The role of Central American barriers in shaping the evolutionary history of the northernmost glassfrog, *Hyalinobatrachium fleischmanni* (Anura: Centrolenidae)

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Abstract

Background. The complex geological history of Central America has been useful for developing an understanding of the effects of different processes and barriers on the distribution and diversity of multiple groups of organisms. Anurans are an excellent choice for such studies because they usually have site fidelity and reduced movement. The objective of this work was to identify the impact of recognized geographic barriers on the genetic structure, phylogeographic patterns and divergence times of a wide-ranging amphibian species, *Hyalinobatrachium fleischmanni*.

Methods. We amplified two coding mitochondrial regions (COI and ND1) and one ribosomal region (16S) in samples collected from the coasts of Veracruz and Guerrero in Mexico to the humid forests of Chocó in Ecuador. We examined the biogeographic history of the species through spatial clustering analysis (BAPS, Geneland and sPCA), Bayesian and maximum likelihood reconstructions, and spatiotemporal diffusion analysis.

Results. Our data suggest a Central American origin of *Hyalinobatrachium fleischmanni* and two posterior independent dispersals towards North and South American regions. The first clade comprises individuals from Colombia, Ecuador, Panama and the sister species *H. tatayoi*; this clade shows little structure, despite the presence of the Andes mountain range and the long distances between sampling sites. The second clade consists of individuals from Costa Rica, Nicaragua, and eastern Honduras, with no apparent structure. The third clade includes individuals from western Honduras, Guatemala, and Mexico and displays deep population structure.

Discussion. Herein, we synthesize the impact of known geographic areas that act as barriers to glassfrog dispersal and have demonstrated their effect in differentiating *H. fleischmanni* into three

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markedly isolated clades. The observed genetic structure is associated with an initial dispersal event from nuclear Central America, followed by vicariance, likely occurring during the Pliocene. The southern samples are characterized by a very recent population expansion, likely related to sea level and climatic oscillations during the Pleistocene, while the structure of the northern clade has probably been driven by dispersal through the Isthmus of Tehuantepec and isolation by the Motagua–Polochic–Jocotán fault system and the Mexican highlands.

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Introduction

Historical biogeography focuses on the role of the geographic space as a driver of biological processes such as speciation, extinction, and diversification (Cox, Lalde & Moore, 2016). Areas with a complex geological history are characterized by the appearance and disappearance of multiple barriers and corridors in their history. These barriers may significantly affect the gene flow of resident species, leading to allopatric speciation by vicariance, whereas corridors may lead to species dispersal and colonization of new areas (Noss, 1991). The use of molecular data for the reconstruction of species relationships, the development of new methods for biogeographic analyses, and the increase in geological studies in complex regions have revolutionized the understanding of such biological processes (Ronquist & Sanmartin, 2011). Biogeographic studies have integrated information regarding the relationships within or between closely related taxa, providing valuable opportunities to understand how patterns of biodiversity may have been shaped, even at short time scales (Crawford, Bermingham & Polanía-S, 2007; Streicher et al., 2014).

Central America is a region with a rather complex biogeographic history and high diversity of habitats and species (Myers et al., 2000; Cavers, Navarro & Lowe, 2003; Iturralde-Vinent, 2006; Daza, Castoe & Parkinson, 2010). The region is delimited to the north by the Isthmus of Tehuantepec (IT) in Mexico and to the south by the Andes in Colombia (Gutiérrez-García & Vázquez-Domínguez, 2012). The geological landscape of Central America has been continuously modified, especially during the last 15 million years (Ma), by major events including the emergence of the Panama Arc (13-15 Ma, Montes et al., 2015), the posterior closure of the Panama Isthmian land bridge when it ceased to function as a seaway (~9-10 Ma, Montes et al., 2012a; Montes et al., 2012b; Ramirez et al., 2016), and the posterior global climatic transitions

during the Plio-Pleistocene (Montes et al., 2015). These events triggered the Great American Biotic Interchange, or GABI, involving the replacement of native taxa (extinctions) and the establishment and diversification of colonizing taxa (speciation) on both continents (Marshall et al., 1982; Stehli & Webb, 1985). Ample phylogeographic research in this region has allowed the effects of geomorphology, topographic barriers, volcanic activity, large climate changes, intermittent connections, and corridors on the biota to be described, aiding in our understanding of the influence of past events on the patterns of genetic structure and the geographic distribution of birds (García-Moreno et al., 2004; Cadena, Klicka & Ricklefs, 2007; Arbeláez-Cortés, Nyári & Navarro-Sigüenza, 2010), plants (Cavers, Navarro & Lowe, 2003; Ornelas, Ruiz-Sánchez & Sosa, 2010; Cavender-Bares et al., 2011), reptiles (Hasbun et al., 2005; Venegas-Anaya et al., 2008), mammals (Eizirik et al., 2001; Ordoñez-Garza et al., 2010; Pérez-Consuegra & Vázquez-Domínguez, 2017), and amphibians (Mulcahy, Morrill & Mendelson, 2006; Crawford, Bermingham & Polanía-S, 2007; Wang, Crawford & Bermingham, 2008; Hauswaldt et al., 2011). As a result, diverse geological factors and major barriers have been more frequently correlated with the evolutionary history and dispersal of species (Bagley & Johnson, 2014).

