analyses. First, we used all species that comprised the final assembled dataset (as explained above), considering all bat families present in this study. Second, we only used species from the Phyllostomidae family, as they are better represented in mist net surveys. Since our main findings did not change by using the two different datasets, we graphically report results of analyses using the first complete dataset with all species, while results for Phyllostomidade can be found in **Supplementary Material**.

We used a consensus phylogenetic tree to represent evolutionary relationships among all species in our dataset from the most recent species-level mammal phylogeny (Upham et al., 2019). If a particular species was not present in the phylogeny, we used its sister-species or, when this was not possible, its closest known relative as a substitute in the phylogeny (Cisneros et al., 2015; de Carvalho et al., 2019). We only had to replace seven species (3.9% of all species in the dataset, **Supplementary Figure 1**), and since phylogenetic placement itself is not used in our analyses, but phylogenetic distance only, this approach should not influence our results. The species names on the phylogeny were updated using their most recent valid synonyms, according to recent taxonomic arrangements for Neotropical bats (Garbino et al., 2020). The tree was pruned to only include the species in our dataset using the package ape (Paradis and Schliep, 2019) in R version 3.6.2 (R Core Team, 2019; **Supplementary Figure 1**).

Statistical Analyses

To identify areas of endemism of bat communities in the Amazon and test our first prediction, we used two metrics available in the Biodiverse software version 3.1 (Laffan et al., 2010). First, we calculated the corrected weighted endemism (CWE), which is less biased by species richness and measures the proportion of endemism in each community (Crisp et al., 2001). Second, we used the CANAPE protocol to measure phylogenetic endemism (PE; Rosauer et al., 2009) and relative phylogenetic endemism (RPE; Mishler et al., 2014). The values of RPE are dependent on species richness because the PE of a set of species naturally increases when new species are added to the phylogeny. To circumvent this problem, we compared the actual PE and RPE values of each grid cell to 999 values of a null distribution (Mishler et al., 2014; Laffan et al., 2016). The *p*-values were estimated from a bi-tailed distribution of values to identify areas with higher (>0.975) or smaller (< 0.025) PE and RPE compared to the null distribution. A PE/RPE ratio higher or smaller than the null distribution indicates, respectively, paleo or neo endemism in a given community. Mixed endemism occurs when PE is significantly higher than the null distribution, but presents intermediate RPE values (i.e., indicating communities with high levels of paleo and neo endemism). We followed Azevedo et al. (2020) to include communities (grid cells) below the 0.01 significance level as mixed endemism, instead of using the term "super endemism" as suggested by Mishler et al. (2014). The final classification of metrics was done in R using a custom script modified from https://github.com/NunzioKnerr/biodiverse_pipeline.

To test our second prediction and infer whether the distribution of each bat species overlaps with interfluve AoEs, we quantified the percentage of area overlap between each species distribution and interfluve AoEs by considering a grid with cells of 0.50° × 0.50° (∼50 km) covering the whole Amazon region. We measured the species distribution fit to interfluve

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[1] http://www.mammaldiversity.org

AoE distribution using an index proposed by Oliveira et al. (2017). The index varies from 0 to 1 (1 = total fit) where higher values indicate that the species is very restricted to the AoE and occupies a large part of its area. For this analysis, we did not apply the threshold used to define communities (as detailed above). In turn, we used all available geographic coordinates for each species, in order to have the best representation of each species distribution. We only considered species with 90% of their distribution within each AoE as a way to exclude species that are not restricted to each particular AoE (Oliveira et al., 2017). Since this analysis demanded the most comprehensive information on species distributions, and not communities (grid cells) as previously defined, we used our whole distribution dataset (i.e., all available individual species records from the above-mentioned data sources, except those outside the AoEs).

To investigate species composition breaks and test our third prediction, we described spatial variation in species composition using a Bray-Curtis dissimilarity matrix transformed into linear values using Non-Metric Multidimensional Scaling (NMDS). These values are, then, geographically interpolated on a map using a Bayesian technique, assuming spatial autocorrelation among the values (Oliveira et al., 2017), which provides a surface map indicating species composition variation. To identify the components that influence bat beta-diversity variation throughout the Amazon, we partitioned beta diversity into turnover and nestedness components (Baselga, 2010). The similarity between the results of each component was measured through a Pearson correlation analysis for each NMDS axis. The values of beta diversity were geographically interpolated with three axes using the Nearest Neighbor technique. This interpolator uses Voronoi polygons to calculate the area of influence around the samples, and all intermediate points are calculated by averaging the neighboring polygons. We chose this interpolation because it does not depend on spatial autocorrelation, like most interpolators. All beta-diversity analyses were performed using the BioDinamica toolkit (Oliveira et al., 2019).

We compared beta diversity between and within each interfluve AoE (fourth prediction) using an univariate ANOVA (Quinn and Keough, 2002). We tested the normality of the mean of the NMDS axes (Shapiro-Wilk test) and homogeneity of variance (Levene test) to satisfy test assumptions. Differences were considered significant when the *p*-value was < 0.05 after Tukey's *post-hoc* test for unequal sample sizes (Zar, 2010). To test our fifth prediction and investigate phylogenetic beta-diversity among all communities in our dataset, while also inferring general similarity patterns among them, we used a clustering analysis implemented in Biodiverse, using the Unweighted Pair Group Method with Arithmetic Mean Averaging (UPGMA). This inference is based on the phylogenetic turnover among communities, which we calculated using the phylogenetic range-weighted turnover index (PhyloRWTurnover). This index measures phylogenetic turnover taking the branch lengths from the phylogenetic tree in consideration while weighting for the shared taxa among the communities, and ultimately down-weights the influence of widespread species with large range sizes (Laffan et al., 2016).

RESULTS

Geographic Patterns of Bat Endemism in the Amazon (CWE, PE, RPE, and CANAPE)

In total, we obtained 5,100 locality records for 182 species belonging to nine families of the order Chiroptera (**Supplementary Figure 2**). After cleaning and excluding duplicate species within each grid cell, our final dataset comprised 3,236 unique species records, totaling 64 communities with 177 bat species. For the Phyllostomidae family, we had a total of 2,317 unique species records, totaling 61 communities with 101 species (**Figure 1** and **Supplementary Table 1**). We report the following results using the geographic locations of interfluve AoEs to facilitate comparisons and interpretation, but we urge readers not to directly interpret our mentions to interfluve AoEs as evidence of support to this hypothesis. In relation to the endemism patterns (CWE, PE and CANAPE) used to test our first prediction, the corrected weighted endemism indicated 13 communities with significant endemism values (**Figure 2A**, in red and blue). We found significant values in the Solimões/Negro/Javari and Tapajós/Amazonas interfluvial zones (**Figure 2A**). In addition to these, in the southern region of Inambari (in blue) and north of Guyana (in red) there are communities with high significant values of endemism. Finally, high values of endemism were also found in communities at the Andean region and north of the Amazon, which lie outside the interfluve AoEs boundaries. The CWE using the Phyllostomidae dataset identified eight communities with significant values in the regions of Inambari, Pantepui Duida, Rondônia (in blue), Xingu, Tapajós and Guyana (in red) (**Supplementary Figure 3A**).

We found Amazonian bat communities where phylogenetic endemism was significantly high (>0.99 and >0.975) in western Amazonia in the Inambari region, in the Xingu region and communities at the Andean region and north of the Amazon, whereas significantly low (<0.01 and < 0.025) where present in the regions of Guiana (Amazon interfluvial zone), Belém, Tapajós, Inambari (Solimões interfluvial zone), Jaú (Negro interfluvial zone), and Napo (Javari interfluvial zone) (**Figure 2B**). The phylogenetic endemism of Phyllostomidae species was significantly high (>0.99 and >0.975) in the south in the Inambari region, Xingu region (Xingu interfluvial zone), communities at the Andean region and north of the Amazon. Significant low PE (<0.01 and <0.025) in the regions of Guyana (Amazonas interfluvial zone) and Napo (Javari interfluvial zone) (**Supplementary Figure 3B**). Significantly high RPE communities included one case in northern Amazonia and in Xingu, while significantly low RPE communities were found in the region of Inambari and Napo (**Figure 2C**). For the Phyllostomidae family, the significantly high RPE is found in the Xingu region and in the Amazonas/Tapajós/Solimões interfluvial

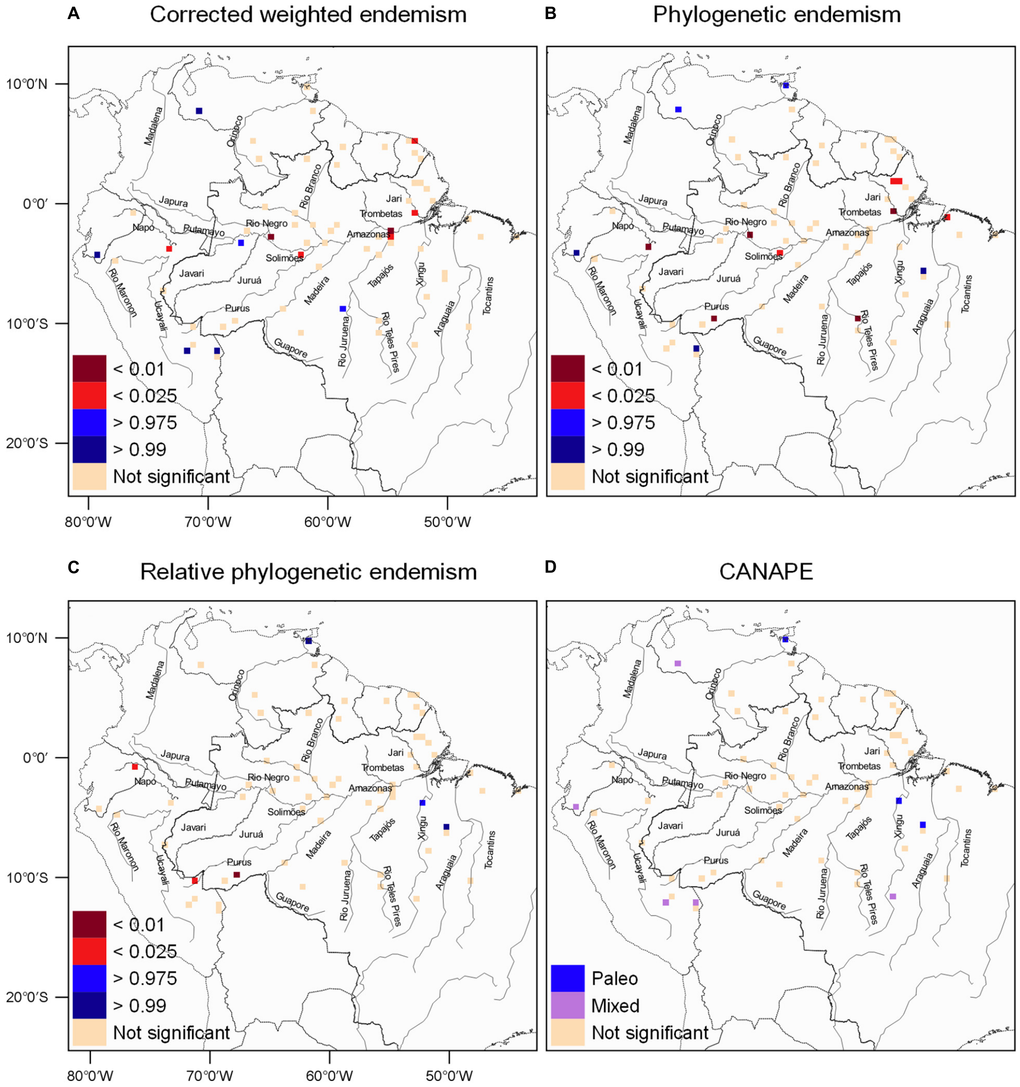

FIGURE 2 | Bat endemism patterns in the Amazon region. **(A)** Results for corrected weighted endemism (CWE). Significance was estimated using two-tailed randomization tests (see section "Materials and Methods"). Beige cells are not significant, red shaded cells depict communities with significantly less CWE than expected, and blue cells depict communities with significantly more CWE than expected. **(B)** Results for phylogenetic endemism (PE). Significance was estimated using two-tailed randomization tests. Beige cells are not significant, red cells contain significantly lower PE than expected, and blue cells contain significantly higher PE than expected. **(C)** Results for relative phylogenetic endemism (RPE). Significance was estimated using two-tailed randomization tests. Beige cells are not significant, red cells contain significantly lower RPE than expected (phylogenetic branch lengths in the grid cells are shorter than expected), and blue cells contain significantly higher RPE (branch lengths in the grid cells are longer than expected). **(D)** Phylogenetic endemism centers derived from the CANAPE analysis. Beige cells are not significant, blue cells indicate centers of paleo-endemism and purple cells are a mix of neo- and paleo-endemism.

zone (in blue), whereas the significantly low RPE was found in the Rondônia and Inambari regions (**Supplementary Figure 3C**). The CANAPE analysis indicated that most communities are composed by mixed endemism (significantly high PE; **Figure 2B** and non-significant RPE; **Figure 2C**), that is, high in paleo and neo endemism, concentrated in the western, east, and northern regions of the Amazon and in Inambari and Xingu (**Figure 2D**). In addition, two communities in the Xingu and another in the northern Amazon region were dominated by paleo-endemisms (**Figure 2D**). The CANAPE results for Phyllostomidae indicated two additional endemism areas, a community dominated by neoendemism in Rondônia and a mixed community in Pantepui Duida (**Supplementary Figure 3D**). Overall, our endemism results seem to be robust considering the inclusion of all species, while also capturing the information from more ancient splits in the bat tree of life.

Bat Species Fit to Interfluve Areas of Endemisms

The fit between the distribution ranges of the species and the interfluve AoEs (Prediction 2) was generally low (mean 0.50)

(**Supplementary Table 2**). Of the total 177 species, only 20 were fully inserted within only one interfluve AoE range; ten of these were exclusive to Inambari AoE, six to Guiana, two to Napo, and one species each to Belém and Rondônia. All species with 100% of the occurrence within one AoE occupied at most 4% of each area. Furthermore, Jaú, Xingu, Tapajós, Pantepui Gran Sabana, and Pantepui Duida did not have exclusive species. In the analysis with the species of the Phyllostomidae family, 12 species were fully inserted within the interfluvial AoEs. This result shows that the Phyllostomidae are responsible for 60% of the species completely inserted within some of the interfluvial AoEs (**Supplementary Table 2**).

Beta-Diversity of Bats in the Amazon: Geographic Patterns of Turnover and Nestedness

The spatial variation in composition breaks of bat species in the Amazon was divided into three clusters (Prediction 3): two clusters separating the east and west regions and a third in northern Amazonia (**Figures 3A–D**). The Phyllostomidae dataset also presented three clusters and, while the overall pattern is similar to the complete dataset, the clusters are predominantly divided in a west to east fashion (**Supplementary Figures 4A,B**). The NMDS analysis showed a high correlation between the observed distance and the ordering distance (non-metric adjustment R^2 = 0.97, linear adjustment R^2 = 0.91), and even more prominent in the Phyllostomidae dataset (0.99, 0.98). The same was observed for the beta-diversity partitioning in the complete dataset turnover (0.93, 0.54) and nestedness (0.99, 0.98), and Phyllostomidae turnover (0.94, 0.61) and nestedness (0.99, 0.99), indicating that the analyses satisfactorily represented the Bray-Curtis distance matrices. The first NMDS axis indicated a division between the western end of the Amazon, along the Maranon and Madalena rivers, and the eastern region contemplating the extension of the Amazon River in the results of both datasets (**Supplementary Figures 5A**, **6A**). The second NMDS axis showed a composition break between the northern region of the Orinoco River and the southern region around the Madeira River (**Supplementary Figures 5B**, **6B**). The third NMDS axis indicated greater dissimilarity in the composition of species from the central Amazonian region around the Madeira and Negro rivers, outwardly to the other regions (**Supplementary Figures 5C**, **6C**). The variation in the composition of bat species in the Amazon was related to both turnover and nestedness but with a higher contribution of the turnover component for both datasets (**Supplementary Figures 5D–F**, **6D–F**). The correlation between the axes of the complete dataset (1, 2, and 3) of the total beta diversity and turnover were 0.80, 0.82, and −0.04, respectively (**Supplementary Figures 6D–F**), whereas the correlation between the axes of total beta diversity and nestedness were 0.62, −0.64, and −0.37 (**Supplementary Figures 5G–I**). The correlation between the axes for Phyllostomidae (1, 2, and 3) of the total beta diversity and turnover were −0.76, −0.30, and 0.69, respectively (**Supplementary Figures 6D–F**), whereas the correlation between the axes of total beta diversity and nestedness were −0.13, −0.28, and 0.42 (**Supplementary Figures 6G–I**).

There was no difference in the NMDS scores of the total beta diversity among the interfluvial AoEs (Prediction 4) (F = 1.593, df = 9, $p > 0.05$, ANOVA) (**Figure 4**). The clustering analysis using the PhyloRWTurnover index (Prediction 5) indicated that most lowland Amazonian communities share similar branches of the phylogenetic tree (**Supplementary Figure 7A**). This phylogenetic turnover analysis did not depict a community geographic structure that could be correlated to the interfluvial AoEs (**Supplementary Figure 7A**). The largest cluster is distributed over all sampled interfluvial AoEs, and the few smaller clusters are found in Inambari, two communities in northern Amazonia (outside the interfluvial AoEs limits) and one Andean community. A similar result was obtained with the Phyllostomidae dataset, the main difference being a cluster in the Pantepui Duida and Inambari regions (**Supplementary Figure 7B**).

DISCUSSION

In this study, we simultaneously assessed the importance of the currently recognized interfluve areas of endemism (AoE) and the patterns of beta-diversity and endemism (taxonomic and phylogenetic) for the assembly of bat communities in *cis*-Andean Amazon. We applied modern statistical approaches that improved our capacity to identify unique areas of evolutionary history (i.e., neo and paleo endemism) in order to better understand the importance of the Amazonian riverine system in structuring bat communities. Our results lend little to no support to the interfluve AoE hypothesis considering the complete dataset with 177 species and also the dataset with 101 phyllostomid species (which represents a very high species richness for one study, including close to 45% of all existing Phyllostomidae species). Furthermore, we did not find a clear geographic pattern pertaining to the influence of rivers in the distribution of phylogenetic and taxonomic endemism of bat communities. Our results also depicted a lack of fit of bat species to each interfluvial AoE, and also no community structure that could be associated to the interfluvial AoEs.

Because the traditional interfluve AoE hypothesis was mainly supported by historical biogeography evidence, no causality is necessarily expected among this body of evidence and processes determining community assembly in Amazonian bats, as recently shown for birds (Oliveira et al., 2017). In other words, there is no strong evidence that rivers are functioning as important ecological or evolutionary processes behind bat endemism patterns in the Amazon forest, which is supported by the main patterns we found following our hypothesis-testing framework. Furthermore, the evidence presented by Ruokolainen et al. (2019) indicate that the courses of many Amazonian rivers have gone through important changes in recent times (Pleistocene and Holocene), calling into question the use of current riverine arrangements to infer biogeographic patterns based on data on the distribution of organisms. Our results reinforce this interpretation, and suggest a geographic regionalization similar to that proposed by Tavares et al. (2017), separating the Amazonian bat biota into three groups that

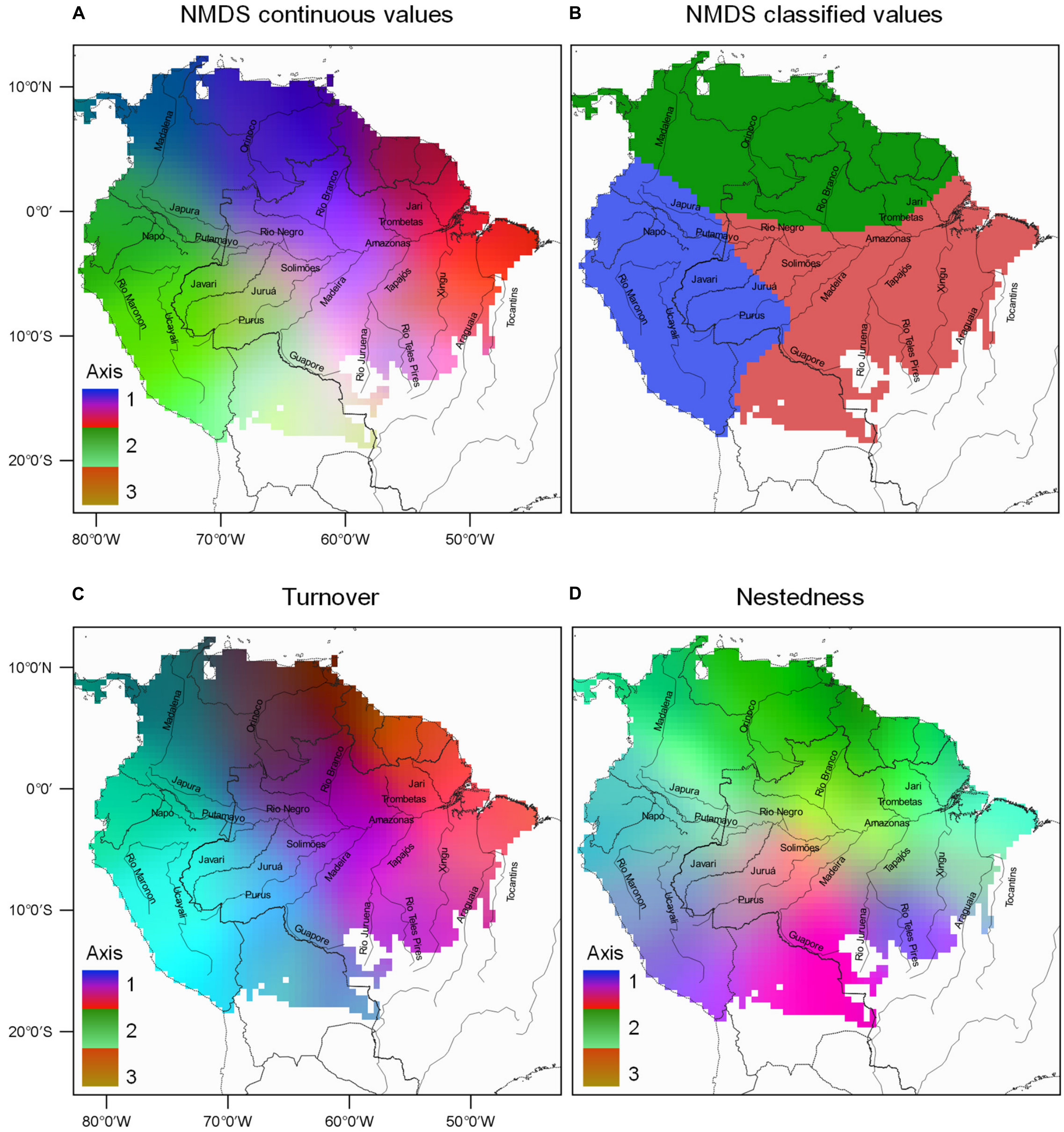

FIGURE 3 | Spatial variation in bats communities composition throughout the Amazon. **(A)** Species composition were obtained by the interpolation of NMDS scores (three axis represented by a RGB scale). **(B)** Classification of the first three breaks (most significant in search order) in species composition. **(C)** Spatial turnover of bat species composition throughout the Amazon. **(D)** Spatial nestedness of bat species composition throughout the Amazon.

are not consistent with the current distribution of Amazonian rivers: Eastern Brazilian Amazon, Guiana Shield, and Western Amazon. Additionally, a recent comparative phylogeography study (Silva et al., 2019) tested alternative diversification models, including the interfluve AoE hypothesis, and concluded that a geomorphological model consisting of roughly these same three geographic groups, as well as climatic history, better describes diversification patterns among upland "terra firme" Amazonian birds. The evidence, thus, indicates that other additional abiotic factors, such as temperature and climate history, and biotic interactions such as competition, might also play important roles in explaining bat diversity patterns in *cis*-Andean Amazon (Santorelli et al., 2018; Dambros et al., 2020).

Our main hypothesis, that Amazonian rivers are not an important ecological or evolutionary process behind bat endemism patterns, was corroborated. Although some rivers seem to be important in determining geographic patterns of species composition, our first prediction was corroborated and the results did not depict a clear influence of interfluvial AoEs in the geographic distribution of unbiased taxonomic endemism (CWE), phylogenetic endemism (PE-RPE), and paleo and neo

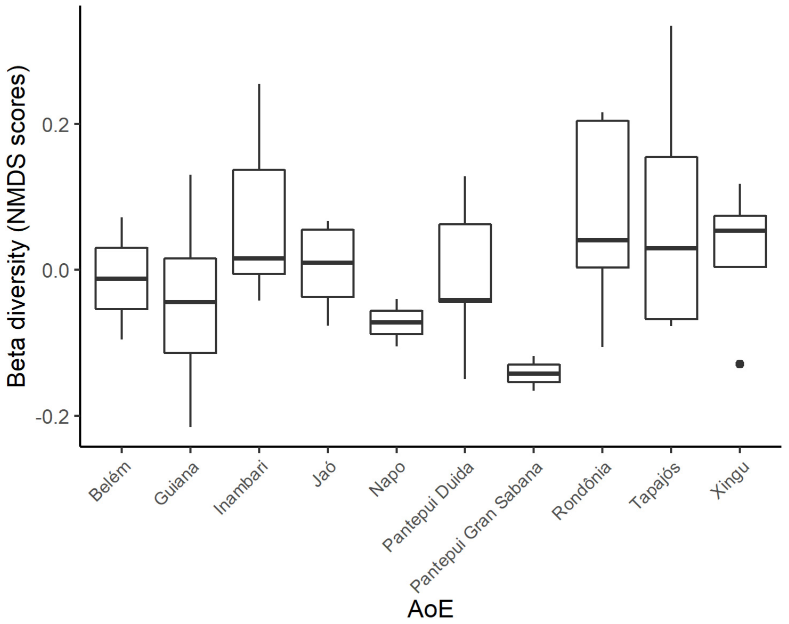

FIGURE 4 | Comparison of total beta diversity (NMDS scores) among interfluvial AoEs. No significant difference was found (ANOVA, $p > 0.05$), meaning that beta-diversity among communities within each interfuvial AoE is not smaller than beta-diversity among communities in different AoEs.

endemism (**Figure 2**). Nonetheless, we found two occasions where CWE regions geographically close to each other were separated by rivers, the Amazon and Tapajós rivers (**Figure 2A**), and one occasion where PE regions were separated by the Amazon river (**Figure 2B**). Thus, our results on bat endemism and distribution do not support the interfluvial AoE hypothesis as currently recognized (Borges and da Silva, 2012).

Our endemism analysis that took into consideration the branch lengths of the species in the phylogeny (CANAPE) showed that communities are mostly composed of mixed endemism, which is defined by the co-occurrence of short and long rare branches narrowly distributed in the landscape (Mishler et al., 2014; López-Aguirre et al., 2018; Azevedo et al., 2020). These communities are mainly distributed south of the Amazon River (**Figure 2D**), and this region is also home to a high proportion of endemic species found on very long branches (paleo-endemism), indicating that they are the current local survivors of clades supposedly older than the current landscape configuration. The ecological processes behind this pattern deserve further investigation, but the historical maintenance of suitable habitats and the species coexistence abilities might play important roles in species persistence in the landscape (Grandcolas et al., 2014; Veron et al., 2019). Sole neo-endemic communities were also less frequent for other taxonomic groups (Mishler et al., 2014; Scherson et al., 2017; Veron et al., 2019), and for New World bat superfamilies (López-Aguirre et al., 2018). Hence, the lack of neo-endemism in our data highlights the Amazon rainforest contribution to long-term species persistence in the landscape in the Neotropical region (Smith et al., 2014; Antonelli et al., 2018). Interestingly, the overall low amount of endemism in our bat communities (paleo, neo or mixed) supports the idea that even though species richness is quite high in the Amazon (Delgado-Jaramillo et al., 2020), most species inhabiting this area are geographically widespread and come from a relatively even sampling of the bat tree of life, with phylogenetic branches that are neither particularly long nor short (Mishler et al., 2014; Scherson et al., 2017).

Another important result corroborating our hypothesis, and in accordance with our second prediction, was the lack of congruence between the distribution limits of species within interfluvial AoEs. Few species occupied only a very small portion (∼4%) of the interfluvial AoEs, and most species (∼70%) occurred in five or more AoEs, a very similar pattern to what was found for the Amazonian avifauna (Oliveira et al., 2017). Moreover for the 22 species that did have 100% of their distribution in only one interfluvial AoEs, 30% are classified as data deficient (DD-six species) by the IUCN (**Supplementary Table 2**), which suggests that their distribution are still largely unknown and can extend far beyond one interfluvial AoE. Thus, their distribution might not necessarily be limited by rivers, considering that our results might be relatively biased by the lack of sampling efforts. Bat species have a high dispersal capacity, and foraging is influenced by the vertical availability of resources in the forest, which makes it difficult to collect species that forage above the canopy (Kalko and Handley, 2001; Carvalho et al., 2013; Farneda et al., 2019). We expected that individual bat species would not be restricted to interfluvial AoEs, due to their mobility and high dispersion capacity, but our results still suggest that larger rivers might act as dispersal barriers for a few species, which was also true for a few Amazonian birds (Oliveira et al., 2017).

