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ORIGINAL PAPER

Genetic diversity and demography of the critically endangered Roberts' false brook salamander (*Pseudoeurycea robertsi*) in Central Mexico

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Abstract

Land use changes are threatening the maintenance of biodiversity. Genetic diversity is one of the main indicators of biological diversity and is highly important as it shapes the capability of populations to respond to environmental changes. We studied eleven populations of *Pseudoeurycea robertsi*, a micro-endemic and critically endangered species from the Nevado de Toluca Volcano, a mountain that is part of the Trans-Mexican Volcanic Belt, Mexico. We sequenced the mitochondrial *cytochrome b* gene from 71 individuals and genotyped 9 microsatellites from 150 individuals. Our results based on the *cytochrome b* showed two divergent lineages, with moderate levels of genetic diversity and a recently historical demographic expansion. Microsatellite-based results indicated low levels of heterozygosity for all populations and few alleles per locus, as compared with other mole salamander species. We identified two genetically differentiated subpopulations with a significant level of genetic structure. These results provide fundamental data for the development of management plans and conservation efforts for this critically endangered species.

Keywords Conservation · Endemic species · Plethodontidae · Population genetics · Nevado de Toluca Volcano · Trans-Mexican Volcanic Belt

Introduction

Genetic diversity is the raw material of evolution; natural selection and adaptation can only occur as long as there is genetic variation in populations. Thus, the maintenance of this diversity becomes highly important as it shapes the capability of populations to respond to environmental changes (Templeton et al. 1990; Young et al. 1996; Frankham et al. 2003; Reed and Frankham 2003; Herrera-Arroyo et al. 2013). Genetic diversity has been recognized by the International Union for the Conservation of Nature (IUCN) as one of the three main levels of biological diversity together with species and ecosystem diversity (Frankham 1998). Land use change is threatening these levels of diversity (Foley et al. 2005; Newbold et al. 2016); especially, habitat loss is considered to be the greatest threat related to land use change for many species (Fahrig 2003). Moreover, fragmentation can also affect population dynamics and species persistence (Barbosa and Marquet 2002; Hanski and Gaggiotti 2004), with impacts that are more insidious than habitat loss alone

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(With 1997). The combination of these factors can lead to reduced gene flow and decreased genetic diversity due to the loss of alleles by the action of genetic drift (Young et al. 1996; Frankham et al. 2005; Lowe et al. 2005; Honnay and Jacquemyn 2007; Rueda-Zozaya et al. 2016; Sunny et al. 2015, 2018). High levels of interpopulation differentiation lead to an increase of genetic structure (Lowe et al. 2005; Honnay and Jacquemyn 2007), as well as inbreeding within patches. In the long term, reductions of the effective population size in each habitat relict, as well fluctuations in other demographic and environmental parameters, can lead wild populations to extinction (Gibbs 1998a, b; Johansson et al. 2006).

Amphibians are the group with the highest proportion of species threatened with extinction (Stuart et al. 2004; Beebee and Griffiths 2005) mainly due to habitat destruction, disturbance, and fragmentation (Mendelson et al. 2006; Wake and Vredenburg 2008; Sodhi et al. 2008; Ochoa-Ochoa et al. 2009; Ducatez and Shine 2017) but also due to other factors like wetland draining, introduction of exotic species, over-exploitation, climate change, UV-B radiation, chemical contaminants and emerging infectious diseases (Carey and Alexander 2003; Daszak et al. 2003; Hirner and Cox 2007; Pearson and Goater 2009; Suislepp et al. 2011). All these factors could work synergistically increasing amphibian populations' declines. Salamanders like other low-vagility ectotherms, are highly vulnerable to habitat disturbance (Nowakowski et al. 2018). They are important as top-down controls of many invertebrate species and can also be a source of high energy prey for other predators (Davic and Welsh 2004). In addition, salamanders can represent an important proportion of the vertebrate biomass in old growth forests (Davic and Welsh 2004), and are thus vital to ecosystem function.

Mexico is ranked fifth in worldwide amphibian diversity (Ochoa-Ochoa et al. 2009; Ordoñez-Ifarraguerri et al. 2017), however, it is the second worldwide country in number of threatened amphibian species (Frías-Alvarez et al. 2010), with 43% of its 376-species threatened (Parra-Olea et al. 2014). Currently, Mexico faces many land use change activities such as agriculture, aquaculture, cattle breeding, logging, wood harvesting, and urban development (Frías-Alvarez et al. 2010). The Trans-Mexican Volcanic Belt (TMVB) is a set of mountains and volcanoes of varying geological ages which cross the Mexican territory from the Pacific coast to the east on the Gulf of Mexico (Mastretta-Yanes et al. 2015). This biogeographic zone possesses a high level of species richness and endemism (Flores-Villela and Canseco-Márquez 2007), and is considered the second biogeographic zone in Mexico with the highest herpetological species-richness and endemicity (Flores-Villela and Canseco-Márquez 2007). However, it is also one of the most disturbed areas of Mexico, with 3.4% of the TMVB highly fragmented by urban settlements (including Mexico City), and 44.7% by agricultural lands (Sunny et al. 2017). The Nevado de Toluca Volcano (NTV) is part of the TMVB and supports populations of a micro-endemic and critically endangered Roberts' False Brook salamander (SEMARNAT 2010; IUCN SSC Amphibian Specialist Group 2016). This species is only present in a small area of 8 km^2 (Lynch et al. 1983; IUCN SSC Amphibian Specialist Group 2016) within a narrow elevation distribution between 2900–3600 meters above sea level (asl) in the NTV near Toluca in the State of Mexico (Lynch et al. 1983; Bille 2009; Van Rooij et al. 2011). Such geographic and ecological restricted distribution makes *P. robertsi* one of the most threatened amphibians in Mexico, with vulnerability score of 18 (Wilson et al. 2013). In 2000, Bille (2009) conducted a survey of *P. robertsi* abundance, revealing it was relatively common in very small areas of certain localities (Raices, Meson Viejo and Lagrimas, Fig. 1). Nevertheless, in 2003 the author was unable to find any individual in Raices locality despite it was a well-preserved *Abies-Pinus* forest (Bille 2009). In this context, knowledge of the genetic diversity of *P. robertsi* is highly important for its conservation. Therefore, the aim of this study was to assess population genetic components including diversity and structure, as well as to infer population parameters such as effective population sizes and potential population bottlenecks in eleven populations of the critically endangered *P. robertsi*. Under this scenario, we expected a general pattern of high genetic structure among populations, low levels of genetic diversity and high levels of inbreeding within populations as well as low populations sizes characterized by a detectable genetic bottleneck. This study provides baseline parameters of genetic diversity and genetic structure for this micro-endemic and critically endangered species, which will allow wildlife managers to develop conservation strategies to conserve threatened *P. robertsi* populations.

