EVOLUTIONARY BIOLOGY

The dual role of Amazonian rivers in the generation and maintenance of avian diversity

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The Amazon River and its major tributaries delimit the distributions of hundreds of terrestrial taxa. It remains unclear whether river-bounded distributions and taxon replacements reflect the historical role of rivers in generating species diversity as vicariant forces, or are the result of their role as secondary barriers, maintaining current levels of species diversity by inhibiting gene flow and population introgression. We use a community-wide comparative phylogeographic and phylogenetic approach to address the roles that the Rio Negro and the Rio Branco play in the avian speciation process in the Guiana Shield. Examining 74 pairs of ecologically similar geographic replacements that turn over across the lower Negro, we found substantial variation in the levels of genetic divergence and the inferred timing of diversification among pairs, ranging from ~0.24 to over 8 million years (Ma ago). The breadth of this variation is inconsistent with a single, shared speciation event. Coalescent simulations also rejected a simultaneous divergence scenario for pairs divided by the Rio Branco but could not reject a single diversification pulse for a subset of 12 pairs of taxa divided by the upper Negro. These results are consistent with recent geomorphological hypotheses regarding the origins of these rivers. Phylogenetically, taxon pairs represent a blend of sister (~40%) and nonsister taxa (~60%), consistent with river-associated allopatric or peripatric speciation and secondary contact, respectively. Our data provide compelling evidence that species turnover across the Rio Negro basin encompasses a mixture of histories, supporting a dual role for Amazonian rivers in the generation and maintenance of biological diversity.

INTRODUCTION

One of the most striking biogeographic patterns in the Amazon basin is the turnover of terrestrial taxa across rivers. The pattern was first noted by Alfred Wallace (1) and has since been widely documented in birds (2–5), primates (6–9), and a diversity of other terrestrial organisms at multiple geographic scales (10). Although some rivers outside the Amazon basin, such as the Congo and the Mississippi, are known to shape the distributions of some organisms (11–14), the scale of the river effect in Amazonia is unparalleled, where hundreds of taxa have river-bounded distributions (15). Extensive river-associated faunal turnover suggests that Amazonian rivers influence the evolutionary process in birds, but the role they play in the generation and maintenance of biological diversity remains unclear (16, 17).

Recent studies suggest that speciation in terrestrial vertebrates occurs primarily by allopatric speciation, with high species richness in the Amazon representing the outcome of repeated cycles of geographic isolation, speciation, and range expansions (18). The first model proposed to explain Amazonian diversification invoked the formation of rivers (19), which may influence all three stages of this process. As dispersal barriers, Amazonian rivers may generate diversity through geographic isolation and allopatric speciation, through either vicariance or founder effects (that is, peripatric speciation). Under a strictly vicariant model, the ancient birth of rivers could have subdivided formerly continuous and widespread ancestral populations into geographically isolated, river-delimited subpopulations that differentiated into distinct taxa (3–5). Under a peripatric speciation model, expanding populations on one river bank could colonize its opposite bank and establish isolated populations. Although there are differences in the range of divergence times predicted by the two models, their salient shared feature is that rivers play a direct role in generating diversity through allopatric speciation (16, 19). On the other hand, rivers may play a key role as barriers to secondary contact, maintaining current levels of species diversity by reducing gene flow and inhibiting homogenization between differentiating populations (20).

The search for common evolutionary histories and unique vicariant explanations to account for current levels of Amazonian diversity has dominated the literature for decades [for example, (2, 3, 5, 7, 9)], yet an increasing number of molecular-based phylogenies suggest high levels of temporal and spatial variance in the historical assembly of avian communities (21, 22). The diversification of extant Amazonian bird lineages, for example, seems to be the result of different processes, including in situ Pleistocene speciation (5, 23), recurrent invasions from non-Amazonian biomes (24, 25), or even more complex histories that initiated as early as the Miocene (26). Whereas time estimates of the origin of each pair of taxa across a common barrier can shed light into the temporal aspect of speciation (27), phylogenies can provide information concerning the potential role of geographic barriers as vicariant forces and offer clues regarding the primary or secondary nature of riverine barriers.

Despite the fact that most phylogeographic and taxonomic breaks within Amazonian avian lineages are associated with rivers, few studies have investigated the phylogenetic relationships of multiple allopatric populations separated by a single riverine barrier. Common distribution patterns (that is, multiple pairs of taxa divided by a common barrier) without temporal and phylogenetic-based spatial information could conceal cases of "pseudo-congruence" (28–30), which may be much more common than previously thought (31). Therefore, understanding the relative timing of major speciation events and whether pairs of taxa across a riverine barrier are each other's closest relatives is helpful in understanding the role of Amazonian rivers in the diversification process. Spatial and temporal congruence among lineage diversification would be consistent with

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a common vicariant event (such as the origin of a river), whereas pseudo-congruence can be interpreted as evidence against a single diversification event, supporting idiosyncratic, species-specific histories. Similarly, under simplifying assumptions, taxon pairs that are sisters are predicted under the vicariant and peripatric models of river-associated divergence, whereas taxa in secondary contact, whose divergence is not directly associated with the river drainage, are predicted to be more distantly related (29).

Using a large database containing georeferenced information from more than 28,000 individual bird records from the Guiana Shield (Fig. 1) and comparative phylogeographic and phylogenetic data, we evaluate the role two major Amazonian rivers play in the generation and maintenance of avian diversity. We do this by conducting a community-wide assessment in the Rio Negro basin, the very same drainage first explored by Wallace nearly 170 years ago. About one-third of all bird species that inhabit lowland terra-firme forests in the Guiana Shield have an ecologically similar geographic replacement on the west side of the lower Rio Negro (Fig. 2) (32). As many as 86 avian taxa (species or subspecies) replace each other along the lower Rio Negro (Fig. 2). In a previous study, analyzing 76 of these pairs, we established that only 21 of these pairs remain bounded by the upper Rio Negro, where the river is much narrower (21). Above the lower portion of the Rio Negro, 42 pairs turn over across another riverine barrier, the Rio Branco (a major whitewater tributary of the Rio Negro), and 13 pairs are not bounded by rivers and turn over at various places within the Branco/Negro interfluve (Fig. 3) (21, 32, 33).

