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Tangled banks: A landscape genomic evaluation of Wallace's Riverine barrier hypothesis for three Amazon plant species

Alison G. Nazareno[1] | Christopher W. Dick[2,3] | Lúcia G. Lohmann[1]

[1]Departamento de Botânica, Universidade de São Paulo, São Paulo, Brazil

[2]Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, Michigan

[3]Smithsonian Tropical Research Institute, Panama City, Panama

Correspondence
Alison Gonçalves Nazareno, Departamento de Botânica, Universidade de São Paulo, São Paulo, SP, Brazil.
Email: alison_nazareno@yahoo.com.br
and
Lúcia G. Lohmann, Departamento de Botânica, Universidade de São Paulo, São Paulo, SP, Brazil.
Email: llohmann@usp.br

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Abstract

Wallace's Riverine Barrier hypothesis is one of the earliest biogeographic explanations for Amazon speciation, but it has rarely been tested in plants. In this study, we used three woody Amazonian plant species to evaluate Wallace's Hypothesis using tools of landscape genomics. We generated unlinked single-nucleotide polymorphism (SNP) data from the nuclear genomes of 234 individuals (78 for each plant species) across 13 sampling sites along the Rio Branco, Brazil, for *Amphirrhox longifolia* (8,075 SNPs), *Psychotria lupulina* (9,501 SNPs) and *Passiflora spinosa* (14,536 SNPs). Although significantly different migration rates were estimated between species, the population structure data do not support the hypothesis that the Rio Branco—an allopatric barrier for primates and birds—is a significant genetic barrier for *Amphirrhox longifolia*, *Passiflora spinosa* or *Psychotria lupulina*. Overall, we demonstrated that medium-sized rivers in the Amazon Basin, such as the Rio Branco, are permeable barriers to gene flow for animal-dispersed and animal-pollinated plant species.

KEYWORDS
Amphirrhox longifolia, double digest RADseq, *Passiflora spinosa*, *Psychotria lupulina*, Rio Branco, single-nucleotide polymorphism

1 | INTRODUCTION

Dispersal barriers have played a pervasive role in species diversification and shaping patterns of genetic diversity for a plethora of species worldwide (e.g., Boubli et al., 2015; Naka, Bechtoldt, Henriques, & Brumfield, 2012; Nazareno, Dick, & Lohmann, 2017; Peres, Patton, & da Silva, 1996). Almost two centuries ago, Wallace (1854) proposed the Riverine Barrier hypothesis based on observations of monkey species distributions in the Amazon Basin. The Amazon River and its tributaries have subsequently been shown to drive allopatric population differentiation for multiple taxa due to their potential role in population isolation (e.g., Boubli et al., 2015; Collevatti, Leoi, Leite, & Gribel, 2009; Gascon et al., 2000; Link et al., 2015; Moraes, Pavan, Barros, & Ribas, 2016; Naka et al., 2012; Nazareno, Dick, et al., 2017; Ribas, Aleixo, Nogueira, Miyaki, & Cracraft, 2012). Indeed, Wallace's "Hypothesis of the Rivers" (1854) presumes that the riverine barriers may explain areas of endemism throughout the Amazon Basin. As such, population structure (i.e., genetic divergence) should be evident across river barriers for species with widespread geographic distributions, representing the first stages of allopatric divergence (e.g., Boubli et al., 2015; Ribas et al., 2012).

In addition to the potential role of rivers as geographic barriers that can limit gene flow and promote genetic drift, intrinsic biological traits of species may predict the magnitude and the spatiotemporal distribution of neutral genetic variation found within and between populations (Burney & Brumfield, 2009; Fouquet, Courtois, Baudain,

& Lima, 2015; Maia, Lima, & Kaefer, 2017). For instance, the ability of frog species to disperse across the Oyapock River—a large, well-channelled Amazonian river on Precambrian rock that drains into the Atlantic Ocean—was highly dependent on their species-specific traits (Fouquet et al., 2015). For plants, the ability to disperse across rivers may vary substantially among species, and likely will also depend on life history traits related to gene dispersal (Lowe et al., 2018).

Comparative population genetics of codistributed species can reveal generalities in patterns of differentiation bearing on hypotheses regarding geographic barriers. In this study, three plant species dispersed and pollinated by animals, that is, *Amphirrhox longifolia* (A. St.-Hil.) Spreng. (Violaceae), *Passiflora spinosa* (Poepp. & Endl.) Mast. (Passifloraceae) and *Psychotria lupulina* Benth. (Rubiaceae), were selected to evaluate Wallace's Riverine Barrier hypothesis in the Amazon Basin. These three plants species were chosen due to their high abundance on both banks (left and right) of the Rio Branco, which is an allopatric river barrier for primates (Boubli et al., 2008, 2015) and birds (Bonvicino et al., 2003; Naka et al., 2012). Each of these plant species are described below.

Amphirrhox longifolia is a small, shrub-like treelet (up to 3 m in height) that is widely distributed in forests from Costa Rica to eastern Brazil (Missouri Botanical Garden, 2009). In the Amazon Basin, this plant species occurs at relatively high densities in floodplains (white-water and black-water) and nonflooding uplands. The species, which has unknown ploidy level, is self-incompatible and has hermaphroditic white flowers that are largely actinomorphic, tubular and spurless (Braun, Dotter, Schlindwein, & Gottsberger, 2012), a floral syndrome associated with pollination by bees and butterflies (Braun et al., 2012). Because of its ballistic dispersal system, the small seeds (ca. 5.0 mm) of *Amphirrhox longifolia* are likely to be dispersed short distances from the maternal tree. However, fruits and seeds were observed in stomach contents of fish in Central Amazonian floodplain forests (Correa, Costa-Pereira, Fleming, Goulding, & Anderson, 2015), suggesting fish dispersal. The weak genetic structure pattern observed for *Amphirrhox longifolia* populations across the Rio Negro in a related study (Nazareno, Dick, et al., 2017) supports dispersal by fish. Beyond the species-specific dispersal traits of *Amphirrhox longifolia*, the strength of riverine barrier in shaping genetic structure seems to be dependent on the width of the Amazonian rivers separating *Amphirrhox longifolia* populations (Nazareno, Dick, et al., 2017).

Passiflora spinosa is a liana that is distributed through forests of the Amazon Basin and also found in the Brazilian savanna. This species produces orange to reddish inflorescences that appear close to the ground or near the forest edges and river banks. Although there is no study regarding the ploidy level of *Passiflora spinosa*, most *Passiflora* species are diploid (Cerqueira-Silva, Jesus, Santos, Corrêa, & Souza, 2014). The flowers of *Passiflora spinosa* have elongated hypanthia and lack fragrance. In addition, the base of the hypanthium is divided into five nectar chambers. These floral features are associated with pollination by hummingbird (Ulmer & MacDougal, 2004). Although there are no studies about the dispersal syndrome for this plant species, a range of *Passiflora* species in the Amazon Basin are dispersed by animals such as fishes (Correa et al., 2015; Valencia, 2002), monkeys (Oliveira, Alfaro, & Veiga, 2014), bats (Bernard, 2002; Oliveira et al., 2014) and tapirs (Morais, 2006). Since the fruit morphology of *Passiflora spinosa* (i.e., yellow fruits with longitudinal green stripes, and thin, brittle pericarps <2.5 mm thick) is similar to that of other *Passiflora* species dispersed by fish (*P. laurifolia*; Valencia, 2002) and monkeys (*P. cirrhiflora*; van Roosmalen, Mittermeier, & Fleagle, 1988), it is very likely that seeds of *Passiflora spinosa* are similarly dispersed.

Psychotria lupulina, the third study species, is a small monoecious, diploid (2n = 22; Corrêa, 2007) shrub (up to 1.5 m in height) that is distributed along forest edges and understories across the Amazon Basin and Guiana shield. The sessile cream flowers are arranged in glomeruli and are visited by small insects (Taylor, 2007). Their ellipsoid fruits become blue at maturity (Taylor, 2007). Fruits of *Psychotria lupulina* are dispersed by fishes and birds (Macedo & Prance, 1978; Valencia, 2002).

Given that *Amphirrhox longifolia*, *Passiflora spinosa* and *Psychotria lupulina* share insect pollination and animal dispersal, we did not expect major differences among species reflecting their life history differences. We did, however, expect the river to play a role in population differentiation at the landscape scale in all three species. According to the Ritland's (1981) unidirectional diversity hypothesis, we expected that the hydrochoric spread of seeds downstream would result in a downstream increase of genetic diversity for *Amphirrhox longifolia*, *Passiflora spinosa* and *Psychotria lupulina* populations. This pattern is expected even for zoochorous riparian plant species that are not adapted to hydrocory (Prots, Omelchuk, & Van Bodegom, 2011). We used a high-throughput sequencing approach (double digest RADseq; Peterson, Weber, Kay, Fisher, & Hoekstra, 2012) to identify informative SNP markers from nuclear genome across all studied populations of *Amphirrhox longifolia*, *Passiflora spinosa* and *Psychotria lupulina* and test the hypotheses outlined above. This study highlights the importance of Amazonian tributaries as barriers for gene flow in the studied animal-dispersed plant species.

2 | MATERIALS AND METHODS

2.1 | Study area and sample collection

The Rio Branco (235,073 km^2) was selected to test the Riverine Barrier hypothesis. Geological analyses indicate relative stability of the Rio Branco, with low lateral mobility of its banks since the Last Glacial Maximum (21,000 years ago; Cremon, 2016). This river is located primarily on the Guiana Shield ecoregion where it is unusual in that it is a sediment-rich “white-water” river. It is 750 km long and flows southward into the Negro-Amazon rivers. Along its course, the Rio Branco can be subdivided into three segments: (a) the upper Rio Branco, from the confluence of the Uraricoera and Tacutu rivers to the Mucajaí river; (b) the middle Rio Branco, which occurs on pre-Cambrian rocks of the Guiana Shield; and (c) the lower Rio Branco, which flows on sedimentary rocks of the Solimões Basin

from Caracaraí to the interfluvium with Rio Negro. Due to its large extension, sampling of plant populations was restricted to the lower Rio Branco, an ecoregion of the Amazon Basin where vegetation structure is directly related to the hydro-edaphic features, supporting a different type of vegetation and plant community structure than that found along the shores of black-water rivers (Worbes, 1997). In the lower Rio Branco, distances across riverbanks can vary from 1.0 to 5.0 km. This area is characterized by a tropical equatorial climate, with mean annual rainfall of 2,200 mm (IBGE). The dry season occurs between June and October, with the rest of the year corresponding to the wet season.

