Isolation by environment is more important than isolation by distance along a tropical gradient in direct developing frogs

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Abstract

To identify which abiotic factors are most important for defining population structure, both spatiotemporal scales and species-specific life history attributes and ecology must be accounted for. Our study aim is to quantify the extent to which divergence is driven by abiotic factors such as temperature, precipitation and elevation. To do so, we used a direct-developing frog species, *Craugastor loki* that occurs along a steep elevation gradient. Using restriction-site associated DNA sequencing (RADseq) from individuals collected from 100 m to 2250 m of elevation at 13 localities at Sierra Madre de Chiapas in southern Mexico we described population structure using a variety of model-based clustering and landscape genomics approaches. We found that populations sampled at higher elevation correspond to an undescribed new species of *Craugastor*, and that populations from *Craugastor loki* between 120 m and 1500 m are clustered in two different genetic groups: a Pacific slope group and a Central Depression slope group. We found that a null model of isolation-by-distance was not supported and divergence is more likely influenced by the interaction between elevation and precipitation, but also temperature. Our results underscore the importance of isolation by environment and highlight that spatial analyses can illuminate fine scale population structure.

Introduction

Biotic and abiotic factors modulate the balance between gene flow and population divergence [1] and can yield a wide range of patterns of genetic and phenotypic variation across the landscape [2]. For example, biotic factors related to life history traits and ecological interactions (e.g., dispersal abilities and generational time) have been shown to determine population structure [3,4]. Likewise abiotic factors, such as geography and environment (e.g., geographic barriers or environmental heterogeneity) also play a key role in population connectivity across a given landscape [2].

Restriction of gene flow across a landscape can often be explained by the ubiquitous pattern of isolation by distance (IBD; [5], where genetic differentiation increases with the increase in geographic differences. However, IBD assumes a uniform landscape and does not account for landscape heterogeneity. In contrast, a model of isolation by resistance (IBR) consider how characteristics of the landscape result in spatial genetic structure [6]. An IBR model predicts a positive relationship between genetic differentiation and resistance to gene flow. Gene flow can also be limited in environmentally heterogeneous landscapes due to divergent selection driving genetic differentiation, leading to isolation by environment (IBE). IBE can be identified by assessing the contribution of environmental factors on genetic variation while controlling for geographic distance [7,8]. IBD, IBR and IBE can easily be confounded with one another and disentangling which of these processes is more important is key to understanding how the landscape influences genetic variation.

Recent studies in amphibians have shown that different climatic variables (e.g., annual mean temperature, isothermality, temperature range and precipitation) function as drivers of population divergence, thus producing a pattern of IBE [9,10]. Thermal

differences can act as an environmental pressure on phenotypic traits through divergent selection and increase population differentiation [10]. Likewise, seasonal variation in temperature influences the movement and behavior of frogs [11] resulting in limited gene flow. Indeed, candidate single nucleotide polymorphisms (SNPs) and structural variants associated with temperature in frogs have been identified and suggest a potential functional role in life history strategies [9,12]. Precipitation can limit amphibian distribution at both the regional and local scales [13]. In dry seasons, frogs tend to move less because their ability to absorb water from the ground decreases when soil water potential decreases [14,15]. Despite an increasing number of studies that explore the contribution of environment on population divergence in ectotherm species, much remains to be explored in the way in which environmental variables shape gene flow and genetic diversity at a fine scale. This is particularly true in tropical forests, where the potential for biodiversity loss is higher.

At small scales, tropical mountains are highly heterogeneous in temperature, precipitation, and humidity [16]. Thus, they are ideal for assessing the potential for IBR and IBE to limit gene flow [17]. Elevation gradients can also be an ideal model to study which factors can lead the adaptive differentiation and identify local adaptation [18,19].

The Sierra Madre de Chiapas is a physiographic region with an ample variety of habitats, where lowland and intermediate habitats are influenced by the orientation of its slopes: the Pacific and the Central Depression. The Pacific slope is more humid in comparison to the Central Depression, due to a continental influence and a rain shadow effect. Furthermore, the region offers a natural elevation gradient in both slopes. Given the natural differences of temperature and precipitation, we can expect that IBE could

contribute more than IBD to population structuring, especially for ectothermic organisms with low vagility and high site fidelity [20].