Our focus in this study is on understanding the roles these two rivers have played in the evolutionary history of the avifauna they currently divide. Specifically, we aim to evaluate to what extent the current evidence supports the hypothesis that rivers acted as primary barriers versus the alternative hypothesis that rivers represent barriers to secondary contact. We obtained molecular data for 74 of the 86 taxon pairs that replace each other along the lower Rio Negro, and we use distributional and phylogeographic data to estimate the precise geographic location of their turnover. We use molecular phylogenetic data to estimate divergence times for each pair of taxa and compare those estimates with current geomorphological models of river genesis. We then use coalescent simulations in a Bayesian framework to compare models of simultaneous divergence for co-distributed pairs of taxa divided by the upper Negro and Branco rivers. Finally, we use phylogenetic reconstructions to determine the relationships of taxon pairs within each clade. In contrast to previous studies, we did not select a subset of exemplar study taxa but instead designed our study (in both the field and the laboratory) to sample all taxon pairs that exhibit phenotypic differentiation across a common riverine barrier. This community-wide analysis provides a unique and comprehensive approach to conduct a nuanced investigation of avian diversification in one of Amazonia's most important suture zones.
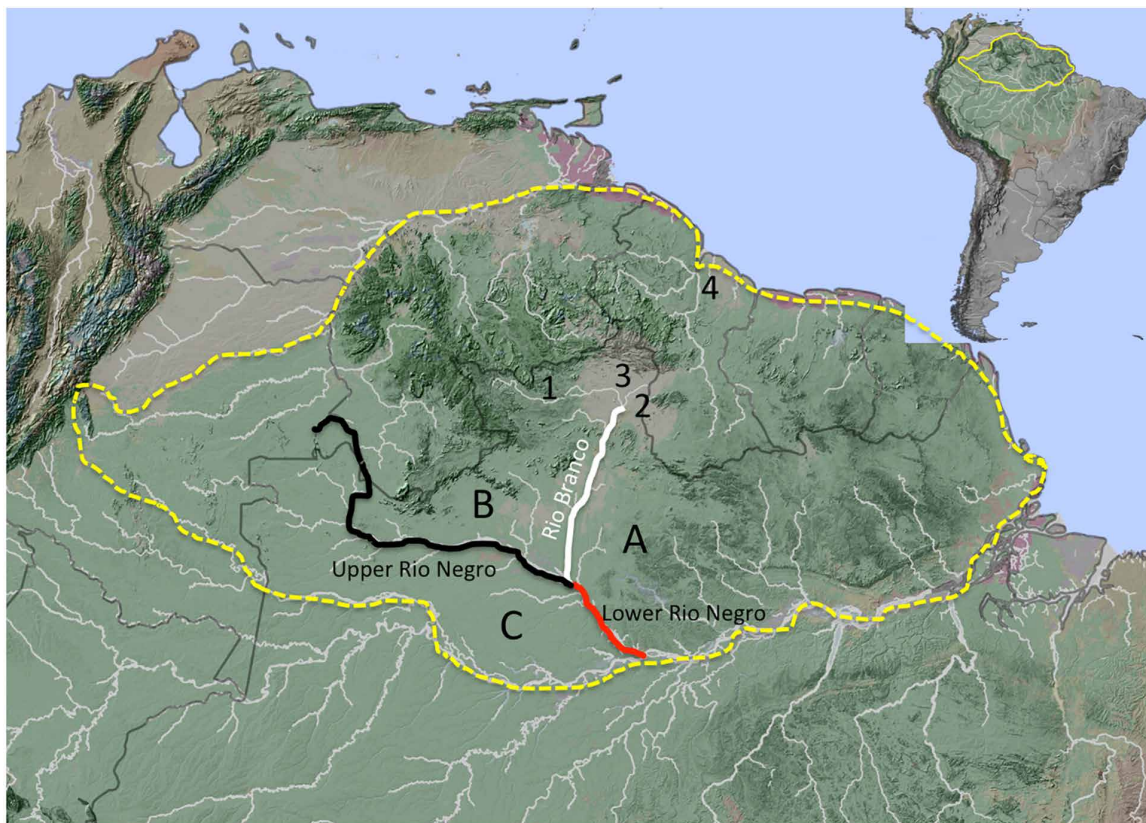

Fig. 1. General location of the Rio Negro basin in northern Amazonia. The Rio Negro is divided into lower (red) and upper (black) segments, indicating their differential effect in taxon turnover. The Guiana Shield, as defined by Osmonson and co-workers (69), is outlined in yellow. Letters represent general biogeographical regions mentioned in the text: (**A**) Guianan area of endemism, (**B**) Branco/Negro interfluve, and (**C**) west bank of the Rio Negro. Numbers represent geographic features mentioned in the text: (1) Rio Uraricoera, (2) Rio Tacutu, (3) Roraima-Rupununi savannas, and (4) Essequibo River.
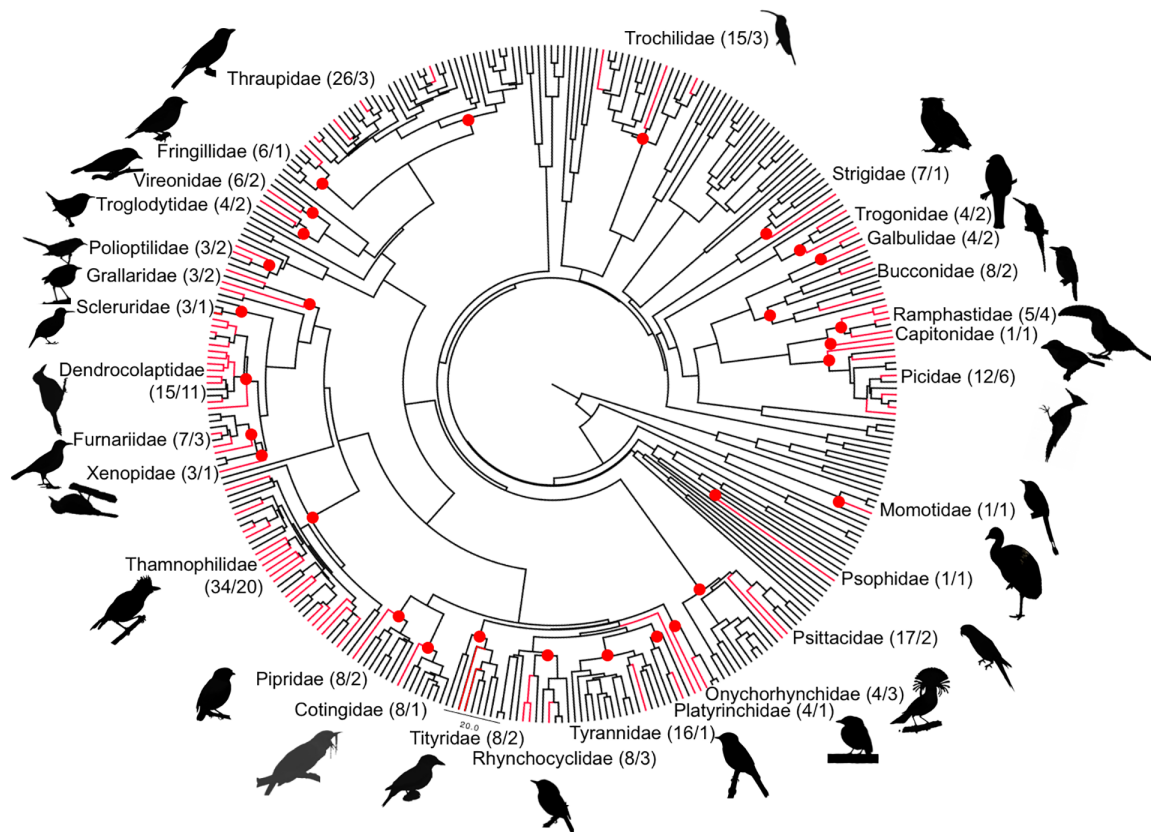
Fig. 2. Phylogenetic tree of a hypothetical terra-firme forest avian community in the Guianan area of endemism. This hypothetical avian community includes 331 species and represents a typical resident avifauna north of the Amazon River and east of the Branco and Negro rivers. This list is based on the study of Cohn-Haft *et al.* (*55*), with minor modifications to include some species absent from central Amazonia but present elsewhere in the Guianan Area of Endemism. In red are 86 avian lineages that have geographic replacements across the lower Rio Negro, 74 of which were included in this study, showing how widespread is the cross-river replacement phenomenon in this region. Red dots were placed at the basal node of each family included in this study. Values following family names represent the number of species expected to occur in our hypothetical avian community and the number of species with cross-river replacements within each family.

