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1 **The influence of environmental variation on the genetic structure of a poison frog**
2 **distributed across continuous Amazonian rainforest**

3

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26 **Abstract**

27 Biogeographic barriers such as rivers have been shown to shape spatial patterns of
28 biodiversity in the Amazon basin, yet relatively little is known about the distribution of
29 genetic variation across continuous rainforest. Here, we characterize the genetic
30 structure of the brilliant-thighed poison frog (*Allobates femoralis*) across an 880 km
31 long transect along the Purus-Madeira interfluvium south of the Amazon river, based on
32 64 individuals genotyped at 7 609 SNP loci. A population tree and clustering analyses
33 revealed four distinct genetic groups, one of which was strongly divergent. These
34 genetic groups were concomitant with femoral spot coloration differences, which was
35 intermediate within a zone of admixture between two of the groups. The location of
36 these genetic groups did not consistently correspond to current ecological transitions
37 between major forest types. A multi-model approach to quantify the relative influence
38 of isolation-by-distance (IBD) and isolation-by-environmental resistance (IBR)
39 nevertheless revealed that, in addition to a strong signal of IBD, spatial genetic
40 differentiation was explained by IBR primarily linked to dry season intensity ($r^2 =$
41 8.4%) and canopy cover ($r^2 = 6.4\%$). We show significant phylogenetic divergence in
42 the absence of obvious biogeographical barriers and that finer-scaled measures of
43 genetic structure show patterns that are associated with environmental variables also
44 known to predict the density of *A. femoralis*.

45 *Keywords:* RADseq, genetic clusters, landscape genetics, Amazonia, amphibians

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51 **Introduction**

52 A key goal in ecology and evolutionary studies is to understand the processes that
53 explain contemporary patterns of genetic diversity. Based on the classic allopatric
54 speciation model, genetic divergence is a consequence of geographic isolation (Wallace
55 1852; Mayr 1963; Coyne and Orr 2004). However, divergence can also arise when
56 isolation is incomplete, under scenarios that may include ecologically-mediated
57 selection triggered by environmental heterogeneity (Nosil 2012; Shafer and Wolf 2013;
58 see also Endler 1977 for an early ‘gradient diversification hypothesis’). Recent evidence
59 that incipient diversification along environmental clines is often associated with
60 secondary contact of already existing ancient lineages (e.g., Dean et al. 2019; Marques
61 et al. 2019) further suggests that, when species’ range expand and contract over time,
62 allopatric and sympatric diversification models are not necessarily mutually exclusive.

63 Neutral genetic population structure arises through the interplay of drift,
64 mutation and migration. Disentangling the legacy of historical events on patterns of
65 genetic structure from more contemporary effects needs to account for the sensitivity of
66 the molecular assays, the analytical approaches employed, as well as recognizing the
67 time required for causal processes to shape genetic structure (Stow et al. 2001;
68 Anderson et al. 2010; Epps and Keyghobadi 2015). While isolation by geographic
69 distance (IBD, Wright 1943; Slatkin 1987) is revealed by most empirical studies (for
70 summaries see e.g. Jenkins et al. 2010; Sexton et al. 2014), gene flow can be further
71 influenced by the landscape matrix where habitat heterogeneity results in different
72 levels of resistance to migration (Manel et al. 2003; Storfer et al. 2010). Because
73 patterns of isolation-by-environmental resistance (IBR) are influenced by species-
74 specific life-history attributes and ecological preferences, such as propensity and ability
75 for migration through given environments, they reveal essential information about

76 habitat relationships of the studied taxa (Balkenhol et al. 2017; Armansin et al. 2020).
77 The spatial scale of sampling is an especially important consideration when testing for
78 IBD and IBR. If the scale of sampling is too small relative to the scale of gene flow of
79 the target species, gene flow from beyond the study area may overwhelm patterns of
80 genetic structure mediated by local environmental variables (Anderson et al. 2010). On
81 the other hand, observed genetic discontinuities may also have arisen from past events
82 rather than contemporary landscapes, due to a time lag between demographic processes
83 and their consequences for population genetic structure (Epps and Keyghobadi 2015).

84 For the world's largest area of continuous rainforest in Amazon basin, the main
85 processes responsible for spatial patterns of biodiversity remain debated (Moritz et al.
86 2000; Hoorn et al. 2010; Ribas et al. 2012; Leite and Rogers 2013). The majority of
87 empirical studies demonstrate that the retraction of past environmental barriers in the
88 Holocene resulted in range expansions of lineages that diverged in isolation up to about
89 0.8 million years ago (Ma), with major rivers often acting as local biogeographic
90 boundaries (e.g. Naka et al. 2012; Nazareno et al. 2017; Ribas et al. 2018; Thom et al.
91 2020). The vast, forested areas between major rivers of the Amazon basin are however
92 also characterized by gradual environmental variation, for which patterns of IBD and,
93 possibly, IBR might be expected for broadly distributed taxa. However, difficulties in
94 systematically sampling the vast, often inaccessible terrain of the Amazon basin has
95 resulted in the gradient hypothesis receiving little attention (Beheregaray et al. 2015).

