Genetic diversity and structure of two populations of *Ambystoma altamirani* and *A. rivulare* of the trans-Mexican volcanic belt.

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Abstract

The most important factor leading to amphibian population declines and extinctions is habitat degradation and destruction. To help prevent further extinctions, studies are needed to make appropriate conservation decisions in small and fragmented populations. The studied mole salamanders are micro-endemic, and their habitat is found in the most ecologically disturbed region in Mexico: The Trans-Mexican Volcanic Belt. The goal of this study was to provide data from the population genetics of two micro-endemic mole salamanders that can be used as a basis for future research and conservation planning of these species and other amphibian species of this region of Mexico. We analysed the genetic diversity and structure, effective population size, the presence of bottlenecks and inbreeding coefficient of 152 individuals from two *Ambystoma* species. For *A. altamirani*, two locations were sampled, as well as for *A. rivualre*; 38 tissues were collected from each locality. We found medium to high levels of genetic diversity expressed as heterozygosity in the populations. However, all the populations presented few alleles per locus and genotypes. Each sampled locality represents a population with a significant level of genetic structure. The effective population size is small but similar to that of the studies from other mole salamanders with restricted distributions or with recently fragmented habitats. Despite the high levels of genetic diversity found, the populations are going through bottleneck processes and their habitats are fragmenting and degrading. Therefore, this study is important to propose better management plans and conservation efforts for these species.

Keywords: Endemic species, Conservation genetics, Microsatellite, Mole salamanders, Endangered species, Conservation.

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Introduction

Mexico is one of the most diverse countries that are losing a large amount of forest due to land use change [1]. Most of the deforestation (80%) is taking place in the central and southern parts of [1,2]. These data put Mexico in fourth place for global deforestation rate [3-5]. The Trans-Mexican Volcanic Belt (TMVB) is one of the most ecologically disturbed regions of the country; 3.4% of the TMVB is highly fragmented by urban settlements and 44.7% by agricultural areas [6,7]. Also, the TMVB is one of the biogeographic zones with the highest species richness and endemism across the country, and it is the most important region in terms of endemic amphibian and reptile species and second most important in terms of species number [8]. However, it is one of the most urbanized areas of the country and is disrupting the natural connectivity of forest landscapes, creating small patches of habitat and reducing the genetic flow between the populations of amphibians and reptiles of the TMVB [9-11].

This loss of genetic connectivity can decrease the genetic diversity and increase the inter-population genetic divergence, while increasing inbreeding levels and loss of alleles due to genetic drift [9,12-15]. This loss results in fluctuations of effective population size and other demographic and environmental parameters that can lead biological populations towards extinction [16-18]. Genetic diversity is highly important because it shapes the ability of populations to respond to environmental changes [11,13,19-22]. Therefore, the International Union for the Conservation of Nature (IUCN) has recognized genetic diversity as one of the three levels of biological diversity necessary to conserve species diversity [23].

It is important to study amphibians because they are among the most threatened vertebrates on Earth [24] and their populations are rapidly declining worldwide due primarily to the loss and degradation of their natural habitats [25-34]. Amphibians are threatened in part because of their low dispersal capacity and small home ranges [35-38]. They are highly sensitive to perturbations in both terrestrial and aquatic environments because of their dual life histories, highly specialized physiological adaptations and specific microhabitat requirements [39-45]. Thus, they have been used as bio indicators of habitat quality for these reasons. The *Ambystoma* populations can be used as a model to infer the quality of the habitat and this study can help us to propose better conservation strategies for these species and their habitat. The two species studied are micro-endemic mountain mole salamanders that inhabit slow-flowing streams within the TMVB [5,46-50]. Both species are endangered according

to Mexican law [51], and their environmental vulnerability scores are 13 on a scale of 3 to 19. This score places them between medium and high vulnerability, primarily because of their restricted geographic and ecological distribution [52,53], as well as the consideration that the areas where these species are distributed are subject to pressures such as illegal logging, introduction of exotic species such as trout, human settlements and pollution of streams.

Therefore, we studied the genetic diversity and structure, effective population size, inbreeding and genetic bottlenecks of two populations of *A. rivulare* and *A. altamirani* in highly disturbed habitats. Under this scenario, we expected that there would be high genetic structure patterns induced by landscape modification. We studied a small population, isolated and subjected to anthropogenic pressures, so these populations would present low levels of genetic diversity, effective population size, and presence of inbreeding and genetic bottlenecks. This information will help raise conservation strategies of these micro-endemic mole salamander species.