Corroborating our third prediction, bat community composition breaks were divided into three clusters separating the east and west regions, with the third cluster in northern Amazonia (**Figure 3B**). Interestingly, the three clusters resemble those estimated for birds by Oliveira et al. (2017), indicating that major biotic regionalization processes might be at play for flying vertebrates in the Amazon (Santorelli et al., 2018; Dambros et al., 2020, but also see Ritter et al., 2019). Although no clear evidence for the role of rivers in the variation of bat communities composition was found, the general compositional changes over the landscape might fit a pattern of rivers acting as dispersal barriers for at least a few bat species. This finding may be related to the persistence of the species and its ability to disperse in the landscape matrix (Smith et al., 2014). In general, flying vertebrates have a large home range, and it would be expected that these species would only be affected by isolation at larger geographic scales (e.g., Trevelin et al., 2013; Aguiar et al., 2014; Arnone et al., 2016). Our results showed that compositional variation is more related to species turnover, which may indicate that some species ranges are indeed influenced by a high dispersion capacity, ultimately leading to lower regional endemism signals (e.g., Peixoto et al., 2014; Varzinczak et al., 2018). In addition, bat nestedness was mostly restricted to northern Amazon (**Figure 3D**), and species composition breaks in this region might be related to the distinct biogeographic history of the Guiana Shield (Fouquet et al., 2012), as well as to historical reasons related to the contraction of the Southern portion of the forest during the last glacial cycles (Rull, 2004; Rull and Nogué, 2007). Moreover, and corroborating our fifth prediction, the phylogenetic turnover analysis indicated that most communities share close related branches in the phylogenetic tree. Hence, this and our previous results indicate that bat communities in the Amazon are not geographically structured when phylogenetic history is taken into account, reinforcing the lack of evidence supporting the interfluvial AoEs hypothesis (**Supplementary Figure 7**).

Finally, we conclude that bat distribution and endemism in the Amazon are likely limited by factors other than rivers. Intraspecific and interspecific interactions, differences in environmental conditions among the regions, and other historical reasons might be more important drivers of bat distribution and endemism in this vast part of the Neotropical region. Here, we show the importance of analytical strategies that take into account the evolutionary history of the species (CANAPE) for inferring community-wide biogeographic patterns. In the future, the data assembled in this paper can also be used to investigate specific community assembly processes of each community based on the putative interaction among species (Ruffley et al., 2019), and the role of the dynamics of the Amazon biogeographic region and its intricate contribution to the long-term persistence of species based on ecological preferences (Crouch et al., 2019). This type of inference might be done in association with information on the biogeographic history of the Amazon, for example considering the Plio-Pleistocene climate change patterns (Hoorn et al., 2010, 2017; Nogueira et al., 2013; Rossetti et al., 2014), which might have promoted limits to dispersion for some species (Naka and Brumfield, 2018), particularly considering that phylogenetic endemism for mammals is mainly associated with energy availability and post-Last Glacial Maximum climate variability (Rosauer and Jetz, 2015). The bioregionalization pattern we found closely resembles that found for birds (Oliveira et al., 2017), and provides further evidence for the pattern suggested by Tavares et al. (2017). It is possible that this pattern reflects geological processes that are older than the formation of the rivers putatively structuring the interfluvial AoEs (Albert et al., 2021; Méndez-Camacho et al., 2021). Furthermore, the fact that we applied metrics that allow for the identification of complementary areas of biodiversity that have unique evolutionary histories, bring into light important geographic locations that might be taken into account in decision-making for conservation policies (Mishler et al., 2014).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

DS, HO, and FD conceived the main ideas, questions, and study design. DS and FD developed specific aims and hypotheses and decided on analytical approaches and methodology. DS and PZ collected the data. DS analyzed the data and led the writing of the manuscript. All authors critically contributed to the drafts and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.774083/full#supplementary-material

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Filtering Effect of Large Rivers on Primate Distribution in the Brazilian Amazonia

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Over a century after Wallace's proposition of the riverine barrier hypothesis, the role of rivers in the diversification of species remains a matter of interest in Amazon biogeography. Amazonian rivers, in particular those large and fast flowing rivers, are widely recognized to act as barriers to the dispersal of some organisms. However, the extent to which primate species changes across interfluves (β-diversity) in response to river features remains to be explicitly tested. In this study, we examine how river characteristics affect the variation in taxonomic and phylogenetic β-diversity to elucidate the ecological processes behind the differences in primate assemblages between neighboring interfluves in the Brazilian Amazonia. We compiled International Union for Conservation of Nature (IUCN) distribution maps of 80 river-bounded primate species in 10 interfluves separated by major rivers throughout the Brazilian Amazonia. We assessed both the taxonomic (disregarding phylogenetic relationships between species) and phylogenetic β-diversities. We applied multiple linear models to evaluate whether annual discharge, sinuosity, and reflectance (as a proxy for amount of sediments) in each river or river section that separates neighboring interfluves make rivers effective barriers to primates. We found that mean discharge has a positive effect, while both sinuosity and amount of sediments have negative effects on primate β-diversity. These variables have significant effects on total taxonomic and phylogenetic β-diversity between neighboring interfluves, and their species turnover components. River features, however, have no effect on species richness differences. Genera are capable of traversing almost all interfluves, but species are replaced by others in opposite interfluves. Discharge affected both small- and large-sized primate total β-diversity, but sinuosity only affected large-sized primate assemblages in neighboring interfluves. Our results indicate that although Amazonian rivers act as barriers for many primate species, this barrier effect seems limited to the short/medium time scales, as primate lineages are able to cross them over long-time scales.

Keywords: Amazonian rivers, biogeography, dispersal, macroecology, species turnover, vicariance, Wallace's riverine barrier hypothesis, β-diversity

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INTRODUCTION

Vicariant events such as the emergence of large rivers or uplift of mountain chains can favor allopatric speciation (Hoorn et al., 2010; Dal Vechio et al., 2019; Mendez-Camacho et al., 2021). In particular, large Amazonian rivers have been considered the most important engines of speciation by vicariance in this biome. Wallace (1852) was the first to notice that large Amazonian rivers delimit the distribution of species and in particular of primates, thus acting as barriers to dispersal in an otherwise featureless landscape. More recently, several authors refined this idea, which became known as the riverine barrier hypothesis (Patton et al., 1994; Gascon et al., 2000). The main prediction of this hypothesis is that congeneric species are separated by large rivers and their evolution was a result of the formation of said river which severed the original population in two which with time, speciate by vicariance (Patton et al., 1994). The riverine barrier hypothesis, which has been extensively tested in the last decades, is currently accepted as an important mechanism of speciation in Amazonia. It was confirmed in several taxonomic groups, including birds, mammals, and some reptiles (Ayres and Clutton-Brock, 1992; Peres et al., 1996; Vallinoto et al., 2006; Naka, 2011; Ribas et al., 2012; Boubli et al., 2015; Lynch Alfaro et al., 2015; Dal Vechio et al., 2019; Fordham et al., 2020). Although the diversification of Amazonian primates has been largely attributed to allopatric speciation by vicariance produced by rivers (Hershkovitz, 1977; Peres et al., 1996; van Roosmalen et al., 2002; Vallinoto et al., 2006; Boubli et al., 2015; Lynch Alfaro et al., 2015), some studies have shown that rivers historically considered as geographic barriers do not isolate primate populations living in their opposite banks (Craul et al., 2008; Link et al., 2015). In addition, little attention is taken on the physical and chemical characteristics of rivers that do act as barriers promoting primate diversification (e.g., Ayres and Clutton-Brock, 1992; Fordham et al., 2020).

The Amazon basin is a transcontinental drainage that holds some of the largest and highest-discharge rivers in the world (Latrubesse et al., 2005; Wohl, 2007). Amazonian rivers are quite distinct and dynamic, showing distinct features, namely river size (i.e., discharge and width), sinuosity, and sediment load (Ayres and Clutton-Brock, 1992; Latrubesse et al., 2005, 2010; Boubli et al., 2015), which can influence species dispersal chances. However, while river size is frequently evaluated as a driver for primate diversification (Ayres and Clutton-Brock, 1992; Fordham et al., 2020), the effects of other variables such as sinuosity and amount of sediments remain elusive. Consequently, additional factors responsible for the differential filtering of species by rivers are still poorly known. For instance, the continuous accumulation of sediments in some river stretches over time can create islands, that even temporarily, act as stepping stones, narrowing river width and ultimately facilitating animal crossing between riverbanks, mainly by those species with lesser swimming abilities (Ayres and Clutton-Brock, 1992; Peres et al., 1996; Sillero et al., 2018). Riverbank erosion can also promote sediment transport between riverbanks causing changes in river form and width, and in the location of islands (Hooke, 2003; Peixoto et al., 2009; Constantine et al., 2014). Sinuosity grows rapidly in sediment-rich rivers, making them more susceptible to such changes (Constantine et al., 2014; Ahmed et al., 2019). Sinuosity may also facilitate the dispersal of individuals between riverbanks given that meander cutoffs can physically transfer masses of land between opposite riverbanks, including their associated fauna and flora (Ayres and Clutton-Brock, 1992; Gascon et al., 2000). Recent accumulating evidences on the formation of paleo-channels throughout Amazonia indicate changes in direction and form of many rivers over time (Cremon et al., 2016; Ruokolainen et al., 2019; Rossetti et al., 2021). All these aforementioned processes can increase opportunities for passive lateral dispersal of individuals between riverbanks (Rabelo et al., 2014; Lynch Alfaro et al., 2015).

Several analytic tools that were recently developed can be used to investigate how large rivers can promote changes in β-diversity on a macrogeographic scale. One of these tools is the partitioning of β-diversity in two components: turnover and nestedness (Baselga, 2010; Carvalho et al., 2012). Turnover represents the replacement of species between communities, while nestedness represents the loss or gain of species between communities (Baselga, 2010; Carvalho et al., 2012). β-diversity partitioning allows the incorporation of phylogenetic trees, which improves our interpretation of the effects of different drivers on biodiversity (Graham and Fine, 2008; Cardoso et al., 2014). Taken together, those approaches that take into account more than one dimension of diversity allow us to generate more robust conclusions about how different biogeographic issues (e.g., barriers to dispersal) act on different processes (e.g., speciation, changes in species composition) or patterns (e.g., species distribution) (Graham and Fine, 2008; Miller et al., 2018). For example, disentangling the nestedness and turnover components of the β-diversity allows us to verify if the barriers completely preclude movements of some lineages, or if they are somewhat permeable in the geologic time scale. The comparison of the taxonomic and phylogenetic β-diversity may also give insights about the timescale in which the barriers preclude dispersal. For instance, while taxonomic β-diversity can inform about changes occurring at short timescales (changes in species occupying different riverbanks), phylogenetic β-diversity can provide information at long timescales (changes at higher taxonomic levels, with greater barriers separating more distant lineages within the genera, and smaller barriers separating closely related species). Barriers that impede movement at longer timescales cause higher effects on the phylogenetic β-diversity, thus generating more contrasting assemblages. Such analytic approaches are still uncommon in biogeographic analysis in Amazonia (e.g., Fluck et al., 2020; Carvalho et al., 2021). Most studies so far have focused only on the taxonomic component of β-diversity, without considering the phylogenetic component or the partitioning of β-diversity (e.g., Ayres and Clutton-Brock, 1992; Dambros et al., 2017, 2020; Fordham et al., 2020).

In this study, we investigated the role of large Amazonian rivers as effective physical barriers to primates, which have limited dispersal abilities (Schloss et al., 2012; Sales et al., 2019). Specifically, we investigated the characteristics of rivers (i.e., discharge, load of sediments, and sinuosity) as drivers of isolation for primate communities distributed among 10 major Amazonian interfluves. To do that, we estimated both taxonomic and phylogenetic β-diversity and their partitioning, turnover and nestedness, between pairs of neighboring interfluves, relating them with the characteristics of the rivers that separate these interfluves. If the hypothesis of rivers as barriers for primates is correct, we expect less similarity (great taxonomic and phylogenetic β-diversity) across riverbanks (Ayres and Clutton-Brock, 1992). Thus, we expect to find a positive relationship between the dissimilarity of primate communities and river discharge (Ayres and Clutton-Brock, 1992; Fordham et al., 2020), and negative relationships with sediment load and river sinuosity (Ayres and Clutton-Brock, 1992; Gascon et al., 2000). Furthermore, considering the phylogenetic dimension, we expect that rivers that act as strong geographic barriers, preclude the crossing of some lineages (i.e., genera), leading to a stronger effect of lineage loss (nestedness) than lineage sharing (turnover). Additionally, assuming the lower chances and lower frequency of crossing in rivers that act as stronger geographic barriers, we expect that lineages on opposite banks of larger rivers will diverge more, and consequently, that the effect of large rivers as geographic barriers will be stronger for phylogenetic β-diversity than for taxonomic β-diversity. Finally, considering the swimming ability of large-sized primates, we expect a lower effect of rivers on them, implying they are less limited by rivers and, consequently, more widely distributed (i.e., occupying more interfluves) than small- and mid-sized species (Ayres and Clutton-Brock, 1992).

MATERIALS AND METHODS

Study Area

The Amazonia is divided by several large rivers that delimit large interfluvial areas (i.e., landmasses between major rivers) which are often considered as areas of endemism for birds and primates (Cracraft, 1985; da Silva and Oren, 1996; da Silva et al., 2005; Naka, 2011; Ribas et al., 2012). The composition of species within these interfluves have provided interesting opportunities to study spatial patterns of species distribution. Here, we used interfluves to investigate the effects of large rivers on the patterns of primate β-diversity in Brazilian Amazonia. We restricted our study to the Brazilian Amazonia because both its primate fauna and river characteristics are well known and freely available (Ayres and Clutton-Brock, 1992; Paglia et al., 2012). Although limited in geographical scope, the Brazilian Amazonia covers over 60% of the entire Amazon basin and all interfluves but Napo have most of their extensions within Brazil (Juen and De Marco, 2012). Additionally, our study area encompasses the mid and lower parts of all major rivers that form this basin, presumably the region where we would expect to see the greatest effect of rivers as barriers for primate dispersal. In contrast, their headwaters are known to have a limited role in isolating species (Ayres and Clutton-Brock, 1992; Peres et al., 1996; Fordham et al., 2020).

We selected interfluves based on previous studies with terrestrial vertebrates, which identified 10 large biogeographic regions separated by major rivers in Amazonia (da Silva et al., 2005; Naka, 2011; Borges and Da Silva, 2012; Ribas et al., 2012; Silva et al., 2019), as follows (**Figure 1**): Guiana (between the Amazon, lower Negro, and Branco rivers), Pantepui-Duida (between Branco and mid and upper Negro rivers), Imeri (between Negro and Uaupés rivers), Napo (between Uaupés, Negro, Uneiuxi, Pucabi, Juami, Içá, Japurá, and Napo rivers), Jaú (between Napo/Solimões, Negro, Uneiuxi, Pucabi, Juami, and Içá rivers), Inambari (between Napo/Solimões, Amazonas, and Madeira rivers), Rondônia (between Madeira and Tapajós rivers), Tapajós (between Tapajós and Xingu rivers), Xingu (between Xingu and Tocantins rivers), and Belém (between Tocantins and Amazon rivers). Our sample units were the pairs of neighboring interfluves distributed along the study area ($N = 17$, **Table 1**).

Data Collection

We included only primate species occurring in the Brazilian Amazonia. Their taxonomy followed Paglia et al. (2012), which was based on previous taxonomic studies of Neotropical primates (Rylands et al., 2000; Rylands and Mittermeier, 2009). However, we also consider recent species descriptions and taxonomic changes (Ferrari et al., 2010, 2014; Boubli et al., 2012; Gualda-Barros et al., 2012; Lynch Alfaro et al., 2012; Dalponte et al., 2014; Byrne et al., 2016; Rylands et al., 2016; Silva et al., 2018; Serrano-Villavicencio et al., 2019). Our database included 80 (65%) out of the 124 primates with confirmed occurrence in the Amazonia (Primate Specialist Group website, 2021) and available in the updated International Union for Conservation of Nature's (IUCN) red list[1] (**Supplementary Table 1**). Although distribution maps such as those obtained by IUCN are not reliable to infer about the presence/absence of species in specific points (Herkt et al., 2017; Fluck et al., 2020), we used them only to obtain species occurrence within a given interfluve, and not to assume their occurrence in specific sites within the interfluves or to run species distribution models. Then, we are confident that their use in this study was appropriate, minimizing possible distribution errors.

Water is considered to pose high resistance to primate dispersal (Rabelo et al., 2019). However, although uncommon, primates occasionally swim to cross rivers (Chaves and Stoner, 2010; Gonzalez-Socoloske and Snarr, 2010; Nunes, 2014). Body size should be a key characteristic influencing the ability of primates to disperse across rivers (Ayres and Clutton-Brock, 1992). In fact, all the available reports come from large-sized atelids, and we are not aware of any report on small- or mid-sized species swimming in the wild. We thus considered primate dispersal ability across rivers as limited and species-specific (Ayres and Clutton-Brock, 1992; Schloss et al., 2012; Rabelo et al., 2019). We thus compiled the average body mass (kg) of the species from Smith and Jungers (1997), classifying them according to their body masses as small- (<1.5 kg), mid- (1.5–4.99 kg), or large-sized species (≥ 5.0 kg).

River Characteristics

We estimated mean annual river discharge by averaging the values recorded in all measuring stations for a given river obtained from the Global Runoff Data Centre (GRDC, Germany[2]) (Fordham et al., 2020), which provides *in situ* annual

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[1] www.iucnredlist.org/

[2] https://www.bafg.de/GRDC/EN/Home/homepage_node.html

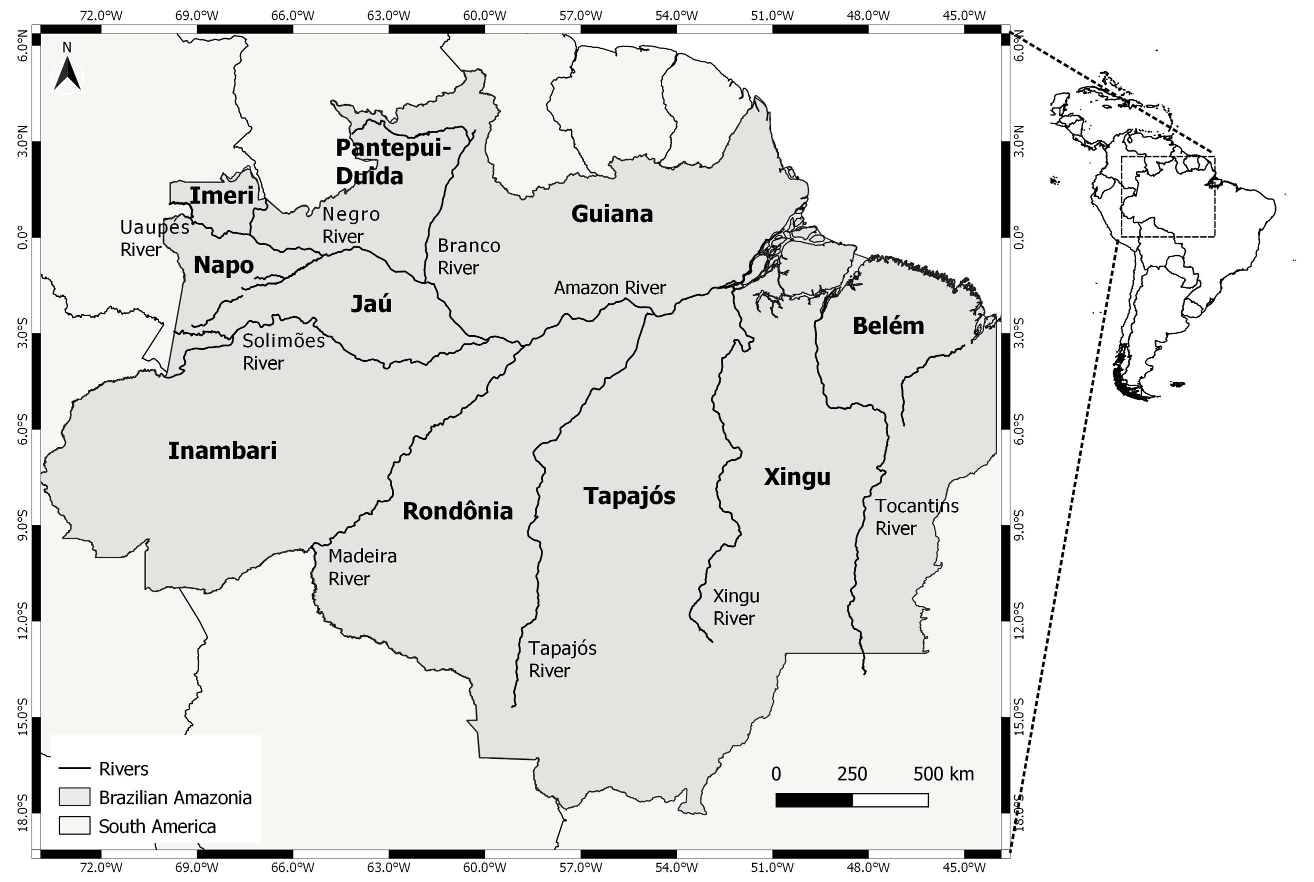

FIGURE 1 | Major interfluves of the Brazilian Amazonia, recognized as different biogeographic provinces.

river discharge times series data for several rivers worldwide. Since the GRDC does not have data for all sections of the Amazon River, the discharge of missing sections were estimated through a simple linear regression between the discharge and the distance from the river beginning (bifurcation between Solimões and Negro rivers) and its estuary (Fordham et al., 2020). Relatively small rivers such as Uneiuxi, Pucabi, and Juami (that separate Napo and Jaú interfluves) have no annual discharge data available were arbitrarily set to 1000 m^3/s.

We established a river sinuosity index (SI) by measuring the straight distance between river headwaters and mouths (D1), and the total length of the rivers (D2) using the length function in the QGIS 3.16 software (QGIS Development Team, 2022). Then, we calculated the SI through the following formula: $SI = 1 - (D1/D2)$. The SI values ranged from 0.064 to 0.306, with higher values representing rivers that are more sinuous. We obtained the sediment load of the rivers based on the empirical correlation between the red light reflectance and the sediment load in sampling stations, provided by Fassoni-Andrade and Paiva (2019) as a high resolution raster file with reflectance values in 250-m pixels. Then, we averaged the sediment load of five points along each river (or river section), following Laranjeiras et al. (2021). River characteristics are described in **Supplementary Table 2**.

Analysis

Based on the species distribution overlaid on a map of the major Amazonian rivers, we built a matrix of primate occurrence (presence/absence) in each interfluve (**Supplementary Table 1**). Since our goal was to evaluate the effect of large rivers on the pattern of β-diversity among Amazonian primates, we disregarded those species that occurred only in the central portion of the interfluve, i.e., those species whose distribution was not limited by any river. Our reasoning was that factors other than rivers, such as vegetation and edaphic variation (habitat type), relief, and/or distance *per se* must limit these species (Emmons, 1984; Gascon et al., 2000). From the occurrence matrix, we calculated the total taxonomic β-diversity ($\beta_{total.tax}$) for each pair of interfluves, and their components, turnover (i.e., replacement of one species by another – $\beta_{turn.tax}$) and nestedness (i.e., richness difference due to gain or loss of species – $\beta_{rich.tax}$) (Carvalho et al., 2012). We also calculated the total phylogenetic β-diversity ($\beta_{total.phy}$) and its components, turnover (i.e., lineage replacement – $\beta_{turn.phy}$) and nestedness (i.e., gain or loss of lineages – $\beta_{rich.phy}$). To calculate phylogenetic β-diversity, we built a phylogenetic tree with all the species included in our analysis from recent phylogenies of all the genus with more than two species (**Supplementary Table 3** and **Supplementary Figure 1**). For *Pithecia*, for which we could not find any

TABLE 1 | Primate β-diversity in the 10 major biogeographic provinces along the Amazonia.

Interfluves separated by rivers[a]	River ID	$\beta_{total.tax}$	$\beta_{turn.tax}$	$\beta_{rich.tax}$	$\beta_{total.phy}$	$\beta_{turn.phy}$	$\beta_{rich.phy}$	$\beta_{tax.small}$	$\beta_{tax.mid}$	$\beta_{tax.large}$
IME-PAN	Negro	0.8125	0.7500	0.0625	0.4660	0.4338	0.0322	0.600	1.000	0.750
PAN-NAP	Negro	0.9630	0.6667	0.2963	0.6472	0.3794	0.2677	0.917	1.000	1.000
PAN-JAU	Negro	0.9630	0.6667	0.2963	0.6166	0.3381	0.2785	0.909	1.000	1.000
NAP-JAU	Uneiuxi-Pucabi-Juami	0.3636	0.3636	0.0000	0.1697	0.1632	0.0065	0.273	0.500	0.250
GUI-JAU	Negro	1.0000	0.8387	0.1613	0.6706	0.4817	0.1889	1.000	0.875	1.000
GUI-PAN	Branco	0.8500	0.7000	0.1500	0.5712	0.4841	0.0871	0.875	0.875	0.667
NAP-IME	Uaupés	0.6500	0.2000	0.4500	0.4101	0.1277	0.2824	0.727	0.600	0.500
INA-JAU	Solimões	0.8372	0.5116	0.3256	0.4656	0.3112	0.1544	0.909	0.714	0.200
NAP-INA	Solimões	0.8636	0.5455	0.3182	0.4989	0.3425	0.1564	0.913	0.750	0.400
GUI-INA	Amazonas	1.0000	0.5778	0.4222	0.7184	0.4117	0.3067	1.000	0.889	1.000
RO-GUI	Amazonas	0.9500	0.5500	0.4000	0.6741	0.4269	0.2472	0.947	0.875	1.000
TAP-GUI	Amazonas	0.9259	0.8148	0.1111	0.6410	0.5765	0.0645	0.929	0.857	1.000
GUI-XIN	Amazonas	0.9000	0.7000	0.2000	0.6137	0.5170	0.0967	0.900	0.833	1.000
INA-RO	Madeira	0.8269	0.7692	0.0577	0.5422	0.4615	0.0806	0.926	0.500	0.200
RO-TAP	Tapajós	0.8462	0.5128	0.3333	0.5172	0.3093	0.2079	0.905	0.250	0.857
XIN-TAP	Xingu	0.6111	0.2222	0.3889	0.3691	0.1785	0.1906	0.600	0.750	0.500
BEL-XIN	Tocantins	0.6667	0.5000	0.1667	0.3635	0.2023	0.1612	0.667	0.750	0.500

[a] IME, Imeri; PAN, Pantepui-Duida; NAP, Napo; JAU, Jaú; GUI, Guiana; INA, Inambari; RO, Rondônia; TAP, Tapajós; XIN, Xingu; BEL, Belém. $\beta_{total.tax}$, total taxonomic beta diversity; $\beta_{turn.tax}$, turnover component of the taxonomic beta diversity; $\beta_{rich.tax}$, richness difference component of the taxonomic beta diversity; $\beta_{total.phy}$, total phylogenetic beta diversity; $\beta_{turn.phy}$, turnover component of the phylogenetic beta diversity; $\beta_{rich.phy}$, richness difference component of the phylogenetic beta diversity; $\beta_{tax.small}$, taxonomic beta diversity of the small-sized primates (<1.5 kg); $\beta_{tax.mid}$, taxonomic beta diversity of the mid-sized primates (1.5–4.99 kg); $\beta_{tax.large}$, taxonomic beta diversity of the large-sized primates (≥5 kg).

phylogeny, and for those genera for which we found two or more inconsistent phylogenies, we used a conservative approach, disregarding any hierarchy for the species/clades involved in the inconsistencies. The phylogenetic relationships of the genera was based on molecular data provided by Perelman et al. (2011). For both dimensions of the β-diversity (taxonomic and phylogenetic) we used the Jaccard distances with 100 resamples. β-diversity was calculated through the function *beta* using the package *BAT* (Cardoso et al., 2021), in R 3.6.3 (R Core Team, 2020).

The β-diversity (both taxonomic and phylogenetic) and its components ($\beta_{total.tax}$, $\beta_{turn.tax}$, $\beta_{rich.tax}$, $\beta_{total.phy}$, $\beta_{turn.phy}$, $\beta_{rich.phy}$, $\beta_{tax.small}$, $\beta_{tax.mid,}$ $\beta_{tax.large}$) between neighboring interfluves were used as response variables to infer which river characteristics made them effective biogeographic barriers using multiple linear regressions. We used mean discharge, sinuosity and sediment load as predictor variables. River discharge was log-transformed for normality. We standardized all predictor variables to zero mean and unit variance. We selected the most parsimonious models based on their Akaike Information Criterion corrected for small samples (AICc). We checked model residuals for spatial autocorrelation using Moran's I. Since our sampling units in the linear models were pairs of interfluves, we built a "distance class connectivity matrix" (Fortin and Dale, 2005) by accessing the shorter connections between pairs of interfluves, considering the number of rivers between them +1. We discarded only one model for which the residuals showed spatial autocorrelation ($\beta_{tax.large}$). We checked the residuals of the models for normality through Shapiro–Wilk's test. Significance level was set at 0.05.

RESULTS

Over half of the 80 Amazonian primate species evaluated ($N = 46$; 58%) were restricted to only 1 of the 10 interfluves defined in this study (**Table 2**). Conversely, eight species (10%) were widely distributed (≥4 interfluves), with *Sapajus apella* occupying nine interfluves (**Table 2** and **Supplementary Table 1**). Contrary to our expectations, only three of the eight species with wider distributions were large-sized (*Alouatta seniculus*, *Ateles chamek*, *Lagothrix lagotricha*, **Supplementary Table 1**).

The Inambari interfluve presented the greatest primate species richness in the Brazilian Amazonia (N = 26 species),

TABLE 2 | Frequency of occurrence of primate species in the major interfluves of the Brazilian Amazonia.

Number of interfluves occupied	Number of species	%
1	46	57
2	19	24
3	8	10
4	5	6
5	2	2
9	1	1
Total	81	100

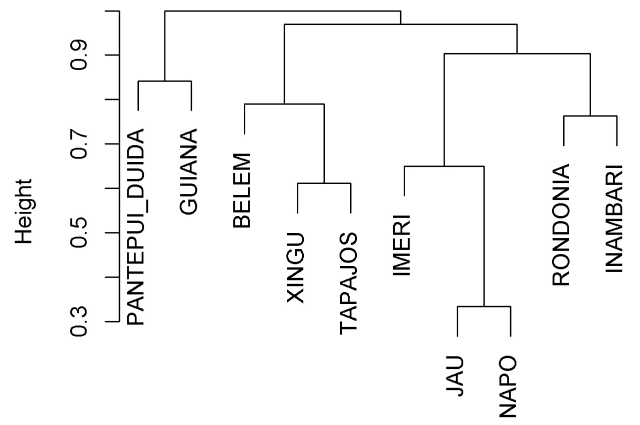

FIGURE 2 | Cluster analysis of the major interfluves in the Brazilian Amazonia based on the primate species composition.

followed by the Rondônia interfluve ($N = 23$). The other interfluves presented from 7 to 18 species (**Supplementary Table 1**). The average dissimilarity between interfluves in the study area was 0.821 (range: 0.333–1.000). We identified three distinct groups of interfluves sharing species among themselves. They can be roughly divided between the northern and southern (plus Imeri) interfluves, with the southern group divided in western and eastern groups (**Figure 2**). Napo and Jaú interfluves presented the lowest dissimilarity (0.333), while the highest dissimilarity was observed between Pantepui-Duida and all southern interfluves (Belém, Xingu, Tapajós, Rondônia, Inambari). Indeed, given the high endemism, most of the interfluve pairs presented high dissimilarity among them (>0.7, **Supplementary Table 4**). Rondônia ($N = 13$) and Inambari ($N = 9$) interfluves presented the highest number of endemic species, followed by Guiana ($N = 7$). Other interfluves presented from zero to three endemic species (**Supplementary Table 1**).