RESULTS

Timing of avian diversification in the Guiana Shield

Levels of genetic divergence and the inferred timing of diversification showed substantial variation among taxon pairs divided by the lower Rio Negro (table S1 and Fig. 4), ranging from ~0.24 (0.09 to 0.48) million years (Ma ago) between two species of *Ramphastos* toucans to over 8 (7.3 to 10.1) Ma ago between two species of *Selenidera* toucanets (Fig. 4). Approximately 63% (46 pairs) of the divergence events occurred during the Pleistocene (last 2.6 Ma), ~34% (25 pairs) during the Pliocene (2.6 to 5.3 Ma ago), and ~4% (3 pairs) during the Late Miocene (5.3 to 11.6 Ma ago) (Fig. 4). As expected, the estimated mean divergence time was older for pairs of species (mean, 2.76 ± 1.90 Ma; range, 0.24 to 5.79) than for pairs of subspecies (mean, 1.87 ± 1.37 Ma; range, 0.54 to 7.21) (Welsh two-sample t test: $t_{54.3} = 2.306$, $P = 0.024$). However, we found no significant difference in the time of divergence between pairs of taxa divided by the three distribution patterns described. The 40 pairs of taxa divided by the lower Rio Negro and the Rio Branco (pattern A in Fig. 3) had a mean divergence time of 2.51 ± 1.75 Ma (range, 0.24 to 8.7); the 22 pairs divided by the upper Rio Negro (pattern B in Fig. 3) had a mean divergence time of 1.89 ± 1.55 Ma (range, 0.58 to 5.79); and the 12 pairs not bounded by rivers within the Branco/ Negro interfluve (pattern C in Fig. 3) had a mean divergence time of

2.08 ± 1.57 Ma (range, 0.52 to 4.64) [analysis of variance (ANOVA): $F_2 = 0.972$, $P = 0.38$].

Test of simultaneous divergence across rivers

The large degree of variation in divergence times among pairs of taxa bounded by a single river (the lower Rio Negro) is inconsistent with a scenario of simultaneous divergence (that is, common vicariance) for all pairs currently divided by that river. We tested scenarios of simultaneous divergence in groups of co-distributed taxa separated by the Rio Branco (28 pairs; pattern A in Fig. 3) versus the upper Rio Negro (16 pairs; pattern B in Fig. 3). A hypothesis of simultaneous divergence was rejected for both subsets of taxa (Fig. 5) on the basis of coalescent estimates of the number of possible divergence times (Ψ) and the degree of discordance of the variance of population divergences (Ω) (Fig. 5 and tables S2 and S3).

Because the geomorphological literature suggests that the ages of both rivers could be relatively recent (less than 1.2 Ma for the Rio Negro and less than 0.02 Ma for the Branco; see Discussion), we assessed whether the lack of support for a single diversification pulse could be due to the presence of older population pairs in the data, some of which dated from the Miocene. We tested for simultaneous divergence using a set of independent simulations in which we excluded the oldest pairs, one at a time. When we excluded the four pairs with
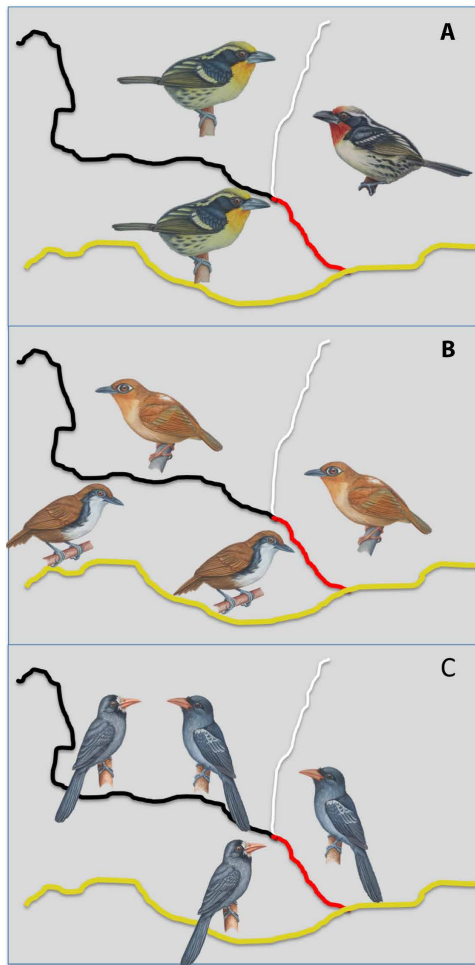
Fig. 3. Avian replacement patterns in the Rio Negro basin. (**A**) "Rio Branco pattern," in which turnover of the taxon pair occurs across the lower Rio Negro (red) and the Rio Branco (white), here represented by two species of barbets, *Capito auratus* (west) and *Capito niger* (east). (**B**) "Rio Negro pattern," in which turnover of the taxon pair occurs across the lower (red) and upper (black) Rio Negro, here represented by two species of antbirds, *Gymnopithys bicolor* (west) and *Gymnopithys rufigula* (east). (**C**) "Branco/Negro interfluve pattern," in which turnover occurs across the lower Rio Negro, but upstream the turnover is not river-bounded and occurs somewhere within the interfluve, here represented by two species of nunbirds, *Monasa morphoeus* (west) and *Monasa atra* (east).

the oldest divergences across the Rio Negro (pairs 13 to 16 in table S2) and then analyzed the remaining 12 pairs, a model with a single divergence pulse was well supported, consistent with simultaneous divergence of these pairs (Fig. 5). In contrast, the exclusion of as many as 23 oldest pairs of taxa across the Rio Branco did not increase support for a simultaneous divergence model (Fig. 5). Together, these results open the possibility of simultaneous divergence for a limited number of taxa across the Rio Negro but argue against the role of the Rio Branco as a primary barrier in the region, despite bounding the distribution of dozens of pairs of avian taxa.

Phylogenetic relationships of pairs of replacing taxa

We reconstructed the phylogenetic relationships of 74 pairs of taxa (figs. S1 to S74). From these, we were able to infer phylogenetic relationships for 66 of the pairs; either the remaining 8 pairs lacked

sampling from key lineages or the particular node of interest was not well supported. We found that the 66 pairs of taxa constituted a blend of sister (25 pairs) and nonsister taxa (41 pairs).

