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Hybridization in headwater regions, and the role of rivers as drivers of speciation in Amazonian birds

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Many understory birds and other groups form genetically differentiated subspecies or closely related species on opposite sides of major rivers of Amazonia, but are proposed to come into geographic contact in headwater regions where narrower river widths may present less of a dispersal barrier. Whether such forms hybridize in headwater regions is generally unknown, but has important implications to our understanding of the role of rivers as drivers of speciation. We used a dataset of several thousand single nucleotide polymorphisms to show that seven taxon pairs that differentiate across a major Amazonian river come into geographic contact and hybridize in headwater regions. All taxon pairs possessed hybrids with low numbers of loci in which alleles were inherited from both parental species, suggesting they are backcrossed with parentals, and indicating gene flow between parental populations. Ongoing gene flow challenges rivers as the sole cause of in situ speciation, but is compatible with the view that the wide river courses in the heart of Amazonia may have driven interfluvial divergence during episodes of wet forest retraction away from headwater regions. Taxa as old as 4 Ma in our Amazonian dataset continue to hybridize at contact zones, suggesting reproductive isolation evolves at a slow pace.

KEY WORDS: biogeography, gene flow, hybrid zone, river barriers.

Despite its exceptional species richness, little is understood about the historical factors that contributed to species accumulation within the Amazon basin. Factors such as repeated immigration into the Amazon from outside sources, and low levels of extinction within the basin could have promoted species accumulation (see Fjeldså 1994). Nevertheless, in situ speciation within the Amazon basin appears to have contributed importantly to species richness. For example, many groups of Amazonian birds, mammals, and other taxa possess widespread superspecies complexes composed of multiple allopatric species or subspecies that occupy different geographic regions within Amazonia (e.g., Haffer 1974). These biogeographic patterns demonstrate that Amazonia is actively producing new taxa (both species and subspecies), which contribute to its species richness. Although the important role of in situ speciation is undisputed, the primary driving mechanisms of speciation within Amazonia are poorly understood.

Early naturalists such as Wallace, Bates, and others made the observation that major Amazonian rivers often formed barriers to geographically replacing taxa, with closely related species or subspecies occupying opposing river banks (Wallace 1853, 1854, 1876; Bates 1863). The subsequent accumulation of museum specimen records since the pioneering efforts in the 19th century have confirmed these early observations (Hellmayr 1910; Hershkovitz 1977; Ayres and Clutton-Brock 1992; Hayes and Seawall 2004), and have further shown that major Amazonian rivers generally form the geographic divisions between adjacent centers of endemism in which multiple codistributed superspecies complexes often share similar geographic patterns of replacement

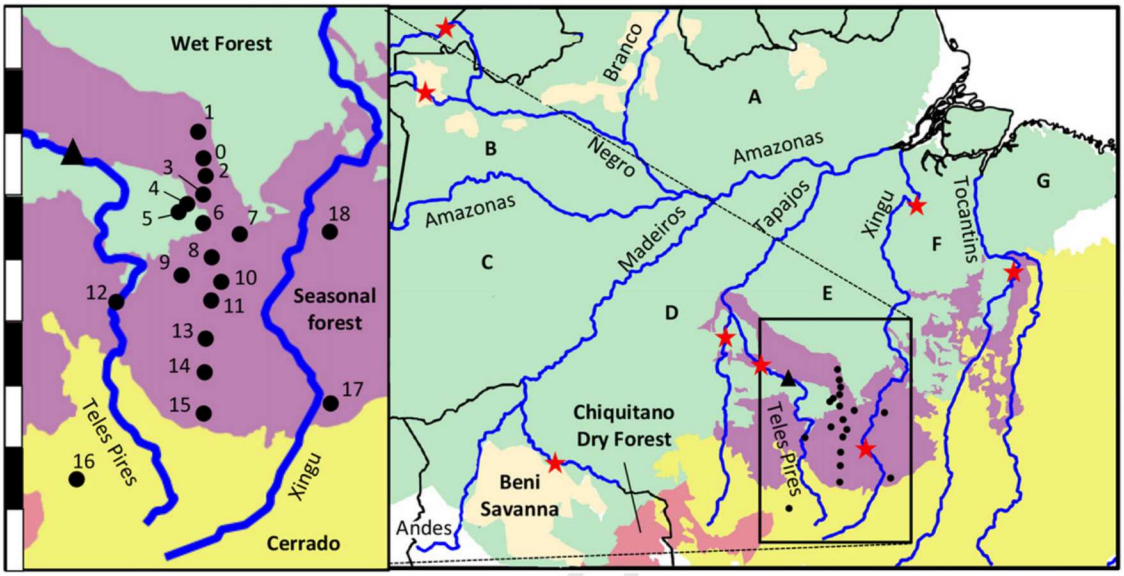

Figure 1. Sampling sites (numbered 0–18; see Table S1) in the headwaters of the Rio Teles Pires and Rio Xingu of the Brazilian Amazon. This headwater region occurs primarily in seasonal forest (dark gray; purple online version), which forms a transition between the wet forests to the north (medium gray; green online version) and the Cerrado savannas (pale gray; yellow online version) and Chiquitano dry forests (pink online version) to the south (drawn from Olson et al. 2001). Other savanna areas shown to the north are also shown in pale gray (pale yellow in online version). Major rivers believed to form important dispersal barriers and which delineate endemism regions for adjacent interfluves are illustrated and labeled. Endemism centers are as follows: (A) Guiana, (B) Napo, (C) Inambari, (D) Rondônia, (E) Tapajós, (F) Xingu, and (G) Belém. Locations where each river first narrows to <100 m is shown by a star (as measured from each rivers main channel in Google Earth). Width of the Rio Xingu greatly exceeds 100 m across most of its length, but has a small region only ca. 200 km from its mouth where it narrows to less than 100 m (northern star). The next region of narrowing occurs in the headwaters (southern star). The black triangle indicates location of the Bates et al. (2004) study of river barriers. Each segment of the vertical scale bar indicates 100 km.

of their respective members across the same rivers. For example, in birds, major Amazonian centers of endemism are largely delimited by the Rio Negro, Rio Amazonas, Rio Madeira, Rio Tapajós, Rio Xingu, Rio Tocantins, and their tributaries, with endemic species occurring in the interfluves between these river systems (Fig. 1; Cracraft 1985; Silva et al. 2005). Similar patterns have been found for Amazonian primates (Ayres and Clutton-Brock 1992), but not for rodents (Patton et al. 1994; but this study investigated a river not generally thought to present a strong barrier to dispersal). These observations led to the suggestion that rivers themselves (and their floodplains) may act as biogeographic barriers, retarding gene flow and promoting allopatric speciation in Amazonia, either through vicariance of once widespread species following formation of rivers, or subsequent to rare dispersal events across or around river barriers (River-barrier hypothesis hereafter, Sick 1967; Willis 1969; Hershkovitz 1977; Capparella 1988).