Primate communities were more similar in interfluves separated by sinuous rivers. Conversely, rivers with greater discharge resulted in less similar communities across their banks. The effect of the sediment load in the role of rivers as barriers was observed only for $\beta_{\text{total.tax}}$ and rivers with lower sediment load separated more dissimilar assemblages (**Table 3**).

As expected, phylogenetic β-diversity ($R^2 = 0.794$) was better explained by river characteristics than taxonomic β-diversity ($R^2 = 0.762$), although the difference was relatively small. Mean discharge had the strongest effect on both taxonomic and phylogenetic components of primate β-diversity in the Brazilian Amazon basin, followed by sinuosity (**Table 3**). Moreover, we found that river characteristics explained better the β-diversity of small-sized ($R^2 = 0.601$) than large-sized primates ($R^2 = 0.542$), although we did not detect any effect for mid-sized primates. Furthermore, river characteristics had an effect on the turnover component of the β-diversity, but they were not good predictors of the nestedness component of the β-diversity at all, a pattern that was consistent for both the taxonomic and phylogenetic β-diversity (**Table 3**).

DISCUSSION

The diversification of Amazonian species is complex (Hoorn et al., 2010; Mendez-Camacho et al., 2021), resulting from multiple mechanisms of vicariance (e.g., Andes uplift, emergence or redirection of large rivers, changes in the vegetation caused by climate change) and non-vicariance (e.g., expansion of species distribution, habitat gradients, human-driven barrier transposition), acting independently or interactively (da Rocha and Kaefer, 2019). Our results support the role of rivers as key agents in spatially structuring Amazonian primate diversity. Confirming our hypothesis, we found significant evidence for a positive effect of mean annual discharge and a negative effect of sinuosity on primate β-diversity. While previous studies have already showed the role of rivers as barriers for Amazonian primates (mainly because of annual discharge: Ayres and Clutton-Brock, 1992; Fordham et al., 2020), our study goes beyond, showing both discharge and sinuosity as the main drivers of changes in primate β-diversity in Amazonia.

Rivers with higher annual discharge have a greater positive effect on primate composition changes between opposite riverbanks, meaning that higher discharge leads to less similar primate communities. It makes sense that discharge act more effectively to prevent primate flux across opposite riverbanks. On one hand, wide, low discharge rivers favor animals crossing using rocks (i.e., stepping stones), islands and/or fallen trees, especially during drier periods (Mamalis et al., 2018). In fact, the resistance caused by discharge to the dispersal of animals is lower during the dry season (Prevedello and Vieira, 2009; Rabelo et al., 2019). On the other hand, high discharge rivers tend to keep higher volumes of water in their main channel even during the driest periods, thus inhibiting primates and other terrestrial animals to cross between riverbanks. Other studies have used width as a measure of river size (Ayres and Clutton-Brock, 1992; Fordham et al., 2020). However, river discharge is probably stronger than river width as a feature promoting the isolation of primates in the Amazonia because width is highly variable (Fordham et al., 2020). Moreover, there are so far many dams built along several Amazonian rivers (Lees et al., 2016), which can artificially input biases on the measurement of river width and, consequently, on the influence of this variable on primate speciation.

Previous studies did not investigate (Ayres and Clutton-Brock, 1992) or find significant results on the role of sinuosity in primate diversification (Fordham et al., 2020). However, we claim that a simple comparison of rivers draining different watersheds, as tested by Fordham et al. (2020), is not appropriate to test this issue because rivers within different watersheds may show similar dynamics (**Supplementary Table 5** and **Supplementary Figure 2**). In fact, mean sinuosity among black water (mean ± SD = 0.819 ± 0.09) and white water rivers (0.817 ± 0.07) are quite similar. For instance, river stretches within the Negro River watershed, which have predominantly black water, are as sinuous as those draining the predominantly white water rivers of the Solimões watershed (see **Supplementary Table 2**). Nevertheless, our results indicate that, independently of basin, sinuous rivers seem to be less effective barriers for primates than straight rivers. The main explanation for the

TABLE 3 | Results of the multiple regression models.

Response variable	R^2	Predictors in the most parsimonious model	Standardized coefficient	SE	*p*
$\beta_{total.tax}$	0.762	Intercept	0.8253	0.0219	<0.001
		Sediment load	−0.0826	0.0352	0.035
		Mean discharge	0.1674	0.0367	<0.001
		Sinuosity	−0.0519	0.0244	0.053
$\beta_{turn.tax}$	0.392	Intercept	0.5817	0.0368	<0.001
		Sinuosity	−0.1179	0.0379	0.007
$\beta_{rich.tax}$*	–	–	–	–	–
$\beta_{total.phy}$	0.794	Intercept	0.5267	0.0171	<0.001
		Sediment load	−0.0556	0.0279	0.067
		Mean discharge	0.1271	0.0291	<0.001
		Sinuosity	−0.0594	0.0193	0.009
$\beta_{turn.phy}$	0.415	Intercept	0.3615	0.0254	<0.001
		Mean discharge	0.0854	0.0261	0.005
$\beta_{rich.phy}$*	–	–	–	–	–
$\beta_{tax.small}$	0.601	Intercept	0.8233	0.0327	<0.001
		Mean discharge	0.1391	0.0337	<0.001
$\beta_{tax.medium}$*	–	–	–	–	–
$\beta_{tax.large}$	0.542	Intercept	0.6955	0.0550	<0.001
		Mean discharge	0.0832	0.0592	0.182
		Sinuosity	−0.1924	0.0592	0.006

**The null model was the most parsimonious model.*

greater permeability of sinuous rivers is the meandric dynamic. Meandering rivers can show relatively frequent cutoffs (Hooke, 2003; Constantine et al., 2014), that increase opportunities for lateral passive dispersal of individuals between opposed riverbanks (Peres et al., 1996). Additionally, recent reports of paleo-channels in Amazonia indicate they were produced by changes in the direction of some large rivers (Ruokolainen et al., 2019; Rossetti et al., 2021). These changes transferred large landmasses from one riverbank to the opposite side together with all animals and plants living there. Moreover, the dynamics of sedimentation and erosion in islands are also greater in sinuous rivers (Peixoto et al., 2009; Ahmed et al., 2019), partly contributing to their filtering effect on primates.

The effects of sediment load on the diversification of primate communities is somewhat controversial and limited to the (total) taxonomic β-diversity (**Table 3**). Although the general idea that sediment load can be related to the sedimentation and erosion dynamics (mainly in islands and riverbanks), thus creating stepping stones that can facilitate species crossing over rivers (Rabelo et al., 2014; Lynch Alfaro et al., 2015), this dynamic can also be related to river sinuosity (Constantine et al., 2014; Ahmed et al., 2019). However, in some cases, sediment load is stronger than river sinuosity *per se* (see **Table 3**). For instance, the high sediment load associated with the long-term dynamic of island formation can be important in the Amazonas River mouth, where some mid- and large-sized species such as *Aotus azarae* and *Alouatta belzebul*, occur in both riverbanks. The Amazon River mouth is characterized by multiple, non-shifting channels with high sediment load, subjected to high and complex sedimentation and erosion dynamics in its islands and banks, thus promoting permeability (Ayres and Clutton-Brock, 1992; Gensac et al., 2016). Although there are no evidence that the Madeira River, which have the second higher sediment load among the large Amazonian rivers, act as an effective barrier for many taxa (Santorelli et al., 2018), it split out several primate species occupying the Inambari and Rondônia interfluves, including *Callibella humilis*, *Cebuella pygmaea*, *Mico rondoni*, *Saguinus labiatus*, *Sapajus apella*, *Cebus unicolor*, and several species of *Plecturocebus* (*P. baptista*, *P. bernhardi*, *P. caligatus*, *P. cinerascens*, *P. dubius*).

Confirming our prediction, rivers characteristics affected more the phylogenetic β-diversity than the taxonomic β-diversity, indicating that the taxa separated by the bulkier and less sinuous rivers are more phylogenetically distant than taxa separated by small and more sinuous rivers. The small difference in the explanatory power of the models can be explained by a possible mismatch in the temporal scale. Rivers act as barriers for primates species or even for divergent populations of some species (e.g., Vallinoto et al., 2006) at shorter temporal scales, while a considerable part of phylogenetic β-diversity occurs at longer temporal scales (i.e., changes at higher taxonomic levels, with greater barriers separating more distant lineages within the genera, and smaller barriers separating closely related species).

The temporal scale can also explain why we do not observe differences in the nestedness component of the β-diversity. In fact, many primate genera are widely distributed across several Amazonian interfluves, indicating that rivers are rarely an insurmountable barrier for some lineages. Although the low, wide river stretches located next to river mouths can be considered as barriers to primate dispersal (but see the case of the Amazon River mouth discussed above), it is unlikely that river headwaters (i.e., high, narrow stretches) fulfill the same role. For instance, some primates occur in both riverbanks, and there is a low genetic divergence between populations living in riverheads, as compared to those located near river mouths (Peres et al., 1996; Vallinoto et al., 2006). Stretches considerably narrow and shallow can allow river crossings, thus increasing dispersal chances. This can be the case of *Mico emiliae*, which appears to have crossed the headwaters of the Iriri River, thus allowing the extension of its distribution as far to the western bank of the Xingu River (Andrade et al., 2018). Considering that most Platyrrhini genera had their origin estimated to at least 6 million years ago (Schneider et al., 2001; Lynch Alfaro et al., 2012; Byrne et al., 2018), there was enough time for them to cross through river waterheads, thus expanding across several Amazonian interfluves in some cases.

Considering that the species of primates are not homogeneously distributed across the Amazonia, the role of rivers as barriers has implications for the conservation of species. The Amazonia, which has the highest primate diversity in the world (Rylands et al., 2000; Rylands and Mittermeier, 2009), also suffers a spatially biased pressure from deforestation. For instance, while the Belém, Xingu, Tapajós, and Rondônia are located at the arc of deforestation, where forest clearance advances at an accelerated pace, other interfluves, such as Guiana, Imeri and Napo are still kept relatively well preserved (da Silva et al., 2005; Fearnside, 2005; Soares-Filho et al., 2006; Broadbent et al., 2008; Alves, 2010; Kalamandeen et al., 2018). Considering the high endemism level in some interfluves (e.g., Rondônia), the loss of forests cannot be compensated by forest maintenance in other interfluves, under penalty of loss of unique endemics. Even in areas with low endemism there is a real risk of loss of the few endemic species present in some highly deforested interfluves, such as Belém and Xingu.

Our results point out to the existence of at least three distinct primate groups in the Brazilian Amazonia, based on the similarity among the communities occurring in each interfluve. The dissimilarity between the northern and southern groups can be explained by the clear geomorphological differences between the Guiana (nutrient-poor sandy soils drained by black water) and Brazilian (rich-nutrient alluvial soils drained by white and clear waters) shields, divided by the Amazon River. On the other hand, the dissimilarity between the western and eastern groups is expected due to marked differences in past geological and climatic events in the Old Amazon basin (da Silva and Oren, 1996; Hoorn et al., 2010; Wesselingh et al., 2010; Mendez-Camacho et al., 2021), coupled with present differences in ecological conditions where the west (west of the Madeira river, is characterized by high rainfall rain, high habitat diversity, eutrophic soils from the Andes runoff (Archer, 2005; Ferrari, 2005; Hoorn et al., 2010; Wesselingh et al., 2010; Juen and De Marco, 2012). In summary, the reconfiguration of the Amazonian landscape in the Neogene, associated with spatial heterogeneity in current ecological conditions across the Amazonian basin may explain the observed differences across these three regions (Ribas et al., 2012; Lynch Alfaro et al., 2015; Lima et al., 2017; Byrne et al., 2018).

Although there is no evidence to accept the generality of the riverine barrier hypothesis to other taxa (Gascon et al., 2000; Santorelli et al., 2018), rivers are important biogeographic barriers for species with limited dispersal ability, such as primates (Ayres and Clutton-Brock, 1992; Boubli et al., 2015; Lynch Alfaro et al., 2015; Santorelli et al., 2018; Fordham et al., 2020; this study). The patterns of β-diversity described here are partly driven by the ability of primates to actively or passively cross rivers. While some primates have limited mobility across rivers, the large atelids, for example, are fully able to cross rivers swimming (Chaves and Stoner, 2010; Gonzalez-Socoloske and Snarr, 2010; Benchimol and Venticinque, 2014; Nunes, 2014). This may partly explain why rivers affected the large-sized primates less than small-sized ones. Rivers can limit primate dispersal, favoring speciation over longer timescales (Ayres and Clutton-Brock, 1992), with several accumulating evidences of rivers acting as effective barriers that promote population isolation and diversification in primates (Peres et al., 1996; Vallinoto et al., 2006; Boubli et al., 2015; Lynch Alfaro et al., 2015). In fact, populations separated by large rivers tend to be divergent. For instance, previous studies showed genetically divergent populations of *Saguinus niger* occurring in opposite banks of Tocantins River, between Belém and Xingu interfluves (Vallinoto et al., 2006), and *Saguinus fuscicollis* occurring in opposite banks of Juruá River (Peres et al., 1996). The isolation promoted by rivers favors the emergence of clades divergent to the original, ultimately favoring speciation. More genetic studies are necessary to disclose the role of rivers as effective barriers for primates and other animals (Vallinoto et al., 2006; Boubli et al., 2015; Lynch Alfaro et al., 2015; Dal Vechio et al., 2019; Naka and Pil, 2020).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because no animal was captured or manipulated.

AUTHOR CONTRIBUTIONS

ÍM and RH designed the study, collected the data, and wrote the manuscript draft. ÍM, RH, and WC performed the analyses. All authors reviewed, edited, and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.857920/full#supplementary-material

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Phylogeography of a Typical Forest Heliothermic Lizard Reveals the Combined Influence of Rivers and Climate Dynamics on Diversification in Eastern Amazonia

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The formation of the Amazon drainage basin has been considered an important driver of speciation of several taxa, promoting vicariant events or reinforcement of barriers that restrict gene flow between opposite river margins. Several recent studies reported a set of miscellaneous events involving climatic fluctuations, geomorphological changes, and dispersal mechanisms as propellers of diversification of Amazonian rainforest taxa. Here, we show the results of dated phylogenetic, biogeographic, and populational analyses to investigate which events could better explain the current distribution of a heliothermic, active foraging lizard in the central and eastern portions of the Amazonian rainforest (besides a disjunct distribution in part of the Atlantic Forest). We sampled *Kentropyx calcarata* from most of its area of occurrence in Amazonia and used mitochondrial and nuclear markers to evaluate if the genetic structure agrees with evolutionary scenarios previously proposed for Amazonia. We performed phylogenetic and populational analyses to better understand the dynamics of this species in the Amazonia rainforest over time. Phylogenetic inference recovered ten *K. calcarata* structured lineages in eastern Amazonia, some of them limited by the Amazon River and its southern tributaries (Tapajós, Xingu, and Tocantins), although we detected occasional haplotype sharing across some of the river banks. According to molecular dating, *K. calcarata* diversified since Miocene–Pliocene, and some of the lineages presented signs of demographic expansion during the Pleistocene, supposedly triggered by climatic dynamics. The putative ancestral lineage of *K. calcarata* was distributed on the Guiana Shield, later spreading south and southeastward by dispersion. Our results indicate that Amazonian rivers acted as barriers to the dispersal of *Kentropyx calcarata*, but they were not the sole drivers of diversification.

Keywords: Amazon basin, biogeography, drainage evolution, *Kentropyx calcarata*, landscape changes, lizard, phylogenetic, population structure

INTRODUCTION

Several phylogeographic studies tried to understand how organisms evolved in Amazonia, testing possible evolutionary scenarios (Moritz et al., 2000; Werneck et al., 2009; Hoorn et al., 2010; Avila-Pires et al., 2012; Leite and Rogers, 2013; Prates et al., 2016a; Ribas and Aleixo, 2019; Silva et al., 2019). The general consensus is that the evolution of the present-day Amazonian landscape was structured by geomorphological and climatic events during the Tertiary and Quaternary, which in turn affected biotic diversification. One of the most important geomorphological events is the Andes uplift, which affected landscape evolution and the river system dynamics. Sea-level fluctuations and other tectonic activities also played a key role in the evolution of Amazonian biodiversity (de Rossetti et al., 2005; Rossetti and De Toledo, 2007; Hoorn et al., 2010).

Climate fluctuations also caused changes in vegetation, although their degree, extension, and impacts on biodiversity are still uncertain (Haffer and Prance, 2001; Bush, 2017). Proposed evolutionary scenarios support that higher levels of precipitation and climatic stability reigned in western Amazonia leading to huge biodiversity in this region, while climatic instability of eastern Amazonia could have made vegetation more susceptible to fragmentation, leading to the possible loss of biodiversity (Cheng et al., 2013). More recently, a synergetic role between climate fluctuations and dynamic drainages through a variation in sedimentary discharge has been proposed (Pupim et al., 2019), which would have also triggered the genetic diversification of species in eastern Amazonia (Silva et al., 2019).

Several biogeographic hypotheses have been proposed based on climatic and geomorphological events, making predictions that are amenable to be tested using molecular data (Moritz et al., 2000; Antonelli et al., 2010; Leite and Rogers, 2013). The riverine hypothesis proposes that the establishment of the Amazon River and its major tributaries fragmented the distribution of species, isolating populations on opposite banks, causing lineage differentiation, and ultimately speciation (Wallace, 1852). Predictions of this hypothesis include the formation of reciprocally monophyletic groups (i.e., sister-taxa or sister lineages) and a strong genetic population structure with little to no migration between opposite banks. On the other hand, this structure may be weaker toward the river headwaters, where it becomes easier to cross (Gascon et al., 2000; Leite and Rogers, 2013). It is also assumed that the populations separated by the rivers remained mostly stable, with no evidence of population expansion related to the differentiation process. Moreover, the diversification times of the sister lineages should be in agreement with the time the respective enclosing river was established (Moritz et al., 2000; Leite and Rogers, 2013).

Studies with amphibians and reptiles in eastern Amazonia have found that some large rivers (Avila-Pires et al., 2012; Dias-Terceiro et al., 2015; Godinho and da Silva, 2018; Moraes et al., 2020) and also smaller rivers (Souza et al., 2013; Fouquet et al., 2015) can act as biogeographic barriers. In general, these and other studies also emphasize the importance of considering the biological specificities of taxa as strong determinants of their distributions (Werneck et al., 2009; Cheng et al., 2013; Prates et al., 2016a,b). Meanwhile, in other studies, rivers are detected not as vicariant (primary) barriers, but as limits of lineages previously differentiated as a result of other factors (e.g., ecological speciation, environmental adaptation, sexual selection, etc.) that eventually became separated by a river (Smith et al., 2014; Naka and Brumfield, 2018; Pirani et al., 2020). Thus, even though the river was not the primary cause of diversification, it could still act to restrict current gene flow (Naka and Brumfield, 2018; Pirani et al., 2020).

In this study, we assessed the relative influence of rivers as barriers and the climatic fluctuation as drivers of genetic diversification using a heliothermic lizard, a forest inhabitant that searches for sunny spots within the forest and tolerates more open types of forest (Vitt, 1991; Ávila-Pires, 1995) as the model system. The teiid lizard *Kentropyx calcarata* Spix, 1825 is widespread in forested areas in Amazonia east of the Negro and Madeira rivers, besides its disjunct occurrence in the Atlantic Forest (Ávila-Pires, 1995; Ribeiro-Júnior and Amaral, 2016). A phylogeographic study focusing on the eastern part of its distribution in Amazonia detected geographically cohesive clades partially separated by some of the Amazon tributaries and the Tocantins River (Avila-Pires et al., 2012). Moreover, Werneck et al. (2009), on the basis of two mitochondrial genes, dated the separation of *K. calcarata* and its sister species, recovered as *Kentropyx pelviceps* Cope, 1868, to the Middle Miocene, while two samples of *K. calcarata*, one from Guyana and the other from Mato Grosso, Brazil, were separated since the Pliocene. A more recent study, involving most *Kentropyx* species and both mitochondrial and nuclear genes, recovered *Kentropyx altamazonica* Cope, 1876 as the sister species of *K. calcarata*, also dating their divergence to the Middle Miocene (Sheu et al., 2020). This study included *K. calcarata* samples from both Amazonia and Atlantic Forest, which formed two lineages that split from each other since the late Miocene/Pliocene. It indicated moreover that ecological niche divergence was an important driver of diversification in the genus. However, in spite of such studies, a better picture of how *K. calcarata* responded to the geomorphological and climatic events that occurred in Amazonia, and which events were the most important for the species diversification, is still lacking. A better knowledge of how widely distributed forest species evolved could help in the prediction of future demographic dynamics and provide a basis for conservation policies.

Here, we investigate how *K. calcarata* diversified in Amazonia through an ancestral area reconstruction and a molecular clock based on a multi-locus dataset. Distribution patterns and historical demographic dynamics of the lineages recovered were also examined. The recognition and arrangement of clades and their diversification times will give us clues about the processes involved in the species' phylogeographic history. Reciprocally, monophyletic sister populations distributed on opposite river banks, with coincident times between clade splits and the arising of rivers, would be an indication that these rivers acted as drivers of populational diversification in *K. calcarata*. On the other hand, splits that occurred during the Pleistocene could be more correlated with forest fragmentation. In addition, the dated demographic analyses may provide clues of bottleneck or population expansion, in agreement with the hypothesis of forest fragmentation by climatic events, if occurred since the end of the Quaternary.

MATERIALS AND METHODS

Sampling and Molecular Data

We used tissue samples (tail, muscle, or liver) from 286 *Kentropyx calcarata* specimens [63 sequences of the mitochondrial genes were obtained from GenBank, from Avila-Pires et al. (2012)], and a total of 12 samples of *Kentropyx striata* (Daudin, 1802), *K. pelviceps*, and *K. altamazonica* were used as outgroups. Samples were obtained through fieldwork and tissue loans from scientific collections, covering most of the wide distribution of the species in Amazonia (**Supplementary Table 1**).

To extract genomic DNA, we used the phenol–chloroform method, following the protocol suggested by Sambrook and Russell (2001), and to maximize the extraction of genomic DNA from small tissue samples, we used the DNeasy Blood and Tissue kit (QIAGEN). Four genes, two being mitochondrial (mtDNA), namely cytochrome b (CYTB) and 16S (Palumbi et al., 1991; Corl et al., 2010), and two nuclear (nuDNA), namely dynein, justaxonemal, heavy chain 3 (DNAH3) and synuclein, alpha interacting protein (SINCAIP; Townsend et al., 2008), were amplified by polymerase chain reaction (PCR), purified with PEG 8000, and sequenced using Big Dye Terminator kit (Perkin-Elmer Corp., Norwalk, CT, United States). Primers and polymerase chain reaction are detailed in **Supplementary Table 2**.

Sequences were edited in BioEdit v.7 (Hall, 1999) and aligned using the Multiple Alignment algorithm in Fast Fourier Transform (MAFFT; Katoh and Standley, 2013). We identified the gametic allele's phase of the heterozygous individuals using a Bayesian approach as implemented in PHASE with a threshold of 90% of posterior probability (Stephens et al., 2001; Stephens and Donnelly, 2003).

Gene Tree Estimates

Sequences were concatenated in the Sequence Matrix software (Vaidya et al., 2011), and samples that failed to amplify for some genes were filled as missing data. We inferred phylogenetic relationships with Bayesian Inference using four independent runs of Markovian chains with 10 million generations and sampling trees every 1,000 generations in MrBayes v.3.1 (Ronquist and Huelsenbeck, 2003). To explain variation in gene sequences, we searched the best-fit partitioning scheme for the data set and the best-fit model of molecular evolution for each subset through Partition Finder (Lanfear et al., 2012). We used Tracer v.1.5 (Rambaut et al., 2018) to evaluate the convergence of sampling parameters and to discard trees sampled before the stability of the log-likelihood values in the Markov chain (first 25% of the trees obtained), as recommended by Huelsenbeck and Hall (2001). Samples remaining after burn-in were used to estimate the values of posterior probability (*pp*), the length of branches, and tree topology. Relations with posterior probabilities ≥90% were considered well supported.

We also performed a maximum likelihood analysis with RAxML-HPC2 program on XSEDE (Miller et al., 2010). The best ML tree was obtained from a heuristic search among 100 trees and branch support with 1,000 bootstrap replicates. Branches with bootstrap (*bs*) values ≥70% were considered well supported, following Hillis and Bull (1993).

Population Structure, Networks, Genetic Polymorphism, and Distances

We investigated the level of populational genetic structure based on a Bayesian clustering analysis inferred with BAPS v.6.0 (Corander et al., 2008). This analysis was performed from a matrix of combined sequences of two mitochondrial markers (16S and CYTB), with a total of 271 samples chosen to encompass those preferentially amplified for both mitochondrial genes and including all the groups recovered in the Bayesian tree. In some samples (HT78_F, HT80_F, HT114_F, HT706_F BPN1887_A, BPN2885_A, BPN2946_A, BPN3796_J, and AMS355_B) data on CYTB were missing. We used a linkage map to indicate the limits of base pairs of each marker. A genetic mixture analysis was performed with 10 runs for each K (number of discrete genetic clusters) that ranged from 1 to 11 (a number slightly above the number of major clades recovered by phylogenetic analyses). After finding the optimal *K* value, an admixture analysis was performed with 100 interactions and a minimum of 5 individuals per population (according to the mixture results). The reference number for each population and the interactions of reference individuals used were, respectively, 200 and 10, following the default values of the software.

We used Haploviewer v.4.1 to determine the number of unique haplotypes and to obtain haplotype networks and the distribution of shared haplotypes based on the maximum likelihood tree (Barrett et al., 2005). We calculated mean pairwise *p*-distances (Nei, 1987) within and between the recovered populations using mtDNA, concatenated mtDNA, and nuDNA datasets in MEGA 6.0 (Tamura et al., 2013).

We calculated DNA polymorphism metrics (i.e., the number of polymorphic sites, number of haplotypes, nucleotide, and haplotype diversity) for the concatenated mtDNA and separately for each nuclear marker dataset with DnaSP v.5 (Librado and Rozas, 2009). Metrics were calculated only for population clusters with a minimum of six samples. Using Arlequin v.3.1 (Excoffier et al., 2005) and the concatenated mtDNA dataset, we calculated fixation indexes (Fst), the Tajima's D (Tajima, 1996), and Fu's Fs (Fu, 1996) values for each group with 1,000 permutations. A three-level hierarchical analysis of molecular variance (AMOVA) was performed using Arlequin v.3.1 to assess genetic diversity among and within the inferred populations, grouped according to the gene tree (**Figure 1**). We also performed this analysis using mtDNA and partitioning groups by the major rivers aiming to test the riverine hypothesis. The populations were grouped according to seven interfluve regions, four of them located south of the Amazon River, following the Rondônia, Tapajós, Xingu, and Belém Areas of Endemism; and the remaining three groups are located north of the Amazon River, with samples from west of Essequibo and Trombetas Rivers, Trombetas-Jari interfluve (including samples from Suriname), and east of Jari River (including samples from French Guiana; see map in **Figure 1**).

Species Tree, Divergence Times, and Biogeographic Reconstruction

To estimate divergence times between the main *K. calcarata* lineages and relate them to the events proposed by the hypotheses of diversification, we estimated a species tree with *BEAST v.1.8.0 (Drummond et al., 2007). For this analysis, we treated each lineage recovered with high support in Bayesian inference (concatenated gene tree, see above) like a distinct species' lineage. We used 277 samples, after having excluded those with missing data for the CYTB gene, and we did not include the SINCAIP gene, in which samples from the J group were not recovered. To calibrate the divergence time estimates, we used the calibration rate of 0.65% change per lineage per million years for mitochondrial markers estimated by Macey et al. (1998) and widely used in squamate reptiles (Glor et al., 2001; Weisrock et al., 2001; Strasburg et al., 2007; Oliver et al., 2009; Torres-Carvajal and de Queiroz, 2009; Gvoždík et al., 2010; Avila-Pires et al., 2012; Pouyani et al., 2012). The rate of the nuclear gene was estimated by the BEAST software relative to the mtDNA rate. We used a coalescent constant size prior and an uncorrelated lognormal relaxed molecular clock, which accommodates the possibility of independent rates of molecular evolution in different branches (Drummond et al., 2007). Analyses were performed with two independent runs for 100 million generations each by sampling trees every 10,000 generations. We checked the ESS values and the convergence of independent runs in Tracer v.1.5 (Rambaut et al., 2018). The mean ages of the nodes were calculated using a burn-in of 2,500. Trees were summarized in a maximum credibility clade (MCC) tree after a burn-in of 10% using the program Tree Annotator and visualized with FigTree 1.4.0 (Drummond and Rambaut, 2007).