Here, we use the amphibian species *Craugastor loki*, a direct-developing frog species found in North and Central America, inhabiting a variety of humid habitats in Chiapas, Mexico. This group of frogs has a wide elevational distribution range extending from sea level up to 2200 meters above sea level (masl), particularly in southern Mexico [21,22], and is often locally abundant. Consequently, this species is particularly suitable for investigating fine-scale genetic differentiation patterns across landscape-level gradients. Its continuous distribution allows for testing abiotic factors, such as the environment, as contributors to genetic differentiation and increased population structure. Our study aims to (1) quantify both fine-scale genetic differentiation and more divergent evolutionary relationships between populations of *C. loki* in the Sierra Madre de Chiapas, (2) test whether patterns of genetic variation in *C. loki* are best explained by geographic distance or environmental conditions, and (3) identify which specific environmental variables might be associated with patterns of genetic differentiation in *C. loki*.

Materials and Methods

Study area and sampling

We collected 90 tissue samples of the direct-developing frog *C. loki* from 13 different localities in the Sierra Madre de Chiapas of southern Mexico (Figure 1, Table S1). Our sampling regime was designed to capture environmental variation along an elevation gradient, with sampling sites ranging from 120 to 2200 masl [23]. We collected mostly toe and, in some cases, liver tissues. Sampling was performed in the rainy season (June to

November in 2017). The number of samples varied from five to eight individuals per site. We included *Craugastor pygmaeus* as an outgroup taxon, as it is closely related to the members of *Craugastor rhodopis* group, which includes *C. loki*. We also included *Craugastor rupinius*, which inhabits the Sierra Madre de Chiapas, is distantly related to *C. loki*, and is nested within the *Craugastor punctariolus* species group [24].

DNA extraction and library preparation

Genomic DNA extractions were obtained using a QIAamp DNA Mini kit. Quality of extractions were checked with agarose gel electrophoresis and the quantity of extractions (20ng/µl) using a Qubit 3 Fluorometric Quantitation Kit (Invitrogen). We sequenced 94 *C. loki* samples using a restriction-site associated DNA sequencing (RAD-seq) method, employing the restriction enzyme *SbfI.* Sequencing libraries were run on two lanes of an Ilumina HiSeq 2000. Library preparation and sequencing were performed at Floragenex Inc (Eugene, Oregon, USA).

Preprocessing and data assembly

We trimmed adaptors, filtered out low-quality data and demultiplexed raw data of the 94 samples using process_radtags in Stacks v.2.5. [25]. We subsequently assembled the data using Ipyrad v.0.7.30 [26], exploring parameter optimization with a focus on the maximum allowed divergence among allelic variants and the number of samples required per locus.

We made two datasets, the first (dataset 1) considered all samples from lowlands and intermediate elevation (120 to 1500 masl), the highlands (samples above to 2000 masl), plus the two outgroup samples of *C. pygmaeus* and *C. rupinius.* The second dataset (dataset 2) only included samples from lowlands to intermediate elevation (100 to 1500 masl).

Samples from higher elevations likely represent a cryptic species. For dataset 1, assembly and variant calling was performed using a clustering threshold of 0.85 and a minimum number of samples per locus of 35. For dataset 2, we used 0.85 as clustering threshold and 30 as the minimum number of samples per locus. We filtered one SNP for each RAD-seq locus using VCFTOOLS v.4.2 [27] and PLINK v.1.0.7 [28], and converted the SNP data to VCF format for landscape genomic analysis. Finally, we filtered dataset 2 allowing for at least 50% missing data, a minimum mean read depth of 26, and a minor allele frequency (MAF) of 0.01.

Phylogenetic analyses

We reconstructed phylogenetic relationships among individuals of *C. loki* along the elevation gradient and outgroup species using dataset 1 and two tree-building methods. First, we used FastTree v.2.1.11 [29] using nearest-neighbor interchange for the topology and subtree-prune and regraft moves (NNI + SPR) for branch length optimization. We used a general time-reversible nucleotide substitution model with a single rate per site (GTR + CAT). Second, we performed a maximum likelihood analysis in RAxML-HPC v.8.2.12 [30] using a GTR + GAMMA nucleotide substitution model and saving the best-scoring tree. For node support we ran 100 bootstrap samples for both analyses. Trees were rooted with our choice of outgroup. Analyses were performed on the CIPRES Science Gateway v.3.3 [31]

Non-spatial population structure analyses

We used two non-spatially aware methods to evaluate population structure. First, we used the non-parametric Discriminant Analysis of Principal Components (DAPC) with the

Adegenet package v.2.1.1 in R v.3.6.1 [32] to analyze both dataset 1 and dataset 2. To determine the number of the groups, we used the find.clusters function and selected the optimal *K* using the lowest BIC value exploring K 1 to 10. The optimal number of PCs to retain was selected using a cross-validation procedure [33]. Second, we used the model-based method Admixture v.13 (Alexander et al., 2009) to analyze dataset 2. We ran *K* from 1 to 5, with 10 iterations per *K* and selected the *K* with the lowest cross-validation error.