A total of 234 flowering individuals of *Amphirrhox longifolia* (*n* = 78), *Psychotria lupulina* (*n* = 78) and *Passiflora spinosa* (*n* = 78) were obtained from 13 sampling locations situated on both banks (left and right) of the Rio Branco during the wet seasons of 2015 and 2016 (Supporting Information Table S1, Figure 1). Specifically, leaves from six individuals were obtained per sampling location. The typical limitations of small sample sizes are offset by large numbers of SNPs (Nazareno, Dick, et al., 2017; Willing, Dreyer, & van Oosterhout, 2012; Senn et al., 2013), which permit high-resolution identification of genetic structure (Brown et al., 2014; Puckett et al., 2016; Trucchi et al., 2016; Kotsakiozi et al., 2017; Nazareno, Bemmels, Dick, & Lohmann, 2017). For example, in a previous study of *Amphirrhox longifolia*, Nazareno, Dick, et al. (2017) reported that even two samples per population were adequate for accurate estimates of F_{ST} when >1,500 SNPs were used. To ensure that this was also valid for *Passiflora spinosa* and *Psychotria lupulina*, we performed genetic structure analyses (e.g.,

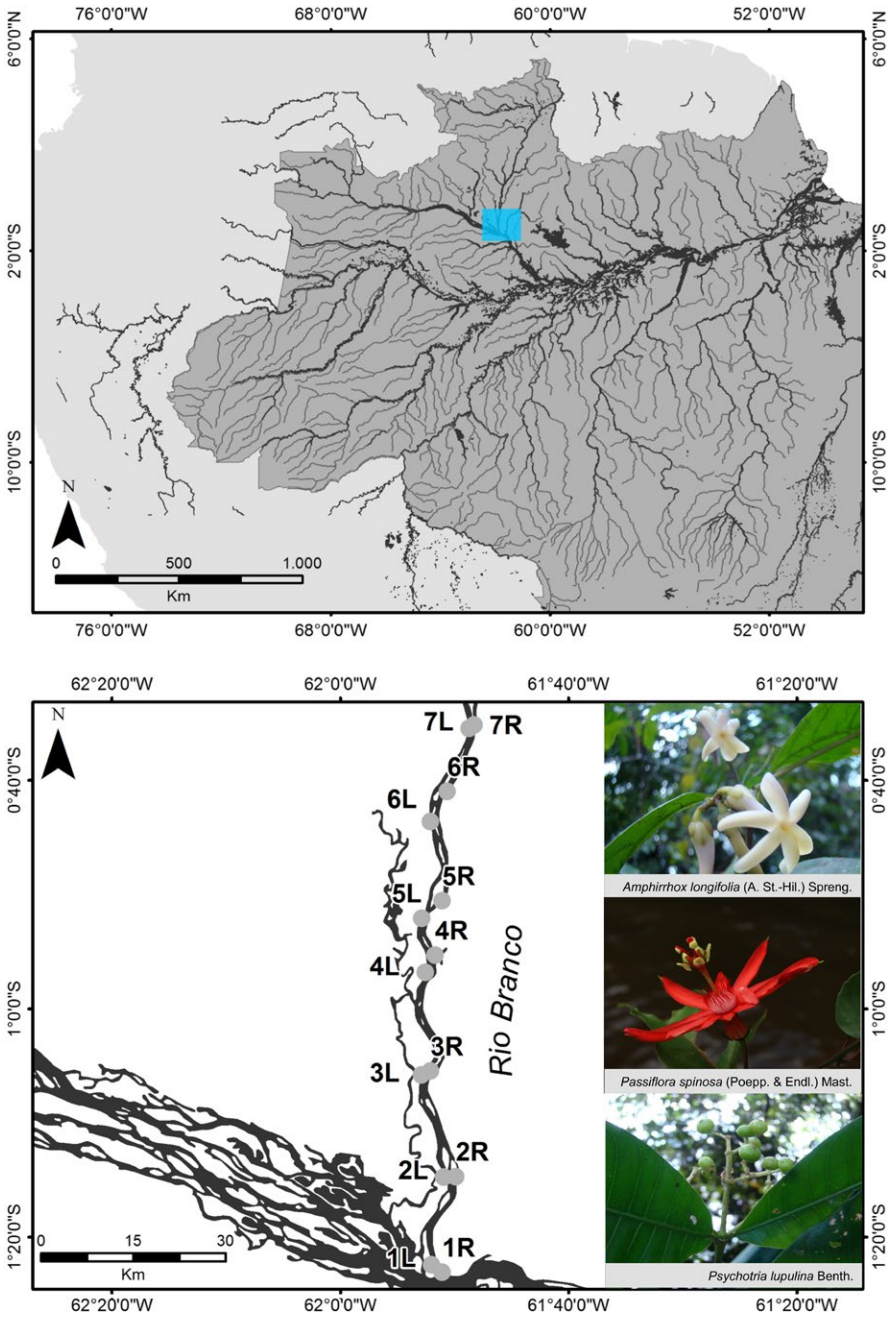


FIGURE 1 Sampling locations (1R – 7R, 1L – 7L) of *Amphirrhox longifolia* (A. St.-Hil.) Spreng, *Passiflora spinosa* (Poepp. & Endl.) and *Psychotria lupulina* Benth. sampled along the left and right banks of the Rio Branco, Amazon Basin, Brazil

pairwise genetic differentiation) while reducing the number of samples, randomly, from six to two. When we compared the mean of the pairwise genetic differentiation between different sample sizes, the results for *Passiflora spinosa* [F_{ST} (n = 6) = 0.019, 95% CI (0.012, 0.025); F_{ST} (n = 2) = 0.010, 95% CI (0.001, 0.014)] and *Psychotria lupulina* [F_{ST} (n = 6) = 0.026, 95% CI (0.018, 0.034); F_{ST} (n = 2) = 0.015, 95% CI (0.010, 0.021)] confirmed that the sample sizes we used are adequate for genetic structure analyses.

All locations were sampled from white-water floodplains. For each sampling location, we identified a corresponding sampling location on the opposite riverbank with distances varying with the width of the river from 1.0 km (Pop7R–Pop7L, Figure 1) to 3.8 km (Pop3R–Pop3L, Figure 1). In addition, distances between sampling locations on the same side of the river varied from 8.3 to 25.0 km. Individuals were sampled at intervals of at least 50 m to avoid sampling close relatives. One voucher specimen was sampled per population (Supporting Information Table S1). All vouchers were deposited at the Herbarium of the University of São Paulo (SPF), São Paulo, Brazil.

2.2 | Library preparation and sequencing

Libraries were prepared for *Passiflora spinosa* and *Psychotria lupulina*, while raw data for *Amphirrhox longifolia* were used from a previous study (Nazareno, Bemmels, et al., 2017). Genomic DNA was extracted from leaf samples of *Passiflora spinosa* and *Psychotria lupulina*, using the Macherey-Nagel kit (Macherey-Nagel GmbH & Co. KG), following the manufacturer's instructions. Four genomic libraries were created using a double digest RADseq protocol (Peterson et al., 2012), with the modifications proposed by Nazareno, Bemmels, et al. (2017) to minimize variance in the number of reads per individual within each pool. Double-stranded DNA concentrations were quantified before digestion reactions using the Qubit dsDNA Assay Kit (Invitrogen). Samples were adjusted to equal molar concentration and the final DNA concentration varied from 350 ng/μl (*Psychotria lupulina* and *Amphirrhox longifolia*) to 500 ng/μl (*Passiflora spinosa*). Each sample was digested with two high-fidelity restriction enzymes EcoRI and MseI (New England Biolabs). Digestion reactions were carried out in a total volume of 20 μl, using 17 μl of resuspended DNA, 5 units of EcoRI, 5 units of MseI and 1× CutSmart buffer (New England Biolabs) for 3 hr at 37°C, ending with a 20 min deactivation step at 65°C. Reactions were then purified with the Agencourt AMPure XP system (Beckman Coulter), following the manufacturer's instructions, with elution in 40 μl TE buffer. In order to standardize the initial DNA mass to be added into an adapter ligation, the cleaned digests were quantified using Qubit. Adapter ligations were carried out in a total volume of 30 μl, combining 42 ng DNA, 0.22 μM of a nonsample specific MseI adaptor (common for all samples), 0.33 μM of a sample specific EcoRI double-strand adaptor for each DNA sample, 1U of T4 DNA ligase (New England BioLabs) and 1.3 × T4 ligase buffer which were incubated at 23°C for 30 min. Reactions were then heat-killed at 65°C for 10 min following a slow cooling to room temperature (23°C). A total of 96 EcoRI double-stranded barcodes with a unique 10 base pair sequence were created using Python; for further details on the barcodes and the MseI oligos sequences, see Nazareno, Bemmels, et al. (2017).

After cleaning the reactions with the Agencourt AMPure XP system, ligation products were amplified in 20 μl PCRs, each containing 13.5 μl of the ligation product, 0.2 μM of Illumina PCR primers, 0.2 mM dNTPs, 1.0 mM $MgCl_2$, 0.5 U of iProof™ High-Fidelity DNA polymerase (BIO-RAD) and 2× of iProof buffer. The PCR protocol (98°C for 30 s, 20 cycles of 98°C for 20 s, 60°C for 30 s and 72°C for 40 s, followed by a final extension at 72°C for 10 min) was carried out in an Eppendorf PCR System. Before pooling samples at each library, each sample was purified using the Agencourt AMPure XP system and the DNA was quantified using Qubit. DNA concentration of each sample ranged from 2.95 ng/μl to 17.00 ng/μl. Multiplexed libraries were prepared with approximately equal amounts of DNA among samples. Automated size-selection was performed using a 2% agarose cartridge (Pippin Prep; Sage Science, Beverly, MA) to select genomic fragments at a target range size of 375–475 bp. Size, quantity and quality of each individual library were measured on the Agilent 2100 Bioanalyzer (Agilent Technologies) using the Agilent DNA 1000 Kit. Each library was sequenced (100-bp single-end reads) in a single lane of an Illumina HiSeq 2000 flow cell (Illumina Inc., San Diego, CA) at The Centre for Applied Genomics in Toronto, Canada (each lane was pooled with 38–42 samples).

