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RESEARCH PAPER

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Testing main Amazonian rivers as barriers across time and space within widespread taxa

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Abstract

Aim: Present Amazonian diversity patterns can result from many different mechanisms and, consequently, the factors contributing to divergence across regions and/or taxa may differ. Nevertheless, the river-barrier hypothesis is still widely invoked as a causal process in divergence of Amazonian species. Here we use model-based phylogeographic analyses to test the extent to which major Amazonian rivers act similarly as barriers across time and space in two broadly distributed Amazonian taxa.

Local: Amazon rain forest.

Taxon: The lizard *Gonatodes humeralis* (Sphaerodactylidae) and the tree frog *Dendropsophus leucophyllatus* (Hylidae).

Methods: We obtained RADseq data for samples distributed across main river barriers, representing main Areas of Endemism previously proposed for the region. We conduct model-based phylogeographic and genetic differentiation analyses across each population pair.

Results: Measures of genetic differentiation (based on F_{ST} calculated from genomic data) show that all rivers are associated with significant genetic differentiation. Parameters estimated under investigated divergence models showed that divergence times for populations separated by each of the 11 bordering rivers were all fairly recent. The degree of differentiation consistently varied between taxa and among rivers, which is not an artifact of any corresponding difference in the genetic diversities of the respective taxa, or to amounts of migration based on analyses of the site-frequency spectrum.

Main conclusions: Taken together, our results support a dispersal (rather than vicariance) history, without strong evidence of congruence between these species and

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rivers. However, once a species crossed a river, populations separated by each and every river have remained isolated—in this sense, rivers act similarly as barriers to any further gene flow. This result suggests differing degrees of persistence and gives rise to the seeming contradiction that the divergence process indeed varies across time, space and species, even though major Amazonian rivers have acted as secondary barriers to gene flow in the focal taxa.

KEYWORDS

Amazonia, comparative phylogeography, *Dendropsophus leucophyllatus*, *Gonatodes humeralis*, neotropical diversity, river-barrier hypothesis

1 | INTRODUCTION

Direct links between speciation and diversity patterns with landscape change, in particular, the formation of large rivers, has been a classic biogeographic explanation for the generation and maintenance of strikingly high Amazonia rain forest diversity (Haffer, 1974; Ribas, Aleixo, Nogueira, Miyaki, & Cracraft, 2012). However, given its large geographic extent, very dynamic geomorphological and historical climate setups, and the distinct habitat preferences of species involved, no single process can fully explain Amazonian diversity (Leite & Rogers, 2013; Moritz, Patton, Schneider, & Smith, 2000). Observed diversity patterns can result from many different mechanisms (Hoorn et al., 2010; Rangel et al., 2018) and, consequently, the factors contributing to divergence across regions and/or taxa may differ. Nevertheless, the river-barrier hypothesis (RBH) is still widely invoked as a causal process in the divergence of Amazonian species and populations.

The traditional RBH posits that river establishment split broadly distributed populations, serving as primary barriers to dispersal and prompting diversification and speciation events (Haffer, 1974; Wallace, 1852). Evidence that rivers have acted as strong dispersal barriers (for example the Amazon river; Pomara, Ruokolainen, & Young, 2014), contrasts with evidence that others may be more permeable (for example the Negro river – Smith et al., 2014; the Tapajós river – Moraes, Pavan, Barros, & Ribas, 2016). Permeability of rivers to dispersal may depend on its physical attributes, such as flow rate and margin properties, as well as the species' intrinsic characteristics, including their dispersal abilities and habitat preferences (see Collevatti, Leoi, Leite, & Gribel, 2009—but also see Naka, Bechtoldt, Henriques, & Brumfield, 2012). Differing support for the RBH across individual taxa (e.g. Fernandes, Wink, & Aleixo, 2012; Nazareno, Dick, & Lohmann, 2017) has begun to highlight the complexities surrounding this hypothesis. This includes uncertainty surrounding the geologic history of the region, including debate over a late Miocene (Figueiredo, Hoorn, van der Ven, & Soares, 2009; Hoorn et al., 2010) versus Plio–Pleistocene establishment for the transcontinental Amazon River drainage (Rossetti et al., 2015). These periods actually correspond to two different developmental phases of the main drainage. In the Plio–Pleistocene, the Amazon river (and its tributaries) would have been more entrenched and likely with vast fluctuations in discharge controlled by Quaternary climate fluctuations (see Irion & Kalliola, 2010). Such debate poses specific challenges to biological interpretations regarding the RBH based on estimates of divergence times. For example, estimated divergence times across species of trumpeter birds (genus *Psophia*) that range between 3 and 0.5 million years ago are consistent with the RBH as the main diversification driver if the Amazon drainage was established during the Plio–Pleistocene (Ribas et al., 2012). On the other hand, if the late Miocene hypothesis is more accurate, as some geochemical and palynological data suggests (Hoorn et al., 2017), then lineage persistence in the landscape after dispersal across rivers would become the de facto process structuring species diversity in Amazonia, with rivers acting mostly as secondary barriers (Cowman & Bellwood, 2013). That is, an alternative to the vicariant model of divergence envisioned under the classic RBH is that divergence may be initiated by dispersal events across rivers (see Smith et al., 2014). Lastly, the same river may be involved in the generation and maintenance of species diversity differently, acting as both a primary and secondary barrier (Naka & Brumfield, 2018).

Here, we leverage the resolution afforded by genomic data and a model-based approach with broad geographic sampling across multiple rivers in two common and widespread Amazonian vertebrate taxa with different ecologies to test if major Amazonian rivers act as barriers. The first focal species, *Gonatodes humeralis* (Sphaerodactylidae, Squamata), is a widely distributed semi-arboreal gecko that occurs in a wide range of ecological conditions in Amazonia and northern Pantanal, and is frequently the most common lizard within local assemblages (Ribeiro-Júnior, 2015). A recent analysis of species delimitation for this taxa demonstrated that it is a unique clade, though the processes responsible for the local patterns of population genetic structure is unclear (Pinto et al., 2019). The second taxon, *Dendropsophus leucophyllatus/D. triangulum* (Hylidae, Amphibia) species complex, which hereafter is referred to simply as *D. leucophyllatus* (see Appendix S1), is a small-sized tree frog associated with temporary and permanent ponds across all of Amazonia (Rodriguez & Duellman, 1994). Although no study specifically addressed its phylogeographic structure, the combination of morphological, bioacoustics and phylogenetic analyses of mtDNA suggests that *D. leucophyllatus* likely represents a species complex (Caminer et al., 2017; Peloso, Orrico, Haddad, Lima-Filho, & Sturaro, 2016).

