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1 **The Role of Rivers as Geographical Barriers in Shaping Genetic Differentiation and**  
2 **Diversity of Neotropical Primates**

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24 **ABSTRACT**

25 We quantitatively tested the riverine barrier hypothesis and its influence on biogeographical  
26 distributions and molecular variation in New World monkeys (Parvorder: Platyrrhini). Using  
27 mitochondrial markers (cytochrome oxidase subunit II and cytochrome b), we analyzed  
28 taxonomic differences and the effects of geographical barriers on molecular patterns across  
29 Central and South America. Nearly 80% of described species are separated by geographical  
30 barriers. River width exhibited a positive correlation with molecular dissimilarity in adjacent  
31 taxa for both molecular markers. Streamflow also showed a positive association, although not  
32 statistically significant, likely due to limited sample size. Several presently described taxa were  
33 not supported based solely on these molecular phylogenetic markers, including *Saimiri*, *Mico*,  
34 *Cebus*, *Sapajus*, and *Cherecebus*. These taxonomic issues are far more common where river  
35 barriers do not exist. In conclusion, we found a significant correlation between river width and  
36 molecular divergence in adjacent taxa, indicating that wider rivers were associated with greater  
37 molecular dissimilarity for two commonly used mitochondrial genes. Species boundaries were  
38 predominantly found at river interfaces, and in the absence of discernable geological barriers,  
39 adjoining species were more likely to exhibit molecular similarity. Our findings suggest that both  
40 river and mountain barriers significantly restrict gene flow for the majority of neotropical taxa,  
41 with geological formation of river barriers coinciding with estimated speciation events.  
42 Additionally, river width proves to be a valuable tool for estimating molecular divergence in  
43 adjacent taxa, particularly in regions with limited sampling.

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47 **KEYWORDS**

48 Amazonia, Neotropics, biogeography, evolutionary history, phylogeography, primates, river  
49 barriers, speciation, molecular phylogenetics, taxonomy

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51 **1. INTRODUCTION**

52

53 The Neotropics, a region encompassing Central America, South America, and the Caribbean  
54 islands, contains approximately one-third of all known primate species (Paglia 2012). This  
55 region is home to a high concentration of unique and native species and is predicted to be an area  
56 of high potential for future discoveries (Moura & Jetz, 2021). This is due to its large diversity of  
57 habitats attributed to its geological and climatic history, resulting in complex ecological barriers  
58 driven by diverse habitats and elevational gradients. The retracted and fragmented forests in the  
59 Amazon basin, resulting from glacial advances during Pleistocene climatic fluctuations and  
60 Pliocene-Miocene orogenic events, has presumably played a critical role in the evolution of these  
61 distinct areas of endemism in accordance with the Refugia hypothesis (Haffner 1969; Prance,  
62 1982; Turchetto-Zolet et al. 2013; Thom et al. 2020), though the degree to which this mechanism  
63 has driven Amazon diversity on its own has been challenged (Bush and Oliveira 2006; Rocha  
64 and Kaefer, 2019). The question remains whether climate fluctuations contributed to either  
65 retraction and expansion of stable forested habitats, or whether areas of endemism were isolated  
66 by climate driven fluvial barriers across the Amazon basin, ultimately contributing to the  
67 considerable diversity of New World monkeys (parvorder: Platyrrhini) (Chapman, 1926; Prance,  
68 1982; Burney and Brumfield, 2009).

69

70 The river barrier hypothesis, proposed by Alfred Wallace in the 19th century  
hypothesizes that rivers limit or even prevent the dispersal of populations, leading to isolation,

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71 and ultimately to divergence through the differential accumulation of mutations, local adaptation,  
72 and genetic drift; a process of speciation known as vicariance (Wallace, 1852; Wallace, 1876;  
73 Lomolino et al., 2010). The river barrier hypothesis proposes that landscape-level drainage  
74 systems can limit the distribution of taxa (Ayers and Clutton-Brock 1992) and supported by  
75 recent geochronological approaches (Pupim et al. 2019; Mourthe et al. 2022), suggesting that  
76 shifting semi-permeable barriers might enable sporadic gene flow, while strong river barrier  
77 formation restricts gene flow and subsequent speciation (Ribas et al. 2012; Musher et al. 2022).  
78 The complexity of successional fluvial deposition and fluctuating landscape connectivity across  
79 geological time explains why some biogeographical patterns are distinctly associated with  
80 riverine barriers, while in other cases the relationship between geological history and taxonomic  
81 diversification is more complicated. Understanding the different factors that contribute to the  
82 high diversity of species in the neotropics and how their influence varies across taxonomic  
83 groups is crucial.

84 In the Amazon basin, the distribution of New World monkey species has been found to  
85 be strongly correlated with the distribution of rivers. Some species are found only on one side of  
86 a river or other body of water, providing evidence for the role of these waterbodies driving  
87 speciation (Lomolino et al., 2010). This is also supported by genetic studies which have revealed  
88 high levels of genetic variation within populations and low levels of variation between  
89 populations separated by rivers, consistent with the idea that these species have evolved  
90 independently (Beaudrot & Marshall, 2011; Harcourt & Wood, 2012). Evidence of rivers  
91 restricting gene flow and leading to differentiation in neotropical primates has also been reported  
92 in several genera including *Saimiri* (Chiou et al., 2011; Alfaro et al., 2015; Ruiz-Garcia et al.,  
93 2015), *Alouatta* (Cortés-Ortiz et al., 2003; Ruiz-Garcia et al., 2017), *Sapajus* (Martins-Junior et

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94 al., 2018; Boubli et al. 2019), *Lagothrix* (Ruiz-Garcia et al., 2019), *Cacajao*, *Callicebus*, *Cebus*,  
95 *Pithecia*, and *Saguinus* (Boubli et al., 2015).

96         The restriction of gene flow due to rivers is influenced by three key factors: the  
97 geographical history of river systems, dispersal ability of organisms, and river characteristics like  
98 size and speed. Firstly, molecular divergence is dependent upon the origin of the river and how  
99 long the barrier has restricted gene flow. For example, recent dynamic tributary rearrangements  
100 have been shown to influence biogeographical observations (Ruokolainen et al. 2019). The  
101 dispersal ability of the organism also plays a role, with larger, more mobile species being more  
102 likely to cross rivers (Lomolino et al., 2010). Furthermore, the restriction of dispersal caused by  
103 river barriers such as width, volume, and flow rate are positively also associated with divergence.  
104 This has been observed in primate species living on opposing sides of Amazonian rivers, where  
105 river strength and width are inversely correlated with species similarity (Ayres & Clutton-Brock,  
106 1992; Fordham et al., 2020). However, despite the evidence in support of the river barrier  
107 hypothesis, further research is needed to fully understand how rivers and other bodies of water  
108 affect gene flow and speciation, and how other factors may also play a role in the process.