2.3 | Identifying and genotyping SNPs

Files containing the raw sequence reads were analysed in STACKS 1.35 (Catchen, Amores, Hohenlohe, Cresko, & Postlethwait, 2011; Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013) using de novo assembly. Initially, we used the process_radtags program in Stacks to assign reads to individuals and eliminate poor quality reads and reads missing the expected EcoRI cut site (options –barcode_dist 4 -q -e ecoRI). All sequences were processed in ustacks to produce consensus sequences of RAD tags. The program ustacks takes a set of short-read sequences from a single sample as input and aligns them into exactly matching stacks. For each species, a maximum-likelihood framework (Hohenlohe et al., 2010) was applied to estimate the diploid genotype for each individual at each nucleotide position. The optimum minimum depth of coverage to create a stack was set at three sequences, the maximum distance allowed between stacks was two nucleotides, and the maximum number of stacks allowed per de novo locus was three. The stacks assembly enabled the Deleveraging algorithm (-d), which resolves overmerged tags, and the Removal algorithm (-*r*), which drops highly repetitive stacks and nearby errors from the algorithm. The alpha value for the SNP model was set at 0.05. Cstacks was used to build a catalogue of consensus loci containing all the loci from all the individuals and merging all alleles together. Each individual genotype was then compared against the catalogue using sstacks. We then used rxstacks to exclude problematic loci with a log-likelihood <–100 and loci that

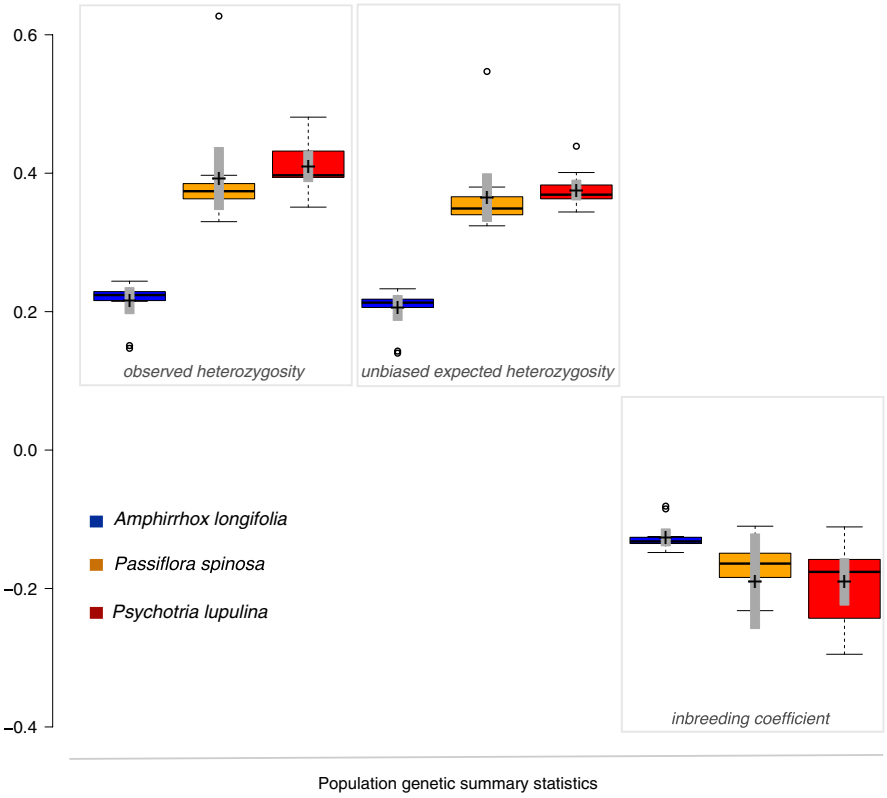
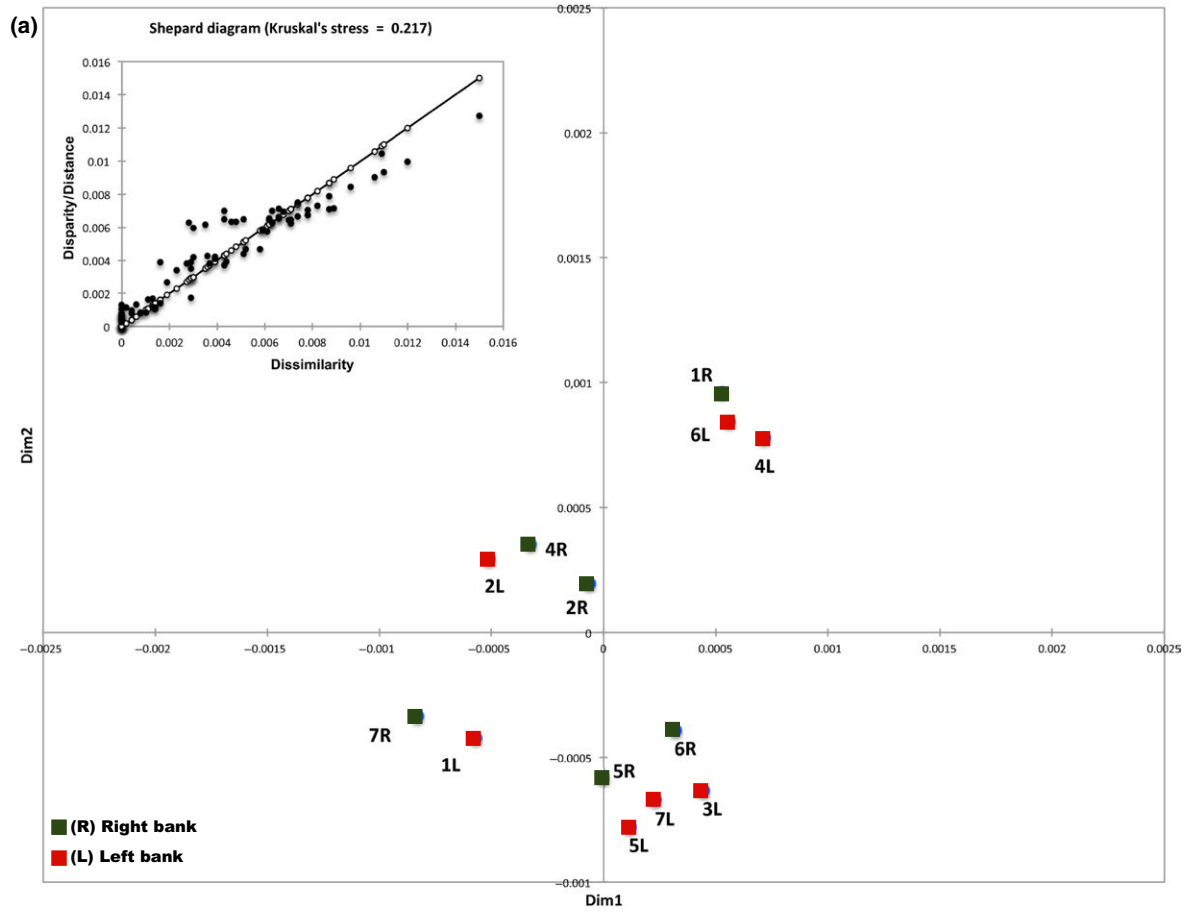


FIGURE 2 Boxplots showing the genetic diversity (uH_E—unbiased expected genetic diversity and H_O—the observed heterozygosity) and inbreeding coefficient for *Amphirrhox longifolia* (A. St.-Hil.) Spreng, *Passiflora spinosa* (Poepp. & Endl.) and *Psychotria lupulina* Benth. Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots; crosses represent sample means; bars indicate 95% confidence intervals of the means

matched a single catalogue locus (conf_limit = 0.25) or any nonbiological haplotypes (–prune_haplo) in more than 25% of individuals. Subsequently, cstacks and sstacks were performed again using the same parameters described above. The POPULATIONS program (Catchen et al., 2011, 2013) was run to obtain the loci that were present in at least 85% of individuals (-*r* 0.85), with a minimum stack depth of 10 (-m 10), and ddRAD tags were requested to be present in all locations (-p 13). In addition, we used a Minor Allele Frequency (MAF) of 1% (–min_maf 0.01) to filter out allelic types—with a count of one—that may mask population structure (e.g., Rodriguez-Ezpeleta et al. 2016). In the end, we only included the first SNP per locus in the final analysis.

2.4 | Quality control of genomic data

For each plant species, the numbers of raw sequence reads and unlinked SNPs were characterized for all populations. We used BAYESCAN 2.1 (Foll & Gaggiotti, 2008) to remove the SNPs potentially under balancing and divergent selection; this software was run with 20 pilot runs of 10,000 iterations, a burn-in of 50,000 iterations and a final run of 100,000 iterations. In order to minimize false-positives, prior odds of the neutral model were set to 10,000 (i.e., the neutral model is 10,000 times more likely than the model with selection; Foll & Gaggiotti, 2008). Furthermore, deviation from Hardy–Weinberg (H-W) equilibrium was assessed using the exact test based on Monte Carlo permutations of alleles—the most appropriate test when small sample sizes are used (Wang & Shete, 2012). The H-W equilibrium tests were done using the adegenet package (Jombart 2008; Jombart & Ahmed 2011) implemented in R (Jombart 2008; Jombart & Ahmed 2011). We also tested for linkage disequilibrium (LD) between loci in each population using GENEPOP 4.0 (Rousset 2008). The exact probabilities were calculated using a Markov Chain consisting of 100 batches and 5,000 iterations per batch. Type I error rates for tests of departure from H-W expectations and linkage disequilibrium were corrected for multiple k tests using the sequential Bonferroni procedure (Rice 1989). After the adjustment of the *p* value, SNPs that failed the H-W equilibrium test and the SNP pairs in LD in at least seven sampling locations were all excluded for further analyses. Considering the final dataset, we calculated minor allele frequencies for each plant species using the package adegenet (Jombart c; Jombart & Ahmed 2011), implemented in R (R Core Team, 2015). We further estimated the unbiased expected genetic diversity (uH_E; Nei & Roychoudhury 1974), the observed heterozygosity (H_O) and the inbreeding coefficient for each population using Wright's Fixation Index *F*. Population genetic statistics were averaged across loci using the R package diveRsity (Keenan, McGinnity, Cross, Crozier, & ProdÃűhl, 2013). For uH_E, H_O and *F*, the 95% confidence intervals were obtained to help evaluate differences between means estimated for all plant species. To assess whether sampling locations along the Rio Branco affected measurements of genetic variation within populations, we performed Spearman correlation test of uH_E and H_O with the geographic distance along the river course using the package GGPUBR (https://CRAN.R-project.org/package=ggpubr) implemented in R (R Development Core Team 2015).


(a)
Shepard diagram (Kruskal's stress = 0.217)
Disparity/Distance
Dissimilarity
Dim2
Dim1
1R
6L
4L
4R
2L
2R
7R
1L
6R
5R
7L
3L
5L
(R) Right bank
(L) Left bank

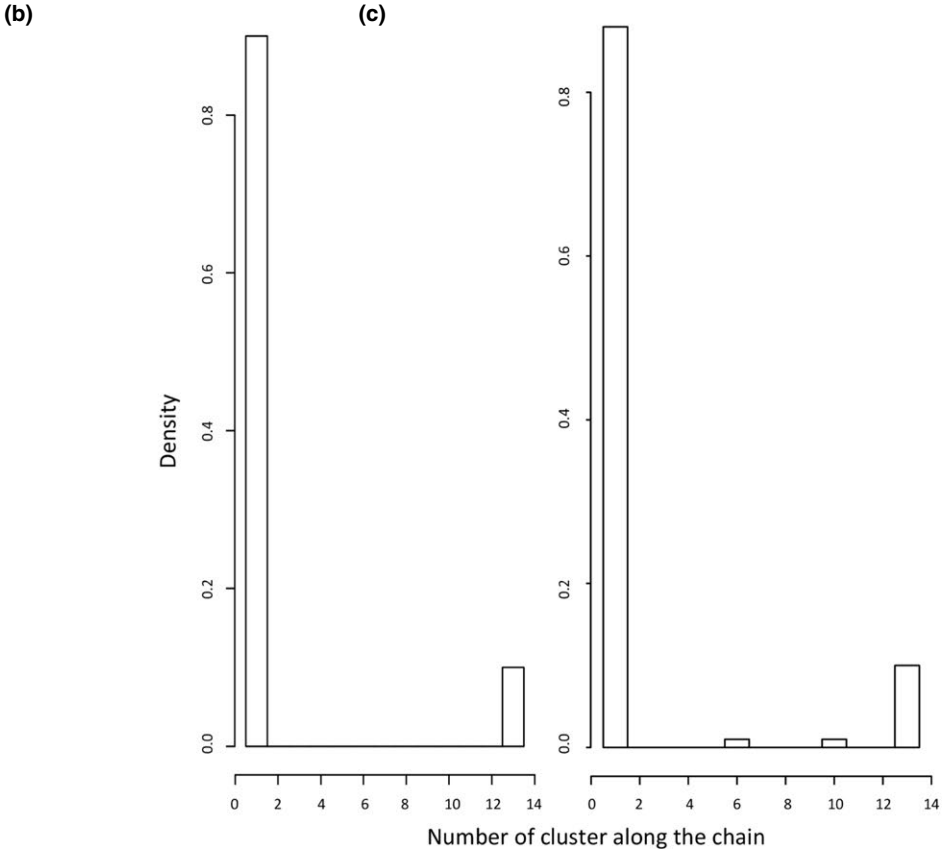

(b)
(c)
Density
Number of cluster along the chain

FIGURE 3 Patterns of geographical structure in *Amphirrhox longifolia* (A. St.-Hil.) Spreng as revealed by multidimensional scaling (MDS) of the matrix of genetic distances are showed in a. The Shepard diagram (inside a) shows the quality of the MDS representation. Population clustering analyses as calculated by GENELAND are also showed. b and c show the density of the estimate of *k* along the Markov chain (after a burn-in of 1,000 × 100 iterations), when spatial data are used (b) or not used (c) in the analyses