Materials and methods

Study area and population sampling

We sampled a total of 150 individuals from 11 populations for the microsatellite data (between 4 and 16 individuals per site via visual encounter surveys Crump and Scott 1994) and for the *cyt* b data set 71 tissues were collected from the eleven populations (between 3 and 6 individuals per site) covering the entire polygon of the NTV from mid-June to mid-August 2016. In particular, during each visit 2 people looked for salamanders under the bark of fallen logs with a diameter of ≥ 5 cm and length of ≥ 30 cm. We started at 9:00 am and spent 2 effective hours of search (i.e., excluding stops), covering an area of about 10 ha in each site. (Fig. 1). We sampled 2 mm of tail tips of adult salamanders for DNA extraction (see below). This low impact methodology does

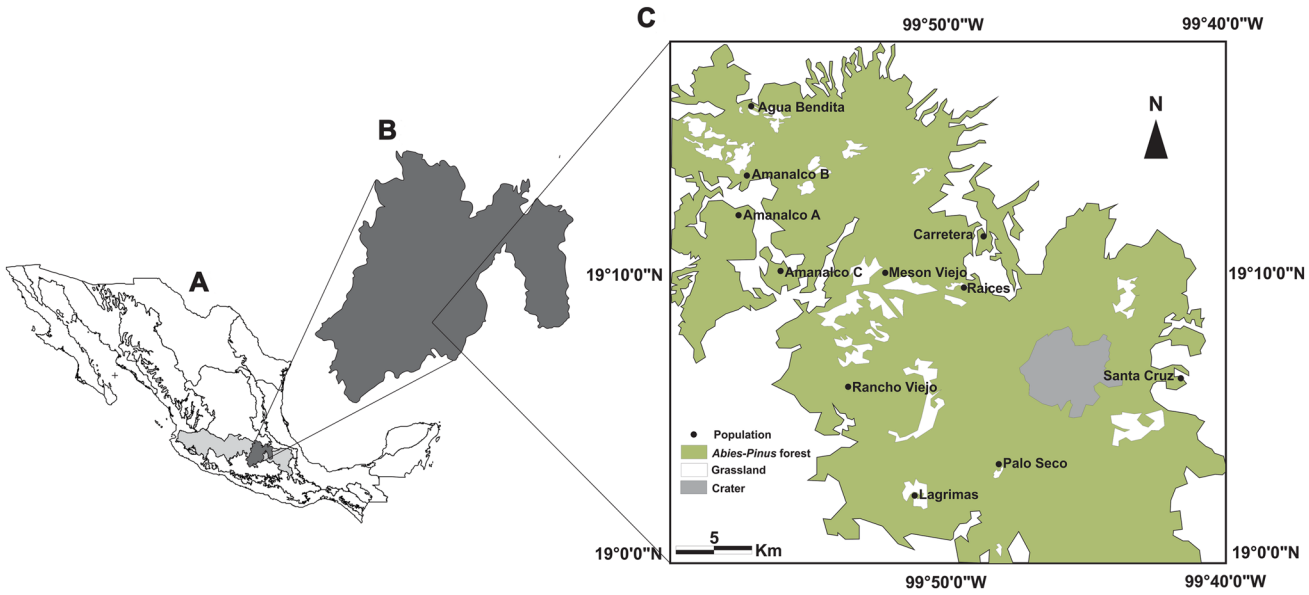


Fig. 1 **a** Map of Mexico showing the Trans-Mexican Volcanic Belt (in grey) and the State of Mexico City (in black). **b** State of Mexico City. **c** Nevado de Toluca Volcano region indicating *P. robertsi* populations sampled

not affect the survival or growth of salamanders (Arntzen et al. 1999; Polich et al. 2013). Tissues were preserved in 90% ethanol and then frozen at − 20 °C until processed. All individuals were released back at their corresponding capture sites.

DNA extraction, amplification and sequencing

We extracted DNA from tail tips with the GF-1 nucleic acid extraction kit (Vivantis) following the manufacturer's instructions, and used it as template for amplification of the mitochondrial *cytochrome b* gene (*cyt b*) with the following primers: MVZ15 (5´G A A C T A A T G G C C C A C A C Q Q T A C G N A A -3´) and MVZ16 (5´A A A T A G G A A R T A T C A Y T C T G G T T T R A T -3) from Moritz et al. (1992). PCR amplifications were performed in a total volume of 15 µL. We used 0.15 µL of 5U Taq polymerase (QIAGEN), 0.75 µL of 10 × PCR buffer with 2.5 mM MgCl2, 0.3 mM of deoxynucleotide triphosphates (dNTP´s) and 0.3 mM of forward and reverse primers. The PCR conditions were as follows: initial 4 min denaturation at 94 °C, followed by 35 cycles, each cycle consisting of 94 °C denaturing for 1 min, 50 °C annealing temperature for 1 min, and extension at 72 °C for 2 min, with a final 72 °C for 3 min. Purification and pair-end sequencing of DNA was performed by the UW High Throughput Genomics Center of the CINVESTAV Irapuato, Mexico. Forward and reverse sequences for each individual were aligned and edited manually using BIOEDIT 7.1.3 (Hall 1999). We also tested nine fluorescently labelled microsatellite primers (Velo-Antón et al. 2009). PCR microsatellite products were multiplexed and run on an ABI Prism3730xl (Applied Biosystems), with Rox-500 as an internal size standard. We obtained allele sizes with PEAKSCANNER 1.0 software (Applied Biosystems) and the fragment lengths with TANDEM 1.08 (Matschiner and Salzburger 2009). In all runs, we included negative controls in at least two runs to guarantee reproducibility.

Potential scoring errors and genotype accumulation curve

The retrieved sequences of *cyt b* were blasted in GenBank to confirm they matched *Pseudoeurycea robertsi*. Sequence alignment was carried out with the CLUSTAL X2 algorithm (Thompson et al. 1997; Larkin et al. 2007) performed in the software GENEIOUS 5.5.7 (Kearse et al. 2012). For microsatellite analysis, we used the software MICROCHECKER 2.2.3 (Van Oosterhout et al. 2004) to test for the presence of null alleles and other typing errors. In addition, we determined the minimum number of loci necessary to discriminate between individuals in a population by creating a genotype accumulation curve (Kamvar et al. 2014) using POPPR 2.4.1 (Kamvar et al. 2014) implemented in the R software (version 3.4.0; R Development Core Team 2017). This function randomly samples loci without replacement and counts the number of observed multi-locus genotypes (Kamvar et al. 2014).

Genetic diversity

For the mtDNA data set, we calculated six genetic diversity estimates with DNASP 5 (Librado and Rozas 2009) for each sampled locality: the number of segregating sites (S), haplotype number (Nh), the nucleotide diversity (π), haplotype diversity (H_d), average number of differences among sequences (k), and Watterson's theta (θ_W). For the microsatellite data set we calculated the observed (H_o) and expected (H_e) heterozygosity, the number of alleles (N_a), effective number of alleles (N_e), number of genotypes, and the number of heterozygote and homozygote genotypes in STRATA G 2.0.2 (Archer et al. 2017) and GENALEX (Peakall and Smouse 2006). We calculated departures from Hardy–Weinberg Equilibrium (HWE) and Linkage Disequilibrium (LD) between pairs of microsatellite loci in the packages PEGAS (Paradis 2010) and LATTICE (Sarkar et al. 2017) respectively, implemented in R. These calculations were evaluated for each sample and locus with a Markov chain approximation considering 10,000 dememorizations, 1000 batches, and 10,000 iterations per batch. In order to correct the P values, we used a False Discovery Rate (FDR) approach according to Benjamini and Hochberg (1995) implemented in the package FDRTOOL 1.2.15 (Strimmer 2008) for R.

Genetic structure and phylogenetic inference

For the mtDNA data set we analyzed the genetic structure using several algorithms. First, the Bayesian approach for assignment of individuals, implemented in the software BAPS v6.0 (Corander et al. 2008). The algorithm provides the posterior probabilities for different numbers of clusters of individuals (K). We performed individual level mixture analysis for multiple defined clusters (K = 2 to 20 clusters), with 10 independent runs for each K values. The K with the highest posterior probability was selected as the most likely data partition.