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Amphibians are excellent systems for studies in which geological and environmental histories are inferred at fine scales, due to their ecology, particularly regarding their terrestrial habits, intolerance to salt water (Beebee, 2005), and marked niche conservatism (Smith, Stephens & Wiens, 2005; Wiens et al., 2006), as well as the restriction of the particular habitats of many species (e.g., Savage, 2002). However, evaluation of the impact of barriers on the phylogeographic patterns of this taxon, extending across the entire Central American region, has been precluded because most amphibians have small ranges.

Glassfrogs (Centrolenidae) comprise a diverse family endemic to the Neotropics, with numerous species and high levels of endemism, mainly distributed among the northern Andes and Central America regions (Castroviejo-Fisher et al., 2014; Mendoza & Arita, 2014). Studies on the dispersal capability of glassfrogs are limited, but these frogs are known to be restricted to streamside habitats (Ruiz-Carranza & Lynch, 1991) and to show site fidelity (Valencia-Aguilar, Castro-Herrera & Ramírez-Pinilla, 2012) and low mobility, with potential genetic subdivision and restricted gene flow (Delia, Bravo-Valencia & McDiarmid, 2017; Robertson, Lips & Heist, 2008). The glassfrog *Hyalinobatrachium fleischmanni* (Boettger, 1893) has one of the widest distributions, ranging from Guerrero and Veracruz states in Mexico, through the lowlands of Central America, to the southernmost limit of its distribution in Ecuador (citations needed). Males of the species call from vegetation along the margins of streams, and egg masses are usually laid on the underside of leaves over a stream. This species exhibits site fidelity and parental care by males, who attend one or more clutches at the same time (Delia et al., 2010; Savage, 2002; Barrera-Rodríguez, 2000). Tadpoles fall from vegetation into the water, where they develop; they are apparently fossorial, living buried in the leaf litter and bank substrate of streams (Villa & Valerio, 1982). Related species are distributed in different regions of South America, including the northern and central Andes, Guiana shield, and Amazon basin, where previous analyses have suggested an Andean origin for *H. fleischmanni* (Castroviejo-Fisher et al., 2014).

Considering its wide distribution, coupled with its site fidelity, *H. fleischmanni* is an ideal organism for studying the role that Central American geographic barriers have played in the dispersal patterns of lowland glassfrog species. In the present study, we had the following objectives: (1) to reconstruct the historical biogeography that has shaped the evolutionary history

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of *H. fleischmanni*, including dispersal or vicariance events and time of divergence; (2) to evaluate the possible presence of multiple isolated lineages within *H. fleischmanni*; and (3) to identify the impact of recognized geographic barriers on the genetic structure and phylogeographic patterns of *H. fleischmanni* over time. Based on known information about the species, we tested the hypothesis that *H. fleischmanni* had a South American origin and subsequently dispersed into the Central American lowlands after the closure of the Isthmus of Panama. Additionally, we hypothesized that the dispersal of this species in Central America has been limited by various high mountain ranges acting as barriers and that changes in sea level during the Pleistocene had an impact on the genetic structure of the lowland populations (Bagley & Johnson 2014). Hence, our prediction is that the current genetic structure of *H. fleischmanni* reflects patterns of vicariance events driven by dispersal barriers.

Materials and methods

Tissue sampling

Genetic material was obtained across the entire distribution area of the species, from both museum collections and fieldwork (Fig. 1). Fieldwork was performed during the rainy season, in which at least three individuals were collected at each locality. Specimen collection permits were provided by Ministerio de Medio Ambiente, Colombia (Resolution 120 of 24 August 2015) and Secretaría del Medio Ambiente y Recursos Naturales, Mexico (office number 00947/16). Captured specimens were euthanized with a 20% lidocaine hydrochloride (Xylocaine) injection, and all efforts were made to minimize suffering. Liver or muscle tissues were collected in the field and were stored in an RNAlater solution until their use in the laboratory. Specimens were

fixed with 10% formalin, stored in 70% ethanol and deposited in biodiversity collections at public research institutions in each country.

Molecular techniques

DNA was obtained from muscle and liver tissues following the phenol-chloroform extraction protocol of Sambrook & Russell (2006). The quantity and quality of the DNA were verified in 1% agarose gels and by measuring absorbance using a NanoDrop spectrometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). Amplification of the mitochondrial COI (658 bp), 16S (895 bp), and ND1 (961 bp) genes was performed following the protocols described by Guayasamin et al. (2008). PCR products were visualized with agarose gels and purified according to the EXO-SAP protocol (GE Healthcare, Piscataway, NJ, USA). DNA sequences were obtained with the BigDye Terminator Cycle Sequencing kit (Applied Biosystems). Sequences from *H. tatayoi* from Venezuela were also included. The sequences were assembled and edited manually and were aligned with Geneious 9.1.2.