2.5 | Population genomic analyses

To investigate the effects of the Rio Branco on the population structure of *Amphirrhox longifolia*, *Passiflora spinosa* and *Psychotria lupulina*, we assessed the genetic structure and the historical and contemporary patterns of connectivity between locations of each plant species using complementary genetic analyses. We calculated genetic distances among locations (DA: Nei, Tajima, & Tateno, 1983) and visualized the results by applying multidimensional scaling (MDS) in XL-STAT (Addinsoft), using the SMACOF method (Scaling by MAjorizing a COnvex Function), which minimizes the "Normalized Stress" (De Leeuw, 1977)—a measure that determines how well a particular configuration reproduces the observed distance matrix. The MDS is an ordination technique that plots location as points in low-dimensional space while attempting to maintain the relative distances between locations as close as possible to the actual rank order of similarities between locations. Thus, locations with similar genetic structure are plotted closer together in ordination space established by a stress factor. MDS requires no assumptions regarding the cause of structure and does not assume Hardy–Weinberg or gametic equilibrium.

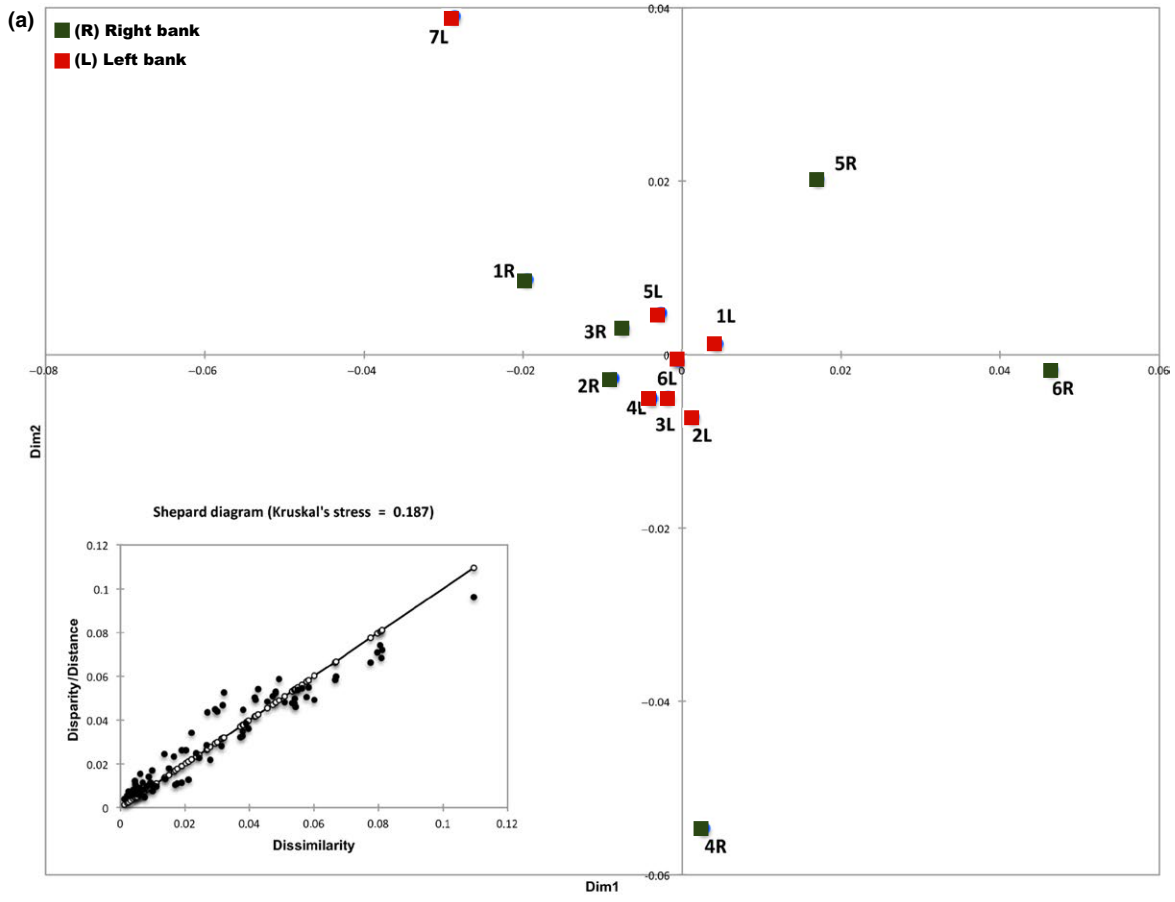
To more precisely understand the geographic distribution of genetic variability, a Bayesian model was developed using a Markov chain Monte Carlo (MCMC), as implemented in the R package GENELAND 4.0.2 (Guillot, 2012). This approach provided an alternative method of clustering populations as it incorporates spatial data in order to identify spatially explicit genetic discontinuities. This method operates by minimizing the Hardy–Weinberg and linkage disequilibrium that would result if individuals from different, randomly mating populations were incorrectly grouped into a population. We used a spatial model with correlated allele frequencies as proposed and implemented by Guillot, Santos, and Estoup (2008), and by Guillot (2012). Spatially explicit models take into account the spatial location of the individuals to improve the inference power of the substructure when differentiation occurs by limited gene flow driven by the presence of physical barriers. We conducted one hundred independent runs of 1,000,000 in length, discarding the first 500,000 iterations (burn-in) in postprocessing. The most likely number of k populations was unknown and hence treated as a simulated variable along with the MCMC simulations ($1 \leq k \leq 13$). The number of genetic clusters (K) was inferred as the modal number of genetic groups of the best run (based on posterior density values).

Pairwise genetic differentiation (F_{ST}) was estimated for each plant species using ANOVA following Weir and Cockerham (1984). We used SPAGeDi (Hardy & Vekemans, 2002) to calculate F_{ST} and estimate the significance of the deviation of F_{ST} values using a jackknife procedure over loci. Although we do not have similar distances between localities separated by the river with those on the same side of the river (Figure 1, Supporting Information Tables S3–S5), we used the nonparametric Wilcoxon–Mann–Whitney test to determine whether levels of genetic structure (F_{ST}) differed between pairs of sampling localities separated by the river (i.e., 1R-1L, 2R-2L, 3R-3L, etc.) and pairs of sampling localities on the same side of the river (i.e., 1R-2R, 2R-3R, 3R-4R, etc.).

In order to investigate the similarity between genetic and geographic distance, we conducted a test for isolation by distance (IBD) to see if this pattern met the expectation of decreased genetic similarity with geographic distance using a Mantel test (Mantel, 1967). Using 10,000 permutation tests of significance for the coefficient of correlation, a single Mantel test between a matrix of pairwise genetic distances [$F_{ST}/(1 - F_{ST})$; Rousset, 1997] and a matrix of Euclidian distances were performed using R (R Core Team, 2015).

A nested hierarchical analysis of molecular variance (AMOVA; — Excoffier, Smouse, & Quattro, 1992) was used to examine the effect of rivers on the partitioning of genetic variation between populations. We defined two hierarchical levels at which we characterized population differentiation: between locations from opposite riverbanks and between locations along each bank. We used ARLEQUIN 3.5.2 (Excoffier & Lischer, 2010) to calculate population differentiation estimates and their statistical significance based on 20,000 random permutations.

Finally, we estimated contemporary directional migration rates (*m*) within the last few generations using Bayesian inference framework implemented in BayesAss (Wilson & Rannala, 2003). As the latest version of BayesAss can read a maximum of 420 SNP loci, for each plant species, we generated five random subsets of our data and ran each individually. In order to ensure that estimates of migration rates can be accurately obtained when small sample sizes are employed, we performed a preliminary analysis with a subset of *Amphirrhox longifolia* individuals from a previous study (Nazareno, Dick, et al., 2017) and compared migration rates obtained from samples sizes of ten, eight, six, and four individuals in two *Amphirrhox longifolia* populations (A and B). The results indicated that there are no differences in the migration rates even when a small number of individuals are used in the analysis [e.g., migration rates (*m*) from population A to population B with sample sizes (*n*) varying from four to 10 individuals per population: $m_{(n=4)} = 0.056$, 95% CI (0.009, 0.121), $m_{(n=6)} = 0.043$, 95% CI (0.009, 0.095), $m_{(n=8)} = 0.042$, 95% CI (0.008, 0.092) and $m_{(n=10)} = 0.039$, 95% CI (0.004, 0.074). Therefore, for each plant species, we conducted the analysis with a sample size of six individuals per sampling location using 2.0×10^7 interactions with a burn-in of 10^7 generations and a sampling frequency of 2.0×10^3. To confirm the consistency of migration rate estimates, we conducted five independent runs of the analysis for each subset of the SNP data. All migration rates whose 95% confidence intervals


(a)
(R) Right bank
(L) Left bank
7L
5R
1R
5L
1L
3R
2R
6L
6R
4L
3L
2L
4R
Dim1
Dim2
Shepard diagram (Kruskal's stress = 0.187)
Disparity/Distance
Dissimilarity