Haffer (1969, 1974) and others (e.g., Vanzolini and Willians 1970; Prance 1973, 1982; Brown et al. 1974) challenged the role of river barriers as the engines of Amazonian speciation. These authors suggested that dry periods, primarily during Pleistocene glacial maxima, resulted in the contraction of Amazonian wet forest into a series of geographically isolated refugia where speciation occurred (Refuge hypothesis). During wetter interglacials, newly diverged species expanded their ranges outwards, with many species expanding until they reached major Amazonian rivers. River barriers were thus considered of secondary importance as speciation had already occurred within refugia. Proponents of the Refuge hypothesis observed that many Amazonian species complexes possess contact zones between geographically replacing species that do not coincide with river barriers, or that coincide with rivers only along part of the geographic ranges (e.g., Haffer 1997). These patterns were interpreted as evidence that range expansion from forest refugia was not always hindered by rivers. Haffer (1997) also pointed to headwater regions as posing a serious problem for the River-barrier hypothesis. Although rivers might be formidable barriers to dispersal along their wide, lower stretches (e.g., the Rio Tapajós can exceed 20 km in width), in headwater regions the width of these rivers often narrows to as little as 70 m (e.g., headwaters of the Rio Teles Pires; see Fig. 1) where they may no longer function as dispersal barriers. Gene flow through headwater regions could prevent differentiation of species in adjacent river interfluves, and has remained a key argument against the River-barrier hypothesis.

