PROCEEDINGS B

royalsocietypublishing.org/journal/rspb

Research

Cite this article: Weir JT, Aleixo A, Pulido-Santacruz P. 2024 Amazonian rivers are leaky barriers to gene flow in forest understory birds. *Proc. R. Soc. B* **291**: 20240795.
https://doi.org/10.1098/rspb.2024.0795

Received: 3 July 2023
Accepted: 29 May 2024

Subject Category:
Evolution

Subject Areas:
evolution

Keywords:
Amazon, river barriers, gene flow, speciation, demographic modelling, birds

Author for correspondence:
Jason T. Weir
e-mail: jason.weir@utoronto.ca

Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.7396502.

THE ROYAL SOCIETY
PUBLISHING

Amazonian rivers are leaky barriers to gene flow in forest understory birds

Jason T. Weir[1,2,3], Alexandre Aleixo[4,5] and Paola Pulido-Santacruz[1,6]

[1]Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, Ontario M5S 3B2, Canada
[2]Department of Biological Sciences, University of Toronto Scarborough, Toronto, Ontario M1C 1A4, Canada
[3]Department of Ornithology, Royal Ontario Museum, Toronto, Ontario M5S 2C6, Canada
[4]Pós-graduação em Biodiversidade e Evolução, Museu Paraense Emílio Goeldi, Belém 66040-170, Brazil
[5]Vale Technology Institute–Sustainable Development (ITV-DS), Belém 66055-090, Brazil
[6]Department of Biology, Faculty of Natural Sciences, Universidad del Rosario, Bogotá 111221, Colombia

JTW, 0000-0001-8372-9937; AA, 0000-0002-7816-9725; PP-S, 0000-0001-7125-0304

Ever since Alfred Russel Wallace's nineteenth-century observation that related terrestrial species are often separated on opposing riverbanks, major Amazonian rivers have been recognized as key drivers of speciation. However, rivers are dynamic entities whose widths and courses may vary through time. It thus remains unknown how effective rivers are at reducing gene flow and promoting speciation over long timescales. We fit demographic models to genomic sequences to reconstruct the history of gene flow in three pairs of avian taxa fully separated by different Amazonian rivers, and whose geographic ranges do not make contact in headwater regions where rivers may cease to be barriers. Models with gene flow were best fit but still supported an initial period without any gene flow, which ranged from 187 000 to over 959 000 years, suggesting that rivers are capable of initiating speciation through long stretches of allopatric divergence. Allopatry was followed by either bursts or prolonged episodes of gene flow that retarded genomic differentiation but did not fully homogenize populations. Our results support Amazonian rivers as key barriers that promoted speciation and the build-up of species richness, but they also suggest that river barriers are often leaky, with genomic divergence accumulating slowly owing to episodes of substantial gene flow.

1. Introduction

Exceptionally high species richness of the Amazon basin is unparalleled in terrestrial vertebrates and many other groups [1], yet the geographic drivers of Amazonian speciation are heavily debated [2,3]. It is now generally agreed that most speciation events in terrestrial vertebrates involve an allopatric phase [4,5] with geographic barriers playing a key role in greatly limiting or preventing gene flow. Yet, there are not a lot of apparent geographic barriers within the Amazon basin that might drive *in situ* speciation. The key exception is the river system that bisects the region. The Amazon River and its major tributaries, the Negro, Madeira, Tapajós and Xingu rivers (figure 1), are generally hundreds of metres to kilometres (up to 20 km) in width. Alfred Russel Wallace was the first to suggest these rivers acted as dispersal barriers promoting allopatric speciation in closely related species distributed on opposing riverbanks [6–8]. Since Wallace, extensive field sampling and biogeographic work have made clear that rivers often form the boundaries between geographically adjacent taxa not only just for butterflies but also for a host of understory forest bird species (and a few canopy ones), as well as primates and other groups [9–14]. Indeed, avian groups that commonly speciate within the Amazon tend to proliferate, with distinct species or subspecies forming on opposite sides of multiple rivers, thereby forming large superspecies complexes with many geographically non-overlapping taxa, each occurring in separate interfluves demarcated by rivers (e.g. figure 1). These biogeographic patterns suggest that rivers do not just promote speciation but that they promote speciation en masse. Many in the field today recognize Amazonian rivers as key barriers driving terrestrial animal speciation [15–19]. Yet, despite this growing recognition of the importance of rivers, there is much we do not understand about how rivers function as barriers that promote speciation.

While rivers often form the division between geographically adjacent taxa, just how effective are they at limiting dispersal? In a classic study, birds were released from a boat at different distances from shore in the Panama Canal to test their ability to cross open water. Individuals from a number of understory species simply crashed into the water when released 100 to 200 m from shoreline demonstrating a poor ability to disperse across open water [20]. A similar study recently conducted on an Amazonian river found similar results [21]. Clear demonstrations like these have cemented the potential for tropical rivers to act as strong dispersal barriers in some species, which is corroborated by an ever-growing number of studies that have demonstrated genetic differentiation across rivers [15,19,22–24]. Still, we do not yet know whether rivers caused a near complete cessation of gene flow between taxa as would be expected if they operated as absolute barriers driving strictly allopatric speciation. Indeed, the river release studies found mixed results for some species with some individuals capable of flying to land and others not [20,21]. These mixed results might support an alternative to rivers acting as absolute barriers, which is that rivers have acted as leaky barriers, reducing but not eliminating gene flow completely.

Gene flow across rivers might be promoted in several ways. First, rivers are dynamic entities whose courses may have changed over time, for example, during tectonic uplift or during the process of oxbow lake formation, thereby passively moving local populations to opposing riverbanks (figure 1*a*; see also [25]). Likewise, extended periods of reduced rainfall (e.g. during prolonged droughts or drier Pleistocene glacial periods) may have resulted in narrower river barriers that were more permeable to gene flow [22]. Finally, some gene flow may occur even when rivers are at their widest, perhaps with the assistance of large river islands as stepping stones (figure 1*b*). These factors leave open the possibility that semipermeable river barriers may provide the perfect opportunity for parapatric speciation scenarios in which divergence occurs, despite ongoing but reduced gene flow connecting populations on opposing riverbanks.

We reconstruct the history of across-river gene flow in three non-flooded (e.g. terra firme) forest understory inhabiting avian species possessing genetically differentiated lineages on either side of three major Amazonian rivers. We tested two subspecies of the Plain-brown Woodcreeper (*Dendrocincla fuliginosa*) that are each other's closest relatives. *Dendrocincla f. fuliginosa* occurs on the north bank of the Amazon River and *D. f. rufoolivacea* on the south bank along the river's lower course in eastern Amazonia (figure 2*a*). This river can be slightly less than 2 km wide at its narrowest point (Obidos, Para State, Brazil) but often exceeds 8 km in width. We also tested two genetically differentiated populations of the black-throated subspecies of the Xingu Scale-backed Antbird (*Willisornis vidua nigrigula*) distributed on either side of the Tapajós River (figure 2*b*). The Tapajós River typically exceeds 1 km in width where these two populations are found on opposing riverbanks but can exceed 19 km near its mouth. Finally, we tested the two genetically differentiated populations of Spix's Woodcreeper (*Xiphorhynchus spixii*) which are distributed on either side of the Xingu River (figure 2*c*), a river which generally exceeds 500 m in width, but can be as wide as 14 km near its mouth. For each of these three species, populations on either side of these rivers have previously been shown to represent genetically unique lineages in both the mitochondrial and nuclear genomes [27–30], and given this we refer to them here as distinct taxa even though in two instances (*X. spixii* and *W. v. nigrigula*), they have not yet been awarded distinct subspecies status.