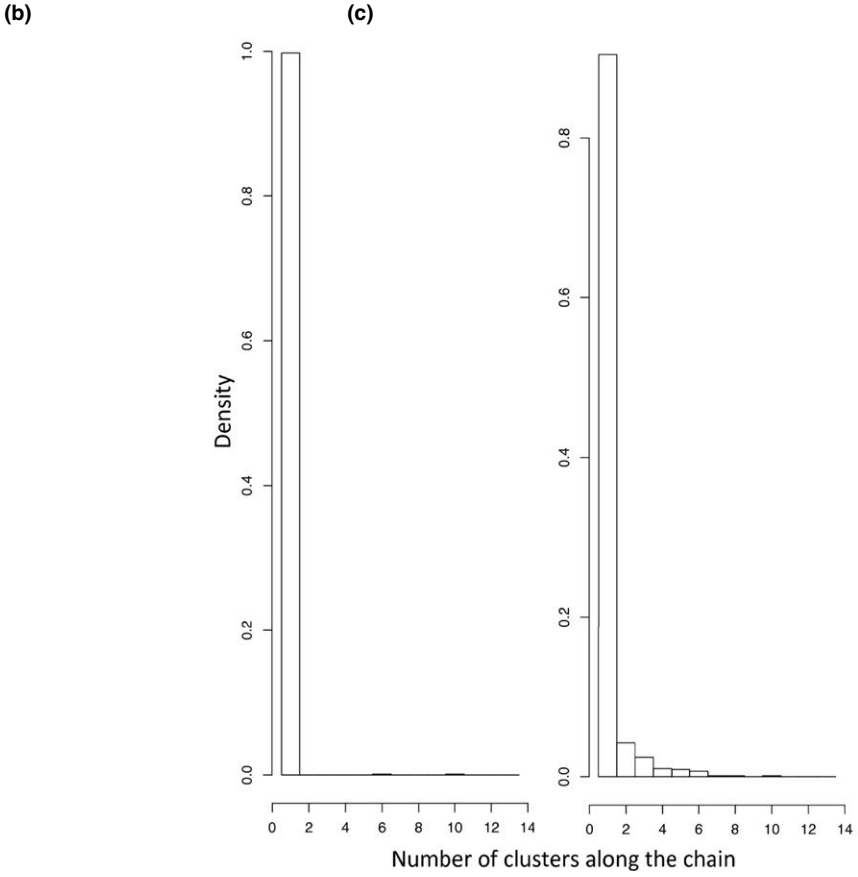

(b)
(c)
Density
Number of clusters along the chain

FIGURE 4 Patterns of geographical structure in *Passiflora spinosa* (Poepp. & Endl.) as revealed by multidimensional scaling (MDS) of the matrix of genetic distances are showed in a. The Shepard diagram (inside a) shows the quality of the MDS representation. Population clustering analyses as calculated by GENELAND are also showed. b and c show the density of the estimate of *k* along the Markov chain (after a burn-in of 1,000 × 100 iterations), when spatial data are used (b) or not used (c) in the analyses

(mean ± [1.96 × Standard Error]) did not include 0 are reported as significant. Significant bidirectional migration rates were visualized as chord diagrams using the software Circos (Krzywinski et al., 2009). We compared the contemporary migration rates between *Amphirrhox longifolia*, *Passiflora spinosa* and *Psychotria lupulina* species applying the Kolmogorov–Smirnov (KS) test. We also used the KS test to compare the average of asymmetrical migration rates between downstream and upstream for *Amphirrhox longifolia*, *Passiflora spinosa* and *Psychotria lupulina*. These statistical analyses were performed in R (R Core Team 2015).

We also examined the proportion of migrants from each ancestral class (e.g., nonmigrants, first- and second-generation migrants) that were assigned to a given class with maximum posterior probability using BayesAss (Wilson & Rannala, 2003).

3 | RESULTS

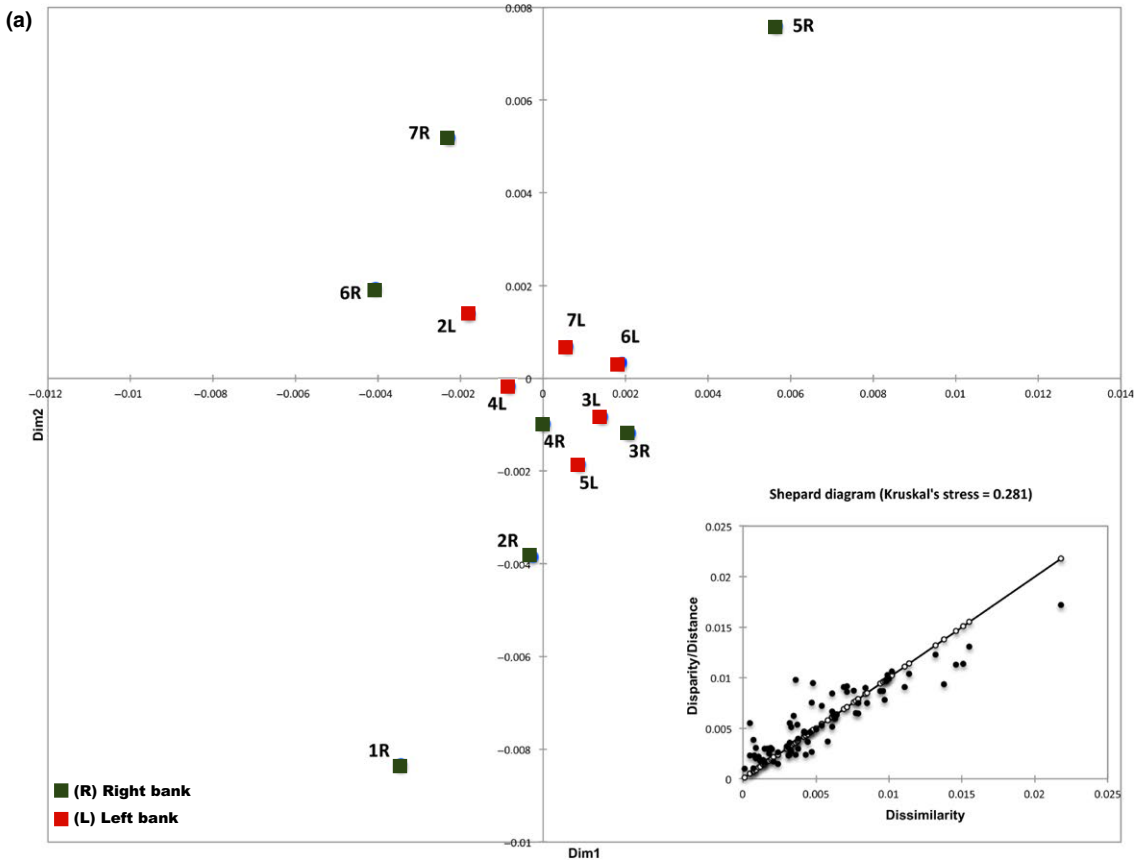
3.1 | Data quality control

The number of single-end raw reads of 101 bp produced for each lane of HiSeq 2000 Illumina varied from 132 million (library with 39 *Passiflora spinosa* samples) to 169 million (library with 65 *A. amphirrhox* samples). Each read starts with a barcode sequence identifying a sample (up to 10 bp long) and the 6 bp restriction site, followed by 85 bp of usable data. The mean number of retained reads that passed the default quality filters, including a Phred quality score >33 and contained an identifiable barcode, for *Amphirrhox longifolia*, *Passiflora spinosa* and *Psychotria lupulina* were 2,192,050 ± 52,090 SE, 2,497,616 ± 405,011 SE and 2,756,427 ± 724,856 SE, respectively. Throughout the *Amphirrhox longifolia*, *Passiflora spinosa and Psychotria lupulina* genomes, further filtering (10-fold coverage; presence in at least 85% of the individuals; MAF >0.01) resulted in 8,098, 14,540 and 9,514 unlinked polymorphic SNP markers, respectively, within the RAD tag sequences for all locations. After a Bonferroni adjustment, no significant departures from H-W equilibrium were observed in any sampling location or species ($p > 6.2 \times 10^{-6}$ for *Amphirrhox longifolia*, $p > 3.4 \times 10^{-6}$ for *Passiflora spinosa* and $p > 5.2 \times 10^{-6}$ for *Amphirrhox longifolia*). In addition, considering each focal species, no linkage disequilibrium was observed after a sequential Bonferroni correction for *k* tests ($k = 3.2 \times 10^{7}$, $p < 1.5 \times 10^{-9}$ for *Amphirrhox longifolia*, $k = 1.05 \times 10^{8}$, $p < 4.7 \times 10^{-10}$ for *Passiflora spinosa* and $k = 4.5 \times 10^{7}$, $p < 1.1 \times 10^{-9}$ for *Psychotria lupulina*).

For *Amphirrhox longifolia*, *Passiflora spinosa* and *Psychotria lupulina* populations, we detected, respectively, ten, four and 13 potential loci that were under diversifying selection with the false discovery rate (FDR) set to 0.05. Thus, a total of 8,075 filtered SNPs for *Amphirrhox longifolia*, 14,536 for *Passiflora spinosa* and 9,501 for *Psychotria lupulina* were used in genomic analyses. Considering the final dataset, minor allele frequency (MAF) averaged 0.1177 ± 0.0220 SD for *Psychotria lupulina*, 0.0901 ± 0.0172 SD for *Passiflora spinosa* and 0.1370 ± 0.0581 SD for *Amphirrhox longifolia*. Genetic diversity parameters did not vary much among populations of *Amphirrhox longifolia*, *Passiflora spinosa* and *Psychotria lupulina* (Supporting Information Table S2). At the species level, the mean unbiased expected genetic diversity varied from 0.205 (*Amphirrhox longifolia*) to 0.376 (*Psychotria lupulina*) and the mean observed heterozygosity ranged from 0.216 (*Amphirrhox longifolia*) to 0.410 (*Psychotria lupulina*; Supporting Information Table S2). These estimates were significantly higher for *Passiflora spinosa* (uH_E —95% CI 0.335, 0.394; H_O—95% CI 0.354, 0.431) and *Psychotria lupulina* (uH_E—95% CI 0.363, 0.388; H_O—95% CI 0.391, 0.429) than for *Amphirrhox longifolia* (uH_E—95% CI 0.190, 0.221; H_O—95% CI 0.200, 0.232; Figure 2). Due to the excess of observed heterozygotes, the mean inbreeding coefficient was negative for all populations (Supporting Information Table S2) but differed significantly between *Amphirrhox longifolia* (= −0.126, 95% CI −0.137, −0.116) and *Passiflora spinosa* (= −0.190, 95% CI −0.249, −0.130) and between *Amphirrhox longifolia* (= −0.126, 95% CI −0.137, −0.116) and *Psychotria lupulina* (= −0.190, 95% CI −0.219, −0.161; Figure 2). No significant Spearman correlations existed between genetic diversity (i.e., unbiased expected genetic diversity and observed heterozygosity) and distance of the sampling location from the streamline, which varied from −0.52 to −0.49 for *Amphirrhox longifolia*, from 0.0 to 0.13 for *Passiflora spinosa* and from −0.09 to −0.05 for *Psychotria lupulina*. These data indicate that genetic diversity is spread without directional pattern along the Rio Branco (Supporting Information Figure S1).