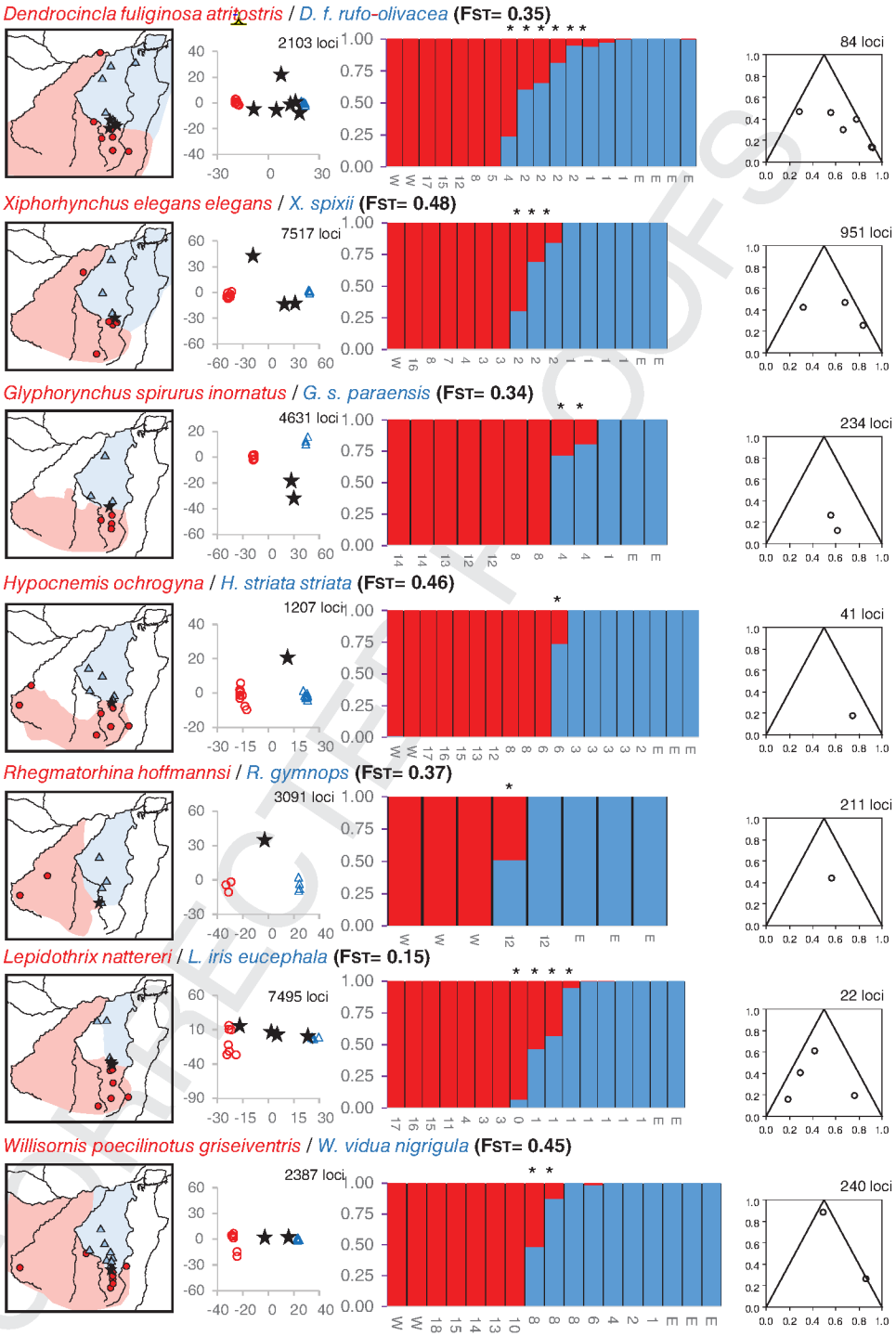

Figure 2. Seven taxon pairs largely separated by the Rio Tapajós (dark gray or red online, taxa west of Tapajós; pale gray or blue online, taxa east of Tapajos) and its tributaries, but which hybridize in headwater regions. Shown are the geographic ranges and sampling localities, PCoA plots (*x*-axis PCo 1, *y*-axis PCo 2), F_{ST} values (calculated from all SNPs), STRUCTURE analysis, and triangle plots of interspecific heterozygosity (*y*-axis) and hybrid index (*x*-axis) based on genome-wide GBS SNP data. Genetically admixed individuals on distribution maps and PCoA analysis are represented by black stars, and on triangle plots by circles. Asterisks above STRUCTURE plots indicate individuals where 95% confidence intervals on admixture proportions did not overlap 0 or 1. The number of SNP loci used in PCoA and STRUCTURE analyses are indicated above the PCoA plots (all SNPs used), and the number of SNPs fixed between parental populations and used in triangle plots are indicated. Numbers along the *x*-axis of STRUCTURE plots indicate the sampling population corresponding to Figure 1 and W and E indicate parental populations outside of our transect from west and east of the Rio Tapajós / Rio Teles Pires, respectively. The two subspecies of *Glyphorynchus spirurus* possess multiple phylogroups based on mitochondrial DNA (sensu Fernandes et al. 2013). We show the ranges only for the two phylogroups that meet in our transect.

There has been limited study of whether gene flow is widespread in headwater regions, and most evidence has been based on morphological patterns hinting at intergradation in these regions for select taxa (see Haffer 1997; Naka et al. 2012). Genetic evidence of gene flow across headwater regions is limited in birds. Based on an analysis of nuclear markers in *Glyphorynchus spirurus* along a section of the Rio Madeira located toward (but still far from) headwater regions, Fernandes et al. (2013) revealed a single genetically admixed individual between deeply diverged genetic phylogroups that occur on opposite banks of this river. Similarly, multilocus data indicate small levels of gene flow in headwater regions of the Rio Xingu between deeply diverged forms of the antshrike *Thamnophilus aethiops* that occur on opposite sides of this river (Thom and Aleixo 2015). A mitochondrial-based genetic study of 76 taxa pairs separated by the Rio Negro in northern Amazonia found that the Rio Negro or its tributary, the Rio Branco, continued to delineate geographic ranges toward headwater regions in all but 13 pairs (Naka et al. 2012). Those results suggest that rivers may continue to be sufficient barriers promoting divergence for most taxa, even toward the northern periphery of wet forest in Amazonia. In Southern Amazonia, Bates et al. (2004) investigated mitochondrial genetic differentiation across a small section of the Rio Teles Pires that approaches headwater regions (Fig. 1). The Rio Teles Pires is the major tributary flowing into the Rio Tapajós, and together this river system delimits the boundaries between the Rondônia and Tapajós centers of endemism, with many endemic species and subspecies demarcated along much of the length of this river system (Fig. 2). The mitochondrial data demonstrated genetic breaks coinciding with the river for six of the 10 species studied, and highlighted the importance of the river-barrier effect (despite the river being as narrow as 100 m at this point) even toward the headwaters. The authors concluded that gene flow in this headwater region seemed unlikely. However, forests capable of supporting many Amazonian wet forest taxa extend another 400 km both to the south and to the east of this Teles Pires study site (an area poorly represented in ornithological collections), and it remains unknown if Amazonian taxa extend into these regions and merge genetically at contact zones (Haffer 1992).

What is needed is a clear assessment of whether taxa come into geographic contact in headwater regions and hybridize there as proposed by Haffer (1997). Here we make use of a next-generation sequencing approach that samples thousands of single nucleotide polymorphisms (SNPs) to study gene flow in birds along the extreme peripheral edges of Amazonian forest in the headwaters of the Rio Teles Pires and Rio Xingu. Haffer (1997) highlighted this headwater region as a suture zone where geographic contact was likely between many pairs of avian species or subspecies indicative of the Rondônia and Tapajós centers of endemism (Fig. 1). This headwater region provided a key example to his argument for the homogenizing effect of gene flow through headwater regions. However, the actual contact zones, while inferred to exist based on current knowledge of geographic ranges and the probable absence of intervening barriers to dispersal, have never been demonstrated conclusively (i.e., with local syntopy). By sampling at regular intervals over a 550 km transect of this headwater region we were able to determine the precise locality of geographic contact for five parapatric pairs of species and two pairs of genetically diverged subspecies that are widespread in the Rondônia or Tapajós centers of endemism and whose geographic ranges are largely separated by the Rio Tapajós and its tributaries. We used several analyses of genetic admixture to test for gene flow at contact zones and to determine if genetically admixed individuals represented only F1 hybrids (which could be sterile, in which case gene flow is not demonstrated) or also included later generation hybrids (e.g., backcrossed individuals) indicative of gene flow. The presence of genetically backcrossed individuals in headwater regions would lend support to Haffer's argument that the River-barrier hypothesis cannot adequately explain the differentiation of taxa in adjacent interfluves.

Methods

SAMPLING DESIGN

We obtained genetic samples (Table S1) from 17 sites along a 550 km north/south transect in the Brazilian states of Pará and Mato Grosso in 2012, one site in 2014, and one site in 2006 and again in 2010 for a total of 19 sites (Fig. 1; Table S2). Sites were spaced every 50–60 km or less over most of the transect. In this region, the Rio Teles Pires and Rio Xingu flow in a primarily south to north direction in parallel to our transect, which lies between the two rivers. Although our transect lies within the Amazonian wet forest biome, the forest in this region has a strong seasonal component, and most of our transect is classified as the "Mato Grosso seasonal forest" by the World Wildlife Fund (Olson et al. 2001). Forest is now heavily degraded along the Rio Teles Pires in this region, but remains intact along most of the Rio Xingu. Most of our sampling sites occurred on privately owned forest contiguous to the pristine forest tracts of Xingu National Park and other protected areas along the Rio Xingu. The transect also included one site (near Nova Mutum) south and west of the Rio Teles Pires along gallery forest and adjacent semideciduous forest within the cerrado savannahs that lie to the south of the wet forest biome, and included two sites in wet forest along the east side of the Rio Xingu headwaters. This sampling design was augmented with samples previously collected further afield in the Rondônia and Tapajós Endemism Centers for a total of 144 individuals (Table S1). Our study focusses on taxon pairs believed by Haffer (1992) to come into geographic contact in this region (*Xiphorhynchus elegans* /