Two key difficulties are involved with reconstructing the history of gene flow across Amazonian rivers. First, gene flow might occur around rivers rather than over them. Many taxon pairs that are separated by rivers over most of their range also extend into headwater regions where rivers narrow and cease to act as barriers to gene flow [10,15,31,32]. For example, many taxon pairs separated by the Tapajós River and its key tributary, the Teles Pires River, form hybrid zones in headwater regions [15,28,33]. Headwater gene flow was previously cited as a key objection to the primary role of rivers in driving speciation [31,32]. Our study limits this confounding factor, as the species we examined do not have geographic contact in headwater regions owing to the presence of reproductively isolated sister species [28,30] inhibiting their presence in these areas (see ranges of sister species in grey in figure 2). The species we study now form very narrow hybrid zones with their sister species that presumably keeps them from expanding into contact in headwater regions. Detailed studies in two of these species pairs indicate strong post-zygotic reproductive barriers and a lack of introgression with their sister species across these hybrid zones [28], suggesting that the sister species are not, at least currently, a conduit for gene flow between populations of our species isolated on either side of rivers. With headwater gene flow inhibited at present, we are able to test whether wide stretches of Amazonian rivers outside of headwater regions were able to eliminate or reduce gene flow at the current time. A second difficulty is that taxon pairs within a genus that are not closely related might have formed somewhere else in Amazonia without rivers playing a role and then expanded their geographic ranges to come into secondary contact along a river. We attempted to avoid this here by focusing on taxon pairs that are true sister taxa (the two subspecies of Plain-brown Woodcreeper and the two unnamed taxa of Spix's Woodcreeper are true sisters [28,30]) or very closely related (the two populations of *W. v. nigrigula* are either true sisters or one may be slightly more closely related to another subspecies, *W. v. vidua* [27]). Their close relationships make it more likely that the rivers separating them represent the primary cause of their divergence, rather than a secondary barrier.

Our earlier work on these three species demonstrated the genetic distinctiveness of populations on either side of major rivers [27,28,30]. Here, we extend our earlier work by using demographic modelling applied to several hundred thousand base pairs of genomic sequence to test whether genetic differentiation across rivers in the taxa pairs in these three species occurs with or without gene flow, and whether gene flow, if present, has been continuous through time or occurred only after a period of initial allopatric differentiation. If rivers functioned as absolute barriers promoting strictly allopatric speciation, then a demographic

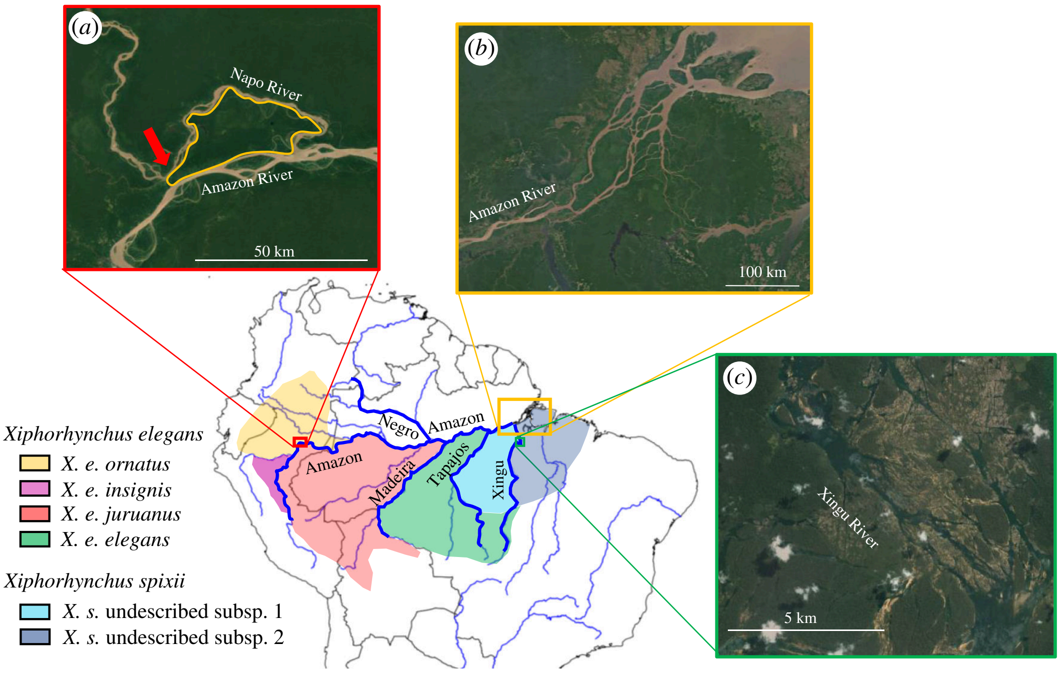


Figure 1. River topologies that might assist across-river migration. (*a*) River course changes may passively move entire populations from one riverbank to the other resulting in a burst of unidirectional gene flow. In this potential future example, the large, forested region (outlined in orange) lying west of the Napo River in Peru where it joins the Amazon River would end up on the eastern side of the Napo River if this river were to carve a new channel to the Amazon (shown by the red arrow). (*b*) River islands may function as dispersal stepping stones such as the series of islands shown here at the mouth of the Amazon River. (*c*) Otherwise, broad rivers may become heavily braided into a series of narrow channels that cease to block migration such as braided sections along the Xingu River where the widest channel in a stretch of river can be less than 50 m across. Geographic ranges of six taxa that make up the Elegant Woodcreeper (*Xiphorhynchus elegans*) and Spix's Woodcreeper (*Xiphorhynchus spixii*) superspecies complex are shown and illustrate how rivers frequently form the boundaries between adjacent taxa in Amazonia. Major rivers that commonly demarcate Amazonian taxa and are proposed to act as barriers are shown in thick blue and are labelled. Satellite imagery obtained from Google Earth. Annotations on these images are our own.

model with no gene flow should be supported. Alternatively, given their dynamic history, we predict that river barriers will be leaky to gene flow. To test this, we fit a series of demographic models which allowed gene flow either continuously through time, as expected under a strictly parapatric model of speciation, or during specific periods in time, with both allopatry (no gene flow) and parapatry (gene flow) playing a role during speciation.

2. Material and methods

See Methods in the electronic supplementary material for full details of methods.

(a) Sample collection and DNA sequencing

We used genotype by sequencing to obtain genome-wide reduced representation sequence data for 40 individuals of Spix's Woodcreeper (32 west and 8 east of the Xingu River), 28 individuals of the Xingu Scale-backed Antbird (7 west and 21 east of the Tapajós River) and 16 individuals of the Plain-brown Woodcreeper (7 of *D. f. fuliginosa* north and 9 of *D. f. rufoolivacea* south of the Amazon River). Sequence data used in this study were previously generated and published by us [28,30]. Reads were aligned to reference genomes and filtered for quality.

(b) Population structure and admixture

We used Structure (v. 2.3.4) [34] to calculate the number of genetically distinctive populations and to estimate genome-wide admixture values between these populations. In all three taxon pairs, previous analyses have uncovered only two mitochondrial haplogroups and two nuclear groupings for each pair that differentiate across their respective rivers [27–30]. After filtering, we used a total of 15 406 single nucleotide polymorphisms (SNPs) for Spix's Woodcreeper, 12 722 for the Plain-brown Woodcreeper and 12 556 for the Xingu Scale-backed Antbird to assess admixture. Ten independent runs of Structure were performed for each population number tested, each with 250 000 samples obtained following a 50 000 step burnin. We used the Evanno method [35] to calculate the number of genetically distinctive populations. As admixture proportions were identical across runs for the best-fit number of populations, we plotted only one run for each group.