3.2 | Population genomic structure and the genetic barrier hypothesis

Based on the MDS and Bayesian clustering methods, we did not identify any potential barrier to gene flow in the Rio Branco for *Amphirrhox longifolia* (Figure 3a–c), *Passiflora spinosa* (Figure 4a–c) or *Psychotria lupulina* (Figure 5a–c). Using Kruskal's stress values (a measure that determines how well a particular configuration reproduces the observed distance matrix), we inferred that two dimensions were sufficient to explain the genetic patterns for *Amphirrhox longifolia* (Figure 3a), *Passiflora spinosa* (Figure 4a) and *Psychotria lupulina* (Figure 5a). The genetic pattern that emerges from our data, as depicted in the MDS plots, grouped together all populations from both banks of the Rio Branco for all plant species studied (Figures 3a, 4a and 5a). The genetic structure patterns from the MDS analyses for *Amphirrhox longifolia*, *Passiflora spinosa* and *Psychotria lupulina* closely matched those obtained using Bayesian clustering analyses. For all plant species, GENELAND results clearly delineated a single group with minimal variance in the posterior probabilities of population


(a)
5R
7R
6R
2L
7L
6L
4L
3L
4R
3R
5L
2R
1R
(R) Right bank
(L) Left bank
Dim1
Dim2
Shepard diagram (Kruskal's stress = 0.281)
Disparity/Distance
Dissimilarity

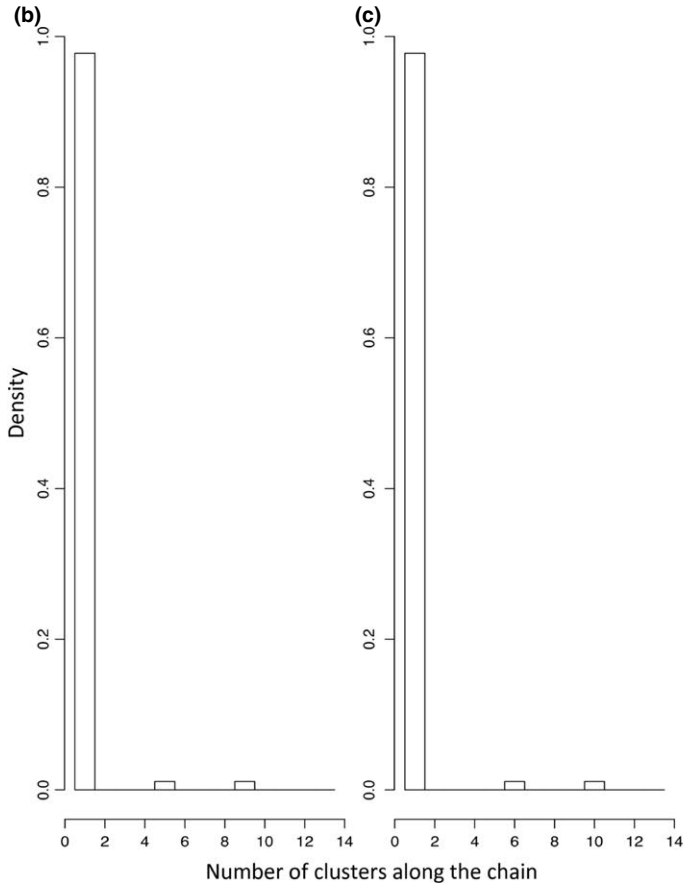

(b)
(c)
Density
Number of clusters along the chain

FIGURE 5 Patterns of geographical structure in *Psychotria lupulina* Benth as revealed by multidimensional scaling (MDS) of the matrix of genetic distances are showed in a. The Shepard diagram (inside a) shows the quality of the MDS representation. Population clustering analyses as calculated by GENELAND are also showed. b and c show the density of the estimate of *k* along the Markov chain (after a burn-in of 1,000 × 100 iterations), when spatial data are used (b) or not used (c) in the analyses

TABLE 1 Analyses of molecular variance (AMOVA) of *Amphirrhox longifolia* (A. St.-Hil.) Spreng, *Passiflora spinosa* (Poepp. & Endl.) and *Psychotria lupulina* Benth. from the Rio Branco, Amazon Basin, Brazil

	Sum of squares	Variance components	% of Variation	*p*-value
Amphirrhox longifolia				
Between banks	77.71	0.01	0.01	0.365
Among populations within banks	842.41	0.86	1.26	<0.001
Within populations	8135.12	67.32	98.73	<0.001
Total	9055.24	68.19		
Passiflora spinosa				
Between banks	1164.15	0.01	0.43	0.322
Among populations within banks	21742.68	10.09	1.00	0.012
Within populations	152112.42	1159.71	98.57	0.007
Total	175019.25	1169.80		
Psychotria lupulina				
Between banks	847.08	0.35	0.05	0.422
Among populations within banks	9062.39	15.70	2.32	0.000
Within populations	80725.85	661.84	97.63	<0.001
Total	90635.32	677.89		

estimation over multiple runs using both spatial (Figures 3b, 4b and 5b) and nonspatial models (Figures 3c, 4c and 5c).

The matrix of geographic distance and the pairwise F_{ST} values quantifying genetic differentiation among sampling sites of *Amphirrhox longifolia*, *Passiflora spinosa* and *Psychotria lupulina* are shown in Supporting Information Tables S3–S5. Pairwise estimates of F_{ST} varied from 0.004 to 0.042, and all but two were statistically significant ($p < 0.05$), indicating low levels of differentiation between *Amphirrhox longifolia* population pairs from the Rio Branco (Supporting Information Table S3). For *Passiflora spinosa*, pairwise estimates of F_{ST} varied from 0.001 to 0.110 and all but 22 were statistically significant ($p < 0.05$), indicating higher levels of historical gene flow between *Passiflora spinosa* population pairs from the Rio Branco (see Supporting Information Table S4). For *Psychotria lupulina*, pairwise estimates of F_{ST} varied from 0.001 to 0.121 and all but fifteen were statistically significant ($p < 0.05$), indicating limited differentiation between *Passiflora spinosa* population pairs from the Rio Branco (see Supporting Information Table S5).

No significant differences in genetic differentiation were observed between localities separated by the river with those on the same side of the river for *Amphirrhox longifolia* (Wilcoxon–Mann–Whitney test, $W = 28$; $p = 0.872$), for *Passiflora spinosa* (Wilcoxon–Mann–Whitney test, $W = 28.5$; $p = 0.873$) and for *Psychotria lupulina* (Wilcoxon–Mann–Whitney test, $W = 30$; $p = 0.802$), indicating that Rio Branco is a permeable barrier for these species. In addition, no correlation between genetic distance and geographical distance was found in any of the three species ($r = 0.176$, $p = 0.2211$ in *Amphirrhox longifolia*, $r = 0.192$, $p = 0.2626$ in *Passiflora spinosa* and $r = 0.2387$, $p = 0.1817$ in *Psychotria lupulina*). Similarly, results of simple matrix correlation between genetic and geographic distance were also not significant when applied separately to both banks of the river ($r = -0.0528$, $p = 0.6243$ for the right bank and $r = -0.1232$, $p = 0.7026$ for the left bank in *Amphirrhox longifolia*; $r = 0.2133$, $p = 0.4862$ for the right bank and $r = 0.4580$, $p = 0.0913$ for the left bank in *Passiflora spinosa*; and $r = 0.4185$, $p = 0.1029$ for the right bank and $r = 0.0520$, $p = 0.8991$ for the left bank in *Psychotria lupulina*). Results were also not significant when applied between pairs of sampling locations on opposite riverbanks ($r = 0.0786$, $p = 0.50$ in *Amphirrhox longifolia*, $r = 0.0957$, $p = 0.4953$ in *Passiflora spinosa* and $r = 0.0930$, $p = 0.5585$ in *Psychotria lupulina*). Altogether, these results indicate that Rio Branco has not acted as a barrier to gene flow for *Amphirrhox longifolia*, *Passiflora spinosa* or *Psychotria lupulina*.

The hierarchical multilocus evaluation of genetic differentiation performed using an AMOVA revealed that a greater proportion of the overall genetic variation exists within populations (98.73%, 98.57% and 97.63%) than between populations from either riverbank (0.01%, 0.43% and 0.05%) or among populations from the same riverbank (1.26%, 1.00% and 2.32%) for *Amphirrhox longifolia*, *Passiflora spinosa* and *Psychotria lupulina*, respectively (Table 1).

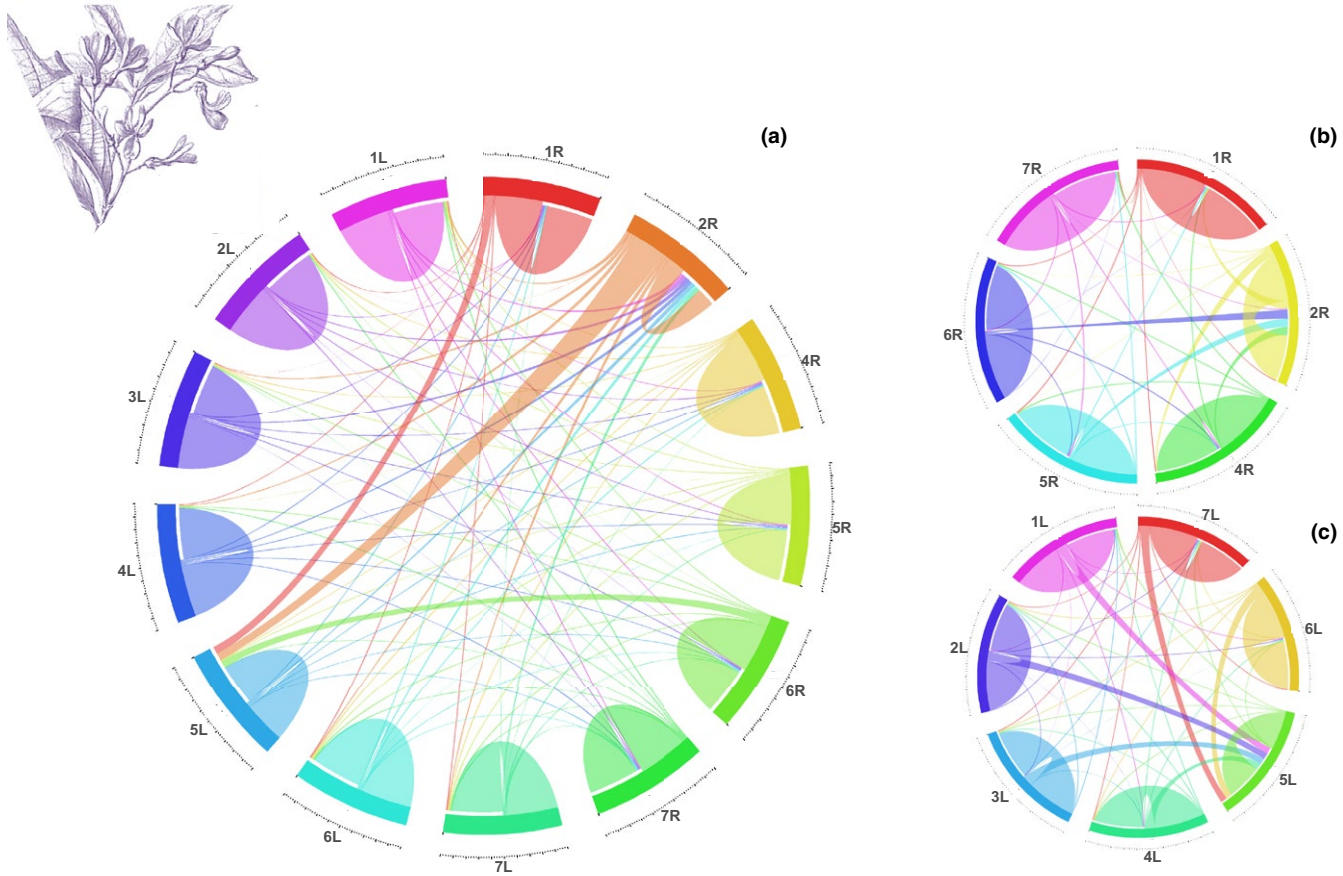


FIGURE 6 Circos plots of contemporary bidirectional migration rates for *Amphirrhox longifolia* (A. St.-Hil.) Spreng sampling locations between banks (a) and for locations in right (b) and left (c) banks of the Rio Branco in the Amazon Basin. Each ribbon has a direction and colour—it starts at the row segment that it touches and ends at the column segment that it does not touch. For instance, in a, sampling location 2R is shown in orange and sampling location 5L is shown in light blue. Hence, the flow from sampling location 2R to sampling location 5L is shown in orange, while the much smaller counter flow from location 5L to location 2R is shown in light blue. The amount of migration rate is indicated by tick marks on the outside of the circle's segments

These results strengthen our findings that the Rio Branco is not a genetic barrier for these three animal-dispersed plant species.

