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Genetic diversity and genetic structure of an endemic Mexican Dusky Rattlesnake (*Crotalus triseriatus*) in a highly modified agricultural landscape: implications for conservation

Armando Sunny[1] · Octavio Monroy-Vilchis[1] · Martha M. Zarco-González[1] · Germán David Mendoza-Martínez[2] · Daniel Martínez-Gómez[2]

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Abstract It is necessary to determine genetic diversity of fragmented populations in highly modified landscapes to understand how populations respond to land-use change. This information will help guide future conservation and management strategies. We conducted a population genetic study on an endemic Mexican Dusky Rattlesnake (*Crotalus triseriatus*) in a highly modified landscape near the Toluca metropolitan area, in order to provide crucial information for the conservation of this species. There was medium levels of genetic diversity, with a few alleles and genotypes. We identified three genetically differentiated clusters, likely as a result of different habitat cover type. We also found evidence of an ancestral genetic bottleneck and medium values of effective population size. Inbreeding coefficients were low and there was a moderate gene flow. Our results can be used as a basis for future research and *C. triseriatus* conservation efforts, particularly considering that the Trans-Mexican Volcanic Belt is heavily impacted by destructive land-use practices.

Keywords Microsatellites · Endemic species · Conservation · Rattlesnake · Habitat fragmentation · Conservation genetics

Introduction

Mexico contains 8–12 % of all species in the world and is one of the five most biologically diverse countries in the world (Mittermeier and Goettsch de Mittermeier 1992; Challenger 1998). It is also ranked second in number of reptile species (Uetz 2015) and first in rattlesnakes, with 31 species (Beaman and Hayes 2008). Unfortunately, most of the country has suffered strong environmental disturbances in the last few decades, mainly due to unsustainable use of natural resources (Sarukhán et al. 2009). Rattlesnakes are abundant in many sites in Mexico, but their populations have declined due to direct hunting and habitat loss, caused by the expansion of urban areas (Campbell and Lamar 2004; Monroy-Vilchis et al. 2008).

The Trans-Mexican Volcanic Belt (TMVB; Fig. 1) is a biogeographic region with more species and endemisms than any other part of the country (León-Paniagua et al. 2007; McCormack et al. 2008; Navarro-Sigüenza et al. 2008; Bryson et al. 2011; Bryson and Riddle 2012). It is the most important region in terms of endemic species of herpetofauna and the second terms of the number of reptile species that inhabit the region (Flores-Villela and Canseco-Márquez 2004). The high diversity of this region is due to its complex geological history and high heterogeneity of climates, soils and vegetation types caused by the convergence of Nearctic and Neotropical regions (Luna et al. 2007; Jiménez-Velázquez 2013). TMVB is a geographical barrier that likely restricted gene flow of several taxa and prevented interaction between species and populations from the north and south

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✉ Octavio Monroy-Vilchis
tavomonroyvilchis@gmail.com; omv@uaemex.mx

[1] Centro de Investigación en Ciencias Biológicas Aplicadas, Universidad Autónoma del Estado de México, Instituto literario # 100, Colonia Centro, CP 50000 Toluca, Estado de México, Mexico

[2] Departamento de Producción Agrícola y Animal, Universidad Autónoma Metropolitana Xochimilco, Calz. Del Hueso 1100, 04960 Col. Villa Quietud, Mexico