Spatially informed population structure analyses

We used multiple approaches to investigate the relationship between geographic and genetic information. First, we inferred the strength of an IBD pattern and compared a spatial model with a non-spatial model using the R package conStruct v.1.3 (Bradburd et al., 2018) implemented in R. conStruct uses an explicit spatial component and models genetic variation in genotyped individuals as partitioned within or admixed specific number of discrete layers, within each layer the relatedness decays as a parameter function of the distance between samples [34]. We analyzed *K* from 1 to 5, each with 10 replicates with 1000 MCMC iterations. We also used a cross-validation function to determine the statistical support for models with and without a spatial component. Because conStruct is sensitive to missing data, we collapsed some closely related individuals from the same population into a single, multi-individual samples (https://github.com/gbradburd/conStruct.git) and ran the cross-validation test using 35 multi-individual samples from 10 localities.

Next, we studied spatial and genomic variation across our sampled elevation gradient using a spatial Principal Component Analysis (sPCA; [35] of dataset 2 implemented in Adegenet v.2.1.1. sPCA relies on an ordination approach for analyzing

spatial and genetic patterns; using Moran's *I* index, it identifies eigenvectors which maximize genetic variation and spatial autocorrelation and maps selected eigenvectors onto geographic space.

Finally, we used the ResistanceGA v.4.0.14 software [36] to associate specific landscape attributes with levels of gene flow. The landscape attributes we used were elevation, temperature, and precipitation (during the rainy season from July to November). ResistanceGA uses pairwise genetic dissimilarity and a genetic algorithm to optimize resistance surfaces. We employed least cost paths to estimate the effective distances across the landscape [36] and used a linear mixed effects model with maximum likelihood to test for landscape effects, where pairwise genetic distance was the response variable and the optimized effective distance was the predictor variable. We used both approaches for each individual variable and for all combinations of the individual surface and null model [36].

Isolation by environment (IBE)

We tested for a signature of IBE using both temperature and precipitation variables, factors highly relevant to amphibian activity. Analyses were performed in the BEDASSLE v1.5 R package [37], which models the covariance of allele frequencies as a Gaussian process and uses a Bayesian model to estimate the contribution of environmental and geographic variables. For environmental variables, we obtained bioclimatic variables available to Mexico from Cuervo-Robayo et al. (2014) interpolated to ~90m resolution. We used annual temperature, annual precipitation, temperature seasonality, and precipitation seasonality, variables that have been associated with genetic differentiation in studies of amphibians [9,10,12]. Pairwise Euclidean distances were generated between sampling sites for environmental variables. Pairwise great-circle geographic distances were generated

between sampling sites using the function rdist.earth with the package field in R. We standardized both distance matrices by dividing values by their standard deviation constants. We performed two replicate Markov chain Monte Carlo runs to ensure convergence of the parameters using the beta-binomial model, running one analysis for 5 million generations, and the second for 8 million generations, sampling every 1000 generations in both runs. Performance of the model was assessed by visualizing plot acceptance rates and parameter trace plots. We discarded the first 50% of samples as the burn-in and estimated the contribution of the environmental distance versus geographic distance to genetic differentiation using the ratio ($\alpha E/\alpha D$).

Results

Sequence data and bioinformatics

We obtained ~661 million reads for the 94 samples, with 6.9 million mean reads per sample. After filtering, we obtained a total of 848 loci for dataset 1(Table 2S) and 1801 loci for dataset 2 (Table 3S). The assembled RADseq data set are available on Dryad repository as VCF files.

Phylogenetics and population structure

Both FastTree and RAxML phylogenetically placed samples from the highlands group (localities from elevation above 2000 masl, Figure 1) in a separate clade with 100% bootstrap support (blue shaded clade in Figure 2a), suggesting that they may belong to a different species of *Craugastor*. Some samples from intermediate elevations (samples from 1000 – 1200 masl) clustered with the outgroup samples: ArrNeg8 clustered with *C.*

pygmaeus and the samples NBra1_1 and NBra1_4 grouped with *C. rupinius*. This is likely explained by the difficulty in differentiating adult members of *C. pygmaeus* from *C. loki*, and the difficulty in differentiating juveniles of *C. rupinius* from *C. loki*. Samples from the low-elevation localities and the majority of samples from intermediate-elevation localities were recovered as a clade of *Craugastor loki* in both analyses (orange and green shaded groups in Figure 2a).