Materials and Methods

Study area and population sampling

We sampled two populations of each species (Figure 1); the first population of *A. altamirani* was in Organels (19° 31' 38.17" N; 99° 28' 39.92" W, with an altitude of 3,335 MASL), and the second population was in Tlazala, both of which are in Isidro Fabela municipality, State of Mexico (19° 31' 31" N, 99° 26' 09.52" W with an altitude of 3,185 MASL). The first population of *A. rivualre* was in Stone Corral (19° 13' 6.60" N; 99° 57' 54.77" W, with an altitude of 2,836 MASL), and the second population was in Raices (19° 9' 37.26" N; 99° 49' 32.11" W, with an altitude of 3,225 MASL), both of which are in the Nevado de Toluca Volcano (NTV).

We collected the individuals with fishing net, and we sampled 2 mm^2 of tail clips of adult mole salamanders. This methodology is a low-impact method that does not affect the survival or growth of the mole salamanders [47,54]. Tissue was preserved in 90% ethanol and then frozen at -20°C until processed. Finally, we released the mole salamanders. Our study received the approval of the ethics committee from Universidad Autónoma del Estado de México (3047-2011E; 9855714) and the collection permits of SEMARNAT (SEMARNAT: SGPA/DGVS/001777/18).

Genetic analysis

We extracted DNA following the manufacturer's instructions for the GF-1 nucleic acid extraction kit (Vivantis), and we used it as a template for amplification of nine loci following published protocols [55]. PCR microsatellite products were multiplexed and run on an ABI Prism 3730 × l (Applied Bio systems) with Rox-500 as an internal size standard. We obtained allele sizes with PEAKSCANNER 1.0 software (Applied Bio systems), and the fragment lengths were obtained with TANDEM 1.08 software [56]. In all runs we included negative controls in at least two runs to guarantee reproducibility.

Potential scoring errors and genotype accumulation curve

In the MICROCHECKER 2.2.3 software [57] we tested the presence of null alleles and large allele dropout. In addition, in POPPR 2.4.1 [58] for R software (version 3.4.0; R Development Core Team 2017), we made an analysis to create a genotype accumulation curve we used for determining the minimum number of loci necessary to discriminate between individuals in each population of the species studied [58]. This function randomly samples loci without replacement and counts the number of observed multi locus genotypes [58].

Genetic diversity

All analyses were done for each study location (it was determined that the sampling sites were independent populations by the STRUCTURE results, see below) and species. We calculated the observed (Ho) and expected (He) heterozygosity, the number

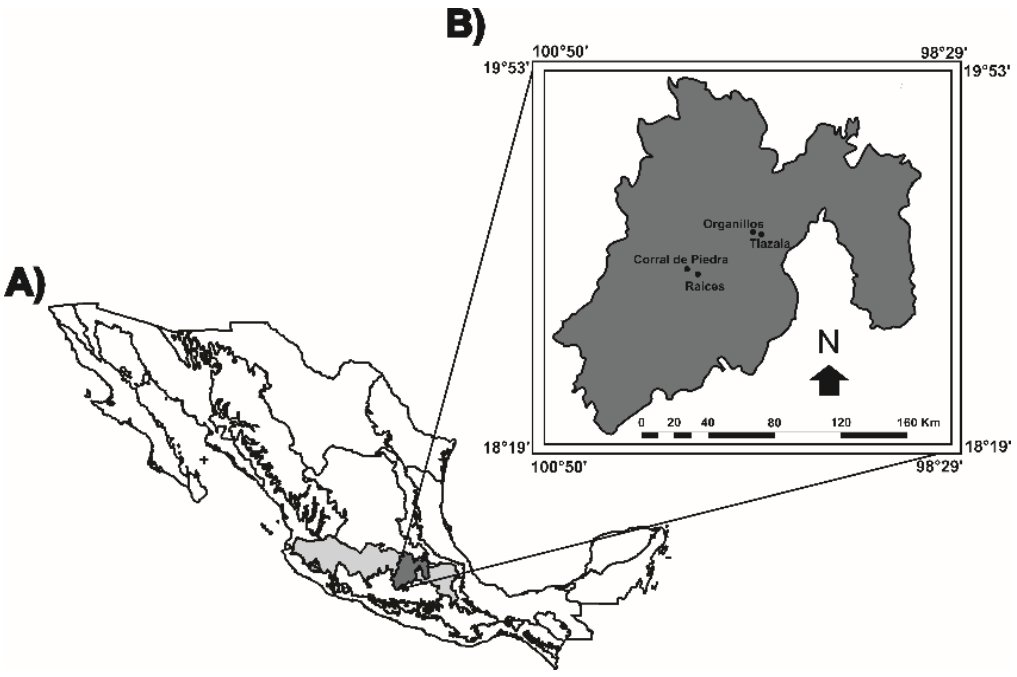

Figure 1. *A) Map of Mexico showing the Trans-Mexican Volcanic Belt and the State of Mexico. B) State of Mexico and the four studied sites.*

of alleles (Na), effective number of alleles (Ne), number of genotypes and the number of heterozygotes and homozygote genotypes in STRATA G 2.0.2 [59] and GENALEX. We calculated departures from Hardy-Weinberg equilibrium (HWE) and Linkage Disequilibrium (LD) between pairs of microsatellite loci in the PEGAS packages [60] implemented in R. These calculations were evaluated for each sampled locality and locus with a Markov chain approximation considering 10,000 de-memorizations, 1,000 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) [61] implemented in the package FDRTOOL 1.2.15 [62,63] for R.