96 Amphibians are well suited to detect environmental and geographic influences
97 on genetic divergence because they have low dispersal abilities and are sensitive to
98 ecological conditions (e.g., Zeisset and Beebee 2008; Pabijan et al. 2020). The brilliant-
99 thighed poison frog *Allobates femoralis* (Dendrobatoidea: Aromobatidae, Grant et al.
100 2017) is a small (~ 33 mm), ground-dwelling, iteroparous diurnal frog commonly

101 distributed throughout primary forest in the Amazon basin (Silverstone 1975;
102 Amézquita et al. 2009), and likely comprises cryptic taxa (Grant et al. 2006, 2017;
103 Fouquet et al. 2007; Santos et al. 2009; Simões et al. 2010). It prefers clay-rich soils and
104 is more abundant in open forest than in forest with closed canopies (Ferreira et al.
105 2018). Males exhibit territorial behavior and signal territory ownership by calling from
106 elevated positions on the forest floor (Roithmair 1994; Montanarin et al. 2011), with
107 their mating success possibly correlated to territory size (about 200 m² maximally,
108 Kaefer et al. 2012). Females lay egg clutches under leaf litter during the rainy season,
109 and tadpoles are usually transported by males to ephemeral puddles in order to complete
110 their development (Ringler et al. 2013). Both sexes are highly polygamous (Ursprung et
111 al. 2011), and life-time dispersal rates are generally low (about 100 m, Ringler et al.
112 2009; Pašukonis et al. 2016). Populations across Amazonia vary in the coloration of a
113 conspicuous femoral spot, which is both an aposematic signal through mimicry with
114 syntopic toxic species as well as sexually selected trait (Amézquita et al. 2009, 2017;
115 Ferreira et al. unpublished).

116 Here, we assess environmental and historical influences on the spatial genetic
117 structure of *A. femoralis* along an ~880 km long transect in the Purus-Madeira interfluve
118 (PMI) south of the Amazon river. We explore the existence of local genetic structure
119 along the transect using clustering techniques, and assess whether the genetic structure
120 of *A. femoralis* conforms to previous studies on other taxa along the same transect
121 (Ortiz et al. 2018; De Abreu et al. 2018). In parallel, we employ landscape genetic
122 inferences to compare the relative contribution of IBD and IBR, predicting that genetic
123 structure will be influenced by landscape variables that have previously been shown to
124 determine the occurrence and abundance of *A. femoralis* along this transect (land cover,
125 silt content, temperature seasonality, and intensity of the dry season; Ferreira et al.

126 2018). We also test whether there are genetic signals for selection associated with these
127 variables. Finally, we examine whether patterns of femoral spot coloration are
128 congruent with distinct genetic lineages and whether there is any evidence of lineage
129 admixture.

130

131 **Materials and Methods**

132 *Study area and sampling*

133 The Purus-Madeira interfluve (PMI) is situated south of the Amazon river and covers
134 approximately 15.4 million hectares, with vegetation, soil and climatic conditions
135 gradually changing along a latitudinal gradient (Cintra et al. 2013; Schietti et al. 2016).

136 The mean annual precipitation varies from 2200 mm to 2800 mm, and is highest in
137 central areas (Alvares et al. 2013; Fick and Hijmans 2017). The northeast of the PMI is
138 characterized by dense lowland rainforest with a mean tree basal area of 56.45 m² ha⁻¹,
139 plinthosols with a predominance of silt, and a complex hydrography with large
140 seasonally flooded areas (Fan and Miguez-Macho 2010; Schietti et al. 2016).

141 Southwestern and central parts are characterized by open lowland rainforest with a
142 mean tree basal area of 19.31 m² ha⁻¹, podzolic soils with high clay content, and small
143 temporary rivers filled during the rainy season (Cintra et al. 2013; Ferreira et al. 2018).
144 Considerable areas of savanna are also present between these two forested regions
145 (IBGE 1997; Figure 1).

146 Between November and March 2010-2015, we collected a total of 66 *A.*
147 *femoralis* individuals from 13 localities along an established 880 km transect which
148 runs in parallel to a federal highway (BR-319), and spans the entire length of the PMI
149 (Figure 1; Table S1). Sampling was carried on regularly spaced biodiversity monitoring
150 plots (modules) constructed by the Rapid Assessment for Long Duration Ecological

151 Projects system (RAPELD; for details see Magnusson et al. 2013). The same sampling
152 design has previously been used to quantify environmental correlates for the occurrence
153 and abundance of *A. femoralis* (Ferreira et al. 2018), and revealed that the species is
154 present in all but three modules (M3-5, see Figure 1). *Allobates femoralis* was sampled
155 by acoustic and visual surveys during the daily periods of peak vocalization (7:00-10:00
156 a.m. and 14:00-18:00 p.m.). We captured frogs by hand and maintained them in sealed
157 plastic bags until arrival in the laboratory, where they were sacrificed and fixed after
158 tissue (leg muscle) was removed for genetic analyses and stored in 96% ethanol. For
159 each captured individual, the femoral spot coloration was noted as yellow, red, or
160 orange.

161

162 *DNA extraction, genotyping and initial filtering*

163 Extraction of DNA and SNP discovery was carried out at Diversity Arrays Technology
164 sequencing Pty. Ltd. (DARTseq) facility (Canberra, Australia; more detail in
165 Supplementary Information Text S1). A modified double-digest restriction-site
166 associated DNA (ddRAD) sequencing protocol was performed on libraries prepared
167 using a combination of *Pst*I-*Hpa*II restriction enzymes (Kilian et al. 2012). The *Pst*I
168 enzyme adaptor also contained an Illumina adaptor sequence, a primer sequence and a
169 variable-length barcode as described by Elshire et al. (2011). The *Hpa*II adaptor
170 contained an Illumina flow cell attachment and overhang sequence. Following
171 enzymatic digestion, fragments were amplified and sequenced on an Illumina
172 HiSeq2500. DNA sequences were aligned via BLAST using the *Nanorana parkeri*
173 reference genome (Sun et al. 2015). To check for contamination, sequences were also
174 blasted to bacterial and fungal genomes (NCBI).