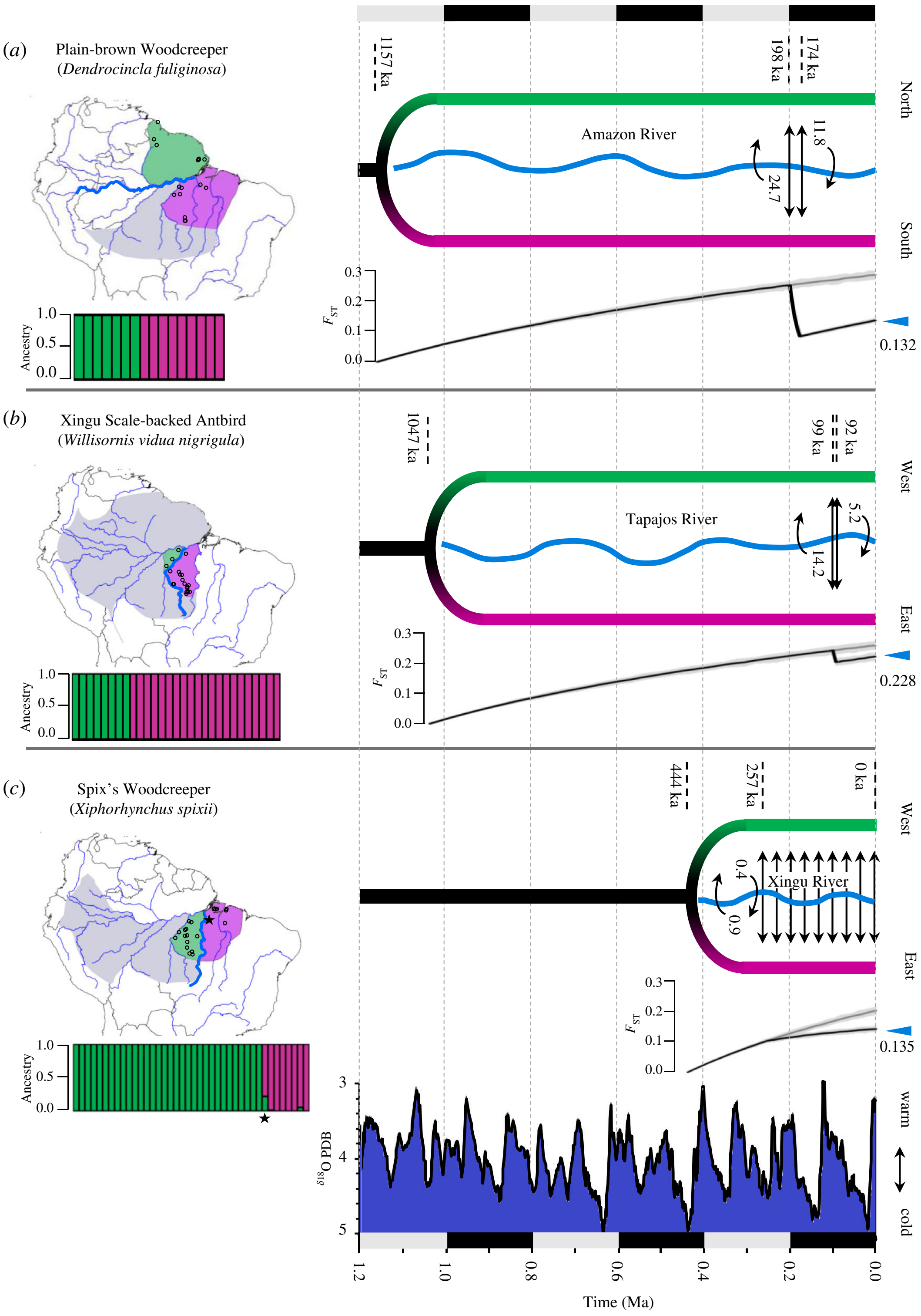


Figure 2. Population structure, history of gene flow and genetic differentiation in three pairs of taxa separated by Amazonian rivers: (*a*) Plain-brown Woodcreeper (*Dendrocincla fuliginosa*), (*b*) Xingu Scale-backed Antbird (*Willisornis vidua nigrigula*) and (*c*) Spix's Woodcreeper (*Xiphorhynchus spixii*). For each species tested, a range map shows the distribution of lineages under study for populations genetically differentiated on either side of rivers (green and magenta) and of reproductively isolated congeners (*Dendrocincla [f.] atrirostris*, *Willisornis poecilinotus*, *Xiphorhynchus elegans*) believed to prevent gene flow through headwater regions in grey. Rivers separating populations are shown by thick blue lines. Bar charts below maps show genomic assignment of individuals to populations and admixture levels. The black star indicates a genomically admixed individual and its corresponding sampling locality on the map. Schematic summary of the best-fit demographic models shows timing of migration (illustrated by vertical black arrows) with curved arrows showing the number of immigrants per generation (but not timing of immigration) that move between populations. The simulated history of genomic differentiation under each demographic model shows the accumulation of Hudson's F_{ST} through time (black) as well as F_{ST} values expected without gene flow in the models (dark grey), with 95% CI shown by pale grey. Empirical Hudson's F_{ST} values at the present time are shown by blue triangles and closely correspond to Hudson's F_{ST} simulated under the best-fit demographic models. Global averaged palaeo-temperature record obtained from the deep-sea oxygen record [26] shows the duration of glacial and interglacial periods over the past one million years. PDB refers to the Pee Dee Belemnite standard used to calibrate isotope ratios.

(c) Demographic modelling

We used demographic modelling applied to several hundred thousand base pairs of filtered genomic sequence (427 415 for Spix's Woodcreeper, 174 230 for Plain-brown Woodcreeper and 458 197 for Xingu Scale-backed Antbird) to test whether genomic differentiation across rivers occurs with or without gene flow, and whether gene flow, if present, has been continuous through time or occurred only after a period of allopatric differentiation. We used fastsimcoal2 v. 27.09 [36] to fit seven sets of models which varied in whether gene flow occurred between our populations on either side of their respective rivers, and if it did, whether gene flow was continuous or episodic since divergence (figure 3).

Purely allopatric differentiation would be supported by a model with no gene flow following divergence (Model 1). This model had an ancestral population which split at time T_1 into two daughter populations—one from each side of the river being assessed—with the ancestral and two daughter populations each having their own effective population size. The remaining models also included migration rate parameters (estimated as a *per capita* probability of migration between populations per generation but reported in figure 2 as the number of migrants per generation). These *per capita* migration rates were symmetrical with the same rate in either direction between populations. Parapatric differentiation would be supported by a model with continuous gene flow to the present (Model 2: with a single constant rate of migration and five parameters) or in which initial rates of gene flow increased after a break point in time as expected if river barriers became weaker through time (Model 3: seven parameters) or in which initial rates slowed after a break point in time (Model 4: seven parameters) as expected if taxa evolved stronger reproductive isolation towards the present or if river barriers became stronger through time but did not eliminate gene flow completely. We also tested a series of models that possessed both periods with and without gene flow. First, we allowed gene flow initially up to a break point in time, followed by no gene flow (Model 5: six parameters), representing a scenario whereby river barriers were initially leaky but then prevented gene flow or where populations diverged initially with gene flow and then evolved reproductive isolation. Next, we allowed an initial period of allopatry with no gene flow, followed by initiation of gene flow towards the present after a time break point (Model 6: six parameters). This scenario models initially strong river barriers that prevent gene flow followed by a weakening of these barriers and initiation of gene flow to the present. Finally, we tested a model in which gene flow was absent except for a single burst occurring between two break points in time (with the timing of each break point estimated as parameters) as might be expected if river barriers were weakened for a period and then re-established at full strength (Model 7: seven parameters). We also tested versions of Models 2 to 7 in which the number of migration rate parameters between populations was doubled to allow migration to be asymmetrical, with different rates in each direction between populations. Asymmetrical versions of Model 2 had six parameters; Models 3, 4 and 7 had eight parameters, and Models 5 and 6 had seven parameters. In addition to rates of gene flow, models estimate a single effective population size for each population (and its ancestor), the timing of divergence between populations separated by rivers, and the time of the initiation and cessation of gene flow.