Genetic structure

We searched for a genetic structure pattern using several algorithms for each of the species and sampling sites. First, we used a Bayesian algorithm implemented in the STRUCTURE 2.3.4 software [64-66]. The chosen parameters were correlated allele frequencies with 1,000,000 burn-in periods and 1,000,000 MCMC iterations [65]. For the degree of admixture, a Dirichlet parameter was applied with correlated allele frequencies. Therefore, the most credible number of populations were estimated using the maximum value of ΔK [67], applied in the STRUCTURE HARVESTER 0.6.92 software [68]. The second method was the analysis of molecular variance (AMOVA) based on FST and RST as implemented by GENALEX 6 [69].

A Wilcoxon test with 30,000 permutations was applied to search significance, using the degree of similarity of the populations based on the populations' genotypes in GENALEX 6. In order to detect the similarity degree of the sampling sites of each species, we applied a Discriminant Principal Components Analysis (DPCA) based on the genotypes in ADEGENET 2.0.1 [70] and ADE4 1.7-6 [71] packages for the R software, and we calculated FST based on Weir and Cockerham (1984) [72] in GENALEX 6. Finally, we tested the existence of the population structure by computing Minimum Spanning Networks (MSN) with Bruvo's [73] and Nei´s distance algorithm Nei (1972) [74] with 1,000 bootstraps in POPPR 2.4.1 and MAGRITTR 1.5 [75] for the R package. This analysis visualizes the relationships among individuals and it can be a more adequate visualization tool than trees [75].

Genetic bottlenecks, effective population size and relatedness

The historical signal of demographic fluctuations was explored by applying a Bayesian algorithm implemented in MSVAR 0.4.1 software [76]. We estimated the rate of change (r) of the effective population size, defined as NCRNT/NSTBL (where NCRNT was the current inbreeding effective population size and NSTBL was the ancestral stable inbreeding effective population size). The r ratio was expressed in $\log_{10}$. Therefore, the population declined if we had a negative r value, was stable if r is equal to zero, and the population was expanded if the r value was positive [77,78]. In order to test for a genetic signature of recent bottlenecks, we used the BOTTLENECK 5.1.26 software [79]. We estimated the observed and expected heterozygosity under the two-phase model (TPM) because the TPM is an intermediate model of evolution which is considered more appropriate for microsatellites. The settings applied were for a 90% step-wise mutation model and 10% variance and were run with 10,000 replicates. Excess heterozygosity was tested using a Wilcoxon test. In order to explore the actual effective population size (Ne), we used the LD method implemented in the NEESTIMATOR 2 software [80]. We calculated the FIT inbreeding values in GENALEX 6. Also as an inbreeding measure, we used the relatedness estimator (rqg) of Amos et al. [81], which was calculated by the GENALEX software. 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 corresponded 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 ML-RELATE software [82].

Results

Population sampling

One hundred and fifty-two individuals were collected from two *Ambystoma* species: for *A. altamirani* and *A. rivualre*, two locations were sampled and 38 tissues were collected from each locality.

Potential scoring errors

We did not find evidence of null alleles or large allele dropout in the populations of each species. The genotype accumulation curve found that the minimum number of loci necessary to discriminate between individuals was eight (Figure S1). Therefore, we concluded that our study has enough loci (N=9).

Genetic diversity

Across the nine loci in the population Organillos we found 3-7 alleles per locus and a total of 30 alleles; Tlazala had 2-7 alleles per locus with a total of 26 alleles (Table 1 and Figure S2). Corral de Piedra had 2-5 alleles per locus with a total of 20 alleles, and Raices had 2-6 alleles per locus with a total of 33 alleles (Table 1 and Figure S3). Organillos had 29 homozygote genotypes and 35 heterozygote genotypes, Tlazala had 16 homozygote genotypes and 22 heterozygote genotypes. Corral de Piedra had 26 homozygote genotypes and 19 heterozygote genotypes, and Raices had 21 Homozygote genotypes and 20 heterozygote genotypes (Table S1). In relation to the observed and expected heterozygosity values, Organillos showed lower observed heterozygosity values (Ho=0.719 ± 0.033) compared to Tlazala (Ho=0.857 ± 0.029); Corral de Piedra showed lower observed heterozygosity values (Ho=0.576 ± 0.034) as compared to Raices (Ho=0.754 ± 0.059; Table 1). False discovery rate correction tests found departures from HWE due to heterozygote deficiency in one locus in the populations of Organillos (*A. altamirani*), Corral de Piedra (*A. rivulare*) and Raices (*A. rivulare*) (Table S2). We did not find LD between any loci of either population for each species.