In order to infer the phylogenetic relationships among haplotypes, first we select the best-fitting models of sequence evolution for our dataset based on the Akaike Information Criterion (AIC; for justification, see: Posada and Crandall 2001; Posada and Buckley 2004) scores as implemented in the software JMODELTEST 0.1.1 (Posada 2008). Subsequently we performed Bayesian phylogenetic inference (BI) analyses with MRBAYES 3.1.2 (Ronquist and Huelsenbeck 2003), and analyses consisted of four runs, each conducted with three heated and one cold Markov chains run for 15,000,000 generations, sampling every 100 generations and initiating with random, unconstrained, starting trees. Heating temperature was set at 0.02 to facilitate greater movement between the four Markov chains (Braun and McAuliffe 2010). Output parameters were visualized using TRACER 1.5 (Rambaut and Drummond 2009). We estimated the 50% majority-rule consensus topology and posterior probabilities for each node with the remaining trees. Third, we implemented ML analyses using PHYML 3.0 (Guindon et al. 2010) using five random starting trees optimized through subtree pruning and regrafting and nearest-neighbor interchange, with four substitution categories. Clade support was assessed by bootstrapping with 500 replicates. Fourth, we constructed unrooted haplotype networks using two methods: The Median-Joining method implemented in POPART 1.7 (Leigh et al. 2016). Also, we performed an analysis of molecular variance (AMOVA) based on F_{ST} as implemented by ARLEQUIN 3.5.2.2 (Excoffier and Lischer 2010). Furthermore, we estimated diversification times for the main mitochondrial lineages obtained, based on a relaxed phylogenetic molecular clock used implemented in BEAST 1.7.4 (Drummond and Rambaut 2007). The time to the most recent common ancestor for the main lineages was obtained using Bayesian Markov chain Monte Carlo (MCMC) searches. We used a GTR + I + G model of evolution across all gene and codon positions and implemented. The time of divergence was estimated considering a Yule Process tree prior, the uncorrelated lognormal relaxed molecular clock method and 50,000,000 generations sampled every 1000th generation, with 10% of the initial samples discarded as burn-in. Convergence and stationarity for both Bayesian analyses were visualized with TRACER 1.5 (Rambaut and Drummond 2009).

For the microsatellite data set, we used a Bayesian algorithm implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000; Falush et al. 2003; Hubisz et al. 2009). The optimal value of K clusters was determined by set the K value from 1 to 10 with 10 iterations per K value, in order to determine the maximum value of posterior likelihood [lnP(D)]. The chosen parameters were correlated allele frequencies with 1,000,000 burn-in periods and 1,000,000 MCMC iterations (Falush et al. 2003). For the degree of admixture, a Dirichlet parameter was applied with correlated allele frequencies assuming an ancestral population. The most credible number of populations were estimated using the maximum value of ΔK (Evanno et al. 2005), as implemented in STRUCTURE HARVESTER 0.6.92 (Earl and vonHoldt 2012). The second method was the AMOVA based on F_{ST} and R_{ST} as implemented by GENALEX 6. A Wilcoxon test with 30,000 permutations was applied to estimate significance using the degree of similarity of populations based on the populations' genotypes in GENALEX 6. In addition, we calculated different estimators of genetic population differentiation: F_{ST}, F_{IS} (Weir and Cockerham 1984), G_{ST} (Nei and Chesser 1983; Hedrick 2011; Jost 2008) and *D* (Jost 2008) in the STRATA G package for the R software. We also estimated Nei's genetic distance (Nei 1972) between sampling localities in GENALEX 6. Finally, we tested the existence of population structure computing Minimum Spanning Networks (MSN)

with the Bruvo's (Bruvo et al. 2004) and Nei´s distance algorithm (Nei 1972) with 1000 bootstraps in POPPR 2.4.1 and MAGRITTR 1.5 (Bache and Wickham 2016) for the R package. This analysis visualizes the relationships among individuals and it can be a more adequate visualization tool than trees (Bache and Wickham 2016).

Isolation by distance and demographic history

For both kind of molecular markers, *cyt b* and microsatellites, we tested the correlation between F_{ST} pairwise genetic and geographical distances with a Mantel test (Mantel 1967) in GENALEX with 10,000 randomizations. To test for population size reduction, we calculated the estimates of neutral tests: Tajima's D (1989), Fu and Li's F (1993) and Fu's F_S (1997). Historical population growth predicts significantly negative D and F_S values. we tested with 10,000 bootstrap replicates, all statistical significance was determined using the coalescent simulator in DNASP. Finally, we examined the historical demography of *P. robertsi* constructing Bayesian skyline plots for each main mitochondrial lineage with BEAST, to infer population fluctuations over time by estimating the posterior distribution of the effective population size at specified intervals along a phylogeny (Drummond and Rambaut 2007). Genealogies and model parameters were sampled every 1000th iteration along 10^7 generations under a relaxed lognormal molecular clock, with uniformly distributed priors and a burn-in of 100 iterations, using coalescent intervals (*m*) of 10. Demographic plots for each analysis were visualized using TRACER.

We estimated a population size reduction with the Garza–Williamson index (M, the ratio of number of alleles to range in allele size) and the critical value (M_c) with the packages STRATA G and Critical M. M values lower than the critical number are indicative of historical population declines. The latter was done based on 10000 simulations and parameters from the two-phase mutation model, as described in Garza and Williamson (2001). We also used the software BOTTLENECK 5.1.26 (Cournet and Luikart 1996; Piry et al. 1999) to test for a genetic signature of recent historical reduction in the effective population size, based on the two-phase model, which is an intermediate model of evolution considered more appropriate for microsatellites (Cournet and Luikart 1996). Accordingly, we estimated the observed and expected heterozygosity under the two-phase model, with settings of 90% stepwise mutation model, 10% infinite allele model, and 10% variance with default values (70% stepwise mutation model, 30% infinite allele model, and 10% variance). Both settings were run with 10,000 replicates. Excess heterozygosity was tested using a Wilcoxon test.

We calculated the effective population size (N_e) with three methods that estimate Ne from (1) LD, (2) heterozygote excess and (3) the molecular co-ancestry method of Nomura (2008), as implemented in the software NEESTIMATOR 2 (Do et al. 2014). Also, as an inbreeding measure, we used the relatedness estimator (rqg) of Queller and Goodnight (1989), calculated by the software GENALEX. To test for significant differences among mean population relatedness, we calculated the upper and lower 95% confidence intervals for the expected range of rqg using 9999 permutations. These intervals correspond to the range of rqg that would be expected if reproduction was random across populations. Additionally, we calculated confidence intervals for estimates of mean relatedness within a population to 95% by bootstrap resampling (9999 permutations). Population rqg values that fall above the 95% expected values indicate that processes such as inbreeding or genetic drift are increasing relatedness. Finally, relatedness among individuals was evaluated using the software ML-RELATE (Kalinowski et al. 2006).

Results

Population sampling

We found individuals in 11 sampling sites of the 14 visited (Table 1), covering the entire polygon of the Nevado Toluca Volcano. Six of these locations represented new records for the species (Rancho Viejo, Amanalco A, B and C, Santa Cruz and Agua Bendita, Palo Seco).

Potential scoring errors and genotype accumulation curve

Significant presence of null alleles was found in two populations Palo Seco (PLT005 and PLT109) and Lagrimas (PLT109). For those two loci, we derived the corrected frequencies from FREENA (Chapuis and Estoup 2006; Chiesa et al. 2016) and used those to recalculate the number of homozygotes and heterozygotes genotypes in each population sample, we compared the F_{ST} values derived from the original dataset. We did not find significant differences consequently, the presumptive null allele at these loci has only a marginal effect on estimates of genetic diversity; therefore, we used the original dataset for all subsequent analyses.