With analyses of ddRADseq, we test the RBH for sampling localities/populations of these two taxa delimited by major rivers whose geographical distribution correspond to the Amazonian Areas of Endemism – AoEs (sensu Silva, Rylands, & da Fonseca, 2005). More specifically, we investigate if there is concordance across both taxa in terms of each river's spatial effectiveness as barrier to gene flow and the divergence times of populations separated by each river. If in fact rivers act as barriers, we expect to find congruent genetic differentiation patterns with limited gene flow between populations separated by rivers in both taxa, although the degree of temporal congruence may differ depending upon when and how differentiation occurred (e.g. a vicariant role of separating previously widespread ancestors vs. an impediment to gene flow following an initial dispersal event across the river; Peres, Patton, & Silva, 1996; Smith et al., 2014). Alternatively, there may be species-specific divergence patterns in which one of the two taxa show a systematic difference in the degree of differentiation across rivers because of divergent traits (ecological, morphological or sexual) that may inhibit gene flow (Zamudio, Bell, & Mason, 2016), or affect population persistence after dispersal across a river (Smith et al., 2014). For example, given that *D. leucophyllatus* exhibits morphological differentiation (Caminer et al., 2017), which is so far unknown for *G. humeralis* (Avila-Pires, 1995; Pinto et al., 2019), the effectiveness of a river barrier may be enhanced by further reductions in gene flow across any common river barrier in the former, but not in the latter. By focusing on broadly distributed taxa, our study avoids the pitfalls of tests that are limited in geographic scope, and with separate analyses for each putative river barrier we avoid the reduced predictive power of generic phylogeographic tests (Papadopoulou & Knowles, 2016).

2 | MATERIALS AND METHODS

2.1 | Areas of Endemism delimited by main Amazonian rivers

Given the focus of our study is on testing the hypothesis of rivers as barriers, we follow the limits of Areas of Endemism (AoEs) as defined by Silva et al. (2005), which are separated by the major rivers and supported by biogeograhic distributions of some terrestrial vertebrates, to refer to specific populations (see Figure 1). These AoEs were used as predefined groups of sampled individuals for subsequent tests of genetic differentiation, and model-based analysis of