Genetic structure

Bayesian assignment analyses corroborated high population divergences among sampling localities (Figure 2). The highest

log likelihood given by STRUCTURE and ΔK method was when K=2 (LnPr=-1793.2) for *A. altamirani* and K=2 (LnPr=-1457.6) for *A. rivulare*. The populations of *A. altamirani* present admixia with low genetic differentiation among them (FST=0.053; Table 2), but the populations of *A. rivulare* do not present admixia with high genetic differentiation among them (FST=0.211; Table 2). In relation to the AMOVA results, genetic variation resided mainly within populations in all the populations studied (Tables S3 and S4). The DPCA of pairwise and the MSN found the same patterns of population structure where the populations of *A. altamirani* are more similar among them, whereas the populations of *A. rivulare* are very different among them (Figures 3 and 4).

Genetic bottlenecks, effective population size and relatedness

MSVAR results suggested that there has been a significant population size reduction in all the studied *Ambystoma* populations: Organillos, r=-0.972; Tlazala, r=-1.403; Corral de Piedra, r=-1.031 and Raices, r=-1.399. The bottleneck analysis detected genetic signs of recent demographic changes typical of bottleneck events, associated with a heterozygote excess in all populations: Organillos, p=0.008; Tlazala, p=0.002; Corral de Piedra, p=0.002; Raices, p=0.002 and Corral de Piedra and Tlazala had a shifted distribution. The effective population size (Ne) estimated from LD was Ne=34.7 (20.9-21.7, 95% CI) for Organillos, Ne=44.1 (21.3-36.0, 95% CI) for Tlazala, Ne=57.6 (21.3-37.6, 95% CI) for Corral de Piedra and Ne=41.5 (20.1-23.6, 95% CI) for Raices.

The FIT statistic as an indicator of inbreeding for the *A. altamirani* populations showed negative and low inbreeding values (FIT=-0.128; Table 2) and showed positive, low inbreeding values for the *A. rivulare* populations (FIT=-0.095; Table 2). We found that mean pairwise relatedness (r) within populations (Figure 5) was generally in accordance with that observed in other *Ambystoma* populations [83-85]. Organillos had low values of inbreeding [mean rqg=0.247, confidence interval (CI =0.024 -(-0.025)], Tlazala had medium to high levels of inbreeding [mean rqg=0.458, confidence interval (CI)=0.029 -(-0.025)], Corral de Piedra had the highest inbreeding values [mean rqg=0.505, confidence interval (CI) =0.028 -(-0.026)] and Raices had medium to high inbreeding values [mean rqg=0.471, confidence interval (CI) =0.032 -(-0.026). In all the populations, the rqg values fell above the 95% expected values from permutations which indicates that inbreeding or drift are increasing the relatedness; they also fell outside the range expected under panmixia. Furthermore, there were low values of inbreeding in the relatedness analysis, the proportion of relatedness of individuals within each population was similar and most of the individuals were unrelated, followed by full siblings, half-siblings and parents/offspring (Table S5).

Discussion

Despite the limited distribution of these species and the anthropogenic activities in the study sites, it is possible that over time there will be a decrease in genetic diversity, habitat fragmentation, river pollution and introduction of exotic species in the sampled areas. If there is a loss of genetic diversity with the passage of time, it could compromise the ability of the population to respond to environmental changes [86-89]. In the present study, we found medium to high levels of genetic diversity expressed as heterozygosity in both species. However, all the populations of each species presented few alleles per locus and genotype. Each sampled locality represents a population with a significant level of genetic structure. The effective population size is small in both species, but it is similar to other mole salamanders with restricted distributions or with recently fragmented habitats. These results are important in order to design better management and conservation strategies to avoid the extinction of these micro-endemic species of the TMVB.

