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

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66

67 **Competing interests**

68 We declare we have no competing interests.

69

70 **Abstract**

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

77 **Local:** Amazon rainforest.

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

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

83 **Results:** Measures of genetic differentiation (based on F_{ST} calculated from genomic data) show
84 that all rivers are associated with significant genetic differentiation. Parameters estimated under
85 investigated divergence models showed that divergence times for populations separated by each

86 of the 11 bordering rivers were all fairly recent. The degree of differentiation consistently varied
87 between taxa and among rivers, which is not an artifact of any corresponding difference in the
88 genetic diversities of the respective taxa, or to amounts of migration based on analyses of the site-
89 frequency-spectrum.

90 **Main conclusions:** Taken together, our results support a dispersal (rather than vicariance)
91 history, without strong evidence of congruence between these species and rivers. However, once
92 a species crossed a river, populations separated by each and every river have remained isolated –
93 in this sense, rivers act similarly as barriers to any further gene flow. This result suggests
94 differing degrees of persistence and gives rise to the seeming contradiction that the divergence
95 process indeed varies across time, space, and species, even though major Amazonian rivers have
96 acted as secondary barriers to gene flow in the focal taxa.

97
98 **Keywords:** river barrier hypothesis, *Dendropsophus leucophyllatus*, *Gonatodes humeralis*,
99 Amazonia, comparative phylogeography, Neotropical diversity.

100

101 INTRODUCTION

102 Direct links between speciation and diversity patterns with landscape change, in
103 particular, the formation of large rivers, has been a classic biogeographic explanation for the
104 generation and maintenance of strikingly high Amazonia rainforest diversity (Haffer, 1974;
105 Ribas, Aleixo, Nogueira, Miyaki, & Cracraft, 2012). However, given its large geographic extent,
106 very dynamic geomorphological and historical climate setups, and the distinct habitat preferences
107 of species involved, no single process can fully explain Amazonian diversity (Leite & Rogers,
108 2013; Moritz, Patton, Schneider, & Smith, 2000). Observed diversity patterns can result from
109 many different mechanisms (Hoorn et al., 2010; Rangel et al., 2018) and, consequently, the
110 factors contributing to divergence across regions and/or taxa may differ. Nevertheless, the river-
111 barrier hypothesis (RBH) is still widely invoked as a causal process in the divergence of
112 Amazonian species and populations.

113 The traditional RBH posits that river establishment split broadly distributed populations,
114 serving as primary barriers to dispersal and prompting diversification and speciation events

115 (Haffer, 1974; Wallace, 1852). Evidence that rivers have acted as strong dispersal barriers (for
116 example the Amazon river; Pomara, Ruokolainen, & Young, 2014), contrasts with evidence that
117 others may be more permeable (for example the Negro river – Smith et al., 2014; the Tapajós
118 river – Moraes, Pavan, Barros, & Ribas, 2016). Permeability of rivers to dispersal may depend on
119 its physical attributes, such as flow rate and margin properties, as well as the species' intrinsic
120 characteristics, including their dispersal abilities and habitat preferences (see Collevatti, Leoi,
121 Leite, & Gribel, 2009 – but also see Naka, Bechtoldt, Henriques, & Brumfield, 2012). Differing
122 support for the RBH across individual taxa (e.g. Fernandes, Wink, & Aleixo, 2012; Nazareno,
123 Dick, & Lohmann, 2017) has begun to highlight the complexities surrounding this hypothesis.
124 This includes uncertainty surrounding the geologic history of the region, including debate over a
125 late Miocene (Figueiredo, Hoorn, van der Ven, & Soares, 2009; Hoorn et al., 2010) versus Plio-
126 Pleistocene establishment for the transcontinental Amazon River drainage (Rossetti et al., 2015).
127 These periods actually correspond to two different developmental phases of the main drainage. In
128 the Plio-Pleistocene the Amazon river (and its tributaries) would have been more entrenched and
129 likely with vast fluctuations in discharge controlled by Quaternary climate fluctuations (see Irion
130 & Kalliola, 2010). Such debate poses specific challenges to biological interpretations regarding
131 the RBH based on estimates of divergence times. For example, estimated divergence times across
132 species of trumpeter birds (genus *Psophia*) that range between 3 and 0.5 million years ago are
133 consistent with the RBH as the main diversification driver if the Amazon drainage was
134 established during the Plio-Pleistocene (Ribas et al., 2012). On the other hand, if the late Miocene
135 hypothesis is more accurate, as some geochemical and palynological data suggests (Hoorn et al.,
136 2017), then lineage persistence in the landscape after dispersal across rivers would become the *de*
137 *facto* process structuring species diversity in Amazonia, with rivers acting mostly as secondary
138 barriers (Cowman & Bellwood, 2013). That is, an alternative to the vicariant model of divergence
139 envisioned under the classic RBH is that divergence may be initiated by dispersal events across
140 rivers (see Smith et al., 2014). Lastly, the same river may be involved in the generation and
141 maintenance of species diversity differently, acting as both a primary and secondary barrier
142 (Naka & Brumfield, 2018).

143 Here, we leverage the resolution afforded by genomic data and a model-based approach
144 with broad geographic sampling across multiple rivers in two common and widespread

145 Amazonian vertebrate taxa with different ecologies to test if major Amazonian rivers act as
146 barriers. The first focal species, *Gonatodes humeralis* (Sphaerodactylidae, Squamata), is a widely
147 distributed semi-arboreal gecko that occurs in a wide range of ecological conditions in Amazonia
148 and northern Pantanal, and is frequently the most common lizard within local assemblages
149 (Ribeiro-Júnior, 2015). A recent analysis of species delimitation for this taxa demonstrated that it
150 is a unique clade, though it is unclear the processes responsible for the local patterns of
151 population genetic structure (Pinto et al., 2019). The second taxon, *Dendropsophus*
152 *leucophyllatus*/*D. triangulum* (Hylidae, Amphibia) species complex, which hereafter is referred
153 to simply as *Dendropsophus leucophyllatus* (see Appendix S1), is a small-sized tree frog
154 associated with temporary and permanent ponds across all of Amazonia (Rodríguez & Duellman,
155 1994). Although no study specifically addressed its phylogeographic structure, the combination
156 of morphological, bioacoustics, and phylogenetic analyses of mtDNA suggests that *D.*
157 *leucophyllatus* likely represents a species complex (Caminer et al., 2017; Peloso, Orrico, Haddad,
158 Lima-Filho, & Sturaro, 2016).