A caveat to testing whether gene flow across rivers is possible is that gene flow might occur with the sister species of each of the species in our study. These sister species are located in headwater regions and appear to retard the range expansion of our focal species through the headwaters. However, it is possible that gene flow may occur via the sister species if full reproductive isolation is not in place (e.g. either historically when reproductive isolation had not yet evolved or at the present also if reproductive isolation is currently incomplete). We test this using three population demographic models. We added genetic samples of the sister species to the best-fit population demographic model (figure 3) for each of our species (see details in the electronic supplementary material) and compared the following three population models: (i) a model in which gene flow occurred only between the two populations of our focal species but not between these and the sister species (Model A), (ii) a model in which no gene flow occurred directly between the two populations of our focal species but occurred only between these populations and the sister species (Model B), and (iii) a model in which gene flow occurred between both populations of our focal species and between each of these and the sister species (Model C). For Models A and C, we used the same modelling of gene flow between our populations on either side of their respective rivers as was used in the best-fit population model for each species (figure 3). However, all parameters of these models were re-estimated when adding the sister species to the model. In all models, *per capita* rates of gene flow involving the sister species were held constant through time and were not asymmetric. Adding the sister species to these models resulted in four additional parameters (two new effective population size parameters, the divergence date between our focal species and the sister species, and one new migration rate parameter involving the sister species).

Support for Model B would indicate that gene flow directly between populations diverging across rivers is not warranted and would instead suggest that gene flow occurred only indirectly around rivers via the headwater inhabiting sister species. Support for Model C would indicate that though some gene flow via the sister species occurred, gene flow still occurred directly between our focal populations distributed on either side of their respective rivers. We used this two-stage approach—testing for the best two population models (Models 1 to 7) first and then conditioning on the best of these to test the effect of gene flow with the sister species (Models A to B)—to keep the total number of models tested to a reasonable number.

Fastsimcoal2 uses coalescent simulation to generate the expected site frequency spectrum (SFS) under a variety of parameter values and then optimizes the set of parameters that generates the SFS most similar to the empirical SFS. For the best-fit models for each species tested, 200 non-parametric bootstraps (each using a different bootstrapped SFS) were performed to estimate 95% CIs for parameter estimates. These were calculated using identical methods reported in Barrera-Guzmán *et al*. [37].

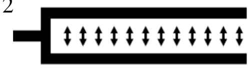
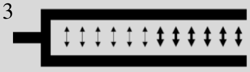
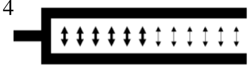
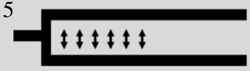
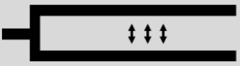
Model	**Demographic scenario**	**gene flow**	**npar**	**Akaike Weights**		
				Plain-brown Woodcreeper	**Xingu Scale-backed Antbird**	**Spix's Woodcreeper**
MODEL 1		none	4	0.000	0.000	0.000
MODEL 2		symmetric	5	0.136	0.000	0.049
		asymmetric	6	0.057	0.000	0.203
MODEL 3		symmetric	7	0.032	0.000	0.013
		asymmetric	8	0.013	0.002	0.157
MODEL 4		symmetric	7	0.016	0.000	0.006
		asymmetric	8	0.006	0.000	0.022
MODEL 5		symmetric	6	0.001	0.000	0.001
		asymmetric	7	0.000	0.000	0.001
MODEL 6		symmetric	6	0.119	0.021	0.038
		asymmetric	7	0.049	0.169	**0.407***
MODEL 7		symmetric	7	**0.392***	0.101	0.014
		asymmetric	8	0.178	**0.706***	0.089
	Akaike Weight for continuous models			0.260	0.002	0.450
	Akaike weight for discontinuous models			0.740	0.998	0.550

Figure 3. Support for alternative demographic models across rivers. Models vary in whether gene flow is present, and if present whether it is continuous through time or discontinuous and symmetrical or asymmetrical. Demographic scenarios have vertical arrows indicating gene flow which may either be symmetrical or asymmetrical. Models 3 and 4 have different rates of gene flow at different time periods as shown by the thickness of the arrows. Npar is the number of model parameters. Akaike weights show relative model support and sum to 1 for each species. The best-fit model with the highest Akaike weight for each species is shown in bold and is followed by an asterisk (*). Akaike weights for continuous models show the combined Akaike weight across Models 2–4 for which gene flow is continuous from divergence to the present as expected under parapatric divergence. Akaike weights for discontinuous models show the combined Akaike weight across Models 5–7 for which gene flow is discontinuous with periods of no gene flow.

(d) Genomic differentiation

We used scripts in Barrera-Guzmán *et al*. [37] to simulate SNPs using fastsimcoal2 under our best-fit demographic model (from T_1 to the present) and to calculate Hudson's F_{ST} from these to determine the extent to which across-river gene flow could be expected to have reversed genomic differentiation. Ninety-five per cent CIs (non-parametric) were obtained from 200 replicate simulations, each obtained from a different bootstrapped SFS. To gauge the accuracy of our simulated values, we compared simulated to empirical F_{ST} at the present using the Fastsimcoal2 biallelic SNP dataset prior to the final thinning step.

3. Results

Two genetically distinctive populations were best supported by the Evanno method [35] for each of our three species (electronic supplementary material, figure S1), with distinct populations separated by their respective barrier rivers in each case (figure 2). These results are in agreement with our previously published Structure analyses for Spix's Woodcreeper [28] and Xingu Scale-backed Antbird [27,28,30], and phylogenetic trees for Plain-brown Woodcreeper [30]. Two individuals in our dataset were reconstructed as hybrids with admixture proportions that were significantly greater than zero. Both admixed individuals came from the population of Spix's Woodcreeper located east of the Xingu River. This included an individual located just east of the Xingu River that was reconstructed to be admixed with 22% (19 to 24%; 95% CI) of its genome derived from the west-bank population. A second individual located further east had 6% (3 to 8%; 95% CI) derived from the west-bank population (figure 2*c*).

For all three pairs of taxa, a demographic model without gene flow (Model 1) was overwhelmingly rejected (Akaike weight <0.001% in all three species; figure 3). Models with initial long periods of allopatry followed by brief or long episodes of gene flow (Models 6 and 7) were best fit (combined Akaike weight: Plain-brown Woodcreeper 74%, Xingu Scale-backed Antbird >99% and Spix's Woodcreeper 55%; figure 3), while those with continuous gene flow from population formation to the present (Models 2, 3 and 4) received very low-to-moderate support (combined Akaike weight: Plain-brown Woodcreeper 26%, Xingu Scale-backed Antbird <0.2% and Spix's Woodcreeper 45%; figure 3). The best-fit model (Model 7; figure 3) for the Plain-brown Woodcreeper supported a divergence date of 1157 thousand years ago (Ka) (1061–1261 Ka, 95% CI) across the Amazon River with no gene flow except during an episode between 174 and 198 Ka when migration greatly exceeded one individual per generation (12–25 individuals; figure 2*a*). Population genetic theory suggests that more than one migrant per generation is sufficient to prevent populations from diverging at neutral sites irrespective of effective population size [38,39]. We thus consider values greater than one migrant per generation to represent high levels of gene flow and values near one migrant to be moderate and below 1 to be low. For the Xingu Scale-backed Antbird, the best-fit model (Model 7) produced similar results with a divergence date of 1047 Ka (983–1097 Ka, 95% CI) across the Tapajós River and high levels of gene flow (5.2–14.2 individuals per generation) only during a very brief window between 92 and 99 Ka (figure 2*b*). The Spix's Woodcreeper supported a different model (Model 6) with a divergence date of 444 Ka (412–470 Ka, 95% CI) across the Xingu River and low-to-moderate gene flow (0.4–0.9 individuals per generation) initiated at 257 Ka and lasting up to the present (figure 2*c*). For all model parameters and their CIs, see electronic supplementary material, figure S2.

For the best-fit models, we also tested the potential effect of gene flow via their sister species in headwater regions. In all cases, the model with gene flow occurring only via the sister species and not directly between populations of each species on either side of their respective rivers (Model B) was strongly rejected with Akaike weights of 0.0 (electronic supplementary material, table S1). However, models with gene flow both between our focal populations and between these and their sister species (Model C) were strongly supported (Akaike weight 1.0 for all three species; electronic supplementary material, table S1) over a model in which gene flow did not occur with the sister (Model A). These results support direct gene flow between populations separated by rivers for each of our focal species despite low background gene flow involving the sister species. The number of migrants per generation was generally one to two orders of magnitude lower between the sister species, and our focal species compared with the rates between the two populations of our focal species (see rates in electronic supplementary material, figure S2).