We are aware that analyses based on mitochondrial DNA (mtDNA) provide a limited view of species evolution (i.e., matrilineal inheritance). However, due to the extreme difficulty of obtaining genetic material from the nuclear Central America region, we could only perform our analyses with sequences that were directly comparable to those from previous studies. However, mtDNA is a robust indicator of population history and species limits (Avise, 2000; Zink & Barrowclough, 2008), and mtDNA markers have helped to decipher genetic structuring in the form of phylogeographic breaks (phylogenetic splits between mostly distinct geographical lineages) in abundant Central American studies (Bagley & Johnson 2014). In addition, our study

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encompasses the widest geographic distribution of the species, allowing us to evaluate the diversification events shaping the evolutionary history of the species.

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Data analysis

Identification of landscape barriers and genetic diversity

The first step in identifying the main barriers to dispersal was to perform spatial and clustering analyses that are commonly applied to mtDNA sequences. First, we conducted a Bayesian analysis of population structure with BAPS v6.0 (Corander et al., 2008), using the spatial clustering of individuals, considering that the spatial prior may strengthen inferences for sparse molecular data. Second, the spatial location of genetic discontinuities was estimated with Geneland (Guillot et al., 2005), which estimates the number of populations within the geographical area of interest, maps borders between populations, assigns individuals to populations, and detects possible migrants. We ran the model in R under the correlated allele frequency model, without uncertainty regarding spatial locations. We generated 10^5 iterations to a thinning of 100, with the maximum rate of the Poisson process fixed as the number of individuals. Third, we performed a spatial principal component analysis (sPCA; Jombart, 2008a) in the package *adegenet* in R (Jombart, 2008b), for which we constructed a neighbor distance net among all coordinates and tested for significant, geographically correlated genetic structure along the main axis, based on a global randomization test. Previous studies have suggested multivariate ordination analyses as an alternative to Bayesian algorithms because they do not make any assumptions about the underlying population genetic model (Jombart, Devillard & Balloux, 2010). Considering that the pool of samples was slightly different for each gene, all analyses were run independently for each gene, with a maximum *k* value of 10 populations per analysis.

We next defined genetically homogeneous regions obtained through landscape analyses for diversity index estimation. We calculated haplotype (*h*) and nucleotide diversity (π), globally and by region, with DNAsp (Libardo & Rozas, 2009). Additionally, the distribution of genetic variability at hierarchical levels was estimated using analysis of molecular variance (AMOVA). Additionally, a median-joining haplotype network for each gene was constructed using PopArt (French et al., 2014). Genetic differentiation among regions was estimated based on the F_{ST} statistic with the *poppr* package (Kamvar, Tabima & Grünwald, 2014) and used corrected distances according to the K2P parameter (Kimura, 1980) in MEGA v.7 (Kumar, Strecher & Tamrura 2016).

Phylogenetic analyses

The sequences of all genes were concatenated, and a phylogenetic tree at the intraspecific level was estimated using both likelihood analysis in RAxML (Stamatakis, 2006) and a Bayesian inference approach implemented in MrBayes (Ronquist & Huelsenbeck, 2003). We rooted our phylogeny using the species *Hyalinobatrachium carlesvilai, H. mondolfii, H. chirripoi* and *H. colymbiphyllum* as outgroups. A list of the specimens and GenBank accession numbers included in this study is presented in Table S1. The best evolutionary model for each non-coding region (16S) and for the coding genes (COI and ND1) was evaluated using PartitionFinder 2 software (Lanfear et al., 2016). Maximum likelihood analysis was conducted using 10,000 rapid bootstrap analyses, the GTR + Γ evolution model and summarized support for the best tree. For Bayesian inference, we ran two independent analyses for 12 million generations, sampling trees and parameter values every 1,000 generations. Burn-in was set to 25%, and the first 3 million generations were therefore discarded.

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212 *Divergence times and Bayesian spatiotemporal diffusion analyses*

213 To estimate diversification times for the different *H. fleischmanni* mitochondrial lineages, we
214 employed BEAST 1.6.2 (Drummond & Rambaut, 2007). The time to the most recent common
215 ancestor for the main lineages was calculated via Bayesian Markov chain Monte Carlo (MCMC)
216 searches. The ultrametric tree was inferred *de novo* using the same partition substitution models.
217 In the absence of a fossil record for glassfrogs, we based our analysis on previously published
218 divergence times. We used three stem ages for *Hyalinobatrachium* species as calibration
219 constraints, following Castroviejo-Fisher et al. (2014). The most recent calibration point was
220 placed at 2.42 Ma (Confidence Interval (C.I.)=1.63–3.37), representing the divergence between
221 *H. fleischmanni* (USNM 559092) and *H. tatayoi* (MHNLS 17174). The following calibration
222 node was placed at 7.65 Ma (C.I.=5.93–9.63), representing the divergence between *H.*
223 *fleischmanni-H. tatayoi* and *H. charlesvilai*. The most ancient calibration point corresponded to
224 the divergence between *H. fleischmanni* and *H. mondolfii* (8.4 Ma, C.I.=6.68–10.52). We
225 implemented an uncorrelated lognormal relaxed molecular clock, and trees were sampled every
226 1,000th iteration for 100,000,000 generations, with 20% of the initial samples being discarded as
227 burn-in, after empirical assessment of appropriate chain convergence and mixing with Tracer 1.4
228 (Rambaut & Drummond, 2007). We constructed the historical demography of the major clades
229 obtained from the phylogenetic results, using Bayesian skyline plots that estimate the posterior
230 distribution of population sizes (Drummond et al., 2005).