We used a probabilistic historical biogeography approach to model ancestral areas in BioGeoBEARS (Matzke, 2013a,b) based on the species tree estimated. We considered five areas recognized as Amazonian areas of endemism–AoEs (Silva et al., 2002), and covering the distribution of *Kentropyx calcarata* lineages we sampled: Guiana (GU), Belém (BE), Xingu (XI), Tapajós (TA), and Rondônia (RO; **Figures 1**, **4**). We considered all points east of the Tocantins–Araguaia rivers system and Marajó Island as part of the Belém AoE. We did not take into account the presence of single individuals marginally present in an area contiguous to the main area of occurrence of a lineage, e.g., the occurrence of one individual from G and C populations (see section "Results") in the Xingu AoE, one individual from E populations in the Tapajós, and two in the Guiana AoE. However, we considered the presence of individuals from B group at the Guiana and Xingu AoEs because of its dense occurrence in both areas. We assumed five as the maximum number of ancestral areas and compared, based on likelihoods, the six biogeographic models allowed in BioGeoBEARS: DEC (Dispersal-Extinction Cladogenesis Model), DIVA (Dispersal-Vicariance Analysis), BAYAREA (Bayesian inference of historical biogeography for discrete areas), plus the same models with the "J" (jump dispersal) parameter that comprises jump dispersals: DEC + J, DIVA + J, and BAYAREA + J (Ree et al., 2005; Ree and Smith, 2008; Matzke, 2013a, 2014). However, given that the population distribution ranges are non-insular-like and to avoid bias in the analysis [as reported in Ree and Sanmartín (2018)], we considered the best fit model without the "J" parameter.

Historical Demography

The demographic history of each population cluster inferred was investigated using the coalescent multi-locus method in Extended Bayesian Skyline Plots (EBSP), which incorporates stochastic differences in the genealogy of genes, estimating changes in population size over time (Drummond, 2005; Heled and Drummond, 2008). We considered separate substitution models for each partition. The number of generations required for the convergence of sampling parameters was 100 million, sampling trees every 10,000 generations. We followed the same calibration scheme as in the species tree analysis.

RESULTS

Phylogenetic and Population Analyses

The resulting dataset included 2,547 base pairs (bp) (16S = 526 bp, 281 samples; CYTB = 782 bp, 284 samples; DNAH3 = 734 bp, 246 samples; SINCAIP = 505 bp, 197 samples). As expected, the two nuclear markers are less variable than the two mitochondrial markers, and CYTB is the most variable marker (highest number of polymorphic sites – K) (**Supplementary Table 3**). The best-fit partitioning scheme was obtained by codon to CYTB and by gene to the other markers. The best-fit evolutionary models were GTR + I + G to 16S and CYTB codon 1; K80 + I + G for DNAH3; HKY + I + G to CYTB codon 2 and SINCAIP; and TrN + G to CYTB codon 3.

Phylogenetic trees resulting from Bayesian and maximum likelihood inferences recovered the same lineages (**Figure 1** and **Supplementary Figure 1**) with high posterior probability and bootstrap supports (except in E and H lineages). Both analyses divided *K. calcarata* into three main clades: J, A–F, and G–I, with clade J, from the northwestern boundary of the Guiana AoE, as sister to the other two (with good support by both methods). The A–F clade, also well supported, grouped all individuals north of the Amazon, and those south of the Amazon and east of the Xingu River. Within this clade, F was recovered with high support as sister to all other lineages (clades A–E), while the relationships between C, D, and E were poorly supported (**Figure 1** and **Supplementary Figure 1**). Clades G–I, which grouped samples from Rondônia and Tapajós AoEs, were well supported only by the Bayesian analysis. The population structure analysis estimated nine population clusters within *K. calcarata* (**Figure 2**). Genetic population results agree with the recovered lineages on the phylogenetic tree (**Figure 1**), except for "F," not recovered as an independent population, because of the high level of admixture in the samples.

The Amazon River and its tributaries Tapajós, Xingu, and Tocantins largely limit some of these populations, but we

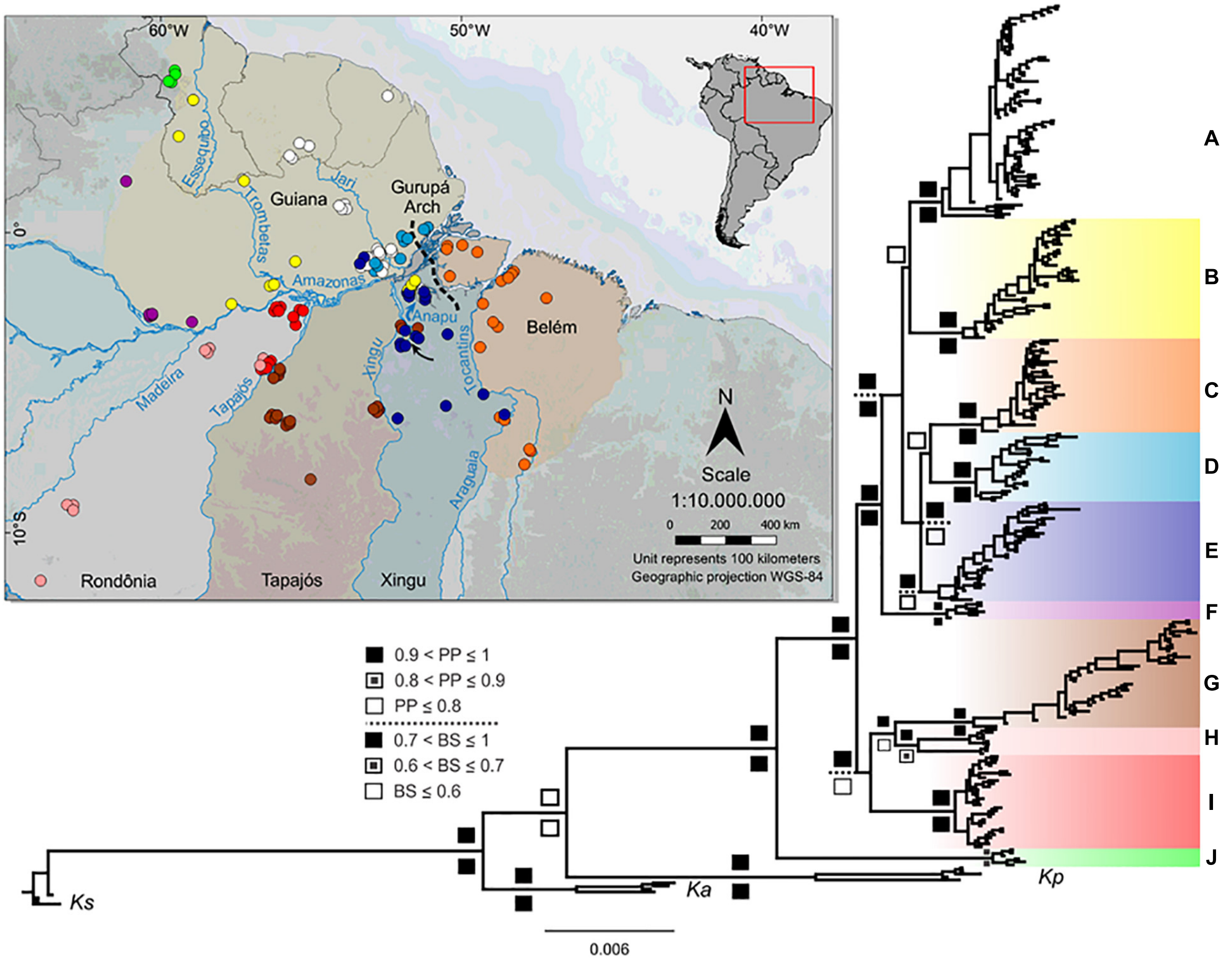

FIGURE 1 | Geographic distribution of *Kentropyx calcarata* sampled locations used in this study and the concatenated phylogenetic tree (Bayesian inference). Inset with South America map indicates the area shown in the larger map. The dashed line indicates the approximate location of the Gurupá Arch. The black arrow shows the Xingu Great Bend region. Each area of endemism (AoE) is defined by highlighted areas on the map (Guiana AoE is only partially delimited, taking into account the western distribution limit of *K. calcarata* samples). Circle colors correspond to the clade colors assigned in the phylogenetic tree. Posterior probability-pp (up) and bootstrap-bs (down) values are represented according to the legend in the figure. Bootstrap supports within the main clades were not represented, since the topology of the likelihood tree in these branches differed from the Bayesian tree. External groups correspond to *Kentropyx striata* (Ks), *K. altamazonica* (Ka), and *K. pelviceps* (Kp).

detected few shared haplotypes on both margins of the Araguaia–Tocantins (C), Xingu (E, G), and Amazon (B, E) (**Figure 1**). In addition, we also found distinct and well-supported lineages without any apparent barrier separating them, for example, within the Guiana and Rondônia AoEs (**Figure 1**).

Mitochondrial haplotype networks show correspondence with the phylogenetic inference (**Figure 3**). CYTB haplotypes (**Figure 3B**) are separated by more mutational steps than 16S (**Figure 3A**) and nuDNA (**Figures 3C,D**), and they form the only network with no shared haplotypes between groups. Both in CYTB and 16S networks, most haplogroups present a radial pattern. In the case of the two nuDNA, most haplotypes are shared between groups, with hardly any geographic structure observed (**Figures 3C,D**). A high number of mutational steps in one sample of the H lineage in the CYTB haplotype network were caused by missing data (**Figure 3B**).

Our results showed, in average, low values (0.001–0.006) of genetic distances within lineages (**Supplementary Table 4**). The largest genetic distances were within D, G, and H lineages, the lowest within the F lineage. In relation to the distances among groups, we recovered high values between lineages from opposite margins of the Amazon River, ranging from 0.022 (comparing F and H) to 0.058 (J and G). However, lineage J (the most divergent lineage in relation to all others), from the north of Amazon River, showed the largest genetic distances in relation to others from the same margin, such as B (the geographically closest lineage, 0.060) and D (eastern Guiana, 0.059). The smallest genetic distances were recovered within clades A–F from Guiana, Xingu, and Belém AoEs (**Supplementary Table 4**).

Similarly, AMOVA revealed a larger molecular variance between populations than within them for mtDNA and DNAH3

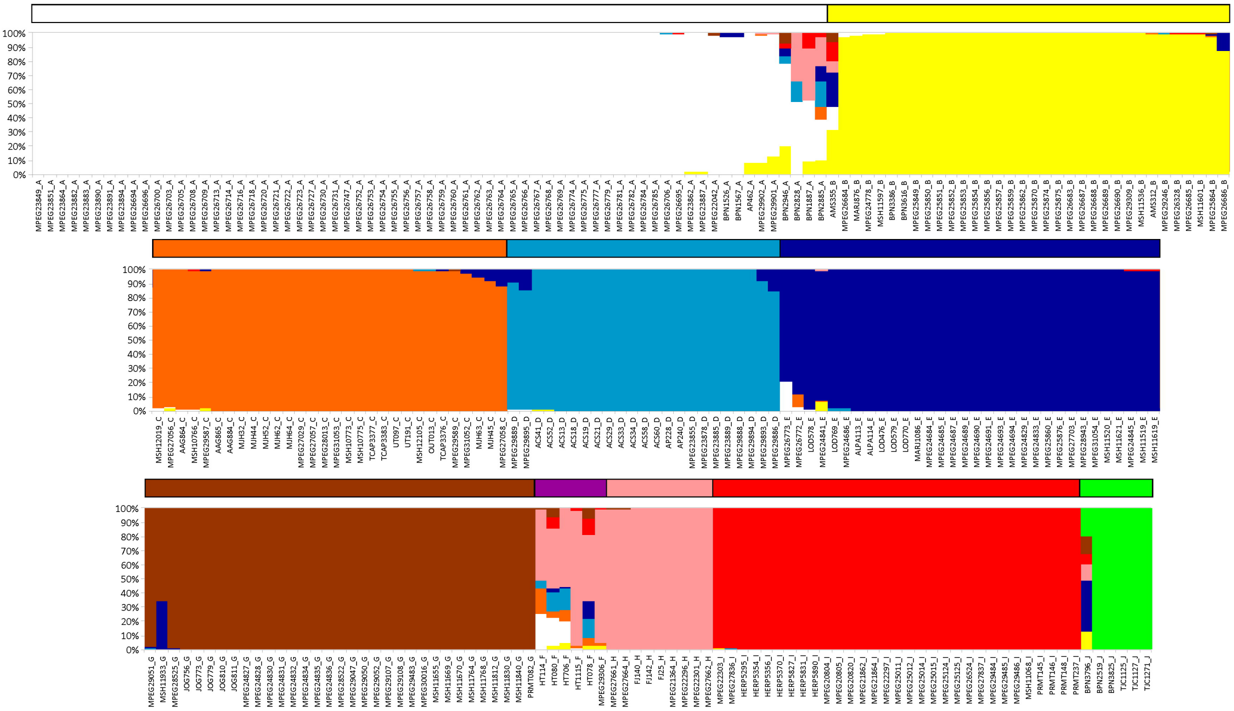

FIGURE 2 | Barplots resulting from populational structure analysis performed on BAPS, based on concatenated mtDNA. Bar colors correspond to the color groups recovered in **Figure 1** (upper = clade AB; middle = clade CDE; lower = J + clade GHI + F).

markers. SINCAIP, in contrast, showed more variation within groups than between groups (**Table 1**). The total fixation index (Fst) was the highest for the concatenated mitochondrial genes (Fst = 0.83787) (**Supplementary Table 5**). The AMOVA was performed to evaluate the riverine hypothesis, and it showed higher mtDNA variation levels among populations from different interfluves (Rondônia, Tapajós, Xingu, Belém, west of Essequibo and Trombetas Rivers, Trombetas-Jari interfluve, and east of Jari River) than from within interfluves (**Table 1**).

Population E (restricted to Xingu AoE) showed the highest haplotype diversity for mtDNA and population H (restricted to Rondônia AoE) the highest nucleotide diversity (**Table 2**). Fst indexes were significant ($p \leq 0.05$) for all populations. The strongest genetic structure was detected when comparing population J from northwest of the Guiana AoE with other groups (**Supplementary Table 5**).

Species Tree, Divergence Times, and Biogeographic Reconstruction

According to the inferred species tree (**Figure 4**), *K. calcarata* divergence started during Early–Middle Miocene (or even in Late Oligocene) with the initial separation of lineage J from NW Guiana, which is sister to all others. The remaining clades diverged into clades (A–F) and (G–I) in Middle–Late Miocene. Splitting within (G–I) and between F and the remaining lineages in this clade occurred during Pliocene, while these latter lineages diverged more recently, in the Plio-Pleistocene (see confidence intervals in **Supplementary Table 6**).

The best model recovered by BioGeoBEARS was DIVALIKE (lnL = −22.06, AIC 48.13) (**Table 3**). The reconstruction based on the best-fit model is represented in **Figure 4**. According to the ancestral reconstruction obtained by this model, the Guiana AoE was considered the most probable ancestral range for *K. calcarata*, with the populations south of the Amazon River in Rondônia + Tapajós, Xingu, and Belém AoEs all originating subsequently by dispersion events from Guiana.

We detected signs of population expansion for populations/lineages distributed in eastern Amazonia—A, C, E (mtDNA), and I (DNAH3) by Tajima's test and for all populations, except D and J by Fu's statistic (mostly mtDNA and/or DNAH3, but only SINCAIP in H; **Table 2**). The EBSP analyses are partly in agreement with those of neutrality deviations. The C (Belém AoE) and E (Xingu AoE) groups showed signals of a rapid and recent expansion between 0 and 1 Ma (**Figure 5**). According to this analysis, the other populations did not show significant changes in demography over time, taking into account the wide ranges of confidence in the graphs (**Figure 5**).

DISCUSSION

Our findings indicate that some of the main Amazonian rivers likely act as diversification barriers among *K. calcarata* populations. The intraspecific structure showed at least nine *K. calcarata* lineages in Amazonia, and the phylogenetic analyses recovered ten lineages, which are supported by high Fst

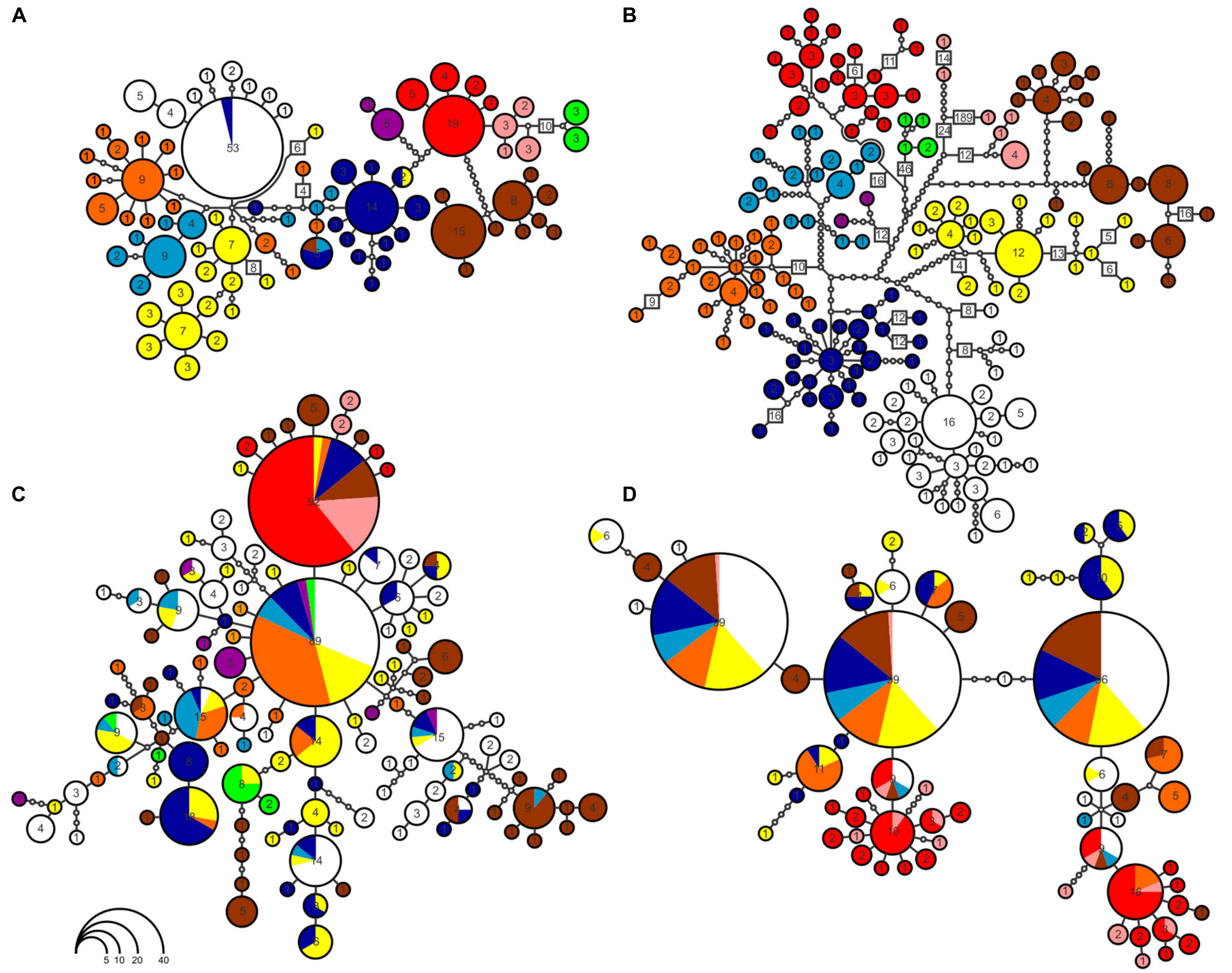

FIGURE 3 | Haplotype networks built for the four markers used in the study: **(A)** 16S; **(B)** CYTB; **(C)** DNAH3; **(D)** SINCAIP. Not all groups were completely sampled in haplotype networks, due to PCR failure. Colors correspond to same scheme of **Figures 1**, **2**. Circle sizes are proportional to the number of individuals of each haplotype. Intermediate points (smallest circles) refer to the number of mutational steps between populations. To facilitate visualization the total number of mutations was indicated in some segments.

indexes and genetic distances (**Supplementary Tables 4, 5**). Four populations (A, D, F, J) are restricted to the north and other four (C, G, H, I) to the south of the Amazon River (**Figure 1**). The Tapajós River separates populations G (east margin) from H and I (west margin), the Xingu River separates almost completely populations E and G, and the Tocantins–Araguaia populations C and E (**Figure 1**). These rivers reduce gene flow and prevent homogenization, although occasionally this barrier effect is incomplete. For example, we observed putative occasional dispersions in some groups (B, C, E, and G) across the Amazon (B and E), Tocantins–Araguaia (C), and Xingu (G and E) rivers (**Figures 1**, **4**). Considering sister groups, the Tapajós River separates G (east margin) and H (**Figure 1** and **Supplementary Figure 1**) or H + I (west margin) (**Figure 4**), and the Amazon River separates, completely or partially, some larger sister clades. These are the cases where the river itself may have acted (depending also on the time scale, discussed below) as a primary barrier, leading to the observed population divergence. On the other hand, strong genetic differences detected in some parapatric *K. calcarata* populations within Guiana and Rondônia AoEs (**Supplementary Tables 4, 5** and **Figure 1**) indicate that the riverine barriers cannot exclusively account for the diversification pattern in these interfluvial areas.

Diversification of *K. calcarata* and Implications for Eastern Amazonian Evolution

Overall, our results support older divergence times (**Supplementary Table 6**) than previously found in genus-level phylogenetic studies including *K. calcarata* sampling (Werneck et al., 2009; Sheu et al., 2020). Differences in sampling density and markers used possibly contributed to these time dissonances. The average splitting time in Sheu et al. (2020)

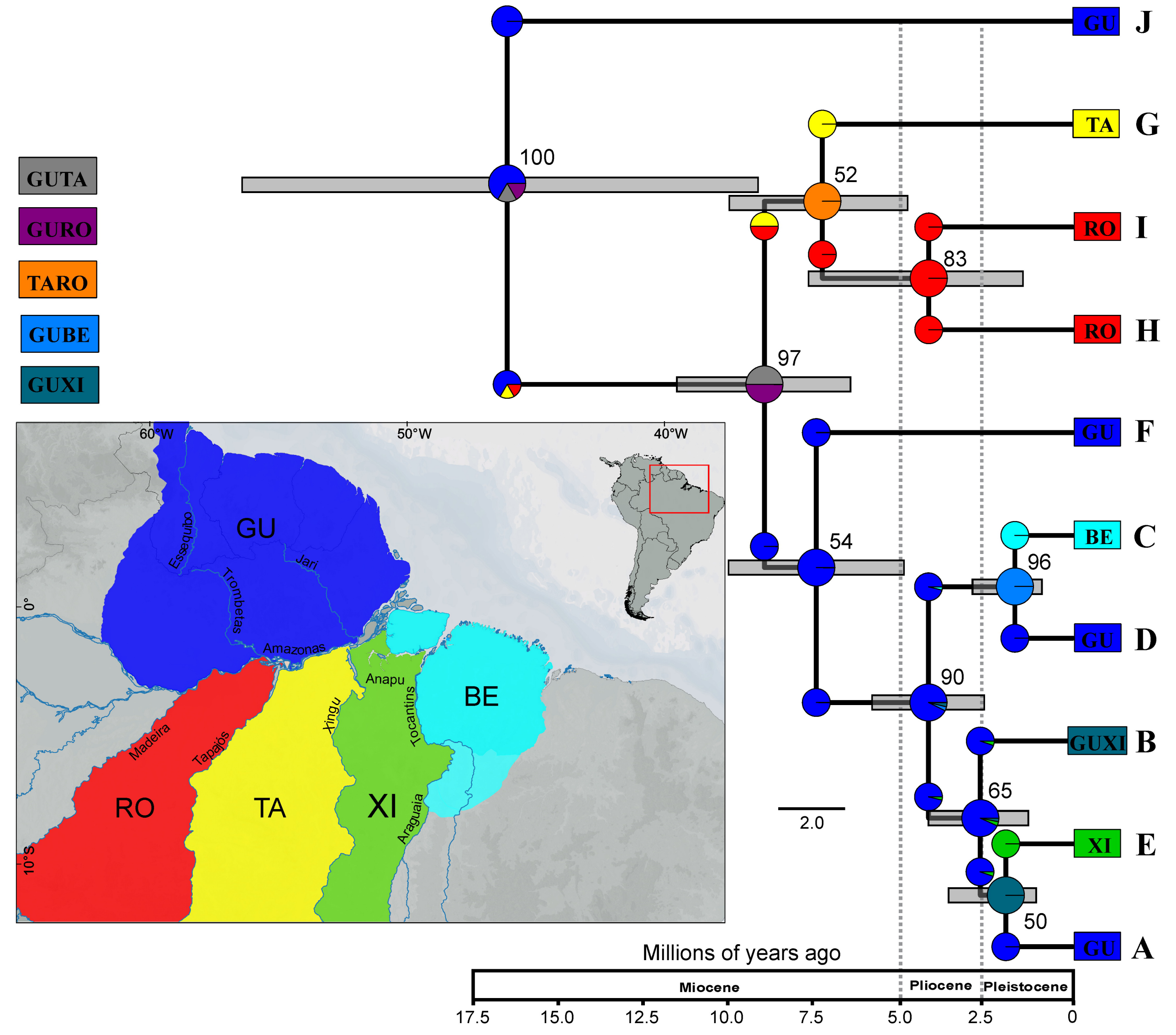

FIGURE 4 | Species tree based on markers CYTB, 16S, and SINCAIP using *BEAST and biogeographic reconstruction inferred using BioGeoBEARS for *Kentropyx calcarata*. We consider five areas: GU–Guiana (dark blue), BE–Belém (light blue), XI–Xingu (green), TA–Tapajós (yellow), and RO–Rondônia (red). The resulting best-fit model for our phylogeny was DIVALIKE (lnL = −22.06, AIC = 48.13). Clades correspond to populations/lineages defined by phylogeographic analysis. The numbers in the nodes correspond to the values of posterior probability (pp). Color of each group correspond to those of **Figures 1**, **2**. Time scale represents millions of years.

between *K. calcarata*, *K. pelviceps,* and K. *altamazonica* occurred more recently (late Miocene) than our findings for the most ancient Amazonian *K. calcarata* population (J) in the early Miocene (**Figure 4**). However, the wide confidence intervals in the species tree (**Figure 4**) could be hiding a more recent average splitting time for J population. Alternatively, the recent average time of splits among the species of the *calcarata* group could be overestimated by the confidence intervals of the species tree in Sheu et al. (2020), and, in this way, the splitting time between *K. calcarata* and their sisters actually would be more ancient. This hypothesis supports the Guiana group J origin during the middle Miocene and the splits in A–F and G–I clades during the late Miocene. In addition, the large divergence in J lineage (**Supplementary Tables 4, 5**) encourages further investigations, using integrative data of morphology and analyses of species delimitation, to access the possibility of this Guianan population being under a speciation process.

According to our results and previous studies, an ancestral origin of *K. calcarata* in the Guiana AoE by Early–Middle Miocene is estimated, with the first intraspecific divergence (between the population from northwestern Guiana AoE, here represented by the green group–J, and all others) occurring by Middle Miocene (**Figure 4**). During the Miocene, recurrent tectonic activities caused marine incursions, trigged Andes uplift phases, caused the formation and subsidence of geological arches and formation of lacustrine and swampy environments

TABLE 1 | Summary statistics of molecular variance (AMOVA) comparing variation among and within phylogenetic clades/populations and comparing variation among and within different interfluve populations.

Variation source	Among populations	Within populations
mtDNA (Fst = 0.83787)		
df	9	261
Var. comp.	15.27008 Va	2.95490 Vb
Var. %	83.79%	16.21%
Interfluve groups (Fst = 0.60339)		
df	7	278
Var. comp.	8.64814 Va	5.68446 Vb
Var. %	60.34%	39.66%
DNAH3 (Fst = 0.24522)		
df	9	482
Var. comp.	0.50047 Va	1.54046 Vb
Var. %	24.52%	7.48%
SINCAIP (Fst = 0.07732)		
df	9	334
Var. comp.	0.14014 Va	1.67236 Vb
Var. %	7.73%	92.27%

Calculations were made based on the concatenated mtDNA and, separately, for each nuclear marker. df, degree of freedom; Var. comp., Variation of the components; Var. %, percentage change; Fst, total fixation index.

(Hoorn et al., 1995, 2010; Caputo and Soares, 2016). An episode of tepuis uplifting, whose eastern border is close to the current area of occurrence of the J lineage, is also believed to have occurred during the Late Tertiary (see Kok, 2013). However, there is no sufficiently detailed information on the geomorphological history of this area to develop further the possibility that this historical event was casual for the divergence of lineage J. Further studies addressing species limits under integrative approaches are advised to address the possibility of this lineage being under an incipient speciation process.

The second division within *K. calcarata* separated the A–F and G–I clades during the Late Miocene (**Figure 4** and **Supplementary Table 6**). The topology, timing, and geographic distribution of these lineages is in agreement with the possibility that it was induced by geomorphological events related to the reversal and establishment of the Amazon River's present course (Figueiredo et al., 2009; Hoorn et al., 2010; Caputo and Soares, 2016; Bicudo et al., 2019, Bicudo et al., 2020). Considering the biogeographic scenario reconstructed, probably at that time *K. calcarata* had a range limited to the western part of its present Guiana distribution. It might have happened as well that its southern limit was such that part of it became "trapped" south of the new course of the Amazon, in the area now recognized as the Rondônia AoE. In this scenario, the Amazon River would have acted as a primary barrier between former continuous populations in the western part of the current distribution of this species in Amazonia.

South of the Amazon River, the species would have spread south and east across the Tapajós River, leading to the formation of lineages (G–I) still during the Late Miocene (**Figure 4** and **Supplementary Table 6**). It is possible therefore that the Tapajós River has acted as an effective barrier between one or both populations to the west (H, I) and the east (G). The Tapajós River limits the distribution of several species of squamates and amphibians (Moraes et al., 2016, 2020). On the other hand, the mouth of the Tapajós River is comprised of a set of geological faults dating to the Pleistocene-Holocene, that form multiple channels incorporated in the present river configuration, at its lower course (Costa et al., 2001). This could have facilitated occasional gene flow between populations from opposite margins, what would explain the uncertain phylogenetic arrangements among lineages from Rondônia and Tapajós AoEs in the gene and species trees (GH-I and G-HI, in respectively, **Figures 2**, **4**).