175 A raw dataset of 147 595 SNPs was filtered for missing data using the *filter_dart*

176 function of the *R* package RADIATOR v. 0.010 (Gosselin 2017). Only individuals and
177 loci with $\geq 95\%$ SNPs genotyped were retained. SNPs were also screened for allele
178 coverage, with any SNPs displaying a local and global minor allele frequency (MAF)
179 threshold of less than 1% removed from the dataset. In cases where multiple SNPs were
180 found within the same read, only one locus was retained (chosen randomly per RAD
181 tag) to avoid statistical bias from physical linkage (Lemay and Russello 2015; Zheng et
182 al. 2012). Two samples from M14 had $< 95\%$ of loci genotyped and were removed,
183 which resulted in 64 individuals from 13 populations genotyped at 10 275 SNPs (see
184 Table S2 for summary of filtering steps). File types required for downstream analyses
185 were created using the RADIATOR package (Gosselin 2017), PGDSpider v. 2.1.1.3
186 (Lischer and Excoffier 2012) and PLINK v. 1.9 (Chang et al. 2015).

187

188 *Phylogenomic relationships*

189 In order to evaluate the evolutionary relationships among *A. femoralis* possessing
190 different femoral spot coloration we constructed a population tree by coalescence using
191 SNAPP v. 1.4.1 (Bryant et al. 2012) implemented in BEAST v. 2.5 (Bouckaert et al.
192 2014). This analysis assumes a lack of gene flow among lineages which is inferred by
193 phenotypic distinctiveness and further tested using clustering analyses. To reduce
194 computational requirements and run times, we selected 2-3 representative individuals
195 per population without signatures of between-population admixture (assessed through
196 femoral spot color). We used our data set of 10 275 SNPs, and mutation rates (u and v)
197 as estimated by SNAPP, with the birth rate (λ) of the Yule prior based on the number of
198 samples used. The trial run for each dataset used a chain length of 1000 000
199 generations, sampling every 1 000 trees. We inspected final log files and created
200 maximum clade credibility trees (median node heights) by combining three independent

201 runs in TreeAnnotator v. 2.5 implemented in BEAST after discarding 25% as burn-in.
202

203 *Detection of SNPs associated with selection*

204 We removed SNPs with evidence of being associated with selection because our
205 population and landscape genetic inferences assume neutral loci (see e.g., Rellstab et al.
206 2015). Analyses to detect loci associated with selection were conducted on the full
207 dataset using two different approaches. First, we detected SNPs under putatively
208 positive or negative selection using F_{ST} outlier analysis (OA) with BayeScan v.2.1 (Foll
209 and Gaggiotti 2008), a Bayesian method based on a logistic regression model which is
210 suited to detecting outliers in scenarios with low-admixed samples while taking into
211 account sample size and genetic structure (Villemereuil et al. 2014; Luu et al. 2017).
212 We ran BayeScan using a prior model (prior odds parametrization) set to 100, thinning
213 interval of 10-20 pilot runs of length 10 000, and burn-in of 50 000 steps. Second, we
214 used Environmental Association Analysis (EAA) with Latent Factors Mixed Models
215 (LFMM), implemented in the *R* package LEA v. 2.1.0 (Frichot and François 2015).
216 LFMM uses a hierarchical Bayesian mixed model based on residuals from PCA that
217 take population genetic structure into account (e.g. Benestan et al. 2016). We ran
218 LFMMs for each of the four environmental variables which were previously identified
219 as predictors of local abundance (Ferreira et al. 2018): land cover, silt content,
220 temperature seasonality, and intensity of the dry season, separately using 10 000
221 iterations, a burn-in of 5 000 steps, and 5 repetitions. We set both BayeScan and LFMM
222 with a false discovery rate of 0.05 (5%). We also investigated whether the SNPs
223 identified as signaling selection could be attributed to a functional part of the genome in
224 order to complement our tests of the influence of landscape variables on gene flow, as
225 variables influencing connectivity may also impose selection (Armansin et al. 2020).

226 Consequently, gene annotations were sought for RAD tags that contained SNPs
227 identified with both BayeScan and LFMM using the NCBI BLAST platform (Johnson
228 et al. 2008). Sequences were annotated to genes classified as ‘*amphibians*’ (taxid:8292),
229 ‘vertebrates’ (taxid:7742) and aligned using the *Nanorana parkeri* (taxid:125878)
230 reference genome (Sun et al. 2015), using BLAST with an E-value threshold of 0.0001.

231 All SNPs that provided evidence for selection were removed from the data set
232 for all downstream analyses of genetic structure. Summary statistics were calculated for
233 each of the modules and any remaining loci that deviated from Hardy-Weinberg
234 Equilibrium at a Bonferroni-correction $\alpha = 0.004$ (1 000 simulations) were also
235 excluded from the dataset. Estimates of observed (H_O) and expected (H_E)
236 heterozygosity, inbreeding coefficients (F_{IS}) and private alleles were calculated using
237 the R-package *diveRsity* v. 1.9.90 (Keenan et al. 2013) with 95% confidence interval
238 calculated with 1 000 bootstraps.