Genetic diversity

The observed heterozygosity values were medium to high, and most of the genotypes were heterozygous with the exception of Corral de Piedra and Raices (Tables 1 and S1). *Ambystoma* species had high levels of genetic diversity [90-94] despite having a limited distribution. However, populations with less Abies forest, more Pinus forest, more urbanization and the presence of trout farms and trout in the streams had lower levels of observed heterozygosity (pers. obs), indicating that maybe

Table 1. *Genetic diversity values in the four Ambystoma populations studied.*

Species	Population	N	Na	Ne	A	Ho	He
Ambystoma altamirani	Organillos	38	5.222	3.623	0.137	0.719	0.706
	Tlazala	38	4.222	3.071	0.111	0.857	0.636
	Total mean	38	4.772	3.347	0.124	0.788	0.671
	SE	0	0.449	0.237	0	0.027	0.026
Ambystoma rivulare	Corral de Piedra	38	3.333	2.513	0.088	0.576	0.562
	Raices	38	3.889	2.815	0.102	0.754	0.617
	Total mean	38	3.611	2.664	0.095	0.665	0.589
	SE	0	0.293	0.193	0	0.039	0.029

N: Sample size, Na: Number of alleles, Ne: Number of effective alleles, A: Allelic richness, H_o: Observed heterozygosity, H_e: Expected heterozygosity.

Table 2. *F_{IS}, F_{ST} and F_{IT} fixation indices estimated according to Weir and Cockerham in the four Ambystoma populations studied.*

Species		F_{IS}	F_{IT}	F_{ST}
Ambystoma altamirani	Total mean	-0.190	-0.128	0.053
	SE	0.049	0.052	0.012
Ambystoma rivulare	Total mean	-0.141	0.095	0.211
	SE	0.051	0.079	0.045

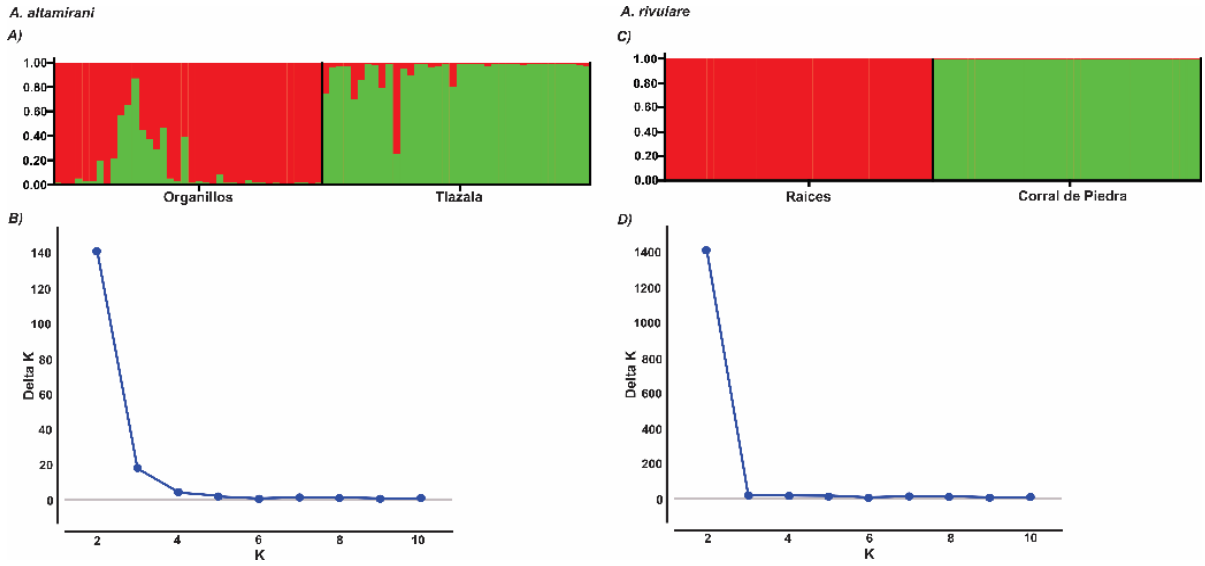

Figure 2. *A) Population genetic structure of Ambystoma altamirani analyzed with STRUCTURE. B) Evanno et al. [62] plots for detecting the number of K groups that best fit the data. C) Population genetic structure of Ambystoma rivulare analyzed with STRUCTURE. D) Evanno et al. [62] plots for detecting the number of K groups that best fit the data.*