109         One approach to studying the impact of riverine barriers on the speciation of New World  
110 primates is to analyze partial mitochondrial genes, cytochrome b (cytb), and cytochrome oxidase  
111 subunit II (COII) genomes. These genes are widely used to trace evolutionary relationships and  
112 genetic diversity among populations and species. Mitochondrial DNA can provide insights into  
113 evolutionary relationships and the timing of divergence. We can compare molecular similarity –  
114 an indicator of gene flow between adjacent species on opposing riverbanks – with measures of  
115 river permeability in order to model the impact of these barriers on taxonomic divergence.  
116 Furthermore, quantifying the correlation between river width and flow rate with molecular

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117 similarity could potentially predict divergence in under-sampled areas. This information can aid  
118 in identifying areas of taxonomic significance, which can then be further explored through  
119 molecular, behavioral, and morphological analyses.

120 Mitochondrial DNA analysis serves as a powerful tool for studying elusive species like  
121 New World primates, where obtaining high-quality tissue samples through non-invasive methods  
122 is challenging. However, the full potential of mitochondrial markers is often hindered by  
123 limitations, including incomplete databases and the absence of crucial sampling details such as  
124 precise locations, posing obstacles in population comparisons. A recent study using mitogenomic  
125 data revealed that while some divergences coincided with river barriers, not all of them occurred  
126 simultaneously or aligned with proposed river formation dates (Janiak et al. 2022). The strongest  
127 evidence of restricted dispersal was found on opposing sides of the Amazon River, particularly  
128 in bearded saki monkeys (*Chiropotes* spp.) and marmosets/tamarins (small platyrrhines), with  
129 limited evidence for the Rio Negro. Surprisingly, large rivers did not act as barriers for certain  
130 primate species, including howler monkeys (*Alouatta* spp.), uakaris (*Cacajao* spp.), sakis  
131 (*Pithecia* spp.), and robust capuchins (*Sapajus* spp.). These findings suggest the involvement of  
132 other evolutionary mechanisms in the diversification of platyrrhine primates. While the study's  
133 findings provide valuable insights, its limitations, such as a small sample size, lack of  
134 quantitative analysis, and focus solely within the Amazon basin, highlight the importance of  
135 conducting further research to deepen our understanding of how riverine barriers contribute to  
136 the diversification of platyrrhine primates.

137 In this study, we conduct a quantitative molecular analysis of the riverine barrier  
138 hypothesis on all new world monkeys across Central and South America. The main goal of the  
139 study is to investigate the role of geographical barriers, particularly rivers, in shaping the

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140 distribution and divergence of Platyrrhine primates in South America. The specific aims are to:  
141 1) identify geographical boundaries such as river and mountain barriers while analyzing  
142 molecular phylogenetics of Platyrrhini across identified barriers 2) assess the relationship of river  
143 width and flow rate to molecular intra and interspecies diversity on opposing riverbanks, and 3)  
144 model geographic regions in need of further taxonomic exploration.

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## 146 **2. METHODS**

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### 148 **2.1 Taxon sampling**

149 IUCN Red List shapefiles were imported into QGIS for mapping All currently described New  
150 World monkey (Platyrrhini) distributions (<https://www.iucnredlist.org/>), representing 161 species  
151 and 34 subspecies. Distinctions were made between points with GPS data, general geographic  
152 descriptions, and those based on IUCN shapefiles (Supplemental Table S1).

### 153 **2.2 Riverine analyses**

154 Geographic boundaries (i.e., rivers, mountains, plateaus, etc.) of all adjacent species and  
155 subspecies were measured using Google satellite maps. Annual river flow rates (m<sup>3</sup>/s) and river  
156 width (km) were used from Ayres & Clutton-Brock (1992) and Fordham et al. (2020), when  
157 available (Supplemental Table S2). We also used the Global Runoff Data Centre database to  
158 ascertain river measurements from streamflow-monitoring stations (GRDC, Germany). River  
159 width was measured in the midpoint of species boundaries from available satellite imagery using  
160 a previously published methodology (Fordham et al. 2020).

### 161 **2.3 Molecular phylogenetics**

162 All molecular data and associated sampling locations used in the main river barrier analysis were  
163 obtained from NCBI (<https://www.ncbi.nlm.nih.gov/>). Partial mitochondrial sequences (i.e.,

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164 cytochrome b and cytochrome oxidase subunit II) were trimmed to the smallest consensus size  
165 for both overall phylogenetic analysis and analysis between adjacent or sympatric taxonomic  
166 groups. A minimum of 200bp sequence and a maximum of 1104bp were available. The two  
167 mitochondrial genes were not concatenated but analyzed separately to independently verify  
168 results since sequence data was not necessarily available from the same individuals and for the  
169 same taxonomic groups. Each outgroup was chosen from the sister group, Catarrhini, using the  
170 closest available sequences for both cytb (NC\_056330.1) and COII (NC\_006901.1). We used  
171 MAFFT (version 7.407\_1) for sequence alignment and curated with trimAI (version 1.41)  
172 (Capella-Gutierrez et al., 2009). The phylogenetic tree was built using distance-based inference  
173 of phylogenetic trees with combined PhyML + SMS programs (version 1.8.1\_1). Molecular  
174 similarity was tested for normality using the Shapiro-Wilk W test, while the Mann-Whitney U  
175 test was used to compare nonparametric data between species and subspecies data for both  
176 molecular markers. Kruskal-Wallis nonparametric analysis of variance was used to compare  
177 molecular similarity values in taxonomic groups constrained by rivers, mountains, and those with  
178 no observable geographical barriers for both molecular markers. River characteristics (i.e., width  
179 and flow rate) were compared to the molecular similarity of adjacent taxa on opposing  
180 riverbanks using Spearman's nonparametric rank coefficient.

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### 182 **3. RESULTS**

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#### 184 **3.1 Geographical barriers**

185 Rivers were found at the interface of species boundaries in 73.9% (198/268) of analyzed cases,  
186 while mountains separated currently described species in 5.2% of cases (14/268). No observable  
187 geographical barriers were seen at the interface of 18.7% of species ranges. The majority



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188 (78.6%) of shared taxonomic boundaries (species and subspecies) were separated by  
189 geographical barriers such as rivers or mountains (236/300); however, several currently  
190 described genera had species interfaces with no geological barrier: *Lagothrix* (1/1 species), *Mico*  
191 (6/22 species), *Callicebus* (4/5 species), *Plecturocebus* (12/43 species), *Cebus* (6/18 species),  
192 and *Sapajus* (5/12 species). At subspecies boundaries, geographical barriers were present in 75%  
193 of cases (24/32 species).