The genotype accumulation curve revealed that the minimum number of loci necessary to discriminate between individuals in our populations was eight (Supplementary Materials, Fig. A1). Therefore, we concluded that our survey of 9 microsatellite loci allowed reliable estimates of genetic diversity.

Table 1 Genetic diversity estimates obtained from microsatellites and mitochondrial *cyt b* DNA sequences (mtDNA)

Population	Microsatellites							mtDNA						
	N	Na	Ne	A	Np	Ho	He	N	π	S	Hd	Nh	k	θ-W
Amanalco A	16	3.000	2.445	0.188	0	0.764	0.564	6	0.026	3	0.800	3	1.600	1.314
Rancho Viejo	16	3.111	2.392	0.194	0	0.708	0.554	6	0.001	1	0.330	2	0.333	0.438
Meson Viejo	16	2.889	2.216	0.181	0	0.729	0.532	7	0	1	0.286	2	0.285	0.408
Amanalco C	16	2.667	2.161	0.167	0	0.729	0.521	9	0.001	4	0.222	2	0.889	1.472
Carretera	16	2.778	2.100	0.174	0	0.569	0.500	8	0.010	13	0.786	4	5.893	5.014
Palo Seco	16	3.222	2.221	0.201	0	0.382	0.488	9	0.002	4	0.861	5	1.333	1.472
Lagrimas	16	3.111	2.260	0.194	0	0.556	0.531	6	0.001	1	0.600	2	0.600	0.438
Amanalco B	16	2.556	2.140	0.160	0	0.722	0.519	6	0	0	0.000	1	0	0
Santa Cruz	11	2.889	2.130	0.263	0	0.556	0.480	7	0.002	4	0.857	5	1.524	1.633
Agua Bendita	7	2.667	2.169	0.381	0	0.556	0.486	3	0	0	0	1	0	0
Raices	4	2.333	1.827	0.583	0	0.306	0.385	4	0.005	5	0.667	2	3.333	2.727
Total population	150	3.667	2.445	0.024	0	0.625	0.561	71	0.010	24	0.40	21	6.081	4.966

Estimates are reported for studied populations of *Pseudoeurycea robertsi* as well as for the total sample (Total population). For microsatellite estimates: *N* sample size, *Na* number of alleles, *Ne* number of effective alleles, *A* allelic richness, N_p number of private alleles, H_o observed heterozygosity, H_e expected heterozygosity. For mtDNA *cyt b: S* number of segregating sites, *Nh* haplotype number, π nucleotide diversity, *Hd* haplotype diversity, *k* average number of nucleotide differences, θ-*W* Watterson's theta

Genetic diversity

We amplified a 617-bp fragment of *cyt* b from 71 individuals, all sequences were deposited in the GenBank database with the access numbers: MK357639-MK357709. The sequences included 24 polymorphic sites, 21 parsimony informative sites, 3 singletons and 21 unique haplotypes, yielding high haplotype diversity ($h = 0.940$) and low nucleotide diversity ($\boldsymbol{\pi} = 0.010$) with an average number of nucleotide differences between haplotypes (k) of 6.081. The population that presented a higher nucleotide diversity was Amanalco A: $\pi = 0.026$, higher Watterson's theta and number of segregating sites values was Carretera: $\theta_W = 5.014$ and $S = 13$ and higher haplotype diversity were: Palo Seco, $H_d = 0.861$, Santa Cruz, $H_d = 0.857$ and Amanalco, $A = 0.800$ (Table 1). With microsatellites, FDR correction tests found departures from HWE in Palo Seco and Raices due to significant deficiency of heterozygosity (Supplementary Materials, Table A1). We found LD between the loci PLT045-PLT039 in the total sample (Supplementary Materials, Table A2). The overall observed heterozygocity, was low: $H_o = 0.625$ and the populations that have the highest Ho are those of Amanalco (Amanalco A: $H_o = 0.764$, Amanalco B: $H_o = 0.722$ and Amanalco C: $H_o = 0.729$), the number of alleles was very similar in all populations, with a range of 2.333 for Raices to 3.222 for Palo Seco and for the total population was 3.667, we did not find private alleles, and the allelic richness were similar in all populations, the allelic richness value for the total population was 0.024 (Table 1). We found in all populations 178 heterozygote genotypes and 161 homozygote genotypes (Supplementary Materials, Table A3), all the populations presented more heterozygous genotypes than homozygotes, except the populations Carretera: $H_{et} = 17$, $Hom = 18$, Palo Seco: $H_{et} = 19$, $H_{om} = 21$ and Raices: $H_{et} = 10$, $H_{om} = 13$. Finally, Agua Bendita had the same number of genotypes heterozygotes and homozygotes ($Het = 13$, $Hom = 13$).

Genetic structure and phylogenetic inference

The genetic population structure analysis using the *cyt b* gene indicated that the optimal number of Clusters was four ($K = 4$, $logML = -335.149$; Fig. 3). The BI and Haplotype Network converged on the same tree topology (Figs. 2, 3), the BI and the Haplotype Network were divided the populations into two clades and two subclades (Figs. 2, 3).

The BI was supported by high posterior probabilities (Fig. 2a) and high boostrap values (Fig. 2b). Clade 1 populations include individuals from Amanalco A, Amanalco B, Amanalco C, Carretera, Meson Viejo, Rancho Viejo, Agua Bendita and Raices, whereas Clade 2 includes individuals from primarily the Lagrimas, Palo Seco and Santa Cruz populations and some individuals of Carretera (Figs. 2, 3). The AMOVA results showed significant levels of genetic variation within (53.83%) and among populations (46.18%), with an F_{ST} fixation index of 0.461 and a p-value <<< 0.0001 (Supplementary Materials, Table A4). Pairwise F_{ST} values ranged from 0.002 to 1, G_{ST} values ranged from 0.034 to 1, D_A values ranged from 0 to 0.018 and D_{XY} values ranged from 0.001 to 0.018 across all population pairs (Supplementary Materials, Table A6), indicating that there are differentiations from low to high between populations. Divergence

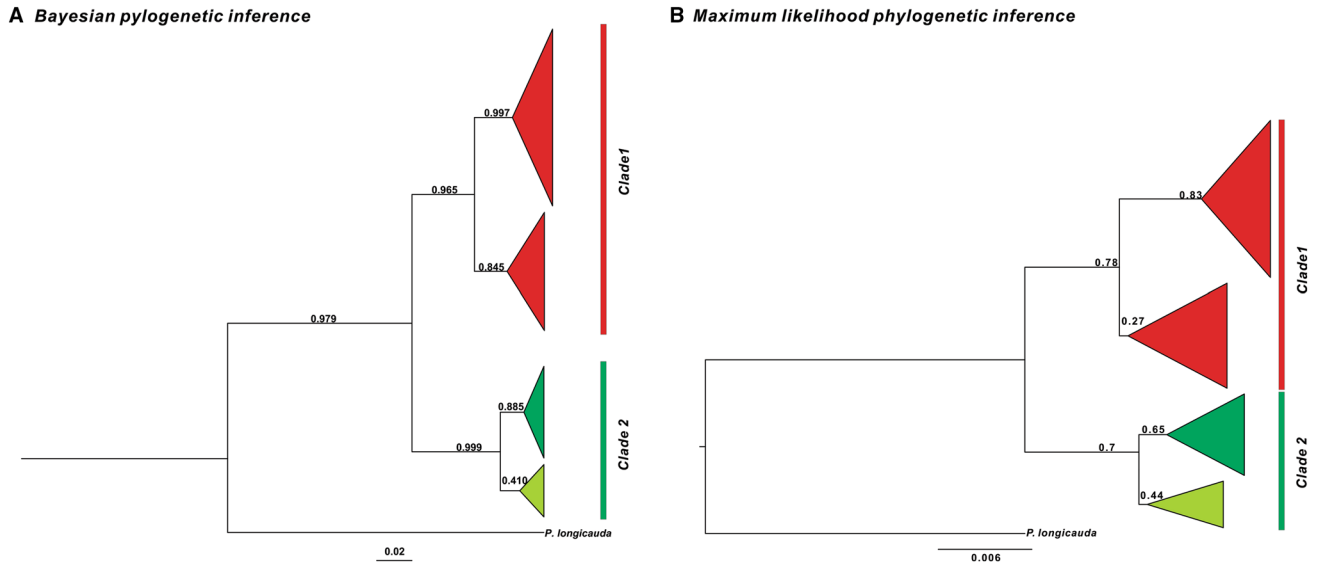


Fig. 2 Phylogenetic analyses of *P. robertsi* based on *cyt* b DNA sequences. **a** Bayesian phylogenetic inference. **b** Maximum likelihood phylogenetic inference