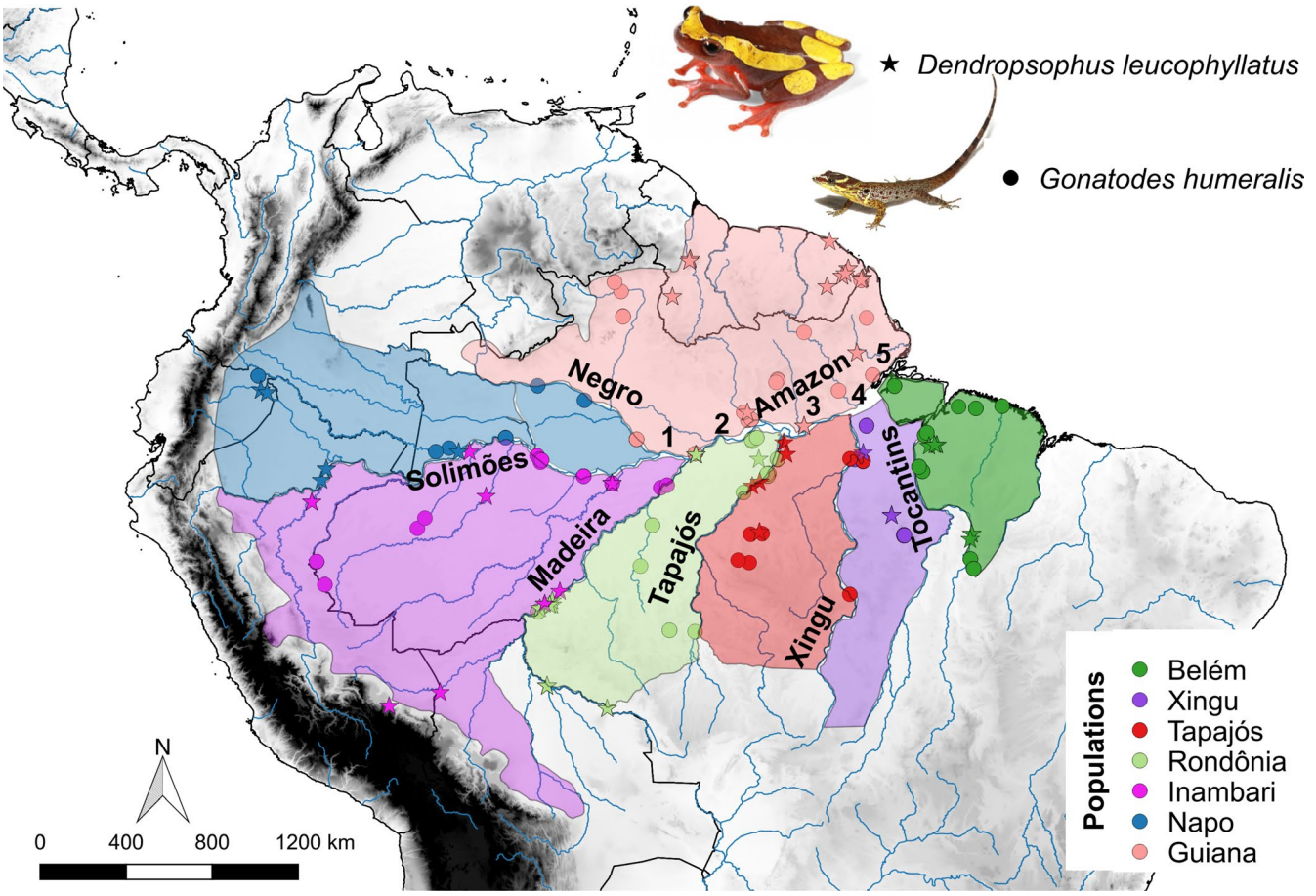

FIGURE 1 Map of sampled localities of *Gonatodes humeralis* (circles) and *Dendropsophus leucophyllatus* (stars) across each species' range, with different colours highlighting populations separated by the main Amazonian rivers; areas are named according to Silva et al. (2005) areas of endemism. Numbers 1–5 represent the five segments of the Amazon River that where considered separately. Photos: tree frog (Santiago Ron), lizard (Rodrigo Tinoco) [Colour figure can be viewed at wileyonlinelibrary.com]

the divergence process. We also verified general agreement between the AoEs limits and population spatial structure (see below).

2.2 | Sampling, ddRADseq genomic data generation and processing

Tissue samples were collected in the field and/or obtained from museum collections of the lizard *G. humeralis* and the tree frog *D. leucophyllatus*. We obtained 194 samples of *G. humeralis* from 37 localities, and 109 samples of *D. leucophyllatus* from 41 localities (Figure 1). Genomic DNA was extracted from the muscle or liver of each individual using the Qiagen DNeasy Blood and Tissue Kit. Two reduced representation libraries for the lizard samples and two for the frog samples were constructed using double digest restriction associated DNA sequencing approach (ddRADseq), following the protocol by Peterson, Weber, Kay, Fisher, and Hoekstra (2012); for details see Appendix S1. Briefly, double digested DNA with ligated unique barcodes from each individual was pooled and 350–450 bp fragments size selected using Pippin Prep (Sage Science). Libraries were sequenced in the Illumina 2,500 platform at The Center for Applied Genomics (Toronto, Canada) to generate 150 bp single-end reads. Genomic data were processed for each taxon separately using STACKS 1.41 pipeline (Catchen, Amores, Hohenlohe, Cresko, & Postlethwait, 2011; Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013) for de novo assembly from the fastQ files from the Illumina sequencing runs. All details regarding bioinformatic processing and data following quality control filters are given in the Appendix S1. All STACKS modules were run under parallel execution with eight threads on the University of Michigan Flux computing cluster.

The software PLINK 1.9 (Chang et al., 2015) was used to filter SNPs and individuals based on the frequency of missing data. For *G. humeralis* we excluded SNPs with more than 10% of missing data, resulting in a final dataset of 160 individuals with 28,474 unlinked SNPs, and a genotyping rate of 0.95. For *D. leucophyllatus*, SNPs with more than 20% of missing data were excluded, resulting in a final dataset of 99 individuals with 1,982 unlinked SNPs, and a genotyping rate of 0.91 (see Table S1 for details in Appendix S1); a higher threshold of missing data was used in the tree frogs given the library was less complete than the lizards.

2.3 | Genetic diversity and genetic differentiation across rivers

Genetic diversity was measured within populations separated by major rivers (Figure 1). Specifically, average nucleotide diversity (π) based on polymorphic sites was calculated directly in STACKS using the POPULATION module (Catchen et al., 2013). To account for potential differences on genetic diversity that could be attributed to an area effect of each AoE, we also investigated for correlation between nucleotide diversity and geographic area in each taxon using R version 3.3.2 (Team, 2016).

Genetic differentiation across each river barrier separating adjacent AoEs was measured by F_{ST}. Specifically, pairwise F_{ST}-values, with their significance assessed from 10,000 bootstrap replicates with a Bonferroni correction for multiple comparisons, were calculated in Arlequin 3.5.2.2 (Excoffier & Lischer, 2010) across a total of 11 river barriers (see Figure 1). Because of insufficient samples, we did not consider the Napo and Imeri as separate AoEs (as defined by Silva et al., 2005); this is a practical decision and does not imply in any questioning about the validity of these two bioregions (e.g. Borges & Silva, 2012). Samples from the Marajó Island were considered as part of the Belém AoE given that geological sediment similarities suggest a shared regional history (Rossetti, Almeida, Amaral, Lima, & Pessenda, 2010). We evaluate genetic differentiation across five different segments of the Amazon River separately (see Figure 1), given that the Amazon River borders several areas dissected by other major rivers. To evaluate the effect of rivers as barriers on the partitioning of genomic variation among populations, within populations and individuals, we used a hierarchical analysis of molecular variance (AMOVA) using Arlequin 3.5.2.2 (Excoffier & Lischer, 2010), by estimating their statistical significance based on 20,000 random permutations.

2.4 | Estimated effective migration surfaces

To evaluate if population structure of both taxa supports the predefined population groups based on the geographic limits of AoEs, we used the Estimated Effective Migration Surfaces method (EEMS; Petkova, Novembre, & Stephens, 2016) that analyses population structure from geo-referenced genetic samples and identifies barriers separating areas where the decay of genomic differences across geographical distances is higher than expectations of a model of isolation by distance. With this approach, a triangular grid spanning the entire geographic range of sampling (i.e. from a sampling area covering most of the Amazonia biome and parts of the Cerrado biome, from −44 to −77 degrees of longitude and −19 to −6 of latitude for both species) divides the distribution of each species, in this case into 600 demes. Each individual is assigned to the nearest vertex of the grid and the migration parameter *m* is estimated by Bayesian inference for every edge of the grid by Markov chain Monte Carlo (MCMC) sampling based on observed genetic dissimilarities based on the unlinked SNPs. When viewed graphically across the species distributions, areas of reduced gene flow can be visualized based on estimates of the posterior probabilities of *m*. We performed 10 independent runs to assess convergence, with 10 million MCMC iterations, with 2 million burn-in and a thinning of 9,999 used for each run. Convergence among runs was accessed with the package in R rEEMSplots, available with the EEMS pipeline.