231 To reconstruct the ancestral distribution and spatial dispersal of the species, we performed a
232 Bayesian spatiotemporal diffusion analysis in BEAST (v.1.8.4), assuming continuous spatial
233 diffusion with a time-heterogeneous random walk model ("Relaxed Random Walk", RRW,
234 Lemey et al. 2010). For this analysis, we used a subset of 34 samples with data for all three genes

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plus samples lacking some genes but originating from intermediate localities, encompassing the entire distribution of the species. We applied a normally distributed diffusion rate, a coalescent Bayesian Skyride model, and SRD06 substitution models (Shapiro, Rambaut & Drummond 2006). We used the jitter option under the TraitLikelihood statistic with a parameter value of 0.1. To summarize the posterior distribution of ancestral ranges using the RRW model, we annotated nodes in a maximum clade credibility tree (MCC) using the program TreeAnnotator v1.7.5. This tree was then used as an input in SpreaD3 (Bielejec et al., 2016) to reconstruct the pattern of spatial diffusion and to visualize lineage diversification across the landscape.

Spatial barriers to dispersal

We contrasted the previous BEAST analysis with a set of possible geographic elements defined *a priori* and based on the four geological blocks across Central America (Maya, Chortis, Chorotega, and Choco) (Marshall, 2007; Gutiérrez-García & Vázquez-Domínguez, 2013), as well as their northern and southern limits the southern Mexico block (SMB) west of the IT and the South American plate (SAP) east of the Andes, respectively. Three highland barriers were evaluated for their effect on species dispersal: the Motagua–Polochic–Jocotán (MPJ) fault system, the Talamanca Cordillera, and the Andes range. Three previously recognized lowland barriers for lowland amphibians (the Hess Escarpment, HE; western Panama isthmus, WPI; and central Panama isthmus, CPI) were also tested as possible barriers during Pleistocene sea level oscillations (Fig. 1).

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Results

We generated a final alignment of 2036 base pairs (bp) for 123 samples from 9 countries, including 13 sequences obtained from the GenBank and BOLD system databases (Table S1). We

did not detect any stop codons in protein-coding genes (COI, ND1). We obtained 25 haplotypes with an overall haplotypic diversity h=0.863 and a nucleotide diversity π=1.282 for the 16S gene. By contrast, we found 63 haplotypes for COI, with h=0.979 and π=0.044, and 45 haplotypes for ND1, with h=0.991 and π=0.042 (Table S2).

Landscape analysis, genetic diversity and structure

The BAPS results depicted six clusters for 16S, seven for COI and five for ND1. Although the number of clusters varied for the northern and southern regions, three clusters in western Chortis, Chorotega, and eastern Chortis were consistently recovered. The best differentiation was obtained with the 16S sequences, which separated the northern clusters, while the COI sequences could differentiate the southern clusters, east-west of the Andes range (Fig. 2a). Geneland showed six, seven and six clusters for 16S, COI and ND1, respectively. Both coding genes showed two clusters on both sides of the Andes range. Separation between the Choco and Chorotega samples was found in all cases (Fig. 2b). sPCA performed on individual genotypes revealed a significant, geographically correlated genetic structure for all three genes (nper=999, P=0.001). Eigenvalues indicated a higher spatial genetic structure for the main axis, related to the global structure. The first sPCA (regional scale) positive axis for all genes exhibited the greatest variation of genetic distance in relation to the distance network (Fig. 2c).

Based on our results, we selected seven genetically homogeneous regions: the North American Pacific, Gulf of Mexico, Maya block, western Chortis, eastern Chortis-Chorotega, Choco block and SAP. The minimum genetic distance between regions was obtained for SAP-Choco (K2P=0.002-0.019), while the maximum was obtained between the North American Pacific and Chorotega (K2P=0.041) for 16S, between Maya and SAP for COI (K2P=0.078), and between

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SAP and Chorotega for ND1 (K2P=0.077) (Table 1). The F_{ST} indices between regions was >0.7 for almost all combinations, except between SAP and Chocó for 16S and among the Gulf, Pacific and Maya regions for ND1 (Table 1).

When comparing the diversity per cluster, the Chorotega population showed the highest haplotypic diversity for all genes, while the Maya populations exhibited the highest nucleotide diversity for coding genes. The Gulf population showed the lowest haplotypic diversity, and SAP exhibited the lowest nucleotide diversity (Table S2).