West of the Tapajós River, two partially overlapping *K. calcarata* lineages are present within the Rondônia AoE (H and I) in the absence of apparent geographic barriers (**Figure 1**). Our dataset is insufficient to suggest monophyly between the two populations of this region or to suggest any hypothesis about the cause of this split. However, signs of population expansion were detected at the Rondônia AoE in lineage H by the nuclear gene SINCAIP (not included in the species tree), the less variable marker, and in the lineage I by the other nuclear gene, DNAH3, which could be linked to the process that led to their divergence and possible secondary contact within Rondônia AoE. This pattern reinforces the complexity of this region, which is considered heterogeneous according to the distribution of other organisms (Geurgas and Rodrigues, 2010; Wilkinson et al., 2010; Fernandes et al., 2013; Souza et al., 2013; Thom and Aleixo, 2015). Local factors acting within interfluves and AoEs, such as local adaptation to ecological selective forces, could also help explain the occurrence of distinct lineages at Rondônia AoE in the absence of apparent geographic barriers (Ortiz et al., 2018). Additional sampling and studies about ecological adaptation and secondary contact are needed to further access this possibility.

The A–F clades occupied originally only the Guiana AoE, where the initial splits within *K. calcarata* occurred (**Figures 1**, **4** and **Supplementary Figure 1**). The westernmost lineage (F) from north of the Amazon River diversified from the other Guianan populations by the Late Miocene (**Figure 4** and **Supplementary Table 6**), possibly due to landscape adjustments following the onset of the transcontinental Amazon River. Later, during the Plio-Pleistocene, other Guianan *K. calcarata* populations (A, B, D) were established. Costa et al. (2001) analyzed the geological faults present along the Amazon River in different periods, including the Late Tertiary and Quaternary, and their influence on the landscape. They recognized both the existence of different domains along the Amazon Basin, as well as temporal variation. Together with surface topographic rearrangements, mantle dynamics were also the key for the formation of the present Amazon River drainage system (Bicudo et al., 2019, Bicudo et al., 2020). These landscape dynamics could have led to the splitting of some populations north of Amazon River, promoting vicariant events and eventually allowing eastward dispersal that could have been congruent with the inferred eastward expansion of *terra firme* forests and várzeas (Bicudo et al., 2019). On the other hand, these populations are currently distributed without evident or strong physical barriers separating them, and they might as well have been influenced by changes

TABLE 2 | Genetic parameters calculated for *K. calcarata* populations.

Pop	Markers	*n*	*h*	Hd ± SD	π ± SD	*k*	Tajima's test	Fu's statistic
A	MtDNA	67	7	0.369 ± 0.074	0.00114 ± 0.00026	0.583	**−2.09059**	**−16.26554**
	DNAH3	116	13	0.680 ± 0.044	0.00287 ± 0.00022	2.083	0.34421	**−22.29128**
	SINCAIP	94	3	0.516 ± 0.013	0.00504 ± 0.00008	2.526	3.40939	2.19301
B	MtDNA	34	6	0.371 ± 0.104	0.00091 ± 0.00030	0.463	−1.67324	**−15.60090**
	DNAH3	72	21	0.888 ± 0.023	0.00381 ± 0.00029	2.773	0.43808	**−24.05887**
	SINCAIP	48	8	0.770 ± 0.034	0.00632 ± 0.00025	3.161	1.26068	−2.13948
C	MtDNA	30	20	0.954 ± 0.026	0.00334 ± 0.00048	4.301	**−2.15110**	**−23.26940**
	DNAH3	56	13	0.642 ± 0.071	0.00220 ± 0.00042	1.612	−1.46253	**−8.10133**
	SINCAIP	44	7	0.847 ± 0.022	0.00760 ± 0.00035	3.839	1.54455	2.71988
D	MtDNA	23	17	0.964 ± 0.026	0.00538 ± 0.00039	6.941	−0.37746	−4.80315
	DNAH3	22	12	0.887 ± 0.048	0.00492 ± 0.00062	3.606	−0.21827	−3.67346
	SINCAIP	18	2	0.529 ± 0.040	0.00526 ± 0.00040	2.647	2.59848	2.49297
E	MtDNA	32	24	0.980 ± 0.013	0.00326 ± 0.00039	4.107	**−2.00913**	**−24.41310**
	DNAH3	60	13	0.812 ± 0.039	0.00424 ± 0.00017	3.099	1.08572	**−8.44958**
	SINCAIP	44	10	0.811 ± 0.036	0.00724 ± 0.00042	3.654	1.72645	0.18862
F	MtDNA	6	2	0.333 ± 0.215	0.00064 ± 0.00042	0.333	−0.93302	**−4.26004**
	DNAH3	12	7	0.833 ± 0.100	0.00368 ± 0.00080	2.697	0.07214	−1.66135
G	MtDNA	33	18	0.934 ± 0.024	0.00741 ± 0.00064	9.513	−0.72676	−1.95804
	DNAH3	64	27	0.906 ± 0.024	0.00574 ± 0.00025	4.199	1.68864	**−17.53535**
	SINCAIP	50	4	0.629 ± 0.038	0.00560 ± 0.00022	2.813	1.22660	0.20952
H	MtDNA	9	4	0.806 ± 0.089	0.00824 ± 0.00180	9	1.40908	0.0625
	DNAH3	18	3	0.392 ± 0.133	0.00078 ± 0.00028	0.575	−0.02647	−0.02696
	SINCAIP	14	6	0.813 ± 0.074	0.00668 ± 0.00064	3.341	1.91021	**−10.37380**
I	MtDNA	31	19	0.946 ± 0.025	0.00379 ± 0.00034	4.869	0.82389	**−14.79771**
	DNAH3	60	4	0.129 ± 0.059	0.00018 ± 0.00008	0.132	**−1.57650**	**−3.92151**
	SINCAIP	52	6	0.645 ± 0.041	0.00571 ± 0.00023	2.849	1.19643	−3.38506
J	MtDNA	6	2	0.600 ± 0.129	0.00232 ± 0.00050	1.2	1.75324	**3.30973**
	DNAH3	12	5	0.742 ± 0.116	0.00341 ± 0.00104	2.500	−0.6541	0.3164

Bold numbers represent significant statistical values. Metrics for SINCAIP of F and J populations are missing due to the small number of sequences available. Pop, populations; n, sample size; h, number of haplotypes; Hd ± SD, haplotype diversity ± standard deviation; π ± SD, nucleotide diversity ± standard deviation; k, number of polymorphic sites; Tajima's test and Fu's statistic, neutral deviation tests.

TABLE 3 | Results of BioGeoBEARS analysis for each model.

Model	lnL	Parameters	*d*	*e*	*j*	AIC	AIC_wt
DEC + J	−17.43	3	0.0038	1.00E-12	0.17	40.86	0.5
DIVALIKE + J	−17.77	3	0.0052	1.00E-12	0.13	41.55	0.35
BAYAREALIKE + J	−18.75	3	0.0036	1.00E-07	0.13	43.5	0.13
DIVALIKE	**−22.06**	**2**	**0.02**	**1.00E-12**	**0**	**48.13**	**0.013**
DEC	−24.02	2	0.027	0.028	0	52.04	0.0019
BAYAREALIKE	−26.43	2	0.044	0.11	0	56.85	0.0002

lnL, Log-Likelihood; d, values of dispersal; e, extinction; j, founder; AIC, Akaike Information Criterion; AIC_wt, Akaike weight. Bold values highlight the best-fit model inferred by BioGeoBEARS.

due to climatic fluctuations (glacial and interglacial periods) during the Plio-Pleistocene, e.g., in the vegetation and river characteristics, in a way that they would act as temporary barriers.

Two populations (B and C) reach the southern margin of the Amazon River in the Xingu and Belém AoE, respectively (**Figure 1**). According to our biogeographic reconstruction, these lineages would have crossed the Amazon River probably by dispersal (**Figure 4**) during Plio-Pleistocene. Even though the Amazon River seems to be a difficult barrier to overcome, other lizard species apparently crossed the river close to its mouth (e.g., *Arthrosaura kockii* and *Tretioscincus agilis*; Ávila-Pires, 1995), the same as with other organisms. For example, Ayres and Clutton-Brock (1992) showed that in primates, the similarity of species composition between margins increases toward the mouth of the Amazon. This is an area dense with islands that was certainly affected by sea-level changes during the Pleistocene (Miller et al., 2011), which may have facilitated biotic dispersal during times of low sea level. Moreover, Marajó Island, at the mouth of the Amazon, is a result of tectonic movements that occurred during the Pleistocene/Holocene (Rossetti and Valeriano, 2007),

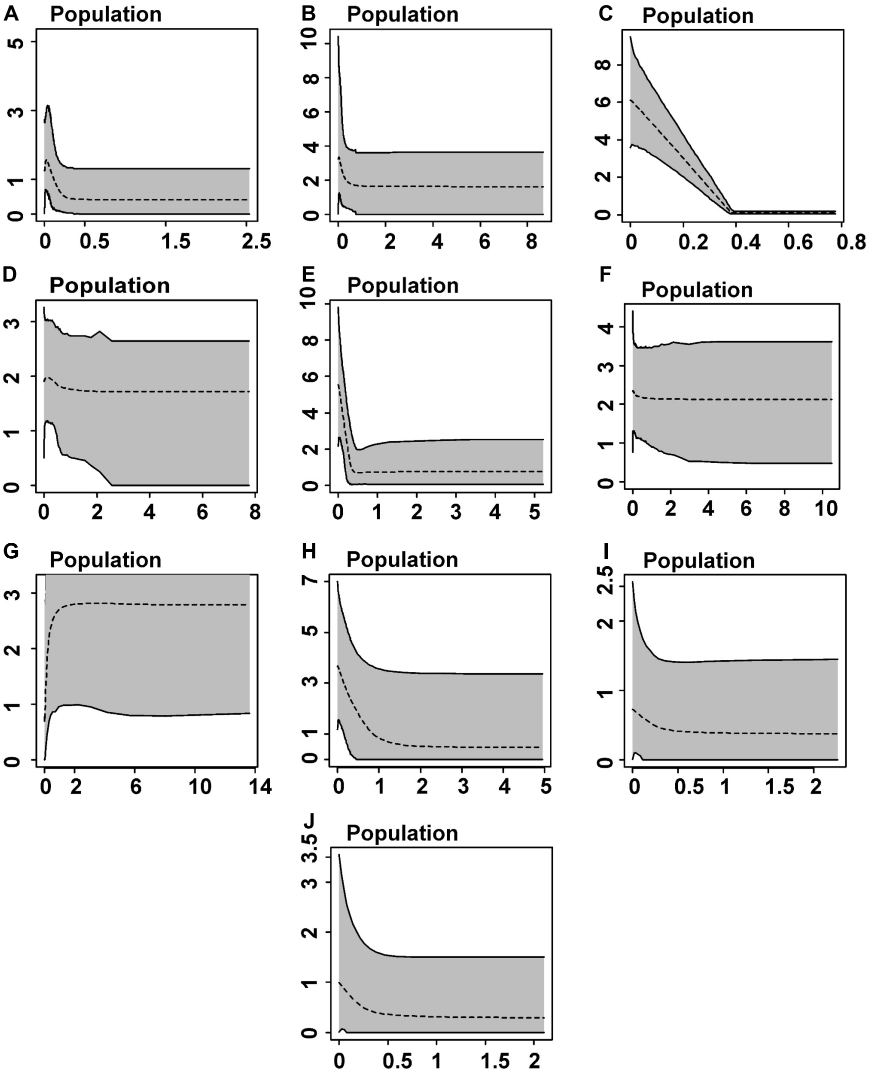

FIGURE 5 | Demographic history estimated by Extended Bayesian Skyline Plots based on mtDNA (CYTB and 16S), and nuDNA (DNAH3 and SINCAIP) for inferred Amazonian populations/lineages of *Kentropyx calcarata*. Dashed lines represent median values. The gray area corresponds to 95% confidence intervals. *X*-axis corresponds to time in millions of years before the present, and *Y* axis represents effective population size scales.

showing that this area was still geologically active in recent times. These movements may have separated previously continuous populations or even facilitated passive dispersal, as for example, in E population. Finally, in lineages B and E there are signs of recent (approximately 0.4 Mya) population expansion, which could have been related to the ecotonal character of the region and the expansion of the forest toward the Caatinga and Cerrado during interglacial periods (Batalha-Filho et al., 2013).

Occasional haplotype sharing across the Xingu River was also detected near the area of the Xingu Great Bend (black arrow in **Figure 1**). This is a segment where the river makes a sharp U-bend to the east, suggesting that in some time of its history it ran straight northward, later being displaced (Sawakuchi et al., 2015). If this is true, it may have allowed the passive migration from the right (east) margin to the left (west) margin (but not in the opposite direction). Moreover, this is a rocky area, and some rocks become apparent during drier periods of the year. In extremely dry years, it may have allowed an occasional crossing of animals through temporary land bridges.

In our reconstruction, we considered the time of the establishment of the Amazon River system proposed by Hoorn et al. (1995, 2010), Figueiredo et al. (2009), Caputo and Soares (2016), and Albert et al. (2018). Other authors (e.g., Latrubesse et al., 2010; Rossetti et al., 2015) postulate that the Amazon River reversal occurred more recently. If this is correct, our scenarios would have to be reconsidered. In general, we consider that our data, especially those of the initial splitting of the species, seem to be more congruent with the older dates for the formation of the present Amazon River system.

The ten lineages of *K. calcarata* recovered in this study were mainly restricted to the major interfluves in eastern Amazonia. Despite the rivers acting as maintainers (and some of them as sources) of population diversity, we also reinforce the effects of climate change during the Quaternary as an actual driver of *K. calcarata* diversification. During this period, when more humid forests probably alternated with a mosaic of humid and dry forests, respectively, in interglacial and glacial periods, we detected expansions, mainly in easternmost populations (from Xingu and Belém AoEs) (**Figure 5**). Climatic fluctuations would have propelled populational expansions and the rivers of the Brazilian Shield then sealed the diversification processes by forming strong secondary barriers in the most downstream regions.

This study represents a substantial effort to evaluate the phylogeography of *K. calcarata,* encompassing a broader geographical sampling in a number of individuals and of markers than any study previously performed with this species. We hope that it also helps to define protection measures for the species, considering that some of its populations were recently detected to be vulnerable to the effects of the ongoing global warming, particularly in eastern Amazonia (Pontes-da-Silva et al., 2018). As the next steps, we suggest testing more deeply (using genomic markers, for example) the different geographic scenarios in eastern Amazonia to better understand the gene flow and the magnitude of climatic and geological events in the diversification of Amazonian lineages of *K. calcarata*; and to incorporate samples from the Atlantic Forest to understand the evolutionary history of this species as a whole.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the GenBank Nucleotide Database, under accession numbers ON160096 – ON160830 and ON025286 – ON025538 can be found in the **Supplementary Material**.

ETHICS STATEMENT

Ethical review and approval was not required for this study in accordance with the local legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

ÁAC, TCÁ-P, and FPW conducted study design and sampling. ÁAC made all laboratory procedures and figures and maps, and performed the analyses. All authors contributed to writing and reviewing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.777172/full#supplementary-material

Supplementary Figure 1 | Maximum likelihood concatenated (mtDNA and nuDNA) gene tree. The numbers represent the bootstraps nodal supports. Phylogroups letter annotations correspond to the same of obtained by Bayesian Inference (**Figure 1**).

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Comparative Phylogeography of Birds Across the Tocantins–Araguaia Interfluve Reveals a New Biogeographic Suture in the Amazon Far East

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Few phylogeographic studies have focused on understanding the role of the Tocantins–Araguaia Interfluve (TAI) in the diversification of Amazonian biodiversity. Herein we evaluate the phylogeographic relationships of 14 avian lineages present in the TAI and its two neighboring Amazonian Areas of Endemism: the Xingu (XAE) and Belém (BAE). Four alternative scenarios coupling degree of genetic differentiation and area relationships were tested: (1) populations distributed in TAI, BAE, and XAE are not genetically differentiated from each other (assumed as the null hypothesis); (2) populations from TAI are more closely related to those from BAE; (3) populations from TAI are more closely related to those from XAE; and (4) TAI populations represent independent or endemic lineages not present in either the BAE or XAE. Molecular analyses considered Bayesian Inference methods and Bayesian Information Criterion (BIC) evolutionary models, haplotype sharing and genetic differentiation estimates. We found three distinct phylogeographic patterns: (i) four lineages presented no population structure across XAE, TAI and BAE; (ii) six lineages were represented in the TAI with distinct phylogroups replacing each other between XAE and BAE, but with varying degrees of contact and evidence of gene-flow within the TAI; and (iii) for four lineages, the Tocantins River acted as a barrier isolating BAE phylogroups from those inhabiting both TAI and XAE. These different patterns demonstrate a heterogeneous response to the barrier effects posed by both the Tocantins and Araguaia rivers on the local fauna. Historical geomorphological and hydromorphological factors, such as the presence and absence of paleochannels and anastomosed channel stretches and variations in sedimentation rates support a dynamic history for the Araguaia-Tocantins floodplains, likely accounting for the observed heterogeneity in species' specific responses. Finally, the scenario of phylogeographic breaks and population subdivision recovered herein along the Tocantins and Araguaia rivers, associated with the existence of contact zones and the occurrence of gene flow, define the TAI as hitherto unknown biogeographic suture zone, localized in southeasternmost Amazonia.

Keywords: Amazonia, contact zones, gene flow, hybridization, river barrier, river dynamics

INTRODUCTION

Biotic diversification in the Amazon is the result of profound morpho-geo-climatic changes in time and space, promoting geographic isolation of populations and species formation (Haffer, 1969, 2008; Cracraft, 1985; Antonelli et al., 2010; Hoorn et al., 2010). These different diversification processes have been associated with one or more scenarios such as those predicted by the refuge (Haffer, 1969, 1992), river barrier (Wallace, 1853; Sick, 1967; Haffer, 1969, 1992, 2001; Cracraft, 1985), ecological gradients (Smith et al., 2001), and maritime incursion (Bates, 2001) hypotheses. More recently, Hoorn et al. (2010) and Silva et al. (2019) presented proposals integrating two of more of these hypotheses, clearly demonstrating the complexity of diversification scenarios across Amazonia.

Phylogeographic studies have helped define interspecific boundaries and shed light on the spatio-temporal patterns of diversification of several Amazonian taxa (Aleixo, 2002, 2004; Marks et al., 2002; Ribas et al., 2006, 2012; Lavergne et al., 2010; Avila-Pires et al., 2012; Carneiro et al., 2012; Batista et al., 2013; Fouquet et al., 2015; Mercês et al., 2015; Thom and Aleixo, 2015; Araújo-Silva et al., 2017; Sampaio et al., 2018; Capurucho et al., 2020; Dantas et al., 2021). These phylogeographic studies confirmed the effect imposed by major Amazonian rivers, inhibiting gene flow between allopatric populations and closely related species, and hence, delimiting interfluves as areas of endemism or endemism center (Silva et al., 2005; Ribas et al., 2012; Batista et al., 2013; Maldonado-Coelho et al., 2013; Thom and Aleixo, 2015). The phylogeographic pattern determined for the genus *Psophia* has been a recurrent example to illustrate the areas of endemism in the Amazon (Ribas et al., 2012).

In parallel, the identification of contact zones and hybridization among Amazonian birds has gained prominence with the phylogeographic approach. Comparative phylogeographic studies showed that pairs of selected parapatric taxa in the Tapajós Area of Endemism (TAE), between the Tapajós/Teles Pires and the Xingu rivers, in the central portion of the Amazon, presented some degree of gene flow around the headwaters of these Amazonian tributaries (Weir et al., 2015). Contact and hybridization zones were also identified between pairs of parapatric species in the headwaters of the Negro and Branco rivers, in the Guiana Shield in northern Amazonia (Naka et al., 2012). These phylogeographic studies confirmed the weakening of the isolation effect of major Amazonian rivers in their headwater portions, by facilitating gene flow between populations on opposite banks in these areas (Haffer, 1969, 1992).

Hence, phylogeography has played a fundamental role in understanding the diversification processes of the Amazonian avifauna. However, there are several regional gaps in Amazonian phylogeographic studies, including the Tocantins–Araguaia Interfluve (hereafter TAI). Haffer (1969, 1992) pointed to the lower Tocantins River as a geographic barrier, promoting the isolation of distinct lineages across their opposite banks, as recently corroborated by studies on *Pyriglena leuconota* (Maldonado-Coelho et al., 2013), *Dendrocolaptes certhia* (Batista et al., 2013), and *Thamnophilus aethiops* (Thom and Aleixo, 2015).

However, thus far, the evolutionary relationships of the TAI with other Amazonian areas of endemism remain obscure, with only a few studies sampling intensively populations in this sector of Amazonia (e.g., Hrbek et al., 2014, Rocha et al., 2015). The results found by Naka et al. (2012), Naka and Brumfield (2018), and Weir et al. (2015), in the northern and central regions of the Amazon, respectively, would also suggest a loss in power of any barrier effect exerted by the Tocantins River toward its headwaters, favoring a scenario of gene flow and little genetic differentiation between the TAI and neighboring areas of endemism. However, in contrast to this expectation, Rocha et al. (2015) showed that structured populations of small marsupials exist between the opposite banks of the middle Araguaia River.

To fill in these gaps, here we evaluate the phylogeographic relationships of at least 21 taxa belonging to 17 species of the avifauna of the TAI associated with humid and ecotonal forests occurring in its central-northern portion, with other Amazonian Areas of Endemism, mainly the neighboring Xingu (XAE) and Belém (BAE) (*sensu* Silva et al., 2005). Considering the relevant role played by major Amazonian rivers as geographic barriers, directly influencing the diversification of neotropical avifauna, we assume that the Araguaia and Tocantins rivers could act in such way, promoting genetic structuring of bird populations within the TAI.

Hence, we tested four alternative predictions concerning comparative levels of genetic differentiation and phylogeographic relationships (**Figure 1**) among the BAE, TAI, and XAE, as follows: (1) populations distributed in the TAI, BAE, and XAE are not genetically differentiated from each other, assumed as our null hypothesis; (2) populations from TAI are more closely related to those from XAE; (3) populations from TAI are more closely related to those from BAE; and (4) TAI populations represent genetically differentiated lineages not shared with the BAE and XAE. These hypotheses examine the possibility of the Tocantins and Araguaia rivers acting as geographical barriers to gene flow and the role of TAI as a contact, hybridization and introgression zone, or even as a new area of endemism for the Amazon. In addition, geological features of the Araguaia and Tocantins rivers are considered to support the discussion on how both rivers have influenced patterns of gene flow between lineages from their opposite banks.

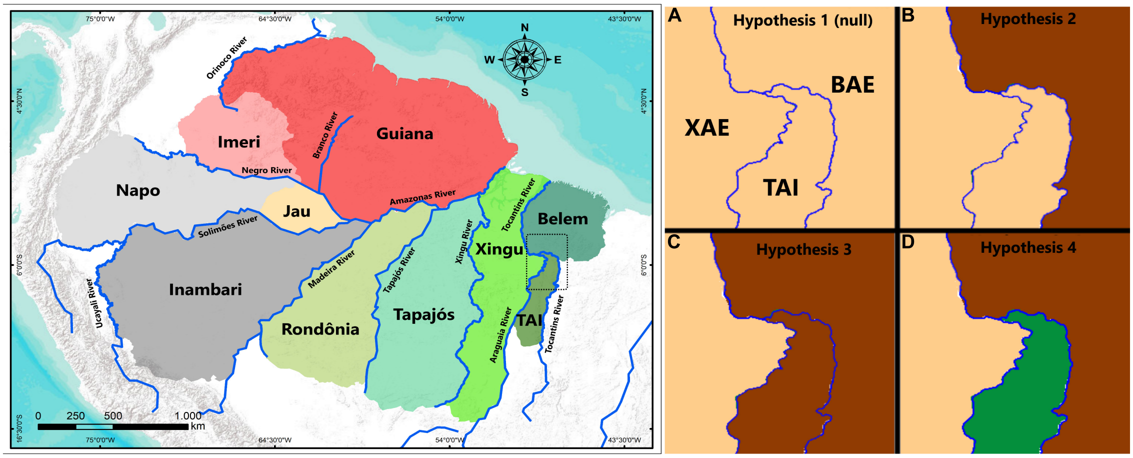

FIGURE 1 | Left: Amazonian areas of endemism according Silva et al. (2019), with the TAI shown in southeasternmost Amazonia. Right and clockwise: frames **(A–D)** of the four hypotheses of expected phylogeographic patterns for the TAI and neighboring areas (Belém Area of Endemism – BAE and Xingu Area of Endemism – XAE). **(A)** Hypothesis 1, all three areas are inhabited by genetically undifferentiated populations (assumed as the null hypothesis). **(B)** Hypothesis 2, TAI and XAE are inhabited by sister populations to the exclusion of BAE populations. **(C)** Hypothesis 3, TAI and BAE are inhabited by sister populations to the exclusion of XAE populations. **(D)** Hypothesis 4, TAI populations are inhabited by reciprocally monophyletic populations with respect to those in both BAE and XAE.

MATERIALS AND METHODS

Study Area

The TAI is located in southeasternmost Amazonian Brazil, inserted predominantly in the limits of the state of Tocantins (**Figure 1**). It is characterized as an ecotonal region with two morpho-phyto-climatic domains, the *Cerrado* and the Amazon (Ab'sáber, 2003; Instituto Brasileiro de Geografia e Estatística [IBGE], 2019). The western and northern regions of the TAI harbor a predominantly Amazonian avifauna, while the eastern, southeastern and central portions support an avifauna typical of the *Cerrado* biome (Dornas et al., in press). Together, these regions present Amazonian forest formations characterized by tropical and ecotonal forests (i.e., forest enclaves surrounded by seasonal semideciduous forests) and savannah vegetation including the *cerrado sensu stricto* and *cerradão* phytophysiognomies (Secretaria e Planejamento do Estado do Tocantins [Seplan], 2012; Haidar et al., 2013; Marques et al., 2020).

Selection of Taxa

To detect broad patterns of avian taxa replacement across the TAI and neighboring interfluves (BAE and XAE) and select the taxa to be sampled in this study, we mapped localities and assessed subspecific taxonomies of specimens from these areas deposited at the Fernando C. Novaes Ornithological Collection at the Museu Paraense Emílio Goeldi (hereafter MPEG), as well as those obtained from the literature. For the BAE, the following references were considered: Roma (1996), Vasconcelos et al. (2007), Novaes and Lima (2009), Portes et al. (2011), Oren and Roma (2012), Lees et al. (2012), Henriques et al. (2021) and the Wikiaves portal.[1] For the XAE, the following references were consulted: Graves and Zusi (1990), Aleixo et al. (2000), Oliveira et al. (2005), Valente (2006), Pacheco et al. (2007), Vasconcelos et al. (2007), Whittaker (2008), Aleixo et al. (2010), Somenzari et al. (2011), Aleixo et al. (2012), Henriques et al. (2021) and the Wikiaves portal. With respect to the TAI, the compilation made by Dornas et al. (in press) was considered. When reporting on our results, we followed the nomenclature and taxonomy contained in Pacheco et al. (2021), complemented by data on species and subspecies distributions found in the E-Bird portal.[2]

Our comparisons revealed which taxa are shared among the three different areas considering the following configuration (i) taxa with allopatric distributions replacing each other across the three areas; (ii) taxa with allopatric occurrences replacing each other between BAE/TAI or XAE/TAI; or (iii) taxa overlapping in distribution across all three areas. In all, 54 taxa belonging to 42 different species (of which 16 and 26 were, respectively, monotypic and polytypic) were identified as candidates for an initial screening (**Supplementary Table 1**). From these, a total of 21 taxa belonging to 17 species (8 monotypic and 9 polytypic) were selected as targets for this study due to tissue availability across all three major interfluves sampled. Together, these taxa comprise a total of 14 lineages, each corresponding to a monotypic/polytypic species or species complex (**Table 1**). Altogether, a total of 397 genetic samples belonging to these taxa were obtained from ornithological tissue collections (**Supplementary Table 2**) and sequenced herein.

Molecular Data

The genetic marker chosen for the molecular analyses was the mitochondrial NADH dehydrogenase subunit 2 gene (ND2). In addition to the 397 samples sequenced, another 141 ND2 sequences belonging to the selected species and respective outgroups were obtained from the GenBank database (**Supplementary Table 2**).

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[1] www.wikiaves.com.br

[2] https://ebird.org/home

TABLE 1 | List containing 14 avian lineages included in the molecular analyses and the taxon present in each of the major areas sampled in this study.

Xingu area of endemism	Tocantins–Araguaia interfluve	Belem area of endemism	Phylogeographic pattern
	Myrmotherula axillaris axillaris		i
Sclateria naevia toddi	*S. naevia* ssp. (single phylogroup)	*Sclateria naevia naevia*	
	Dendrocincla fuliginosa rufoolivacea		
Manacus manacus longibarbatus	*M. manacus* ssp. (single phylogroup)	*Manacus manacus purissimus*	
XAE phylogroup	*Campylopterus obscurus*	BAE phylogroup	ii
Pyrrhura anerythra	*Pyrrhura* spp.	*P. coerulescens*	
Thamnophilus amazonicus obscurus	*T. amazonicus* ssp.	*Thamnophilus amazonicus paraensis*	
XAE phylogroup	*Willisornis vidua* (contact by apparent parapatry)	BAE phylogroup	
Dendrocolaptes retentus	*Dendrocolaptes* spp.	*D. medius*	
XAE phylogroup	*Taeniotriccus andrei klagesi* (contact by apparent parapathy)	BAE phylogroup	
*Psophia interjecta**		*P. obscura*	iii
Formicarius colma amazonicus (XAE + TAI phylogroup)		*F. colma amazonicus* (BAE phylogroup)	
Schiffornis turdina wallacii (XAE + TAI phylogroup)		*S. turdina wallacii* (BAE phylogroup)	
Granatellus pelzelni pelzelni		*Granatellus pelzelni paraensis*	

Key to the recovered phylogeographic patterns are as follows: (i) no population structure across XAE, TAI, and BAE; (ii) TAI as a contact zone between distinct species/phylogroups inhabiting XAE and BAE; and (iii) the Tocantins River as a barrier isolating BAE phylogroups from those inhabiting both TAI and XAE.
**See Dornas et al. (2017).*

Total DNA was extracted following standard procedures of the phenol-chloroform technique (Sambrook et al., 1989). The amplification of the ND2 gene occurred through the Polymerase Chain Reaction (PCR). Primers used for gene amplification were H6313: CCTTGAAGCACTTCTGGGAATCAGA (Sorenson et al., 1999) and L5215: TATCGGGCCCATACCCCGAAAAT (Hackett, 1996). The total volume of PCR reactions was 25 μL containing: 12.4 μL of Master Mix (50 U/mL, 400 μL of each dNTP and 3 mM of MgCl2), 9.8 μL of ultra-pure water, 0.4 μL of each of the primers (200 ng/μL) and, finally, 2 μL of genomic DNA.