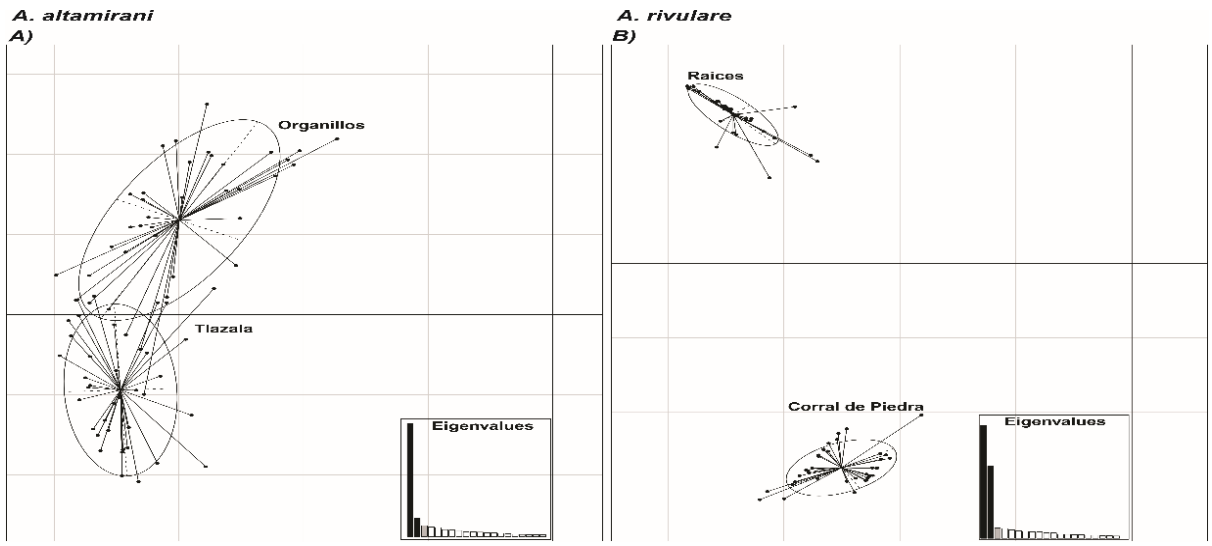

Figure 3. *Scatterplot showing the relationships among the populations of each species based on a discriminant principal components analysis of 9 microsatellite genotypes.*

all these factors could be acting synergistically to increase the loss of genetic diversity. The Abies forest is important for these species for the microclimatic conditions that it generates, such as high humidity and low temperatures, characteristics that the high-mountain *Ambystoma*s are adapted to [5,46,84].

The human population growth in the towns around Corral de Piedra and Organillos has been increasing in the last decades, causing an influx of sewage, waste from local agriculture and pollutants from trout farms into the rivers. Also, in Corral de Piedra we did not find larvae, and each mole salamander had tail bites. This could be due to trout predation [95] because of trout escape from farms and the exotic fish favour native species reduction [96-99]. Introduced fish species have been linked to reductions in amphibians' population sizes [100-102], sometimes to the point of extinction from direct. All these factors could lead to less observed heterozygosity values and fewer alleles. We found a significant deviation from the HWE proportions due to a heterozygote deficiency in Organillos, Corral de Piedra and Raices. This is a common result for threatened species with fragmented populations [84,103-106].

The explanation for the observed deviation from HWE could be genetic drift [107], and it is important to consider genetic drift in conservation plans since it's the main cause of long-term loss of genetic diversity which leads to an increased chance of inbreeding, the foremost genetic factor threatening the short-term survival of populataions [84,108,109]. Therefore, urgent implementation of conservation plans is needed in order to avoid the threats that the studied populations of mole salamanders are facing, including the introduction of trout, high rates of logging, livestock, fires and ecotourism activities [92]. However, this is not exclusive to the populations mentioned above, this situation is occurring throughout the TMVB, putting different degrees of pressure on each population of mole salamanders for this biogeographic province. These human activities are fragmenting