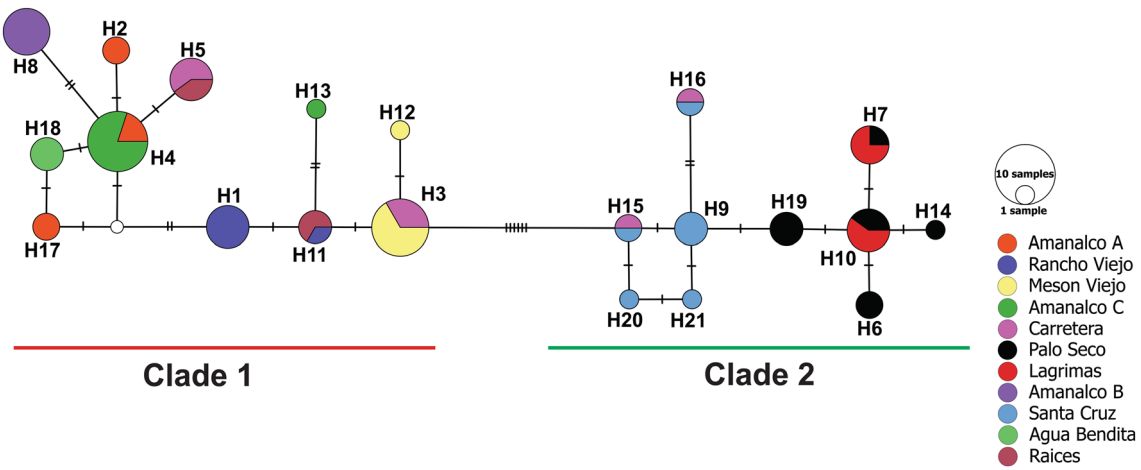


Fig. 3 Haplotype network of *cyt b* DNA sequences from *P. robertsi* obtained using the Median-joining method

time estimation based on the Yule process tree method, indicated that haplotypes from clades 1 and 2 diverged about 1.96 million years ago (95% HPD: 1.03–3.14; Fig. 4; Table 2).

With the microsatellite data, the maximum log likelihood assumed by STRUCTURE was K = 2 (LnPr (k = 2) = − 2625.650), and a ΔK = 19.401. (Fig. 5 and Fig. A2). Pairwise F_{ST} values ranged from − 0.031 to 0.303, G_{ST} values ranged from − 0.032 to 0.175, D values ranged from 0 to 0.012 and F_{IS} values ranged from − 0.068 to 0.062 across all population pairs (Supplementary Materials, Table A5). In relation with the AMOVA results, genetic variation resided mainly within populations, as shown by both F_{ST} and R_{ST} fixation indices (Supplementary Materials, Table A6). Finally, the MSN considering the Bruvo's distance and the Nei's distance found a low pattern of population structure (Fig. 6).

Isolation by distance and demographic history

For the *cyt* b data the Mantel test found a low but significant correlation between the pairwise genetic and geographical distances $R^2 = 0.118$ distance, $P = 0.04$ (Fig. A4). The estimates of the neutral tests found for the total population a Tajima's D (D = 0.695; P > 0.1), Fu and Li's F (F = 0.853; P > 0.1) and Fu's FS tests (FS = − 2.517;