The nonsister relationship between Guiana Shield endemics and their replacement west of the Rio Negro was most often due to phylogenetic subdivision within the western taxon (24 of 41 or ~58%) rather than within the eastern taxon (7 of 41 or ~17%). In the remaining 10 nonsister pairs (~25%), both the eastern and western taxa had closer phylogenetic relationships to other Neotropical taxa. Pairs of sister taxa had a mean divergence time of 1.57 ± 1.24 (range, 0.24 to 4.59) Ma, significantly younger than nonsister pairs, which had a mean divergence time of 2.75 ± 1.76 (range, 0.3 to 7.7) Ma (Welsh two-sample t test: $t_{62.1} = 3.14$, $P = 0.002$). Sister and nonsister pairs were evenly divided across distribution patterns; 36% of the pairs were sister taxa across the Branco and the Branco/Negro interfluve (patterns A and C in Fig. 3), and ~42% were sister taxa among the pairs divided by the Rio Negro (pattern B in Fig. 3) (table S1).

DISCUSSION

The role of Amazonian rivers in the diversification process

The evolutionary role of Amazonian rivers in the assembly of the Neotropical biota remains one of the most contentious topics in tropical evolutionary biology ([15–17], [34]). The growing number of biogeographical and molecular studies of Amazonian organisms during the last two decades has enlightened our views of the processes behind current distribution patterns. Although single-lineage studies provide the building blocks for comparative phylogeography, they cannot elucidate general trends on their own ([35]). By using a community-wide comparative approach in one of Amazonia's largest and most biogeographically important river basins, we were able to dissect the evolutionary histories of dozens of co-distributed pairs of avian taxa divided by common riverine barriers. We showed the dual role of rivers in the evolutionary process. Rivers maintain diversity by inhibiting gene flow and introgression for taxa in secondary contact, and they likely generate diversity by acting as a dispersal barrier in allopatric speciation.

Three key results from our study demonstrate that diverse histories underlie the "shared" biogeographic pattern of taxon turnover across Amazonian rivers. First, we showed that the divergence times of taxon pairs divided by two riverine barriers are not clustered in a particular time period, and instead span a more or less continuous distribution of ages beginning in the Late Miocene (~8 Ma ago) and ending as recently as the Late Pleistocene (~0.24 Ma ago). Younger diversifications are likely happening across these barriers, but we lack the molecular resolution to accurately date them. Second, coalescent simulations indicated that the probability of single diversification pulses having shaped current distribution patterns across the Rio Branco and the Rio Negro was low when all pairs were included in the analyses. Third, phylogenetic reconstructions showed that most parapatric replacing pairs of taxa divided by common riverine barriers were not sister taxa, even when the taxon pairs represented clear ecological and biogeographical replacements. Given these results, we conclude that the current spatial configuration of river-bounded pairs of taxa is primarily the result of secondary contact, with some possible cases of primary diversification.

In 58% of the nonsister pairs, the nonsister relationship was due to phylogenetic subdivisions within the western taxon. That is, it is often the western taxon that has a closer relationship to a lineage that
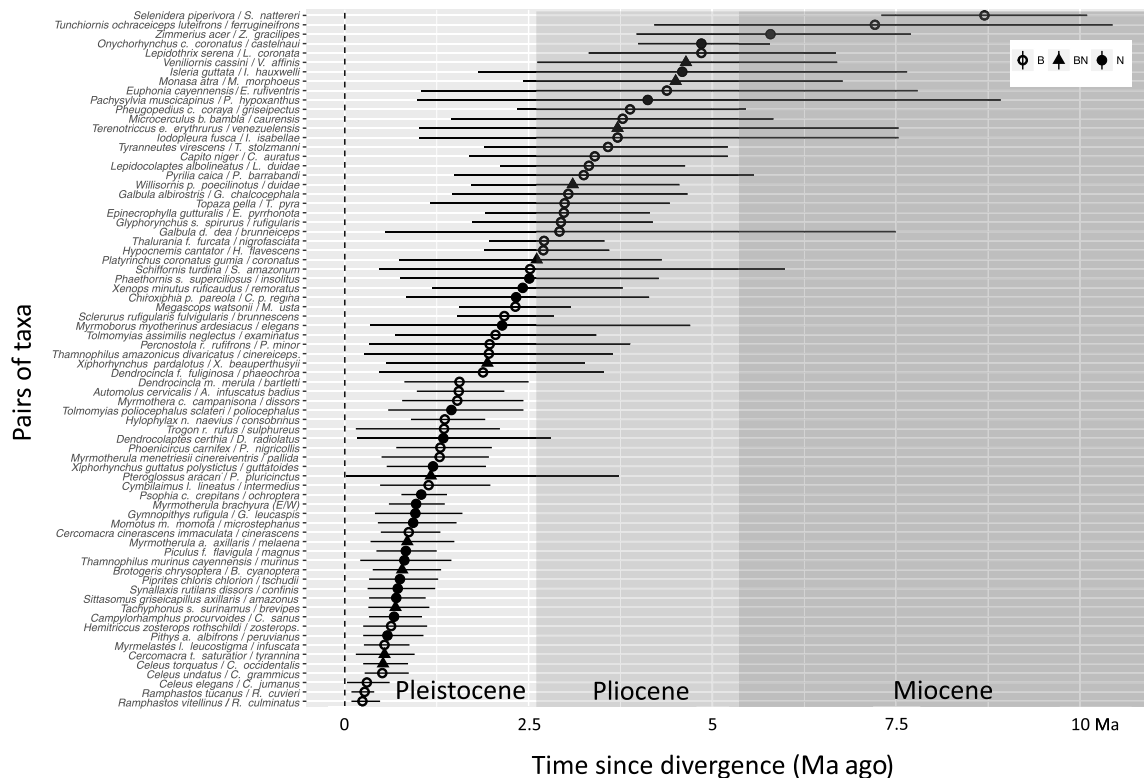
Fig. 4. Time since divergence of 74 pairs of avian taxa (species and subspecies) that replace across the lower Rio Negro and other barriers upstream. Open circles refer to pairs divided by the Rio Branco (pattern A in Fig. 3), solid circles refer to pairs divided by the lower and upper Rio Negro (pattern B in Fig. 3), and triangles refer to pairs divided by the lower Rio Negro but not river-bounded within the Branco/Negro interfluvium (pattern C in Fig. 3). Symbols represent mean time of divergence, and lines represent their respective credible intervals (see table S1).

is not found on the eastern side of the lower Rio Negro. This pattern is consistent with previous studies that found that more dynamic areas in western Amazonia could be responsible for much of the speciation that occurred during the Neogene (*36*). Although a nonsister relationship between parapatric pairs of taxa does not reject a history of river-caused primary diversification (subsequent diversification events could blur the original signature of an initial population split), it is difficult to envision a primary barrier bounding so many nonsister pairs of taxa of so many different ages. This supports the idea that taxon evolution in this assemblage of birds is not the result of a particular geological event but rather the result of idiosyncratic histories in a dynamic landscape (*37*). These results are also consistent with the geomorphological history of the rivers, the details of which are only starting to be revealed by geological studies (see below).