2.5 | Divergence history for each river barrier and species

For each river barrier, parameterized divergence models were estimated using a composite-likelihood method based on the joint site frequency spectrum (SFS) implemented in FASTSIMCOAL2 (Excoffier, Dupanloup, Huerta-Sanchez, Sousa, & Foll, 2013). Datasets were

reprocessed for each river barrier separately using the POPULATION module from STACKS pipeline (Catchen et al., 2013) and PLINK software (Chang et al., 2015) to maximize the number of loci for each separate analysis. Specifically, POPULATION was run to obtain vcf files with unlinked SNPs and the folded joint SFS (i.e. minor allele) using a python script for each separate FASTSIMCOAL analysis (available on Github/KnowlesLab; He & Knowles, 2016). Note that the requirement of no missing data precluded a global analysis considering all barriers in a single model (see Excoffier et al., 2013). For *G. humeralis*, the SFS was calculated based on 20–30 individuals per analysis, whereas 10–20 individuals were analysed for *D. leucophyllatus* to maximize the number of loci that could be included in each analysis, given *D. leucophyllatus* had fewer individuals with a common set of loci.

Divergence models were estimated with and without a migration parameter. The time of divergence, T_{DIV}, the population size of the other population, N_2, the ancestral population size N_{ANC}, and migration, m (for those models that included migration), were estimated from the SFS using uniform priors (see Table 1 for details). For all divergence models, the effective population size of one population (N_1) was fixed to improve the accuracy of parameter estimates from the SFS—following the recommendations of the program (Excoffier & Foll, 2011). Specifically, N_1 was calculated directly from the empirical data based on nucleotide diversity (π) of variant and invariant sites, where $\pi = 4N\mu$, assuming a mutation rate of 3.25×10^{-8} for *G. humeralis* and 3.46×10^{-8} for *D. leucophyllatus*. These rates were estimated from the regression formula for cellular organisms (Lynch, 2010) based on genome sizes estimated in related species (i.e. *Teratoscincus scincus* for *G. humeralis* and *Dendropsophus microcephalus* for *D. leucophyllatus*; www.genomesize.com), and considering one generation per year (Crump, 1974; Vitt, Magnusson, Ávila-Pires, & Lima, 2008). To account for potential errors in mutation rates estimates derived from estimates of genome sizes, we also conducted another set of analyses using a lower mutation rate from the literature based on estimates from mitochondrial DNA (i.e. 6.45×10^{-10} for *G. humeralis* Prates, Rivera, Rodrigues, & Carnaval, 2016 and 7.35×10^{-9} for *D. leucophyllatus* Gehara et al., 2014). A total of 40 runs were conducted for each river barrier and we present the point estimate of the highest likelihood across runs, as well as 95% confidence intervals on the parameter estimates calculated using a parametric bootstrap of 100 simulated datasets. Each analysis was based on 100,000–250,000 simulations for likelihood estimation with a stopping criterion of 0.001, and 10–40 expectation-conditional cycles (ECM).

3 | RESULTS

3.1 | Genetic diversity and differentiation across rivers

Genetic differentiation (F_{ST}) was generally high in both the gecko and the frog, although F_{ST}-values varied across river barriers, and *D. leucophyllatus* consistently had higher levels of genetic differentiation between populations across rivers than *G. humeralis* (Figure 2a), with an average F_{ST} of 0.3924 (±0.1421 *SE*) and 0.1324 (±0.0532 *SE*), respectively (see Tables S2 and S3 in Appendix S1). The only river barriers where populations exhibited similar levels of genetic differentiation in both taxa were the Madeira and Solimões rivers (Figure 2a).

Estimates of genetic diversity (π) were comparable across populations in each species (Figure 2b). This indicates that the consistently lower F_{ST}-values among populations of *G. humeralis* were not due to a systematically higher genetic diversity in this species, which would confound comparisons of F_{ST} between the taxa (see Cruickshank & Hahn, 2014). Instead, the only substantial difference in genetic diversity between the taxa was in two populations—the Inambari and Napo. However, in both cases diversity was slightly lower in *G. humeralis* than in *D. leucophyllatus*, indicating that the higher differentiation observed in *D. leucophyllatus* is not an artifact of depressed genetic diversity. In fact, the higher genetic diversity observed in *D. leucophyllatus* in the Inambari and Napo populations (Figure 2b) contributes to the fairly similar levels of genetic differentiation in both species for the Solimões and Madeira river barriers (Figure 2a), the only two cases in which F_{ST}-values did not differ between taxa. Genetic diversity was not significantly correlated with the geographic area of a region in either taxa ($p = .87$ for *G. humeralis* and $p = .17$ for *D. leucophyllatus*; see Figure S2 in Appendix S1). An AMOVA showed significant structuring of genetic variation among populations in both species (Table S4 in Appendix S1), explaining 19.19% and 22.26% of the variance in *G. humeralis* and *D. leucophyllatus*, with the bulk of the variance attributed to variation among and within individuals (for details see Table S4 in Appendix S1).

Evidence of significant genetic structuring among populations identified in the AMOVA (Table S4 in Appendix S1) was supported, in part, by the Bayesian analysis EEMS (i.e. the inferred contours of barriers correspond to the rivers; Figure 3). We see reduced gene flow corresponding to most, but not all the rivers. Notably, reduced migration was not inferred for the Western Amazonian region in the lizard *G. humeralis*, in contrast to the strong correspondence between inferred areas of reduced migration and the geographic position of rivers. In the frog *D. leucophyllatus* the EEMS map showed some, but less correspondence than in the lizards, between areas of inferred reduced migration and the geographic position of rivers, most notably for the Xingu and Tapajós rivers (Figure 3) to the south of the Amazon River (Figure 1). In both species, we note that the inferred reduced migration areas do not match exactly the geographic coordinates of the rivers; however, given the method divides the species' distributions into triangular grids that may or may not match exactly the position of rivers, this is not all that unexpected. For this reason, we focus more on whether there are areas of inferred migration that resemble the geographic configuration of rivers in our analyses.