159 With analyses of ddRADseq, we test the RBH for sampling localities/populations of these
160 herpetofauna taxa delimited by major rivers whose geographical distribution correspond to the
161 Amazonian Areas of Endemism – AoEs (*sensu* Da Silva, Rylands, & da Fonseca, 2005). More
162 specifically, we investigate if there is concordance across both taxa in terms of each river's
163 spatial effectiveness as barrier to gene flow and the divergence times of populations separated by
164 each river. If in fact rivers act as barriers, we expect to find congruent genetic differentiation
165 patterns with limited gene flow between populations separated by rivers in both taxa, although
166 the degree of temporal congruence may differ depending upon when and how differentiation
167 occurred (e.g., a vicariant role of separating previously widespread ancestors versus an
168 impediment to gene flow following an initial dispersal event across the river; Peres, Patton, &
169 Silva, 1996; Smith et al., 2014). Alternatively, there may be species-specific divergence patterns
170 in which one of the two taxa show a systematic difference in the degree of differentiation across
171 rivers because of divergent traits (ecological, morphological or sexual) that may inhibit gene flow
172 (Zamudio, Bell, & Mason, 2016), or affect population persistence after dispersal across a river
173 (Smith et al., 2014). For example, given that *D. leucophyllatus* exhibits morphological
174 differentiation (Caminer et al., 2017), which is so far unknown for *G. humeralis* (Ávila-Pires,

175 1995; Pinto et al., 2019), the effectiveness of a river barrier may be enhanced by further
176 reductions in gene flow across any common river barrier in the former, but not in the latter. By
177 focusing on broadly distributed taxa, our study avoids the pitfalls of tests that are limited in
178 geographic scope, and with separate analyses for each putative river barrier we avoid the reduced
179 predictive power of generic phylogeographic tests (Papadopoulou & Knowles, 2016).

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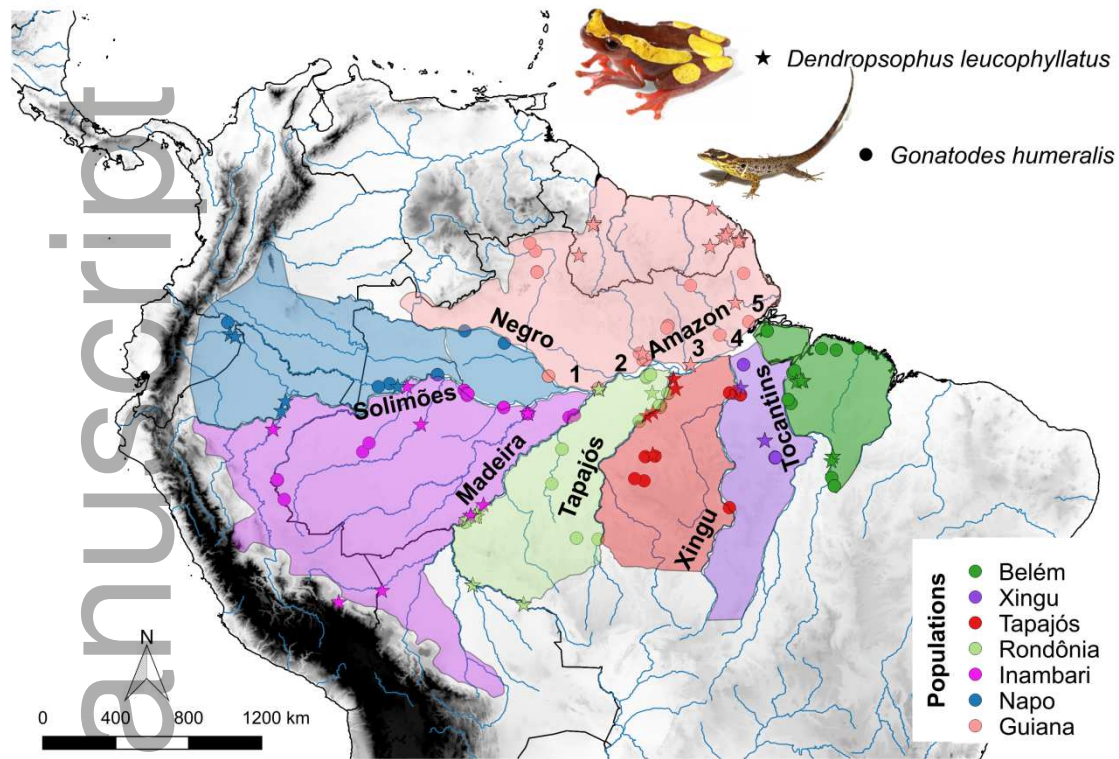
181 **METHODS**

182

183 *Areas of Endemism delimited by main Amazonian rivers*

184 Given the focus of our study is on testing the hypothesis of river as barriers, we follow the limits
185 of Areas of Endemism (AoEs) as defined by Da Silva et al. (2005), which are separated by the
186 major rivers and supported by biogeographic distributions of some terrestrial vertebrates, to refer
187 to specific populations (see Figure 1). These AoEs were used as pre-defined groups of sampled
188 individuals for subsequent tests of genetic differentiation, and model-based analysis of the
189 divergence process. We also verified general agreement between the AoEs limits and population
190 spatial structure (see below).

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191
 192 **Figure 1.** Map of sampled localities of *Gonatodes humeralis* (circles) and *Dendropsophus*
 193 *leucophyllatus* (stars) across each species' range, with different colors highlighting populations
 194 separated by the main Amazonian rivers; areas are named according to Da Silva et al., (2005)
 195 areas of endemism. Numbers 1–5 represent the five segments of the Amazon River that were
 196 considered separately. Photos: tree frog (Santiago Ron), lizard (Rodrigo Tinoco).

