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A role of asynchrony of seasons in explaining genetic differentiation in a Neotropical toad

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The process of diversification can be studied at the phylogeographic level by attempting to identify the environmental features that promote and maintain population divergence. Here we investigate diversification in *Rhinella granulosa*, a Neotropical toad from northeastern Brazil, by testing a range of hypotheses that encompass different putative mechanisms reducing gene flow among populations. We sequenced single nucleotide polymorphisms and examined individual predictions related to the role of geographic barriers (rivers), ecological gradients, historical habitat stability, and spatial variation in climate seasonality, also known as the asynchrony of seasons hypothesis. This hypothesis postulates that temporal asynchrony of wet and dry seasons over short distances causes parapatric populations to become isolated by time. After determining genetic structure, inferring past distributions, ranking demographic models, and estimating the power of monthly climatic variables, our results identified two populations that are not associated with geographic barriers, biome gradients, or historical refugia. Instead, they are predicted by spatial variation in monthly rainfall and minimum temperature, consistent with the asynchrony of seasons hypothesis, supported also by our comparative framework using multiple matrix regression and linear mixed effects modeling. Due to the toad's life history, climate likely mediates gene flow directly, with genetic differentiation being provoked by neutral mechanisms related to climate driven population isolation, and/or by natural selection against migrants from populations with different breeding times. The asynchrony of seasons hypothesis is seldom considered in phylogeographic studies, but our results indicate that it should be tested in systems where breeding is tightly coupled with climate.

INTRODUCTION

Organismal diversification can be studied at the phylogeographic level by investigating the mechanisms causing populations to diverge. Phylogeographic diversification hypotheses can be tested individually with the aid of well-defined expectations for genealogical patterns (e.g., Avise 2000) or through tests of a null hypothesis (e.g. Knowles 2001). However, in regions where diversification is likely to be complex, multiple hypotheses should be considered. This is the case in the Neotropics, where high levels of species richness seems to have accumulated continuously in time through different concomitant processes (Rull 2020). Multiple hypotheses can then be simultaneously approached through ranking (e.g. Jackson et al. 2017) or methods assessing relative explanatory power (e.g. Wang 2013), the latter being of special interest for non-mutually exclusive hypotheses.

In the Neotropics, traditional explanations for population divergence are vicariance-based and frequently invoke geographic barriers (Turchetto-Zolet et al. 2013). One classic example is Wallace's riverine barrier hypothesis (Wallace 1876), first proposed to explain the distribution of monkey species in the Amazon. This hypothesis invokes the vicariant effect of rivers as the cause for reduced migration, and from a phylogeographic

perspective it predicts that genetic discontinuities within a species should correspond to rivers. Other hypotheses are based in historical habitat instability, which would cause vicariance through climate-induced habitat fragmentation (Hewitt 2001). The classic Neotropical example also comes from the Amazon and was firstly proposed to explain bird speciation (Haffer 1969). In short, cold phases of Milankovitch cycles would have caused the Amazon to contract, provoking range fragmentation and population isolation in rainforest-associated organisms. From a phylogeographic perspective, hypotheses based in historical habitat instability differ from the riverine barrier hypothesis because they predict that population sizes should be impacted by Plio-Pleistocene habitat dynamics; isolated populations are expected to have expanded following recovery of their habitat when the climate becomes suitable again, which can be detected as exponential population growth. Ecological niche modeling (ENM) can be used to establish geographically explicit hypotheses for species ranges during extreme climate conditions (Vasconcellos et al. 2019), most typically the last glacial maximum (LGM) and last interglacial (LIG).

Non-allopatric models of diversification are seldom considered in Neotropical diversification studies (but see Beheregaray et al.

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2015). In continuous populations, individuals can become isolated by geographic distance alone (Wright 1943), a pattern that implies no deterministic evolutionary processes. The existence of different biomes in the distribution of a species raises the possibility of isolation by environment, that is, when genetic distance increases with environmental distance regardless of geography (Orsini et al. 2013; Wang and Bradburd 2014). This class of models is difficult to explore in non-model organisms and through phylogeographic methods as it implies population differentiation is caused by selective processes and adaptation (see reviews in Rundle and Nosil 2005 and Funk et al. 2006). Nevertheless, genetic discontinuities that are geographically concordant with ecological gradients and diversification under the presence of gene flow are measurable expectations of this hypothesis (Camurugi et al. 2021). In the Neotropics, major gradients are found between contrasting widespread biomes; for example the two main South American rainforests, the Amazon and the Atlantic Forest, are separated by an intervening belt of open vegetation including the Caatinga seasonally dry tropical forest, the Cerrado savannas, and the Chaco (Olson et al. 2001; Costa et al. 2018). However, in the Neotropics and elsewhere diversification studies are mostly focused on species that are endemic to a particular biome (Turchetto-Zolet et al. 2013).

A class of hypotheses that remains unexplored in the phylogeographic literature in general, but especially in the Neotropical region, invokes isolation by time. Most commonly associated with sympatric speciation and selective processes (e.g. allochronic speciation, see review in Taylor and Friesen 2017), isolation by time can be expected in continuous populations of widespread organisms because they are often dependant on environmental cues to regulate breeding times and are thus under selective pressure to synchronize life cycles with their environment, particularly when variation occurs in a predictable manner. At higher latitudes, many organisms synchronize reproduction over vast areas in response to changes in photoperiod and insolation (Karsch et al. 1984). However, since tropical regions experience only slight changes in photoperiod, adaptations related to the timing of reproduction must rely on other cues such as temperature, rainfall, and food supply (James and Shine 1985; Moore et al. 2005). Compared to hemispherical patterns of photoperiod and insolation, these cues vary over much shorter distances (Schwerdtfeger 1972). Based on this, the asynchrony of seasons hypothesis postulates that higher tropical diversity could be explained by spatial variation in climate seasonality, which would cause geographically continuous populations to reproduce out of phase even across short distances, thus becoming isolated by time of breeding (Martin et al. 2009; Moore et al. 2005). Asynchrony of seasons is predicted to be particularly important in tropical seasonal environments, where the temporal distribution of rainfall dictates the seasons, and expectations under this hypothesis should mirror the degree of season overlap.