The DAPC results for dataset 1 identified a $K = 3$ as the best-fitting model (Figure S1a). The first axis accounted for 39.1% of the variation and identified the greatest separation between a cluster containing samples from the highlands, the outgroup samples (*C. pygmaeus* and *C. rupinius*), and the intermediate-elevation samples associated with those clades in the phylogenetic trees, and two clusters containing samples from the lowland and intermediate elevations (Figure 2b). Interestingly, four additional samples (GnNB2_4 LaFlor2, LaLoma1, LaLoma7) from intermediate elevations were placed closer to the "highland" cluster in ordination space. To verify the identity of these four samples, we reviewed fieldnotes and confirmed uncertainty in our taxonomic identification; thus, we omitted these individuals from dataset 2.

DAPC analysis of dataset 2 identified three groups, one of which is represented by the orange cluster that corresponds to the Central Depression slope (Figure S1b). The other two groups (light green and dark green) identified in discriminant space are samples from the lowlands and intermediate elevations of the Pacific slope (Figure S1b), accounting for 16.26% of the conserved variance. BIC scores stabilized at values of $K = 3$ (Figure S1c).

Admixture analysis of dataset 2 identifying two groups with cross-validation support for $K = 2$ as the best fit model but additional structure was identified at $K = 3$

(Figure 2c). One genetic cluster recovered samples from the Central Depression slope and the other clustered the lowland and intermediate samples from the Pacific slope (Figure 2c).

conStruct analyses recovered two genetic clusters (Figure 2c): Central Depression slope (orange color) and Pacific slope (green color) including samples from low and intermediate elevations. However, there was no difference between non-spatial model and spatial model of conStruct (Figure S4).

Landscape genomics

The sPCA detected a significant pattern both globally (P = 0.003) and locally (P = 0.002). The first axis identified a break between the clusters (Figure S5) associated with the Central Depression slope and the Pacific slope (Figure 3). However, some sampled localities fall at the boundary of the genetic break, corresponding to a lower elevation area.

The resistance surface analysis with ResistanceGA found the highest support for a model containing rainy season precipitation and elevation, although a competing model containing only temperature was nearly as well-supported (Table 1). Little to no support was found for a null model of no geographic structuring or for isolation-by-distance.

Isolation by environment

Bayesian analysis of the relative contribution of IBE and IBD to genetic differentiation in BEDASSLE produced evidence of IBE when elevation was considered. Both temperature seasonality and precipitation seasonality show an important contribution on genetic differentiation across elevation more than the geographic distance (Table 2, Figure S6). The annual mean temperature and annual precipitation never reached convergence despite running for 8 million MCMC iterations. The mean of the α_E/α_D ratio

can be interpreted as the relative effect of changing one degree Celsius or one millimeter precipitation with one meter elevation distance between populations when the IBD has the same effect size of IBE. That is, for elevation, we would expect that 1°C change of the variation of temperature to have a similar effect on genetic divergence as a shift of 857.98 m.

Discussion

Disentangling which abiotic factors have an important role in population connectivity and limiting gene flow can be challenging, particularly given that abiotic environmental variables such as temperature and precipitation are usually autocorrelated with geography [37], and frequently also with elevation [38]. Here, we found significant genetic differentiation between populations of the direct-developing frog *Craugastor loki,* and further found that patterns of gene flow were best explained by the interaction between temperature and precipitation, with elevation suggesting a pattern of isolation by environment.