3.2 | Parameterized divergence models

Several generalizations emerge from comparing the divergence model parameters estimated for each separate river barrier for *G. humeralis* and *D. leucophyllatus* (Table 1). First, estimated divergence times were relatively recent for each of the 11 rivers

TABLE 1 Parameter estimates for divergence models of each putative river barrier listed in order from the west to east, as well as the number of loci analysed for each test (see methods for details)

	Gonatodes humeralis					*Dendropsophus leucophyllatus*				
Rivers	**Loci**	N_1	N_{ANC}	N_2	T_{DIV}	***Loci***	N_1	N_{ANC}	N_2	T_{DIV}
Solimões	20,640	24,615	3,303 (3,073–3,456)	10,134 (10,124–11,314)	1,314 (1,291–1,439)	1,731	52,023	22,051 (16,901–24,529)	28,436 (24,581–35,287)	13,568 (12,076–16,126)
Negro	16,895	18,462	19,268 (17,311–19,767)	130,381 (120,975–137,223)	12,836 (12,640–13,432)	1,388	40,462	32,968 (25,101–37,540)	35,056 (30,158–42,031)	21,791 (20,361–25,064)
Amazon 1	16,732	24,615	7,448 (6,691–10,546)	47,728 (44,527–197,192)	4,630 (4,485–7,210)	1,362	33,237	17,945 (9,376–23,843)	21,740 (19,112–25,229)	44,011 (41,642–51,610)
Madeira	17,666	35,385	6,524 (5,987–6,724)	27,854 (26,671–30,270)	3,896 (3,805–4,141)	1,257	39,017	46,711 (37,194–54,154)	218,803 (172,007–286,268)	18,593 (16,931–21,223)
Amazon 2	14,580	35,385	8,361 (1,705–10,777)	60,325 (56,642–440,863)	4,259 (4,105–426,533)	2,396	39,017	41,931 (32,246–43,453)	41,110 (36,771–47,054)	27,949 (26,697–31,713)
Tapajós	10,502	35,385	12,389 (11,058–13,039)	51,629 (47,488–56,603)	7,142 (6,890–7,717)	1,460	39,017	73,558 (53,952–79,708)	82,053 (75,195–95,965)	41,816 (40,271–47,834)
Amazon 3	11,657	29,231	10,775 (9,745–11,125)	74,159 (69,453–84,456)	6,177 (5,949–6,614)	1,229	33,237	30,143 (1,418–42,233)	50,845 (44,578–58,235)	96,906 (89,812–140,218)
Xingu	10,245	29,231	12,539 (11,025–13,038)	42,683 (40,000–46,299)	7,379 (7,166–7,999)	10,181	27,457	11,761 (10,211–11,964)	10,849 (10,307–11,869)	8,035 (7,936–8,808)
Amazon 4	5,902	29,231	14,511 (12,932–15,881)	122,242 (110,313–142,178)	8,782 (8,335–9,461)	1,328	33,237	20,863 (8,052–25,789)	32,281 (28,621–37,146)	55,355 (53,002–66,591)
Tocantins	11,036	29,231	6,899 (6,259–7,413)	18,187 (17,063–20,008)	3,508 (3,379–3,824)	10,729	15,896	20,641 (17,746–21,443)	102,797 (94,194–111,828)	18,755 (18,488–20,010)
Amazon 5	15,265	29,231	8,973 (1,485–9,608)	68,324 (63,930–542,516)	6,128 (5,924–358,416)	1,308	33,237	66,923 (1,776–86,050)	79,125 (68,380–89,123)	116,698 (110,045–170,130)

Note: The highest likelihood, as well as the 95% confidence interval for each parameter estimate (in parentheses) across 40 runs of FASTSIMCOAL2 (Excoffier et al., 2013) are shown for the models without migration. Note that the population size N_1 was calculated directly from the empirical data (i.e. it is a fixed parameter in the model) to improve the accuracy of the other estimated parameters (i.e. the second population size, N_2, and the ancestral population size, N_{ANC}, and divergence time, T_{DIV}) from the SFS (following the recommendations for the program; see Excoffier & Foll, 2011). Divergence models were also estimated with migration, m; however, estimates of m were 0 or always <0.00001, so only results from the divergence model without migration are reported.