The amplification profile proceeded in the following order: an initial 5 min step at 95°C for block temperature homogenization; followed by 35 cycles of 3 min each, separated by 1 min at 95°C, 1 min at the initiator association temperature for each selected species, and 1 min at 72°C; finally, a final step of 5 min, at 72°C, for the polymerization of eventual molecules, from which the polymerase has dissociated before the end of the total synthesis of the fragment. The association temperatures of the primers of each species were determined by a temperature gradient PCR (from 50 to 63°C). The amplified samples were checked in electrophoresis using a 1% agarose gel and purified following the Polyethylene Glycol (PEG-8000) protocol. The products of the amplifications and purifications were sequenced, directly and automatically, using the Kit-Big Dye Terminator Cycle Sequencing Standard Version 3.1‖on the ABI 3130 Sequencer from Applied Biosystems according to the manufacturer's specifications.

After DNA extraction, amplification and sequencing processes, the sequences were edited and aligned by the ClustalW method using the BioEdit software (Hall, 1999). The saturation in the number of mutations was evaluated through graphs of transitions plotted against transversions as a function of genetic distances using the DAMBE program (Xia and Xie, 2001).

Molecular Analyses

Phylogenetic analyses were carried out using Bayesian Inference (BI), as implemented in the MrBayes 3.1.2 program (Ronquist and Huelsenbeck, 2003). The molecular model of evolution best fitting each dataset was obtained based on the Bayesian Information Criterion (BIC) estimated with JModelTest (Posada, 2008). The BI proceeded with two independent runs of 10,000,000 generations each, with the sampling of one tree every 1,000 generations. The TRACER 1.5 software (Rambaut and Drummond, 2009) was used to verify whether posterior distributions of parameters reached the minimum threshold (ESS >200). Trees obtained before the Markov chain reached stable and convergent likelihood values were discarded. Haplotype networks were estimated for all species with HaploViewer (Salzburger et al., 2011).

The population genetics fixation index *Fst* was calculated using the Arlequin 3.0 program (Excoffier et al., 2005). Following Wright (1978), Hartl and Clark (2007), and Frankham et al. (2008), *Fst* values smaller than 0.49 were interpreted as representing low differentiation, between 0.5 and 0.69, moderate differentiation and above 0.7 high genetic differentiation.

RESULTS

The superposition of distributions of the XAE, BAE, and TAI target taxa belonging to the 14 lineages selected for the phylogeographic analyses revealed the following patterns: (1) 7 pairs of taxa replace each other between XAE and BAE, with populations of unknown subspecific identity present in the TAI; and (2) 7 taxa occur indistinctly throughout all three areas (**Table 1**). Among the 14 lineages selected, the lower Tocantins River was a barrier separating 10 pairs of differentiated phylogroups belonging to thirteen species or species complexes (**Table 1**). In contrast, only four lineages belonging each to a distinct species did not show signs of geographic structuring across the Tocantins and Araguaia rivers. Below, we describe the results of the phylogeographic analyses in detail.

Phylogeographic Patterns

The phylogenetic and population genetic analyses obtained for the different lineages sampled uncovered three main distinct phylogeographic patterns (**Figure 2**), as follows: i) no population structure across XAE, TAI, and BAE, found in *Mymotherula axillaris, Sclateria naevia, Manacus manacus*, and *Dendrocincla fuliginosa*; (ii) TAI as a contact zone with evidence of gene-flow of lack thereof between distinct species/phylogroups replacing each other in the XAE and BAE, as in *Pyrrhura anerythra/Pyrrhura coerulescens*, *Campylopterus obscurus*, *Dendrocolaptes medius/Dendrocolaptes retentus*, *Thamnophilus amazonicus, Willisornis vidua*, and *Taenotriccus andrei*; and (iii) the Tocantins River as the barrier isolating BAE phylogroups from those inhabiting both TAI and XAE, as in *Psophia interjecta/Psophia obscura, Formicarius colma, Granatellus pelzelni/Granatellus paraensis*, and *Schiffornis turdina*. We found no support among the sampled taxa for two of our *a priori* hypotheses; i.e., no TAI lineages/populations grouped as sister to those in the BAE, to the exclusion of XAE phylogroups (as postulated by hypothesis 3), and no reciprocally monophyletic (endemic) phylogroups on the TAI were recovered (as predicted by hypothesis 4).

Pattern "i" corroborated hypothesis 1, assumed as the null, that populations distributed in TAI, BAE, and XAE are not genetically differentiated, demonstrating no barrier effect posed by the Araguaia River or the Tocantins River on the gene flow between populations of species present in all sampled areas. The topologies of the phylogenetic trees indicated non-reciprocal monophyly among the TAI and the two neighboring endemic areas (**Figure 3**) which, added to the absence of population structure indicated by the extensive sharing of haplotypes and reduced *Fst* values (smaller than 0.49), demonstrated absence of genetic structure, supporting regular levels of gene flow between populations of *M. axillaris, S. naevia, M. manacus*, and *D. fuliginosa* across these three different areas (**Supplementary Figures 1–4** and **Supplementary Table 3**). For *D. fuliginosa*, the absence of genetic differentiation seemed to also include the Tapajós Area of Endemism (TAE), suggesting a lack of phylogeographic structure across southeastern Amazonia as a whole, but strong genetic differentiation with respect to lineages occurring in western Amazonia and the Guiana Shield (see also Schultz et al., 2019).

For pattern "ii," the phylogenetic trees presented two statistically well-supported and reciprocally monophyletic clades associated with XAE and BAE, respectively, with TAI populations grouping in both clades (**Figure 3** and **Supplementary Figures 5–8**). The haplotype networks also confirmed this genetic structure, indicating the presence of haplotypes otherwise restricted to both the XAE and BAE in the interior of the TAI (**Supplementary Figures 5–9**). These phylogeographic results showed, therefore, that there are representatives of both XAE and BAE in the TAI, which constitutes a wide contact zone between pairs of distinct taxa endemic to those areas of endemism. In addition to the contact zones, our phylogenetic trees and haplotypes networks also suggest the existence of hybrid individuals, at least between the closely related species pairs *P. anerythra/P. coerulescens* and *D. retentus/D. medius* (**Supplementary Figures 5, 6**). In other cases, such as in *C. obscurus*, specimens occurring in syntopy (e.g., AGUA 97 and AGUA 47) belonged to these two distinct mitochondrial clades, reinforcing the TAI as a wide contact zone between otherwise genetically structured populations across the lower Tocantins River, a situation clearly not maintained across the Araguaia River.

In *Willisornis vidua*, BAE populations clustered in a well-supported clade along with those from the northernmost part of the TAI, whereas XAE specimens did not cluster in a single clade, but with some of them being closely related to several individuals occurring throughout most of the TAI, as also supported by the haplotype networks (**Supplementary Figure 9**). These two genetically structured *W. vidua* populations within the TAI are apparently parapatric, and no evidence of widespread gene flow between them has been recovered so far (see Quaresma et al., 2022). Preliminary phylogeographic analyses focusing on *T. andrei* showed a similar north-south replacement across the TAI involving distinct phylogroups and haplotype network arrangements clustering with the BAE or XAE clades (**Supplementary Figure 10**). However, as we had access to few samples, this phylogeographic structure was not well supported statistically and could just be a sampling artifact. In this case, it is necessary to increase the sampling of *T. andrei*.

Pattern "iii" supports the Tocantins River as a barrier separating phylogroups restricted to the TAI and XAE from those in BAE (**Figure 3**), as predicted by hypothesis 2. The topologies of phylogenetic trees, the sharing of haplotypes and the *Fst* values recovered for *F. colma, G. pelzelni, S. turdina*, and *P. interjecta/P. obscura* are consistent with a stronger barrier effect played by the Tocantins River (**Supplementary Figures 11–13** and **Supplementary Table 3**; see Dornas et al., 2017 for *P. interjecta*).

DISCUSSION

The determination of phylogeographic patterns in the Amazon involving the sampling of the TAI area has been limited (Hrbek et al., 2014; Rocha et al., 2015) or completely absent (e.g. Aleixo, 2002; Ribas et al., 2006, 2012; Patané et al., 2009; Batista et al., 2013; Rodrigues et al., 2013; Soares et al., 2019). This lack of TAI samples in Amazonian phylogeographic studies precludes postulating any preliminary hypothesis regarding the historical relationships between this eastern Amazon interfluve and other regions.

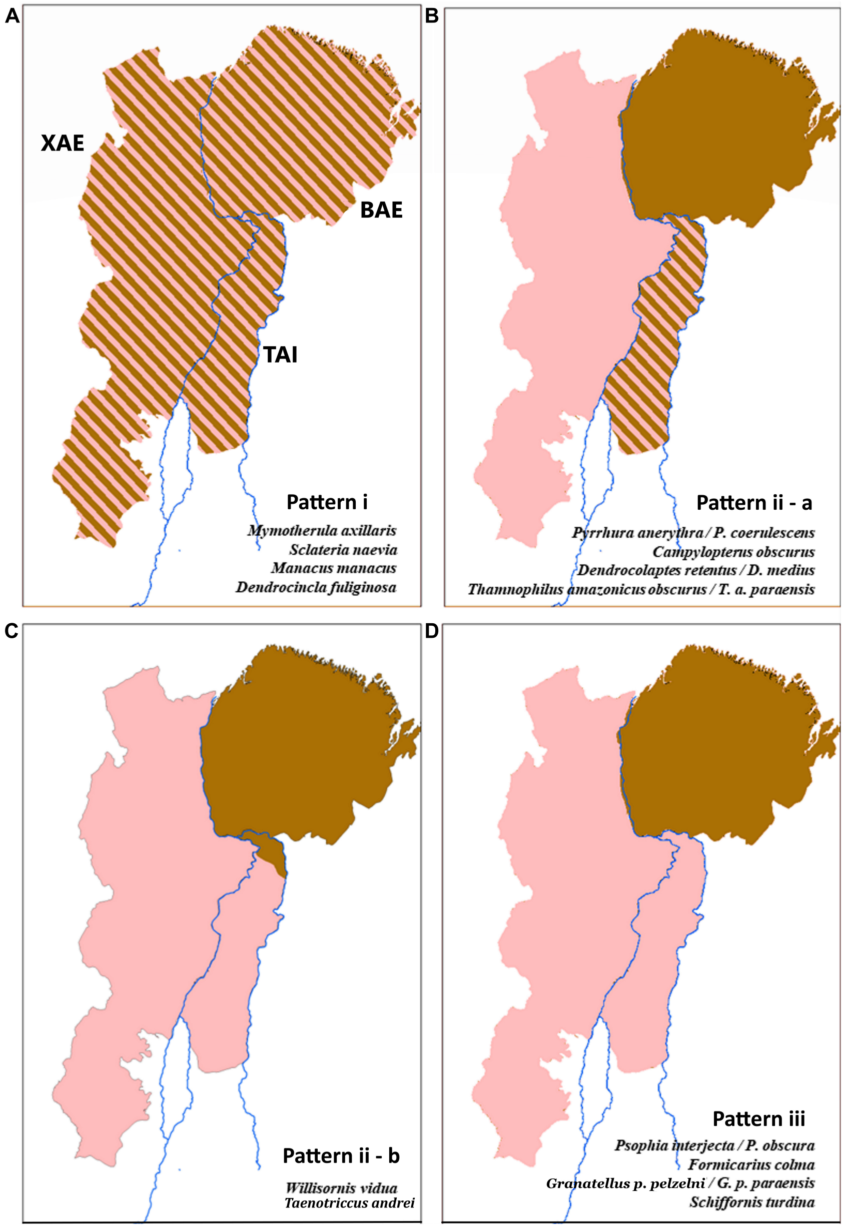

FIGURE 2 | Phylogenetic and haplotype network analyses and *Fst* indexes revealed three distinct phylogeographic patterns shared by genetically sampled avian lineages in the TAI, as follows: pattern i **(A)**, no population structure across the Xingu Area of Endemism (XAE), TAI, and Belem Area of Endemism (BAE); pattern ii, two clades associated each with XAE and BAE, with TAI populations grouping in both clades, but with two alternative spatial distributions, defined as "a" – TAI as a contact zone with evidence of gene-flow and sympatry between distinct species/phylogroups inhabiting XAE and BAE **(B)**, and "b" – two distinct phylogroups without evidence of gene-flow, associated with XAE and BAE and distributed in a parapatric fashion within the TAI **(C)**; and pattern iii **(D)**, the Tocantins River as the barrier isolating BAE phylogroups from those inhabiting both TAI and XAE. See **Supplementary Material** for details.

Here, we assessed for the first time the comparative barrier effects posed by both the Tocantins and Araguaia rivers on 14 avian linages in the southeasternmost Amazonian biota. With the caveat that we sampled only one mitochondrial locus, which is insufficient to estimate accurate rates of gene flow and hybridization, our data showed the TAI as a contact zone between the rather differentiated avifauna of the neighboring BAE and XAE, providing further support to the overall view that the upper reaches of major Amazonian rivers are more permeable to gene flow and faunal exchange than their lowermost parts.

Effect of Geographic Barrier of the Tocantins and Araguaia Rivers

The effect of large Amazonian rivers as primary or secondary geographic barriers to the local avifauna, delimiting the

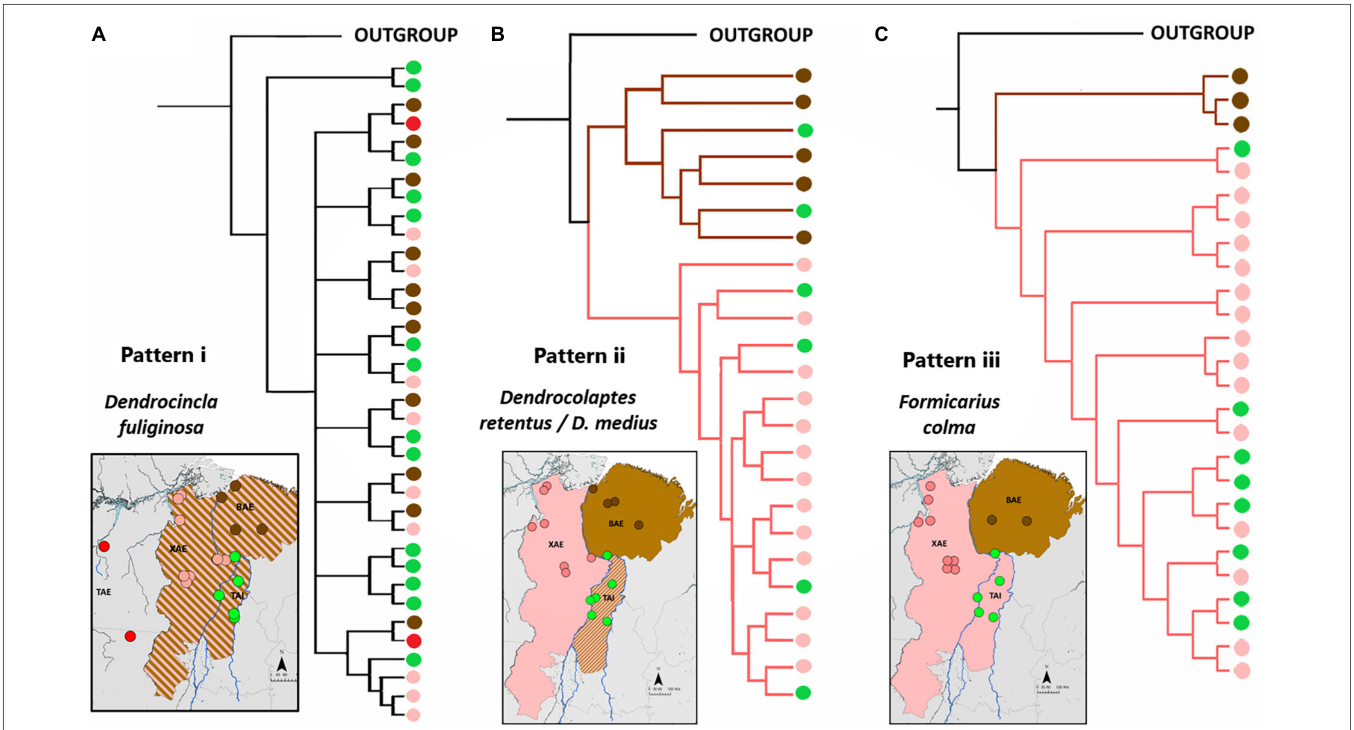

FIGURE 3 | Topologies of phylogenetic trees representative of each of the three phylogeographic patterns recovered among lineages of the sampled taxa inhabiting the XAE, BAE, and TAI. Black circles represent outgroup samples, whereas red, green, pink, and brown circles refer respectively to the TAE, TAI, XAE, and BAE samples. **(A)** Phylogenetic tree obtained for *Dendrocincla fuliginosa* illustrating pattern i (null hypothesis), whereby no geographically structured was recovered. **(B)** Phylogenetic tree of samples of the allopatric *Dendrocolaptes retentus* (whose distribution is centered in the XAE) and *D. medius* (distributed mainly in the BAE) illustrating pattern ii, in which the riverine barrier effect is present only in the lower Tocantins River, with the TAI consisting a contact zone between otherwise allopatric lineages replacing each other in the XAE and BAE. **(C)** Phylogenetic tree recovered for *Formicarius colma* samples illustrating pattern iii, in which the barrier effect is present along the entire Tocantins River, implying in a sister relationship between XAE and TAI lineages to the exclusion of those in the BAE. See **Supplementary Material** for details on these and the other sampled species.

distribution of independent evolutionary lineages among interfluves, is widespread (Wallace, 1853; Sick, 1967; Haffer, 1969, 1992; Naka, 2011; Fernandes et al., 2012; Naka et al., 2012; Ribas et al., 2012; Naka and Brumfield, 2018; Silva et al., 2019). The phylogeographic patterns documented herein demonstrated that TAI lineages are closely related to those in the neighboring XAE and BAE. However, the different patterns of genetic diversity and area relationships found revealed that taxa have had distinct evolutionary histories in the TAI.

Pattern "I," consistent with the assumed null hypothesis, given the expected outcome of a lack of genetic differentiation in the absence of a river-barrier effect, was shared by four species and indicated an absence of genetic structuring between TAI, XAE, and BAE. This supports a lack of barrier effects imposed by the Araguaia and Tocantins rivers, consistent with gene flow between populations of the sampled lineages across the TAI, XAE, and BAE (**Figure 2**). Natural history attributes of the avian lineages sampled, alongside with the history of formation of the Araguaia and Tocantins rivers may explain these inferred high rates of gene flow. The four species included in pattern "i" inhabit environments of flooded forest or floodplain, typical of river banks (Remsen and Parker, 1983), as well as river islands (Sick, 1997; Ridgely and Tudor, 2009; Kirwan and Green, 2011; Sigrist, 2013), which may have facilitated their dispersal across both the Araguaia and Tocantins rivers. Species in this habitat are known to have different population genetic histories than those in *terra firme* habitats (Harvey et al., 2017), which may explain this pattern.

In contrast, pattern "ii" reflect an important biogeographic characteristic of the Amazon: the reduction of the barrier effect of Amazonian rivers toward their headwaters (see Weir et al., 2015). Disregarding the four species sharing the phylogeographic pattern "i" discussed above, the remaining thirteen species targeted for molecular analysis presented genetically structured populations along the opposite banks of the lower Tocantins River (**Table 1**). This role played by the lower Tocantins River in isolating populations of reciprocally monophyletic and allopatric taxa between the XAE and BAE had already been documented for several avian lineages associated mainly with upland *terra-firme* forest (Ribas et al., 2012; Batista et al., 2013; Maldonado-Coelho et al., 2013; Thom and Aleixo, 2015; Silva et al., 2019). This variation in strength of the barrier effect played by the Tocantins River is consistent with the narrowing of its course upstream from its confluence with the Araguaia River, which can be two to three times narrower compared to its lower course. This is clearly demonstrated by the phylogeographic patterns detected for the allopatric species pairs *P. anerythra*/*P. coerulescens*, *D. retentus*/*D. medius, Campylopyerus obscurus*, and *Thamnophilus amazonicus*.

This gradual reduction in the barrier effect between the lower Tocantins River and its middle and upper portions together with the Araguaia River is similar to the conditions described for the Negro and Branco rivers on the Guiana shield (Naka et al., 2012; Naka and Brumfield, 2018). The lower Rio Negro acts as a phylogeographic break for more than 40 pairs of allopatric taxa among those 69 sampled (Naka et al., 2012; Naka and Brumfield, 2018). For the region of the Tocantins–Araguaia basin, the lower Tocantins River isolated seven pairs of allopatric taxa between XAE and BAE and five more taxa whose populations showed genetic structure between those areas of endemism (patterns "ii" and "iii"). In addition to these allopatric populations and taxa for which we obtained genetic data, another nine pairs of taxa are separated by the lower Tocantins River (**Supplementary Table 1**), but were not sampled genetically by us.

In addition to its narrower course when compared to the lower Tocantins, glaciation cycles between 1 million and 20,000 years before present, when dry and cold periods alternated with hot and humid periods (Hoorn et al., 2010; Meyer et al., 2014) might be related to the lower intensity of the barrier effect exerted by the Araguaia River. Ecological niche modeling showed that during the driest and coldest glacial periods (such as the Last Glacial Maximum), forests were likely replaced by savanna and non-humid forest phytophysiognomies in southeastern Amazonia (Aleixo et al., 2014; Silva et al., 2019). If this scenario is correct, then it would have caused a reduction in the geographic distribution of bird species associated with humid forests in southeastern most Amazonia, probably accompanied by the extinction of several taxa, which nevertheless might have persisted in the central-western portions of the biome (Silva et al., 2019). Although in the wettest and hottest periods of these glacial cycles there were probably successive retakes of forest cover into the extreme east of the Amazon, it is assumed that on these occasions the Araguaia River acted as a secondary geographic barrier, preventing the transposition of many typical upland *terra-firme* species, which are present in the XAE but do not enter the TAI (such as *Cercomacra cinerascens, T. aethiops, Monasa morphoeus, P. leuconota, Galbula dea, Thamnophilus palliatus, Myrmoborus myotherinus, Glyphorynchus spirurus, Vireolanius leucotis, Conopophaga aurita, Hylopezus paraensis, Synallaxis rutilans, Lamprospiza melanoleuca, Ramphocaenus melanurus*, and *Piprites chloris*) (Ridgely and Tudor, 2009; Billerman et al., 2020; Birdlife International, 2020; **Supplementary Figure 14**).

The current patterns of geographic distributions of this set of bird species could reflect a response to paleoenvironmental conditions affecting southeastern Amazonia as a whole and the upper stretches of the Tocantins and Araguaia rivers in a similar way as discussed for the genera *Hylexetastes* and *Xiphocolaptes*, which are widely present in Amazonia, but absent in the BAE, the easternmost Amazonian area of endemism (Silva et al., 2002; Azuaje-Rodríguez et al., 2020). Therefore, the hypothesis 3 that postulated the Araguaia River as a geographic barrier impeding the gene flow between TAI and XAE, albeit not corroborated by our molecular analyses, appears nevertheless supported by the geographic distributions of *Ortalis superciliaris,* shared between TAI and BAE, but absent in XAE (**Supplementary Figure 14**; Grantsau, 2010; Pascoal et al., 2016; Del Hoyo and Kirwan, 2020), and *Piculus paraensis*, also absent in XAE, but confirmed in the BAE (Del-Rio et al., 2013) and suspected within the TAI (Dornas et al., in press).

The phylogeographic pattern documented here for *W. vidua*, whereby two parapatric genetic lineages exist in the TAI could also be related to Late Pleistocene climatic-vegetational changes, which could have resulted in the presence of large savanna blocks between the northern and central-southern parts of the TAI separating two patches of fully forested areas. The first to the north of the TAI, geographically close to the BAE and adjacent to the left (western) bank of the Tocantins River, and the second to the south, geographically close to the XAE and adjacent to the right (eastern) bank of the Araguaia River (**Supplementary Figure 15**). These genetically distinct populations of *W. vidua* are apparently not in direct contact and exchanging genes due to the presence of an intervening modern ecological barrier, at the same time that their establishment in different parts of the TAI reflects distinct origins and possible independent colonization events into this interfluve.

In contrast to pattern "ii," pattern "iii" documented herein for five species supported an extended barrier effect along the Tocantins River upstream from its confluence with the Araguaia River, highlighting the variability in inter-specific responses to a single riverine barrier, as verified for the middle and upper portions of the Negro and Branco rivers (Naka et al., 2012; Naka and Brumfield, 2018). Similarly, the proximity of middle and upper Tocantins River to the Amazon – Cerrado ecotone may enhance the barrier effect posed by the physical course of the river itself, which could "stabilize" the southeasternmost limits of ranges of several humid forest taxa within the TAI.

Finally, the phylogeographic analyses of the selected taxa did not demonstrate the presence of any genetically differentiated lineage restricted to the TAI. Evolutionarily, avian endemic lineages to the easternmost portion of the Amazon have shown very recent times of diversification coupled with smaller genetic distances separating them from other closely related south-central Amazonian lineages (Ribas et al., 2012; Batista et al., 2013; Aleixo et al., 2014; Thom and Aleixo, 2015; Silva et al., 2019). The continuing existence of gene flow together with the variation in the intensity of the barrier effect of the Tocantins and Araguaia rivers show that the time of diversification of these populations present in the TAI is still very recent, not favoring local coalescent processes.

Phylogenetic systematic studies supported lineages associated to the BAE and XAE, as single evolutionary units (e.g., *Hylopezus macularius* complex), hence, corroborating a scenario of reduced genetic diversification in the far east of the Amazon (Carneiro et al., 2012; Rodrigues et al., 2013; Silva et al., 2019). However, the presence of some species with populations showing high and significant *Fst* values between the TAI and XAE and also between TAI and BAE (**Supplementary Table 3**), demonstrate that genetic differentiation is underway inside the TAI. Until now, the only endemism of the TAI supported by molecular analyses is a didelphid marsupial described from the right bank of the Araguaia River (Rocha et al., 2015).

Contact Zones and Gene Flow

Contact zones represent areas where there is an overlap between parapatric populations of different taxa (Haffer, 1997; Aleixo, 2007). The recovered phylogeographic pattern “ii” demonstrated that within the TAI there is the concomitant occurrence of distinct populations or lineages otherwise endemic to the XAE and BAE, as verified for *D. retentus*/*D. medius*, *P. anerythra*/*P. coerulescens*, and *C. obscurus*.

The comparison of plumages of the specimens collected in the TAI for *D. medius* and *D. retentus* and *P. anerythra* and *P. coerulescens*, with those obtained from XAE and BAE indicated the apparent occurrence of hybridization events (**Supplementary Table 2** and **Supplementary Figures 5, 6**). In *Dendrocolaptes*, the sharing of haplotypes from both endemic areas within the TAI, associated with the intermediate phenotypes between *D. retentus* and *D. medius*, support this area as a contact and hybridization zone between these species (*Fst* XAE and TAI = 0.23660/*Fst* BAE and TAI = 0.41681/$P > 0.05$), which nevertheless sort out completely across the lower Tocantins River (Batista et al., 2013).

The hybridization between *P. anerythra* and *P. coerulescens* had been initially suggested for the lower Tocantins River (Somenzari and Silveira, 2015) and later confirmed within the TAI by morphological comparisons among three specimens collected in the region (Brito et al., 2016). The three specimens collected in the TAI by Brito et al. (2016) were sampled in the molecular analyses (**Supplementary Table 2**) and had their hybridization also corroborated by genetic characters (**Supplementary Figure 5**). However, one specimen syntopic with the three hybrid specimens mentioned above (Dornas et al., in press), presented haplotype and morphological diagnoses of *P. anerythra*, supporting the phenotypic and genotypic occurrence of the species in the TAI. The confirmation of *P. coerulescens* records within the limits of the TAI is provided only through photographic records WA2759636 (Corrêa, 2017) and WA1882554 (Pacheco, 2005). The low and non-significant *Fst* values between XAE and TAI (*Fst* = 0.157), and between BAE and TAI (*Fst* = 0.368) *P. anerythra*/*P. coerulescens* lineages support a scenario of ongoing gene flow between them.

In turn, *C. obscurus* is a recently split species recognized by recent taxonomic revisions within the *Campylopterus largipennis* complex (Lopes et al., 2017). Our phylogenetic analyses recovered structure between XAE and BAE populations of this species, but which was not maintained in the TAI, where specimens of both lineages were found in syntopy (**Supplementary Figure 7**). Despite the marked molecular divergences observed between BAE and XAE *C. obscurus* populations, no apparent diagnosable morphological differences between were observed. Therefore, the extension of gene flow between these distinct populations must be assessed with a wider spectrum of genetic markers.

The occurrence of these contact and hybridization zones within the TAI (see also Areta et al., 2017) can be related to fluvial morphodynamic processes, such as the reduction of the isolation effect of the Tocantins and Araguaia rivers upstream from their confluence, due to geological and geomorphological characteristics. The widths of the Tocantins and Araguaia rivers gradually reduce upstream of their confluence (**Supplementary Figure 16**), being up to 10 times narrowed than the width of stretches of the Tocantins River downstream of the confluence with the Araguaia River (Agência Nacional das Águas [ANA], 2020). Moreover, the presence of extensive and forested river islands along the headwaters of the Tocantins and Araguaia rivers favors the establishment of several species of birds associated with humid forest habitats. These islands may facilitate the crossing between banks, promoting more frequent gene flow between populations on these upper stretches than in the Lower Tocantins area (**Supplementary Figure 16**).