197
 198
 199 *Sampling, ddRADseq genomic data generation, and processing*

200 Tissue samples were collected in the field and/or obtained from museum collections of
 201 the lizard *Gonatodes humeralis* and the tree frog *Dendropsophus leucophyllatus*. We obtained
 202 194 samples of *G. humeralis* from 37 localities, and 109 samples of *D. leucophyllatus* from 41
 203 localities (Figure 1). Genomic DNA was extracted from the muscle or liver of each individual
 204 using the Qiagen DNeasy Blood and Tissue Kit. Two reduced representation libraries for the

205 lizard samples and two for the frog samples were constructed using double digest restriction
206 associated DNA sequencing approach (ddRADseq), following the protocol by Peterson, Weber,
207 Kay, Fisher, and Hoekstra (2012); for details see Appendix S1. Briefly, double digested DNA
208 with ligated unique barcodes from each individual was pooled and 350–450 bp fragments size
209 selected using Pippin Prep (Sage Science). Libraries were sequenced in the Illumina 2500
210 platform at The Center for Applied Genomics (Toronto, Canada) to generate 150 bp single-end
211 reads. Genomic data were processed for each taxon separately using STACKS 1.41 pipeline
212 (Catchen, Amores, Hohenlohe, Cresko, & Postlethwait, 2011; Catchen, Hohenlohe, Bassham,
213 Amores, & Cresko, 2013) for *de novo* assembly from the fastQ files from the Illumina
214 sequencing runs. All details regarding bioinformatic processing and data following quality
215 control filters are given in the Appedix S1. All STACKS modules were run under parallel
216 execution with 8 threads on the University of Michigan Flux computing cluster.

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

224

225 *Genetic diversity and genetic differentiation across rivers*

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

232 Genetic differentiation across each river barrier separating adjacent AoEs was measured
233 by F_{ST} . Specifically, pairwise F_{ST} -values, with their significance assessed from 10,000 bootstrap
234 replicates with a Bonferroni correction for multiple comparisons, were calculated in Arlequin

235 3.5.2.2 (Excoffier & Lischer, 2010) across a total of 11 river barriers (see Figure 1). Because of
236 insufficient samples, we did not consider the Napo and Imeri as separate AoEs (as defined by Da
237 Silva et al., 2005); this is a practical decision and does not implies in any questioning about the
238 validity of these two bioregions (e.g., Borges & Da Silva, 2012). Samples from the Marajó Island
239 were considered as part of the Belém AoE given that geological sediment similarities suggest a
240 shared regional history (Rossetti, Almeida, Amaral, Lima, & Pessenda, 2010). We evaluate
241 genetic differentiation across 5 different segments of the Amazon River separately (see Figure 1),
242 given that the Amazon River borders several areas dissected by other major rivers. To evaluate
243 the effect of rivers as barriers on the partitioning of genomic variation among populations, within
244 populations and individuals we used a hierarchical analysis of molecular variance (AMOVA)
245 using Arlequin 3.5.2.2 (Excoffier & Lischer, 2010), by estimating their statistical significance
246 based on 20,000 random permutations.

247

248 *Estimated Effective Migration Surfaces*

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

264 run. Convergence among runs was accessed with the package in R `rEEMSplots`, available with
265 the EEMS pipeline.

266

267 *Divergence history for each river barrier and species*

268 For each river barrier, parameterized divergence models were estimated using a
269 composite-likelihood method based on the joint site frequency spectrum (SFS) implemented in
270 FASTSIMCOAL2 (Excoffier, Dupanloup, Huerta-Sanchez, Sousa, & Foll, 2013). Datasets were
271 reprocessed for each river barrier separately using the POPULATION module from STACKS
272 pipeline (Catchen et al., 2013) and PLINK software (Chang et al., 2015) to maximize the number
273 of loci for each separate analysis. Specifically, POPULATION was run to obtain vcf files with
274 unlinked SNPs and the folded joint SFS (i.e., minor allele) using a python script for each separate
275 FASTSIMCOAL analysis (available on Github/KnowlesLab; He & Knowles, 2016). Note that
276 the requirement of no missing data precluded a global analysis considering all barriers in a single
277 model (see Excoffier et al., 2013). For *G. humeralis*, the SFS was calculated based on 20 to 30
278 individuals per analysis, whereas 10 to 20 individuals were analyzed for *D. leucophyllatus* to
279 maximize the number of loci that could be included in each analysis, given *D. leucophyllatus* had
280 fewer individuals with a common set of loci.

281 Divergence models were estimated with and without a migration parameter. The time of
282 divergence, T_{DIV} , the population size of the other population, N_2 , the ancestral population size
283 N_{ANC} , and migration, m (for those models that included migration), were estimated from the SFS
284 using uniform priors (see Table 1 for details). For all divergence models the effective population
285 size of one population (N_I) was fixed to improve the accuracy of parameter estimates from the
286 SFS - following the recommendations of the program (Excoffier & Foll, 2011). Specifically, N_I
287 was calculated directly from the empirical data based on nucleotide diversity (π) of variant and
288 invariant sites, where $\pi = 4N\mu$, assuming a mutation rate of 3.25×10^{-8} for *G. humeralis* and 3.46
289 $\times 10^{-8}$ for *D. leucophyllatus*. These rates were estimated from the regression formula for cellular
290 organisms (Lynch, 2010) based on genome sizes estimated in related species (i.e., *Teratoscincus*
291 *scincus* for *G. humeralis* and *Dendropsophus microcephalus* for *D. leucophyllatus*;
292 www.genomesize.com), and considering one generation per year (Crump, 1974; Vitt,
293 Magnusson, Ávila-Pires, & Lima, 2008). To account for potential errors in mutation rates

294 estimates derived from estimates of genome sizes, we also conducted another set of analyses
295 using a lower mutation rate from the literature based on estimates from mitochondrial DNA (i.e.,
296 6.45×10^{-10} for *G. humeralis* (Prates, Rivera, Rodrigues, & Carnaval, 2016) and 7.35×10^{-9} for *D.*
297 *leucophyllatus* (Gehara et al., 2014)). A total of 40 runs were conducted for each river barrier and
298 we present the point estimate of the highest likelihood across runs, as well as 95% confidence
299 intervals on the parameter estimates calculated using a parametric bootstrap of 100 simulated
300 datasets. Each analysis was based on 100,000 to 250,000 simulations for likelihood estimation
301 with a stopping criterion of 0.001, and 10 to 40 expectation-conditional cycles (ECM).

302

303 RESULTS

304

305 *Genetic diversity and differentiation across rivers*

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

Author

312

313

314

316 **Figure 2.**

317 Estimated

318 pairwise

320 values

322 between

324 separated

326 major river

327 labels

328 identify the

330 barrier)

332 with (b)

334 estimated

335 diversity, as

336 measured

338 average

339 nucleotide

340 diversity, π ,

342 *Gonatodes*

343 *humeralis*

344 (circles) and *Dendropsophus leucophyllatus* (stars).