Simulated Hudson's F_{ST} (figure 2) through time under the best-fit demographic models (figure 3 for the best-fit models) illustrates the expected effect of periods of gene flow on genetic differentiation and produced almost identical F_{ST} at the present (Plain-brown Woodcreeper; mean = 0.132, 95% CI = 0.124–0.141; the Xingu Scale-backed Antbird = 0.220, 95% CI = 0.206–0.233; Spix's Woodcreeper simulated = 0.143, 95% CI = 0.132–0.153) as our empirical estimates (Plain-brown Woodcreeper: mean = 0.132, 95% CI = 0.124–0.140; the Xingu Scale-backed Antbird: mean = 0.228, 95% CI = 0.223–0.232; Spix's Woodcreeper: mean = 0.135, 95% CI = 0.131–0.139), suggesting that our models do a reasonably good job of reflecting reality.

4. Discussion

A major objection to rivers as engines of *in situ* Amazonian diversification is the potential for ongoing gene flow to occur through headwater regions, where rivers narrow to streams that fail to act as barriers [15]. Gene flow through headwater regions could homogenize populations on either side of rivers, preventing speciation. However, not all Amazonian taxa pairs separated by rivers extend their geographic ranges into headwater regions. Here, we focus on three such taxon pairs which differentiate across three different major Amazonian rivers but for which range expansion and geographic contact through headwater regions are, at least currently, blocked by the presence of geographically excluding and reproductively isolated sister species occupying headwater regions. Though our modelling suggests that some gene flow may occur indirectly through headwater regions via hybridization with the sister species, our results continue to strongly support direct gene flow between our taxon pairs (electronic supplementary material, figure S3). Our model-based estimates of gene flow at the present thus capture the effect of river barriers at limiting across-river gene flow in the heart of Amazonia where rivers are at their widest. Our best-fit demographic models suggest that no detectable recent gene flow for the Plain-brown Woodcreeper differentiating across the Amazon River and the Xingu Scale-backed Antbird across the Tapajós River (figure 2*a,b*) has occurred in the past several tens of thousands of years. In contrast, moderate levels (0.4–0.9 individuals per generation in each direction) of contemporary across-river gene flow occur in the Spix's Woodcreeper differentiating on either bank of the Xingu River (figure 2*c*). Our results thus suggest that some Amazonian rivers, outside of headwater regions, are currently acting as near-absolute barriers to gene flow, whereas others are not. However, our modelling also strongly supports periods of gene flow across all rivers tested, indicating that over longer time periods, Amazonian rivers have been leaky barriers to gene flow (figures 2 and 3) and have not acted as strictly allopatric drivers of speciation. Here, we discuss the implications of our model fitting both for how speciation is initiated across rivers, whether speciation across rivers is parapatric with ongoing gene flow or whether periods of allopatry with no gene flow are still instrumental in initiating speciation. We also discuss the timeline to speciation with gene flow across semipermeable river barriers.

We begin by asking whether rivers initiate speciation through vicariance or dispersal. According to our demographic modelling (figure 2) estimates, our taxon pairs have been diverging for half a million (Spix's Woodcreeper) to almost a million years (Plain-brown Woodcreeper and Xingu Scale-backed Antbird). In the case of the Plain-brown Woodcreeper, the date greatly postdates the origin of the eastward Atlantic-draining Amazon River around 11.8 Ma and the deep channelling of the river around 5 Ma [40], suggesting that these subspecies formed either through active dispersal or passive transport (via channel movement) across an already established Amazon River. The ages of formation of the Tapajós and Xingu Rivers are less well understood, with evidence that the Tapajós shifted to its current north-eastward course sometime during the past 2 million years, as indicated by both geological evidence [41] and the estimated divergence time between other avian sister taxa replacing each other across the river [42,43]. Hence, it remains possible that populations of Spix's Woodcreeper and Xingu Scale-backed Antbird may have formed either via vicariance when these rivers acquired their modern configurations or via dispersal across rivers thereafter.

A key question, both for the role of Amazonian rivers and more generally for other geographic barriers driving vertebrate speciation, is whether barriers need to be absolute, with no gene flow, or whether speciation can occur parapatrically with ongoing, albeit reduced gene flow over the partial barrier. The poor fit of models with continuous gene flow from population divergence to the present suggests that rivers are not commonly driving strictly parapatric speciation. Instead, the best-supported model for each species (Model 7 for the Plain-brown Woodcreeper and the Xingu Scale-backed Antbird; Model 6 for Spix's Woodcreeper) suggests a long period without gene flow following divergence, consistent with population differentiation initiated in allopatry across strong river barriers. This period of allopatry, estimated to be 187 kyr in Spix's Woodcreeper, 959 kyr in the Plain-brown Woodcreeper and 948 kyr in the Xingu Scale-backed Antbird (figure 2), provided time for at least some genetic differentiation (but not complete reproductive isolation) between taxa before the onset of gene flow. Our results thus suggest that rivers are capable of providing long periods of allopatric divergence without gene flow and contribute to a growing consensus that periods of allopatry are fundamental to most vertebrate speciation events [5,44] including those with current parapatric distributions [45,46].

For both the Plain-brown Woodcreeper and the Xingu Scale-backed Antbird, gene flow was reconstructed as having occurred as a single, several thousand-year burst during the mid- to late-Pleistocene. There could be several causes for such a focused burst in time. A change in river course—whereby a river switches to a new carved channel, resulting in a geographic region that once occurred on one side of the river to now occur on the other—might have transferred an entire population passively from one riverbank to the adjacent one as a single event in time (e.g. figure 1*a*; see also [25]). Such passive transport events might have resulted in a short-lived burst of gene flow as the transferred population merged genomically with its conspecific sister lineage. Such events have previously been proposed for the Tapajós River [33]. Such a course change should result in strongly unidirectional gene flow and can thus be tested by our demographic models. A model in which *per capita* rates of gene flow were asymmetric was strongly supported for the Xingu Scale-backed Antbird but not for the Plain-brown Woodcreeper. Nevertheless, the absolute number of migrants per generation was still highly asymmetrical in both species, with more individuals dispersing from south to north across the Amazon River for the Plain-brown Woodcreeper, or from the east to west across the Tapajós River for the Xingu Scale-backed Antbird. Despite this asymmetry, gene flow was still bidirectional with elevated rates (i.e. >1 individual per generation) in both directions (figure 2*a,b*). The presence of bidirectional gene flow argues against a single major change in river course as the key driving cause of gene flow, though more complex changes in river courses involving population transfer in both directions across these rivers at specific times remains possible. Another possible cause for a burst of bidirectional gene flow would be reduced river volume and flow rate during a severe drought or during periods of reduced rainfall across the Amazon basin, such as during cool periods of the middle and late Pleistocene glacial cycles [47,48]. The Plain-brown Woodcreeper had its burst overlapping with the second to last glacial maxima, and the Xingu Scale-backed Antbird had its short-lived burst of gene flow during the early part of the most recent glacial cycle. River widths may have been affected at these times.

In contrast to the Plain-brown Woodcreeper and the Xingu Scale-backed Antbird, the best-fit model for Spix's Woodcreeper supported a much shorter initial period of allopatry without gene flow and a much longer period with gene flow (initiated 257 Ka), which continues to the present (figures 2*c* and 3). Though the exact cause of gene flow initiation is not known, a change in river course *ca* 257 Ka might have permanently weakened the Xingu River's ability to act as an absolute barrier. A likely location for sustained gene flow across the Xingu River over the past several hundred thousand years is along a series of braided river channels, each with a narrow width (the widest of which can be less than 50 m), where the Xingu River descends from the Brazilian shield to the Amazonian lowlands downriver from the confluence with its largest tributary, the Iriri River (figures 1*c* and 4). This braided section occurs about 210 km south from where the Xingu empties into the Amazon River and persists upstream for an additional 200 km. The individual of the eastern lineage sampled on the eastern bank near this braided channel region showed 22% ancestry from the western lineage (figure 4), whereas the remaining individuals of the eastern lineage were sampled further away from the Xingu River exhibited only 0 to 5% admixture. These patterns of introgression are consistent with ongoing gene flow occurring primarily through the braided channel regions in the north with much less (if any) gene flow occurring across the river further south. Dense sampling along both riverbanks above, below and within the braided channel region would help confirm the role of the braided channels in promoting gene flow.