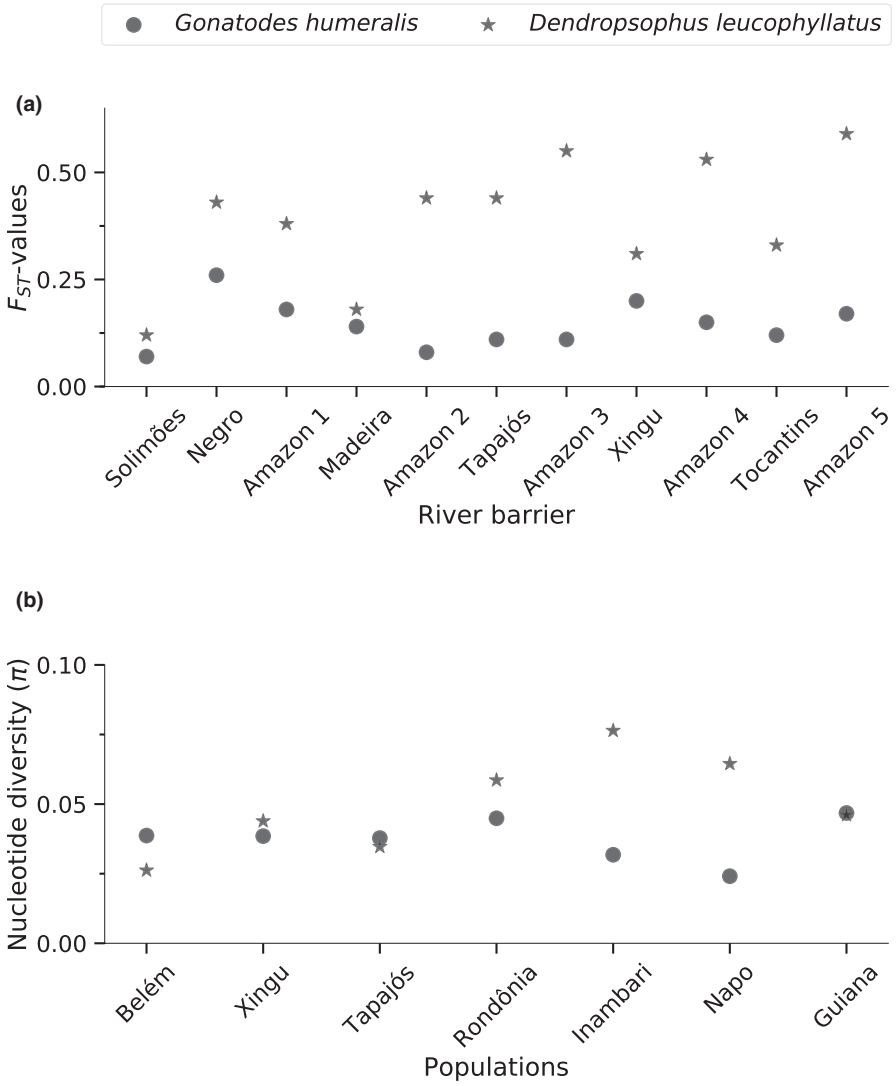

FIGURE 2 (a) Estimated pairwise F_{ST}-values between areas separated by a major river (i.e. labels identify the river barrier) along with (b) estimated genetic diversity, as measured by average nucleotide diversity, π, in *Gonatodes humeralis* (circles) and *Dendropsophus leucophyllatus* (stars)

separating populations in both species, with all divergence occurring within the last 116 kya (see Table 1), or within the 650 kya when applying a lower mutation rate (see Table S5 in Appendix S1). However, divergence times varied among river barriers and differed between the species (Table 1). For example, a divergence time of 4,630 years was estimated in *G. humeralis* for segment 1 of the Amazon river (Figure 1) compared to 44,011 years in *D. leucophyllatus*.

Although for any given river barrier the timing of divergence differed between species, there was a consistent pattern with respect to the relative timing of divergence across river barriers. Specifically, as with the patterns of genetic differentiation based on F_{ST}-analyses (Figure 2a), parameterized divergence models consistently estimated older divergence times for *D. leucophyllatus* compared with *G. humeralis* (Table 1), except for the Xingu river. Both species also showed no evidence of ongoing gene flow among populations separated by the different river barriers. Parameter estimates of migration, *m*, were always 0 or <0.0001, indicating negligible gene flow; therefore, only the results from divergence models without migration are presented (Table 1). Note that the prior on *m* spanned very low to relatively high levels of migration to accommodate a range of values (i.e. a log-uniform prior of 1e-8 to 1e2).

Consideration of the relative divergence times estimated for each pair of populations separated by a river barrier showed the order of divergence times was not congruent between species. For example, the oldest divergence time for *G. humeralis* was associated with the Negro river, followed by Amazon 4, Xingu, Tapajós, Amazon 3, Amazon 5, Amazon 1, Amazon 2, Madeira and the Tocantins followed by the Solimões with the most recent divergence time. For *D. leucophyllatus* the order differed, with the oldest divergence time associated with the Amazon segment 5, followed by Amazon 3, Amazon 4, Amazon 1, Tapajós, Amazon 2, Negro, Tocantins, Madeira, and the Solimões followed by the Xingu with the most recent divergence time. In general, there was not any obvious consistency in divergence between species with respect to river size (Table S6), or flow direction (e.g. headwater to the mouth of Amazon river; Table 1).

4 | DISCUSSION

By considering the divergence history associated with different rivers separately, our study tested not only for similarities, but also for how rivers might differ as barriers across time, space and

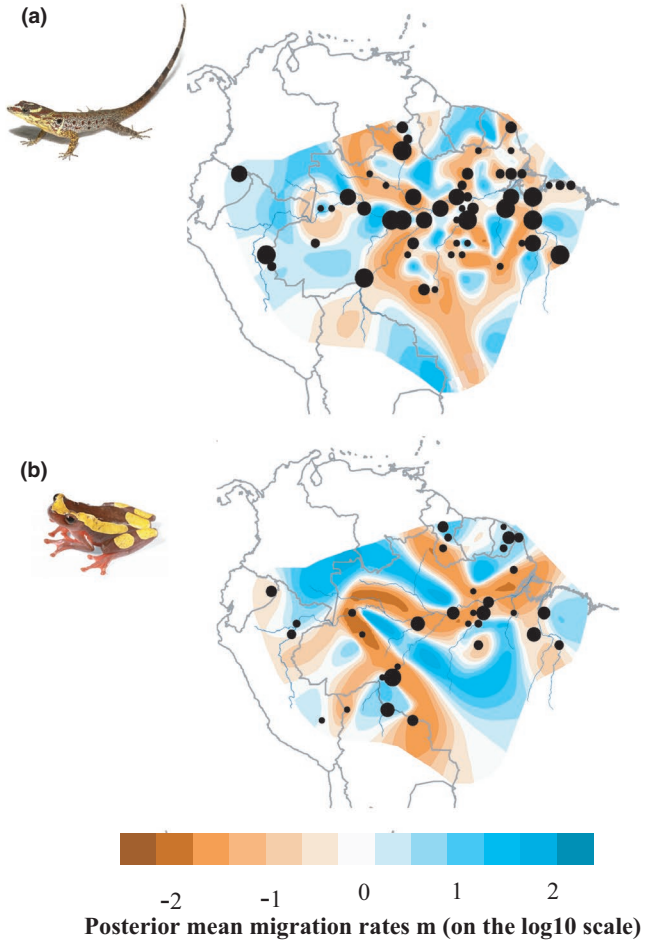

FIGURE 3 Estimated effective migration surfaces (EEMS) plots showing the effective migration rates (*m*) on a $\log_{10}$ scale between all samples of (a) *Gonatodes humeralis* and (b) *Dendropsophus leucophyllatus*. Geographic regions of low migration are shown in orange, representing migration barriers. Areas in blue represent geographic regions where the genetic similarity is higher, or in other words, where samples are connected by migration rates higher than that expected under isolation by distance. The dots represent the sampled localities, and show localities with more samples as the difference in the relative size of the dots. The mean migration across the 10 independent EMMS runs is presented in each species. Photos: lizard (Rodrigo Tinoco), tree frog (Santiago Ron) [Colour figure can be viewed at wileyonlinelibrary.com]