194 The average width of rivers at all adjacent species boundaries was 1.0 km while  
195 subspecies had an average river width of 0.12 km. Subspecies were separated by rivers no larger  
196 than 0.22 km in width (Table 1). In general, wider rivers were associated with an increased  
197 molecular dissimilarity. The greatest average molecular dissimilarity (90%: cytb) was found on  
198 the Negro River, with an average width of 1.95 km at adjacent taxa boundaries. The rivers  
199 Branco, Jiparana, and Amazon all had average molecular similarity equal or less than 95% (cytb)  
200 while only the Juruá River had 95% similarity or less (COII). Several rivers were found  
201 separating multiple Platyrrhini taxa: the Negro River (1.95 km width; N= 2 genera, 2 species);  
202 Amazon (1.11 km; N=4,10), Madeira (1.2 km; N=1,3); Japurá (0.71 km; N=2,3); Tapajós (2.23  
203 km; N=4,4), Marañón (0.61 km; N=3,3); Huallaga (0.58 km; N=2, 2); São Francisco (1.02 km;  
204 N=2,3); Purus (0.3 km; N=2,2); Juruá (0.28 km; 3,3), Jequitinhonha (0.23 km; N=2,2).

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210 Table 1: River characteristics and molecular similarity for adjacent taxonomic groups on  
211 opposing riverbanks.

River names	<sup>1</sup> Updated annual streamflow (m <sup>3</sup> /s)	<sup>2</sup> Range of River width (km) <sup>3</sup>	Mean molecular similarity (%; COII)	Mean molecular similarity (%; cyt b)	Genera
Abacaxis	1670	0.22	-	95.2	<i>Plecturocebus</i>
Abacaxis	1670	0.22	98.1	-	<i>Mico</i>
Amazon	191354	1.79	-	95.4	<i>Alouatta</i>
Amazon	60868-154860	1.12-13.6	95.4-97.6	92.7-94.2	<i>Aotus</i> (N=3)
Amazon	52008-147785	1.2-8.6	96.8-98.1	94.0-95.0	<i>Ateles</i> (N=2)
Amazon	60868-191354	1.6	-	97.6-97.8	<i>Cheracebus</i> (N=2)
Amazon	93826-191354	1.1-1.4	-	96.4-96.8	<i>Saimiri</i> (N=2)
Amazon	93826	2.2	97.4	-	<i>Lagothrix</i>
Aripuanã	1713	0.19	-	96.1	<i>Plecturocebus</i>
Branco	2912	0.93	-	94.3	<i>Ateles</i>
Casanare	-	0.14	-	95.5	<i>Ateles</i>
Chagras	-	0.12	-	99.5	<i>Alouatta</i>
Coco	-	0.14	-	99.8	<i>Ateles</i> *
Corixo Grande	-	0.22	99.5	97.1	<i>Aotus</i> *
Doce	120	0.44	-	95.6	<i>Sapajus</i>
Grijalva	2750 <sup>^</sup>	0.14	-	96.1	<i>Alouatta</i>
Guasaule	-	0.05	-	99.8	<i>Ateles</i> *
Guayabero	-	0.15	-	96.9	<i>Saimiri</i> *
Heath	-	0.1	-	99.7	<i>Alouatta</i>
Huallaga	3500 <sup>^</sup>	0.58	-	95.0	<i>Ateles</i>
Huallaga	3500 <sup>^</sup>	0.58	-	97.0	<i>Saimiri</i>
Japurá	12714-13824	0.71-1.73	-	95.9-97.6	<i>Cheracebus</i> (N=2)
Japurá	12714	1.56	-	97.1	<i>Saimiri</i>
Jequitinhonha	203	0.23	98.0	98.6	<i>Callithrix</i>
Jequitinhonha	203	0.27	-	99.6	<i>Sapajus</i>
Jiparaná	711	0.23	-	95.1	<i>Plecturocebus</i>
Juruá	1561	0.34	95.0	92.1	<i>Aotus</i>
Juruá	3859	0.35	-	98.9	<i>Cheracebus</i>
Juruá	1561	0.28	98.2	95.8	<i>Saimiri</i> (N=2)
Juruá	4541	0.34	98.5	-	<i>Lagothrix</i>
Juruena	1427	0.96	-	96.3	<i>Plecturocebus</i>
Madeira	18203-37759	1.2-1.8	-	94.9-97.1	<i>Plecturocebus</i> (N=3)
Madre de Dios	9350 <sup>^</sup>	0.44	-	96.1	<i>Aotus</i>
Magdalena	7200 <sup>^</sup>	0.33	-	94.4	<i>Ateles</i>

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Marañón	16708 <sup>^</sup>	0.61	-	94.2	<i>Aotus</i>
Marañón	16708 <sup>^</sup>	0.61	-	95.0	<i>Ateles</i>
Marañón	16708 <sup>^</sup>	0.61	99.4	97.0	<i>Saimiri</i>
Nangaritza	-	0.05	95.2	-	<i>Aotus</i>
Negro	19753	1.95	95.2	94.1	<i>Aotus</i>
Negro	19753	3.65	-	85.9	<i>Saguinus</i>
Paraná	19183	0.82	-	94.5	<i>Alouatta</i>
Purus	6537	0.3	-	97.1	<i>Plecturocebus</i>
Purus	6537	0.53	-	96.1	<i>Saimiri</i>
Riacho do Fazendao	-	0.02	-	98.9	<i>Callithrix</i>
Río Motagua	209 <sup>^</sup>	0.2	-	95.8	<i>Alouatta</i>
Santiago	-	0.06	-	99.5	<i>Ateles</i> *
São Francisco	1205	0.56-0.9	-	95.5-95.6	<i>Sapajus</i> (N=2)
Tapajós	13349	0.66	99.0	95.8	<i>Aotus</i>
Tapajós	13349	2.23	98.4	94.3	<i>Ateles</i>
Tapajós	13349	1.7	96.9	-	<i>Mico</i>
Tapajós	13349	1.95	-	94.7	<i>Plecturocebus</i>
Tapajós	13349	2.06	-	98.0	<i>Saimiri</i>
Tempisque	-	0.05	-	99.3	<i>Ateles</i> *
Uatuma	134	0.12	-	92.5	<i>Saguinus</i>

212 Note: <sup>1</sup>Average monthly discharge from last 12 months from Global Runoff Data Centre. <sup>2</sup>River

213 width was calculated as the mid-way point on a river for two adjacent ranges using satellite

214 imagery. Currently described subspecies included (asterisk). If updated data was not available for

215 a particular river, then original Fordham et al. (2020)<sup>^</sup> data was used.