In this study we investigated population diversification in *Rhinella granulosa*, a widely distributed toad in northeastern Brazil, in the light of the aforementioned hypotheses. The riverine barrier hypothesis is based on the river drainages that flow through the region, including the São Francisco, which is postulated to represent a geographic barrier to several taxa (references in Werneck et al. 2015). Isolation by historical climate instability is supported by geologic evidence for variation in rainfall (Auler et al. 2004; Wang et al. 2004) and habitat type (de Oliveira et al. 1999), with several co-distributed taxa showing signatures of population size changes (Gehara et al. 2017). Isolation by environment is also consistent with the distribution of *R. granulosa*, which encompasses severe environmental gradients related to the contrasting biomes of Atlantic Forest and Caatinga. Finally, asynchrony of seasons is predicted to affect northeastern Brazil following spatial variation in climate seasonality (Fig. 2 in Martin et al. 2009). We collected samples from the whole of the toad's distribution and

sequenced single nucleotide polymorphism markers (SNP) to infer its genetic structure. We first addressed hypotheses individually, with their respective expectations regarding demographic aspects of population diversification, historical habitat ranges and the power of monthly climatic variables in predicting genetic structure. We then considered hypotheses simultaneously, accessing their significance and relative importance using pairwise distance matrix approaches.

MATERIALS AND METHODS
Genome and taxon sampling
We extracted total DNA from liver and muscle preserved in ethanol using DNeasy Blood & Tissue kits (Qiagen, Venlo, Netherlands) and used double digest restriction-site associated DNA sequencing (Peterson et al. 2012) to obtain SNP markers from the nuclear genome. Briefly, we digested samples individually with the enzymes Sbf1-HF and MspI and barcoded the samples using the ligation enzyme Ligase T4 and eight different Illumina adaptors. We pooled barcoded samples in groups of eight prior to amplification using six indexed primers and allocated these pools into four libraries of 48 samples (including samples from other projects). We size-selected fragments of 300–550 bp with Blue Pippin Prep prior to sequencing and sequenced one library per lane (100 bp single end) in the Illumina HiSEq 2000 platform. Bioinformatics was conducted using Pyrad v. 3.0.66 (Eaton 2014) to filter reads, assemble loci and extract alignments. Sequence reads with Phred quality scores below 99% were discarded. We used the following clustering parameters: 6× for minimal coverage, minimum similarity of 0.90, and maximum proportion of 0.25 shared polymorphic sites. Other parameter settings used the default values. We retained polymorphic loci present at a minimum of 70% of the samples and considered one SNP per locus (except when stated otherwise).

We initially sequenced 142 samples from the entire distribution of *R. granulosa* in the Caatinga and Atlantic Forest biomes (Fig. 1A, B). Due to variation in the number of loci obtained per sample we used the Matrix condenser tool (de Medeiros and Farrell 2018) to downsample our data. We ranked samples according to number of shared loci, then discarded poorly sequenced samples up to the point that elimination of a sample would result in elimination of a sampled locality. This resulted in 80 samples from 51 localities (Supplementary Table 1) sequenced for 7 688 loci (9.4% missing data).

Isolation by environment and by geographic barrier
We first tested for a pattern of isolation by distance with the *mantel. randtest* function of the R package adegenet v. 2.0 (Jombart 2008). We then inferred population structure with the spatial clustering algorithm implemented in BAPS (Corander et al. 2008; Cheng et al. 2013) using the Log(marginal likelihood) as criterion for inferring the number of populations (*k*). We ran the analysis ten times considering a maximum value of *k* = 10. We followed with admixture analysis (Corander and Marttinen 2006) for obtaining individual assignment coefficients using a minimum of one individual per population, 100 iterations 200 reference individuals and 20 repetitions. We plotted population structure across space by interpolating individual admixture coefficients with the R script 'POPSutilities.r' (http://membres-timc.imag.fr/Olivier.Francois/POPSutilities.R) using the option 'threshold' of the 'maps' function.

We examined the hypothesis of isolation by environment by assessing concordance of population limits with the Caatinga and Atlantic Forest boundaries. If biome boundaries promote genetic differentiation, we expect that population genetic structure should roughly correspond to these regions. Likewise, we examined isolation by geographic barrier by searching for concordance of populations with the course of the São Francisco river.

Isolation by instability
To test the isolation by climate instability hypothesis we estimated past putative ranges for the focal species and explored demographic aspects of population diversification.