species. Specifically, genomic samples of the two broadly distributed Amazonian taxa we studied (the lizard *G. humeralis* and the tree frog *D. leucophyllatus*) show significant genetic differentiation associated with each of the 11 tested rivers borders separating populations, and with little to no ongoing migration across rivers (Table 1), the data are consistent with rivers acting similarly as barriers to gene flow (see Figure 2 and Table S3 in Appendix S1). However, divergence times were too recent to support a vicariant history directly linked to the formation and establishment of Amazonian rivers (even if mutation rates were an order of magnitude lower than the ones applied here). Moreover, the relative timing of divergence differed among taxa, suggesting the lack of a common history affecting them at the local level of individual rivers (Table 1; see also Naka & Brumfield, 2018). Interestingly, the degree of differentiation consistently varied between taxa, which is not an artifact of any corresponding difference in the genetic diversities of the respective taxa (Figure 2 and Table S4 in Appendix S1). Consequently, even though rivers are effective secondary barriers to gene flow, they differ in terms of when they became a barrier and taxon-specific histories are an important consideration for understanding temporal differences among rivers regarding when divergence was initiated (i.e. impediments to dispersal, instead of vicariant barriers; Cowman & Bellwood, 2013; Naka & Brumfield, 2018).

4.1 | Similar but different

There is no doubt that Amazonian rivers can act as barriers to gene flow in some instances. Support for the RBH ranges from classic studies based on distributional data or divergence times across river banks estimated from a few sequenced loci (Aleixo, 2004; Boubli et al., 2015; Foley et al., 2014; Funk et al., 2007) to recent analyses based on multiple loci (e.g. Lutz, Weckstein, Patane, Bates, & Aleixo, 2013; Naka & Brumfield, 2018; Nazareno et al., 2017). However, once studies move beyond a focus on a specific river, and as data accumulate across more taxa, support for rivers as either primary or secondary barriers became mixed (e.g. Nazareno et al., 2017; Solomon, Bacci, Martins, Vinha, & Mueller, 2008). As such, it is difficult to draw generalizations about why one river, but not another, may impede gene flow, and only in certain taxa (see Ayres & Clutton-Brock, 1992). As we advocate here, to maximize insights, we need to move beyond concordant or discordant divergence histories. Instead, through the study of multiple, widespread species we can consider the diversity of processes that might underlie the observed genetic differentiation associated with river barriers without the confounding factors that arise from tests in which the constituent set of taxa used in tests differ across rivers (e.g. Smith et al., 2014).

Amazonian rivers have different characteristics such as pH, type of water, stability and origin and geomorphological history (see Table S6 in Appendix S1). Such differences may influence the propensity for species dispersal across rivers (e.g. Ayres & Clutton-Brock, 1992; Naka et al., 2012). In this study, and as secondary barriers, the rivers seem to act in a similar way across taxa (i.e. they impede ongoing gene flow and partially structure populations). Nonetheless, there are notable differences across rivers and taxa: the timing of divergence varies among rivers and there is no chronological order of these divergences shared between the analysed species (Table 1). When these similarities and differences are considered jointly, they point to avenues of further exploration to improve our understanding of the varying role of rivers in the divergence history of Amazonian taxa.

As widespread taxa with divergence times that post-date the rivers' origins (i.e. independent of the geological scenario for the establishment for the transcontinental Amazon River drainage divergence times are more recent), both *G. humeralis* and *D. leucophyllatus* must have crossed rivers at least once as they obtained their current ranges, which encompass all of Amazonia (Figures

1 and 3). Empirical evidence suggest that a simple colonization with range expansion is unlikely to have occurred (e.g. Lima et al., 2017). The relative timing of divergence across rivers differs between taxa, which, unlike absolute divergence time estimates (see Table 1), is not contingent upon specific mutation rates used with applications of the molecular clock, and the timing of divergence does not follow an obvious geographic pattern. This points us to a divergence process with cyclical periods of connections and isolations (Haffer, 2008), where previous dispersal events across rivers prompted by recent meander belt dynamics are likely. Indeed, recent studies suggest a very dynamic drainage network in the Amazonian lowlands during the Quaternary, with frequent river captures and fluvial changes affecting the barriers' stability and permeability (Pupim et al., 2019; Ruokolainen, Massaine Moulatlet, Zuquim, Hoorn, & Tuomisto, 2018). Moreover, changes in water availability and vegetation distribution caused by climatic changes might also have promoted repeated periods of gene flow and isolation of populations separated by the river barriers (Cheng et al., 2013; Haffer, 2008). Admittedly, with any genetic signature of this older history overridden by the divergence in allopatry that dates to a relative recent past (Table 1), it is not clear why earlier dispersal events were not associated with long-term isolation, especially considering the persistence of isolation over the tens to hundreds of thousands of years documented here in both species, and even longer in some cases within *D. leucophyllatus*. Given the analysed taxa are widespread and common where they occur, it is unlikely that local extinctions (at least at the geographic scale examined here; Figure 1), are driving colonization dynamics (see Papadopoulou & Knowles, 2017). Instead, it is more likely that abiotic factors associated with shifting river physiography (e.g. flood-pulse patterns, Junk et al. (2011), and river captures, Rossetti, Bertani, Zani, Cremon, and Hayakawa (2012)) might be at play, for some, but most likely not all rivers. For example, recent divergences were observed for the Solimões in both species, whereas relatively older divergences were observed for the Tapajós, Amazon 4 and Amazon 3 in both species, which suggest that patterns of genetic divergence could be reflecting a shared response to a common abiotic factor (see Table S6 in Appendix S1). In contrast, the Negro and the rest of the Amazon river segments show opposing divergence patterns between the taxa, with very recent divergence times in *G. humeralis* but some of the oldest divergence events estimated in *D. leucophyllatus*. We note that these contrasting divergence times cannot be explained by difference in mutation rates between the lizard and frog taxa given the covarying divergence patterns between the taxa for the other aforementioned rivers (Figure 1), but the very recent divergence times in *G. humeralis* might explain the highest portion of the genetic variation being allocated within individuals of *G. humeralis* (Table S4 in Appendix S1).