For a dozen pairs of taxa bounded by the upper Rio Negro, our results do support a single diversification pulse (table S4). With one exception, the mean time of origin estimated for those avian lineages (of seven different families) is consistent with recent geomorphological models proposing that most of the current course of the Rio Negro formed between 0.7 and 1.2 Ma ago (*5*). Phylogenetic analyses for those 12 pairs showed that half of them are each other's closest relatives and 3 are nonsisters (we could not define the relationship of 3 pairs) (table S4). For those pairs divided by the upper Rio Negro that are not sister taxa, secondary splits have occurred very recently within the western taxon, after the inferred temporal formation of the Rio Negro (figs. S7, S40, and S50). A recent analysis of the spatial

and temporal diversification patterns of primates in the Guiana Shield found similar results. At least three primate genera that are currently divided by the upper Rio Negro (*Cacajao*, *Callicebus*, and *Cebus*) have sister populations across this river (*9*), and estimated divergence times are relatively recent and consistent with those reported here. Although not conclusive, these results suggest that the Rio Negro may have played a key role in the diversification process as a primary barrier, through either vicariance or peripatric speciation, for a handful of bird lineages in the Guiana Shield.

Our results are consistent with the view that single hypotheses will likely fail to explain the origins of current levels of Amazonian biological diversity and that more complex models will be required to account for current avian distribution patterns in the Guiana Shield (*38*). Collectively, our results illustrate that taxon turnover across a major Amazonian suture zone, in this case the lower Rio Negro, encompasses a diversity of histories. It seems clear that the role of rivers in the evolutionary process of the Amazonian biota is mainly due to their efficacy as dispersal barriers, and that taxon turnover includes taxon pairs that diversified elsewhere and are in secondary contact at the river, but also some that probably diverged directly because of the river.

The history of riverine barriers
One key aspect that is often overlooked is whether the geomorphological history of rivers supports the evolutionary scenarios inferred from the molecular data. This is largely due to geological uncertainties
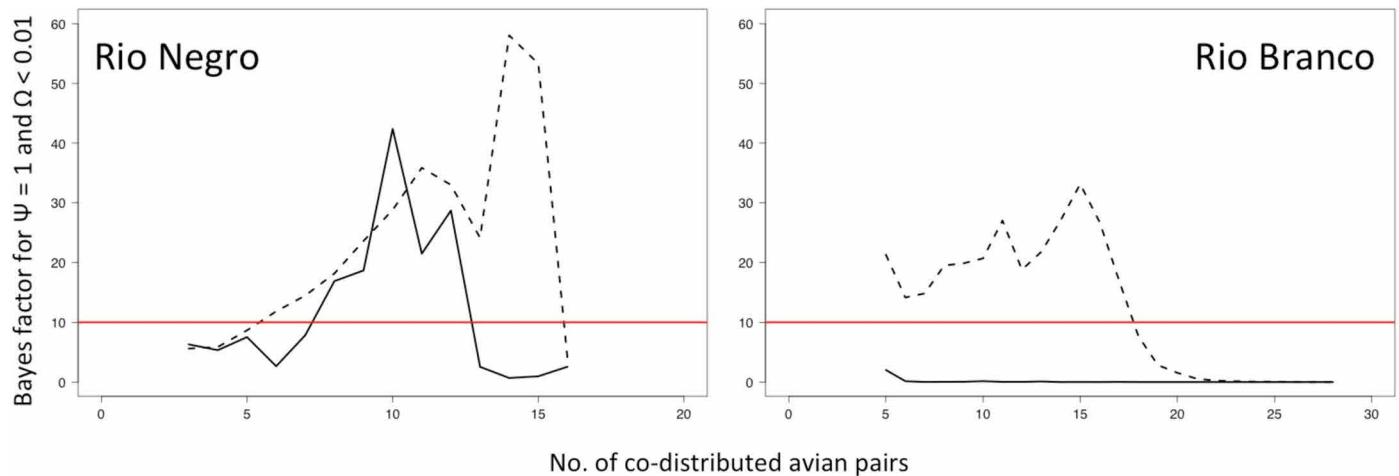
Fig. 5. Bayesian model support for independent comparisons of simultaneous divergence in multiple pairs of co-distributed avian taxa across the Rio Negro (3 to 16 pairs) and the Rio Branco (5 to 28 pairs). Solid line represents the Bayes factor value for $\Omega < 0.01$ (degree of discordance of the variance of population divergences), where the dashed line represents the value for $\Psi = 1$ (number of divergence times or speciation pulses). Strong evidence for a single diversification pulse is indicated by Bayes factor values > 10 in models where both hyperparameters (Ψ and Ω) are consistent with a single pulse (that is, $\Psi = 1$ and $\Omega < 0.01$). The red horizontal line indicates this threshold ($K = 10$). The data are consistent with a single diversification pulse for 8 to 12 pairs across the Rio Negro but reject a common diversification scenario for pairs across the Rio Branco.

related to the origin of Amazonian rivers (*39*). In recent years, however, the histories of some rivers in the region have been clarified, including that of the Rio Branco. Previous work suggested that before flowing southward into the Rio Negro, the Tacutu and Uraricoera rivers (the two rivers that form the Branco) were part of the Essequibo basin, flowing toward the Caribbean in a fluvial system called the Proto-Berbice (*40, 41*). A recent change of direction in this river is evident from dated paleochannels and the current geometry of several tributaries that flow in a northeast direction and change southward when they meet the present course of the Rio Branco (*41–43*). To geologists, this is evidence that the Rio Branco was captured by the Rio Negro drainage relatively recently, possibly during the Late Pleistocene (*41*). A chronological framework based on radiocarbon and optically stimulated luminescence dating showed that the sedimentary deposits in the current valley of the Rio Branco include two alluvial plains that are as young as 18.7 thousand years and that intersect a Late Pleistocene residual megafan (*43*). These results suggest the Rio Branco acquired its current southward direction only a few thousand years ago. Therefore, despite representing a major phylogeographic break, bounding the distributions of at least 40 pairs of avian taxa, our molecular results are consistent with a scenario where this river played no primary role in the original diversification of the pairs whose distributions it currently bounds.

If the Rio Branco is too recent to have acted as a primary barrier for taxa that diverged millions of years ago, as the new geological data seem to suggest, what was responsible for separating so many pairs of avian taxa in the Guiana Shield? Although Haffers's Pleistocene refugia hypothesis has been criticized in the past, mostly because of temporal and climatic inconsistencies (*44–46*), climatic fluctuations and subsequent vegetation changes at the edges of Amazonia, where modern forest/savanna ecotones thrive, have received some support (*47*). Located at the northern edge of the Amazon, the Rio Branco basin is characterized by large savannas (the largest in Amazonia) and extensive areas of white sands and paleodunes (*48*), suggesting that the area has long been exposed to climatic fluctuations and consid-

erable changes in vegetation cover (*49*). In view of the putative recent origin of the current configuration of the Rio Branco, and the fact that most avian taxa divided by this river are considerably older than the Late Pleistocene, it seems very likely that the current limits provided by the Branco are the result of secondary contact between taxa that diverged elsewhere. Hence, our results are consistent with the view that (at least some) Amazonian rivers may represent natural suture zones for populations in secondary contact, as suggested by Haffer throughout his career (*2, 15, 20, 50, 51*). On the other hand, the data from the upper Rio Negro partially support the riverine barrier hypothesis for a small number of taxa. How avian dispersal abilities, species-specific functional and ecological traits, and population parameters affected the current distribution of these species remains to be explored.