We found that *Craugastor loki* inhabits areas from sea level to ~1500 masl. Previous work on the evolutionary relationship of the *Craugastor rhodopis* group (including *C. rhodopis, C. occidentalis* and *C. loki*) using mitochondrial DNA suggested three clades of *C. loki* occurring from Mexico to Central America, with the southern clade inhabiting mainly lowland areas [22]. Here we confirm that *C. loki* occupies mainly lowland and intermediate elevations and that samples above 1500 masl possibly correspond to another species. This group of frogs are highly polytypic and field identification is challenging due to a lack of diagnostic characters [39,40]. Here, both phylogenetic and clustering results of our genomic data show that the members of *Craugastor* found above

1500 masl are relatively distinct from lower elevation populations of *C. loki*, indicating that they either belong to another species that we did not include in the analysis (e.g., *C. matudai*, *C. montanus*, *C. greggi*) or possibly are an unidentified species. Our sampling covers different localities that occur near the type locality of these species, like Cerro Ovando (Figure 1), the type locality where *C. matudai* [41] and *C. montanus* occur [42]. Both species were described by museum specimens as *C. loki*. *Craugastor greggi* [43] is also supposed to be present in this area, inhabiting cloud forest like the highland ecosystem where we sampled. Even if all those species represent different groups in the taxonomical classification of *Craugastor*, in the field these frogs could not to be distinguished from each other and the original descriptions did not describe the vivid coloration of live individuals. Further work and a geographic more extensive sampling are needed to clarify the evolutionary relationships among these taxa.

Within *Craugastor loki*, we identified two main groups: one cluster from the Central Depression slope and the other from the Pacific slope. Spatial analyses (e.g., sPCA) show a discontinuity in the area sampled that matches a valley that breaks the range of the Sierra Madre de Chiapas (Figure 3). It may be that the dispersal of frogs along the Sierra Madre de Chiapas could be restricted by this break in elevation. Frogs often have low dispersal capacity and limited movement up and down elevation gradients [44]. In addition, it seems that the break identified by our spatial analysis is reflected in local forest communities. To the southeast of where the range breaks the local conditions become more humid. In contrast, to the northwest the conditions of the Sierra Madre de Chiapas are usually drier and more influenced by the Central Depression, resulting in a continuous dry forest habitat.

Our landscape resistance and IBE analysis both suggest an effect of climatic variables on spatial genetic differentiation. Precipitation and temperature are important

factors influencing the natural history and ecology of these direct-developing frogs, characterized by the lack of a water-dwelling larval phase and their use of substrate humidity for reproduction [44]. The Central Depression slope cluster presents a thermal landscape different from the Pacific slope, where the former is characterized mainly by temperature seasonality [23]. Hence, our results suggest that the abiotic variables considered in our study, temperature and precipitation, have an important role in the pattern of IBE.

Several studies on amphibians have documented patterns of genetic differentiation across elevation gradients [17,45] and have found evidence of phenotypic divergence associated with temperature and elevation, explained by a pattern of IBE [9]. In addition, Medina et al. (2021) found a clinal pattern of genomic differentiation associated with temperature and identified some candidate SNPs associated with temperature and body size across the clinal gradient. Our results suggest a pattern of IBR and IBE. When we used specific layers of temperature and precipitation, we found that the seasonality of both parameters show signatures of IBE when elevation is taken into account. Both environmental variables are intrinsically associated with elevation and the orientation of the slope. In a previous study, Percino et al. (2021), studied some physiological traits from the same localities of this study. The frogs that inhabit at the Central Depression slope showed a different thermal sensitivity compared with the populations from Pacific slope. First, they exhibit high thermal accuracy, meaning that their body temperature is close to the microenvironmental temperature, and high thermal quality, that is, the thermal landscape offers suitable microhabitats with temperature close to body temperature. Thus, it is suggested that the landscape plays an important role on frog populations and eventually could drive phenotypic and probably genotypic divergence as a result of potential local

adaptation. Further work is necessary to specifically study the role of local adaptation or thermal plasticity coupled with evapotranspiration rates across the elevation of the Sierra Madre de Chiapas.

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Competing interests

The authors declare no conflicts of interests exist.

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Data Accessibility

The data are accessible to the repository in Dryad (XXXXXXX). The repository contains the data in vcf format.

Author Contributions

RPD and DP designed the research. RPD performed the fieldwork and lab work. RPD, KJ, TM analyzed the data. DP, DW contributed with analytical tools. RPD lead the manuscript writing with input of all authors.

Table 1. Summary of model selection for the generalized linear mixed-effects models carried out in ResistanceGA. The null model assumes no geographic structure and the alternative models performed with environmental surfaces using temperature, precipitation, and elevation, where the interaction between elevation and precipitation is well fitted to the model.