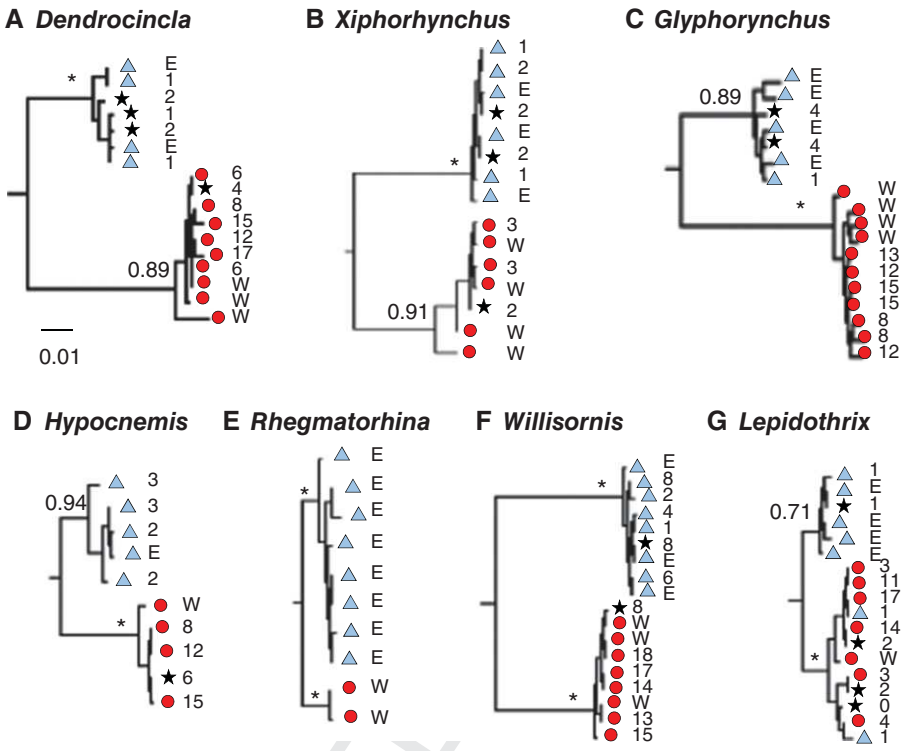

Figure 3. **Bayesian phylogenetic trees generated from 970 bp of mitochondrial DNA for taxon pairs largely separated by the Rio Tapajós (dark gray or red online, taxa west of Tapajós; pale gray or blue online, taxa east of Tapajos) and its tributaries. Individuals highlighted as significantly admixed in genome-wide SNP datasets are shown by black stars. Posterior probabilities are shown at the basal node for each taxon (asterisk (*) indicates >0.95). Individuals are numbered according to the sampling population corresponding to Figure 1 and W and E indicate parental populations outside of our transect from west and east of the Rio Tapajós / Rio Teles Pires, respectively. All taxon pairs are drawn to scale (scale bar is show under the first taxon pair).**

X. spixii, *Rhegmatorhina hoffmannsi* / *R. gymnops*, *Willisornis poecilinotus* / *W. vidua*, and *Lepidothrix nattereri* / *L. iris*), as well as subspecies pairs in *Dendrocincla fuliginosa*, *G. spirurus*, and the species pair *Hypocnemis ochrogyna* / *H. striata*, which we found across the transect.

DNA SEQUENCING

Whole genomic DNA was extracted from pectoral muscle using the Omega Bio-tek E.Z.N.A DNA Extraction Kit. We sent these samples to the Cornell Institute of Genomic Diversity (IGD) to obtain SNP datasets through a genotyping by sequencing (GBS) protocol described in Elshire et al. (2011). This protocol uses the PstI enzyme to produce reduced representation single-end libraries, which were sequenced on multiple lanes of the Illumina HiSeq 2000 platform with 95 individuals uniquely barcoded and multiplexed for each lane. We processed the resulting 100 base pair (bp) reads through the nonreference genome version of the Universal Network Enabled Analysis Kit (UNEAK) pipeline (Lu et al. 2013) implemented in TASSEL 3.0 (Bradbury et al. 2007). The pipeline was run separately for each taxon pair. The pipeline trims reads to 64 bp, merges identical reads into tags within each barcoded individual, and uses pairwise alignment to identify tag pairs with 1 bp mismatch (while probabilistically correcting sequencing errors using the error tolerance rate parameter which we set to 0.03), which are retained as candidate SNPs. For each taxon pair, only loci with one SNP are retained. Candidate SNPs present in fewer than 70% of individuals were excluded; then individuals possessing fewer than 10% of candidate SNPs were excluded. Next, a maximum-likelihood method (Lynch 2009) was used to reconstruct genotypes based on sequencing coverage using the scripts provided in White et al. (2013) (we used an updated version of the PairDuplicates.pl script provided by T. White, which corrected an earlier programming bug that resulted in incorrect genotype calls at some SNPs). This method retains SNPs with an Akaike information criterion ≥ 4 units lower than the next best reconstructed genotype. SNPs with observed heterozygosities greater than 0.75 were excluded (this functions to exclude likely paralogs). Other studies have found that varying the heterozygosity threshold cutoff between 0.5 and 1.0 had little effect (Baldassarre et al. 2014), so we used the recommended 0.75 threshold. This was followed by a final filtering step in which SNPs present in fewer than 75–90% (depending on the taxon pair) of individuals were excluded and individuals with fewer than 40–75% (depending on the taxon pair) of SNPs were excluded.

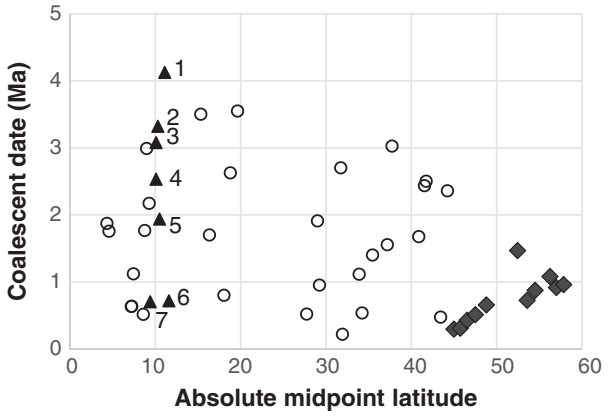

Figure 4. A comparison of mitochondrial cytochrome b coalescent dates (estimated using a 2.1% molecular clock applied to GTR-y distances; Weir and Schluter 2008) for our Amazonian taxon pairs and for other New World terrestrial birds that possess hybrid zones. Amazonian birds studied here are indicated by black triangles (taxa pairs as in Fig. 2: (1) *Willisornis*, (2) *Glyphorynchus*, (3) *Dendrocincla*, (4) *Xiphorhynchus*, (5) *Hypocnemis*, (6) *Rhegmatorhina*, (7) *Lepidothrix*). Boreal birds (i.e., above 45°N) are indicated by gray diamonds, all other hybrid zones are indicated by open circles. The absolute midpoint latitude of each hybrid zone is plotted on the *x*-axis. Non-Amazonian species and dates taken from Weir and Price (2011).