The isolation by instability hypothesis predicts that genetic structure will correspond to regions of historically stable climate (Vasconcellos et al. 2019). To obtain maps of past putative ranges we used ENM with the geographic coordinates of sampled localities and bioclimatic variables available at the BioClim database (Fick and Hijmans 2017) at 10" resolution
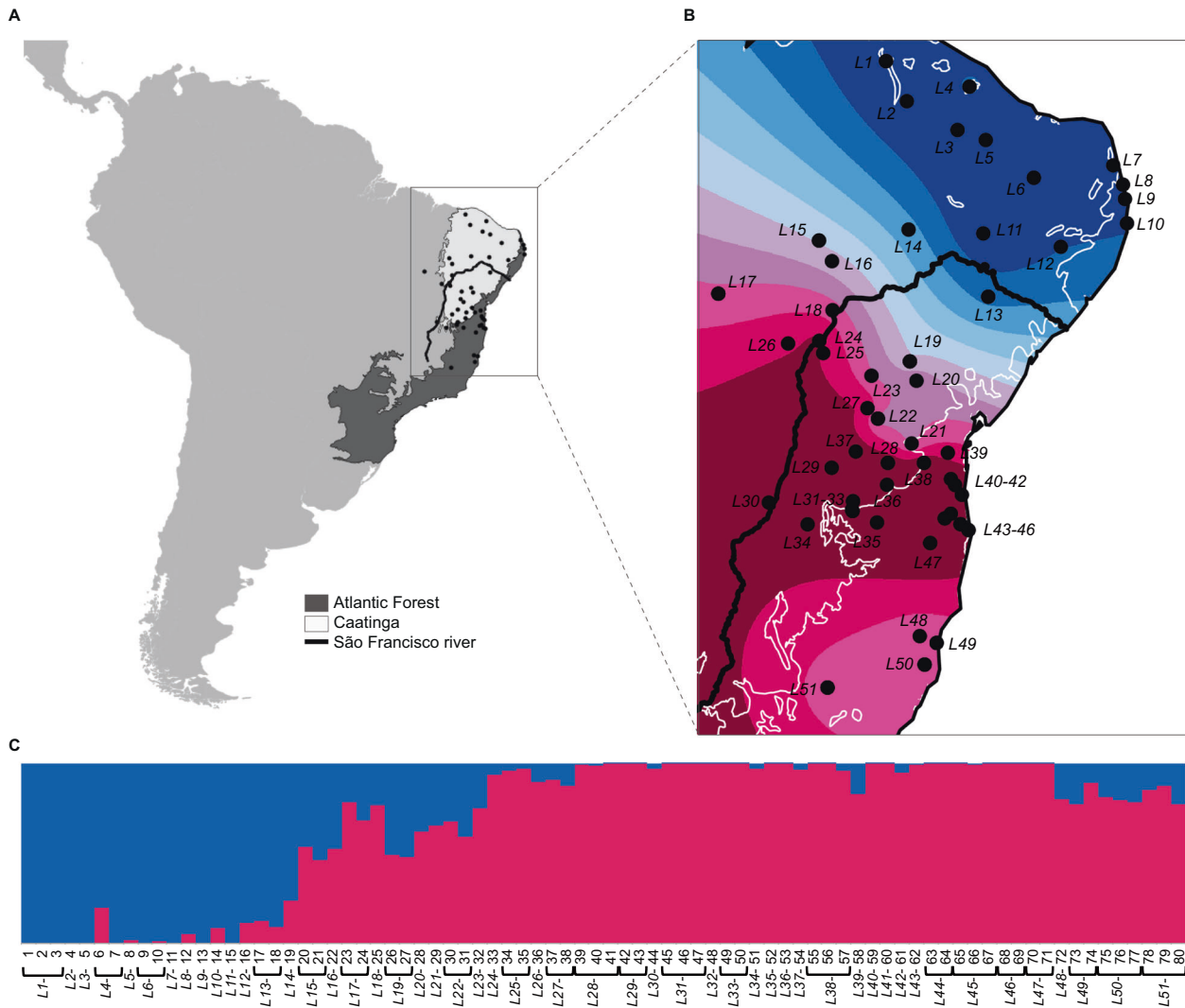
Fig. 1 Sampling and genetic structure in *Rhinella granulosa* based in SNP data. A Distribution of the Atlantic Forest and Caatinga biomes and the São Francisco river on the South American continent. **B** Localities sampled and distribution of the Northern and Southern populations according to interpolated assignment coefficients. **C** Barplot showing assignment coefficients for individual samples.

plus elevation. Sampled localities were required to be separated by 20 km in order to avoid spatial autocorrelation. We used principal component analysis (PCA) in the R package FactoMineR v. 1.39 (Lê et al. 2008, available in CRAN) to reduce the number of variables to the number of principal components showing eigenvalues greater than 1, and selected the variable that was the most correlated with each particular principal component to use in the ENMs. We used the maximum entropy algorithm MaxEnt v. 3.4.1 (Phillips et al. 2017) implemented in the R package biomod2 (Thuiller et al. 2014) to construct 100 models using 10,000 pseudo-absences randomly sampled from a rectangular background (latitude −35° to 10°, longitude −70° to −33°). We used 10,000 maximum iterations and the 'autofeatures on' setting to perform runs using 70% of the coordinates for calibration, and then evaluated the models with the area under the curve (AUC) statistic. We projected models with AUC greater than 0.9 and obtained presence-absence only maps with the *binary.meth* option, which uses the threshold that maximizes AUC evaluation scores. We considered past climatic conditions reconstructed for the LGM (~21,000 ya) and mid-Holocene (~6000 ya) obtained for three general circulation models, the Community Climate System Model (CCSM4; Gent and Danabasoglu 2011), the Model for Interdisciplinary Research on Climate (MIROC-ESM; Watanabe et al. 2011), and the model of the Max Planck Institute for Meteorology (MPI-ESM-P; Stevens et al. 2013). We also considered climatic conditions reconstructed for the LIG (~120,000 ya) obtained with the climatic model of Otto-Bliesner et al. (2006).

Under the isolation by instability hypothesis, population divergence should occur in the absence of gene flow and is expected to leave a

signature of historic demographic expansion. To explore demographic aspects of population diversification we used fastsimcoal2 (Excoffier and Foll 2011) to generate data under eight models representing possible diversification scenarios (Fig. 2). Note that we assume that chromosomes are sampled at random in a given population, so a sample of five diploid toads will include ten samples of their respective chromosomes and be represented by ten sets of simulated data. All models included two populations with individuals assigned to populations following results from genetic structure analysis. Briefly, we modeled demographic stability and population isolation (model 1), demographic stability and migration between populations (model 2), exponential demographic growth in one of the populations and migration between populations (models 3 and 4), exponential demographic growth in both populations and migration between populations (model 5), exponential demographic growth independently in each population (model 6), or exponential demographic growth in one of the populations and isolation (models 7 and 8). For each model we estimated the composite likelihood of the probability of the observed data (joint site frequency spectrum, jSFS) 30 times using fastsimcoal v. 2.6.0.3 (Excoffier and Foll 2011). Each replicate consisted of 100,000 iterations for the calculation of the composite likelihood and 50 cycles of the Brent algorithm for parameter optimization. We selected the best replicate of each model and applied information theory to rank models (Burnham and Anderson 2004).