Riverine barriers and the generation and maintenance of biodiversity

Large Amazonian rivers are extraordinary biogeographical barriers to the avifauna and other terrestrial biota, and their efficacy as dispersal barriers represents an effective mechanism for maintaining current levels of biological diversity. Our data suggest that the number of avian taxa bounded by an Amazonian river does not depend entirely on its physical features or history. Our results provide a clear example of how two very different Amazonian rivers (in this case, the Branco and Negro) may be involved in both the generation and the maintenance of species diversity. Considerable attention has been given in the past to the physical or ecological features related to the dissecting power of Amazonian rivers (*7, 34*). Studies on primate communities suggested that annual river discharge and river width were both good predictors of the amount of ecological dissimilarities across rivers, and that opposite banks of fast-flowing and nutrient-poor rivers (such as the Negro) show consistently lower levels of community similarities than slow-flowing, nutrient-rich, meandering rivers (*7*). Although the Rio Branco is not a meandering river, and according to a recent analysis could be considered a river of "clear waters" during

the dry season (*52*), it has a considerable sediment load and yet remains an important biogeographical barrier. Whether nutrient-rich rivers are areas of secondary contact, and nutrient-poor craton rivers are potential vicariant forces, is open to investigation. However, it is quite remarkable that two rivers that differ in virtually every possible ecological and physical aspect (for example, size, hydrology, color, type and extent of associated flooded forests, amounts of sediments, and neotectonics) (*53*) both have a common dissecting biogeographic effect on the avifauna.

Discussions regarding the origin of the Amazonian biota have focused on the mechanisms behind the generation of new species (*3*, *4*, *20*), and it took decades for Amazonian biogeographers to realize that both dispersal and vicariance may act simultaneously in a complex and changing landscape (*18*). We found evidence consistent with the vicariant role of the Rio Negro for a limited, yet significant, number of pairs of avian taxa but also with the role of the Rio Branco as a barrier to secondary contact. A recent comparative phylogeographic study of widespread, co-distributed avian lineages suggested that the number of species within a lineage may be a function of its age and the dispersal ability of each clade (*18*). While this may be true, our study still opens the possibility for common vicariant forces, albeit for a limited number of taxa. Whereas taxon-specific histories are likely involved, the quest for common evolutionary patterns may not be completely lost. Using community-wide approaches that capture the full demographic history of individual taxa, at the scale currently being done in population genomic studies of human evolution (*54*), it will be possible to disentangle the proportion of cases explained by different processes acting in concert to shape current avian distribution patterns in Amazonia.

Finally, the high interpair variance in cross-river divergence times suggests that the common distribution patterns found in 74 pairs of taxa across the lower Rio Negro are (at least to some extent) the result of pseudo-congruence, where general distribution patterns found among multiple clades are the result of idiosyncratic histories, rather than vestiges of common diversification events (*31*). It is possible that other Amazonian suture zones also represent cases of pseudo-congruence, and particular attention needs to be given in comparative studies across common biogeographical barriers. These results come from only one, albeit important, Amazonian region, and similar studies from other river basins will likely place our results into a broader context.

MATERIALS AND METHODS
Avian distribution patterns and taxon sampling
Lowland terra-firme forests in the Guiana Shield are among the world's most species-rich habitats. Alpha diversity at any single locality in the Guiana Shield often harbors as many as 260 resident bird species (*55*). One-third of these (86 species) have a closely related and ecologically similar replacement taxon (species or subspecies) across the region's main river, the lower Rio Negro (Fig. 2) (*21*, *32*). Here, we compiled specimen record data and collected molecular data for 74 of these pairs, representing 29 different avian families (table S1 and Fig. 2). The 74 pairs present varying degrees of phenotypic and genotypic differentiation and, with one exception, are distinct, named taxa. More than half of these pairs (55%) currently represent allopatric subspecies in more widespread polytypic species. The rest of the pairs represent closely related species, many of which were considered conspecific until recently (for example, *Capito niger* and *C. auratus*, *Hypocnemis*

cantator and *H. flavescens*, *Lepidocolaptes albolineatus* and *L. duidae*, *Zimmerius acer* and *Z. gracilipes*) or are part of well-established species complexes (for example, the genus *Selenidera*, *Pyrilia*, *Ramphastos*, or *Epinecrophylla*). Finally, one pair (*Myrmotherula brachyura*) currently represents a single taxon (unpublished data indicate that two species are likely involved). A previous study in the region showed that haplotypes of most of the individual taxa (irrespective of their current taxonomic status) are monophyletic on the basis of mitochondrial DNA (mtDNA) (*21*). To avoid biases from subjective taxonomic decisions, we treated the 74 pairs equally as "pairs of taxa" irrespective of their current taxonomic status, but we are confident that each of the pairs selected and evaluated in this study represents meaningful evolutionary lineages that are relevant to the question being asked.

We characterized geographic patterns of taxon replacements using distribution maps constructed from a 28,000-record distributional data set of museum specimens, published sources, audio recordings, and observations from our fieldwork in the region. A detailed description of the geographic database was published previously (*32*). All 74 taxon replacements occur at the lower Rio Negro, but above this region, we used specimen records and phylogeographic data (described below) to pinpoint whether taxon turnover occurred at the upper Rio Negro, the Rio Branco, or within the interfluve of the two rivers (see Fig. 3).

Most tissue samples used in this study are from the Guiana Shield, north of the Amazon River, and have associated voucher specimens (Supplementary Appendix). We included samples from other regions to build the phylogenetic trees included in this study (figs. S1 to S74). To identify whether the pairs of taxa divided by rivers represent sister lineages, we either reconstructed our own trees or consulted published phylogenies (table S1).

Molecular data
To obtain a standard measure of genetic divergence across many different pairs of taxa, we sequenced the complete (1041 base pairs) protein-coding mitochondrial gene *ND2* [NADH (reduced form of nicotinamide adenine dinucleotide) dehydrogenase subunit 2], which has proven to be well suited for assessing population structure at both deep and shallow divergence levels (*56*). For two pairs (*Cymbilaimus l. lineatus/intermedius* and *Myrmotherula a. axillaris/melaena*), we used cytochrome b sequences, which were already available (*18*). Although single-locus data have inherent limitations, particularly because of the stochastic nature of gene coalescence (*57–59*), we opted to maximize taxonomic breadth and number of samples over collecting data from multiple loci. A recent study found that multilocus data from next-generation sequences produced approximate Bayesian computation results (see below) similar to those found with mitochondrial markers (*18*). Although we do not advocate for single-locus studies in the genomic era, we agree with Bowen *et al.* (*35*) that the signal observed in the mtDNA offers a useful proxy of genetic differentiation and geographic structure of avian populations, particularly when used in multiple-species comparative approaches. We obtained DNA sequences following standard extraction, amplification, and sequencing protocols, which are described in detail in the Supplementary Materials.