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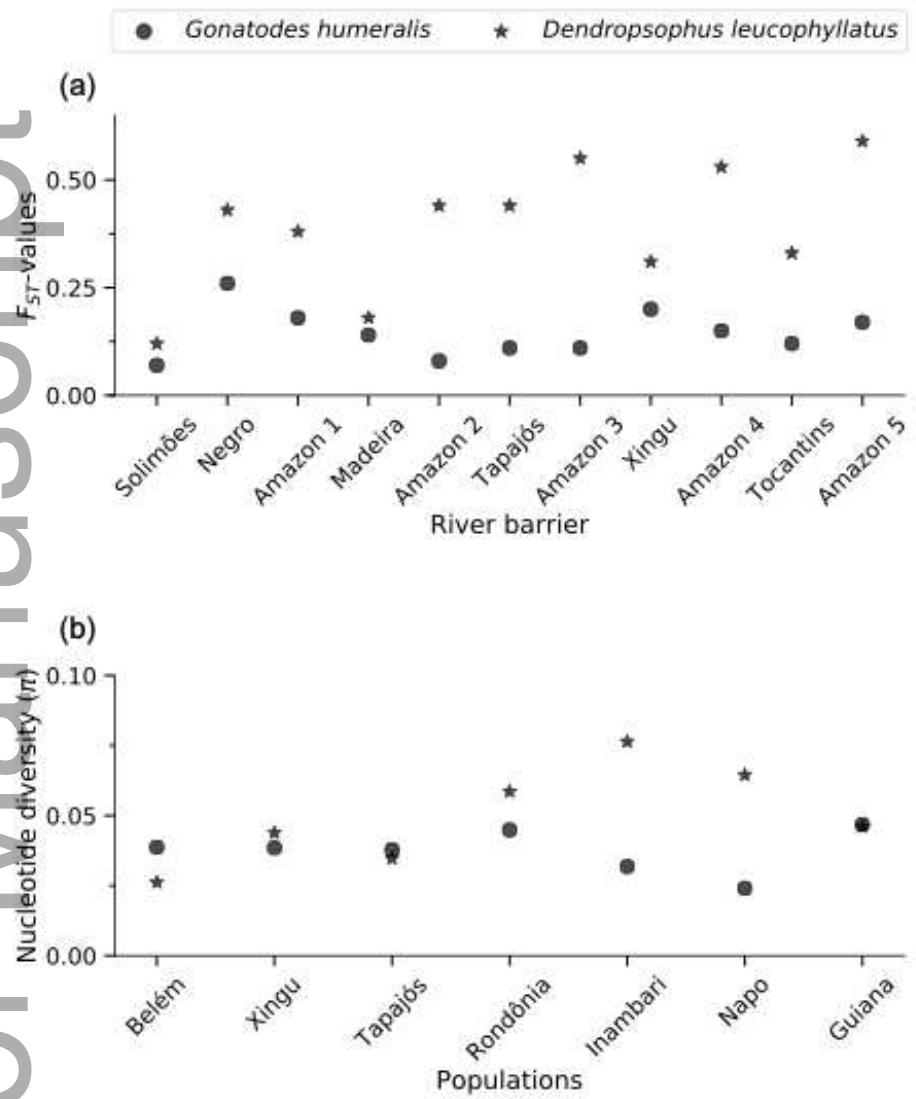
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Estimates of genetic diversity (π) were comparable across populations in each species (Figure 2b). This indicates that the consistently lower F_{ST} -values among populations of *G. humeralis* were not due to a systematically higher genetic diversity 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



(a)

F_{ST} -

areas

by a

(i.e.

river

along

genetic

by

in

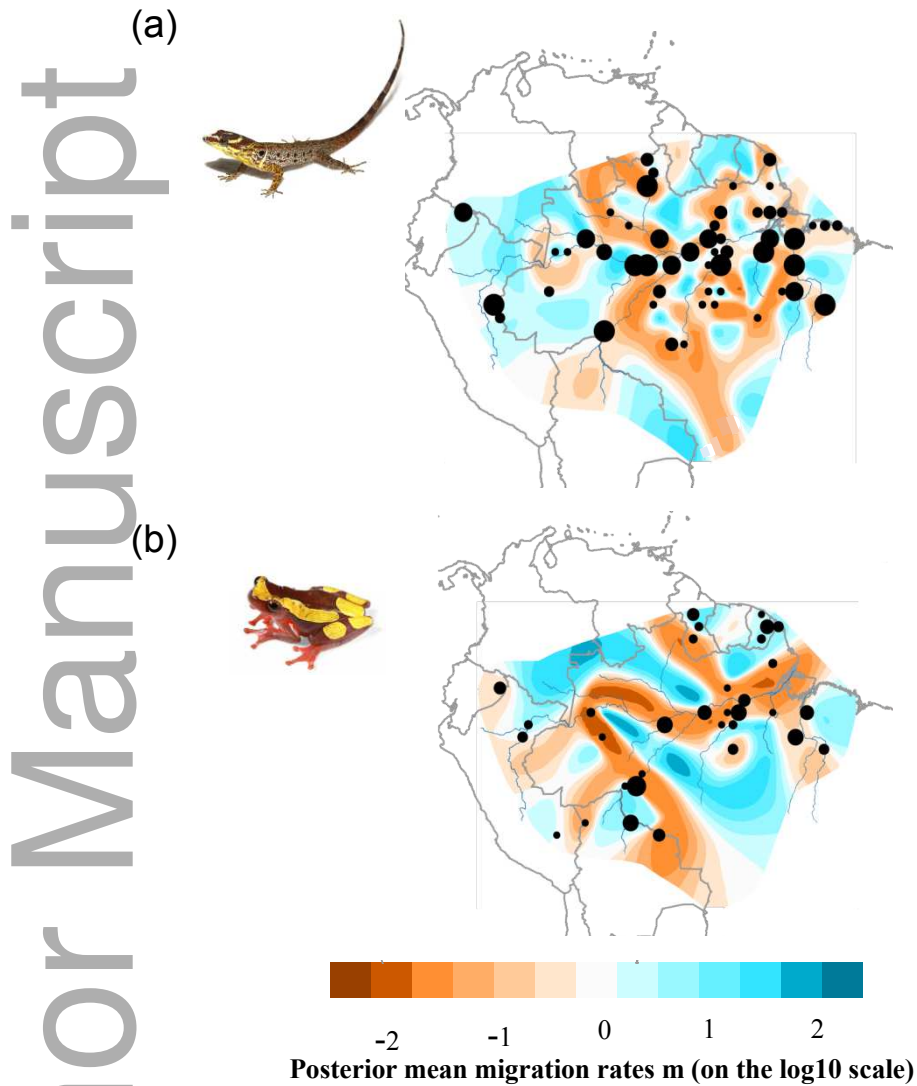
352 *D. leucophyllatus*, indicating that the higher differentiation observed in *D. leucophyllatus* is not
353 an artifact of depressed genetic diversity. In fact, the higher genetic diversity observed in *D.*
354 *leucophyllatus* in the Inambari and Napo populations (Figure 2b) contributes to the fairly similar
355 levels of genetic differentiation in both species for the Solimões and Madeira river barriers
356 (Figure 2a), the only two cases in which F_{ST} -values did not differ between taxa. Genetic diversity
357 was not significantly correlated with the geographic area of a region in either taxa ($p=0.87$ for *G.*
358 *humeralis* and $p=0.17$ for *D. leucophyllatus*; see Figure S2 in Appendix S1). An AMOVA
359 showed significant structuring of genetic variation among populations in both species (Table S4
360 in Appendix S1), explaining 19.19% and 22.26% of the variance in *G. humeralis* and *D.*
361 *leucophyllatus*, with the bulk of the variance attributed to variation among and within individuals
362 (for details see Table S4 in Appendix S1).