Once the best model was determined, we estimated parameter values and confidence intervals using all SNPs in a nonparametric bootstrapping procedure: we subsampled individuals and alleles to produce 60 different
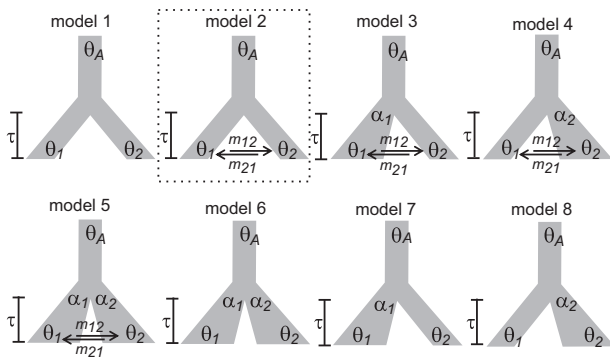
Fig. 2 Demographic models considered for *Rhinella granulosa*. θ_A—ancestral population size, θ_1—northern population size, θ_2—southern population size, α_1—exponential rate of expansion for northern population, α_2—exponential rate of expansion for southern population, m_{21}—migration rate from Northern to Southern population, m_{12}—migration rate from Southern to Northern population, τ—divergence time between northern and southern population.

jSFS, randomly selecting SNPs scored in at least 75% of the individuals in each population. For each subsampled jSFS we ran 30 replicate analyses as above, totalling 1800 runs. Confidence intervals were drawn based on the estimates of the best run of each subsampled jSFS. Initial values for parameters were drawn from uniform distributions with intervals as follows: population sizes (θ_a, θ_1, θ_2) and time for divergence (τ) ranged from 1000 to 1,000,000 and migration rates (m_{12}, m_{21}) ranged from 1×10^{-9} to 1×10^{-3}. For models including exponential growth (α_1, α_2), populations changed to 1×10^{-7} to 1×10^{-2} of the original size. These values correspond to those used in other demographic modeling applications in Neotropical amphibians (Thomé and Carstens 2016; Gehara et al. 2017; Lanna et al. 2020). Population sizes are measured in genes, migration rates represent the probability of a gene to be sent from one population to the other, and times are measured in years assuming a mutation rate of 2.1×10^{-9} substitutions per site per generation (Gottscho et al. 2014). Because the generation time of our focal species is unknown, we considered a generation time of 1 year based in general anuran biology and following other studies (Carnaval et al. 2009; Thomé and Carstens 2016).

Asynchrony of seasons
To examine the asynchrony of seasons hypothesis we used linear discriminant analyses (LDA) to quantify the performance of monthly climatic variables in explaining genetic structure. Briefly, we constructed linear discriminant models with monthly averages of rainfall, minimum and maximum temperatures as predictors, and population identity as a categorical variable. Localities were attributed to populations based on average assignment coefficients. We constructed models with all variables conjunctly, and separately for rainfall, minimum and maximum temperatures. We conducted the LDA in the R package flipMultivariates (available at https://github.com/Displayr/flipMultivariates). To examine the possibility of overfitting the model, we used the *trainControl* and *train* functions of the R package caret v. 6.0.86 (Kuhn 2008) to perform a five-fold cross validation.

We further inspected potential variation in climate in the multivariate space with PCA in FactoMineR v. 1.39 (Lê et al. 2008).

Comparison of hypotheses
To evaluate and compare the different hypotheses conjunctly, we calculated pairwise distance matrices to be used as independent variables explaining genetic distance in the multiple matrix regression approach (MMRR) of Wang (2013). This approach calculates individual regression coefficients for each variable and their significance through permutations (999). For isolation by environment, we calculated environmental distances based on main bioclimatic variables determining the Caatinga and Atlantic Forest biomes (Cole 1960; Costa et al. 2018): Annual Mean Temperature (BIO1), Temperature Seasonality (BIO4), Annual Precipitation (BIO12), and Precipitation Seasonality (BIO15). For isolation by geographic barrier and isolation by instability, we estimated resistance surfaces with Circuitscape

(Shah and McRae 2008) in julia v. 5.0.0 (Hall et al. 2021); for isolation by geographic barrier, we used the river drainage available in https://www.naturalearthdata.com/ considering upstream catchment area as a measure of river width (and thus resistance). For isolation by instability we used the obtained ENM layers and considered areas of maximum suitability to reflect minimal resistance, first building intersection layers for periods represented by more than one layer, and then combining layers from different periods to achieve a single surface with different cell values. For both isolation by environment and instability, we scaled resistance values to vary from zero to one, and further replaced zeros by 0.0001. For the asynchrony of seasons hypothesis, we calculated environmental distances based on the monthly variables that were significant ($p < 0.05$) in the LDA. Finally, we also considered isolation by distance by calculating Euclidian distances based on the coordinates of each location. We checked for collinearity of variables using Mantel tests and variance inflation factors (VIF). When significant variables were correlated, we applied information theory to rank models based on scores obtained through linear mixed effects modeling (Clarke et al. 2002), using the MLPE.lmm function of the R package resistanceGA v. 4.1.0.46 (Peterman 2018) with the option REML = FALSE to obtain valid scores fit with maximum likelihood.

RESULTS
Isolation by environment and by geographic barrier
The Mantel test resulted significant (R-Squared = 0.626; $p = 0.001$; 999 permutations). The results of the ten runs of the BAPS spatial algorithm were nearly identical and indicate genetic diversity is best allocated into two populations separated by a gradient of individual assignment coefficient values (Fig. 1C). The second and third best were $k = 3$ and $k = 4$. Interpolation over geographic space reveals one population occupying a more northern position (hereafter Northern population), one population spreading across the southern range of the species (hereafter Southern population), and a contact zone where admixed samples are found (Fig. 1B). Population boundaries did not closely match biome distributions or the course of the São Francisco river.