Two other morphodynamic processes linked to fluvial systems in the Tocantins and Araguaia rivers can be related to the transposition of the banks by birds and consequent gene flow. The first is a succession of large oxbow lakes, with their surroundings taken over by floodplain forests. These lakes signal the old lines of the riverbed, known as paleochannels and characterize a meandrite fluvial system (**Supplementary Figure 16**), in which the riverbed is strongly sinuous, constituting an anastomosed fluvial system formed by numerous fluvial islands of varying sizes, generating a fluvial landscape with a highly ramified (Riccomini and Coimbra, 1993; Latrubesse and Stevaux, 2002, 2006; Morais et al., 2005; Rocha, 2011; Fryirs and Brierley, 2018). The second is the presence of straits, known also as pinched channels. Straits are drainage anomalies characterized by places where there is a narrowing of the river banks, with a marked reduction in the width of the riverbed, from the order of kilometers to a few hundred meters, usually resulting from local structural geological control, such as superimposition on dikes or embankments conditioned by geological faults (Howard, 1967; Summerfield, 2014; Barros and Magalhães Junior, 2020). Consequently, they can represent a relevant crossing point for the local biota between opposite river banks in closer proximity.

In the Tocantins River, one of the most remarkable straits is located in front of the municipality of Estreito. This river section presents an abrupt funneling of the riverbed from a predominant width of 1 km to about 150 m (6°33′42″S, 47°27′36″ W). On the Araguaia River, one of the main existing straits is located near the city of Xambioá (6°22′S; 48°23′W). The Pre-cambrian rock matrix of highly resistant quartzites, prominent in the region of Serra das Andorinhas (left bank) called the Morro Grande Formation, crosses the Araguaia River, entering the limits of the TAI (Figueiredo and Sousa, 2009). This geological continuity originates a very tapered strait, regionally called Remanso dos Botos (**Supplementary Figure 16**). The reduction in the width of the river in this stretch goes from 1.2 km to ca. 400–500 m. In sum, geomorphological fluvial processes support a strong physical historical connection between XAE and BAE biotas in the TAI, which is consistent with the documented contact zones and hybridization and gene flow events documented herein between lineages isolated in the BAE and XAE across the Lower Tocantins River.

Tocantins–Araguaia Interfluve as an Eastern Amazon Suture Zone

The disproportionate presence of a high number of phylogeographic breaks, contact zones and hybridization events configure the definition of biogeographic suture zones (Remington, 1968; Swenson and Howard, 2004, 2005). In the Amazon, the Negro–Branco interfluve has been characterized as a biogeographic suture zone for birds (Naka et al., 2012; Naka and Brumfield, 2018), as also verified for the upper reaches of the Tapajós River (Haffer, 1992).

Similarly, within the TAI, the recovered pattern "iii" supports the Tocantins River as a barrier separating genetically differentiated populations distributed in the XAE and TAI from those occurring in the BAE. In turn, pattern "ii" indicates the joint occurrence within the TAI of taxa otherwise distributed allopatrically in the XAE and BAE, hence, characterizing the existence of a contact zone. As discussed above, these contact zones involve either (1) an instance of apparent parapatry between two genetically distinct populations occurring in different parts of the TAI, and separated by the original presence of savanna formations (such as in *W. vidua*), or (2) taxa in apparent direct contact and which hybridize with each other within the TAI (such as in *D. retentus*/*D. medius* and *P. anerythra*/*P. coerulescens*).

Thus, the high number of phylogeographic breaks and contact zones within a single interfluve, as described for the Negro-Branco rivers by Naka et al. (2012) is equivalent to the one we documented herein for the TAI. Similarly, Weir et al. (2015) show several instances of hybridization between taxa distributed otherwise parapatrically across the headwaters of the Tapajós/Teles Pires and Xingu rivers, hence, further supporting a suture zone in south-central Amazonia. Both sutures zones documented by Naka et al. (2012) and Weir et al. (2015) overlap to some extent with ecotonal transitions between forest and savanna habitats located in the middle and upper portions of their respective hydrographic basins. These characteristics are also shared by the TAI, where another ecotonal transition between Amazonian forests and Cerrado savannas occur (Haidar et al., 2013; Marques et al., 2020). Therefore, all these characteristics combined support the TAI as a novel biogeographic suture zone, localized in the Eastern Amazon.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the GenBank Nucleotide Database repository, accession numbers: ON137233–ON137450 and ON157084–ON157237.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because there was no handling of birds, just traditional laboratory work involving tissue aliquots, reagents, electronic machinery, and computer software. The source specimens of these aliquots were collected before the present study, by field expeditions of different teams from the consulted ornithological collections.

AUTHOR CONTRIBUTIONS

TD and AA conceived the study. TD and SD analyzed the specimens in ornithological collection. TD, SD, and LA-S performed the laboratory work referring to molecular analyses. TD, LA-S, and AA applied and analyzed the data in computer applications. TD and FM analyzed the data under geological and fluvial morphology perspectives. TD wrote the manuscript. AA and SD reviewed and improved the English version of manuscript. All authors reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.826394/full#supplementary-material

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Riverine Barriers as Obstacles to Dispersal in Amazonian Birds

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Amazonian rivers represent known barriers for avian dispersal, reducing gene flow and enhancing differentiation. Despite the importance of rivers in the avian evolutionary process, we have made only minor advances in understanding the limitations imposed by rivers on flying birds. To fill that gap, we conducted dispersal-challenge experiments over water, assessing the flying capabilities of 84 tropical bird species of 22 different avian families. We mist-netted and released 484 birds from a stationary boat on the Rio Branco, northern Amazonia, at increasing distances from the shore, including 249 individuals at 100; 219 at 200; 8 at 300; and 5 at 400 m. A successful trial was represented by a bird reaching the riverbank, whereas a failure would refer to birds not reaching the shore and landing on the water, when they were rescued by our team. Our main goal was to understand if the outcome in the experiments could be predicted by (i) phylogenetic constraints, (ii) morphology (body mass and wing shape), (iii) flight speed, (iv) ecological preferences (stratum, habitat, and river-island specialization), and (v) psychological reluctance to fly. Nearly two thirds of the individuals (332) were successful in reaching the riverbank, whereas 152 failed. We found significant differences among lineages. Whereas seven avian families succeeded in all of the trials, two families (antbirds and wrens) were particularly bad dispersers (<40% success). The hand-wing index (HWI) was the single most powerful predictor of trial success. Flying speed was also a significant predictor of success. Overall, ecological attributes had a low explanatory power. Only forest stratum preference had a significant, although weak, effect on dispersal ability: canopy- and ground-dwellers performed better than understory birds. However, we found no effect of habitat preference or river-island specialization on dispersal ability. Our speed estimates for 64 bird species are among the first produced for the tropics and suggest slower flying speeds than those reported from temperate migratory birds. Although birds showed behavioral differences when presented with the opportunity to fly away from the boat, we found no evidence that their reluctance to fly could predict the outcome in the experiments. This represents the first experimental study evaluating the riverine effect through dispersal ability of Amazonian birds, providing important insights to better understand dispersal limitations provided by riverine barriers.

Keywords: avian flight, riverine barriers, dispersal experiments, flight speed, ecological predictors

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INTRODUCTION

Given their ability to fly, birds are considered the most vagile class of terrestrial vertebrates, evidenced by their capacity to colonize even the most remote oceanic islands (Lees and Gilroy, 2014). However, it is well known that many avian taxa have river-bounded distributions, particularly in the Amazon basin, where dozens of avian lineages have morphologically and genetically distinct close relatives separated by rivers (Haffer, 1992; Naka et al., 2012). In fact, Amazonian rivers are known to represent important biogeographical boundaries (Haffer, 1969) and are traditionally used to delimit areas of endemism (Cracraft, 1985). First described for primates and birds (Wallace, 1852; Ayres and Clutton-Brock, 1992), the riverine effect on biodiversity has now been documented in other groups, such as butterflies (Brown, 1982; Rosser et al., 2021), frogs (Fouquet et al., 2012), lizards (Ávila-Pires, 1995; Pirani et al., 2019), and plants (Nazareno et al., 2021). However, it is among birds that this phenomenon is best known and best documented. River-bounded pairs of taxa are often represented by different subspecies in polytypic species or by allospecies in species complexes. The riverine effect on birds was first described for mighty Amazonian rivers such as the Amazon itself and its larger tributaries, like the Madeira, or the Negro, but we now know that even relatively small rivers, such as the Branco, the Aripuanã, and the Jiparanã also define the distributions of several pairs of avian taxa (Naka, 2011; Fernandes et al., 2014).

Although the role of rivers as primary or secondary barriers in the avian evolutionary process remains controversial (Naka and Brumfield, 2018), the fact that rivers do somehow limit dispersal and gene flow is well accepted (Haffer, 1992; Musher et al., 2022). In the last three decades, we have advanced considerably in gathering genetic evidence showing gene flow restrictions across rivers (Capparella, 1988, 1991; Ribas et al., 2012), but we had made only minor advances in understanding the real limitations that current rivers impose on flying birds.

Dispersal is one of the main forces of evolution and a key factor for understanding current and historic patterns of gene flow and differentiation. Dispersal also may affect community structure and composition of species assemblages. Therefore, understanding the capacity of species to move through the landscape can have direct implications for both evolutionary biology and ecology, and can be used to inform models of differentiation, community ecology, and biological conservation. For instance, high dispersal ability may provide the means to conquer new areas, stimulating peripatric speciation, but it could also facilitate gene flow and population homogenization, preventing species differentiation (Claramunt et al., 2012). Dispersal ability can also define which species colonize islands (MacArthur and Wilson, 1967; Lees and Gilroy, 2014) or which species are able to persist in fragmented landscapes (Burkey, 1989; Moore et al., 2008; Lees and Peres, 2009). Likewise, population connectivity in fragmented landscapes depends on species dispersal ability, which are mostly unreported for most tropical bird species (Hartfelder et al., 2020; Tourinho et al., 2022; Claramunt et al., 2022).

Given the flying capacity of birds, it is often assumed that features other than their flying apparatus should be involved in limiting species dispersal (Diamond, 1981). For instance, forest-interior species could be less prone to cross a river than open habitat birds due to their inexperience in dealing with heavily illuminated environments. In fact, some forest-interior Amazonian species exhibit higher dispersal abilities than non-specialized birds. Finally, behavioral reticence or reluctance to even attempt gap-crossing flights may also play an important role (Diamond, 1981; Laurance et al., 2004). From a bird's perspective, crossing an open gap of water may represent more than a physical challenge, it may also mean venturing into a harsh, hostile environment.

On the other hand, recent studies have shown that flight capabilities and long-distance flight efficiency likely represent a key aspect for avian dispersal and gap-crossing tendencies (Moore et al., 2008; Ibarra-Macias et al., 2011; Claramunt et al., 2012; Claramunt, 2021; Claramunt et al., 2022). Therefore, flight speed could also represent an important variable, as faster birds may be more prone to successfully cross a riverine barrier than slower birds. Unfortunately, data on avian flying speed are not only rare, but mostly restricted to temperate migratory non-passerines (Alerstam et al., 2007; Pennycuick et al., 2013), and are virtually non-existent for tropical birds. Therefore, while multiple factors may be involved in determining a species ability to cross an inhospitable barrier, it is important to evaluate both physical, ecological, and behavioral constraints.

Moore et al. (2008) conducted one of the first experiments of dispersal limitation in tropical birds, assessing the ability of 10 forest-dwelling species to fly over a known distance in a lake in Panama. In that study, mist-netted birds were released at different distances from the shore, and the trial outcomes (success or failure) were correlated with species extinctions and colonization in forest fragments. This novel experimental approach has provided empirical evidence on the flight capabilities and limitations of tropical forest birds.

In this study, we used dispersal-challenge experiments over water to assess the barrier crossing capabilities of 84 tropical bird species widely distributed throughout the avian tree of life. Specifically, we aimed at understanding if success or failure in the dispersal experiments could be predicted by (i) phylogenetic constraints, (ii) morphology (body mass and wing shape), (iii) flight speed, (iv) habitat and microhabitat preferences, and (v) psychological reluctance to fly. To answer these questions, we mist-netted wild birds in riparian habitats along the Rio Branco, a medium-sized river in northern Amazonia, and released them from an anchored boat at known distances to the shore. We assessed success or failure in the experiments, and measured flight speed and an estimation of an individual's reluctance to fly once they were released. We described the experimental results, evaluating differences among species and families, and correlated those results with the potential effect of dispersal ability on the evolutionary process. This represents the first experimental study to evaluate the effect of a riverine barrier through the dispersal ability of Amazonian birds and provides important insights to better understand the limitations provided by riverine barriers in the tropics.

MATERIALS AND METHODS

Study Area

This study was conducted along the Rio Branco, located in the Brazilian state of Roraima, northern Amazonia. Despite its relatively short length (∼550 km), the Rio Branco represents the largest tributary of the Rio Negro (Goulding et al., 2003). The Rio Branco has a complicated hydrological setting, with blackwater, whitewater and clearwater tributaries, but generally it is considered a sediment-rich white-water river, particularly during the rainy season (Ríos-Villamizar et al., 2013). Geologically and biogeographically, the Rio Branco can be divided in the lower and upper sections, which host different avifaunas (Naka et al., 2007, 2020). All of our sampling was conducted on riparian habitats on the lower Rio Branco, where tall flooded forests, Cecropia-dominated riverine forests, and sandbar scrub are all intermingled throughout the landscape (Naka et al., 2020).

With over 430 bird species recorded, the avifauna of the Rio Branco is possibly the best documented for any Amazonian river (Naka et al., 2006, 2007, 2020; Laranjeiras et al., 2014, 2019, 2021). The river has been referred to as a biogeographical and ecological hotspot, due to the diversity of habitats along its margins and the powerful biogeographical effect that this river has on the non-flooded terra-firme forest avifauna (Naka et al., 2020). Phenotypic and genotypic studies have shown that the river represents a biogeographical barrier for at least 40 pairs of avian taxa (Naka, 2011; Naka et al., 2012; Naka and Brumfield, 2018), offering a unique opportunity to test avian dispersal abilities in a real biogeographical scenario.

Dispersal Experiments

We conducted dispersal experiments during two dry seasons (October 2013 and September–October 2014) using 511 birds (157 and 354, respectively). We adopted the 'dispersal challenge' approach used by Moore et al. (2008), where birds were captured using mist-nets and released from a stationary boat at specified distances from the shore (100, 200, 300, and 400 m). Only species that succeeded at 100 m were challenged to cross 200 m and so on. Only two species were used in trials at 300 and 400 m. These distances (100–400 m) are conservative, as large Amazonian rivers are generally much wider, but may account for the use of river islands as stepping-stones to cross a riverine barrier.

We mist-netted birds in various riparian habitats, including sandbar scrubs, Cecropia-dominated riverine forests, tall flooded (*várzea*) forests, and tall transitional forest. We minimized bird handling time to a minimum, checking mist-nets every 30 min in shaded tall forests and every 15 min on more exposed river islands. Upon capture, birds were identified, photographed, and visually aged and sexed, when possible. Birds were manipulated only to obtain photographs and measurements of body mass and wingspan, and then placed in individual cloth bags. Birds were marked by partially clipping the tip of one of the rectrices to avoid using the same bird in subsequent trials. Birds were transported to our release station (an anchored boat) within 30 min of capture. Prior to being released, birds were held in a box for 1 min, and then were allowed to fly back to the river bank. Upon release, at least two people followed the birds with binoculars and a third member of the team recorded the duration of flight with a digital stopwatch. Birds usually flew straight to the closest bank. When this was not the case, and birds covered longer distances, we measured the actual distance flown with the aid of a GPS. A second (non-anchored boat) was always ready to rescue birds that "failed" the dispersal trial and landed on the water. Birds that showed obvious signs of stress (weak, panting) were not used in the experiments. We measured wing speed prior to each release session using a Instrutemp Icel anemometer (model 3010), avoiding releasing birds under strong wind conditions ($>$ 3 m/sec). We measured flight speed by quantifying flight duration over a known distance. For the speed estimates, we excluded birds that (i) did not fly at all, (ii) landed on the water, (iii) did not fly to the shore's nearest point, (iv) did not fly in a straight line (performed zigzags or detours), (v) performed circles around the boat, or (vi) did not go to the shore (for ex., swallows). Birds were captured and released under a Research License granted to LNN by the Chico Mendes Institute for Biodiversity Conservation (ICMBio) from the Brazilian Ministry of the Environment (license no. 30112-1 and subsequent renewals).

Morphological Predictors

We obtained morphological measurements exclusively from museum specimens. The overwhelming majority of specimens measured for this study were collected from the Rio Branco, minimizing geographical variation within species. Most of these specimens are currently held at the Universidad Federal de Pernambuco's ornithological collection. To avoid potential biases, a single person (BC) was responsible for measuring all birds. In a few cases, for species with a single sample, we included measurements from specimens held at the Royal Ontario Museum, which were measured by SC and collaborators.

From these specimens we estimated the hand-wing index (HWI), which reflects the degree of elongation of the distal portion of the wing (Kipp, 1959; Claramunt and Wright, 2017). The HWI is a proxy for the aspect ratio of the wing, and thus reflects the energetic efficiency of forward flight in birds (Norberg, 1990; Pennycuick, 2008). We calculated the HWI as $100^{*}K/WL$, where K is Kipp's distance, the distance between the tip of the longest primary and the tip of secondary 1 on the closed wing, and WL is the traditional wing length measured from the carpal joint to the tip of the longest primary (Claramunt and Wright, 2017). Alternatively, we estimated K as $WL - S1$, where $S1$ is the distance between the carpal joint and the tip of the first secondary feather, the most distal flight feather of the forearm, which produces very similar results (Claramunt and Wright, 2017).

Ecological Predictors

We tested as ecological predictors: (i) foraging stratum, (ii) habitat preference, and (iii) river-island specialization. Species categorizations presented in **Table 1** were obtained from our own experience on riparian habitats on the Rio Branco, which we have studied for over a decade (Naka et al., 2020). We understand that in a few cases, these may diverge from what species do elsewhere in Amazonia, but to the best of our knowledge, they represent the best categorization for this avifauna. We obtained data on habitat preference from hundreds of point counts conducted on the Rio Branco and its tributaries, which is available in the Supplementary Material of our recent publication (Naka et al., 2020).

Psychological Predictors

Upon release (i.e., opening the concealing box), not all birds flew immediately. Whereas some birds flew immediately upon the opening of the box, other individuals took their time. The time that each bird took to fly off the boat differed among individuals. We considered this waiting period as the reluctance to fly (measured in seconds). This period ranged from 0 to 5 min. When a bird refuse to take off after the 5-min period, the dispersal experiment was canceled, and the bird was taken back to the shore and released in its natural habitat.

Statistical Analyses

We estimated the phylogenetic signal or phylogenetic inertia in variables using the *phylosig* function in the *phytools* 0.7 library (Revell, 2012). Phylogenetic signal is assessed through a "lambda" transformation of internal branch lengths (Pagel, 1999). Lambda approximates zero when there is no or low phylogenetic signal and approximates to 1 when there is a high phylogenetic signal, showing Brownian motion-like phylogenetic inertia. We estimated lambda via maximum likelihood and present tests of whether lambda is equal to 0, using likelihood ratio tests.

We analyzed the relationship between morphological and ecological predictors and river-crossing success during the dispersal experiments using phylogenetic logistic regression models, which were implemented in the generalized estimating equation function *compar.gee* (Paradis and Claude, 2002) in the *ape* 5.4 R package (Paradis and Schliep, 2019). We modeled the frequency of success and failure by specifying a binomial distribution for the response and used a phylogenetic correlation structure to model phylogenetic non-independence among species.

The species phylogeny was obtained from birdtree.org using the Hackett et al. (2008) backbone topology and the *V2.iii* calibrations (Jetz et al., 2014). The final, maximum clade credibility tree, was then obtained from the sample of 1000 trees using the function *maxCladeCred* in *phangorn* 2.6.3 (Schliep, 2011).

Phylogenetic non-independence was taken into account by correlation structures that model the evolution of residuals along the branches of the phylogeny. We evaluated the performance of four phylogenetic correlation structures available in the *ape* library, including Brownian, Lambda, Martins, and Grafen (Grafen, 1989; Martins and Hansen, 1997; Pagel, 1999; Freckleton et al., 2002). We chose the correlation structure that minimized the quasi-likelihood information criterion (QIC, Paradis and Claude, 2002). We also used QIC to evaluate the relative fit of different models. To evaluate whether predictor coefficients were different from zero, we used *t* tests with phylogenetically adjusted degrees of freedom (Paradis and Claude, 2002). As a measure of absolute model fit, we estimated coefficients of determination based on squared correlation coefficients between the observed frequency of success and the predicted probabilities estimated by the models (Agresti, 2007), with weights determined by the number of experiments done for each species.

For our speed estimates, we only used experiments at 100 and 200 m from the coast, which represented more than 95% of the sample. Given that the average flight speeds were nearly identical at 100 and 200 m, we lumped those results, obtaining a single speed estimate per species, which is presented in **Table 1**.

RESULTS

Dispersal Experiments

We conducted dispersal experiments on 484 individuals, representing 84 species, belonging to six avian orders and 22 families (**Table 1**). We captured an additional 27 birds, which were excluded from the analyses for various reasons (explained in section **Materials and Methods**), but mostly because they refused to fly off the boat. Most of our sample (85%) was composed of passerine birds of 16 different families. Six of these families (Thamnophilidae, 130 spp; Thraupidae, 76 spp.; Furnariidae, 63 spp.; Tyrannidae, 48 spp.; Pipridae, 41 spp. and Dendrocolaptidae, 28 spp.) accounted for 75% (386 ind.) of the trials. On average, we sampled 6.6 birds per species (± 7.8), with a median of 4 birds. Fifteen species were represented by singletons and 14 by doubletons, whereas 30 species had at least 5 replicates, and 5 species participated in more than 20 experiments (**Table 1**). A total of 249 birds of 68 species were released 100 m from the shore, 219 of 58 species at 200 m, 8 of 2 species (*Pipra filicauda* and *Ramphocelus carbo*) at 300 m and 4 individuals of a single species (*Pipra filicauda*) at 400 m (**Supplementary Table S1**).

About two thirds of the birds (172 individuals of 56 species) were successful in reaching the river bank at 100 m, whereas the other third (77 individuals of 29 species) failed to do so. An almost identical rate of success was apparent on the 200 m trials, where 146 individuals of 42 species managed to cross the water gap and 73 of 30 species failed. All 13 birds that were released at 300 and 400 m were successful, but they only represented two species. We found a clear variation in trial success among species; a total of 36 bird species (43%) succeeded in all their challenges, whereas 14 species (17%) failed in all of theirs (**Table 1**). On the other hand, 34 species (40%) had both successes and failures in the experiments. This variation was also evident at the family level (**Figure 1**). Seven of the 22 avian families tested, succeeded in 100% of the trials (Columbidae, Alcedinidae, Galbulidae, Picidae, Formicariidae, Tytiridae, Hirundinidae, and Parulidae), although two of these included less than 5 trials (Formicariidae and Tytiridae). Seven families succeeded in 75–90% of the trials (Cuculidae, Trochilidae, Dendrocolaptidae, Furnariidae, Tyrannidae, Pipridae, and Thraupidae). Another six families succeeded in 50–70% of the trials (Onychorhynchidae, Rhynchocyclidae, Columbidae, Vireonidae, Turdidae, and Passerellidae), although the last four families had fewer than 5

TABLE 1 | Eighty-four (84) bird species included in the dispersal experiments, including rates of success and failure, average speed, and main habitats, microhabitat and stratum used based by each species (based on Naka et al., 2020). Numbers in parentheses after family names indicate the number of experiments per family.

Family/Species	Succ-Fail	Ave. speed (m/sec)	Mass (g)	Kipp's index	Stratum[1]	Microhabitat and specialization[2]	Habitats[3]
Columbidae (3)							
Patagioenas subvinacea	1-0	12.1	228	26.4	C	F. edge	GF VF Tr
Geotrygon montana	0-1	NA	130	28.7	G	F. interior	VF Tr
Leptotila rufaxilla	1-0	10.9	137	24.1	G	F. edge	S RF GF VF Ig
Cuculidae (5)							
Crotophaga ani	4-1	7.5	80.6	22.4	U	Open habs.	S GF RF VF
Trochilidae (16)							
Glaucis hirsutus	4-0	12.8	5.1	56.6	U	F. interior	VF Tr
Phaethornis rupurumii	7-3	10.1	2.5	62.5	U	F. interior	GF VF
Chlorestes notata	2-0	8.8	3.5	56.7	U	F. edge	RF VF Ig
Alcedinidae (7)							
Chloroceryle aenea	7-0	8.6	14.5	22.9	U	F. edge	W VF
Galbulidae (2)							
Galbula galbula	2-0	NA	19.4	20.4	U	F. edge	GF RF VF Ig
Picidae (2)							
Celeus elegans	1-0	5.6	123.5	19.2	C	F. interior	VF Tr
Colaptes punctigula	1-0	9.9	63	17.1	C	F. edge	GF RF VF
Thamnophilidae (120)							
Myrmotherula axillaris	4-5	6.6	12.5	13	U	F. interior	VF Tr Ig
Myrmotherula assimilis	0-1	NA	10	12.2	U	F. interior (RI)	VF Ig
Isleria guttata	0-1	NA	10.5	11.2	U	F. interior	Tr
Thamnomanes caesius	1-1	7.1	17.2	14	U	F. interior	VF Tr
Sakesphorus canadensis	1-3	NA	26.4	7.1	U	F. edge	S GF RF VF Ig
Thamnophilus doliatus	0-1	NA	26.5	9.2	U	F. edge	S GF RF Ig
Thamnophilus murinus	0-1	NA	17	11.6	U	F. interior	Tr
Thamnophilus punctatus	1-0	6.3	21	7.6	U	F. interior	Tr
Thamnophilus aethiops	1-3	9.4	26.2	9.8	U	F. interior	Tr
Taraba major	1-0	NA	53	9.7	U	F. edge	GF RF VF
Hypocnemoides melanopogon	7-18	8.4	12.3	15.9	U	F. interior	VF Ig
Hylophylax naevius	1-0	NA	12.3	NA	U	F. interior	VF
Hylophylax punctulatus	14-8	7.9	11.5	12	U	F. interior	VF
Myrmoborus leucophrys	0-10	NA	18.5	12.1	U	F. interior	GF VF
Myrmoborus lugubris	3-5	7.9	20	12.3	U	F. interior (RI)	RF VF
Percnostola rufifrons	0-2	NA	29.7	10.1	U	F. interior	Tr Ig
Cercomacra carbonaria	5-13	5.5	14.7	13.3	U	F. edge (RI)	GF RF
Cercomacra tyrannina	1-2	6.3	16.8	12.3	U	F. interior	VF Tr
Hypocnemis cantator	0-1	NA	12	9.7	U	F. interior	Tr
Hypocnemis flavescens	0-1	NA	12	10	U	F. interior	Tr
Gymnopithys rufigula	3-1	5.2	29.5	12.1	U	F. interior	Tr
Formicariidae (1)							
Formicarius colma	1-0	8	55	13.5	G	F. interior	VF Tr
Dendrocolaptidae (27)							
Glyphorynchus spirurus	1-0	5.5	12	22.5	U	F. interior	Tr
Nasica longirostris	2-0	7.6	53.5	16.4	C	F. edge	GF RF VF Ig
Dendrocolaptes certhia	0-1	NA	63	16.6	C	F. interior	Tr
Xiphorhynchus obsoletus	11-2	7.8	29.2	18.9	U	F. interior	RF VF Ig
Xiphorhynchus guttatus	7-2	7.9	46.5	15.7	C	F. interior	GF VF Tr
Dendroplex kienerii	1-0	6.7	42	19.6	C	F. interior	RF VF Ig
Furnariidae (60)							
Furnarius leucopus	14-0	8	33.4	13.4	G	F. edge	S GF RF

(Continued)

TABLE 1 | (Continued)

Family/Species	Succ-Fail	Ave. speed (m/sec)	Mass (g)	Kipp's index	Stratum[1]	Microhabitat and specialization[2]	Habitats[3]
Philydor pyrrhodes	2-0	9.8	28	19.3	U	F. interior	VF Tr
Cranioleuca vulpina	5-1	8	16.1	16.8	U	F. edge	S RF VF
Mazaria propinqua	3-7	4.6	17.0	10	U	F. edge (RI)	S
Synallaxis gujanensis	21-7	7.3	16.2	12.3	G	F. interior	S RF VF
Onychorhynchidae (2)							
Myiobius barbatus	1-1	5.9	13.5	14.5	U	F. interior	VF
Pipridae (41)							
Pipra filicauda	36-5	10.4	13.9	17.3	U	F. interior	GF VF
Tityridae (5)							
Schiffornis major	2-0	8.8	27.5	16.8	U	F. interior	VF
Pachyramphus rufus	3-0	7.8	17	18.5	U	F. edge	GF RF
Rhynchocyclidae (22)							
Mionectes oleagineus	5-4	5.6	9.7	14.8	U	F. edge	VF
Tolmomyias poliocephalus	1-1	7.5	13	12.44	C	F. edge	RF VF Tr Ig
Tolmomyias flaviventris	0-1	NA	11	13.5	C	F. edge	GF RF VF
Todirostrum maculatum	5-3	8	7	11.8	U	F. edge	S GF RF VF Ig
Poecilotriccus sylvia	1-0	NA	8	13.3	U	F. interior	GF VF
Lophotriccus galeatus	0-1	NA	7	18.2	C	F. edge	VF Tr
Tyrannidae (47)							
Stigmatura napensis	3-1	6	10.1	12.1	U	F. interior (RI)	S
Camptostoma obsoletum	0-1	NA	8	18.1	U	F. interior	S GF RF Ig
Elaenia spectabilis	1-0	6.8	14	19.02	U	F. edge (RI)	S
Myiopagis flavivertex	1-0	5.7	11.5	9.4	U	F. interior	VF Ig
Ramphotrigon ruficauda	1-2	4.6	17.7	24.3	U	F. interior	Tr
Myiarchus tuberculifer	3-0	5.6	24	11.6	U	F. edge	RF VF Ig
Pitangus sulphuratus	3-0	7	49.8	19.8	C	Open habs.	S GF RF Ig
Myiozetetes cayanensis	2-1	8.3	24.3	16.7	C	Open habs.	S GF RF Ig
Arundinicola leucocephala	1-0	6.3	11	11.9	U	Open habs. (RI)	W
Cnemotriccus fuscatus	25-2	7.5	13.7	14.7	U	F. interior (RI)	RF
Vireonidae (4)							
Cyclarhis gujanensis	2-0	6.9	25	13.3	C	F. interior	GF RF VF Ig
Hylophilus pectoralis	0-2	NA	10.5	11.2	C	F. edge	S GF RF VF
Hirundinidae (5)							
Tachycineta albiventer	5-0	NA	14.4	52.1	NA	Open habs.	W B Ig
Troglodytidae (23)							
Troglodytes musculus	1-0	6.4	10.5	8.5	U	Open habs.	S GF RF Ig
Phaegopedius coraya	5-4	11.2	12.0	9.4	U	F. interior	Tr
Cantorchilus leucotis	3-10	6.9	17.7	9.5	U	F. edge	GF RF VF
Turdidae (2)							
Turdus fumigatus	1-1	6.8	64	21.3	C	F. interior	VF
Passerellidae (19)							
Ammodramus aurifrons	8-0	7	15.6	12.1	G	Open habs. (RI)	B S
Arremonops conirostris	5-6	7.5	29.0	10.8	G	F. interior (RI)	RF
Parulidae (2)							
Setophaga petechia	2-0	5.3	9	27.9	C	F. edge (RI)	S GF RF
Thraupidae (71)							
Saltator coerulescens	5-1	8	48	15.4	C	F. edge	GF RF
Eucometis penicillata	2-3	6.8	27	19	U	F. interior	VF
Ramphocelus carbo	22-5	8.3	22.4	15,5	U	F. edge	S GF RF VF
Sporophila lineola	4-0	7.7	11.5	23.8	U	Open habs. (RI)	S
Sporophila intermedia	2-0	7.2	10.5	15.3	U	Open habs. (RI)	GF S
Sporophila castaneiventris	4-0	6.9	9	11.7	U	Open habs. (RI)	S

(Continued)

TABLE 1 | (Continued)

Family/Species	Succ-Fail	Ave. speed (m/sec)	Mass (g)	Kipp's index	Stratum[1]	Microhabitat and specialization[2]	Habitats[3]
Sporophila angolensis	4-1	7.2	11.3	14.6	U	Open habs. (RI)	GF VF S
Conirostrum bicolor	10-0	7.6	8.9	18.5	C	F. edge (RI)	S RF
Paroaria gularis	7-0	7.5	24.8	17.6	U	Open habs.	W GF RF VF S Ig
Thraupis episcopus	1-0	8.2	35.5	21	C	Open habs.	S GF RF Ig

River-island specialization refers exclusively for our study area. Taxonomy and systematic order follow Pacheco et al., 2021).