216

### 217 3.2 River width and molecular dissimilarity

218 River width was positively correlated with the molecular dissimilarity of adjacent taxa on

219 opposing riverbanks using both cytb (non-parametric Spearman's Rank Order Correlation:

220 N=59; r=0-.46; p=0.00; Figure 1a), and a smaller set of COII sequences which were not

221 significant (non-parametric Spearman's Rank Order: N=18; r=-0.23; p=0.36; Figure 1c). Annual

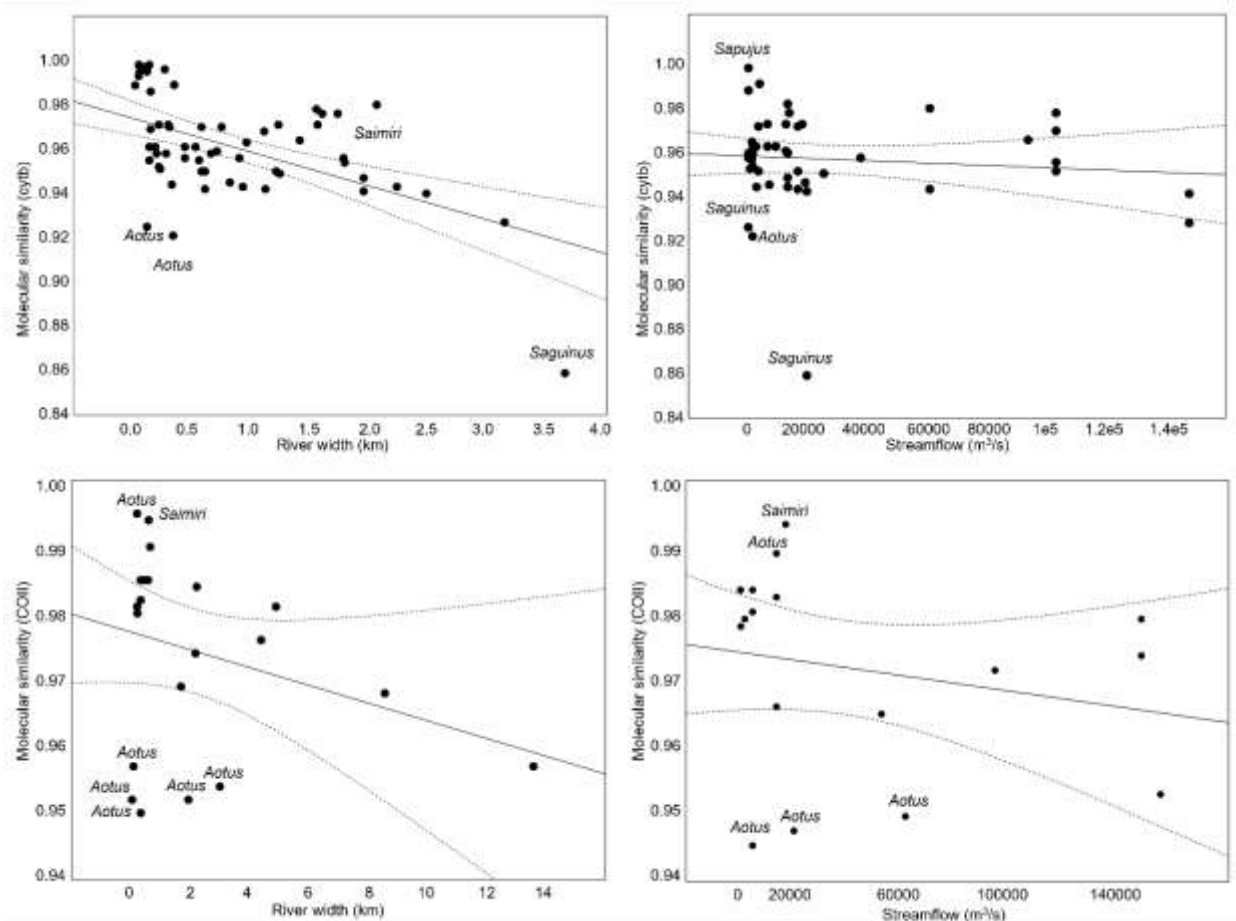
222 streamflow (m<sup>3</sup>/s) was not significantly associated with molecular dissimilarity in adjacent taxa

223 on opposite riverbanks for neither cytb (Spearman's Rank Order Correlation: N=46; r=0-.21;

224 p=0.16) nor COII (N=16; r=-0.30; p=0.26; Figure 1b,c), though there was a generally positive

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225 relationship. Several data points in all four analyses fell above and below the 95% confidence  
226 interval. However, when data from adjacent taxa with no river barrier were included along with  
227 unsupported samples (i.e., 100% molecular similarity between adjacent taxa), there was a similar  
228 – but significant - trend for both *cytb* (N=92;  $r=-0.37$ ;  $p=0.00$ ) and *COII* (N=31;  $r=-0.42$ ;  
229  $p=0.02$ ).  
230