Isolation by instability
Variable selection for ENM resulted in the following: Maximum Temperature of Warmest Month (BIO5), Minimum Temperature of Coldest Month (BIO6), Precipitation of Wettest Month (BIO13), and Precipitation of Warmest Quarter (BIO18). Of the 100 ecological niche models generated, 87 showed AUC values exceeding 0.9 and were included in the projections. The projection for present time conditions resulted in a map encompassing most of the Caatinga and northeastern Atlantic Forest (Fig. 1A). Projections for the past show no evidence for historical habitat fragmentation, showing instead mainly continuous distributions for the LGM and mid Holocene. Differences between maps based on the two general circulation models were minor (Fig. 3). Increase in the total ranges of suitability areas is observed for all LGM maps.

Demographic modeling found little support for models that included exponential demographic growth, but did identify Model 2 (i.e., isolation with migration and no exponential growth) as the model that best explains population diversification (Table 1). Estimated parameter values are shown in Table 2 and indicate a Pleistocene age for population divergence and variation in population sizes.

Asynchrony of seasons
The performance of the LDA models constructed with all variables together, and with rainfall and minimum temperature separately, reached 100% correct predictions. For maximum temperature, the percentage of correct predictions was 98.04%. The five-fold cross validation showed 85% of correct predictions for the model with all variables, 100% for the model including rainfall only, 95% for minimum temperature and 92% for maximum temperature, indicating that LDA models may slightly overfit the data in some cases.
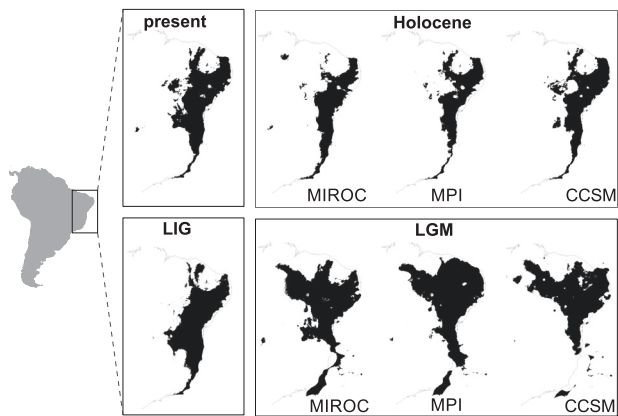
Fig. 3 Ecological niche models projected to the present, Holocene, last glacial maximum (LGM), and last interglacial maximum (LIG). MIROC, MPI, and CCSM refer to atmospheric circulation models (see text).

Table 1. Model selection for demographic models simulating population divergence in *Rhinella granulosa*.

Models	EstL	k	AIC	Δi	wi
Model 1	−14,184.80	4	28,377.60	1231.81	3.29×10^{-269}
Model 2	−13,566.90	6	27,145.80	0	0.9998
Model 3	−13,574.58	7	27,163.17	17.37	1.69×10^{-5}
Model 4	−13,583.19	7	27,180.37	34.57	3.11×10^{-8}
Model 5	−13,595.59	8	27,207.18	61.39	4.68×10^{-15}
Model 6	−14,181.70	6	28,375.40	1229.60	9.91×10^{-269}
Model 7	−14,182.31	5	28,374.62	1228.83	1.46×10^{-268}
Model 8	−14,182.53	5	28,375.07	1229.27	1.17×10^{-268}

The maximum likelihood for observed data was −13,144.32.
EstL maximum likelihood estimated for the model, *k* the number of parameters included in the model, *AIC* the Akaike Information Criterion, *Δi* Akaike differences, *w*i model probabilities.

Rainfall in October, November, and December showed the highest R-Squared values (Table 3). In the PCA with the monthly averages per locality, the first two principal components explain 87.18% of the variation. The biplot of these components showed two separate groups with no overlap, corresponding to the localities from the Northern and Southern populations (Fig. 4A). Distribution of raw rainfall averages shows differences in the regimes for each population, indicating a temporal dislocation of the rainy season in the region occupied by the two populations (Fig. 4B, C).

Comparison of hypotheses
The overall model in the MMRR analysis was significant, with the independent variables explaining ~45% of the genetic variation (R-Squared = 0.447, F statistic = 205.08, p = 0.001). Two individual hypotheses, asynchrony of seasons and isolation by the environment, were significant whereas isolation by distance was not significant (Table 4). Among these, the regression coefficients indicate asynchrony of seasons best explains genetic variation, followed by isolation by distance. Isolation by environment resulted in a negative coefficient, indicating an inverse relationship between genetic variation and biome. The Mantel tests showed a correlation between geographic distance (isolation by distance) and asynchrony of seasons (Table 5), but VIF values were moderate (Table 4). The linear mixed effects analyses indicate that models excluding isolation by distance perform better than

models excluding asynchrony of seasons. The model including both was ranked as the best model (Table 4).

DISCUSSION
We investigated the possible mechanisms behind population differentiation in *R. granulosa*, a widely distributed toad occurring in northeastern Brazil. Genetic diversity is allocated into two populations whose distributions are not explained by biome boundaries, suspected geographic barriers, or historical refugia. Instead, genetic structure corresponds to regional differences in climate, as predicted by the asynchrony of seasons hypothesis, with geographic distance likely also playing a role. Assuming the biology of *R. granulosa* is similar to other tropical explosive breeding amphibians, climate acts as a mediator of gene flow, leading to asynchronous breeding in populations located in regions with unphased climates. This is consistent with neutral and non-neutral mechanisms promoting genetic differentiation. Future research should test this hypothesis via the collection of data on the timing of and environmental cues for breeding.

Isolation by environment and by geographic barrier
Genetic variation in *R. granulosa* is best structured into two populations separated by a NW-SE oriented contact zone where samples show gradually decreasing assignment coefficients. This pattern was obtained considering the geographic origin of samples. Such population structure is clearly not consistent with biome distributions in this region; the xeric Caatinga has mostly an inland distribution whereas the wet Atlantic Forest occurs along the coastline and, due to the shape of South America in that region, the boundary between the two roughly assumes a NE-SW orientation, opposite to that of the contact zone. This geographic configuration likely explains the inverse relationship between environmental and genetic distance in the MMRR analysis. While it is possible that biome boundaries have changed over time, there is no evidence that these regions have shifted in the manner that would be required to explain this contact zone. Thus, we found no evidence for a role of environmental gradients related to contrasting biomes in causing ecological differentiation of populations in this species. The existence of an ecological barrier between *R. granulosa* from the Caatinga and Atlantic Forest was refuted before by Prates et al. (2013) based on physiological aspects of locomotor performance.