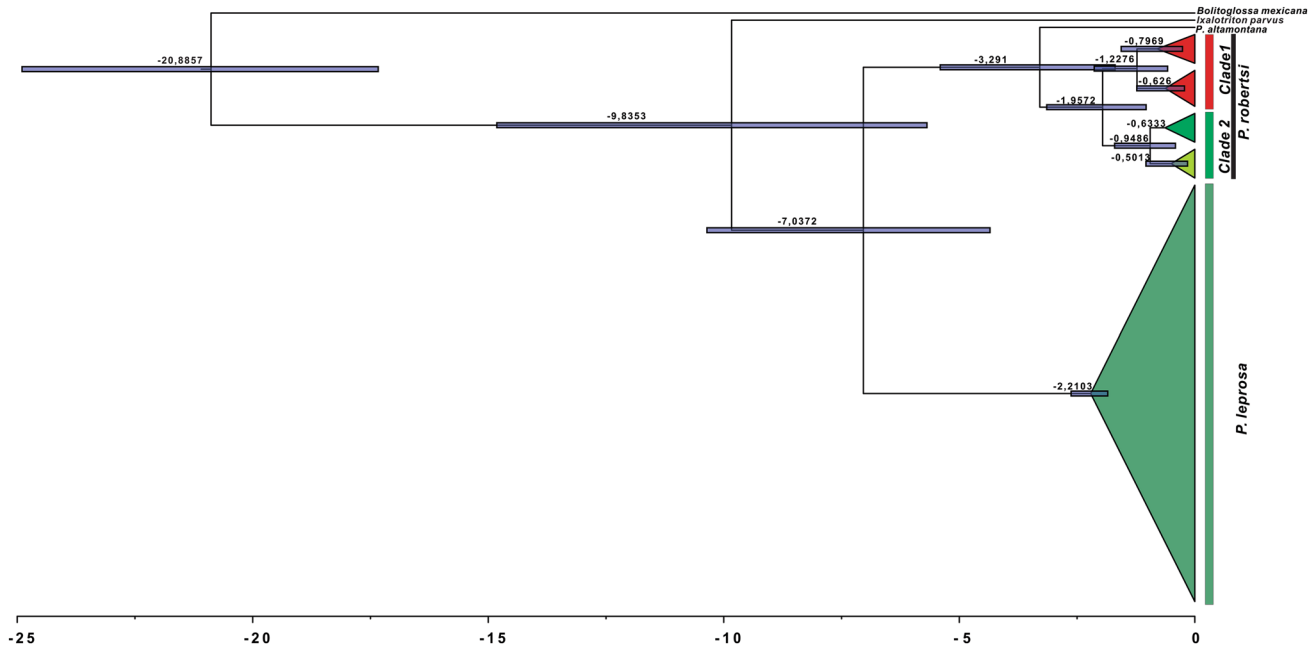


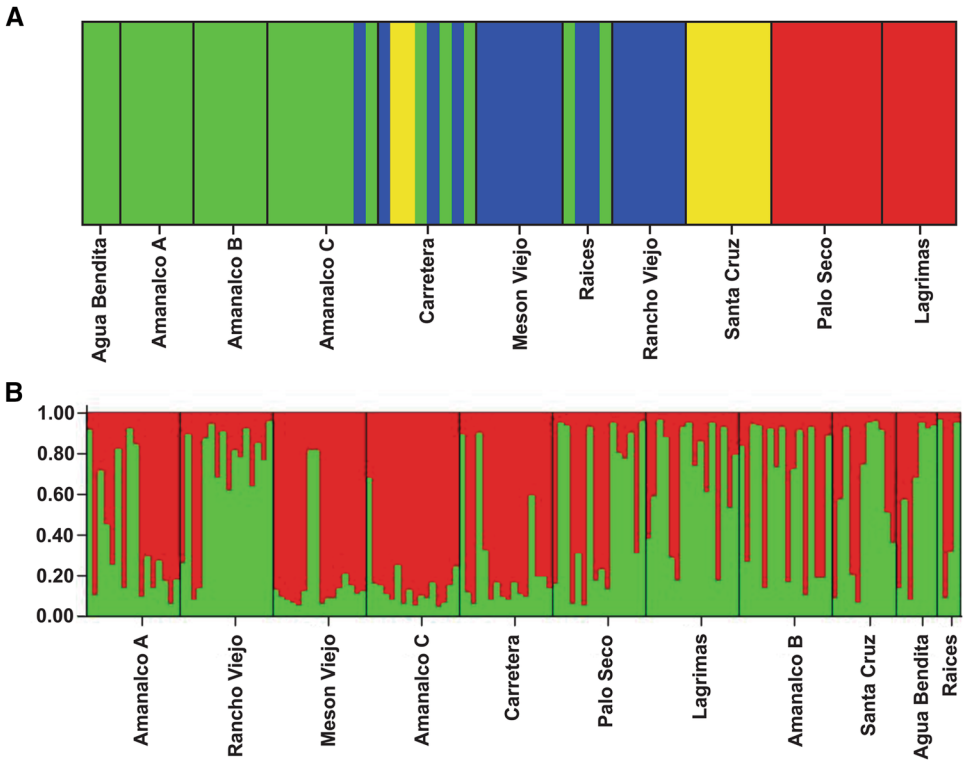
Fig. 4 BEAST divergence-time estimation of *P. robertsi* (Yule's tree method) indicated that haplotypes from Clades 1 and 2 diverged about 1.96 million years ago (95% HPD: 1.03–3.14)

Table 2 Calibration points used in BEAST analyses based on mitochondrial *cyt b* sequencs of *Pseudoeurycea robertsi* from the Nevado de Toluca Volcano, indicating calibrated clades, the corresponding reference, mean estimated date in million years (Ma), the associated standard deviations (SD), and the 95% highest posterior density (HPD) under two model priors

Calibration points	Reference	Mean estimated date (Ma)	SD	95% HPD under Yule speciation tree prior (Ma)	95% HPD under Coalescent constant population size tree prior (Ma)
Split of *Bolitoglossa* from *Ixalotriton* + *Pseudoeurycea* clade	Rovito et al. 2015	28	0.1	(17.34–34.09)	(22.63–32.57)
Time of the Last Common Ancestor of all *P. leprosa*	Parra-Olea et al. 2012a, b	2.3	0.1	(1.85–2.63)	(1.85–2.34)
Time of the Last Common Ancestor of SE + Central TVB Clade of *P. leprosa*	Parra-Olea et al. 2012a, b	1.16	0.05	(0.9–1.95)	(0.81–1.16)

Each calibration point was tested separately using three different combinations and length runs

$P > 0.1$) indicated departures from neutrality in molecular variation (Supplementary Materials, Table A7). Fu's Fs statistic was significantly negative for the total population, supporting the general inference of recent population expansion; however, the Tajima's D statistic does not support this hypothesis. Regarding the Bayesian Skyline demographic reconstructions based on mtDNA, the two main clades indicated different historical scenarios. While Clade 1 showed a pattern of population growth though time (from 1.9 until 0.2 Ma, Fig. 7a), Clade 2 indicated a constant effective population size with a drastic increase in size around 500,000 years before de present (Fig. 7b). Skyline reconstruction also indicated contemporary extremely reduced female effective population sizes (*Ne*) of 31.6 individuals (12.5-125.9, 95% HPD) for Clade 1 and 15.8 (1.2–79.4, 95% HPD) individuals for Clade 2. With the microsatellite data, The Mantel test for isolation-by-distance found a positive and significant relationship between geographic and population genetic distance, but R^2 is very low ($R^2 = 0.118$, $P = 0.030$; Supplementary Materials, Fig. A5) as the correlation just explained 10% of the observed variation. For the total population, there was no evidence of a recent genetic bottleneck under the SMM model with the two variances and probabilities tested (Supplementary Materials, Table A8). However, in some populations we found evidence of recent genetic bottlenecks under the SMM model: Rancho Viejo, Amanalco C, Carretera, Amanalco B, Agua Bendita and Raices.



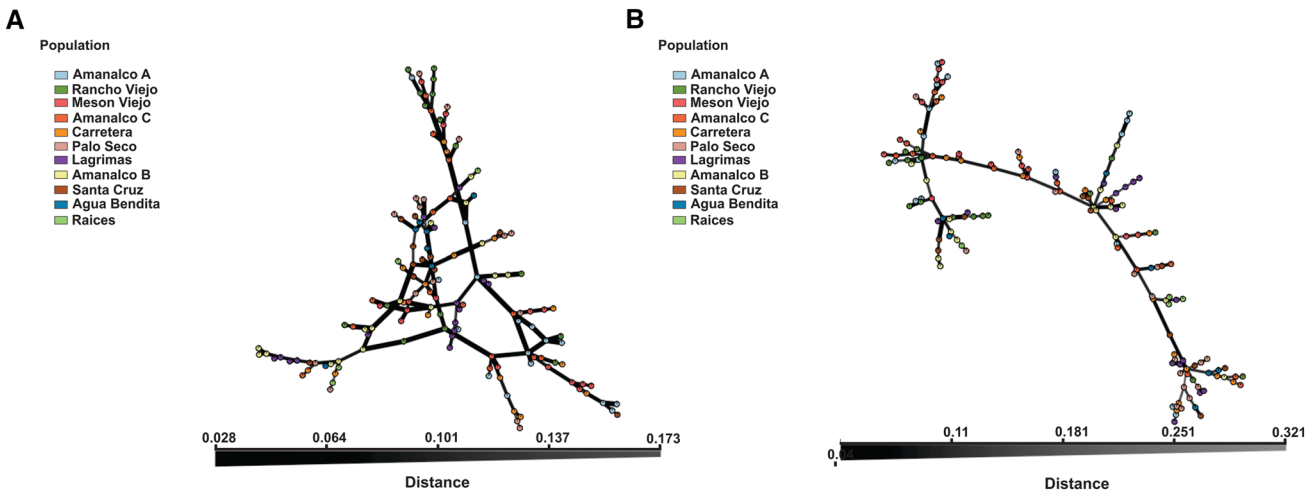


Fig. 5 Population genetic structure of *P. robertsi*. **a** BAPS results based on *cyt b* data. **b** STRUCTURE results based on Microsatellite data; individual bars represent assignment coefficients for each individual to each genetic population

Fig. 6 Minimum Spanning Networks representing the relationships among individuals and Populations based on Bruvo's (**a**) and Nei´s (**b**) distance algorithms

The effective population size (Ne) estimated from microsatellite loci with the LD method was medium to low in the total population Ne = 76.5, and ranged from 4.8 to 33.7 in the subpopulations (Supplementary Materials, Table A9). However, in some populations we were not able to obtain *Ne* estimates due to infinity values. The results from the Garza–Williamson test showed empirical *M* values significantly higher than *Mc* for all the populations, suggesting no evidence of a historical bottleneck (Supplementary Materials, Table A10 and Table A11).