Biotic factors might also influence the genetic divergence associated with rivers, and in particular, the effectiveness of the barrier itself (Nosil, Harmon, & Seehausen, 2009). However, in the case of our focal taxa, both are found in different Amazonian forest types, including "*várzea*" (floodplain forest) and "*terra firme*" (upland forest; Ribeiro-Júnior, 2015; Rodriguez & Duellman, 1994, personal observations). This suggests no obvious differences in the restrictions imposed by the landscape on their respective dispersal abilities, unlike other taxa where dispersal across rivers is influenced by the surrounding habitat (Collevatti et al., 2009). However, lizards may be more capable of dispersal in general than frogs, since they have fewer environmental restrictions (Duellman,), which might contribute to the consistently lower F_{ST}-values observed in *G. humeralis* (Figure 2). For instance, *G. humeralis* is a semi-arboreal lizard easily observed on tree trunks where they inhabit and reproduce (Vitt et al., 2008), and could have passively dispersed more frequently between river banks with floating vegetation (a pattern not unexpected for the group, since even trans-Atlantic dispersal has been documented for related gecko lizards; Gamble et al., 2011). *Dendropsophus leucophyllatus*, though also a common species, has a reproduction mode directly related to lentic water systems (temporary and permanent ponds), where the eggs are deposited for the hatching of the tadpoles (Rodriguez & Duellman, 1994). It is also possible that the consistent tendency of greater genetic differentiation (Figure 2) and older divergence times in *D. leucophyllatus* than *G. humeralis* (Table 1) may reflect the decreased gene flow and/or more long-term effectiveness of the barrier because of local adaptation within each of the AoEs/river interfluves (Ortiz, Lima, & Werneck, 2018). For example, the tree frog, unlike the lizard, exhibits phenotypic differentiation across its range that might impact interbreeding (Caminer et al., 2017).

4.2 | Rivers as drivers of species diversity

The role of rivers in preventing gene flow and promoting diversification is supported by the existence of AoEs, such as those delimited by the borders of major Amazonian rivers, and which are supported by diversity patterns of different taxonomic groups (Fernandes et al., 2012; Godinho & da Silva, 2018; Lynch Alfaro et al., 2015). Our work extends these findings to population divergence of frogs and lizards, but we cannot predict if the observed genetic structure of *D. leucophyllatus* and *G. humeralis* associated with river barriers could promote speciation in these taxa over time (Sukumaran & Knowles, 2017). Dates based on phylogenetic studies suggest that species diversification predates population divergence by hundreds of thousands to millions of years in both species (e.g. Duellman, Marion, & Hedges, 2016; Gamble, Simons, Colli, & Vitt, 2008).

In several Amazonian taxa, population differentiation within species is commonly correlated with their speciation rates (Harvey et al., 2017). To the extent that this finding is generalizable, the significant genetic structure and relatively older divergence times between *D. leucophyllatus* populations compared to those of *G. humeralis* suggests that genetic divergence has proceeded further along the speciation continuum (see Huang & Knowles, 2016) in the former. Indeed, recent taxonomic work suggests that *D. leucophyllatus* constitutes a species complex (see supplemental information: *D. leucophyllatus* taxonomy; Caminer et al., 2017). However, we note that genetic differentiation associated with the proposed new putative species (i.e. Inambari population; Caminer et al., 2017) is not any

more pronounced than the intraspecific differentiation associated with other river barriers (see Table S2, S3 and S6 in Appendix S1).

5 | CONCLUSION

It is well known that large rivers can limit the distribution of some species without necessarily indicating that they represent vicariant barriers that caused allopatric speciation (Losos & Glor, 2003), but few studies focus on widespread taxa, where the primary role of rivers is likely as secondary barriers (i.e. impediments to dispersal; Cowman & Bellwood, 2013), at least for taxa whose current ranges post-date the origin of the river barriers (e.g. Moraes et al., 2016; Naka & Brumfield, 2018). Our work shows that the rivers indeed act as barriers restricting gene flow (Cowman & Bellwood, 2013; Naka & Brumfield, 2018). However, with the divergence times of each river varying, and the relative divergence times differing between species, our work supports the contention that the barriers represented by rivers may be much more dynamic than classic views of vicariant histories promoted by river formation (Lynch et al., 2015; Ribas et al., 2012). Moreover, by detailing how genetic divergence varies temporally, spatially and across taxa, our work lends support to the hypothesized proposal of cyclical periods of connections and isolations (i.e. a transient barrier; Cowman & Bellwood, 2013), that could be caused by both climatic fluctuations and recent river dynamics. Moreover, with consistent differences in the degree of differentiation between these two common and widespread Amazonian taxa, ecological preferences and local adaptation may potentially influence the long-term effectiveness of river barriers by further reducing gene flow during the divergence history of the tree frog *D. leucophyllatus* relative to the gecko *G. humeralis*. Only with future studies that consider support for these alternative modes of divergences, as opposed to focusing exclusively on tests of concordance across taxa or vicariance, will the diversity of processes associated with rivers as barriers begin to be better understood.

CONFLICT OF INTEREST

We declare we have no competing interests.

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DATA AVAILABILITY STATEMENT

Input files for all analyses have been deposited in the Dryad Digital Repository (https://doi.org/10.5061/dryad.ck223mq).

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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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