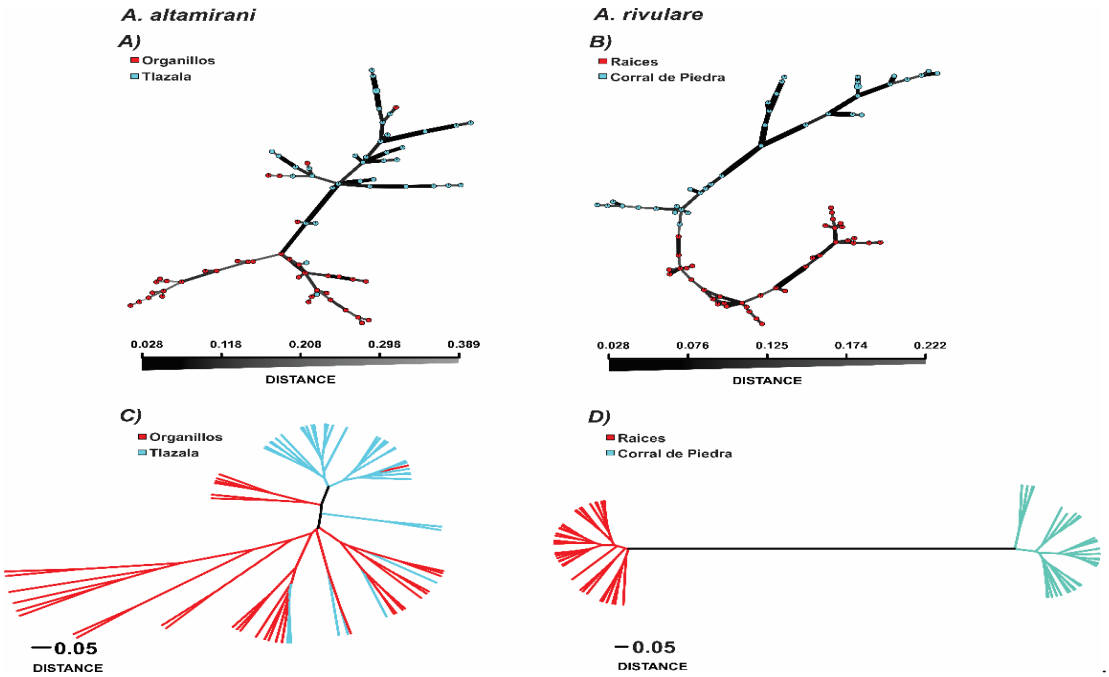

Figure 4. *A) and B) Minimum spanning networks with the Bruvo's distance algorithm, representing the relationships among individuals and populations of each species. C) and D) Tree constructed by the NJ method using the estimated standardized genetic distances using the Nei´s distance algorithm [69] with 1,000 bootstraps.*

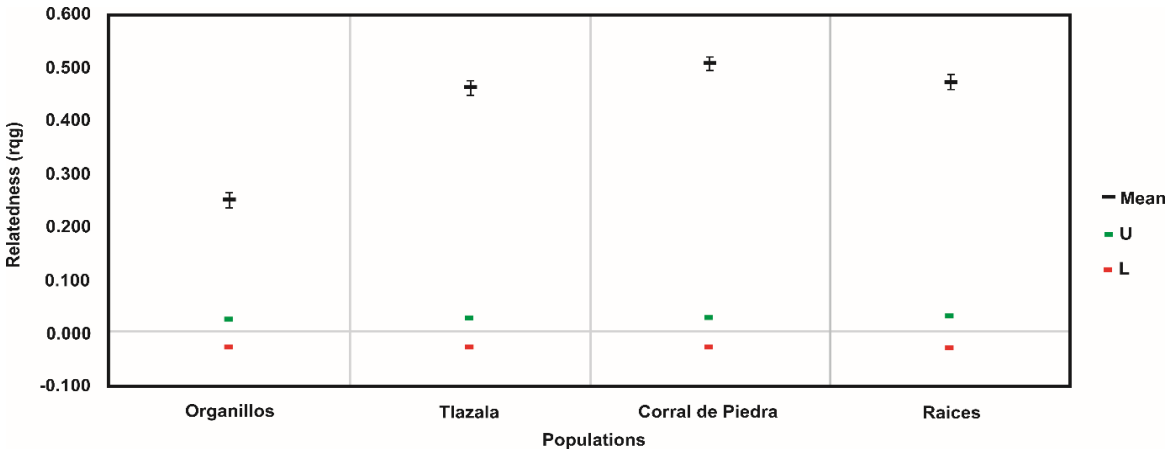

Figure 5. *Mean within-lake pairwise relatedness coefficient rqg across the four Ambystoma populations studied. The green bars are 95% upper and lower expected values for a null distribution generated from 9999 permutations of data from all populations, and enclose the values expected if breeding were panmictic across all populations; relatedness in all sampled populations fell outside the range expected under panmixia. Black bar represents the observed mean relatedness in each population; the black bars are the upper and lower bootstrap value for each population.*

and isolating the populations of mole salamanders, leading to population declines and isolation, which in turn could cause genetic drift and loss of genetic diversity and heterozygosity [15,92].

Genetic structure

The structural analysis found four populations with varying levels of diversity. The populations of *A. rivulare* show no signs of admixia, although the populations of *A. altamirani* are more similar to some admixia (Figures 2-4). The populations of *A. altamirani* and *A. rivulare* we studied are ~67 km apart in linear distance, and the two populations of *A. rivulare* are ~16 km apart in linear distance, so the genetic exchange is extremely reduced. The two populations of *A. altamirani* are closer, ~2.5 km apart in linear distance so gene flow could take place. In other studies, mole salamander migrations occur between temporal ponds and lakes as individuals look for food-rich habitats [85]. The known maximum dispersal distance in mole salamanders is less than 2 km [110,111]. Migration among other populations could be limited as a result of physical barriers like discontinuity of forests, roads, and towns, as well as the strong philopatric tendencies of the mole salamanders for breeding sites favouring high genetic structuring, even at small scales [112,113].