A similar conclusion is drawn about population divergence being caused by the river network in the region, including the São Francisco river. This river shows some geographic coincidence with the contact zone at its oriental end, which is lost as its course deviates south towards its springs in southeastern Brazil. The lack of sharp genetic boundaries between populations and our demographic modeling results (further discussed below) also support the interpretation that a traditional vicariant barrier (such as this river) is not the main cause of population diversification in this species. Parameter estimates suggest divergence occurred in the mid Pleistocene under bidirectional gene flow, and although the estimated age of the river varies, Werneck et al. (2015) attributed mid Pleistocene divergence in the lizard *Tropidurus semitaeniatus* to the role of historical river drainages in the region (see Thomé et al. (2021) for a discussion on the geologic evidence on the age of this river). For *R. granulosa*, similar (as opposed to asymmetrical) estimates of migration are also not consistent with this hypothesis. Finally, this hypothesis received insignificant support when all hypotheses were considered together in the MMRR.

Isolation by instability
Only one demographic model received appreciable probabilities (model 2; $w_i = 0.999$), indicating that there is virtually no support in the data for models that include exponential expansion of

Table 2. For each parameter in the best model (see Fig. 2) the following estimates are provided: the point estimate from the best replicate, minimum and maximum range of values across replicates, mean estimate across replicates, and 90% confidence interval (CI).

Parameter	Point estimate	Minimum estimate	Maximum estimate	Mean estimate	90% CI
θ_a	6.57×10^3	1.38×10^3	1.64×10^5	6.22×10^3	2.97×10^3–9.47×10^3
θ_1	1.09×10^6	1.03×10^6	1.15×10^6	1.08×10^6	1.06×10^6–1.10×10^6
θ_2	1.50×10^6	1.48×10^6	1.59×10^6	1.53×10^6	1.50×10^6–1.55×10^6
τ	1.09×10^6	1.07×10^6	1.13×10^6	1.10×10^6	1.08×10^6–1.11×10^6
m_{21}	1.38×10^{-7}	1.27×10^{-7}	1.56×10^{-7}	1.41×10^{-7}	1.27×10^{-7}–1.56×10^{-7}
m_{12}	1.31×10^{-7}	1.17×10^{-7}	1.36×10^{-7}	1.25×10^{-7}	1.20×10^{-7}–1.29×10^{-7}

Table 3. Results of linear discriminant analysis of genetic structure in *Rhinella granulosa* conducted separately for monthly rainfall, minimum and maximum temperatures.

	Rainfall		Tmin		Tmax	
Month	R-Squared	*p*	R-Squared	*p*	R-Squared	*p*
July	0.08	0.069	0.21	<0.001	0.04	1
August	0.01	0.601	0.23	0.005	0.03	1
September	0.03	0.308	0.29	0.006	0.04	1
October	0.45	<0.001	0.36	0.025	0.07	0.413
November	0.77	<0.001	0.40	0.017	0.17	0.014
December	0.75	<0.001	0.43	0.002	0.23	0.002
January	0.32	<0.001	0.32	0.009	0.12	0.078
February	0.03	0.308	0.25	0.015	0.04	0.824
March	0.44	<0.001	0.21	0.005	0.03	1
April	0.50	<0.001	0.23	<0.001	0.03	1
May	0.34	<0.001	0.23	<0.001	0.03	1
June	0.15	0.007	0.22	<0.001	0.03	1

R-Squared values indicates the proportion of variance explained by the northern and southern population categories in each model; *p* value refers to the significance of monthly variables in each model.
Tmin minimum temperature, *Tmax* maximum temperature.

either population or that do not include gene flow between populations. Presence/absence maps of past distributions show no evidence for significant historical range fragmentation at any time and thus no support for the hypothesis of population differentiation following isolation in multiple refugia. Instead, binary maps showed a single continuous range for all times, with minor variations according to the global circulation model. In consonance, the MMRR found no significant role of isolation by instability in explaining genetic distances.

Maps from the ENM (Fig. 2) indicate the range of the species varied across time slices, with narrower ranges during interglacial time periods (i.e., present time and LIG), suggesting demographic change possibly occurred. Although our demographic modeling results clearly do not support exponential expansions, some sort of increase in population sizes may be occurring (following differences in current population sizes and ancestral population sizes), possibly at the LGM. A number of taxa, either at the Caatinga or northern Atlantic Forest, seem to have experienced intense demographic/range dynamics (Thomé et al. 2016; Gehara et al. 2017; Silveira et al. 2019), and palaeoclimatic evidences do support historical climate variation for northeastern Brazil (Auler et al. 2004; Wang et al. 2004). The supposedly antagonist responses of the wet and dry biomes are poorly documented both in timing and geographic extent and, considering that the current distribution of *R. granulosa* encompasses both Caatinga and Atlantic Forest, we hypothesize responses for past climate changes should vary independently of past biome distributions in this species.

Asynchrony of seasons

The asynchrony of seasons hypothesis postulates that spatial variation in the seasonality of a climatic variable across a species range would pose the same effects of a temporal barrier, causing parapatric populations to breed out-of-phase. The asynchrony would occur more often in seasonal environments of tropical regions, where organismal phenologies are less influenced by photoperiod and more influenced by climatic factors that vary at regional scale, particularly rainfall (Martin et al. 2009; Moore et al. 2005). As opposed to the distribution of biomes, geographic barriers and historically stable areas, temporal patterns of climate variation explained with accuracy the genetic structure in *R. granulosa*, despite of some possible overfitting in the LDA analysis. Monthly rainfall and monthly minimum temperature showed the highest predictive power. Over multivariate space, monthly climate variables mirror genetic structure perfectly, with well defined, nonoverlapping polygons in the PCA biplot. Such a match between genetic structure and the temporal distribution of climate is paramount and supports a strong role of spatial variation in seasonality in causing diversification in *R. granulosa*. Monthly rainfall peaks with a temporal shift in regions occupied by the different populations, with the Northern population clearly experiencing a delayed wet season.