239

240 *Genetic Structure*

241 Genetic structure was described with putatively neutral loci using the model-based
242 clustering approaches implemented by ADMIXTURE (Alexander et al. 2009) and
243 sNMF in the R package LEA v. 2.1.0 (Frichot et al. 2014). To ensure that the
244 underlying genetic structure was not violating the assumptions of these models, we also
245 carried out Discriminant Analysis of Principal Components (DAPC) calculated using
246 the R package *adegenet* v. 2.1.1 (Jombart et al. 2010). Genetic partitioning was further
247 described by calculating pairwise F_{ST} between 11 sites in the R-package *adegenet* v.
248 1.3.1 (Jombart and Ahmed 2011).

249 sNMF is a method based on sparse non-negative Matrix Factorization
250 algorithms (NMF) and least-squares optimization (Frichot et al. 2014). We tested the

251 number of genetic clusters (K) ranging from 1 to 11 (upper limit equal to the number of
252 sampling localities) with 20 independent runs per test, alpha set at 100, a tolerance error
253 of 0.00001, entropy set as true (where the cross-entropy criterion is calculated), a
254 random seed of 50, and 10 000 interactions in the algorithm. The best-supported K was
255 determined by the lowest error value of ancestry through the cross-entropy criterion.
256 ADMIXTURE simultaneously estimates the probability of the observed genotypes
257 using ancestry proportions and population allele frequencies (Alexander et al. 2009).
258 Significance was defined at $p < 0.05$, above which individuals were considered pure.
259 We ran ADMIXTURE using a cross-validation with a random seed as 43, the block
260 relaxation algorithm as the point estimation method, QuasiNewton as the convergence
261 acceleration algorithm, and a delta of < 0.0001 to terminate point estimations. The
262 number of K was determined by the lowest cross-validation error value. DAPC is a
263 multivariate method that performs discriminant functions to describe the relationships
264 between clusters as well as membership probabilities of each individual for different
265 groups, optimizing variance between groups while minimizing variance within groups
266 (Jombart et al. 2010). We used cross-validation to define the number of principal
267 components (PCs) retained in the analysis, identifying the optimal point in the trade-off
268 between retaining too few and too many PCs in the model. We used the number of PCs
269 associated with the lowest Root Mean Squared Error - RMSE as the optimum number
270 for the PCA in the DAPC analysis. Eight PCs and two DAs were retained for the
271 analyses, and explained 41% of the total variance. To test whether the number of
272 sampled individuals in each module was sufficient for the inferences of genetic
273 structure, we ran the above analyses with two alternative datasets: all individuals
274 sampled, and three randomly chosen individuals for each module only.
275

276 *Construction of Environmental Resistance Surfaces*

277 To test the effects of landscape variables on genetic connectivity in *A. femoralis*, we
 278 used four environmental variables with ecological effects for the species as predictors of
 279 local abundance (see Ferreira et al. 2018): land cover, silt content, temperature
 280 seasonality (representing the annual range in temperatures) and the Walsh index, a
 281 measure of the intensity and duration of the dry season (Walsh 1996). Environmental
 282 data were obtained from the public repository Ambdata (www.dpi.inpe.br/Ambdata;
 283 Amaral et al. 2013), and converted to raster format using the *R* package *raster* v. 2.6.7
 284 (Hijmans 2017) with a cell resolution of 30 arcsecond (1 km²). To avoid model
 285 overparameterization, we tested for collinearity between variables through pairwise
 286 Pearson's correlations analyses based on values extracted of each sampling location.
 287 The four variables were not strongly correlated with each other ($r < 0.65$ in all cases)
 288 and were therefore retained. To facilitate comparisons among surfaces, we standardized
 289 all raster files to values between 1 and 100 (following Row et al. 2017, see Figure 2).

290 We generated multiple resistance surfaces from our environmental variables to
 291 test multiple hypotheses about their effects on genetic distance following Yadav et al.
 292 (2019), evaluating each resistance surface model separately. We assumed that resistance
 293 in each raster cell was a function of environmental variables as follows:

$$294 \quad r_i = 1 + \alpha \left(\frac{v_i - 1}{max - 1} \right)^\gamma,$$

295 where r_i is the resistance of raster cell i , v_i is the environmental variables value in cell
 296 i , and max is the maximum value of the raster surface (in our case 100, see above).

297 Furthermore, α is a parameter that determines the maximum possible resistance value,
 298 and γ is an exponent that determines the shape of the relationship (slope) between
 299 environmental variable values (v_i) and resistance (r_i), being linear when $\gamma = 1$ and
 300 nonlinear when $\gamma \neq 1$ (Shirk et al. 2010; Dudaniec et al. 2013, 2016). This approach has

301 been shown to effectively identify IBR including linear and non-linear relationships
302 (Shirk et al. 2010; Dudaniec et al. 2013, 2016; Yadav et al. 2019). The equation
303 expresses resistance as a function of the effect of landscape features. Based on previous
304 information (Ferreira et al. 2018), we assume that the effects of land cover and
305 temperature seasonality on resistance are negative and positive, respectively (Figure 3).

306 We used values of 0, 5, 10, 100, 1000 for intercept (α), and values of 0.01, 0.1,
307 0.5, 1, 5, 10, 100 for slope (γ) to create linear and non-linear resistance surfaces. Models
308 where α is equal to zero (seven models for each landscape feature) are identical
309 regardless of γ values, indicating no influence of resistance on genetic connectivity,
310 which reduced the resistance surfaces for each dataset to 29 unique models. Values of γ
311 < 1 represent resistance surfaces with increased sensitivity, $\gamma = 1$ represents a linear
312 resistance relationship and $\gamma > 1$ are resistance surfaces with reduced sensitivity (Figure
313 2). We calculated pairwise resistance distance matrices for all landscape features using
314 circuit theory (Hanks and Hooten 2013; McRae et al. 2008) as implemented in
315 CIRCUITSCAPE v. 4.0.5 (McRae 2006). This approach identifies all possible pathways
316 of movement between focal points across a given raster dataset and calculates average
317 cumulative resistance between all pairwise sampling sites.