The AMOVA results (Table 2) for all genes indicated that 79-88% of the observed genetic variability was partitioned between regions, compared with 12-20% within regions (all $P <$ 0.001). Overall, the haplotype networks for the three genes were concordant, with higher diversity and structure being revealed for COI and ND1 than for 16S. Four mitochondrial haplotype groups were detectable among the entire distribution (Fig. 3), where the concordance between the haplotype network and the species distribution suggested a deep pattern of geographic structuring and differentiation across the complete range. The SAP and Choco regions shared the same 16S haplotype but showed differences in the COI and ND1 coding genes.

Phylogenetic patterns, times of divergence, and demography

The PartitionFinder output indicated that HKY+I+G, GTR+G, and GTR+G+I were the best models for 16S, COI, and ND1 respectively. The phylogenetic relationships based on the Bayesian approach and maximum likelihood showed three main, well-supported clades, although their relative positions were not fully resolved (Fig. 4). The first clade, designated the "Northern clade", was divided in two lineages: a large lineage containing all samples from the SMB and Maya regions (pp=1) and a smaller one from the western Chortis region (pp=1). The second

Comment [Office16]: Since these three genes are linked, shouldn't they be analyzed together?

Comment [Office17]: When presenting the information by gene, the reader is under the impression that they are independent sources of evidence. However, since the genes are links, perhaps it makes sense to present the results of concatenated sequences.

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clade, the “Central clade”, was comprised of samples from the eastern Chortis and Chorotega regions (pp=1). The third clade, the “Southern clade”, consisted of samples from the Choco and SAP regions, including the sister species *H. tatayoi* from Venezuela (pp=1). The Southern clade did not show any structure, displaying a polytomic topology.

The results regarding the estimation of divergence time showed a pattern in which divergence among the three main clades occurred during the Pliocene (~3.40 Ma, HPD=2.25-4.56 Ma; Fig. 3). With respect to the Northern clade, the split between the lineage from West Chortis and the remaining samples occurred also during the Pleistocene, in the Gelasian age (~2.19 Ma, HPD=1.38-3.53), while separation between samples from the Pacific and Gulf +Maya regions occurred at the beginning of the Calabrian age (~1.76 Ma, HPD=0.86–2.23). Finally, the split between lineages from the Gulf and Maya regions occurred near the end of the Calabrian age (~1.51 Ma, HPD=0.62-1.73). The divergence between the Central and Southern clades occurred at the end of the Pliocene (~2.64 Ma, HPD=1.50-3.68), while splits within each clade began at the end of the Calabrian age for the Central clade (~0.81 Ma, HPD=0.12-0.71) and during the Gelasian age (~1.64 Ma, HPD=0.63-1.89) for the Southern clade (Fig. 4).

The 95% credible intervals of the effective population size (BSP results) overlapped along the entire time period in the Northern clade (Fig. 5a). In the Southern clade (Fig. 5b), the effective population size did not overlap; this clade exhibited a constant population size and posterior expansion at approximately 0.1-0.3 Ma. The lack of 95% CI overlap of the more ancient and the more recent population with a Bayesian posterior probability >0.95 showed that the female effective sizes did not overlap, providing significant support for a population size change.

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The results regarding Bayesian spatiotemporal diffusion (Fig. 6) highlight the Chorotega and West Chortis region as the most likely ancestral geographic area for *H. fleischmanni*, suggesting two subsequent dispersal events, in which the most recent common ancestor (MRCA) of the Northern clade was distributed in the environs of the Chortis and Maya regions, whereas that of the Southern clade (stem node) was distributed around the Chorotega and Chocó regions. Our results also reflect independent dispersal for samples west of the MPJ fault system and later divergence of the three remaining clusters in the Northern clade around the IT. For the Central clade, an initial range in the Chorotega region south of the Talamanca range was observed, with posterior dispersal towards eastern Chortis. Furthermore, an ancestral range was detected in the Choco block within the Southern clade, with subsequent dispersal towards the south and east, reaching the lowlands east of the Andes range to the south, while two lineages dispersed northwards independently, reaching the southern limit of the Choco region.

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Discussion

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The complex geologic and geographic history of Central America has long intrigued researchers, who have aimed to decipher how different features that act as barriers to or corridors for dispersal have affected the distribution and diversity of multiple taxa (Gutierrez-García & Vázquez-Dominguez, 2013; Bagley & Johnson, 2014). Our results show a deep phylogenetic structure of *H. fleischmanni*, which has differentiated as three well-supported clades, revealing old divergence events dating back to the Pliocene and younger divergence events within clades during the Pleistocene (Fig. 3). Additionally, our results show *H. fleischmanni* to be paraphyletic with *H. tatayoi*, nested within the South American clade.