[1] *Stratum G: ground; U: understory; C: canopy.*

[2] *Microhabitat and specialization: F. edge (forest edge); F. interior (forest interior); Open habs (Open habitats); RI (River-island specialist).*

[3] *Habitat W: water (rivers and lakes); B: beaches and sandbars; S: Sandbar scrub; VF: Varzea Forest; RF: River-edge (or Cecropia-dominated) Forest; Ig: Black-water Igapo Forest; Tr: Transitional Forest; GF: Gallery Forest.*

motion mode of evolution. The HWI was a significant predictor of trial success, both at the species level (R^2 = 0.29, t = 3.5, p = 0.0019; **Figure 2A**) and at the family level (R^2 = 0.60; F = 22.5; p = 0.0004; **Figure 2B**), for which the predictive power was particularly strong. In general, families with HWIs higher than 15 were successful in more than 80% of the trials, whereas those with lower values fared worse in the dispersal experiments.

Body mass ranged from 2.5 g (*Phaethornis rupurumii*) to 228 g (*Patagioenas subvinacea*), with a median of 16.9 g (**Table 1**). In contrast to the HWI, we found no significant effect of mass on trial success (R^2 = 0.001, t = 0.36, p = 0.72); smaller birds had the same chance of success, compared to larger ones, in the experimental trials (**Figure 3A**).

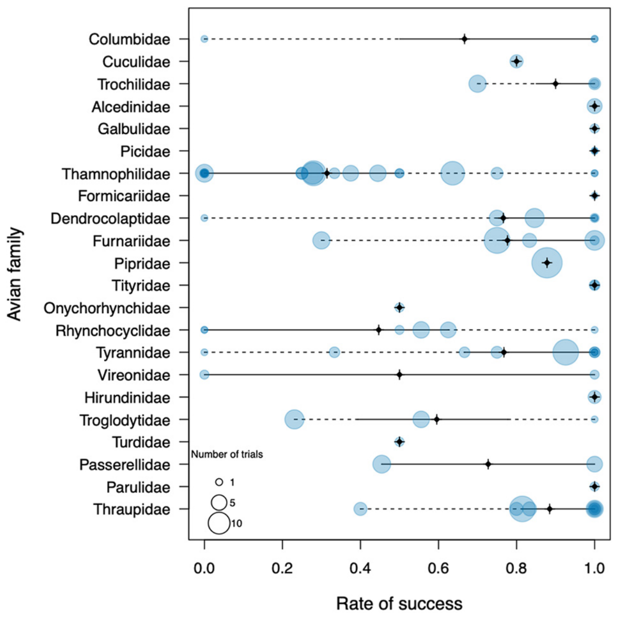

FIGURE 1 | General results of the dispersal trial experiments conducted in the Rio Branco in northern Amazonia, grouped per family. Each bubble represents one species within the family, whereas the size of the bubble represents the number of individuals used in the experiments. The black cross marks the family median, solid lines span the interquartile range, and dotted lines span the total range.

samples. Finally, the antbirds (Thamnophilidae) and the wrens (Troglodytidae) were particularly bad dispersers, attaining only 38 and 39% of success, respectively. Phylogenetic signal in river-crossing success rate was relatively low (lambda = 0.34) but significantly different from zero (Likelihood ratio = 12.8, p = 0.0003).

Morphological Predictors

We evaluated two morphological predictors of dispersal success: the hand-wing index (HWI) and body mass. The HWI ranged from 7.1 (*Sakesphorus canadensis*) to 62.5 (*Phaethornis rupurumii*), and had a very strong phylogenetic signal (lambda = 0.99), significantly different from zero (Likelihood ratio = 111.8, p < 0.001), suggesting a Brownian motion mode of evolution.

Speed

We obtained flight speed estimates of 269 individuals of 64 bird species (**Table 1** and **Supplementary Table S1**). There was a medium phylogenetic signal in speed (lambda = 0.53), which was significantly different from zero (Likelihood ratio = 9.08, p = 0.0002). Average flight speeds were nearly identical at 100 and 200 m, averaging 7.6 m/sec (**Table 2**). The two species that were exposed to longer distances flew faster when crossing 300 and 400 m (**Table 2**). The slowest species in our sample that managed to cross the river gap presented in the experiments were the White-bellied Spinetail (*Mazaria propinqua*) and the Rufous-tailed Flatbill (*Ramphotrigon ruficauda*) which flew at 4.6 m/s, both of which had more failures than successes (3–7 and 1–2, respectively). On the other hand, the fastest species was a hummingbird, the Rufous-breasted Hermit (*Glaucis hirsutus*), which flew at 12.8 m/s (**Table 2**). For the one species for which we had trials at all distances, the Wire-tailed Manakin (*Pipra filicauda*), we found a significant and positive effect on distance over speed (R^2 = 0.23; F = 9.54, p = 0.004), flying faster when covering longer distances. We found that flight speed is a significant predictor of trial success (R^2 = 0.138; t = 2.62; p = 0.017, **Figure 3B**), with the caveat that speed was only measured for species that successfully crossed the riverine gap at least once.

Ecological Predictors

Most species involved in the experiments were from the understory (57 species), followed by the canopy (19 species),

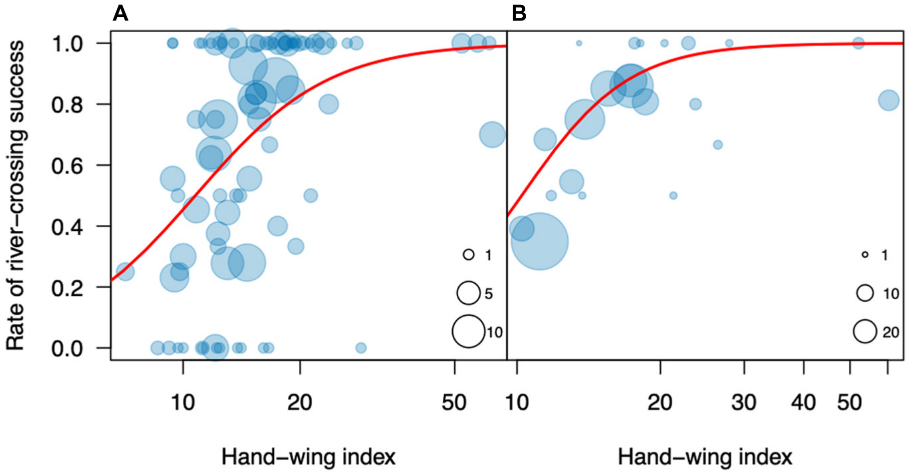

FIGURE 2 | Rate of river-crossing success in the dispersal experiments on the Rio Branco in relation to the Hand-wing index at the species **(A)** and family **(B)** levels. In panel **(A)**, each circle represents one species, and its diameter represents the number of individuals for that species. In panel **(B)**, each circle represents one family, and its diameter represents the number of species for that family.

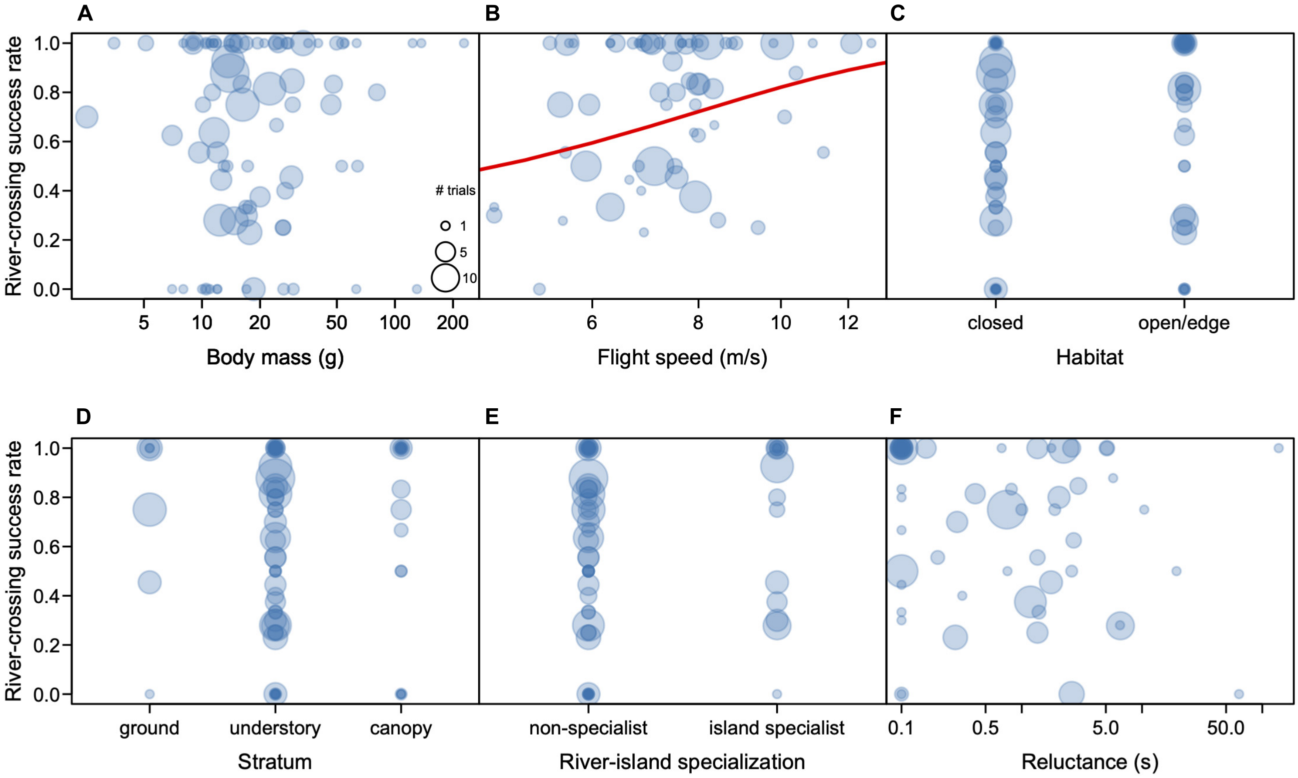

FIGURE 3 | Rate of success in the dispersal experiments on the Rio Branco in relation to body mass **(A)**, flight speed **(B)**, habitat **(C)**, Stratum **(D)**, river island specialization **(E)**, and reluctance to fly **(F)**. Each circle represents one species, and its diameter represents the number of individuals for that species.

the ground (7 species), and the open airspace (1 species) (**Table 1**). About half (42) of the species were typical of open habitats (20 species) or forest edge (22 species) and the other half (40 species) was represented by forest interior birds (**Table 1**). At least 15 of the 84 species subjected to the experiments can be considered river-island specialists at our study site (**Table 1**). We found a significant effect of forest stratum preference on river-crossing success ($R^2 = 0.052$, $F = 4.2$, $p = 0.029$) (**Figure 3D**), but not on habitat preferences ($R^2 = 0.027$, $t = 1.5$, $p = 0.15$) (**Figure 3C**), or river-island

TABLE 2 | Summary of flight speed estimates obtained during the dispersal experiments on the Rio Branco.

Distance	Number of individuals	Number of species	Average speed (SD)	Median speed (m/sec)	Slowest (m/sec)	Fastest (m/sec)
100	152	53	7.5 (1.6)	7.7	4.5	12.7
200	105	40	7.6 (1.5)	7.5	4.7	12.7
300	8	2	12.7 (3.2)	12.7	12.7	12.7
400	4	1	11.8 (1.7)	11.8	11.8	11.8
Total	269	64				

specialization ($R^2 < 0.001$, $t = 0.23$, $p = 0.81$) (**Figure 3E**). Contrary to our predictions, birds from open habitats and the forest edge, and river-island specialists did not perform better in the trials compared to forest-interior and non-specialists of islands.

Psychological Predictors

We measured the reluctance to fly (measured in seconds) in 354 individuals of 65 species. Most individuals (256 or 72%) of 29 species flew immediately upon release, but nearly 30% of the individuals took longer to fly or did not fly at all. Although we didn't find an effect of the time taken to take off and the rate of success ($R^2 = 0.006$, $t = 0.67$, $p = 0.51$, **Figure 3F**), we observed that certain families where more reluctant to fly than others. For example, individuals of eight species refused to take off from the boat (staying for more than 5 min), six of which were antbirds (Family Thamnophilidae), including the Dot-backed Antbird (*Hylophylax punctulatus*, 2 ind.), the Black-chinned Antbird (*Hypocnemoides melanopogon,* 2 ind.), the Guianan Warbling Antbird (*Hypocnemis cantator*, 2 ind.), the White-browed Antbird (*Myrmoborus leucophrys*, 3 ind.), the Ash-breasted Antbird (*Myrmoborus lugubris*, 1 ind.), and the Black-headed Antbird (*Percnostola rufifrons*, 1 ind.). The two other individuals that refused to fly belonged to the Ochre-bellied Flycatcher (*Mionectes oleagineus* (1 ind.) and the Coraya Wren (*Pheugopedius coraya*, 1 ind.).

DISCUSSION

In this study, we present experimental evidence showing that riverine barriers may represent significant obstacles to avian dispersal. Our results are novel in several ways, representing the first dispersal experiments on birds in Amazonia, the first empirical dataset showing differences in the dispersal abilities of species and families, and the first estimates of avian flight speeds reported from the tropics. Despite the many confounding factors that are inheritably present in controlled experiments in the wild, several conclusions can be drawn from this study. First and most importantly, we have documented differences in the ability of bird species to cross a body of water, where even relatively narrow gaps (100 m) may represent an unsurmountable barrier for many species of birds. This differential ability seems to be related to flight performance, rather than body size or ecological and psychological factors. Specifically, we found significant effects of wing shape and flight speed on the ability of species to succeed in the dispersal challenges, both of which have a significant phylogenetic component. Among the ecological attributes tested, only stratum preference was a significant, albeit weak, predictor, whereas habitat preference and river island specialization did not prove to be important factors. Behavioral reluctance to fly is harder to evaluate in experimental conditions but deserves further investigation.

Morphological Predictors of Cross-River Dispersal

We assessed the effect of the hand-wind index (also known as Kipp's Index) and body mass in predicting species outcomes in the dispersal experiments. The hand-wind index, which has a very strong phylogenetic signal, was the strongest predictor found in this study, both at the species and family levels. These results are consistent with theoretical expectations of increased long-distance flight efficiency with higher aspect ratio wings (Norberg, 1990; Pennycuick, 2008) and adds to the mounting evidence of the usefulness of the hand-wing index as a proxy for avian dispersal ability (Claramunt et al., 2012, 2022; Claramunt and Wright, 2017; Sheard et al., 2020; Claramunt, 2021).

On the other hand, we found no effect of body mass on dispersal capacity within our sample. Prior expectations were ambiguous. On the one hand, there are some empirical reasons to expect that larger birds could have better dispersal abilities than smaller birds, as predation and range size have been reported to be size-dependent (Suhonen et al., 1994; Ottaviani et al., 2006). Although some studies reported significant effects of body size on the capacity of tropical birds to move through fragmented landscapes (Lees and Peres, 2008, 2009; Neuschulz et al., 2013) these studies did not analyze indicators of flight efficiency, potentially confounding these two factors. On the other hand, recent phylogenetically controlled comparisons found no effect of body mass on dispersal distances in Holarctic birds (Claramunt, 2021) or in movements across habitat gaps in Amazonian birds (Claramunt et al., 2022). It is possible that our result is sample-dependent, as our sample lacks some large birds such as raptors and parrots, which are expected to be good flyers. But, we also lacked large nearly flightless species such as tinamous and trumpeters that are expected to have poor dispersal capabilities. Ultimately, dispersal abilities in birds depend on their flight abilities, and long-distance flight performance depends more on the morphology of the flight apparatus than on body size. Strong-flying and mobile species can be found along the entire spectrum of body sizes in birds from the smallest species (hummingbirds, swifts) to the largest (albatrosses, condors), and the same is true for nearly flightless sedentary species.

Flight Velocity and Dispersal Ability

Flight velocity was a significant predictor of species success in the dispersal challenges. Faster species had a higher probability of succeeding in the dispersal experiments. As with the HWI, flight velocity can be considered a measurement of flight efficiency, and both variables seem to be important in defining which species were able to cross open gaps of water, suggesting that species performance is directly related to their morphology.

Being the first large dataset on flight speeds ever produced for tropical birds, there is little data warranting direct comparisons. The best datasets available include those gathered using ornithodolites on 38 non-passerine migratory birds in Sweden (Pennycuick, 1982; Pennycuick et al., 2013) and radars on 138 species on migration in the Western Palearctic (Bruderer and Boldt, 2001; Alerstam et al., 2007). Our flight speed estimates seem low compared to those reported there, which ranged from 11 to 22 m/s in Sweden (Pennycuick et al., 2013) and from 8 to 23 m/s in the Western Palearctic (Bruderer and Boldt, 2001; Alerstam et al., 2007). Our mean values were similar to the slowest speeds in those studies, and our slowest values (4.6 m/s) were nearly half the slowest species in the temperate region. These values could indicate that flight speeds among tropical species is comparatively low, but most species in available datasets included many large non-passerine species such as ducks, swans, cranes, and waders and only a handful of passerines. The genus *Turdus*, however, was sampled both in the Palearctic and the Rio Branco, and allows a direct comparison. The average speed of four species of Palearctic thrushes averaged 12.3 m/s, whereas our only flight speed estimate of a thrush (*Turdus fumigatus*) was nearly half that (6.8 m/s).

These datasets, however, are not completely comparable, as Bruderer and Boldt's (2001) measurements were obtained on migrating birds flying at constant speeds and using tracking radar. Migratory flight speeds are inherently faster than short distance flights, even for temperate birds. Furthermore, our estimates include the acceleration phase, which is composed of more powerful strokes, yet slower speeds, which would necessarily bias our estimates to slower speed estimates. However, having very similar estimates of 100 and 200 m suggests that this acceleration phase does not account for such large differences. Quite interestingly, Bruderer and Boldt (2001) mentioned that released birds were apparently reluctant to depart with migratory speed, flying at considerably lower speeds than migrating conspecifics. These authors showed that whereas migrating passerines fly at around 10–15 m/s, most released birds flew at a speed of 5 to 10 m/s, which is quite close to our estimated ranges.

Our method for measuring flight speed relied on measuring flight time over a known distance (i.e., shortest distance to the riverbank). Despite representing a direct measurement, our estimates are not devoid of potential biases. Although we avoided releasing birds under strong winds (> 3 m/s), even mild winds could influence speed estimates (Nilsson et al., 2014). Furthermore, although we measured wind speed prior to each release session, we did not note wind direction, which is an obvious aspect to take into consideration. Despite these potential biases, our measurements are quite consistent across species and families. For instance, hummingbirds ranged from 8.8 to 12.8 m/s and the four species of seedeaters tested, had very similar flying speeds (6.9 to 7.7 m/s).

Ecological Traits Associated With Dispersal

The role of ecological attributes on dispersal remains elusive. Despite having clear predictions in terms of species success in the dispersal experiments in relation to stratum and habitat preference, and river-island specialization, none of the attributes included in this study showed a strong effect of ecology. Favoring one of our predictions, we found a significant, although weak, effect of foraging stratum on the rate of success in the dispersal experiments. Both canopy and ground-dwelling species were more likely to succeed in reaching the river bank than understory birds. This result is concordant with previous studies showing lower genetic differentiation across rivers (Burney and Brumfield, 2009) and higher demographic connectivity across habitat gaps (Bradfer-Lawrence et al., 2018) in canopy birds compared to those from the understory. That ground dwelling birds may have higher flight capabilities than understory birds is an interesting result. This result is at odds with the Barro Colorado experience, where many ground-dwelling species have disappeared from islands and their populations have not been replenished (Willis, 1974). Although ground dwelling birds do not fly much during foraging, strong flight capabilities may be important for escaping predators and making larger-scale movements within the forest, such as that documented for *Geotrygon montana* (Stouffer and Bierregaard, 1993).

Empirical evidence suggests that forest-edge and open habitat birds are more likely to cross forest gaps than forest-interior species (Laurance et al., 2004; Lees and Peres, 2009). Birds of the forest edge, which are often compared to birds from open-habitats due to the high levels of solar irradiation (Foggo et al., 2001), are also expected to have enhanced mobility, and were expected to perform well in our dispersal experiments. However, we found no effect of habitat preference (forest interior vs. forest-edge and open areas) on crossing capabilities. This result is likely due to the large variation found among forest-edge/open areas birds, which includes some excellent flyers but also species such as the Black-crested Antshrike (*Sakesphorus canadensis*), the Plain-crowned Spinetail (*Synallaxis gujanensis*), and the Buff-breasted Wren (*Cantorchilus leucotis*), which fared poorly in our experiments likely because of their low flight performance.

Similarly, we hypothesized that river-island specialists should demonstrate higher dispersal capabilities than birds that do not need to relocate to other environments on a seasonal basis due to the ephemeral nature of river islands (Rosenberg, 1990; Zimmer and Leisler, 2003). However, we found no significant effect of this kind of specialization in relation to their success in the experiments. Recent ecological data on some of these river-island flooded forests specialists suggests that birds do not necessarily relocate to unflooded forests. Quite the contrary, they seem to perform vertical displacements along the vegetation, making use of the available space during the flooding period, allowing them year-round territoriality without major habitat shifts (Rowedder et al., 2021). These results do agree with recent genetic data that found stronger population structure among river island specialists, when compared to floodplain specialists (Choueri et al., 2017; Thom et al., 2018), which included some of our relatively bad dispersers (e.g., *Myrmoborus lugubris*).

Psychological Constraints

Empirical evidence suggests that the dispersal ability of birds may be limited, at least in part, by psychological constraints. Data from Amazonian forest fragments indicate that the dispersal of forest-interior birds may be limited by tree-fall gaps, clearings or relatively narrow roads (Stouffer and Bierregaard, 1995; Laurance et al., 2004), a distance that is unlikely to represent a physical challenge for a bird. Whether these limitations are related to the "fear of flying" (Diamond, 1981) or a physical limitation due to their sensory system (Ausprey et al., 2021) remains to be tested. However, flying over an open gap of water represents a very different challenge than crossing a forest gap or even a road, as birds need to cover the entire distance in a single bout, without pausing or resting along the way. We hypothesized that birds that were more reluctant to fly would have a lower success in the dispersal experiments. Although the time taken by each bird to fly was unrelated to individual success, our empirical data suggests that most species that refused to even attempt crossing the water gap were represented by either antbirds or other low success species. Those birds preferred to remain in their boxes rather than venturing into the unknown. We suspect that under natural circumstances, these species will likely avoid crossing a river, which may have long-term effects on the genetic structure of these populations. In fact, river crossing is clearly not devoted of risks, even for those that can cross the gap of water successfully. Among these dangers, we witnessed aerial attacks on some species by White-winged Swallows (*Tachycineta albiventer*), as birds were allegedly invading their aerial space, and a predation event where a Rufous-throated Antbird (*Gymopithys rufigula*) was attacked in flight and caught by a Bat Falcon (*Falco rufigularis*) during what otherwise looked like a potentially successful river crossing.

Dispersal Ability of Amazonian Birds

Results from experimental studies on dispersal could be readily applied in at least two different fields, including biological conservation and evolutionary biology. Recent studies have successfully explored links between dispersal capacity and fragment recolonization and extinctions (Bates, 2002; Moore et al., 2008), but little has been done to bridge the gap between dispersal ability and the avian evolutionary process, particularly in Amazonia, where riverine barriers define the distribution of multiple avian lineages. In fact, establishing a link between dispersal ability and avian distribution patterns in Amazonia has proven to be a challenging task. For example, nearly a third of the species that occur in the terra-firme forests of the Guiana Shield in northern Amazonia have taxon replacements across the lower Rio Negro, and those include groups generally considered to be good flyers, such as hummingbirds and parrots (Naka et al., 2012). Furthermore, the woodcreepers (Dendrocolaptidae), which responded very well to the dispersal experiments with > 80% of success rate, have more taxon replacements across the lower Rio Negro (73% of the species have an allospecies or different subspecies) than the antbirds (Thamnophilidae), which fared poorly in the experiments with a low success rate (< 40%), but have a lower (58%) taxon replacement across that same river (Naka and Brumfield, 2018).

There are few potential explanations behind these apparent contradictions. On the one hand, our results are highly conservative, because 100 or even 400 m represent a much lower distance than most Amazonian rivers currently impose on birds. On the other hand, at an evolutionary scale, the presence of river islands as well as rare seasonal droughts or even reductions in river discharge during dry Glacial cycles could make it easier for poor dispersers to manage to cross a river gap. However, simulations have shown that reduced dispersal across rivers can maintain two species in allopatry for many generations, despite repeated river crossings by few individuals (Santorelli et al., 2022).

It is important to note that the riverine barrier effect is best documented for terra-firme birds, which inhabit upland forests that never get flooded by the seasonal flood pulse of Amazonian rivers. These species demonstrate greater genetic diversity and levels of divergence across the landscape than floodplain species (Harvey et al., 2017). Therefore, although it could be argued that our sample obtained in riparian habitats may not be representative of the terra-firme forest avifauna, we believe our results are important for this discussion. First, nearly a third of our sample is represented by species which also occur in terra-firme forests and are readily found on tall transitional forests. Second, given the strong effect of morphology on dispersal success and the pervasive effect of the phylogeny on species dispersal abilities, it seems reasonable to expect similar results when including a larger sample of terra-firme forest species. In fact, our results seem to downplay the role of ecology on dispersal, suggesting that avian morphology may be more important than being a terra-firme or a flooded forest species. Finally, although it is possible that bird handling had an effect on flight capacity (mist-netted birds may be subject to temporary wing-strain) which might explain some failures in species with low sample sizes, we believe our large sample and consistent results at the species and family levels are strong enough to show a clear underlining pattern.

FINAL CONCLUSION

Our data shows that dispersal abilities are not homogeneous across the avian tree of life, and some lineages are more likely to successfully cross a riverine barrier than others. So far, variables associated with flight performance, such as the hand-wing index and flight speed, seem to be the best predictors of success in dispersal-challenge experiments. Our results also show that although there are good reasons for ecological attributes to have an effect, they do not seem to have a strong predictive power, suggesting that flight efficiency may represent a much more direct proxy to estimate species' dispersal capabilities. We foresee that experimental data on dispersal abilities can shed light onto important aspects of avian ecology and evolution, including patterns of gene flow and genetic differentiation, as well as patterns of functional landscape connectivity in fragmented habitats. These two different aspects of avian biology will broaden our understanding on the role of riverine barriers in the evolution of the Amazonian biota and the effect of dispersal in species occupancy and recolonization in fragmented habitats.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article are included in the article or as **Supplementary Material** (**Supplementary Table S1**).

ETHICS STATEMENT

We followed standard ethical guidelines for conducting research on wild birds (Fair et al., 2010). Procedures for capturing wild birds were approved by the Chico Mendes Institute for Biodiversity Conservation (ICMBio), Brazilian Ministry of the Environment (Research License no. 30112-1 granted to LN and subsequent renewals).

AUTHOR CONTRIBUTIONS

LN and BC envisioned and designed this study. BC, GL, and LN conducted the experiments. LN, SC, and BC wrote the manuscript. SC, LN, and BC analyzed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.846975/full#supplementary-material

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