318

319 *Landscape genetic resistance modelling*

320 To evaluate the contribution of landscape features in explaining genetic differentiation,
321 we fitted a Maximum-Likelihood Population-Effects (MLPE) mixed-effects model as
322 implemented within the *mlpe_rga* function using the R package *ResistanceGA* v. 4.0-4
323 (Peterman 2018). This model uses individual pairwise metrics for genetic differentiation
324 and landscape resistance, considering each pairwise data point as an observation. The
325 lack of independence is incorporated as a population-level factor which distinguishes

326 between data points that share a common deme, and those that do not (Clarke et al.
327 2002; Row et al. 2017). Individual based pairwise genetic distance was measured as
328 $F_{ST}/(1-F_{ST})$ and used as the dependent variable, resistance distance as the independent
329 variable, and population as the random variable. We fitted the mixed-effects models
330 using parameterization to account for the non-independence of values within pairwise
331 distance matrices without restricting maximum-likelihood (Clarke et al. 2002; Van
332 Strien et al. 2012). Next, to identify which model best described genetic distance among
333 sites, we performed a model selection approach using Akaike Information Criteria
334 (AICc). We then calculated the difference between the AIC of each model and the
335 minimum AIC value found (Burnham and Anderson 2002; Diniz-Filho et al. 2008) with
336 the lowest change in AICc score ($\Delta AICc=0$) and the largest AIC weight ($wAICc$)
337 considered the most parsimonious model. These analyses were performed using the R
338 package *ResistanceGA* v. 4.0-4 (Peterman 2018), with MLPE models fitted with
339 *mlpe_rga* using the standard *lme4* v. 1.1-17 formula interface (Clarke et al. 2002; Bates
340 et al. 2015), *magrittr* v. 1.5 (Bache and Wickham 2014), and *dplyr* v. 0.7.4 (Wickham et
341 al. 2017).

342

343 *Effects of IBD and IBR on genetic differentiation*

344 We used a Mantel test (Mantel 1967) to estimate the significance of any relationship
345 between pairwise F_{ST} and geographic distance (km) using the function *mantel.randtest*
346 implemented in the *ade4* v. 1.7-11 R-package (Dray and Dufour 2017), with 10 000
347 permutations. We also carried out an independent test for spatial autocorrelation
348 between geographic and genetic distance using a Mantel correlogram (Oden and Sokal
349 1986), computed using the function *mantel.correlog* with 10 000 permutations. The
350 number of geographic distance classes was selected by the Struges equation, Pearson

351 correlation and correction of p values through FDR in the *R* package *vegan* v. 2.5.1
352 (Oksanen et al. 2018).

353 The effect of IBR decoupled from IBD was calculated using distance-based
354 redundancy analysis (dbRDA) using *vegan* v. 2.5.1 (Oksanen et al. 2018). dbRDA is a
355 direct extension of a multiple regression to model multivariate response data (Legendre
356 and Gallagher 2001; Benestan et al. 2016), and was used to quantify the correlation
357 between the best MLPE model for each landscape variable and $F_{ST}/(1-F_{ST})$, assuming
358 models with genetic differentiation as the dependent variable and cost distances as
359 independent variables, conditioned on IBD. We obtained statistical significance from
360 each dbRDA model using Analyses of Variance (ANOVA; 1 000 permutations).

361 To verify that our limited sample size did not affected the MLPE and dbRDA
362 inferences, we sub-sampled our data with three random individuals for each module, re-
363 calculated F_{ST} values, and correlated the complete and sub-sampled F_{ST} matrices against
364 each other. A correlation coefficient of 1.00 suggested that the sample sizes in the
365 analyses provided reliable estimates.

366

367 **Results**

368 *F_{ST} Outlier Analysis and Environmental Association Analysis*

369 Outlier analysis with BayeScan detected 174 SNPs with significantly high F_{ST} (2.28%).
370 The analysis with LFMM identified 1281, 912, 859 and 689 SNPs associated with land
371 cover, the Walsh index, silt content and temperature seasonality, respectively. Of these,
372 43 SNPs were associated with each of the four environmental variables (Figure S1).
373 Twenty-three outliers were in common for the BayeScan and LFMM analyses, none of
374 which resulted in significant matches to either the *N. parkeri* genome or during BLAST
375 searches using Genbank.

376 We removed the 23 loci in consensus between EAA and outlier approaches to
377 produce an approximately neutral data set for population and landscape genetic
378 analyses. Preliminary analyses indicated that inclusion or exclusion of these loci
379 deviating from neutral expectations made no detectable difference to the results.
380 Because of the strong genetic divergence of modules 1 and 2 from the remaining
381 modules (see SNAPP analysis below), these two modules were excluded from the
382 landscape genetic analyses to allow for subtle environmental influences on genetic
383 structure to be detected. With the exclusion of the SNPs with signatures of selection and
384 data from M1 and M2, a total of 7 609 SNPs were available for analysis. Summary
385 statistics for modules M6-M14 are provided in Table 1.