Layer	k	AIC	AICc	R2m	R2c	LL	delta.AICc	weight
Elevation and Precipitation	7	-7893.07	-7891.07	0.0516	0.1276	3953.53	0	0.3965
Temperature	4	-7891.19	-7890.51	0.0425	0.1129	3949.60	0.5546	0.3005
Precipitation and temperature	7	-7891.14	-7889.14	0.0487	0.1247	3952.57	1.9287	0.1512
Elevation and temperature	7	-7890.51	-7888.51	0.0486	0.1213	3952.25	2.5596	0.1103
Precipitation	4	-7885.98	-7885.30	0.0513	0.1389	3946.99	5.7669	0.0222
Distance	2	-7884.71	-7884.51	0.0260	0.0950	3944.35	6.5607	0.0150
Elevation, Precipitation, and temperature	10	-7885.42	-7881.27	0.0494	0.1256	3952.71	9.7999	0.0030
Elevation	4	-7880.71	-7880.02	0.0260	0.0949	3944.35	11.0420	0.0016
Null	1	-7836.83	-7836.77	0	0.0630	3919.41	54.3006	6.41E-13

Table 2. Results of the BEDASSLE analysis. We ran two replicates for each analysis. The first runs compared the relative effect of the precipitation seasonality (αE) and geographical distance (αD) on genetic differentiation. The third and fourth runs compared the relative effect of the temperature seasonality (αE) and geographical distance (αD). The fifth and sixth runs compared the relative effect of the precipitation seasonality (αE) and elevation distance (αD) on genetic differentiation, while the last two runs compared the relative effect of the precipitation seasonality (αE) and elevational distance (αD).

	Ratio (αE/αD)	**95% Confidence Intervals**
Precipitation seasonality and geographical distance	0.428	(0.212 – 0.695)
Precipitation seasonality (replicate)	0.390	(0.192 – 0.621)
Temperature seasonality and geographical distance	0.483	(0.052 – 1.196)
Temperature seasonality (replicate)	0.436	(0.214 – 0.710)
Precipitation seasonality and elevation distance	12.674	(1.192 – 29.929)
Precipitation seasonality (replicate)	10.323	(1.119 – 25.548)
Temperature seasonality and elevation distance	857.980	(446.588 – 1629.37)
Temperature seasonality (replicate)	932.536	(453.102 – 1826.62)