3.3 | Contemporary levels of gene flow in the Rio Branco

Contemporary and bidirectional migration rates estimated between all sampling populations in BayesAss suggested an average migration rate of 0.027 ± 0.037 SE (range 0.014–0.146) for *Amphirrhox longifolia* (Supporting Information Table S6), 0.027 ± 0.021 SE (range 0.015–0.094) for *Passiflora spinosa* (Supporting Information Table S7) and 0.024 ± 0.019 SE (range 0.015–0.093) for *Psychotria lupulina* (Supporting Information Table S8). When all population pairs were considered, migration rates were significantly different between *Amphirrhox longifolia* and *Psychotria lupulina* ($D = 0.455$, $p < 0.001$), and between *Amphirrhox longifolia* and *Passiflora spinosa* ($D = 0.518$, $p < 0.001$). However, there were no differences of the migration rates between *Passiflora spinosa* and *Psychotria lupulina* ($D = 0.127$, $p = 0.335$).

Estimates of short-term gene flow were symmetric for almost all sampling locations in Rio Branco. However, asymmetrical migration rates were detected among some sampling location pairs for *Amphirrhox longifolia* (Supporting Information Table S6), *Passiflora spinosa* (Supporting Information Table S7) and *Psychotria lupulina* (Supporting Information Table S8). For all plant species studied, contemporary gene flow occurred both in upstream and downstream direction. Upstream migration must have taken place mainly by animals for *Amphirrhox longifolia* and *Passiflora spinosa*, since we did not observe any differences in the average of asymmetrical migration rates between upstream and downstream for *Amphirrhox longifolia* (Kolmogorov–Smirnov test = 0.454, $p = 0.2058$) or for *Passiflora spinosa* (Kolmogorov–Smirnov test = 0.556, $p = 0.1243$). On the other hand, significant differences of the average of asymmetrical migration rates between downstream (= 0.0582) and upstream (= 0.0275) (Kolmogorov–Smirnov test = 0.583, $p = 0.034$) indicated that hydrochory can also play an important role in the dispersal of *Psychotria lupulina*.

Migration rates (i.e., the proportion of individuals that move from the corresponding source population) were highest between populations Pop2R and Pop5L for *Amphirrhox longifolia* (Supporting Information Table S6, Figure 6), Pop5R and Pop6R for *Passiflora spinosa* (Supporting Information Table S7, Figure 7) and Pop5R and Pop3L for *Psychotria*

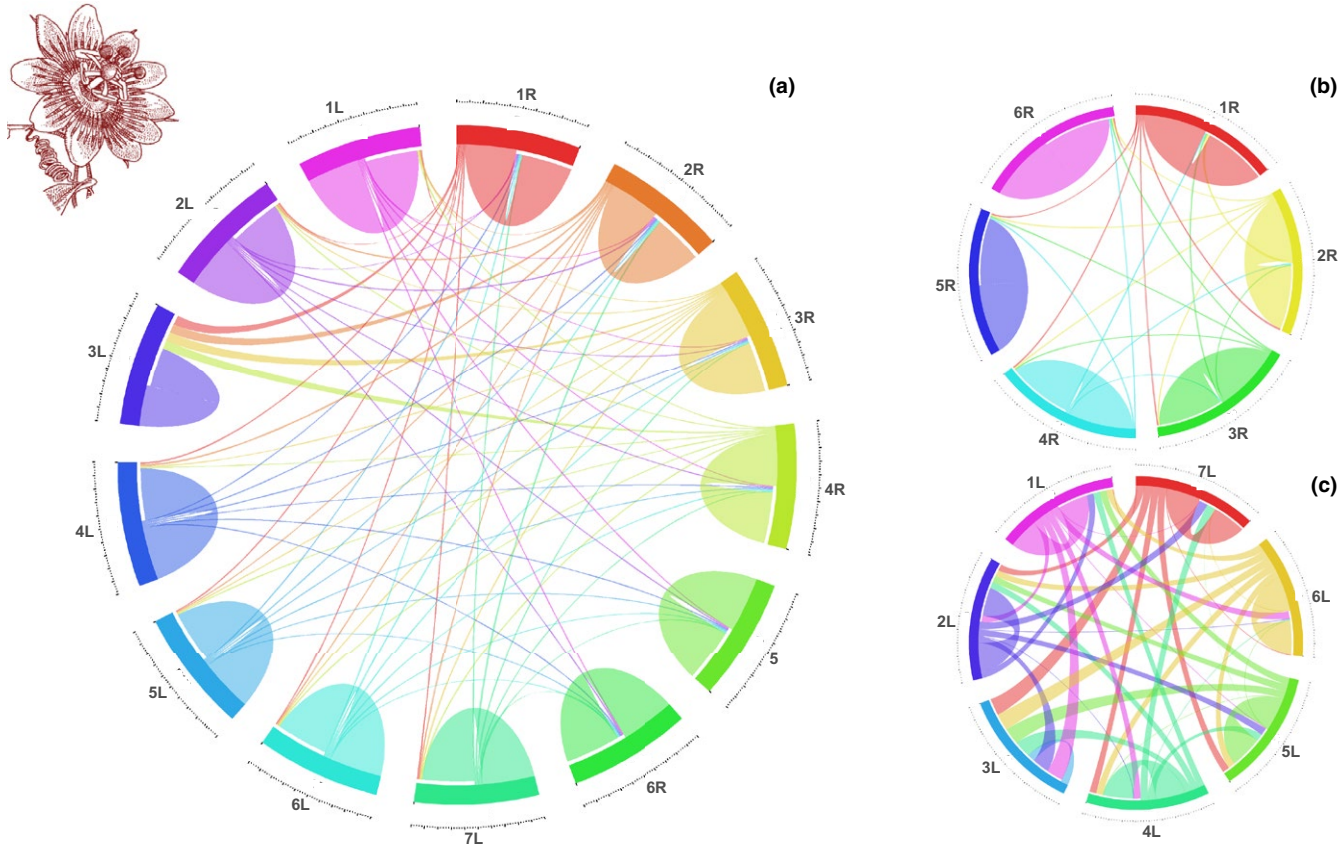


FIGURE 7 Circos plots of contemporary bidirectional migration rates for *Passiflora spinosa* (Poepp. & Endl.) sampling locations between banks (a) and for locations in right (b) and left (c) banks of the Rio Branco in the Amazon Basin. Each ribbon has a direction—it starts at the row segment that it touches and ends at the column segment that it does not touch. In left bank of the Rio Branco (c), for example, sampling location 2L is shown in dark blue and sampling location 7L is shown in red. Hence, the flow from sampling location 2L to sampling location 7L is shown in dark blue, while the much smaller counter flow from location 7L to location 2L is shown in red. The amount of migration rate is indicated by tick marks on the outside of the circle's segments

lupulina (Supporting Information Table S8, Figure 8). Population Pop5L was the greatest sink of migrants for *Amphirrhox longifolia* (Figure 6), while Pop3L was the greatest sink of migrants for *Passiflora spinosa* and *Psychotria lupulina* (Figures 7 and 8). The posterior probabilities observed for *Passiflora spinosa* and *Psychotria lupulina* indicate that a greater proportion of individuals (83% for *Passiflora spinosa* and 67% for *Psychotria lupulina*) have closer migrant ancestry (i.e., the mode of the posterior proportion of first-migrants is much higher than that for the posterior distribution of the proportion of either nonmigrants and second-generation migrants). *Amphirrhox longifolia*, however, presented a different pattern, with a large proportion of individuals within each sampling locality showing estimated ancestry coefficients consistent with 2nd generation migrants (Supporting Information Figure S2). This pattern is expected when high levels of gene flow are observed (Wilson & Rannala, 2003).

4 | DISCUSSION

The population structure observed for the three ecologically similar plant species studied here (i.e., *Amphirrhox longifolia*, *Passiflora spinosa* and *Psychotria lupulina*) showed a congruent lack of genetic structure throughout Rio Branco. The Bayesian and genetic distance-based clustering analyses grouped populations separated by the river into the same groups (Figures 3–5). In the AMOVA analysis, a low proportion of the total variance attributed to the variance across banks strengthened our conclusion that this tributary is not a genetic barrier for *Amphirrhox longifolia*, *Passiflora spinosa* or *Psychotria lupulina*. In addition, for all plant species studied here, the lack of relationship between genetic and geographical distance matrices indicated that historical gene flow via seeds and/or pollen occurred frequently along the two banks of this river. Species-specific traits, such as dispersal abilities, can effect species responses to biogeographical barriers and patterns of genetic and/or species diversity (Ditchfield, 2000). Animal-mediated seed dispersal is considered the most prevalent dispersal syndrome for lowland rain forest tree plant species (Howe & Smallwood, 1982; Willson, Irvine, & Walsh, 1989). Although correlations of dispersal mode with levels of genetic structure for riverine plant species are weak (e.g., Fér & Hroudová, 2008; Nazareno, Dick, et al., 2017; Wei, Meng, Bao, & Jiang, 2015; Zellmer, Hanes, Hird, & Carstens, 2012), plant species that are animal-dispersed tend to have lower levels of genetic structure than species dispersed by other syndromes (Collevatti et al.,