To what extent did these shorter bursts of gene flow in the Plain-brown Woodcreeper and the Xingu Scale-backed Antbird, or prolonged episode of gene flow in Spix's Woodcreeper, retard the process of genomic differentiation and speciation? To address this, we simulated SNPs along our best-fit demographic models to various time points following population divergence and calculated F_{ST} at these time points from these. We found that simulated Hudson's F_{ST} increased steadily, as expected during periods without gene flow, but declined precipitously by 55% during the 24 000 years of gene flow in the Plain-brown Woodcreeper and modestly by 16% during the 7000 years of gene flow in the Xingu Scale-backed Antbird (figure 2*a,b*). Following the cessation of gene flow, simulated F_{ST} continued to increase in these taxa, but at the present time, they have not yet recovered levels obtained prior to the initiation of gene flow. These results demonstrate that bouts of gene flow can rapidly initiate genomic homogenization, at least at neutral sites, of taxa separated by rivers. The species we are studying here are examples where gene flow was not successful in fully homogenizing taxa, but presumably there could have been examples where episodes of dispersal have resulted in complete genomic homogenization (i.e. lineage extinction through fusion), despite having been differentiated at some point in the past. In contrast, simulated F_{ST} in Spix's Woodcreeper did not decline following the initiation of gene flow, but continued to increase, albeit at a much-reduced rate, compared with its rate of increase prior to gene flow (figure 2*c*). Spix's Woodcreeper thus represents a case of ongoing genomic differentiation despite moderate levels of gene flow, and illustrates that even the weaker river barrier effect of the Xingu may promote genetic differentiation and presumably speciation once enough time has passed. Nevertheless, the Xingu River appears to separate relatively few species pairs compared with other major rivers such as the Amazon, Tapajós, Madeira and Negro (e.g. [49]), suggesting few taxa pairs are able to persist long enough as distinct lineages on either side of the Xingu for reproductive isolation to take hold. The permeability of the Xingu River to gene flow thus renders it less effective at driving speciation in birds.

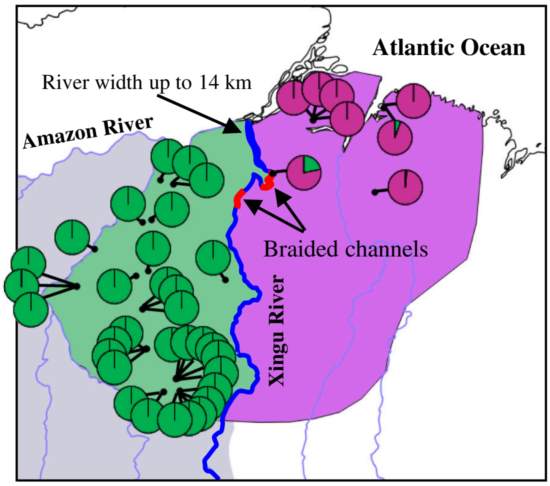


Figure 4. Population structure of Spix's Woodcreeper (*Xiphorhynchus spixii*) across the Xingu River. Population differentiation was constructed from a genome-wide sample of SNPs using the program Structure and shows two distinct lineages ($k = 2$) separated east (magenta) and west (green) by the Xingu River. One individual from the eastern lineage had 22% of its genome derived from the western population. This individual was collected from the eastern bank of the Xingu River along a stretch of narrow braided channels (figure 1*c*) highlighted in red. To the north of these braided channels, the river expands into a broad lake-like barrier as wide as 14 km before emptying into the Amazon River. To the south, the river's main channel is generally a few hundred metres to more than a kilometre wide all the way to headwater regions beyond the southern limits of the range of Spix's Woodcreeper. Note that the Xingu River is exaggerated in this figure relative to other Amazonia rivers.

What are the implications of our results for the timeline of speciation in forest understory birds of Amazonia? The period of initial divergence across rivers without gene flow supported by our models was relatively long, lasting anywhere from 187 to 959 kyr depending on the species (figure 2). Nevertheless, these long durations without gene flow were insufficient to drive high levels of reproductive isolation as evidenced by the moderate (0.9 individuals per generation) to very high levels of gene flow (up to 25 individuals per generation) that retarded or eroded genomic differentiation in each pair tested once river barriers became leaky. They also failed to consistently drive substantial plumage differentiation, with two of our three pairs of taxa having not previously been recognized as distinct subspecies presumably because of their plumage similarity. Our results imply that strong reproductive isolation in Amazonian birds separated by occasionally permeable rivers may require considerably more than a million years before complete reproductive isolation is achieved. In contrast, parapatric pairs of Amazonian taxa estimated to be two or more million years since divergence, and for which rivers may have also played a key role in speciation, failed to homogenize at neutral genomic regions upon secondary contact (*Xiphorhynchus* and *Willisornis* [28] and *Hypocnemis* [50]) demonstrating that reproductive isolation was completed over these longer timescales. Complete speciation in forest bird taxa separated by episodically permeable barriers may thus be a drawn-out process, requiring more than a million years.

How do our results regarding the role of river barriers relate to competing models of terrestrial vertebrate speciation in Amazonia? Two key theories emphasize the role of river barriers in speciation. These are the River Barrier Hypothesis [31,51], which emphasizes only the role of the rivers, and the River Refuge Hypothesis [12,51,52], which emphasizes that river barriers were most effective during periods when the periphery of wet forest in Amazonia retracted inwards away from headwater regions (e.g. during glacial cycles of drying/cooling). Rivers become narrower and may cease to be effective barriers in headwater regions [15,32,52]. Retraction of wet forest away from headwater regions (well supported by palaeo-pollen records during the last glacial maximum and presumably would have also occurred during earlier glacial cycles; [50]) into the heart of the Amazon basin where rivers are broader would have aided genetic differentiation leading to speciation according to this theory. In contrast, a third theory, the Forest Refuge Hypothesis, at first tried to do away with the need for rivers at all. It took forest retraction to the extreme and suggested that wet forests retracted even within the heart of Amazonia into tiny islands of habitat (an idea for which palaeo-pollen evidence is largely lacking [53]). It was these forest refugia, rather than wide rivers that initiated population divergence under this model. Back when this hypothesis was first coined, speciation was imagined as having occurred within the timeline of a single refugial cycle (i.e. 100 kyr assuming it was driven by mid- to late-Pleistocene glacial events) and completely within forest refugia. The rivers played no role in speciation itself but did form secondary barriers to range expansion for already reproductively isolated species once forest re-expanded following each glacial cycle. Molecular dating subsequently demonstrated that reproductive isolation in birds generally requires a much longer process than afforded by the 100 kyr duration of a mid- to late-Pleistocene glacial cycle [54,55]. The lack of well-developed reproductive isolation in the taxon pairs we studied here, despite having diverged half a million to a million years ago, is in agreement with a long timeline for species formation (figure 2). Many glacial cycles would be needed. The implication for the Forest Refuge Hypothesis is that river barriers would be important in retarding gene flow during periods of forest expansion between subsequent refugial events. Over the million plus year duration of a typical speciation event, there would have been about seven or eight major interglacials during the mid- to late-Pleistocene (back to *ca* 0.8 Ma) and many more minor ones during the weaker cycles of the early Pleistocene (0.8–2.5 Ma). River barriers would be essential at preventing the genetic collapse of diverging populations at these times even under the Forest Refuge Hypothesis. Because river barriers are key to all three leading models of Amazonian terrestrial speciation, our evidence for river barriers uncovered here using demographic modelling does not help us discriminate between them. Retraction of forest away from headwater regions and forest refugia could have played a role together with river barriers in driving speciation over long time periods in Amazonia.