Estimation of divergence times
We estimated the time since divergence between pairs of populations on opposite sides of the lower Rio Negro using a Bayesian relaxed phylogenetic approach (*60*) implemented in BEAST v1.8.2 (*61*). We assumed an uncorrelated relaxed substitution rate (where the rate at

each branch is drawn independently from a lognormal distribution) based on an avian molecular clock estimated specifically for the mitochondrial marker used (ND2) by Smith and Klicka (*62*). For the cytochrome b samples (*Myrmotherula axillaris* and *Cymbilaimus lineatus*), we used a specific calibration obtained by Weir and Schluter (*63*). The selected prior distribution for the mean (ucld.mean) was set to 0.0125 for ND2 and 0.0105 for cytochrome b, with an SD of 0.1. The SD parameter (ucld.stdev) followed an exponential distribution with mean equaling 1. Because we were working at the population level (species and subspecies pairs), we used a population constant size tree prior. However, in some cases where we had phylogenies above the species level, we used a birth-death evolutionary process, following a uniform birth rate distribution [0,1000] and a uniform relative dead rate [0,1] (table S1). The choice of this particular prior had a minor effect on our time estimates; a constant size tree prior yielded slightly younger estimates than a birth-death model (~10% younger). We determined the best-fit finite-sites nucleotide substitution model for each pair of taxa under the Bayesian information criterion, as implemented in jModelTest 2 (table S1) (*64*). In BEAST, we ran analyses for at least 30 million generations [higher in some cases with low effective sample size (ESS) values], sampling every 5000 generations. We verified Markov chain Monte Carlo convergence, ESS, and posterior intervals spanning the 95% highest posterior density using Tracer v1.6.0 (*65*).

Simultaneous divergence

Parts of the following methods sections have been reproduced from (*21*). We compared models of simultaneous versus nonsimultaneous divergence among pairs of co-distributed taxa using a hierarchical approximate Bayesian computation (hABC) approach implemented in MTML-MSBAYES, which works under a coalescent model using multiple co-distributed population pairs. Instead of conducting independent analyses on every population pair and testing the hypothesis of temporal concordance, hABC tests for simultaneous divergence using all the data in a single analysis [see (*65*, *66*) for a complete rationale on the advantages and limitations of this method]. The subparameters of each pair (Φ) are allowed to vary independently across all populations, and include parameters such as divergence times, current and ancestral population sizes, post-divergence founding population sizes, recombination rates, and post-divergence migration rates. MSBAYES estimates a set of three hyperparameters (φ) that characterize and quantify the degree of variability in divergence times across populations while allowing variation in various within population-pair demographic parameters (subparameters) that can affect the coalescent (*67*). Rather than directly calculating likelihood expressions, it uses simulation-based summary statistics that approach an approximate likelihood, which can be sampled from the posterior probability. These summary statistics are as follows: (i) π, the average number of pairwise differences among all sequences within each population pair; (ii) θw, the number of segregating sites within each population, normalized for sample size; (iii) Var (π = θw) in each pair; (iv) πnet, Nei and Li's net nucleotide divergence between each pair of populations. Two of these hyperparameters calculated include (i) Ψ, the number of possible discrete co-divergence times, and (ii) Ω, the degree of discordance of divergence times, measured as the variance of population divergence times, divided by the mean divergence time of all population pairs [Var (τ)/E(τ)]. Strong model support for Ψ = 1 and for very low values of Ω (<0.01) are consistent with scenarios of simultaneous divergence. As a general rule, values of Ω are more conservative than values of Ψ (that is, less prone to type 1 errors). Therefore, we only considered as compelling evidence of simultaneous divergence those models where both hyperparameters (Ψ and Ω) support such scenario. We tested alternative models by estimating the Bayes factor of Ψ = 1 versus Ψ > 1 and Ω < 0.01 versus Ω > 0.01 and accepted values of Bf (*K*) > 10 as strong evidence for a single divergence event, following Jeffreys's support criterion (*68*). For Ψ, the Bf was calculated as [(Ψ = 1/Ψ > 1)/(*P*(Ψ = 1)/*P*(Ψ > 1)], where the numerator represents the number of values = 1, divided by the number of values > 1 from the 1000 accepted random draws from the posterior, and the denominator is a function of the number of species in the simulations [for example, if there are five species in the simulation, then *P*(Ψ = 1) equals 0.2 and *P*(Ψ > 1) equals 0.8]. For Ω, the Bf was calculated as [(Ω < 0.01/Ω > 0.01)/(*P*(Ω < 0.01)/*P*(Ω > 0.01)], where the numerator was represented by the number of values < 0.01, divided by the number of values > 0.01 from the 1000 random samples obtained from the posterior, and the denominator was represented by the number of values < 0.01 from a sample of 100,000 values randomly taken from the prior, divided by the number of values > 0.01 in the same sample.

We were particularly interested in whether taxon pairs separated by the upper Rio Negro showed a different pattern of divergence in comparison to taxon pairs separated by the Rio Branco. We used hABC to test for simultaneous divergence in 16 pairs of taxa across the Rio Negro (a total of 268 individuals, with an average of 10.3 ± 5.4 samples from the west bank and 6.3 ± 2.9 samples from the east bank) and in 28 pairs of taxa across the Rio Branco (a total of 422 individuals, with an average of 6.4 ± 3.8 samples from the west bank and 8.6 ± 4.5 samples from the east bank) (tables S2 and S3). We included 16 of 22 pairs bounded by the upper Negro and 28 of 40 pairs bounded by the Rio Branco, excluding 6 and 12 pairs, respectively, because of reduced sample sizes; we only selected taxon pairs for which we had sufficient samples to run the simulations following Hickerson *et al.* (*67*). For pairs divided by the upper Rio Negro, we only selected those populations that are separated by the entire extension of the river, including the lower and upper sections (pattern B in Fig. 3). For this river, only *M. brachyura* represents a single taxon. For the Rio Branco, we selected species bounded by the lower Rio Negro and the Rio Branco (pattern A in Fig. 3). For this specific analysis, we complemented our already extensive sampling with three pairs of unnamed populations, but which exhibit cryptic genetic structure across these rivers (*Thamnomanes caesius*, *Herpsilochmus dorsimaculatus*, and *Formicarius colma*).

We ran two types of analyses. First, we conducted analyses including all available taxon pairs (28 for the Rio Branco and 16 for the upper Rio Negro). We then implemented a sequential exclusion of older taxon pairs as a way to assess the influence of individual taxon pairs on the analysis. In these analyses, we created subsets of pairs, excluding one pair (the oldest) at the time, and repeated the analyses. We excluded older pairs sequentially and repeated the analysis until we were left with three pairs across the upper Rio Negro and five pairs across the Rio Branco. We calculated Bayesian support (by means of Bayes factors) for simultaneous divergence including all 14 independent simulations for the Rio Negro and 24 simulations for the Rio Branco. In total, we ran 1,000,000 simulations (which is the number of random draws from the hyperprior) for each independent simulation. We set the tolerance to 0.001 (the proportion of accepted draws from the prior), which provides the 1000 closest matches between the summary statistic calculated

from the observed and simulated data to sample from the posterior distribution.