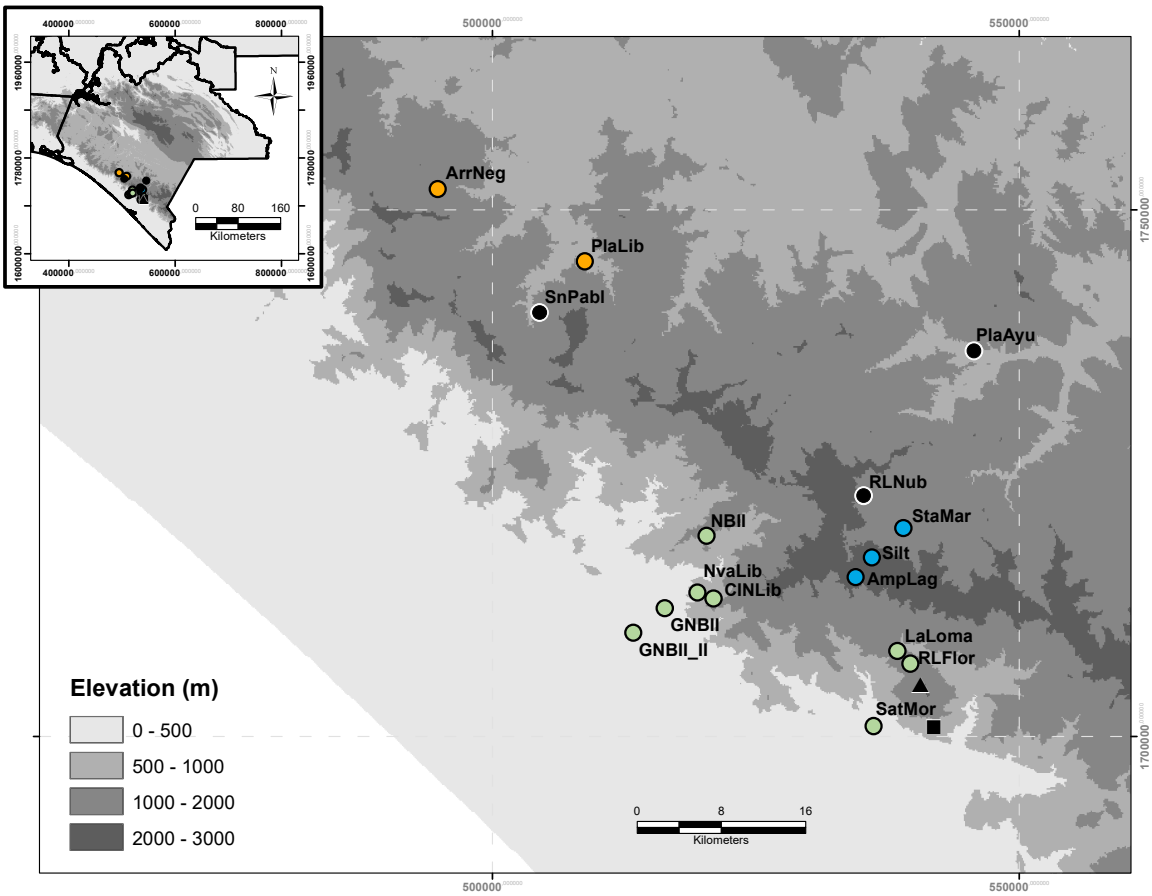

Figure 1. Sampled localities of *Craugastor* in Chiapas, Mexico. The orange and green circles correspond to the two genetic clusters of *C. loki* identified by Admixture and conStruct analysis, as seen in Figure 2. Blue circles correspond to the localities of *Craugastor* sp. from the highlands (see Supplementary material). Black circles with white outline are visited localities where frogs were not found. Triangle and square symbols correspond to the type localities of the *C. montanus* and *C. matudai,* respectively.