In conclusion, we have demonstrated that Amazonian rivers represent leaky barriers allowing episodes of gene flow between taxa that lasted just a few thousand to several hundred thousand years in duration. However, we did not find strong support for a scenario where population differentiation was initiated with ongoing gene flow from population divergence to the present, as expected if rivers have commonly promoted strictly parapatric speciation. Instead, our models suggest that rivers may have acted as absolute barriers to gene flow initially, followed by subsequent episodes where rivers permitted moderate-to-strong gene flow. This interplay between long periods with no gene flow interspersed with episodic bursts of gene flow may often prolong the speciation process to greatly exceed a million years in duration. Thus, while rivers appear to contribute importantly to speciation in many Amazonian birds and other groups, the formation of fully reproductively isolated new species through river barriers appears to be a protracted process that often involves episodes of gene flow.

Ethics. Fieldwork to obtain genetic samples followed procedures in the University of Toronto animal ethics protocol 2 00 10 519. The Brazilian National Research Council (CNPq) granted field research permits (4253–1, 40173–1 and 6581–1).

Data accessibility. Raw sequence reads are deposited in the Short Reads Archive (BioProjects PRJNA 433557, 433538 and 600749). Demographic models and site frequency spectra are located in Dryad [56].

Supplementary material is available online [57].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. J.T.W.: conceptualization, formal analysis, funding acquisition, investigation, writing—original draft; A.A.: resources, writing—review and editing; P.P.-S.: investigation, resources, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Competing interests. We declare we have no competing interests.

Funding. Funding was supplied by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) to J.T.W. (Discovery Grant RGPIN-2022-04817 and Accelerator Grant 492890), CNPq ('INCT em Biodiversidade Uso da Terra da Amazonia' #306843/2016-1; 'Productivity Fellowship' #309243/2023-8) and the Amazonia Paraense Foundation—FAPESPA (ICAAF 023/2011) to A.A. and from a fellowship (Colciencias Francisco Jose de Caladas no. 20110582) to P.P.-S.

Acknowledgements. The authors thank many landowners of Brazil who generously allowed us to sample on their properties. Alfredo Barrera-Guzman and Maya Faccio assisted with field collection. Ellen Nikelski, Else Mikkelsen and Vanessa Luzuriaga-Aveiga provided comments on earlier drafts and two anonymous reviewers helped to improve the manuscript.

References

1. Jenkins CN, Pimm SL, Joppa LN. 2013 Global patterns of terrestrial vertebrate diversity and conservation. *Proc. Natl Acad. Sci. USA* **110**, E2602–E210. (doi:10.1073/pnas.1302251110)
2. Haffer J. 2008 Hypotheses to explain the origin of species in Amazonia. *Braz. J. Biol*. **68**, 917–947. (doi:10.1590/s1519-69842008000500003)
3. Hoorn C *et al*. 2010 Amazonia through time: Andean uplift, climate change, landscape evolution, and biodiversity. *Science* **330**, 927–931. (doi:10.1126/science.1194585)
4. Bolnick DI, Fitzpatrick BM. 2007 Sympatric speciation: models and empirical evidence. *Annu. Rev. Ecol. Evol. Syst.* **38**, 459–487. (doi:10.1146/annurev.ecolsys.38.091206.095804)
5. Coyne JA, Orr HA. 2004 *Speciation*, p. 545. Sunderland, MA: Sinauer Associates.
6. Wallace AR. 1876 *The geographical distribution of animals*. London, UK: Macmillan.
7. Wallace AR. 1854 On the monkeys of the Amazon. *Ann. Mag. Nat. Hist*. **14**, 451–454. (doi:10.1080/037454809494374)
8. Wallace AR. 1853 *A narrative of travels on the Amazon and Rio Negro*. London, UK: Reeve.
9. Patton JL, da Silva MNF. 1998 Rivers, refuges and ridges: the geography of speciation of Amazonian mammals. In *Endless form: species and Speciation* (eds DJ Howard, ST Berlocher), pp. 202–213. New York, NY: Oxford University Press.
10. Naka LN, Bechtoldt CL, Henriques LMP, Brumfield RT. 2012 The role of physical barriers in the location of avian Suture zones in the Guiana shield, northern Amazonia. *Am. Nat*. **179**, E115–32. (doi:10.1086/664627)
11. Hershkovitz P. 1977 *Living new world monkeys (Platyrrhini)*. Chicago, IL: University of Chicago Press.
12. Ayres JM, Clutton-Brock TH. 1992 River boundaries and species range size in Amazonian primates. *Am. Nat.* **140**, 531–537. (doi:10.1086/285427)
13. Hayes FE, Sewlal JAN. 2004 The Amazon river as a dispersal barrier to passerine birds: effects of river width, habitat and taxonomy. *J. Biogeogr.* **31**, 1809–1818. (doi:10.1111/j.1365-2699.2004.01139.x)
14. Hellmayr CE. 1910 The birds of the Rio Madeira. *Novit. Zool.* **17**, 257–428. (doi:10.5962/BHL.TITLE.875)
15. Weir JT, Faccio MS, Pulido-Santacruz P, Barrera-Guzmán AO, Aleixo A. 2015 Hybridization in headwater regions, and the role of rivers as drivers of speciation in Amazonian birds. *Evolution*. **69**, 1823–1834. (doi:10.1111/evo.12696)
16. Sick H. 1967 Rios e enchentes na amazonia como obstáculo para a avifauna. *Simpósio Sobre Biota Amaz Atas Zool.* **5**, 495–520.
17. Willis EO. 1969 On the behavior of five species of Rhegmatorhina, ant-following antbirds of the Amazon basin. *W. Bull.* **81**, 363–395.
18. Capparella AP. 1988 Genetic variation in neotropical birds: implications for the speciation process. *Proc. Int. Ornithol. Congr.***19**, 1658–1664.
19. Ribas CC, Aleixo A, Nogueira ACR, Miyaki CY, Cracraft J. 2012 A palaeobiogeographic model for biotic diversification within Amazonia over the past three million years. *Proc. R. Soc. B* **279**, 681–689. (doi:10.1098/rspb.2011.1120)
20. Moore RP, Robinson WD, Lovette IJ, Robinson TR. 2008 Experimental evidence for extreme dispersal limitation in tropical forest birds. *Ecol. Lett*. **11**, 960–968. (doi:10.1111/j.1461-0248.2008.01196.x)
21. Naka LN, Costa B da S, Lima GR, Claramunt S. Riverine barriers as obstacles to dispersal in Amazonian birds. *Front. Ecol. Evol.* **10**. (doi:10.3389/fevo.2022.846975)
22. Dornas T, Dantas SM, Araújo-Silva LE, Morais F, Aleixo A. Comparative phylogeography of birds across the tocantins–araguaia interfluve reveals a new biogeographic suture in the Amazon far east. *Front. Ecol. Evol* **10**. (doi:10.3389/fevo.2022.826394)
23. Naka LN, Brumfield RT. 2018 The dual role of Amazonian rivers in the generation and maintenance of avian diversity. *Sci. Adv*. **4**, eaar8575. (doi:10.1126/sciadv.aar8575)