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

377

378

379



380 **Figure 3.** Estimated effective migration surfaces (EEMS) plots showing the effective migration
381 rates (m) on a log₁₀ scale between all samples of (a) *G. humeralis* and (b) *D. leucophyllatus*.
382 Geographic regions of low migration are shown in orange, representing migration barriers. Areas
383 in blue represent geographic regions where the genetic similarity is higher, or in other words,
384 where samples are connected by migration rates higher than expected under IBD. The dots
385 represent the samples localities, and shows localities with more samples as the difference in the

386 relative size of the dots. The mean migration across the 10 independent EMMS runs is presented
387 in each species. Photos: lizard (Rodrigo Tinoco), tree frog (Santiago Ron).

388

389 *Parameterized divergence models*

390 Several generalizations emerge from comparing the divergence model parameters
391 estimated for each separate river barrier for *G. humeralis* and *D. leucophyllatus* (Table 1). First,
392 estimated divergence times were relatively recent for each of the 11 rivers separating populations
393 in both species, with all divergence occurring within the last 116 kya (see Table 1), or within the
394 650 kya when applying a lower mutation rate (see Table S5 in Appendix S1). However,
395 divergence times varied among river barriers and differed between the species (Table 1). For
396 example, a divergence time of 4,630 years was estimated in *G. humeralis* for segment 1 of the
397 Amazon river (Figure 1) compared to 44,011 years in *D. leucophyllatus*.

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

408 Consideration of the relative divergence times estimated for each pair of populations
409 separated by a river barrier showed the order of divergence times was not congruent between
410 species. For example, the oldest divergence time for *G. humeralis* was associated with the Negro
411 river, followed by Amazon 4, Xingu, Tapajós, Amazon 3, Amazon 5, Amazon 1, Amazon 2,
412 Madeira, and the Tocantins followed by the Solimões with the most recent divergence time. For
413 *D. leucophyllatus* the order differed, with the oldest divergence time associated with the Amazon
414 segment 5, followed by Amazon 3, Amazon 4, Amazon 1, Tapajós, Amazon 2, Negro, Tocantins,
415 Madeira, and the Solimões followed by the Xingu with the most recent divergence time. In

416 general, there was not any obvious consistency in divergence between species with respect to
417 river size (Table S6), or flow direction (e.g., headwater to the mouth of Amazon river; Table 1).

418

419 **DISCUSSION**

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

438

439 *Similar, but different*

440 There is no doubt that Amazonian rivers can act as barriers to gene flow in some
441 instances. Support for the RBH ranges from classic studies based on distributional data or
442 divergence times across river banks estimated from a few sequenced loci (Aleixo, 2004; Boubli et
443 al., 2015; Foley et al., 2014; Funk et al., 2007) to recent analyses based on multiple loci (e.g.,
444 Lutz, Weckstein, Patane, Bates, & Aleixo, 2013; Naka & Brumfield, 2018; Nazareno et al.,
445 2017). However, once studies move beyond a focus on a specific river, and as data accumulates

446 across more taxa, support for rivers as either primary or secondary barriers became mixed (e.g.,
447 Nazareno et al., 2017; Solomon, Bacci, Martins, Vinha, & Mueller, 2008). As such, it is difficult
448 to draw generalizations about why one river, but not another, may impede gene flow, and only in
449 certain taxa (see Ayres & Clutton-Brock, 1992). As we advocate here, to maximize insights, we
450 need to move beyond concordant or discordant divergence histories. Instead, through the study of
451 multiple, widespread species we can consider the diversity of processes that might underlie the
452 observed genetic differentiation associated with river barriers without the confounding factors
453 that arise from tests in which the constituent set of taxa used in tests differ across rivers (e.g.,
454 Smith et al., 2014).

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

465 As widespread taxa with divergence times that post-date the rivers origins (i.e.,
466 independent of the geological scenario for the establishment for the transcontinental Amazon
467 River drainage divergence times are more recent), both *G. humeralis* and *D. leucophyllatus* must
468 have crossed rivers at least once as they obtained their current ranges, which encompass all of
469 Amazonia (Figure 1 and Figure 3). Empirical evidence suggest that a simple colonization with
470 range expansion is unlikely to have occurred (e.g., Lima et al., 2017) . The relative timing of
471 divergence across rivers differs between taxa, which, unlike absolute divergence time estimates
472 (see Table 1), is not contingent upon specific mutation rates used with applications of the
473 molecular clock, and the timing of divergence does not follow an obvious geographic pattern.
474 This points us to a divergence process with cyclical periods of connections and isolations (Haffer,
475 2008), where previous dispersal events across rivers prompted by recent meander belt dynamics