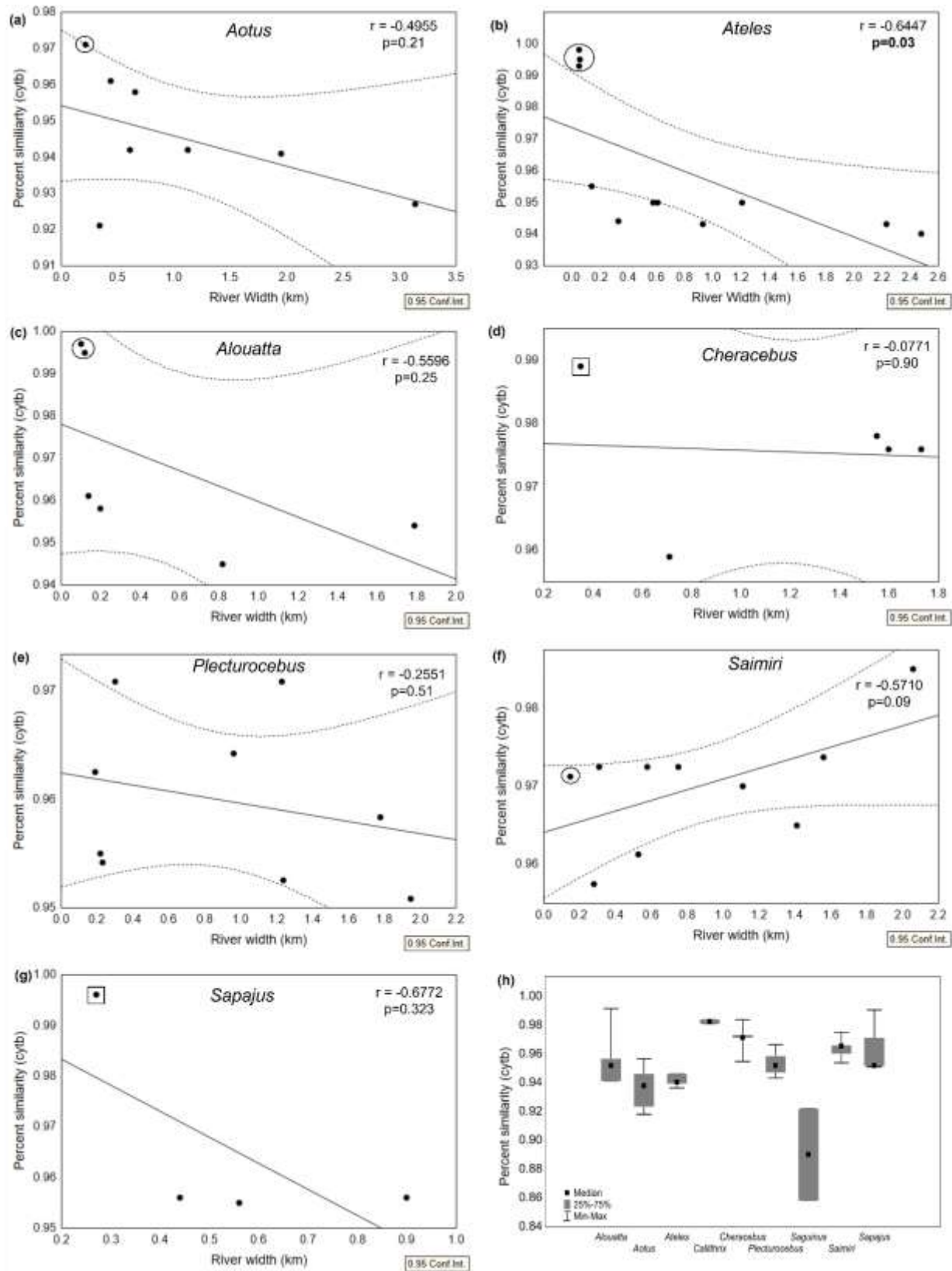


231  
232 Figure 1. Comparison of molecular similarity of adjacent taxa on opposing sides of river  
233 coupled with river width (km). Upper figures (a,b) represent analysis using partial cytochrome b  
234 (*cytb*) sequence and lower figures (c,d) use available cytochrome oxidase subunit II (*COII*) data.  
235 Ninety-five percent confidence interval represented by dashed lines.

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236  
237           Molecular similarity within each genus was negatively correlated with river width in all  
238 cases except for *Saimiri* (cytb; Spearman  $R = 0.53$ ,  $N=10$ ,  $p=0.11$ ; Figure 2). However, only  
239 *Alouatta* (cytb, Spearman  $R = -0.94$ ;  $N=6$ ;  $p<0.05$ ; Figure 2c) and *Ateles* (cytb, Spearman  $R = -$   
240  $0.87$ ;  $N=12$ ;  $p=0.00$ ; Figure 2b) exhibited a significant intra-genus negative relationship between  
241 wider river and increased molecular dissimilarity while *Callithrix* and *Saguinus* both had  
242 insufficient data for both molecular markers. *Saimiri* exhibited a similar trend for COII sequence.  
243

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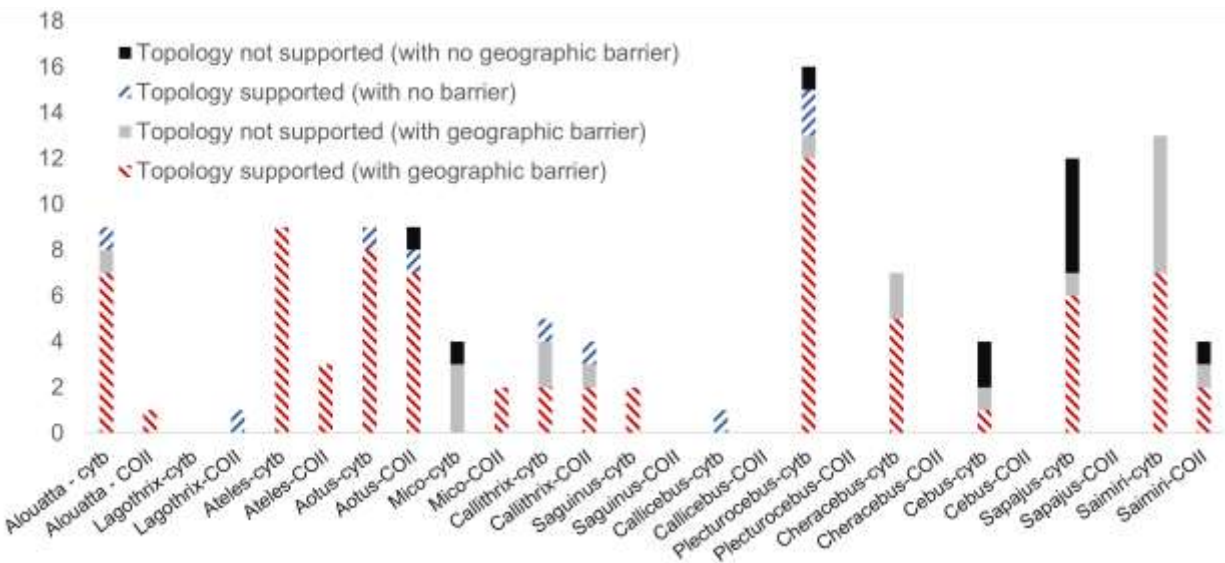
245  
246 Figure 2 (a-g) Association of river width (km) with cytb sequence similarity (y-axis) of adjacent  
247 species on opposing sides of barrier for each Platyrrhini genus with available data. *Saguinus* only  
248 included two data points and was not included. (h) Descriptive statistics for cytb sequence  
249 similarity data from (a-g), depicting those taxa with far lower median values compared to others  
250 (e.g. *Saguinus* and *Aotus*) and those taxa with upper ranges indicative of subspecies or possibly  
251 misclassified taxa (e.g., *Callithrix*, *Cheracebus*, *Saimiri*, and *Sapajus*). Dotted lines represent  
252 linear trendlines and include the correlation coefficient (r). Curved outer bands represent 95%  
253 confidence interval. Encircled points represent presently described subspecies while squares  
254 depict taxa presently described as species that have similar phylogenetic similarities consistent  
255 with subspecies classification.

256  
257 Several genera exhibited greater molecular divergence (i.e., lower similarity) on opposing  
258 riverbanks than others. *Saguinus* exhibited a far lower average molecular similarity range of  
259 values (Figure 2h: cytb mean: 89.2%; range: 85.9-92.5%) compared to most other genera though  
260 only statistically different from *Cherecebus* (cytb mean: 97.6%; range: 95.9-98.9%;  $p < 0.05$ ). The  
261 range of *Aotus* molecular similarity values from taxa on opposing riverbanks (Figure 2a: cytb  
262 mean: 94.0%; range: 92.1-96.1%) was also significantly lower than *Cheracebus* (Figure 2d: cytb  
263 mean: 97.6%; range: 95.9-98.9%;  $p < 0.05$ ) and *Saimiri* (Figure 2f: cytb mean: 96.9%; range:  
264 95.8-98.0%;  $p < 0.05$ ). COII data set was comparatively smaller and no statistically significant  
265 results were found between genera.

### 266 **3.3 Molecular phylogenetic trees**

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267 Seventy-five percent (Figure 3: cytb: 66/88) and 90.5% (COII: 19/21) of phylogenetic  
268 trees comparing all sequences within two adjacent taxonomic groups were supported.  
269 Unsupported molecular differentiation between adjacent taxonomic groups separated by rivers  
270 was 24.2% (cytb: N=66) and 10.5% (COII: N=19) of cases compared to adjacent taxonomic  
271 groups with no observable geographical barrier (cytb: 55.6%; N=18 and COII: 11.1%; N=9). All  
272 taxonomic groups separated by mountains were supported molecularly for both mitochondrial  
273 genes.



274  
275 Figure 3. Phylogenetic support for adjacent species with geographic barriers versus those without  
276 any river or mountain barrier. Does not include subspecies analysis. Molecular data was not  
277 available for *Brachyteles*, *Cebuella*, *Leontopithecus*, *Leontocebus*, and *Pithecia*.