386

387 *Population Tree*

388 The population tree constructed with SNAPP showed that individuals from the northern
389 modules M1 and M2 (yellow femoral spot) belong to a strongly divergent lineage
390 (Figure 4, Figure S2), consistent with the relatively high pairwise F_{ST} values found
391 between M1 or M2 and the other localities (F_{ST} range 0.72-0.83). The remaining
392 modules were split into three markedly shallower but distinct individual clades
393 (posterior probability = 1.00 in all cases), with Cluster C formed by the most distal node
394 (Figure 4).

395 Corresponding with the genetic lineages identified using SNAPP, the population
396 genetic inferences with ADMIXTURE, sNMF and DAPC produced a congruent result
397 of three inferred genetic clusters from Module 6 onward (Figure 5, see also Figure S3).
398 The first Cluster A comprised 14 individuals with red femoral spots across modules
399 M6-M8 in dense forest. It was distinct from a second Cluster B, which comprised 24
400 individuals from five populations (BM8_M9 - M11) across dense and open forest. This

401 cluster largely comprised individuals with yellow femoral spots, with the exception of
402 population BM8_9 with an intermediate (orange) coloration and evidence of genetic
403 admixture (Figure 5). A third cluster (C, characterized by red femoral spots) was
404 confined to 16 individuals from the eastern bank of the upper Madeira river (M12 to
405 M14), an open forest area separated from the remainder of the transect by patches of
406 savannah. Reducing the dataset to three individuals for all modules did not alter the
407 genetic partitioning revealed by each of the three clustering methods, demonstrating that
408 the sampling regime was sufficient to resolve genetic structure (Supplementary Figure
409 S4).

410

411 *Isolation by geographic distance (IBD) and environmental resistance (IBR)*

412 Pairwise genetic distances (F_{ST}) across modules M6 to M14 ranged from 0.020 (M13
413 and M14) to 0.207 (M6 and M14; Table 2), with a strong association between genetic
414 and geographic distances and therefore IBD (Mantel test: $p < 0.0001$, $r^2 = 0.96$, Figure
415 6). The Mantel correlograms calculated for seven classes of geographic distance
416 revealed spatial autocorrelation in four cases: positively at geographic distances to 60
417 km ($r = 0.67$, $p < 0.001$) and 143 km ($r = 0.24$, $p = 0.02$), and negatively at distances of
418 476 km ($r = -0.61$, $p = 0.03$) and 560 km ($r = -0.61$, $p < 0.001$; Figure S5).

419 Our MLPE analysis showed that a land cover model with $\alpha = 5$ and $\gamma = 10$
420 explained 98% of the genetic variation (Table 3). The Walsh index explained 96% of
421 the genetic variation at $\alpha = 100$ and $\gamma = 5$, and temperature seasonality and silt content
422 explained 95% of the genetic variation each, at $\alpha = 10$ and 1000, and at $\gamma = 5$ and 1,
423 respectively (Table 3). The α values determine the maximum resistance of the variables
424 (e.g., in the case of Walsh index, $\alpha = 100$ suggests that landscape resistance to gene
425 flow is 100 times greater than zero), and the γ values indicate whether the variable

426 influenced genetic connectivity linearly or non-linearly. Silt presented a value of $\gamma = 1$,
427 suggesting a linear resistance relationship. All other confidence sets of resistance
428 surfaces presented values $\gamma > 1$, supporting resistance surfaces with reduced resistance
429 sensitivity. ΔAIC values were identical for the four landscape features (0.00),
430 supporting the maximum-likelihood models. In the dbRDA models, the Walsh index
431 captured 8.4% of the observed genetic variation ($F_{1,52} = 41.72$; $p = 0.001$), followed by
432 land cover (6.4%; $F_{1,52} = 26.85$, $p = 0.001$), temperature seasonality (5.3%; $F_{1,52} = 20.54$,
433 $p = 0.001$) and silt content (3.5%; $F_{1,52} = 11.79$, $p = 0.001$; Table 3; Figure S6).

434

435 **Discussion**

436 We characterized patterns of genetic structure and femoral spot coloration for the
437 brilliant-thighed poison frog *A. femoralis* that was sampled along an 880 km transect
438 through continuous rainforest in a major Amazonian interfluve. We revealed four
439 genetically distinct clusters, one derived from a deep lineage divergence, and each
440 corresponding with femoral spot coloration that differed between individuals from
441 adjacent clusters. Transitions between major forest types were not consistently
442 associated with the boundaries of genetic clusters. Genetic variation was characterized
443 by a pattern of IBD across hundreds of kilometers, and subtle but significant effects of
444 contemporary landscape features on the distribution of individual measures of genetic
445 variation.

446 Under a pronounced pattern of IBD, as is the case for our study system, genetic
447 clustering algorithms can overestimate the number of partitions or lead to misleading
448 admixture inferences (Frantz et al. 2009; Garcia-Erill and Albrechtsen 2020). We
449 nevertheless argue that the clusters identified along our *A. femoralis* transect represent
450 biologically meaningful entities, as they were identified through four independent

451 approaches and conform with a phenotypic trait (femoral spot coloration). While precise
452 time calibrations are beyond the scope of the present study, the latter also suggests that
453 the clusters have arisen from past rather than contemporary phenomena, addressing the
454 ‘time lag problem’ of landscape genetic inferences (see e.g. Epps and Keyghobadi
455 2015). That the DAPC approach failed to identify the zone of admixture is not overly
456 surprising, as it does not assess differential ancestry proportions for each individual (see
457 also Miller et al. 2020).