476 are likely. Indeed, recent studies suggest a very dynamic drainage network in the Amazonian
477 lowlands during the Quaternary, with frequent river captures and fluvial changes affecting
478 barriers stability and permeability (Pupim et al., 2019; Ruokolainen, Massaine Moulatlet,
479 Zuquim, Hoorn, & Tuomisto, 2018). Moreover, changes in water availability and vegetation
480 distribution caused by climatic changes might also have promoted repeated periods of gene flow
481 and isolation of populations separated by the river barriers (Cheng et al., 2013; Haffer, 2008).
482 Admittedly, with any genetic signature of this older history over-ridden by the divergence in
483 allopatry that dates to a relative recent past (Table 1), it is not clear why earlier dispersal events
484 were not associated with long term isolation, especially considering the persistence of isolation
485 over the tens to hundreds of thousands of years documented here in both species, and even longer
486 in some cases within *D. leucophyllatus*. Given the analyzed taxa are widespread and common
487 where they occur, it is unlikely that local extinctions (at least at the geographic scale examined
488 here; Figure 1), are driving colonization dynamics (see Papadopoulou & Knowles, 2017).
489 Instead, it is more likely that abiotic factors associated with shifting river physiography (e.g.,
490 flood-pulse patterns, Junk et al. (2011), and river captures, Rossetti, Bertani, Zani, Cremon, and
491 Hayakawa (2012)) might be at play, for some, but most likely not all rivers. For example, recent
492 divergences were observed for the Solimões in both species, whereas relatively older divergences
493 were observed for the Tapajós, Amazon 4, and Amazon 3 in both species, which suggest that
494 patterns of genetic divergence could be reflecting a shared response to a common abiotic factor
495 (see Table S6 in Appendix S1). In contrast, the Negro and the rest of the Amazon river segments
496 show opposing divergence patterns between the taxa, with very recent divergence times in *G.*
497 *humeralis* but some of the oldest divergence events estimated in *D. leucophyllatus*. We note that
498 these contrasting divergence times cannot be explained by difference in mutation rates between
499 the lizard and frog taxa given the co-varying divergence patterns between the taxa for the other
500 aforementioned rivers (Figure 1), but the very recent divergence times in *G. humeralis* might
501 explain the highest portion of the genetic variation being allocated within individuals of *G.*
502 *humeralis* (Table S4 in Appendix S1).

503 Biotic factors might also influence the genetic divergence associated with rivers, and in
504 particular, the effectiveness of the barrier itself (Nosil, Harmon, & Seehausen, 2009). However,
505 in the case of our focal taxa, both are found in different Amazonian forest types, including

506 “várzea” (floodplain forest) and “terra firme” (upland forest) (Ribeiro-Júnior, 2015; Rodriguez &
507 Duellman, 1994, personal observations). This suggests no obvious differences in the restrictions
508 imposed by the landscape on their respective dispersal abilities, unlike other taxa where dispersal
509 across rivers is influenced by the surrounding habitat (Collevatti et al., 2009). However, lizards
510 may be more capable of dispersal in general than frogs, since they have fewer environmental
511 restrictions (Duellman, 1979), which might contribute to the consistently lower F_{ST} -values
512 observed in *G. humeralis* (Figure 2). For instance, *G. humeralis* is a semi-arboreal lizard easily
513 observed on tree trunks where they inhabit and reproduce (Vitt et al., 2008), and could have
514 passively dispersed more frequently between river banks with floating vegetation (a pattern not
515 unexpected for the group, since even trans-Atlantic dispersal has been documented for related
516 gecko lizards; Gamble et al., 2011). *Dendropsophus leucophyllatus*, though also a common
517 species, has a reproduction mode directly related to lentic water systems (temporary and
518 permanent ponds), where the eggs are deposited for the hatching of the tadpoles (Rodriguez &
519 Duellman, 1994). It is also possible that the consistent tendency of greater genetic differentiation
520 (Figure 2) and older divergence times in *D. leucophyllatus* than *G. humeralis* (Table 1) may
521 reflect the decreased gene flow and/or more long-term effectiveness of the barrier because of
522 local adaptation within each of the AoEs/river interfluves (Ortiz, Lima, & Werneck, 2018). For
523 example, the tree frog, unlike the lizard, exhibits phenotypic differentiation across its range that
524 might impact interbreeding (Caminer et al., 2017).

525

526 *Rivers as drivers of species diversity*

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

535 years in both species (e.g., Duellman, Marion, & Hedges, 2016; Gamble, Simons, Colli, & Vitt,
536 2008).

537 In several Amazonian taxa, population differentiation within species is commonly
538 correlated with their speciation rates (Harvey et al., 2017). To the extent that this finding is
539 generalizable, the significant genetic structure and relatively older divergence times between *D.*
540 *leucophyllatus* populations compared to those of *G. humeralis* suggests that genetic divergence
541 has proceeded further along the speciation continuum (see Huang & Knowles, 2016) in the
542 former. Indeed, recent taxonomic work suggests that *D. leucophyllatus* constitutes a species
543 complex (see supplemental information: *Dendropsophus leucophyllatus* taxonomy; Caminer et
544 al., 2017). However, we note that genetic differentiation associated with the proposed new
545 putative species (i.e., Inambari population; Caminer et al., 2017) is not any more pronounced than
546 the intraspecific differentiation associated with other river barriers (see Table S2, S3 and S6 in
547 Appendix S1).

548

549

550 CONCLUSION

551 It is well known that large rivers can limit the distribution of some species without
552 necessarily indicating that they represent vicariant barriers that caused allopatric speciation
553 (Losos & Glor, 2003), but few studies focus on widespread taxa, where the primary role of rivers
554 is likely as secondary barriers (i.e., impediments to dispersal; Cowman & Bellwood, 2013), at
555 least for taxa whose current ranges postdate the origin of the river barriers (e.g., Moraes et al.,
556 2016; Naka & Brumfield, 2018). Our work shows that the rivers indeed act as barriers restricting
557 gene flow (Cowman & Bellwood, 2013; Naka & Brumfield, 2018). However, with the
558 divergence times of each river varying, and the relative divergence times differing between
559 species, our work supports the contention that the barriers represented by rivers may be much
560 more dynamic than classic views of vicariant histories promoted by rivers formation (Lynch et
561 al., 2015; Ribas et al., 2012). Moreover, by detailing how genetic divergence varies temporally,
562 spatially, and across taxa, our work lends support to the hypothesized proposal of cyclical periods
563 of connections and isolations (i.e., a transient barrier; Cowman & Bellwood, 2013), that could be
564 caused by both climatic fluctuations and recent river dynamics. Moreover, with consistent

565 differences in the degree of differentiation between these two common and widespread
566 Amazonian taxa, ecological preferences and local adaptation may potentially influence the long-
567 term effectiveness of river barriers by further reducing gene flow during the divergence history of
568 the tree frog *D. leucophyllatus* relative to the gecko *G. humeralis*. Only with future studies that
569 considers support for these alternative modes of divergences, as opposed to focusing exclusively
570 on tests of concordance across taxa or vicariance, will the diversity of processes associated with
571 rivers as barriers begin to be better understood.