278  
279 Several individual river barriers had statistically higher levels of molecular dissimilarity  
280 than anticipated. *Saguinus inustus* and *S. midas* only shared 85.9% molecular similarity (cytb)  
281 despite being adjacent species, though they were separated by one of the largest rivers in the  
282 Amazon Basin, the Negro River. Two other barriers had higher molecular dissimilarity than



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283 expected based on narrow river width: *Aotus nancymae* and *A. nigriceps* were 92.1%  
284 molecularly similar despite being separated by the Juruá River which is only 0.34 km wide, and  
285 *Saguinus midas* and *S. bicolor* were only 92.5% similar despite being separated by the Uatuma  
286 river with a width of only 0.12 km. Conversely, unexpectedly high molecular dissimilarity on  
287 opposing riverbanks was found for *Saimiri ustus* and *S. collinsi*. For example, the Tapajós River  
288 was over 2 km wide at a minimum, and yet these two species had 98% molecular similarity.

289 Well-resolved phylogenetic trees were found in 71.7% of cases using partial cytb  
290 sequences and 87.0% of cases using partial COII sequences – irrespective of whether physical  
291 barriers were present or not. For all adjacent species comparisons, mean molecular similarities  
292 were 95.6% (cytb: N=51; Interquartile range 50%: 94.5-97.0%) and 97.2% (COII: N=16;  
293 Interquartile range 50%: 95.6-98.2); while subspecies level differences were 98.5% (cytb: N=8;  
294 Median 99.4%; Interquartile range 50%: 97.0-99.65%) and 99% (COII: N=2; Interquartile range  
295 50%: 98.5-99.5%). The difference in mean molecular similarity between species versus  
296 subspecies similarity was significant using cytb (Mann-Whitney:  $p < 0.001$ ). Mean COII  
297 molecular similarity between species and subspecies was not significant (Mann-Whitney:  $p = 0.06$ ).

298 No statistical difference was found between molecular similarity across all taxonomic  
299 groups separated by rivers and mountains versus those with no discernable geographical barrier  
300 (Kruskal-Wallis (cytb:  $\chi^2 = 0.39$ ,  $p = 0.82$ ,  $df = 2$ ; COII:  $\chi^2 = 3.67$ ,  $p = 0.16$ ,  $df = 2$ ). An average  
301 molecular similarity was found of 95.5% (cytb: N=53) and 96.9% (COII: N=17) when river  
302 boundaries were present (range=89.2-98.8%), 96.3% (cytb) and 94.3% (COII) when mountain  
303 barriers were present (range=94.3-98.5%), and 95.6% (cytb) and 96.5% (COII) where no  
304 observable geological barrier was present (range=93.6-98.2%). Among subspecies, molecular  
305 similarity among adjacent taxa was on average 98.3% similar (cytb) and 96.7% (COII) on

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306 opposing geographical barriers, while adjacent taxa where no observable geographical barrier  
307 existed were 96.2% (cytb; N=2) and 99.0% (COII; N=1) similar. No subspecies were found to be  
308 separated by mountains.

### 309 **3.5 Suspect taxonomy**

310 Species delimitation was not supported in taxa on opposing river barriers in 25.8% (cytb)  
311 and 10.5% (COII) of analyzed cases (Table 2). In cases with no geographical barrier between  
312 presently described adjacent taxa species delimitation was not supported in 62.5% (cytb) and  
313 11.1% (COII) of cases. Unsupported phylogenetic relationships were found in only two instances  
314 where mountains separated taxonomic groups. Adjacent taxa that were not supported  
315 phylogenetically for either molecular marker were *Aotus azarai infulatus* and *A. azarai*  
316 *boliviensis* (now a proposed species by Martins-Junior 2022), *Callithrix geoffroyi* and *C.*  
317 *penicillata*, *Saimiri boliviensis boliviensis* and *S. b. peruviansis*, and *Saimiri oerstedii oerstedii*  
318 and *S. o. citrinellus*. Numerous other adjacent taxa were supported phylogenetically only at a  
319 single molecular marker, while for many other relationships data wasn't available for both  
320 species involved.

321 Of note, *Lagothrix* had three unsupported adjacent taxa (COII) out of seven interspecies  
322 borders. No cytb data was available for *Lagothrix*. All four bordering *Mico* adjacent species were  
323 phylogenetically unsupported (cytb only), though one of these cases was supported using COII.  
324 Three of these mixed results were at river boundaries and one with no geographical barrier.  
325 *Saimiri* had nine unsupported phylogenetic cases. No shared *Saimiri* species borders were  
326 supported molecularly using cytb, though two adjacent taxa were supported using COII data. Six  
327 bordering *Saimiri* had river barriers while one subspecies case had a mountain range, and another  
328 had no geographical barrier. Of four bordering *Cebus* adjacent species with available data (only

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329 cytb), only one barrier was supported (mountains), while two had no geographical barrier and  
330 another was separated by the Branco River (0.6km width). Finally, six bordering *Sapajus* species  
331 were not supported phylogenetically (only cytb available) for which five had no geographical  
332 barrier.

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350 Table 2: Evidence of unsupported topologies for at least one molecular marker (or both) between  
 351 presently described adjacent taxonomic groups. cytb and COII values represent percent sequence  
 352 similarity. All *Saguinus* relationships were supported.

Genus	Species sharing adjacent ranges	cytb	COII	River Width (km)	Fordham 2020 Methodology*
<i>Alouatta</i>	<i>A. macconnelli</i> and <i>A. seniculus</i>	Unsupported	No data	1.95	19753
<i>Aotus</i>	<i>A. azarai infulatus</i> and <i>A. a. boliviensis</i>	Unsupported	Unsupported	0	0
	<i>A. azarai azarai</i> and <i>A. a. boliviensis</i>	97.7%	Mixed	0	0
<i>Callithrix</i>	<i>C. geoffroyi</i> and <i>C. penicillata</i>	Unsupported	Unsupported	0.07	No data
	<i>C. penicillata</i> and <i>C. aurita</i>	Unsupported	94.3%	0	NA
<i>Cheracebus</i>	<i>C. purinus</i> and <i>C. torquatus</i>	Unsupported	No data	1.23	No data
	<i>C. lugens</i> and <i>C. torquatus</i>	Unsupported	No data	0.06	No data
<i>Cebus</i>	<i>C. albifrons</i> and <i>C. castaneus</i>	Unsupported	No data	0.93	2912
	<i>C. albifrons</i> and <i>C. olivaceus</i>	Unsupported	No data	0	0
	<i>C. olivaceus</i> and <i>C. castaneus</i>	Unsupported	No data	0	0
<i>Lagothrix</i>	<i>L. lagotricha poeppigii</i> and <i>L. l. tschudii</i>	No data	Unsupported	0.67	No data
	<i>L. lagotricha lugens</i> and <i>L. l. lagotricha</i>	No data	Unsupported	0	0
	<i>L. lagotricha lagotricha</i> and <i>L. l. poeppigii</i>	No data	Unsupported	0	0
<i>Mico</i>	<i>Mico humeralifer</i> and <i>M. argentatus</i>	Unsupported	96.9%	0.96	13349
	<i>Mico mauesi</i> and <i>M. humeralifer</i>	Unsupported	No data	0.18	No data
	<i>Mico mauesi</i> and <i>M. chrysoleucos</i>	Unsupported	No data	0.48	No data
	<i>Mico mauesi</i> and <i>M. melanurus</i>	Unsupported	No data	0	0
<i>Plecturocebus</i>	<i>Plecturocebus discolor</i> and <i>P. cupreus</i>	Unsupported	No data	0.67	No data