For genome-wide SNP datasets, we used F_{ST} to determine genetic differentiation between the two taxa in each taxon pair. F_{ST} was calculated in the R package Adegenet version 1.4-2 (Jombart & Ahmed 2011). Hybrid individuals from contact zones (diagnosed using STRUCTURE; Pritchard et al. 2000, see details below) were not included in F_{ST} measurements. Because only loci with a single SNP for each taxon pair were retained by the UNEAK pipeline, our estimates of F_{ST} are likely to be somewhat inaccurate, and are used with caution here simply to provide an approximate estimate for genome-wide genetic differentiation.

A 970 bp region of the mitochondrial gene cytochrome b was sequenced using standard protocols (Weir and Price 2011a) to date when taxon pairs diverged from a common ancestor and to determine whether taxa within taxon pairs are reciprocally monophyletic. GTR-y distances were calculated in PAUP version 4.0 (Swofford 2002), and a 2.1% molecular clock applicable to Passerine birds (Weir and Schluter 2008) was used to provide approximate dates of divergence between members of each taxon pair. Bayesian phylogenetic trees were constructed in MrBayes 3.2 (Ronquist and Huelsenbeck 2003) using the best fit model of sequence evolution returned by MrModelTest 2 (excluding models that make a correction for invariant sites; Nylander 2004) and were rooted with closely related outgroups (see Table S2). Phylogenies were run for two million generations, were sampled every 200 generations, and majority-rule consensus phylogenies were generated from samples following a 0.5 million generation burn-in.

ADMIXTURE ANALYSIS

We use the term hybrid to describe genetically admixed individuals between pairs of subspecies and species. Principal coordinate analysis (PCoA) in R (cmdscale function; R Core Team 2014) and admixture analysis in STRUCTURE 2.3.4 (Pritchard et al. 2000) were used to classify individuals as parental or hybrid. Both analyses were performed on the entire postfiltered SNP datasets for each species pair. Each individual's genotype at each SNP was coded as 0 or 1 for homozygotes, and 0.5 for heterozygotes for the PCoA analysis. PCoA was performed using Euclidean distances. STRUCTURE was run with the number of populations set at $k = 2$ because, in each case, we are dealing with two taxa that are genetically diverged in both SNPS (with two distinct parental clusters occurring in PCoA space) and in mitochondrial DNA (with deep divergences between, but not within, each cluster in mitochondrial DNA; see Results). For each taxon pair, eight independent STRUCTURE runs (each with a different starting seed) were performed for 100,000 generations following a 100,000 generation burn-in. Poststructure results were pooled across runs to determine admixture proportions and their 95% confidence intervals.

Individuals highlighted as parental populations in the STRUCTURE and PCoA analyses were then used as reference populations to calculate the hybrid index of each admixed individual using the R package INTROGRESS (Gompert and Buerkle 2010). Parental populations from the Rondônia Endemism Center were set to have a hybrid index of 0, and those from the Tapajós Center were set to an index of 1. Interspecific heterozygosity—the proportion of loci in an admixed individual's genome with alleles inherited from both parental species—was calculated for each admixed individual using INTROGRESS. These values range from 0 (no excess heterozygosity) to 1. Values near 1 are obtained for F1 hybrids, whereas values less than 1 indicate later generation hybrids that have either backcrossed with the parentals and/or with other hybrids (Fitzpatrick 2012). Triangle plots, in which hybrid index is plotted on the *x*-axis and interspecific heterozygosity along the *y*, were used to visualize these results. We used either all SNPs or only SNPs with fixed differences between each of the two parental populations for calculation of the hybrid index and interspecific heterozygosity. Because sample sizes of parental populations were small, SNPs in the fixed SNP dataset were not treated as fixed in the analysis of hybrid index and maximum-likelihood methods implemented in INTROGRESS were instead used to estimate parental allele frequencies. Currently available methods for calculating interspecific heterozygosity assume parental allele frequencies are known. Results were similar for almost all admixed individuals when all SNPs or only fixed SNPs were used, and we report results for the fixed SNP dataset only.

Results

Our dataset was incorporated into multiple Illumina libraries, each of which also had individuals from unrelated projects. Each Illumina library of 95 multiplexed individuals had on average ca. 2.0×10^8 raw reads, thus each individual had on average ca. 2.1×10^6 raw reads. Our postfiltered datasets included between 1207 and 7517 SNPS depending on the taxon pair (Fig. 2). A recently published passerine GBS dataset of ca. 68,000 SNPs reported a low Burrow's composite measure of inter- and intralocus disequilibria, indicating that SNPs are largely independent for datasets of this size (Parchman et al. 2013). We lacked reference genomes for our taxon pairs and so have not performed a similar analysis, but given we used far fewer SNPs, we expect most SNPs to likewise be independent. Our postfiltering SNP datasets had an average depth of coverage of 20.9 per locus (range of averages: 5.9–303.8) and of 21.4 per individual (range of averages: 8.9–71.1).

PCoA analyses of SNPS (Fig. 2) revealed two distinct clusters for each taxon pair that correspond to parental taxa endemic or largely endemic to the Rondônia and Tapajós endemism center. Genome-wide F_{ST} values between the two taxa in each taxon pair ranged from 0.15 in *Lepidothrix* to 0.48 in *Xiphorhynchus* (Fig. 2). All but one of these F_{ST} values were higher than the only other genome-wide F_{ST} value previously reported for closely related sister species of birds in the Neotropics (*Manacus vitellinus* and *M. candei* with an F_{ST} of 0.26; Parchman et al. 2013). With the exception of the lower genetic differentiation in *Lepidothrix*, F_{ST} values suggest moderate to strong genetic differentiation between taxa in our taxon pairs. Mitochondrial DNA phylogenies (Fig. 3) and genetic distances (Table S1) showed a similar pattern with divergence between parental taxa much greater than within each taxa and with a range of genetic differentiation (Fig. 4). GTR-gamma divergence in mtDNA and genome-wide SNP-based F_{ST} values were strongly correlated (Pearson's $r = 0.48$) but not significantly ($P = 0.13$) correlated.