The MMRR also supports asynchrony of seasons as the hypothesis that accounts for most of the genetic variation in this species. VIF were only moderate, rendering the regression coefficients reliable. The analysis also highlights a possible effect of isolation by distance (detected as nearly significant) but model
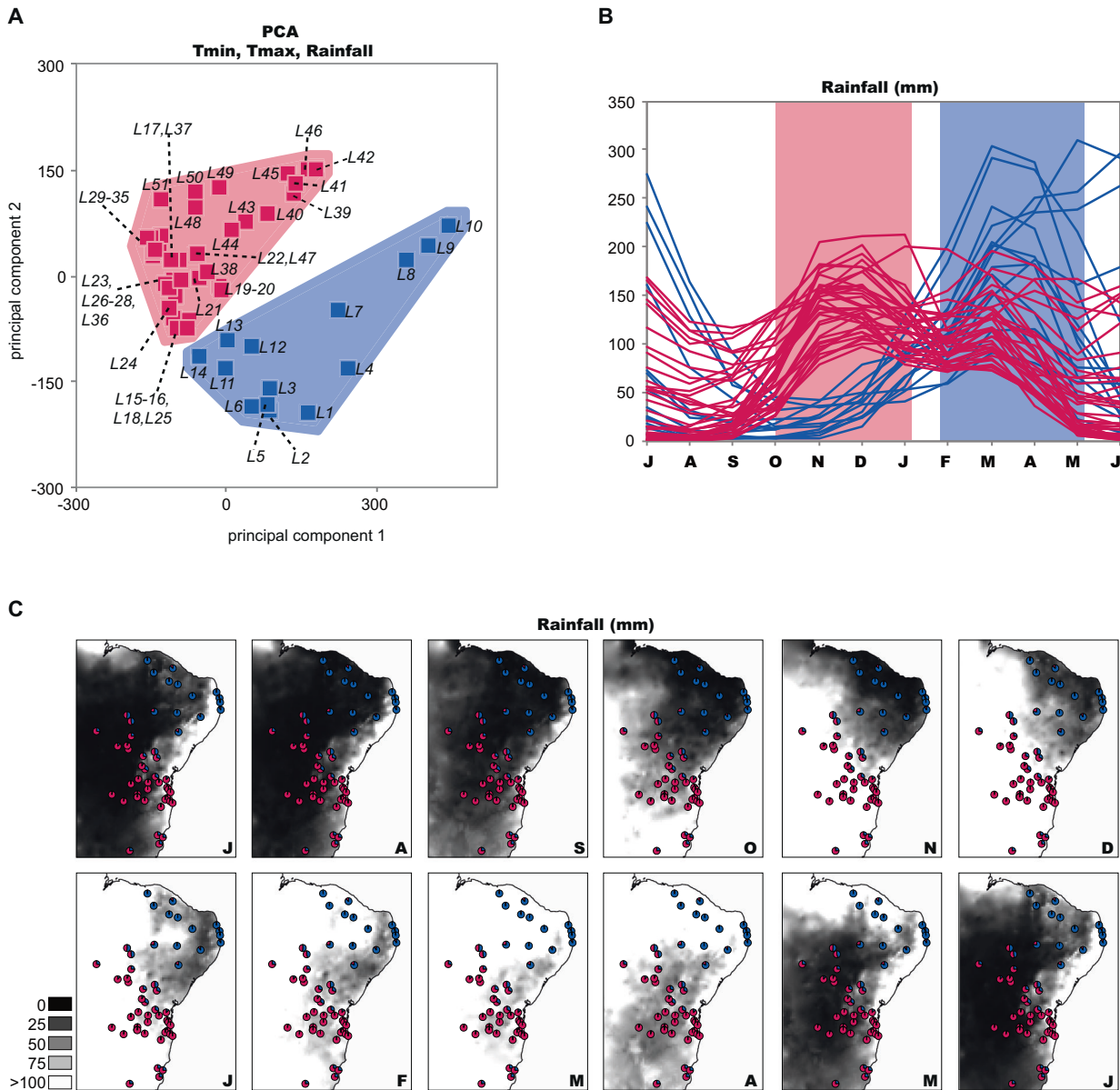
Fig. 4 Climate variation in localities sampled for *Rhinella granulosa*. Locality codes and population colors match Fig. 1. **A** Principal component analysis of monthly averages for rainfall, minimum and maximum temperatures. **B** Monthly averages of rainfall per locality, with the rainy season for each population highlighted in the background. **C** Monthly averages of rainfall over space and average assignment coefficients per location.

ranking based in mixed linear effects modeling confirmed a larger explanatory power of the asynchrony of seasons compared to isolation by distance: removing the asynchrony of seasons hypothesis has a larger impact than removing isolation by distance. Regardless, both are included in the best model. Finally, the matrices representing both hypotheses are correlated, which indicates that the effects of geographic and temporal distancing are likely impossible to disentangle. While other research has reported diversification related to asynchrony in breeding times (reviewed in Taylor and Friesen 2017), the prevalence of this phenomenon is likely underestimated due to the confounding effect of isolation by distance (Maes et al. 2006).