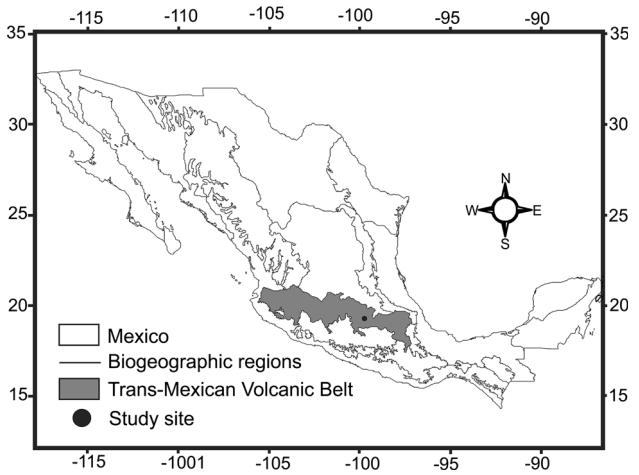

Fig. 1 Map of Mexico showing the Trans-Mexican Volcanic Belt and the study site

(Ruiz-Sanchez and Specht 2013). In addition, the TMVB contains several types of subtropical ecosystems that are separated by high mountains. The mountains act as ecological islands to restrict gene flow among lineages, and therefore promote diversification and speciation (Ruiz-Sanchez and Specht 2013). The TMVB is composed of 12,492.9 km^2, but is the most disturbed region in Mexico, with only 1346.9 km^2 (1.1 %) of *Abies* forest and 6507.7 km^2 (5.4 %) of *Pinus* forest remaining, the two principle habitats for the species, *Crotalus triseriatus,* a Mexican Dusky Rattlesnake. These habitats are also highly fragmented by agriculture (44.7 %) and urban settlements (3.4 %); in fact, some of the largest metropolitan areas in Mexico (such as Mexico City, Toluca, Puebla, Morelia and Guadalajara) are located in the TMVB. The valley of Mexico City is one of the largest metropolitan areas in the world (CONAPO 2010), with 2557.4 people per km^2. Reptiles are extremely sensitive to local habitat changes (Castellano and Valone 2006; Ribeiro et al. 2009) due to their ecological and physiological constraints, low dispersal capacity and small home ranges (Huey 1982). In fact, some studies found that reptiles and amphibians were more susceptible to landscape changes than other vertebrate taxa (White et al. 1997; Ribeiro et al. 2009).

C. triseriatus is endemic to Mexico and its distribution is restricted to the highlands, along the TMVB in eastern Michoacán, Estado de México, Morelos, Distrito Federal, Hidalgo, Tlaxcala, Puebla, and Veracruz states (Bryson et al. 2014). This species can be found in *Pinus-Abies* forests and grasslands associated with this type of forest, in altitudes from 2500 to 4572 MASL (Campbell and Lamar 2004). Although *C. triseriatus* is considered in the category of "least concern" (Canseco-Márquez and Mendoza-Quijano 2007) according to the IUCN, along with 29 other species of the genus *Crotalus*, this assignment may be due to a lack of knowledge on its distribution, ecology, natural history and genetic diversity (Canseco-Márquez and Mendoza-Quijano 2007; Bryson et al. 2014). Therefore, the aim of this study was to assess *C. triseriatus* genetic diversity and inbreeding, its effective population size, and the presence of bottlenecks in a highly modified landscape. These results will provide valuable information for management decisions to help preserve this endemic species. We expected, based on the species' life history, the presence of isolated and fragmented populations, and its endemic status, that the *C. triseriatus* study population would have low genetic variability, high genetic structure and low gene flow.

Materials and methods

Study area and population sampling

Sampling was conducted in a small and highly fragmented area (Figs. 1, 2) near the Toluca metropolitan area (19°24′54″N; 99°41′15″W, with an altitude of 2606 MASL) in the State of Mexico. In this area, crop fields, livestock and human settlements have replaced the majority of the native vegetation, and remnant populations of *C. triseriatus* persist in pastures, crops, and along the margin of crop fields. The study area had three different types of vegetation and land use; the first area had crop fields and wetlands, the second area had a fauna protection area and several areas of urbanization, and the third area had agricultural fields and livestock areas. 98 rattlesnakes were captured between February 2012 and August 2014. Tissue samples (ventral scale) were obtained and immediately placed in 90 % ethanol. All rattlesnakes were released immediately at the point of capture.

Habitat description

Satellite photographs of the study area were obtained from Google Earth (Google Inc.) to obtain spatial information about microhabitats of genetic clusters. In each image pixels were changed to meters using the software Image J 1.64r (Rasband 2012) and the area of each cover type was measured in each microhabitat. Cover types included water (W), grassland (G), agriculture (A), urbanization (U), minor road (MR), major road (MaR), water canal (WC), cattle grassland (CG), sheep grassland (SG) and protected wildlife area (PWA). In each locality, the percentage of each cover type was determined for multivariate analysis using a Spearman's correlation with a 95 % confidence level, to determine if microhabitat cover types (variables) were correlated with each other. In order to reduce the number of microhabitat variables, a principal components