STRUCTURE analysis of the SNP data highlighted between one and six individuals in each taxon pair as being significantly genetically admixed (i.e., 95% confidence intervals did not overlap 0 or 1; Fig 2), and these occurred at intermediate positions along PCo 1 between parental taxa as expected if they were hybrids. All seven taxa pairs studied had at least one individual with more than 25% admixture (Fig. 2). In all cases, individuals reconstructed as genetically admixed came from geographic regions precisely at the contact zones between taxa endemic to the Rondônia and Tapajós Endemism Centers as expected if they represent hybrids (Fig. 2). In five of the seven taxa pairs studied, sites with hybrids also had individuals genetically typical of one of the two parental taxa (parental taxa syntopic with hybrids: *D. fuliginosa atrirostris* and *D. f. rufo-olivacea*, *W. v. nigrigula, H. ochrogyna, R. gymnops, L. iris eucephala*). However, at no sites did we record both parental taxa syntopically. The geographic regions in which hybridization occurred corresponded closely across most of our contact zones (Fig. 2), all of which appear to occur within a 270 km region, and thus represent a narrow suture zone (sensu Remington 1968). Finer-scaled geographic sampling is necessary to determine the widths of individual hybrid zones, but our preliminary sampling suggests that they are less than 200 km wide for most taxa pairs.

The level of missing loci per individual ranged from 0 to 52% for individuals not identified by STRUCTURE as hybrids (mean = 7.6%) and 0 to 61% (mean = 11.4%) for hybrids, and the differences were not significant (*t*-test; $P = 0.40$). The higher values for the hybrids were due to two individuals with high levels of missing data. One of the two hybrid individuals in *Glyphorynchus* and the sole *Hypocnemis* hybrid had 58% and 61% of loci missing, respectively. When these two individuals are excluded, then the percentage of missing data in the remaining hybrid individuals is similar (mean = 5.4%, range = 0–18%) to that for parentals. To be certain that the high level of missing loci were not biasing the admixture proportions for *Glyphorynchus* and *Hypocnemis*, we filtered datasets for these species so that only SNPs present in the hybrid individual were retained. STRUCTURE analyses were then repeated using identical methods as above. Admixture proportions were almost identical for the two filtering strategies with admixture proportions for the hybrid individuals differing by less than 1% for the two approaches. This result demonstrates that admixture proportions were not biased by the level of missing loci.

We used triangle plots of interspecific heterozygosity versus hybrid index for diagnostic SNPs fixed in our sample from parental populations to determine if hybrids represented F1's or later generation hybrids. Only one individual was a likely F1 hybrid (*W. poecilinotus*), as evidence by a HI close to 0.5 and an interspecific heterozygosity score close to 1 (Fig. 2). Low values of heterozygosity in the remaining hybrid individuals suggest these individuals represent latter generation hybrids that have crossed with other hybrids and/or with parentals (individuals lying along the diagonal lines in the triangle plots are likely backcrosses with parentals; Fitzpatrick 2012). Results were similar when all SNPs (not just those that are diagnostic) were used, with the exception that the likely F1 hybrid for *W. poecilinotus* now had a much lower interspecific heterozygosity.

Bayesian phylogenetic trees generated from mitochondrial cytochrome b (Fig. 3) indicate that each of the taxon pairs formed two distinct clades, which correspond closely to the two groups recovered by the PCoA and STRUCTURE analysis of the SNP datasets (Fig. 2). The SNP and mitochondrial datasets thus support the distinctness of the two named taxa within the taxon pairs. The only lack of correspondence between the mitochondrial and SNP datasets occurred in two individuals of *Lepidothrix* from

population 1 (toward the northern edge of the contact zone) in which the SNP dataset classified them as *L. iris eucephala* (this classification agrees with the male plumage of this population), but the mitochondrial analysis grouped them with *L. nattereri* (Fig. 3). However, one of the five individuals sampled from population 1 had a small, but significant signature of admixture in the SNP dataset, thus mitochondrial introgression into some of the individuals of this population is not surprising.

Without fail, individuals with a significant signature of admixture in the SNP dataset all occur at the populations immediately at the contact zones between the taxa in each taxon pair. These admixed individuals group with both parental taxa in Bayesian mitochondrial phylogenies in *Dendrocincla, Xiphorhynchus, Lepidothrix*, and *Willisornis*, or with just one of the two parental taxa in *Hypocnemis* (only one admixed individual detected in this pairs) and *Glyphorynchus* (Fig. 3). We have not yet successfully sequenced the mitochondrial DNA of the only genetically admixed *Rhegmatorhina* sample. For two taxa pairs, the population immediately at the contact had individuals grouping with both of the mitochondrial clades (*Xiphorhynchus* and *Willisornis*).

In each of the two mitochondrial clades detected for each taxon pair, average mitochondrial cytochrome b based GTR-γ distances within clades were small (0.0–0.7%) compared to between the clades (1.5–8.67%; Table S3). Mitochondrial sequence based estimates of gene coalescence times between parental populations from the Rondônia and Tapajós endemism centers ranged from just 0.7 Ma in *L. iris* / *L. nattereri* and *R. hoffmannsi* / *R. gymnops* to 4.1 Ma in *W. poecilinota* / *W. vidua* (Fig. 4). Coalescent dates are estimated to predate population splitting dates on average by only about two to three hundred thousand years in Neotropical birds (see Weir 2006), and the low levels of genetic distance within our taxa support this.