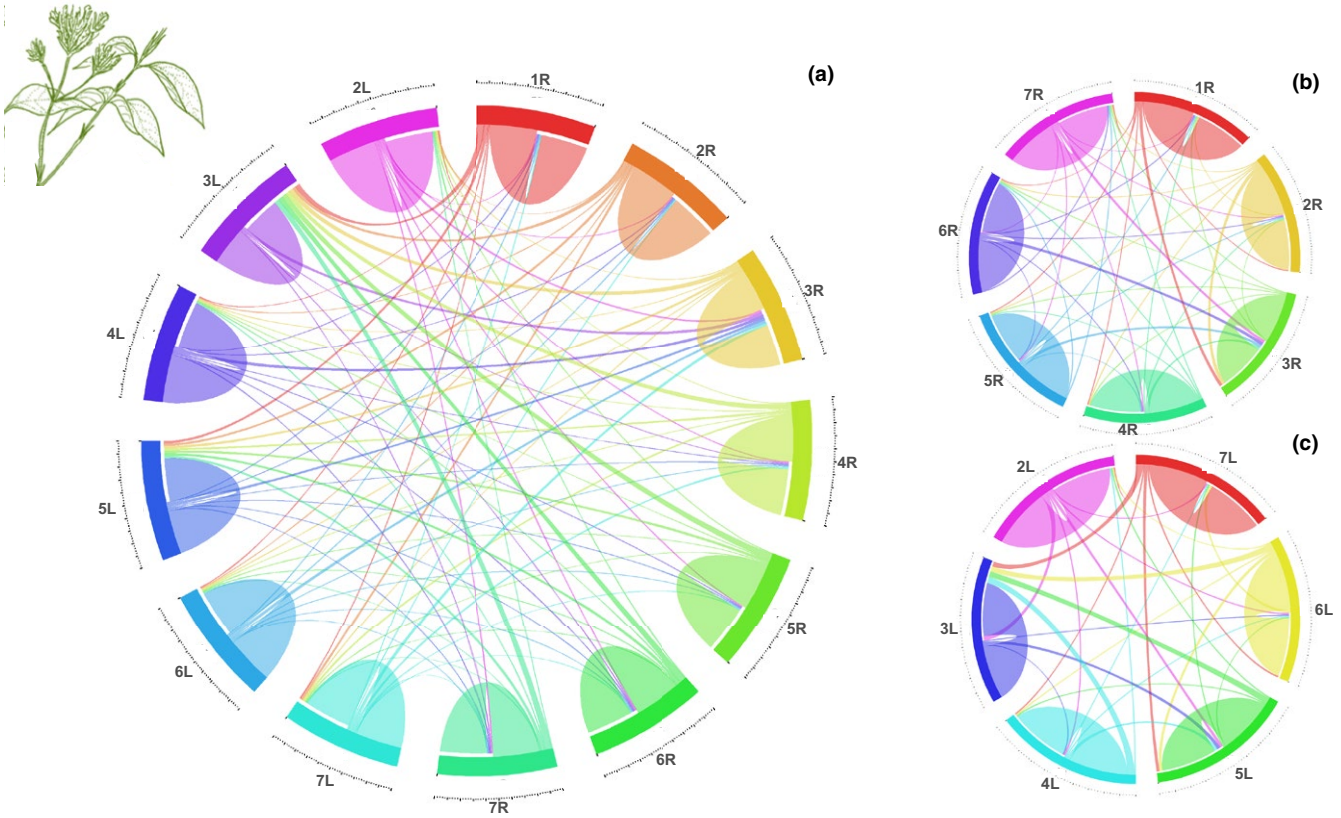


FIGURE 8 Circos plots of contemporary bidirectional migration rates for *Psychotria lupulina* Benth sampling locations between banks (a) and for locations in right (b) and left (c) banks of the Rio Branco in the Amazon Basin. Each ribbon has a direction—it starts at the row segment, which it touches, and ends at the column segment, which it does not touch. For instance, in left bank of the Rio Branco (c), sampling location 5L is shown in green and sampling location 3L is shown in blue. Hence, the flow from sampling location 5L to sampling location 3L is shown in green, while the much smaller counter flow from location 3L to location 5L is shown in blue. The amount of migration rate is indicated by tick marks on the outside of the circle's segments

2009; Fér & Hroudová, 2008, 2009; Hamrick & Godt, 1990; Ray & Excoffier, 2010), slowing down population differentiation (Linhart & Grant, 1996).

As far as within-population genetic diversity is concerned, high levels of H_E were observed mainly for *Passiflora spinosa* (= 0.365) and *Psychotria lupulina* (= 0.376) as the maximum gene diversity observable with biallelic markers such as SNPs is 0.5. At the population level, *Passiflora spinosa*—a liana likely pollinated by hummingbirds and potentially dispersed by fishes and mammals—showed as much genetic variation (i.e., H_E and H_O) as *Psychotria lupulina*—a shrub pollinated by small bees and dispersed by fishes and birds (Macedo & Prance, 1978; Valencia, 2002). The genetic diversity levels reported for *Passiflora spinosa* and *Psychotria lupulina* are in line with those observed for other zoophilous and entomophilous plant species (Ballesteros-Mejia, Lima, Lima-Ribeiro, & Collevatti, 2016). However, the lower levels of genetic diversity observed for *Amphirrhox longifolia*—a plant species potentially dispersed by fishes and with floral traits compatible with pollination by bees and butterflies—suggested that other factors (e.g., mating and breeding systems, growth form, habitat, plant density, lifespan, taxon age) beyond pollination and seed dispersal can be affecting the levels of genetic diversity of this plant species. Although no inbreeding was detected for *Amphirrhox longifolia*, *Passiflora spinosa* or *Psychotria lupulina* in the Rio Branco, mating system should affect the observed levels of genetic diversity. Indeed, mating systems determine how genes are recombined and maintained by individual species, which, in turn, represents the basis of much of their evolution (Ritland & Jain, 1981; Sork et al., 2002). However, in order to fully understand how mating systems affect genetic diversity in the study species, it is important to understand patterns of multilocus and single-locus outcrossing, as well as biparental inbreeding rates.

Contrary to Ritland's (1981) unidirectional diversity hypothesis, the genetic diversity of populations of *Amphirrhox longifolia*, *Passiflora spinosa* and *Psychotria lupulina* did not show a directional pattern. Ritland's hypothesis (1981) predicts that the hydrochoric spread of seeds downstream should result in a downstream increase of genetic diversity, with less diverse upstream populations. This pattern is expected even for zoochorous riparian plant species that are not adapted to hydrocory as these taxa can also be dispersed by water (Boedeltje, Bakker, Bekker, Van Groenendael, & Soesbergen, 2003; Prots et al., 2011). While few studies have

documented a downstream increase of genetic diversity (e.g., Liu, Wang, & Huang, 2006; Pollux, Luteijn, Van Groenendael, & Ouborg, 2009), a meta-analysis using more than 20 riparian plant species reported no evidence of downstream accumulation of genetic diversity (Honnay, Jacquemyn, Nackaerts, Breyne, & Van Looy, 2010). In *Amphirrhox longifolia* and *Passiflora spinosa*, seed dispersal by fishes may explain the lack of higher downstream genetic diversity. Indeed, upstream migration events between populations within and among banks of the Rio Branco were detected, even among distant populations. In *Passiflora spinosa*, for example (Figure 7), the highest upstream migration rates were detected between populations separated by 58.0 km (Pop1R-Pop5L) and 42.1 km (Pop2R-Pop5L). The large-bodied characid *Colossoma macropomum* (i.e., a frugivorous fish and putative disperser of *Passiflora spinosa*) disperses seeds at long distances (>5.0 km) and may have contributed to the long seed dispersal events in *Passiflora spinosa*. Considering that asymmetrical gene flow was observed for *Amphirrhox longifolia*, *Passiflora spinosa* and *Psychotria lupulina*, it is possible that water may have played an important role in long-distance dispersal, impacting seed dispersal of these three animal-dispersed plant species. Indeed, significant differences between the average asymmetrical migration rates downstream and upstream were observed for *Psychotria lupulina*, with gene flow being approximately two times higher downstream. In addition to seed dispersal, gene flow by pollen may also lead to bidirectional gene flow if populations are not too distant from each other. Further studies using molecular markers with different modes of inheritance may contribute to an improved understanding of the role of seed dispersal and pollen movement for the genetic patterns of these riparian plant species.

Overall, our results indicate that the Rio Branco does not seem to represent a current barrier for gene flow nor to have represented a barrier in the past for plant species such as *Amphirrhox longifolia*, *Passiflora spinosa* and *Psychotria lupulina*. Indeed, the frequent gene flow slowed population differentiation within the Rio Branco by promoting genetic admixtures and concomitant population homogenization. Nonetheless, zoological studies also focused on the Rio Branco found support for the Riverine Barrier hypothesis (Bonvicino et al., 2003; Boubli et al., 2008, 2015; Naka et al., 2012). For instance, Boubli et al. (2015), based on mitochondrial cytochrome b DNA sequences, demonstrated that the Rio Branco was an important geographical barrier, limiting the distribution of three primate genera (*Cacajao*, *Callicebus* and *Cebus*) to the western riverbanks and three other genera to the eastern riverbanks (*Pithecia*, *Saguinus* and *Sapajus*). However, the primate species *Alouatta macconnelli* is found on both banks of the Rio Branco and no obvious genetic structure was recovered (Boubli et al., 2015).

Organisms with low dispersal ability are more likely to show hierarchical genetic structure (e.g., Hopken, Douglas, & Douglas, 2013; Mullen, Woods, Schwartz, Sepulveda, & Lowe, 2010; Phillipsen & Lytle, 2013; Ritland, 1989) than plants that can move extensively across rivers (e.g., Collevatti et al., 2009; Fér & Hroudová, 2008, 2009). Although our study was restricted to three plant species and focused on plants with similar dispersal syndromes, the patterns of genetic structure observed here seem to have resulted from species-specific traits. However, this trend is not consistent with what has been observed in other animal-dispersed plant species in wider Amazon rivers, suggesting that the strength of riverine barriers for Amazon plants is also dependent on the width of the rivers separating populations. Indeed, the Rio Negro, a wider Amazonian river than the Rio Branco, can represent a barrier to dispersal in *Amphirrhox longifolia* (Nazareno, Dick, et al., 2017). However, the Rio Negro does not seem to represent a barrier for the low-density and widely distributed canopy-emergent tree species *Caryocar villosum* (Caryocaraceae) that grows in the upland forests, nor to a habitat-specific tree (*C. microcarpum*) that grows in seasonally flooded black-water forests (Collevatti et al., 2009). These results are expected given the long distances of gene flow associated with bat-pollination and seed dispersal by strong swimming tapirs and fish (Collevatti et al., 2009).

Comparative population genomics studies can provide key information for comprehensive assessments of the role of Amazonian waterways on the genetic structure of Amazonian plant species. Our study constitutes the first attempt to document patterns of genetic differentiation for Neotropical plants using a genomic approach. Overall, we demonstrated that medium-sized rivers in the Amazon Basin, such as the Rio Branco, are not important barriers to gene flow for animal-dispersed plant species. Comparative population genomic studies focused on riverine plant species with different life history traits (e.g., mating system, habitat, pollination and dispersal modes), as well as upland plant species, would bring important new insights into this puzzle.

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DATA ACCESSIBILITY

SNP datasets for the *Amphirrhox longifolia*, *Passiflora spinosa* and *Psychotria lupulina* populations are available for download from the Dryad Digital Repository (https://doi.org/10.5061/dryad.f53j0dk).

AUTHOR CONTRIBUTIONS

A.G.N. and L.G.L. designed the study and coordinated sample collection. A.G.N. conducted molecular work, performed analyses, and led the writing of the manuscript with input from all co-authors. C.W.D. provided laboratory assistance, analytical input and troubleshooting.

ORCID

Alison G. Nazareno https://orcid.org/0000-0001-9681-5877
Christopher W. Dick https://orcid.org/0000-0001-8745-9137
Lúcia G. Lohmann https://orcid.org/0000-0003-4960-0587

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SUPPORTING INFORMATION

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

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