Quintero et al. (2014) found support for the asynchrony of seasons hypothesis by correlating genetic distance in 57 New World bird species with rainfall asynchrony. Whereas the rationale in this case is that rainfall would affect organismal life cycles by influencing resources storage, the mechanism is more

straightforward for *R. granulosa*. Neotropical amphibians at seasonal environments show a tightly coupled relationship between reproduction and climate (Schalk and Saenz 2016). While we are unaware of phenology studies conducted for this species in the field, this toad is considered an explosive breeder, meaning its reproduction occurs in bouts that are tightly associated with heavy rainfall (Narvaes and Rodrigues 2009). In fact, its reproductive physiology was demonstrated similar to that of typical desert anurans (Madelaire and Gomes 2016), which also show explosive breeding (Wells 2007). Moreover, after amplected females lay their aquatic eggs, the temporary ponds formed by heavy rains must last long enough for aquatic larvae to undergo metamorphosis prior to moving into terrestrial habitat (see references gathered in Pereyra et al. 2016). As with minimum temperature, rainfall is believed to act as a limiting factor (Wells 2007) and was shown to correlate with reproductive activity in other Neotropical frogs (Brasileiro and Martins 2006; Thomé and

Table 4. Results of the multiple matrix regression (MMRR) comparing pairwise distance matrices representing different hypotheses for the diversification of *Rhinella granulosa*, and of the ranking of models through mixed linear effects modeling (MLPE).

MMRR	Regression coefficients	t-statistic	p	VIF
Intercept	0.000	0.000	0.577	
IBD	0.283	6.313	0.055	4.600
AOF	0.369	7.901	0.007	5.001
IBGB	0.095	2.915	0.504	2.445
IBE	−0.244	−7.958	0.005	2.151
IBI	0.105	4.278	0.239	1.370
MLPE	**k**	**AIC**	**Δi**	**wi**
AOS, IBE, IBD	3	−4754.261	0	0.265
AOS, IBE	2	−4754.205	0.056	0.258
IBE, IBD	2	−4753.050	1.211	0.145
All	5	−4752.524	1.737	0.111
All minus IBD	4	−4752.523	1.738	0.111
All minus AOS	4	−4752.498	1.763	0.110

IBD isolation by distance, *AOF* asynchrony of seasons, *IBGB* isolation by geographic barrier (rivers), *IBE* isolation by environment, *IBI* isolation by instability, *VIF* variance inflation factor, *k* the number of parameters included in the model, *AIC* the Akaike Information Criterion, *Δi* Akaike differences, *wi* model probabilities.

Table 5. Mantel tests (999 permutations) between pairwise distance matrices representing different hypotheses for the diversification of *Rhinella granulosa*.

	IBD	IBGB	IBE	AOS	IBI
IBD	1	0.765	0.173	0.735	0.393
IBGB	0.001	1	0.131	0.536	0.236
IBE	0.007	0.030	1	0.600	0.157
AOS	0.001	0.001	0.001	1	0.485
IBI	0.001	0.024	0.037	0.001	1

R-squared values are at the top right of the diagonal whereas p values are at the bottom left of the diagonal.
IBD isolation by distance, *AOF* asynchrony of seasons, *IBGB* isolation by geographic barrier (rivers), *IBE* isolation by environment, *IBI* isolation by instability.

Brasileiro 2007). Because locomotor performance in *R. granulosa* was demonstrated to be highly sensitive to lower temperatures (Titon and Gomes 2017), we expect this variable to affect calling activity, which is also energetically demanding (Wells 2007). Thus, temporally displaced climatic regimes will most directly affect mate encounters between reproductive individuals (and reproductive success), directly mediating gene flow between populations. Nevertheless, it is plausible that asynchronic rainfall also influences *R. granulosa* indirectly by affecting available resources as in birds and other less water-dependent organisms.

That natural selection directly contributes to a reduction of gene flow between populations is a prediction of the asynchrony of seasons hypothesis because migrants among populations should have lower fitness (Nosil et al. 2005). In *R. granulosa*, migrants would likely arrive at a different stage of sexual maturity and, if able to mate, their offspring's viability could be compromised at early stages of development. Nevertheless, following regional patterns of climate is no evidence for local adaptation if conditions only vary temporally (i.e., the absolute

climate is similar). Also arguing against local adaptation is a renowned ability of amphibians to cope with unstable environments, with desert amphibians showing extreme plasticity in reproductive behavior, including breeding times (Wells 2007). The physiological profile of *R. granulosa* indicates this should be the case (Madelaire and Gomes 2016).

Regardless of whether diversification is promoted by neutral or selective factors, the male advertisement call was reported to differ between individuals from the Southern and Northern populations of *R. granulosa* (Giaretta et al. 2018). As advertisement calls are the main form of species recognition in anurans (Wells 2007), this suggests a mechanism for the formation of barriers to reproduction. Further experimental studies are necessary to confirm differences are relevant for the toads themselves, as the advertisement call may be less important in explosively breeding amphibians (Forester and Thompson 1998).

Demographic inference and phylogeographic research have benefited from a dramatic increase in the amount of data that can be generated from non-model systems (Garrick et al. 2015), but research in the discipline remains challenging due to the complex forces that influence genetic diversity within species. For example, both historical and contemporary processes may have influenced the diversity within *R. granulosa*. Moreover, it is challenging to disentangle the role of putative processes that overlap geographically such as isolation by distance and asynchrony of seasons. Also, it is possible that the São Francisco river plays some role in population divergence, but as a soft barrier instead of a vicariant agent (Lanna et al. 2020, Thomé et al. 2021).

CONCLUSIONS

Genetic structure in *R. granulosa* suggests the existence of two geographically cohesive populations. Biome-related environmental gradients, geographic barriers, and historical habitat instability do not appear to have influenced diversification in *R. granulosa*. However, we were not able to reject a role of climate through spatial variation in seasonality—the asynchrony of seasons hypothesis. Instead, our results are consistent with the predictions of this hypothesis: population distributions follow the geographic structure in the timing of seasons, with monthly climatic variables showing full power in predicting genetic structure. We suggest that genetic differentiation within *R. granulosa* can be provoked by neutral mechanisms related to climate driven population isolation, and/or by natural selection against migrants from populations with different breeding times. The Neotropics appears to be a region where asynchrony of seasons is both expected (Martin et al. 2009) and underexplored as a potential explanation for biodiversity patterns. Because this hypothesis is seldom considered in phylogeographic studies in general, we hope our results can encourage researchers to incorporate it in future diversification investigations in the region and elsewhere, especially in systems where breeding is tightly coupled with climate.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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