The F_{IT} statistic as an indicator of inbreeding for the whole population showed low inbreeding values (F_{IT}= -0.097). We found that mean pairwise relatedness (r) within populations found low values of inbreeding in all the populations. Populations Rancho Viejo, Meson Viejo, Amanalco C, Carretera, Lagrimas, Amanalco B, Santa Cruz and Agua

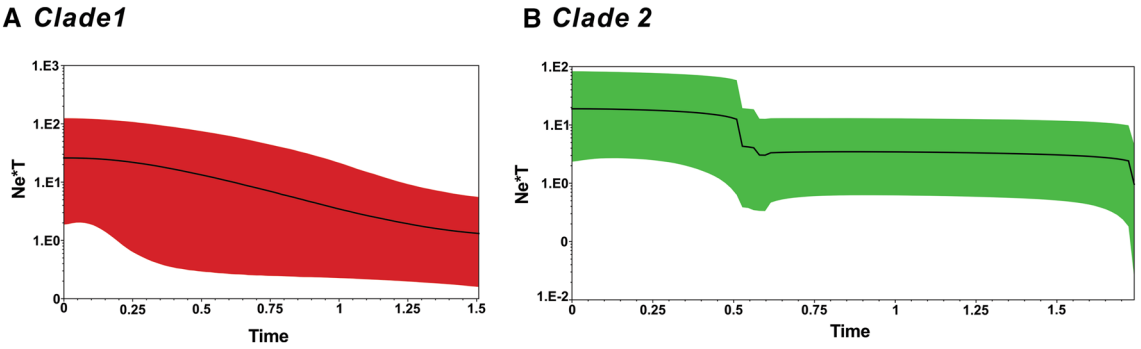


Fig. 7 Bayesian skyline plots for each main mitochondrial lineage used to assess the historical demography of *P. robertsi* clades 1 and 2

Bendita, revealed rqg values above the 95% expected values from permutations, indicating that inbreeding or genetic drift are increasing levels of relatedness between individuals. No population fell outside the range expected under panmixia (Fig. 8). The proportion of relationships of individuals within each population was similar in all populations, with most individuals being unrelated, followed in decreasing prevalence by half-siblings, full siblings, and parent/offspring (Supplementary Materials, Table A12).

Discussion

Our study reports the first comprehensive characterization of population genetics of *Pseudoeurycea robertsi*, one of the most threatened and extremely endemic salamanders in Mexico, based on nine microsatellite loci and mitochondrial *cyt* b. This represents the most extensive dataset recorded in terms of number of samples (N = 150) and localities (11) using two molecular markers with different modes of inheritance. Historically, phylogenetic and ecological studies on this species have considered a significant lesser number of individuals (Parra-Olea 2002) and localities (Bille 2009; IUCN SSC Amphibian Specialist Group 2016). Furthermore, our microsatellite data allowed estimates of genetic diversity and effective population size, critical parameters to inform management decisions aimed at the conservation of this micro-endemic species.

Genetic diversity

For the *cyt* b data we found high haplotype diversity and low nucleotide diversity values. This trend is commonly observed when there is evidence of a population expansion associated with negative values of Tajima's D and Fu's F (Zeng et al. 2006). Estimates of genetic diversity reported here were very similar to those reported for *P. leprosa* (Windfield-Pérez 2008; Parra-Olea et al. 2012a, b), a salamander that is also distributed in the NTV region

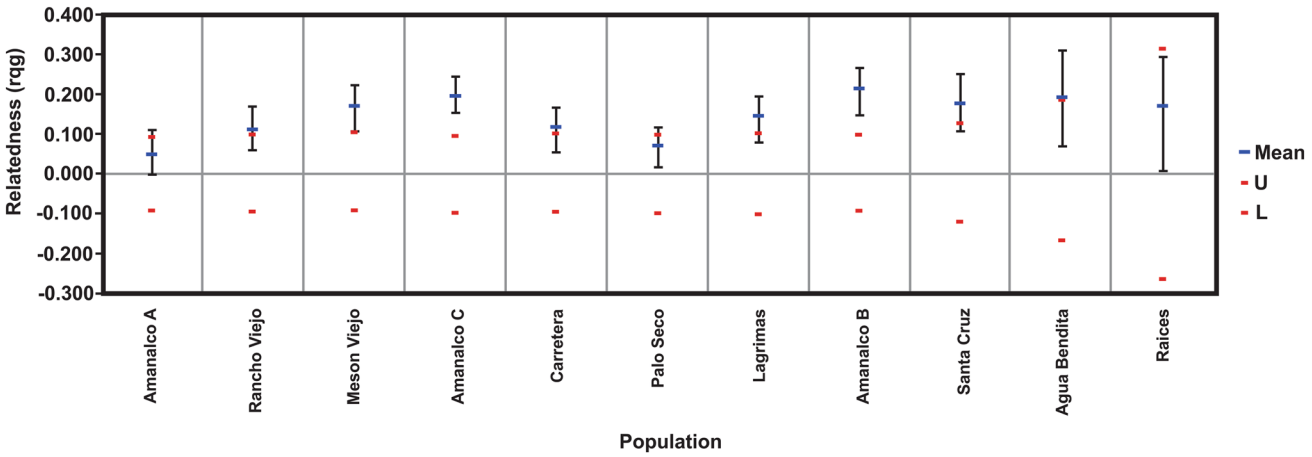


Fig. 8 Mean within-populations pairwise relatedness coefficient (rqg) between the *P. robertsi* populations studied. The red bars are 95% upper and lower expected values for a null distribution generated from 999 permutations of the data between populations. Blue bars represent the observed mean relatedness in each population, with upper and lower bootstrap value for each population

but has a much wider distribution (Parra-Olea et al. 2012a, b; Velo-Antón et al. 2013).

With the microsatellite data, our results revealed deviations from HWE for some loci in two populations (Palo Seco and Raices), as an outcome of heterozygote deficiency. This is not uncommon to detect in threatened species with fragmented populations (Degner et al. 2007; Lowe et al. 2005; Spear and Storfer 2010; Vázquez-Domínguez et al. 2012; Velo-Antón et al. 2013). A possible explanation for the observed decrease in heterozygosity could be associated to the effects of genetic drift (Hedrick 2011), which are more prominent in small populations and lead to both short- and long-term losses of genetic diversity. Furthermore, small population size may also increase levels of inbreeding, one of the main genetic factors threatening the short-term survival of populations (Frankham et al. 2005; Vega et al. 2007; Heredia-Bobadilla et al. 2016, 2017). In addition to these genetic factors, other ecological or behavioral factors such as habitat loss, fragmentation, or changes in social structure, may affect movement, levels of isolation, and ultimately reproductive success, increasing the probability of extinction of this species. Although the presence of null alleles could potentially be associated with deviations from HWE, this was not the case in our study since neither F_{ST} values nor genetic distances were significantly affected by the elimination of null alleles from the analysis. Small sample sizes (e.g., $N < 10$) may also affect estimates of genetic diversity, genetic distances and phylograms. In our study, most populations had samples sizes above 10, which are not uncommon for amphibian species with small populations and limited distributions, as in most threatened species. Furthermore, although some sample sizes were relatively small, we increased the number of nuclear loci analyzed, which has been shown to have a stronger effect than the number of individuals sampled in producing more reliable estimates of genetic diversity (Parra-Olea et al. 2012a, b; Vázquez-Domínguez et al. 2012; Percino-Daniel et al. 2016).