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	<i>Plecturocebus moloch</i> and <i>P. vieirai</i>	Unsupported	No data	0	0
<i>Saimiri</i>	<i>Saimiri boliviensis</i> <i>boliviensis</i> and <i>S. b.</i> <i>peruviensis</i>	Unsupported	Unsupported	0	0
	<i>Saimiri cassiquiarensis</i> <i>cassiquiarensis</i> and <i>S.</i> <i>sciureus</i>	Unsupported	No data	2.35	19753
	<i>Saimiri cassiquiarensis</i> <i>cassiquiarensis</i> and <i>S.</i> <i>ustus</i>	Unsupported	No data	2.73	191354
	<i>Saimiri cassiquiarensis</i> <i>macrodon</i> and <i>S.</i> <i>vanzolinii</i>	Unsupported	No data	0.76	No data
	<i>Saimiri ustus</i> and <i>S.</i> <i>sciureus</i>	Unsupported	No data	2.5	191354
	<i>Saimiri sciureus</i> and <i>S.</i> <i>collinsi</i>	Unsupported	No data	4.16	191354
	<i>Saimiri oerstedii</i> <i>oerstedii</i> and <i>S. o</i> <i>citrinellus</i>	Unsupported	Unsupported	0.09	No data
	<i>Saimiri ustus</i> and <i>S.</i> <i>vanzolinii</i>	Unsupported	No data	1.2	No data
	<i>Saimiri cassiquiarensis</i> <i>macrodon</i> and <i>S. c.</i> <i>albigena</i>	Unsupported	No data	0	NA
<i>Sapajus</i>	<i>Sapajus nigrinus</i> and <i>S. cay</i>	Unsupported	No data	0.82	19183
	<i>Sapajus apella apella</i> and <i>S. libidinosus</i>	Unsupported	No data	0	0
	<i>Sapajus apella apella</i> and <i>S. cay</i>	Unsupported	No data	0	0
	<i>Sapajus flavius</i> and <i>S. libidinosus</i>	Unsupported	No data	0	0
	<i>Sapajus nigrinus</i> and <i>S. libidinosus</i>	Unsupported	No data	0	0
	<i>Sapajus libidinosus</i> and <i>S. cay</i>	Unsupported	No data	0	0

353 \*Annual streamflow (m<sup>3</sup>/s) using average monthly discharge from last 12 months.

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355 **4. DISCUSSION**

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357           The findings of this study demonstrate the significant role of rivers as primary  
358 geographical barriers that separate species and subspecies of Platyrrhini primates. Rivers were  
359 frequently observed at species boundaries, while mountains played a lesser role in species  
360 separation. Specifically, we identified 35 rivers across Central and South America that  
361 effectively restricted gene flow between adjacent pairs of New World monkey species. Larger  
362 rivers such as the Amazon, Japura, Madeira, and Tapajós were found to separate three or more  
363 genera of primates and exhibited greater molecular dissimilarity on opposing riverbanks. Smaller  
364 rivers like the Riacho do Fazendao and the Nangaritzza also showed associations with divergent  
365 molecular signatures in adjacent species. These results highlight the crucial role of rivers in  
366 shaping the genetic differentiation and diversity of Platyrrhini primates.

367           Our findings are consistent with previous research conducted by Ayres & Clutton-Brock  
368 (1992) and Fordham et al. (2019), which demonstrated a negative correlation between river size  
369 and species composition similarity on opposite riverbanks. Similar patterns have been observed  
370 in other taxa, such as plants (Nazareno et al., 2019), amphibians (Moraes et al., 2016;  
371 Figueiredo-Vázquez et al., 2021), and birds (e.g., Capparella, 1987; Hayes & Sewlal, 2004;  
372 Fernandes et al., 2012; Ribas et al., 2012; Pomara et al., 2014; Naka & Brumfield, 2018;  
373 Kopuchian et al., 2020), where larger river widths are associated with higher levels of genetic  
374 differentiation. Additionally, when comparing molecular similarity between taxa on opposing  
375 riverbanks, we observed varying levels of molecular divergence among genera. *Saguinus*  
376 exhibited significantly lower average molecular similarity compared to most other genera,  
377 indicating higher genetic differentiation. *Aotus* also showed lower molecular similarity values  
378 compared to *Cheracebus* and *Saimiri*. Furthermore, our study revealed that certain genera like  
379 *Lagothrix*, *Mico*, *Callicebus*, *Plecturocebus*, *Cebus*, and *Sapajus* exhibited species interfaces

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380 despite the absence of geological barriers, suggesting that other factors, such as ecological or  
381 behavioral differences, contribute to maintaining species boundaries in these cases.

382 Our findings revealed a positive correlation between river width and molecular  
383 dissimilarity across all genera, indicating that wider rivers act as stronger barriers to gene flow.  
384 The width of rivers at species boundaries varied, with an average width of 1.0 km for all adjacent  
385 species boundaries and 0.12 km for subspecies boundaries. Notably, the Negro River, with the  
386 widest average width (1.95 km) at adjacent taxa boundaries, exhibited the greatest average  
387 molecular dissimilarity. Other rivers such as the Branco, Jiparana, and Amazon also showed high  
388 molecular dissimilarity, suggesting their role as barriers to gene flow. Although the association  
389 between annual streamflow and molecular dissimilarity was not significant, a general positive  
390 pattern was observed when considering unsupported phylogenetic results and adjacent species  
391 similarity with no river barrier.

392 We further investigated the relationship between river width and molecular dissimilarity  
393 within each genus. Except for *Saimiri*, all other genera showed a negative correlation between  
394 river width and molecular similarity, indicating that wider rivers are associated with increased  
395 molecular dissimilarity within genera. However, presumably due to a small number of species,  
396 all but *Ateles* was insignificant within genera. Notably, *Alouatta* and *Ateles* exhibited significant  
397 intra-genus negative relationships, indicating a strong impact of wider rivers on genetic  
398 differentiation within these genera – despite their high level of mobility. Some specific cases  
399 highlighted unexpected levels of molecular dissimilarity between adjacent taxa separated by  
400 geographical barriers. For example, *Saguinus inustus* and *S. midas* exhibited particularly low  
401 molecular similarity (e.g., *cytb*: 85.9% similarity) even for a relatively wide river (i.e., Negro  
402 River: 3.65 km width). A limited number of data points were available for *Callithrix* and

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403 *Saguinus*, preventing definitive conclusions. These findings underscore the genus-specific  
404 differences in the influence of river width on genetic differentiation.