458 Possible taxonomic implications of the deeply diverged population of *A.*
459 *femorialis* from the northeast of the PMI (localities M1 and M2) will require further
460 work. Timing the divergence is needed to evaluate the role of historical processes in
461 isolating these localities from the remainder of the PMI. The northeast of the PMI is
462 well drained, of young sedimentary origin (Late Pleistocene-Early Holocene, see e.g.
463 Sombroek 2001) and due to the proximity to the Amazon river subject to rapid changes
464 in topography and hydrology that might have resulted in periods of isolation (Hoorn et
465 al. 2010; Latrubesse et al. 2010; Pupim et al. 2019). At present, the populations from
466 M1 and M2 are also separated from the remainder of the transect by approximately 150
467 km of lowland dense forest unoccupied by *A. femoralis* (Ferreira et al. 2018). Isolation
468 by unsuitable habitat is also suggested for cluster C (M12-M14, red femoral spots),
469 which is separated from the remainder of the modules by secondary vegetation,
470 including intervening savannah over about 150 km along the transect, an ecological
471 barrier that is likely to have been further strengthened during the glacial periods in the
472 late Pleistocene (Cohen et al. 2014).

473 In contrast to the association of Clusters A and C, the area of contact between
474 Clusters A and B (M8-M9) does not occur at the location of a current ecotone. This
475 implies that the divergence of Clusters A and B might be linked to a barrier which is no

476 longer present. Our finding for *A. femoralis* contrasts with recent data on the genetic
477 structure of a treefrog (Ortiz et al. 2018) and with plumage coloration in birds (De
478 Abreu et al. 2018) along the same transect, which both reveal a zone of divergence
479 spatially matching with the ecotone between open and closed forest (M10 and M11).
480 For these species, it was concluded that present day environmental differences were
481 responsible for the genetic partitioning.

482 Individuals in Cluster A possess different femoral spot coloration (red) from
483 those in Cluster B (yellow), except in a relatively narrow (~100 km) zone of admixture
484 where individuals possess orange femoral spots. This color transition mirrors a well-
485 studied model hybrid zone system between the European red (fire)-bellied toad
486 *Bombina bombina* and the yellow-bellied toad *B. variegata*, that form orange-bellied
487 hybrids in parapatry (e.g. Szymura and Barton 1986, 1991). In this system, spatial
488 separation through differential habitat preferences leads to a narrow zone of admixture
489 despite the lack of pronounced postzygotic mating barriers (Vines et al. 2003). Another
490 mechanism that can lead to narrow zones of admixture is sexual selection, and
491 assortative mating in accordance with red or yellow femoral spot coloration has indeed
492 been demonstrated with *A. femoralis* mate choice experiments (Ferreira et al.
493 forthcoming). In addition, femoral spot coloration in *A. femoralis* also spatially varies in
494 association with mimicry with syntopic toxic species (Amézquita et al. 2017), and
495 evaluating locally co-occurring taxa to investigate such relationships may help shed
496 light on the mechanisms underpinning the distribution of color variation at this locality.

497 Spatially structured transitions of coloration across an area of genetic admixture
498 could serve as a mechanism to generate new phenotypes (Stelkens and Seehausen 2009;
499 Sefc et al. 2017). In other poison dart frogs, hybridisation has indeed been shown to
500 result in independent aposematic lineages and novel colors morphs (Medina et al. 2013;

501 Ebersbach et al. 2020). Examining the evolutionary history of admixed individuals with
502 color variation across the wider distribution of *A. femoralis* in the Amazon basin will
503 help establish the role of hybridization in generating this polymorphism. In addition,
504 testing for assortative mating particularly for individuals possessing the orange
505 phenotype and conditions allowing disassortative mating (e.g., low mate availability;
506 Medina et al. 2013) will contribute towards a better understanding of the isolating
507 processes involved.

508 Although contemporary environmental variation was not consistently associated
509 with the four distinct genetic clusters we have described, genetic connectivity still varies
510 with environmental conditions. Environmental variables have been shown to influence
511 gene flow in other anurans. For example, IBR contributed an additional 10-20% in
512 variation to models governed by IBD for the European common frog *Rana temporaria*
513 (Van Buskirk and Jansen van Rensburg 2020). Given that this study was conducted in
514 rugged, alpine terrain, such values are consistent with the environmental influence that
515 we measured in a more gradually varying environment. We found that the influence of
516 land cover was strongly supported by our MLPE models, confirming previous evidence
517 that dense forest flooded by streams and overflowing rivers are not favorable habitats
518 for *A. femoralis* (Ferreira et al. 2018). Our dbRDA analysis showed that the Walsh
519 index was also associated with less connectivity. A possible explanation is that rainfall
520 strongly determines the existence and persistence of water-filled ditches on the forest
521 floor, a requirement for reproduction for many amphibians including *A. femoralis*
522 (Menin et al. 2011; Ringler et al. 2015). Rainfall gradients and the two dominant forest
523 phytophysionomies in the PMI are autocorrelated, which likely explains the
524 inconsistency with the highest ranking variable resolved with the MLPE and dbRDA
525 analyses (forest cover versus Walsh Index, respectively). Open forests in the drier,

526 southwestern areas of the PMI are more seasonal and have lower stem densities and
527 higher tree mass compared to wetter, dense forest at northeastern parts (Sombroek 2001;
528 Cintra et al. 2013; Schietti et al. 2016).