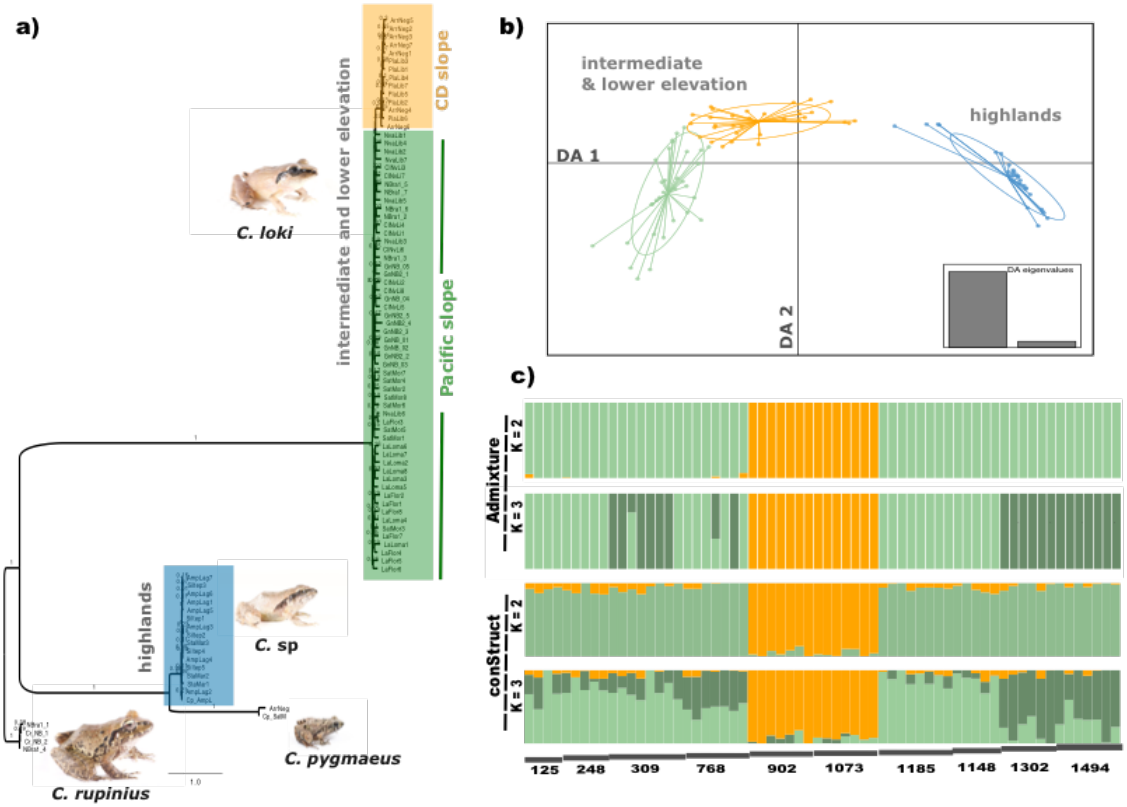

Figure 2. (a) Results from phylogenetic analysis using maximum likelihood performed in RAXML and (b) discriminant analysis of principle components of the entire genomic data set. The individuals from higher elevation populations may correspond to other species of *Craugastor*. Colors correspond to the individuals sampled in the different localities shown in Figure 1. (c) Genetic assignment of the sampled populations using Admixture (from K = 2 to K = 3) and non-spatial model performed in conStruct. Individuals are ordered from lower to higher elevation. Elevation (in meters) is represented by horizontal bars below the assignment plots. Central Depression cluster and Pacific slope are shown in orange and green respectively. Photos in Fig. 1 by R. Percino-Daniel (*C. rupinius* & C. sp) and J. E. Pérez Sanchez (*C. pygmaeus*).

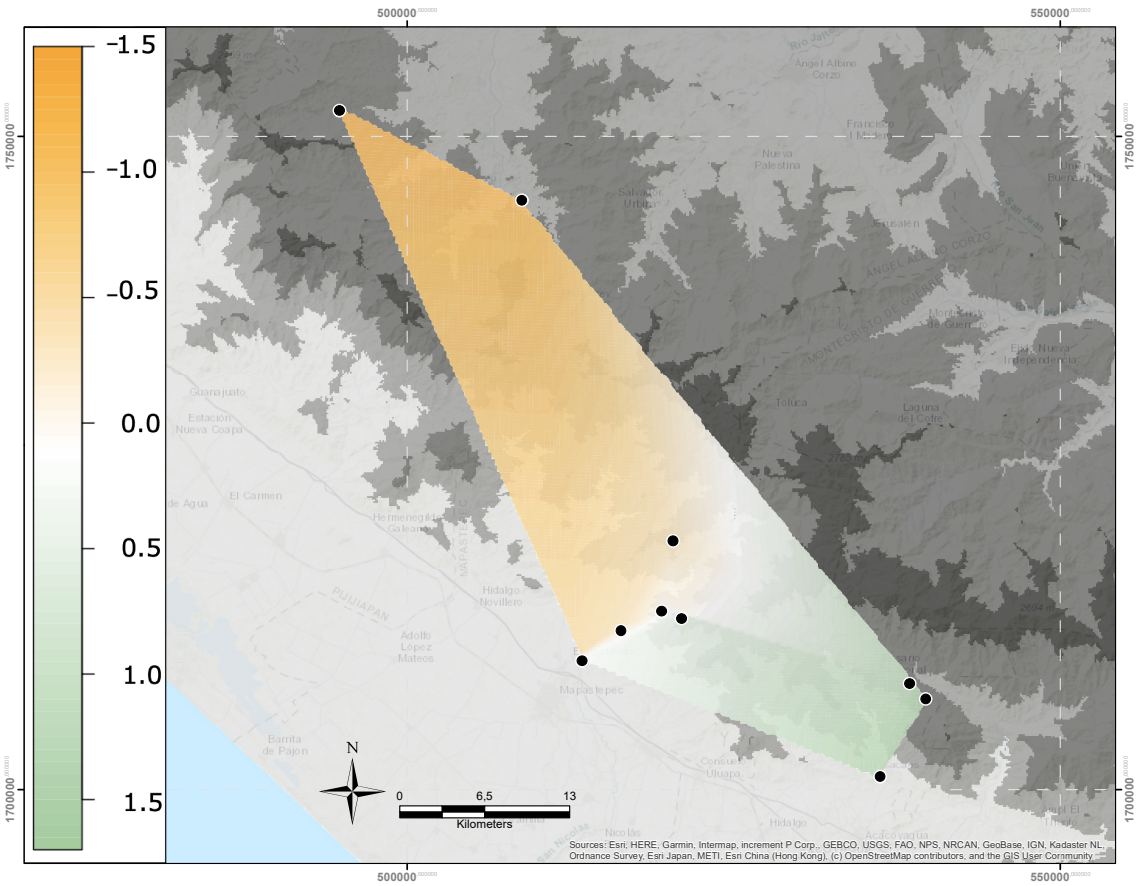

Figure 3. Interpolated spatial genetic structure based on sPCA superimposed over elevation map. The bar at left represents the interpolated vector scores, which corresponds to the degree of differentiation between individuals. The white color represents the break of the mountains edge of the highlands at Sierra Madre de Chiapas (lower elevation area). Black dots represent sampled sites.