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The Southern clade

The Southern clade encompasses samples from the Choco and SAP regions, including the sister species *H. tatayoi* from Venezuela. Samples ranging from Panama to Venezuela and Ecuador grouped together, with no clear phylogenetic separation among them. Nonetheless, haplotype networks and landscape analyses allowed us to identify a partition on both sides of the Andes range for the coding genes (Figs. 2 and 3). The lack of significant structure for the Southern clade is remarkable, considering that the distances between populations reach 1,600 km. In addition, geographic barriers along the Western and Central Panama Isthmus and the Andes range, which are widely recognized as speciation drivers for both highland and lowland species (Bagley & Johnson 2014, Mendoza et al., 2015), seem to not have exerted any effect on the genetic structure of this clade. Notably, the genetic distance observed on both sides of the Andes range (COI K2P=1.9%; Table 1) is lower compared to the distances reported for lowland species with a higher dispersal capacity from the same region, such as the hummingbird *Amazilia amabilis* (K2P=2.06%, Mendoza et al., 2016). These results contrast with previous knowledge of the ecology of glassfrog species, which have been found to be characterized by site fidelity (Valencia-Aguilar, Castro-Herrera & Ramírez-Pinilla, 2012), low mobility and restricted gene flow even at local scales (Delia, Bravo-Valencia & McDiarmid, 2017; Robertson, Lips & Heist, 2008). However, most of the previous research on this frog has focused on calling males and reproductive behavior (Delia, Bravo-Valencia, & McDiarmid, 2017), while the dispersal capability of females and tadpoles, which can have a significant impact on mtDNA genetic structure, is still unknown. Thus, it is possible that the Choco and SAP regions present adequate conditions for tadpole dispersal, allowing range expansion. However, this hypothesis needs to be evaluated based on additional demographic studies and a greater sample size per site.

Comment [Office23]: H. tatayoi is not sister to H. fleischmanni.

Comment [Office24]: The study includes only two samples from Venezuela and one from Ecuador. The conclusions would be much stronger if the authors could include more samples from these two countries.

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Our results do not support our initial hypothesis that the species was originally from South America and then dispersed through the Isthmus of Panama. Indeed, the Southern clade is rather young, and the various populations it encompasses differentiated during the last million years (middle Pleistocene). Our results show that this clade has experienced a recent population expansion during the last 100,000-300,000 years, reaching a relatively final stable population size, exhibiting a dispersal route from Central Panama to South America (Figs. 5, 6). Based on the genetic and phylogenetic results for this clade, we suggest that its dispersal towards South America and on both sides of the Andes occurred very recently, likely as a consequence of climatic oscillations during glacial periods (Smith, Amei & Kickla 2012). Under this scenario, there has been insufficient time for effective genetic differentiation to occur, and the phylogenetic reconstruction therefore failed to resolve the divergence detected in the spatial analysis.

The Northern clade

Comment [Office28]: Given the results, the authors should mention the possibility of cryptic species in H. fleischmanni.

Unlike the Southern clade, the Northern clade shows significant genetic structure in different lineages, ranging from western Chortis (Central America) to lowland forests in Veracruz and Guerrero (Mexico). One remarkable finding was the split between samples from either side of the MPJ fault system, where individuals separated by only 60 km exhibit great genetic distances (K2P=1.6% for 16S, 5.2% for COI and 6.1% for ND1, Table 2), even reaching the limit of the barcode gap for Neotropical amphibians (6% for COI; Lyra et al., 2017). The calibration results showed that samples from these localities have been isolated since the Gelasian age (early Pleistocene; ~2.19 Ma, HPD=1.38-3.53). The MPJ fault system has been recognized as the main barrier to dispersal for multiple species ranging from the Maya to the Chortis blocks (Barrera-Guzman et al., 2012, Rovito et al., 2015, Ornelas, Ruiz-Sánchez & Sosa, 2010), which have effectively acted as a barrier for *H. fleischmanni*.

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Within this clade, we found that samples from SMB do not group as a single lineage but instead display a paraphyletic position in relation to the lineage from the Maya region. These two regions present the lowest K2P among all comparisons performed in this analysis, except for samples from either side of the Andes range. The BAPS and sPCA results for COI and ND1 also grouped samples from these two regions. One possible reason for this finding is that the IT has not acted as a significant barrier for *H. fleischmanni.* It is important to highlight that unlike the known impact of the Western and Central Panama Isthmus, the effect of the IT has mostly been defined based on montane species (Bryson, García-Vázquez & Riddle, 2011; Jiménez & Ornelas, 2016), for which it represents a barrier that limits dispersal. Hence, in the case of lowland species such as *H. fleischmanni*, the IT might act more as a corridor than a barrier, which is supported here by the Bayesian spatiotemporal diffusion results (Fig. 6).

On the other hand, the Geneland and phylogenetic results showed a more complex scenario, in agreement with the presence of three well-defined lineages within the Northern clade, one of which is located in the north of the SMB, another southward the SMB, and the last in the Maya block (Fig 2b). This genetic structure is very similar to that observed for the brush-finch *Arremon brunneinucha*, distributed in humid montane forests (Navarro-Sigüenza et al., 2008). Samples from the Gulf of Mexico and Pacific are clearly isolated by the highlands of the Sierra Madre Oriental and the Sierra Madre Occidental (Fig. 1), indicating a divergence pattern that is frequently detected for lowland species (Mulcahy, Morrill, & Mendelson, 2006; Rivera-Ortíz et al., 2016) and species groups (Streicher et al., 2014; Palacios et al., 2016).