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572 **TABLES**

573 **Table 1.** Parameter estimates for divergence models of each putative river barrier listed in order from the west to east, as well as
574 the number of loci analyzed for each test (see methods for details). The highest likelihood, as well as the 95% confidence interval
575 for each parameter estimate (in parentheses) across 40 runs of FASTSIMCOAL2 (Excoffier et al., 2013) are shown for the
576 models without migration. Note that the population size N_1 was calculated directly from the empirical data (i.e., it is a fixed
577 parameter in the model) to improve the accuracy of the other estimated parameters (i.e., the second population size, N_2 , and the
578 ancestral population size, N_{ANC} , and divergence time, T_{DIV}) from the SFS (following the recommendations for the program; see
579 Excoffier & Foll, 2011). Divergence models were also estimated with migration, m ; however, estimates of m were 0 or always
580 less than 0.00001, so only results from the divergence model without migration are reported.

<i>Gonatodes humeralis</i>						<i>Dendropsophus leucophyllatus</i>				
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 (5,3002 – 66,591)
Tocantins	11,036	29,231	6,899 (6,259 – 7,413)	18,187 (17063 – 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)

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822 **Biosketch**

823

824 The authors are broadly interested in biogeographical and evolutionary history of amphibians and
825 reptiles in the Neotropics. This work was part of a collaboration between MPEG, INPA, UFPA
826 and the University of Michigan.

827

828 RMP, FPW, ATT and LLK designed the study, which was part of a larger funded project of MJS,
829 TCAP and LLK. MJS, PLVP, TCAP and MTR contributed samples. RMP carried out the
830 molecular lab work and data analysis, with help from ATT and MLK. RMP, ATT, FPW and LLK
831 drafted the manuscript. All authors reviewed and approved the manuscript.

832

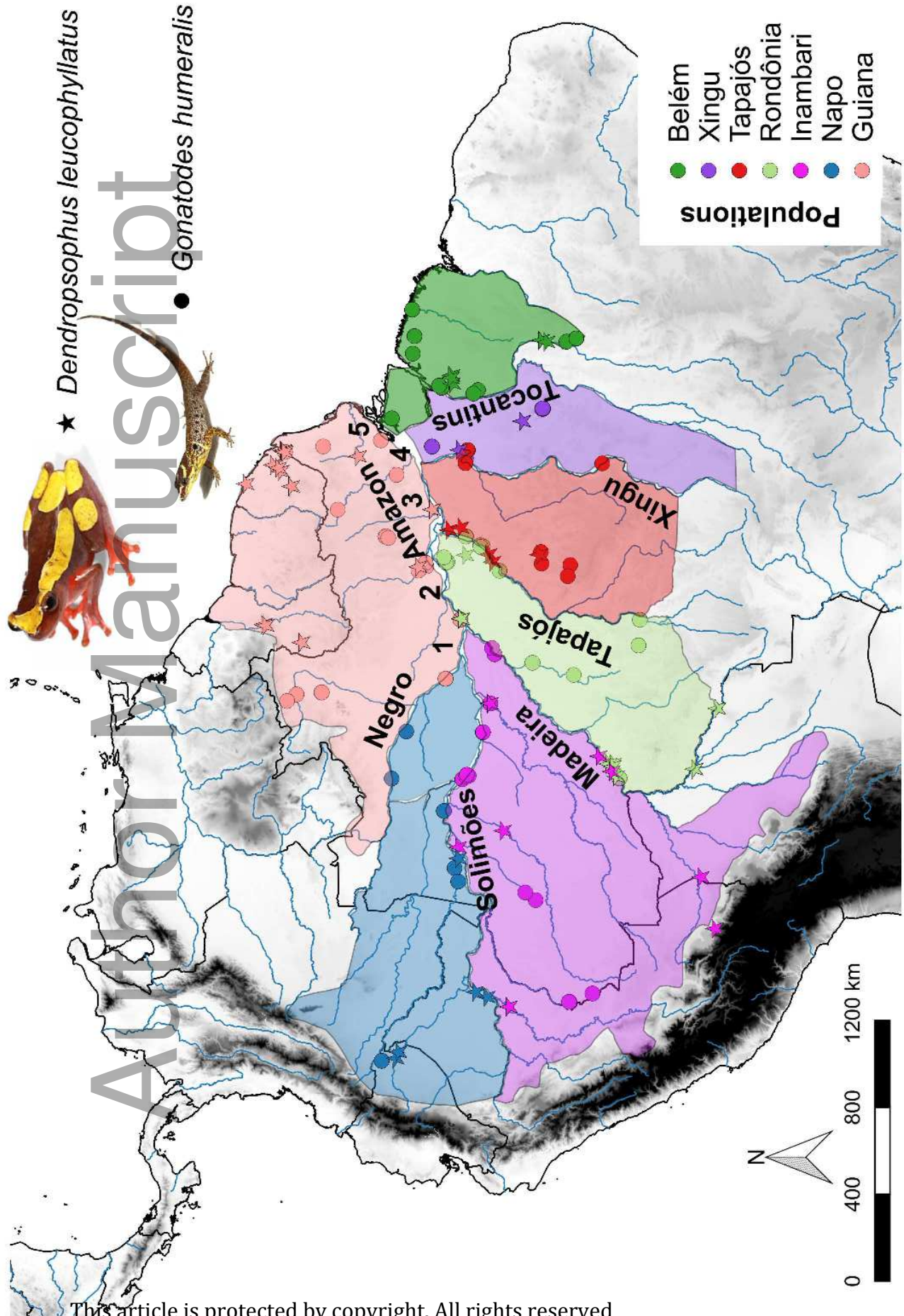
833 **Data Accessibility**

834 All data will be freely available at the University of Michigan archive DeepBlue ([https:// xxxx](https://xxxx))
835 pending acceptance.