405         The ability of Platyrrhini taxa to traverse rivers or occupy different ecological niches  
406 associated with dispersal such as canopy and river edge species reported in birds (Burney &  
407 Brumfield, 2009), may explain why some species can cross larger rivers while others cannot.  
408 This alone may not be sufficient to explain why some species are capable of crossing larger  
409 rivers. Body size alone does not appear to be a determining factor, as relatively smaller species  
410 like capuchins (i.e., *Sapajus* and *Cebus*) are equally capable of crossing larger rivers compared to  
411 larger species like *Ateles*. For example, the relatively smaller, lower canopy capuchin species are  
412 not restricted by larger rivers while the large and very mobile *Ateles* is often found in the upper  
413 canopy but is limited by the Tapajos and Amazon Rivers (Lima et al., 2018). *Cheracebus* (mean  
414 male weight: 1.5 kg) had the highest molecular similarity when controlling for river width (cytb:  
415 97.7%) despite being far smaller than *Alouatta* (mean male weight: 9.8 kg) or *Ateles* (mean male  
416 weight: 11 kg). However, further research is needed to elucidate the specific mechanisms behind  
417 this phenomenon.

418         While hybridization and introgression are common along species edges of some  
419 Platyrrhini genera (e.g., Bicca-Marques et al., 2008; Mercês et al., 2015; Carneiro et al., 2016),  
420 many currently described adjacent species were not supported molecularly. Nevertheless,  
421 adjacent species were more phylogenetically similar when no geographical barrier was evident.  
422 This suggests the importance of incorporating geographic barriers into taxonomic descriptions.  
423 However, it is important to note that the two molecular markers used in this study may not be  
424 sufficient for distinguishing adjacent species. The intention was not to describe new species



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425 solely on two partial sequences, but as a quantitative measure of divergence. Further evidence  
426 from multiple disciplines should therefore be considered.

427 *Aotus* and *Saguinus* were far more dissimilar on opposing riverbanks than expected based  
428 solely on river size. Small rivers were sufficient to restrict dispersal in both genera, leading to  
429 higher molecular dissimilarity than expected based on overall relationship across all other New  
430 World monkey adjacent species despite their proficient arboreal nature. As an example, the Jurua  
431 River is only 0.34 km in width and was a barrier between adjacent species of *Aotus* (cytb and  
432 COII), *Cheracebus* (cytb and COII), *Saimiri* (cytb and COII), and *Lagothrix* (COII). However,  
433 *Aotus* showed the lowest molecular similarity between adjacent species on opposing riverbanks  
434 (cytb: 92.1% and 95.0%: COII). *Lagothrix* and *Saimiri* had the highest molecular similarity on  
435 opposing riverbanks (COII: 98.5% and 98.2%, respectively). The potential existence of new  
436 taxonomic diversity within several genera, including *Aotus*, has important conservation  
437 implications. The range of *Aotus nigriceps*, for example, encompasses a vast area in the Amazon  
438 basin that is currently facing significant deforestation and wildfires (Helenbrook & Valdez  
439 2021). The description of additional taxonomic diversity can provide important information for  
440 conservation planning and maximize protections for neotropical primate species and intraspecies  
441 diversity in these highly threatened habitats.

442 This study has several limitations that should be considered. Comparisons within genera  
443 were constrained by limited sample sizes, and a significant number of Platyrrhini species lacked  
444 available data for molecular analysis. In some instances, sequence data was accessible, but  
445 associated geographic coordinates were missing. Nonetheless, it may be feasible to deduce  
446 molecular divergence from geographical barriers, which could aid in identifying potential areas  
447 that might harbor new distinct taxa by incorporating biogeographical evidence. However, it is

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448 crucial to recognize that predictions of molecular divergence inferred solely from biogeography  
449 do not suffice to describe new taxa, and further research is necessary. The integration of multiple  
450 lines of evidence, including morphology, paleontology, behavior, and ecology, is crucial for a  
451 comprehensive understanding of species boundaries and genetic differentiation within Platyrrhini  
452 primates (Costa-Araujo et al., 2021).

453         Regarding the relationship between geographic barriers and speciation, there are  
454 significant questions regarding the history of riverine formation in the neotropics and their  
455 contribution to species richness and maintenance of contemporary species (e.g., Naka and  
456 Brumfield 2018; Méndez-Camacho et al. 2021). Estimates of river formation in the Amazon  
457 basin, ranging from 2-12 million years contribute to uncertainty (Rossetti et al., 2005; Campbell  
458 et al., 2006; Figueiredo et al., 2009; Hoorn et al. 2010; Latrubesse et al. 2010; Latrubesse et al.  
459 2010; Hoorn 2010; Ribas et al. 2012; Méndez-Camacho et al. 2021). While the formation of a  
460 river barrier preceding the origin of taxonomic divergence is not sufficient evidence to infer  
461 causation, additional evidence of endemism and other biogeographical patterns can provide  
462 further insights (Silva et al. 2002; Borges and Silva 2012).

463         Nonetheless, it is important to consider evidence of endemism that does not always align  
464 with river boundaries, as demonstrated by studies conducted by Hall and Harvey (2002) and  
465 Oliveira et al. (2017). Discrepancies may in part be explained by fluctuating river characteristics  
466 both due to sediment deposition in upper headwaters which allow dispersal, rearrangement of  
467 river networks (Ruokolainen et al. 2019), or more complex evolutionary processes. Nonetheless,  
468 our results quantitatively support the River Barrier Hypothesis despite these potentially  
469 confounding processes. Rather this study points to the importance of geological barriers in

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470 restricting dispersal, and how it could be used as a tool to predict additional taxonomic  
471 boundaries.

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## 473 **5. CONCLUSIONS**

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475 This study emphasizes the crucial role of rivers as primary geographical barriers that  
476 shape the genetic differentiation and diversity of Platyrrhini primates. In light of the alarming  
477 deforestation rates in New World monkey habitats, driven by agricultural expansion and fires in  
478 the Amazon rainforest (Cardil et al., 2020; Helenbrook & Valdez, 2021), urgent action is needed  
479 to protect these highly vulnerable primate species. Adopting a systematic approach that  
480 integrates statistical biogeography, molecular phylogenetics, and multiple lines of evidence is  
481 essential for better understanding species boundaries, genetic differentiation, and guiding  
482 targeted conservation efforts. Furthermore, the potential existence of new taxonomic diversity  
483 within Platyrrhini genera underscores the importance of comprehensive species descriptions and  
484 conservation planning to safeguard neotropical primate diversity.

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#### 715 **CONFLICT OF INTEREST**

716 The author acknowledges no potential conflict of interest.

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718 **DATA AVAILABILITY**

719 The raw data supporting the conclusions of this article are included in the article or as

720 Supplementary Material (Supplemental Table T1 and T2).

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722 **BIOSKETCH**

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