529 Environmental variation also appears to impose different selective pressures
530 along the PMI, with environmental association analyses showing the largest number of
531 SNP loci associated with the Walsh index and forest cover. Further work with greater
532 SNP densities and a reference genome will contribute towards the identification of
533 genes under selection. Nonetheless, our existing results suggest that both the levels of
534 connectivity and differences in fitness that are associated with environmental variation
535 may contribute to the observed fine-scale patterns of genetic variation. We reduced the
536 risk of false positives in such inferences (see Hoban et al. 2016; Ahrens et al. 2018) by
537 considering only those loci which were identified by both BayeScan and LFMM.

538 Although strong IBD and environmental-based selection are conditions that may
539 lead to divergence in accordance with the gradient diversification hypothesis (Endler
540 1977), our data also suggest a role of historical processes in the generation of the
541 patterns of genetic divergence we describe for *A. femoralis*. In particular, the relatively
542 rapid restructuring of the Amazon region may give rise to conditions where historical
543 isolation and processes associated with secondary contact reduce the potential for
544 environmental gradients to strongly influence genetic and phenotypic variation. For
545 example, reinforcement by the development of reproductive character displacement
546 could potentially be a stronger influence on gene flow than the effects of environmental
547 gradients. Accumulating genetic data from additional species using the standardized
548 sampling system along the PMI provides a unique opportunity to look for traits (e.g.,
549 variation in mating cues) that predict whether current environmental transitions or

550 mechanisms associated with past landscapes generate diversity in areas of continuous
551 habitat.

552

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576

577 **Data Availability**

578 In accordance with the Journal of Heredity data archiving policy, we will have
579 submitted all the data and R scripts to Dryad.

580

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1020 **Table and figure captions**

1021

1022 **Table 1.** Number of sampled individuals (N_{TOTAL}) and summary genetic data at each
1023 sampling site for *Allobates femoralis* along the Purus-Madeira interfluve in central-
1024 southern Amazonia. Heterozygosity (H_O), expected heterozygosity (H_E), inbreeding
1025 coefficient (F_{IS}) and their low and high values (95%), number of private alleles (PA)
1026 and probability of deviating from Hardy–Weinberg equilibrium (HWE) are provided.

1027

1028 **Table 2.** Pairwise genetic distances F_{ST} (below diagonal) and geographic distance (in
1029 Km) between *Allobates femoralis* sampling locations (above diagonal) within the Purus-
1030 Madeira interfluve.

1031

1032 **Table 3.** Summary of model selection using MLPE and dbRDA that evaluated the
1033 effects of isolation by resistance (IBR) on genetic distance ($\log(F_{ST}/1-F_{ST})$). For MLPE,
1034 the Akaike Information Criteria (AIC), r^2 value, standard error (SE) and the parameter
1035 combination (α and γ) is given for the best models for each landscape variable. For
1036 dbRDA the magnitude of difference is given by the t -value and the F and p values were
1037 obtained by ANOVA. Bolded p values show significant effects of IBR on genetic
1038 distance.

1039

1040 **Figure 1.** The distribution of modules from which samples of *Allobates femoralis* were
1041 collected in the Purus-Madeira interfluve, central-southern Amazonia, Brazil. White
1042 circles indicate absence of *A. femoralis*. For sample sizes at each module see Table 1.
1043 See online version for full colors.

1044

1045 **Figure 2.** Rasters capturing each of the four environmental variables used in
1046 CIRCUITSCAPE to generate resistance distance matrices between each pair of

1047 sampling locations a) land cover, b) silt content, c) temperature seasonality - Bio4 and
1048 d) Walsh index. See online version for full colors.

1049

1050 **Figure 3.** The isolation-by-resistance (IBR) relationships tested for the effect of land
1051 cover and temperature seasonality on genetic distance $F_{ST}/(1-F_{ST})$ using seven values of
1052 γ (0.01, 0.1, 0.5, 1, 5, 10, 100). The different slopes are not shown (α values) and are
1053 displayed here for $\alpha = 5$ here for simplicity. The curves show decreasing landscape
1054 resistances from right to left for land cover (A) and left to right for temperature
1055 seasonality (B).

1056

1057 **Figure 4.** A population tree generated using SNAPP, and a histogram showing
1058 individual ancestry proportions color coded to correspond to each genetic cluster,
1059 estimated using ADMIXTURE. The location of the collection modules are color coded
1060 to reflect the color assigned to each genetic cluster in the ADMIXTURE plot (the white
1061 circles for M3-M5 indicate the absence of *A. femoralis*). Posterior probabilities obtained
1062 at each node are shown on the tree. Cluster 1 corresponds to individuals with yellow
1063 femoral spots, Cluster A corresponds to individuals with red femoral spots, Cluster B
1064 corresponds to individuals with yellow femoral spots, with a zone of admixture between
1065 Cluster A-B (BM8-9) with an intermediate color phenotype (orange), and Cluster C
1066 corresponds to individuals with red femoral spot. See online version for full colors.

1067

1068 **Figure 5.** Histograms for individual *A. femoralis* sampled along the Purus-Madeira
1069 interfluvium using three different clustering approaches a) ADMIXTURE, b) sNMF and c)
1070 DAPC. Each individual is represented by a bar partitioned into different colors to
1071 represent individual ancestry proportions. K represents the most likely number of
1072 genetic clusters. See online version for full colors.

1073

1074 **Figure 6.** Relationship between genetic and geographic distance in *A. femoralis* across
1075 the Purus-Madeira interfluvium.

1076