The Central clade

Our results revealed a Central clade without any deep geographic structure, expanding along both the Chortis and Chorotega blocks, which separated from the Southern clade around the end of the Pliocene (~2.64 Ma). We did not find any evidence suggesting differentiation between samples from the Chortis and Chorotega regions, suggesting that the Hess Escarpment has not acted as a significant barrier. However, we must consider the small sample sizes from Honduras and Nicaragua (n=10 samples), which likely limits detailed structural evaluation for this region. Most phylogeographic studies performed in the region known as nuclear Central America face similar problems, with limited or null sampling from northern Honduras (Mulcahy, Morrill, & Mendelson 2006; Castoe et al., 2003; Strecker et al., 2004; Crawford & Smith, 2005) or sampling that is biased towards the dry forests of the Pacific coast (Parkinson, Zamudio, & Greene 2000; Hasbún et al., 2005; Vázquez-Miranda, Navarro-Sigüenza & Omland, 2009; Poelchau & Hamrick, 2011), where *H. fleischmanni* has not been recorded. Our Bayesian spatiotemporal diffusion results showed rapid dispersal from the Chorotega to Chortis blocks, with no apparent impact on the genetic structure of these populations (Fig. 6). Nevertheless, additional work is needed to confirm whether the main geographic features present in this region have driven the dispersal of low-mobility species such as *H. fleischmanni* in humid forests.

Phylogeographic patterns

The three main clades that we identified for *H. fleischmanni* show deep intraspecific divergence, with genetic distance values greater than 2% (16S) and 5% (COI and ND1). Indeed, the landscape analysis, Bayesian spatiotemporal diffusion analyses, and estimated divergence time revealed interesting patterns that allowed us to reconstruct the historical biogeography of these frogs and to identify the impact of different geographic barriers on the genetic structure and phylogeographic patterns of *H. fleischmanni*. Although the main phylogenetic topology and the

three major clades were well supported in Bayesian analyses, the maximum likelihood phylogenetic reconstruction did not resolve these relationships (see Supplementary Material).

The Bayesian spatiotemporal diffusion analyses suggest that *H. fleischmanni* originated along the region encompassing the Chorotega and eastern Chortis elements, which contrasts with the South American origin proposed by Castroviejo-Fisher et al. (2014). Interestingly, we found that *H. fleischmanni* has undergone two dispersal events: one southward to the Chocó region and one northward, reaching the Maya region, followed by vicariance events driven by the effect of the Chortis highlands and the Talamanca range. Considering that divergence times among the three clades are similar (i.e., the isolation of the Northern clade occurred ca. 3.40 Ma (HPD=2.25-4.56 Ma), while that between the two other clades occurred 2.64 Ma (HPD=1.50-3.68)), it is likely that the ancestor of *H. fleischmanni* arrived in Central America during the Pliocene, soon after the closure of the Isthmus of Panama (Montes et al., 2015). Accordingly, the dispersal-vicariance events among the main clades potentially occurred simultaneously or over a very short time, which might explain why the position of the clades and their internal structure were not consistent between the Bayesian and maximum likelihood approaches.

Regarding the vicariance events for the Central and Southern clades, multiple elements need to be revised. The samples from each cluster that are geographically closest are located on opposite sides of the Talamanca Cordillera in the Chorotega block. The time of divergence of the MRCA for these clades (3.28 Ma, HPD=1.59-3.86) coincides with the estimated age of the intervening mountains (1-2 Ma; Denyer, Alvarado & Aguilar 2000; Marshall et al., 2003), which are recognized as a main driver of speciation (Savage, 2002). The time of divergence also coincides with the rise of the sea level during the mid-late Pliocene (~3.5–3 Ma), which generated a seaway, likely reinforcing the western Isthmus of Panama break and therefore acting as a barrier

Comment [Office29]: Note that all species that are closely related to H. fleischmanni are endemic to South America. Thus, a South American origin of fleischmanni makes a lot of biogeographic sense. The authors should include this in their discussion.

Comment [Office30]: Arrived from where? South America, right?

462 across the Pacific region (Cronin & Dowsett, 1996; Bagley, Hickerson & Johnson 2018). Hence,
463 the central mountain ranges on Costa Rica and Panama and the eustatic sea levels around the
464 western Isthmus of Panama might have increased divergence, as documented for multiple spatial
465 divergence patterns of amphibian species (Crawford Bermingham & Polanía-S; Wang, Crawford
466 & Bermingham., 2008; Bagley, Hickerson & Johnson, 2018).

467 The isolation of the Northern clade from the other two does not entirely correspond to our
468 hypothesis of geographical barriers. Populations from both the Northern and Central clades are
469 distributed throughout the Chortis block, indicating that the MPJ fault system was not the main
470 driver of divergence between clades. On the other hand, our structure (Geneland) results suggest
471 a frontier at the center of the Chortis block, near northeastern Honduras (Fig. 2b). Similar
472 divergence patterns have been observed between two water-dependent subspecies of *Caiman*
473 *crocodilus* (Venegas-Anaya et al., 2008), in agreement with the eastern limit of the Chortis
474 highlands (Morrone, 2014; Townsend, 2014). Here, the complex topography resulting from
475 multiple volcanic activities along the Chortis highlands during the last 2 Ma and the presence of
476 dry habitats in the Pacific region (Savage, 2002) may have isolated the *H. fleischmanni*
477 populations during the late Pliocene. This hypothesis is in agreement with the high species
478 endemism recognized for the region (Anderson et al., 2010; Townsend et al., 2012), in which
479 intensive study is required to evaluate the underestimated regional taxonomic diversity
480 (Townsend & Wilson 2016).