24. Silva SM *et al*. 2019 A dynamic continental moisture gradient drove Amazonian bird diversification. *Sci. Adv*. **5**, eaat5752. (doi:10.1126/sciadv.aat5752)
25. Musher LJ *et al*. 2022 River network rearrangements promote speciation in lowland Amazonian birds. *Sci. Adv*. **8**, eabn1099. (doi:10.1126/sciadv.abn1099)
26. Zachos J, Pagani M, Sloan L, Thomas E, Billups K. 2001 Trends, rhythms, and aberrations in global climate 65 ma to present. *Science* **292**, 686–693. (doi:10.1126/science.1059412)
27. Quaresma TF, Cronemberger ÁA, Batista R, Aleixo A. 2022 Diversification and species limits in scale-backed Antbirds (Willisornis: Thamnophilidae), an Amazonian Endemic lineage. *Zool. J. Linn. Soc*. **196**, 1408–1430. (doi:10.1093/zoolinnean/zlac011)
28. Pulido-Santacruz P, Aleixo A, Weir JT. 2018 Morphologically cryptic Amazonian bird species pairs exhibit strong postzygotic reproductive isolation. *Proc. R. Soc. B* **285**, 20172081. (doi:10.1098/rspb.2017.2081)
29. Schultz ED, Pérez-Emán J, Aleixo A, Miyaki CY, Brumfield RT, Cracraft J, Ribas CC. 2019 Diversification history in the Dendrocincla Fuliginosa complex (Aves: Dendrocolaptidae): insights from broad geographic sampling. *Mol. Phylogenet. Evol*. **140**, 106581. (doi:10.1016/j.ympev.2019.106581)
30. Pulido-Santacruz P, Aleixo A, Weir JT. 2020 Genomic data reveal a protracted window of introgression during the diversification of a Neotropical Woodcreeper radiation. *Evolution* **74**, 842–858. (doi:10.1111/evo.13902)
31. Haffer J. 1997 Contact zones between birds of southern amazonia. *Ornithol. Monogr.* **48**, 281–305. (doi:10.2307/40157539)
32. Haffer J. 1997 Alternative models of vertebrate speciation in Amazonia: an overview. *Biodivers. Conserv*. **6**, 451–476. (doi:10.1023/A:1018320925954)
33. Barrera-Guzmán AO, Aleixo A, Shawkey MD, Weir JT. 2018 Hybrid speciation leads to novel male secondary sexual ornamentation of an Amazonian bird. *Proc. Natl Acad. Sci. USA* **115**, E218–E225. (doi:10.1073/pnas.1717319115)
34. Pritchard JK, Stephens M, Donnelly P. 2000 Inference of population structure using multilocus genotype data. *Genetics* **155**, 945–959. (doi:10.1093/genetics/155.2.945)
35. Evanno G, Regnaut S, Goudet J. 2005 Detecting the number of clusters of individuals using the software structure: a simulation study. *Mol. Ecol*. **14**, 2611–2620. (doi:10.1111/j.1365-294X.2005.02553.x)
36. Excoffier L, Marchi N, Marques DA, Matthey-Doret R, Gouy A, Sousa VC. 2021 Fastsimcoal2: demographic inference under complex evolutionary scenarios. *Bioinformatics* **37**, 4882–4885. (doi:10.1093/bioinformatics/btab468)
37. Barrera-Guzmán AO, Aleixo A, Faccio M, Dantas S de M, Weir JT. 2022 Gene flow, genomic homogenization and the timeline to speciation in Amazonian Manakins. *Mol. Ecol*. **31**, 4050–4066. (doi:10.1111/mec.16562)
38. Mills LS, Allendorf FW. 1996 The one-migrant-per-generation rule in conservation and management. *Conserv. Biol*. **10**, 1509–1518. (doi:10.1046/j.1523-1739.1996.10061509.x)
39. Spieth PT. 1974 Gene flow and genetic differentiation. *Genetics* **78**, 961–965. (doi:10.1093/genetics/78.3.961)
40. Figueiredo J, Hoorn C, van der Ven P, Soares E. 2009 Late Miocene onset of the Amazon river and the Amazon deep-sea fan: evidence from the Foz do Amazonas Basin. *Geology* **37**, 619–622. (doi:10.1130/G25567A.1)
41. Costa JBS, Léa Bemerguy R, Hasui Y, da Silva Borges M. 2001 Tectonics and paleogeography along the Amazon river. *J. South Am. Earth Sci*. **14**, 335–347. (doi:10.1016/S0895-9811(01)00025-6)
42. Ribas CC, Aleixo A, Gubili C, d'Horta FM, Brumfield RT, Cracraft J. 2018 Biogeography and diversification of Rhegmatorhina (Aves: Thamnophilidae): implications for the evolution of Amazonian landscapes during the Quaternary. *J. Biogeogr*. **45**, 917–928. (doi:10.1111/jbi.13169)
43. Fernandes AM, Gonzalez J, Wink M, Aleixo A. 2013 Multilocus phylogeography of the wedge-billed Woodcreeper Glyphorynchus Spirurus (Aves, Furnariidae) in lowland Amazonia: widespread cryptic diversity and paraphyly reveal a complex diversification pattern. *Mol. Phylogenet. Evol*. **66**, 270–282. (doi:10.1016/j.ympev.2012.09.033)
44. Phillimore AB, Orme CDL, Thomas GH, Blackburn TM, Bennett PM, Gaston KJ, Owens IPF. 2008 Sympatric speciation in birds is rare: insights from range data and simulations. *Am. Nat*. **171**, 646–657. (doi:10.1086/587074)
45. Linck E, Freeman BG, Dumbacher JP. 2020 Speciation and gene flow across an elevational gradient in New Guinea Kingfishers. *J. Evol. Biol*. **33**, 1643–1652. (doi:10.1111/jeb.13698)
46. E. Luzuriaga-Aveiga V, Ugarte M, Weir JT. 2021 Distinguishing genomic homogenization from parapatric speciation in an elevationally replacing pair of Ramphocelus Tanagers. *Mol. Ecol*. **30**, 5517–5529. (doi:10.1111/mec.16128)
47. Luna LW, Naka LN, Thom G, Knowles LL, Sawakuchi AO, Aleixo A, Ribas CC. 2023 Late Pleistocene landscape changes and habitat specialization as promoters of population genomic divergence in Amazonian floodplain birds. *Mol. Ecol*. **32**, 214–228. (doi:10.1111/mec.16741)
48. Thom G, Xue AT, Sawakuchi AO, Ribas CC, Hickerson MJ, Aleixo A, Miyaki C. 2020 Quaternary climate changes as speciation drivers in the Amazon Floodplains. *Sci. Adv*. **6**, eaax4718. (doi:10.1126/sciadv.aax4718)
49. Cracraft J. 1985 Historical biogeography and patterns of differentiation within the South American Avifauna: areas of Endemism. *Ornithol. Monogr*. 49–84. (doi:10.2307/40168278)
50. Cronemberger ÁA, Aleixo A, Mikkelsen EK, Weir JT. 2020 Postzygotic isolation drives genomic speciation between highly cryptic hypocnemis antbirds from Amazonia. *Evolution*. **74**, 2512–2525. (doi:10.1111/evo.14103)
51. Haffer J. 1969 Speciation in Amazonian forest birds. *Science* **165**, 131–137. (doi:10.1126/science.165.3889.131)
52. Haffer J. 1992 On the "river effect" in some forest birds of southern Amazonia. *Bol. Mus. Para Emilio Goeldi Ser. Zool.* **8**, 217–245.
53. Bush MB, Oliveira P de. 2006 The rise and fall of the refugial hypothesis of Amazonian speciation: a paleoecological perspective. *Biota Neotrop.* **6**. (doi:10.1590/S1676-06032006000100002)
54. Weir JT, Price TD, Boughman AEJ, McPeek EMA. 2011 Limits to speciation inferred from times to secondary sympatry and ages of hybridizing species along a latitudinal gradient. *Am. Nat*. **177**, 462–469. (doi:10.1086/658910)
55. Weir JT, Schluter D. 2007 The latitudinal gradient in recent speciation and extinction rates of birds and mammals. *Science* **315**, 1574–1576. (doi:10.1126/science.1135590)
56. Weir J, Aleixo A, Pulido-Santacruz P. 2024 Data from: Amazonian rivers are leaky barriers to gene flow in forest Understory birds. Dryad Digital Repository. (doi:10.5061/dryad.z34tmpgnr)
57. Weir J, Aleixo A, Pulido-Santacruz P. 2024 Data from: Amazonian rivers are leaky barriers to gene flow in forest understory birds. Figshare. (doi:10.6084/m9.figshare.c.7396502)