We used the following prior parameters for all simulations: (i) bounds of θ per site: upper θ = 0.1, lower θ = 4×10^{-8}; (ii) upper limit of τ (time of divergence) = 1.0; (iii) the number of τ classes (Ψ, possible times of divergence) was set to 0, which means that there could be as many differentiation events as pairs of populations in the comparison; (iv) migration rate = 0 (which disables migration); and upper ancestral population sizes were set to 0.025, and (v) subparameters unconstrained (0). These prior parameters have worked well in simulations and are expected to perform well under a wide number of cases.

Phylogenetic analyses

To infer the phylogenetic relationship of taxon pairs, we reconstructed phylogenies for clades within which the taxon pairs belonged. We considered sister taxa those pairs that were more closely related to one another in the phylogenetic tree than to any other named or unnamed monophyletic population. Nonsister taxa were those pairs that included another taxon (species or subspecies) or another monophyletic population (possibly an undescribed lineage) as more closely related to either one of the two populations under analysis. In some cases, where we lacked samples from particularly important populations, we were unable to determine sister relationships.

We assume that a sister relationship between a taxon pair indicates support for the river having a direct effect on their divergence and that a nonsister relationship indicates support for the river representing a zone of secondary contact. We acknowledge that nonsister relationships between cross-river taxon pairs could occur if there have been divergence events on either side of the river subsequent to the cross-river divergence event. To address this, we inspected every nonsister pair and evaluated whether their most recent split was recent enough (less than 1 Ma) to represent a secondary split in relation to the origin of the river that bounded these two forms, assuming in these cases that this population split likely occurred after the river-bounded populations were separated (see Discussion).

We reconstructed mitochondrial gene trees for 60 taxon pairs using new data in combination with additional sequence data available in GenBank. We also included published phylogenies of 14 avian lineages with a pair of taxa bounded by the lower Rio Negro. Even when published phylogenies were already available, we tried to build our own trees to facilitate comparisons in timing methods. In these cases, we confirmed that our results were similar to those originally published.

Phylogenetic trees were built in BEAST v1.8 (61) following the same parameters presented above to estimate divergence times. Majority rule consensus trees were built using TreeAnnotator, excluding 20% of the trees as burn-in, resulting in a total of ~5000 trees. In total, we obtained well-supported trees with high ESS values (>300) for 60 pairs of taxa known to exhibit phenotypic and genotypic differentiation across the lower Rio Negro.

SUPPLEMENTARY MATERIALS

Fig. S28. Time-calibrated tree of the genus Mouse-colored Antshrike (*Thamnophilus murinus*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S29. Time-calibrated tree of the genus Amazonian Antshrike (*Thamnophilus amazonicus*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S30. Time-calibrated tree of the Fasciated Antshrike (*C. lineatus*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S31. Time-calibrated tree of the Spot-backed Antbird (*Hylophylax naevius*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S32. Time-calibrated tree of the Spot-winged Antbird (*Myrmelastes leucostigma*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S33. Time-calibrated tree of the Black-faced Antbird (*Myrmoborus myotherinus*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S34. Time-calibrated tree of the genus *Percnostola*, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S35. Time-calibrated tree of the genus *Cercomacra* with emphasis on *C. cinerascens*, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S36. Time-calibrated tree of the genus *Cercomacroides* with emphasis on *C. tyrannina*, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S37. Time-calibrated tree of the genus *Hypocnemis*, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S38. Time-calibrated tree of the genus *Pithys*, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S39. Time-calibrated tree of the *Willisornis* complex, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S40. Time-calibrated tree of the genus *Gymnopithys*, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S41. Time-calibrated tree of the genus *Myrmothera*, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S42. Time-calibrated tree of the genus *Dendrocincla* with emphasis on *D. fuliginosa*, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S43. Time-calibrated tree of the White-chinned Woodcreeper (*Dendrocincla merula*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S44. Time-calibrated tree of the Olivaceous Woodcreeper (*Sittasomus griseicapillus*) species complex, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S45. Time-calibrated tree of the Wedge-billed Woodcreeper (*Glyphorynchus spirurus*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S46. Time-calibrated tree of the *Xiphorhynchus ocellatus* species complex, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S47. Time-calibrated tree of the *Xiphorhynchus guttatus* complex, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S48. Time-calibrated tree of the genus *Campylorhamphus*, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S49. Time-calibrated tree of the *L. albolineatus* complex, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S50. Time-calibrated tree of the *Dendrocolaptes certhia* complex, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S51. Time-calibrated tree of the Short-billed Leaftosser (*Sclerurus rufigularis*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S52. Time-calibrated tree of the Plain Xenops (*Xenops minutus*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S53. Time-calibrated tree of the genus *Automolus*, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S54. Time-calibrated tree of the Ruddy Spinetail (*Synallaxis rutilans*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S55. Time-calibrated tree of the genus *Tyranneutes*, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S56. Time-calibrated tree of the genus *Lepidothrix*, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S57. Time-calibrated tree of the Blue-backed Manakin (*Chiroxiphia pareola*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S58. Time-calibrated tree of the Royal Flycatcher (*Onychorhynchus coronatus*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S59. Time-calibrated tree of the Ruddy-tailed Flycatcher (*Terenotriccus erythrurus*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S60. Time-calibrated tree of the *Schiffornis turdina* species complex, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S61. Time-calibrated tree of the genus *Iodopleura*, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S62. Time-calibrated tree of the genus *Phoenicircus*, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S63. Time-calibrated tree of the Wing-barred Piprites (*Piprites chloris*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S64. Time-calibrated tree of the Golden-crowned Spadebill (*Platyrinchus coronatus*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S65. Time-calibrated tree of the Yellow-margined Flycatcher (*Tolmomyias assimilis*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S66. Time-calibrated tree of the Grey-crowned Flycatcher (*Tolmomyias poliocephalus*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S67. Time-calibrated tree of the White-eyed Tody-tyrant (*Hemitriccus zosterops*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S68. Time-calibrated tree of the genus *Zimmerius*, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S69. Time-calibrated tree of the Tawny-crowned Greenlet (*Tunchiornis ochraceiceps*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S70. Time-calibrated tree of the genus *Pachysylvia*, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S71. Time-calibrated tree of the genus *Microcerculus*, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S72. Time-calibrated tree of the Coraya Wren (*Pheugopedius coraya*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S73. Time-calibrated tree of the Fulvous-crested Tanager (*Tachyphonus surinamus*), indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Fig. S74. Time-calibrated tree of the *Euphonia cayennensis* species complex, indicating crown age, phylogenetic relationship, and location of geographic break of the two replacing forms in the Guiana Shield.

Supplementary Appendix. List of tissue samples and voucher specimens for each taxon (including subspecies when relevant) with their general collecting locality.

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