481 *Taxonomic implications*

482 Previous studies have suggested that *H. fleischmanni* is a paraphyletic species in relation to its
483 sister species *H. tatayoi* (Castroviejo-Fisher et al., 2011; Delia, Alvarado & Aguilar, 2017). Here,

Comment [Office31]: The other possibility that should be discussed is that H. tatayoi is a synonym of H. fleischmanni. Are there good morphological and/or acoustic traits that differentiate these species?

484 we confirm the paraphyly of the species, as the *H. tatayoi* samples are grouped within the
485 Southern clade, lacking significant genetic differences from the western Andes samples.
486 Furthermore, our overall Bayesian topology coincides with the results obtained by Delia,
487 Alvarado & Aguilar (2017) for 12S sequences; consequently, we identified three main isolated
488 lineages with large genetic distances that could be considered as candidate species. However,
489 because our analyses are based only on mtDNA differences, we recognize that other sources of
490 evidence (morphologic, acoustic, and/or ecological) are needed to confirm the proposed potential
491 species (Padial et al., 2014).

492 **Conclusions**

493 We have conducted the most comprehensive analysis of genetic variation and divergence within
494 *H. fleischmanni* to date, producing one of the few phylogenetic and phylogeographic studies for
495 glassfrogs, with the exception of a few studies from Guyana (Castroviejo-Fisher et al., 2011;
496 Jowers et al., 2015), and this is the first such study of a Central American species. Moreover, our
497 results aided in the successful reconstruction of the historical biogeography of these frogs and
498 dispersal and vicariance events during the history *H. fleischmanni* lineages, revealing a higher
499 complexity for the species than expected, especially for the Northern lineage, in which significant
500 population structure was found. Indeed, our results support the Talamanca range, the MPJ fault
501 system, and the Chortis highlands as significant factors exerting effects on the dispersal of
502 lowland amphibians during the late Pliocene and early Pleistocene. Additionally, we suggest that
503 the IT acted as a corridor, rather than a barrier for *H. fleischmanni* during the early Pleistocene,
504 while the Hess Escarpment and the Andes range did not play a significant role as barriers. The
505 complementary use of phylogenetic and landscape analyses allowed us to perform an adequate

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evaluation of dispersal patterns and potential barriers within this region; hence, our approach can be applied in biogeographic and phylogeographic studies of different taxa.

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Figure Captions

Fig. 1. Geographic distribution of *H. fleischmanni* samples. Main geological blocks, delimited by geological barriers, are shown. Sample origins are indicated by black dots, while gray polygons show the species distribution according to IUCN. SMB=South Mexican block, IT=Isthmus of Tehuantepec, SAP=South American Plate, MPJ=Motagua-Polochic-Jocotán.

Fig. 2. Results of the Bayesian (BAPS, Geneland) and multivariate spatial analyses (sPCA) for *H. fleischmanni* population clustering based on 16S (first column), COI (second column) and ND1 (third column) sequences. For the sPCA analysis, the color of each point is determined in the red-green-blue (RGB) system based on each individual's score on the first (translated to a red channel) and second axes (translated to green) of the sPCA.

Fig 3. Haplotype networks of mitochondrial DNA haplotypes for 16S (A) COI (B) and ND1 (C) from *H. fleischmanni.* Hatch marks represent inferred mutational steps. The size of the circle is proportional to the number of individuals found for each haplotype.

Fig 4. Time-calibrated tree of *H. fleischmanni* unique haplotypes, inferred from BEAST based on the combined ribosomal (16S) and protein-coding (COI, ND1) mitochondrial sequences, with calibration on three nodes indicated by green bars (see Materials and Methods section for details). Blue rectangles over key nodes indicate the 95% highest posterior densities (HPD) of the estimated times of divergence events (in Ma). Clade support is indicated by *posterior* BI values in BEAST and Mr Bayes and by RAxML Bootstrap analysis and is presented in this order separated by a slash. Asterisks at tips represent *H. tatayoi* samples included in the analysis. The inner map shows the geographic locations of haplotype lineages. Each color in the map coincides with the haplogroup obtained in the phylogenetic reconstruction.

Fig. 5. Bayesian skyline plots for Northern (A) and Southern (B) clades generated through phylogenetic reconstruction.

Fig. 6. Spatial projection of the Bayesian spatiotemporal diffusion analysis of *H. fleischmanni* lineages for three time points, based on the maximum clade credibility (MCC) tree estimated with a "Relaxed Random Walk" model. Lines represent branches of the MCC tree; shaded areas indicate the 95%-HPD uncertainty for the ancestral branches; the shading gradient indicates older (lighter) versus younger (darker) events; and dot color represents the ages of older nodes (darker) and younger tips (lighter).