The genetic diversity values reported in this study are similar to those reported for species with small and fragmented populations or slightly higher than those reported in other studies with salamanders and other amphibian species (Zamudio and Wieczorek 2007; Rhoads 2011; Parra-Olea et al. 2012a, b; Velo-Antón et al. 2013; Percino-Daniel et al. 2016). However, the average number of alleles (Na = 2.333–3.222; Table 2) tend to be lower in most other salamander and amphibian studies (Greenwald et al. 2009; Rhoads 2011; Parra-Olea et al. 2012a, b; Velo-Antón et al. 2013; Zhang et al. 2015; Percino-Daniel et al. 2016). It is important to consider these results since populations that are small and isolated, and that live in fragmented areas affected by anthropogenic activities may be more at risk of extinction if they present low levels of genetic diversity.

Genetic Structure and phylogenetic inference

For the *cyt* b data the different analyses of genetic structure found four distinct clusters (K = 4), which consisted of two major clades, each with two sublcades. Clade 1 (north part of the NTV) is composed of the western populations and Clade 2 (south part of the NTV) of the eastern populations. On the north and south sides of the volcano there are historical lineages of *P. robertsi*; however, Clade 1 and 2 present the greatest divergence between their haplotypes, and the larger number of mutational steps between haplogroups, which makes them the most distant genealogical lineages. These two genealogical groups, with the highest percentage of haplotypic variants, are found in areas dominated by *Abies-Pinus* (Clade 1) and *Pinus-Abies* (Clade 2) forests, which may have promoted genetic differentiation between groups. Interestingly, the study site Carretera, which showed the greatest levels of genetic diversity among their haplotypes, is located in the inter-highway zone, with evidence of admixia between the two major genetic clades.

The structure analysis using microsatellite data reveled two clusters, with a weak population structure ($F_{ST} = -0.011$ to 0.303, $G_{ST} = 0.002$ to 0.17 and $D_{xy} = 0.005$–0.026). These results may suggest a metapopulation dynamics, a common pattern in amphibian populations with reduced and fragmented distributions at small geographical scales (Giordano et al. 2007; Noël et al. 2007; Zamudio and Wieczorek 2007; Purrenhage et al. 2009; Sunny et al. 2014a, b; Heredia-Bobadilla et al. 2016, 2017). The little levels of genetic structure found may be unexpected, since it is known that salamanders are usually restricted to woodlands (Petranka et al. 1993; Welsh and Droege 2001) and have limited dispersal ability and small home ranges (Kleeberger and Werner 1982; Ovaska 1988; Gergits and Jaeger 1990; Gibbs 1998a, b; Marvin 1998). However, it has been found that male salamanders can disperse through harsh habitats (Marsh et al. 2005); in fact, in some cases dispersion of males is favored when there are high levels of competition (Liebgold et al. 2011).

The difference in the number of clusters identified with the two different type of markers used (mitochondrial versus nuclear) may be due to their distinct modes of inheritance and evolutionary dymanics. Whereas mitochondrial markers reflect evolutionary processes operating through the maternal germline, microsatellites reflect evolutionary processes of both sexes that have occurred more recently, e.g., over a few thousand years. We found that estimates of genetic divergence were higher for the mtDNA than for the microsatellite data set (Supplementary Materials, Table A6). This was expected based on the lack of sex-biased dispersal and

the fact that the matrilineal inheritance and haploid nature of mtDNA reduces the effective population size to one-fourth of that of nuclear genes (DeSalle et al. 1987; Gariboldi et al. 2016). Likewise, the genetic structure found may also be influenced by the migratory patterns of females, which suggests that they are scarce, infrequent, or of very short distances. This has been reported in *Plethodon cinereus* and *P. vehiculum* in which most females are highly philopatric, showing significant positive genetic structure at a small geographic scale (Ovaska 1988; Liebgol et al. 2011). In contrast, males of these species showed no spatial genetic structure over short geographic distances (Ovaska 1988; Liebgol et al. 2011).

Both *cyt* b and microsatellites did not show significant correlations between genetic and geographic distances, which suggests that other factors than geographic distance may be limiting dispersal. Previous studies on amphibian populations have documented that the genetic differentiation may be driven by other environmental variables, including landscape topography, habitat continuity, riparian corridors, elevation, and the presence of roads (Funk et al. 2005; Arens et al. 2007; Purrenhage et al. 2009; Wang 2009).

Our results showed no generalized evidence of inbreeding. Previous studies established that a minimum of one to ten migrants per generation are necessary to prevent inbreeding (Vucetich and Waite 2000; Heredia-Bobadilla et al. 2016). Low values of Ne may result from population bottlenecks, genetic isolation, asymmetry in the proportions of males and females, and differences in reproductive success between individuals (Tennessen and Zamudio 2003; Myers and Zamudio 2004; Semlitsch 2008; Wang 2009). Salamanders tend to have low Ne values (Savage et al. 2010; Parra-Olea et al. 2012a, b; Sunny et al. 2014a; Heredia-Bobadilla et al. 2016; Percino-Daniel et al. 2016) resulting from high asymmetry in reproductive success among individuals (Savage et al. 2010; Heredia-Bobadilla et al. 2016). If only a few individuals successfully breed each year, the variance in mating success may contribute strongly to low overall effective population sizes (Savage et al. 2010; Heredia-Bobadilla et al. 2016). Populations Rancho Viejo, Meson Viejo, Amanalco C, Carretera, Lagrimas, Amanalco B, Santa Cruz and Agua Bendita showed all relatively high rqg values (above 95%), possibly due to the fact that these areas have been subjected to anthropogenic disturbance. Although at this moment levels of inbreeding reported in this study are relatively low compared to previous studies (Parra-Olea et al. 2012a, b), these values may increase if anthropogenic activities continue affecting these populations.

Conservation Implications

The preservation of proper levels of genetic diversity in *P. robertsi* may require the protection of hotspots where we found high genetic diversity with sufficient levels of gene flow, adequate vegetation cover, and different types of environmental gradients (Moritz 2002; Domínguez-Domínguez and Vázquez-Domínguez 2009). We recommend that the areas of Amanalco, Meson Viejo and Rancho Viejo should be prioritized in conservation programs, since these areas revealed higher levels of genetic diversity for populations of *P. robertsi*. These areas also have the most pristine *Abies-Pinus* forests of the Nevado de Toluca Volcano, a major habitat for *P. robertsi*. It is worth notice that the *Abies* forests of the volcano have remained constant between 1972 and 2000 (Franco-Maass et al. 2006), and that between 2002 and 2011 *Abies* forest areas have remained relatively constant throughout the country (González-Fernández et al. 2018). However, governmental laws have recently changed the protection status of the Nevado de Toluca Volcano (DOF 2013), which allow commercial logging of almost all *Abies* forests (Mastretta-Yanes et al. 2014), further threatening the already critically endangered *P. robertsi*. Therefore, it would be important that new regulations are implemented to avoid deforestation and limit the use of fallen logs, since some studies have provided evidence that timber harvesting may influence the genetic diversity of local populations (Stiven and Bruce 1988).

Future studies on landscape genetics may facilitate the identification of potential forest corridors, which may help maintaining levels of dispersal among *P. robertsi* populations and, thus, promote a metapopulation dynamics for the preservation of genetic diversity. As a final note, we would like to emphasize that a successful conservation strategy for the long-term conservation of the *Abies-Pinus* forest and their associated *P. robertsi* populations will only be possible if effective communication is developed between scientists, managers and the local people. Otherwise, these salamanders will not have enough genetic diversity to face future environmental changes (Lande 1988; DeYoung and Honneycutt 2005; Frankham et al. 2005; Hedrick 2011).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. Our study received the field permits and the approval of the ethics committee from Universidad Autónoma del Estado de México and SEMARNAT (SGPA/DGVS/05701/16).

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