Discussion

Our data provide the first evidence that multiple, codistributed taxa endemic to adjacent interfluves in Amazonia, and whose geographic ranges are largely separated by river barriers, nevertheless come into geographic contact in the headwater regions where they hybridize. In all seven taxon pairs studied, we found two distinct clades in mitochondrial DNA (Fig. 3), most of which had moderate to high levels of genome-wide F_{ST}, and which correspond closely to two distinct clusters in PCoA plots based on the genome-wide SNP datasets (Fig. 2). These clades demonstrate genetic differentiation between the taxa in each pair largely separated by the Tapajos and Teles Pires rivers. For each pair, PCoA and STRUCTURE analyses detected between one and six genetically admixed individuals from headwater localities, which lie precisely at the contact zones between taxa. These results support the key argument of opponents of the River-barrier hypothesis—namely that geographic contact and gene flow in headwater regions should prevent speciation from occurring between adjacent interfluves if river barriers were the only feature driving speciation in these taxa. Rather, other factors—probably in combination with river barriers—must play a role in genetically isolating taxa long enough for speciation to occur. Here we discuss other possible factors, and the implications of ongoing gene flow.

Our results could be interpreted as being consistent with (though not proof of) the Refuge hypothesis that forest refugia, rather than river barriers, drove Amazonian diversification. However, both paleopollen data and molecular dating of phylogenetic splitting events generally argue against the Refuge hypothesis as a general explanation of Amazonian speciation. Although paleopollen data have demonstrated the expansion of drier forest types along the northern and southern periphery of Amazonia during cooler/dryer periods (e.g., Mayle et al. 2004), it has not supported the widespread fragmentation of Amazonian forest into a large series of refugia as previously proposed by the Refuge hypothesis (Bush and de Oliveira 2006). Likewise, dating of phylogenetic splitting events in the Amazon has shown evidence that many (but not all) Amazonian species predate the Pleistocene glacial cycles, especially the major cycles of the past one million when forest refugia where deemed most likely (e.g., Moritz et al. 2000; Weir 2006; Rull 2008, 2011; Hoorn et al. 2010; Ribas et al. 2012). The seven taxon pairs studied here have coalescent dates ranging between 0.7 and 4.1 Ma, with three pairs predating the Pleistocene glacial periods altogether, and most pairs predating the severe glacial cycles of the last one million years of the Pleistocene (Fig. 4). While we acknowledge the imprecision of phylogenetic dating, these results nevertheless suggest that at least some of the taxon pairs studied predate periods when forest refugia seemed likely to have initiated speciation. The span of dates also argues against a single biogeographic event, such as the initial formation of the Rio Tapajós river system, as causing the simultaneous origin of taxon pairs endemic to the Rondônian and Tapajós endemism centers (see also Smith et al. 2014 who argue more generally against river-based vicariance as a key driver of Amazonian diversification).

The lack of evidence for the Refuge hypothesis and the apparent failings of the River hypothesis given gene flow in headwater regions are addressed by a hybrid of these two known as the River-refuge hypothesis (Capparella 1991; Ayres and Clutton-brock 1992; Haffer 1992). This hypothesis acknowledges that rivers in headwater regions fail to provide barriers sufficient to promote speciation, but builds on paleopollen records that do support periods of past retraction of wet forest at the edges of Amazonia toward its center (reviewed in Mayle et al. 2004). Rivers are wider toward the center of Amazonia and, in combination with the retraction of wet forest out of headwater regions, are believed to

provide sufficient barriers to promote speciation under this model. For example, given that the Rio Teles Pires delimits taxa boundaries just 170 km to the west of the northern part of our transect (Haffer 1997; Bates et al. 2004), it seems likely that a retraction of wet forest by just two or three hundred kilometers in the Rio Teles Pires area would probably be sufficient to isolate many populations on opposite sides of this river, leading to their divergence. Although paleopollen records from the Rio Teles Pires headwaters have not been studied, those at the edge of Amazonian wet forest to the west in Bolivia (Mayle et al. 2000; Burbridge et al. 2004) and to the east in the Brazilian state of Pará (Sifeddine et al. 2001) support expansion of dry forest into these regions during the last glacial maxima, and climatic models likewise support dry forest expansion along the entire southern periphery of the Amazon, but without necessarily bisecting Amazonian wet forest (Mayle et al. 2004).

Other alternatives to the River-refuge hypothesis could also explain in situ Amazonian speciation despite gene flow in headwater regions. First, speciation might occur despite continual parapatric contact through headwater regions if populations in each interfluve became differentially adapted to local ecological conditions, and headwater regions represented a strong ecotone (Endler 1977, 1982). Selection against hybrid individuals outside of ecotones could be strong and sufficient to maintain species differences despite local genetic admixture at the ecotone. We have not performed a formal assessment of habitat or climatic differentiation between our taxa pairs endemic to the Rondônia and Tapajós endemism centers. Nevertheless, we feel that species in these centers are unlikely to be adapted to greatly different environments given that their ranges are separated only by a river barrier, with presumably similar forest types, climates, and ecological communities on either bank (see Lees et al. 2013 for a comparison of bird communities across the Rio Teles Pires). As such, parapatric speciation via differentiation across an ecological gradient seems unlikely for our birds, although it has been suggested for Amazonian fish across gradients of water chemistry (Cooke et al. 2014).

Second, headwater regions may possess sink populations, which, despite gene flow into them, fail to prevent sister taxa from diverging in adjacent interfluves. Our transect occurs at the very southern edge of Amazonian wet forest extent. Much of this region represents a mosaic of interdigitating wet forest types (e.g., primarily along streams and rivers) and semideciduous drier forest (e.g., away from water courses) before finally transitioning to the Chiquitano dry forests and Cerrado savannahs that lie to the south (Fig. 1). Although many of our study species also occur in the semideciduous forest types, some appear to do so in low numbers. *R. gymnops*, *D. fuliginosa*, and *G. spirurus* are generally abundant in typical wet forest habitats toward the center of Amazonia, but were captured in low numbers across our transect (*Rhegmatorhina* only in the northern half), while other wet forest taxa were not detected at all (e.g., their ranges have not expanded this far into headwater regions). Clearly, transitional forests at headwater regions may prevent some wet forest species from expanding into these areas, while others occur in low numbers and represent sink populations, which fail to retard divergence outside of headwater regions. Despite the transitional nature of forest at headwater regions, some of our study species were abundant along the entire length of the transect, and commonly occurred away from lush streamside vegetation (*W. poecilinotus* / *W. vidua*, *L. nattereri, X. elegans*/*X. spixii*). These latter examples suggest that sink dynamics in headwater regions alone cannot explain the ongoing divergence between interfluves in these groups, and we feel that the River-refuge hypothesis—consistent with paleopollen data—may provide the best explanation in such examples.