These populations in the TMVB occupy some of the most disturbed areas of the country, mainly due to habitat fragmentation by agriculture (44.7%), roads (25.3%), urban settlements (3.4%), stream pollution and fish introduction [109,114-116]. Since there are different factors affecting *Ambystoma* populations, we recommend some measures to try to minimize the impact of the anthropogenic activities on these amphibian populations. We must conserve the endangered Abies-Pinus forest. In the TMVB, there are only 1346.9 km^2 of Abies

forest (1.1%) and 6507.7 km^2 of Pinus forest (5.4%); therefore, it is extremely important to conserve these forests, considering that Mexico is currently fourth in global deforestation rates [112,113]. Likewise, the amphibians and reptiles have a very limited distribution, sometimes in very small areas with special characteristics that generate certain microhabitats suitable for their survival and reproduction [104].

Therefore, it is necessary to start considering the creation of natural reserves with corridors that include these microhabitats. Also, it's important to implement better reforestation and assisted regeneration practices, with germplasm of the area. Currently, it is still a common practice that authorities reforest the areas of Abies forests with other conifer species, such as *Pinus ayacahuite*, *Pinus pseudostrobus*, *Pinus patula* or *Cupressus lusitanica*, which in turn can change the microclimate and the environmental and habitat conditions necessary for these species and others to survive and persist.

Genetic bottlenecks, effective population size and relatedness

The studied populations are isolated from other populations of mole salamanders. This phenomenon could explain the low Ne values found in all populations and the asymmetry in the proportions of males and females and differences in the reproductive success between individuals. These intrinsic characteristics of mole salamanders favour low Ne values. Some years, only a few individuals successfully breed, and the variance in mating success may contribute strongly to lower overall effective population sizes [89]. Another explanation of the low Ne values found could be the bottleneck effect caused by the introduction of trout; however, we do not know when this introduction happened, as the trout predate the early stages of development of *Ambystoma*s and also eat the tail of adult *Ambystomas*, a phenomenon which has already been reported by other studies [102]. It has also been reported that trout can transmit pathogens and certain emerging infectious diseases [105]. Likewise, trout can compete for food with *Ambystomas*. All these features can lead the population to a process of genetic bottleneck or genetic drift, which in turn reduces genetic diversity and makes the population lose fitness and the ability to adapt to changes in the environment [108]. In all populations, the rqg values were above the 95% expected values from permutations (Figure 5), indicating that inbreeding or genetic drift is increasing the relatedness, and they fell outside the expected range under panmixia. Furthermore, there were low values of inbreeding in the relatedness analysis; the proportion of relatedness of individuals within each population was similar. Despite the lack of strong signs of inbreeding and relatedness, inbreeding and genetic drift are acting in these populations. Therefore, we suggest habitat restoration, increasing Abies forest, decreasing the construction of more trout farms in rivers with *Ambystoma* and preventing trout from escaping, since mole salamanders do not recognize trout as predators and their survival decreases proportionately [113]. Therefore, we encourage streams to be restored to a fishless state, stop the influx of sewage, and decrease waste from local agriculture and pollutants from trout farms into the rivers in order to avoid the loss of alleles through genetic drift and inbreeding.

Conservation implications

In order to conserve this species and all the species that live in the coniferous forests of TMVB, it is necessary to avoid excessive logging and give support to the local communities with incentives such as payments for ecosystem services. Also, we consider the implementation of an environmental education program to be fundamental to avoid excess logging, pollution of the rivers and the use of *Ambystoma* salamanders as traditional medicine for the lungs or traditional food [78]. It is also necessary to have further control trout breeding because trout escape from the farms to the steams, due primarily to low-quality fences and mesh dividing the farms from the streams.

We recommend divisions to be built higher and periodically revised to avoid holes where trout can escape. Trout farms are affecting the populations of mole salamanders; several studies have demonstrated that the predation by introduced fishes has been linked to reductions in the population's size, survival, growth, egg predation, reproduction and organic waste pollution of the rivers [116]. Trout farms have been encouraged by Mexican governmental agencies and recommended as a potential conservation tool for native forests so the best strategies may include isolating trout farms from streams containing native amphibians, increasing efforts to prevent trout escape and attempting to eradicate populations of escaped trout from streams, thereby balancing the economic and conservation value of trout farms with their potential negative effects on native amphibians. Also, it is necessary that each trout farm has an area of wastewater treatment since these waters when released to the stream, can change the physical, chemical and bacteriological parameters and contaminate the stream for up to 3-12 km [104].

Conclusion

Certain sediments turn completely anoxic and, with hydrogen sulfide by-products of microbial sulfate reduction, increase to toxic level. Also, it is very important that management agencies restore a fishless state to all lentic habitats that have the potential to host mole salamanders. Finally, additional research is needed, as well as effective communication between scientists and managers and sensible management actions to carry out a coordinated strategy between scientists, managers, local people and fishermen to ensure the long-term conservation of the mole salamanders. If these conservation strategies are not carried out, the expected heterozygosity values found will continue to decline, the inbreeding will be higher, the effective population size will be lower and the mole salamanders will not have enough genetic diversity to be able to adapt to changes in the environment.

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