836

837 **Supporting information**

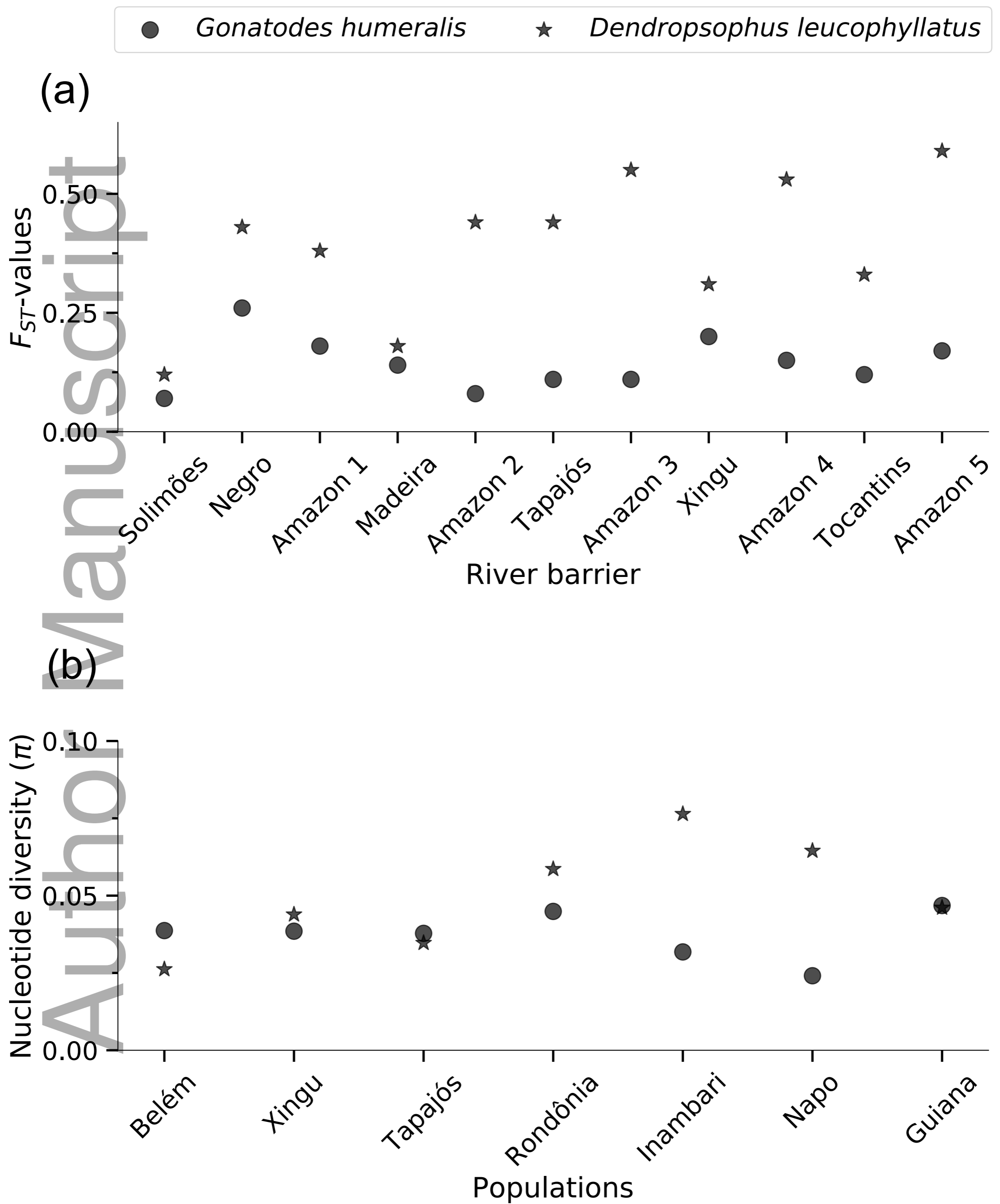
838 Additional supporting information may be found online in the Appendix S1 section at the end of
839 the article.

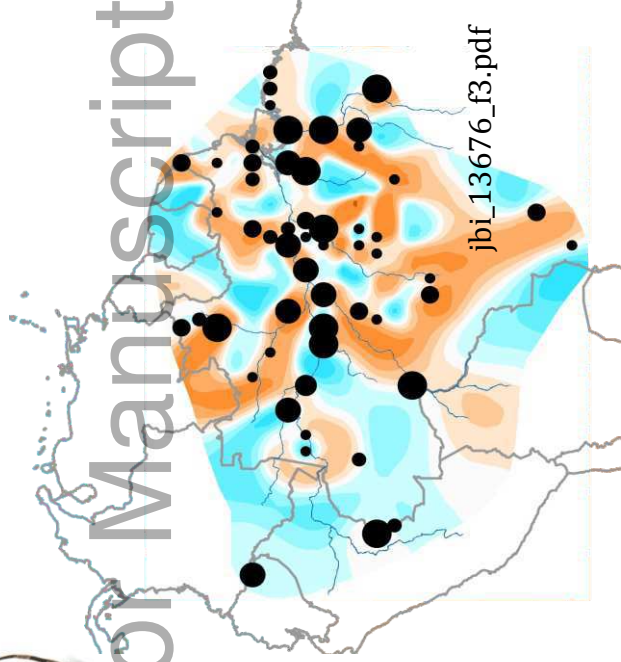


★ *Dendropsophus leucophyllatus*

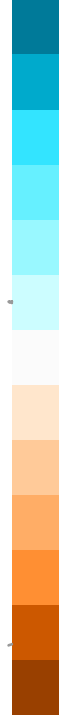
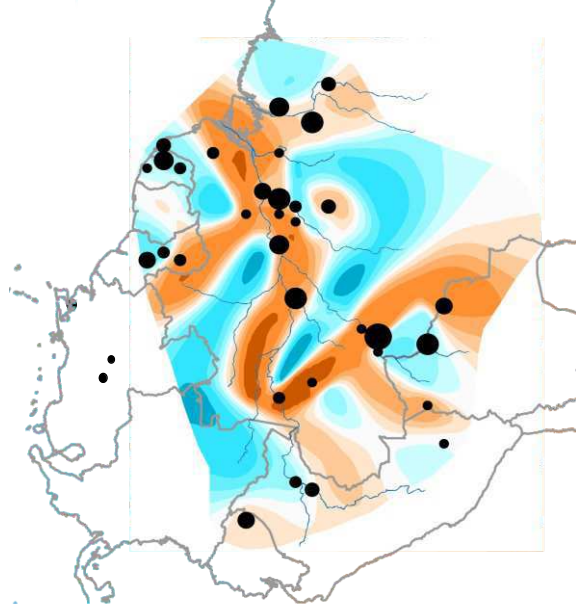
● *Gonatodes humeralis*

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Posterior mean migration rates m (on the log10 scale)



Posterior mean migration rates m (on the log10 scale)

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1
0
-1
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