Regardless of the precise dynamics under which rivers and forests interacted to promote interfluvial differentiation, our data demonstrate that hybridization continues to occur despite one to four million years of divergence because taxa pairs split from a common ancestor (Fig. 4). If the River-refuge hypothesis is correct, these results demonstrate that a single vicariant event driven by wet forest retraction along the periphery of Amazonia was not sufficient to generate enough reproductive isolation to prevent gene flow between parental species. Multiple episodes of retraction and divergence across the wider river courses that characterize the heart of the Amazon basin, followed by expansion and contact in headwater regions seems likely given the ages of these taxon pairs. In this respect, these results for the Amazon basin mirror those in the boreal zone of North America, where, despite multiple episodes of boreal forest retraction into refugia during glacials, followed by expansion during interglacials, many boreal birds as old as one to 1.5 million years (Fig. 4), as well as mammals, continue to form hybrid zones where their geographic ranges currently come into contact (Arbogast and Kenagy 2001; Weir and Schluter 2004; Toews et al. 2011; Irwin et al. 2009; Seneviratne et al. 2012). The comparison suggests that differentiation seems possible despite the slow rate at which reproductive isolation accumulates (about a million years at high latitudes and considerably longer in the tropics for birds; Weir and Price 2011b; this study) and despite repeated episodes of gene flow during periods of contact. One possibility is that genetically introgressed populations in contact zone regions go extinct with every repetition of forest expansion and retraction without ever having sufficient time to cause diverging populations to become homogenized genetically (Weir and Schluter 2004). In this way, ongoing divergence is possible despite repeated episodes of parapatric contact. For example, model-based estimates of migration rates are zero between parental populations of genetically diverged forms of *T. aethiops* from the Rondônia and Tapajós centers of endemism (Thom and Aleixo 2015), suggesting that

away from the vicinity of contact zones in the headwaters, gene flow has limited effect.

Hybridization does not necessarily imply a lack of complete reproductive isolation (e.g., Coyne and Orr 2004; Price 2008). For example, hybrids might be sterile, in which case contact regions should possess only F1 progeny (i.e., offspring from direct mating of parentals of each species) and parentals would be fully reproductively isolated. However, low levels of interspecific heterozygosity in all but one of our hybrid individuals suggest that genetically admixed individuals represent later generation hybrids, which have crossed with each other and/or with parentals. Admixed individuals falling along the diagonal lines in the triangle plots of heterozygosity and hybrid index (e.g., *D. fuliginosa*, *X. spixii/X. elegans*, *W. poecilinotus/W. vidua*, and *L. nattereri/L. iris*) are expected to result from the direct crossing of a hybrid with a parental (Fitzpatrick 2012). Such individuals suggest gene flow into parental populations, and a lack of complete reproductive isolation. These results contrast with interspecific heterozygosity for hybrids between recently diverged sibling species of flycatcher (*Ficedula albicollis* and *F. hypoleuca*) from high latitude regions in Europe where all hybrids tested had F1 genotypes and suggest strong selection against hybrid offspring (Kawakami et al. 2014). Although our data demonstrate that reproductive isolation is incomplete, small sample size limits the scope of our inferences regarding the width of hybrid zones and levels of selection against admixed individuals. Some of our taxon pairs are very old (e.g., 4 Ma; Fig 4.), and are noticeably differentiated in vocalizations (*W. poecilinotus/W. vidua*, *X. spixii/X. elegans*), suggesting partial reproductive isolation might be in place despite hybridization. However, other taxon pairs do not show marked differentiation in plumage or song (*G. spirurus*, *D. fuliginosa*; the latter of which we could not differentiate even in the hand) despite having diverged from a common ancestor approximately 3 Ma. Ironically, the taxon pairs with the most clearly differentiated plumage patterns, and which have always been recognized as specifically distinct (*R. hoffmannsi/R. gymnops*; *L. nattereri/L. iris*), are also the youngest (<1 Ma) in our sample. These observations suggest that plumage differentiation evolves at different rates in different species in our sample.

The ages of our Amazonian taxon pairs are similar to those reported for other species with hybrid zones from across the New World tropics (Weir and Price 2011b), with *Willisornis* representing the oldest taxon pair (ca. 4 Ma) with a hybrid zone that we are aware of for birds. The average and maximum age of taxa pairs with hybrid zones diminishes with increasing latitude in the New World (Fig. 4). At boreal latitudes above 45°N, all taxon pairs with hybrid zones diverged from a common ancestor less than 1.5 Ma, suggesting that complete reproductive isolation is in place by one to two million years. Indeed, high latitude species generally have achieved sufficient differentiation, that by about 1.7 million years on average, they are capable of expanding their geographic ranges into sympatry (Weir and Price 2011bb). Our results for the Amazon suggest that some taxa pairs as old as three to four million years have still experienced insufficient time to achieve complete reproductive isolation, and continue to exclude each other geographically across hybrid zones. Tropical birds also are reported to evolve more slowly than high latitude species in song, plumage coloration, body mass, and climatic niche (Martin et al. 2010; Weir and Wheatcroft 2011; Weir et al. 2012; Lawson and Weir 2014)—traits important for reproductive isolation and ecological differentiation. Together, these results suggest a slower pace for differentiation in lowland regions of the tropics, despite their high species richness. In the case of Amazonian taxa differentiating on opposite sides of rivers, the apparent lack of strong ecological differences on adjacent river banks is likely to result in lower rates of divergent natural selection between them, when compared to high latitude species (Lawson and Weir 2014).

In conclusion, our results confirm the presence of gene flow across headwater regions of Amazonia, despite the very old ages of some of the taxon pairs involved. Our current level of sampling is insufficient to measure hybrid zone width or test for levels of selection against hybrids, and further sampling is necessary to address hybrid zone dynamics in each of these taxon pairs in further detail. Nevertheless, our data do demonstrate low levels of interspecific heterozygosity and suggest the presence of hybrid individuals that have backcrossed with parental populations. These results demonstrate incomplete reproductive isolation and challenge the generality of the River-barrier hypothesis as a driver of in situ speciation in the Amazon.

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DATA ARCHIVING

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Locality information for GBS and cytochrome b sequences.
Table S2. Geographic coordinates of the 18 localities in our transect as shown in Figures 1 and 2.
Table S3. GTR-gamma distances within taxa and between hybridizing pairs of taxa for the mitochondrial gene cytochrome b.