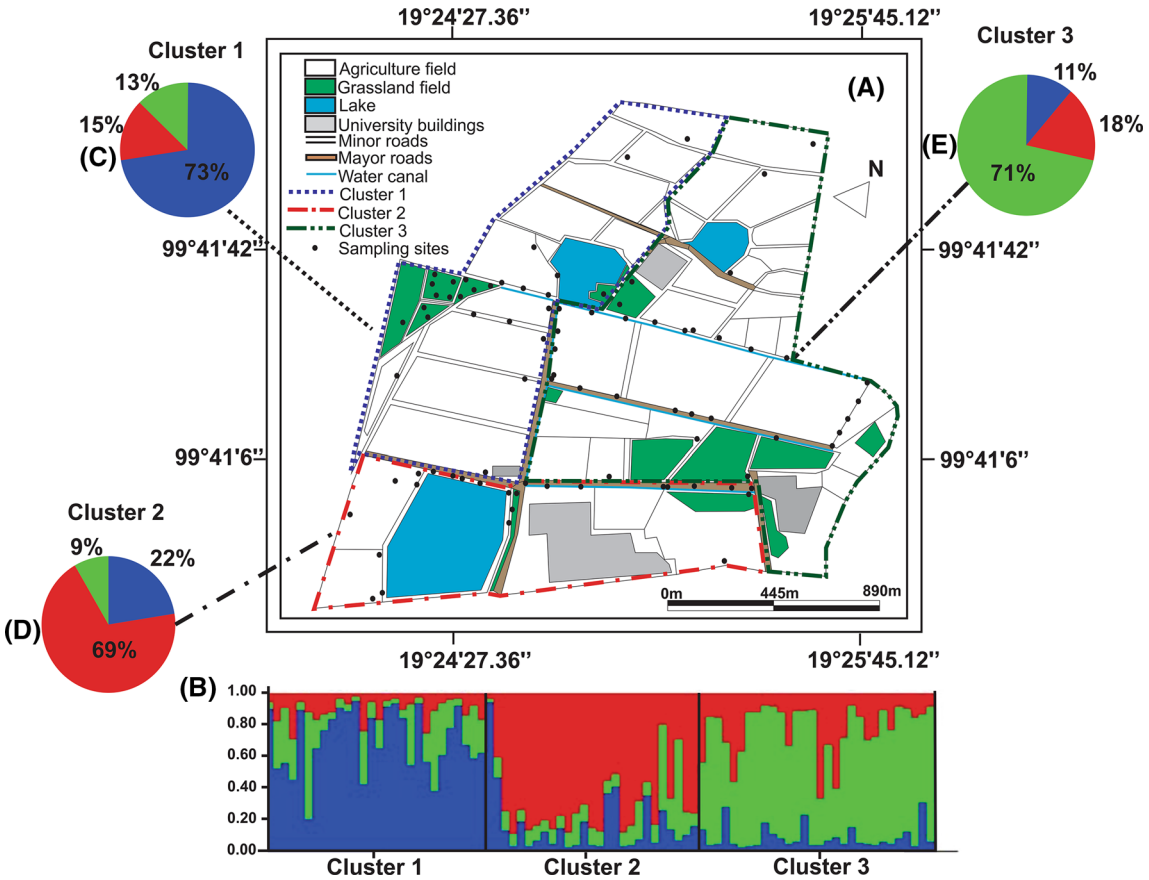

Fig. 2 **a** Study site: Cluster 1: *blue dotted line*, Cluster 2: *red line with one dot*, Cluster 3: *green line with two dots*. **b** Distribution of the three genetic clusters inferred from STRUCTURE; assignment graphs represent the mean membership coefficient for each individual to each genetic cluster. *c* Proportions of the three genetic clusters assigned by STRUCTURE to Cluster 1. *d* Proportions of the three genetic clusters assigned by STRUCTURE to Cluster 2. *e* Proportions of the three genetic clusters assigned by STRUCTURE to Cluster 3. (Color figure online)

analysis (PCA) was performed. Using the most important variables found in the PCA, we created a dendrogram with 1000 replicates to estimate bootstrap values using the Wards algorithm with a Euclidean similarity index, to determine microhabitat similarities. All statistical analyses were performed using the software Paleontological Statistics "Past" 3.06 (Hammer 2015).

Genetic analysis

DNA was extracted from rattlesnake scales using a commercial GF-1 nucleic acid extraction kit (Vivantis), following the manufacturer's instructions. Briefly, added 60 uL DTT (5 %), 3× of proteinase K, and lysis enhancer were added to rattlesnake scales, and the eluted DNA was used directly as a template for polymerase chain reaction (PCR). Eleven fluorescently labeled microsatellite loci were amplified: CWA29 and CWB6 (Holycross et al. 2002), 5A and 7-87 (Villarreal et al. 1996), CRTI09, CRTI05, CRTI08 and CRTI10 (Goldberg et al. 2003), CC1110 (Pozarowski et al. 2012), and MFRD5 and MFR15 (Oyler-McCance et al. 2005). PCR microsatellite products were multiplexed and run on an ABI Prism3730xl (Applied Biosystems), with Rox-500 as an internal size standard. Allele size was determined with the software PEAKSCANNER 1.0 (Applied Biosystems), and fragment lengths were measured and binned with TANDEM 1.08 (Matschiner and Salzburger 2009). Negative controls were included in all runs and standards were included in at least two runs to assure accuracy, precision, and reproducibility.

Statistical analyses

Identification of duplicate genotypes and potential scoring errors

GIMLET 1.3.2 (Valière 2002) was used to identify recaptured rattlesnakes and reduce error in the interpretation of the population's genetic diversity (Kohn et al. 1999). The presence of null alleles and other typing errors were determined using MICROCHECKER 2.2.3 (Van Oosterhout et al. 2004).

Genetic structure

Several approaches were used to assess the degree of genetic structure. First, genetic structure was defined with a Bayesian clustering method, which uses multilocus genotype data and identifies the number of K clusters (genetic groups) with the software STRUCTURE 2.3.4 (Pritchard et al. 2000; Falush et al. 2003; Hubisz et al. 2009). The number of K was determined by testing the K value from 1 to 8 and running the analysis ten times per K value in order to determine the maximum value of posterior likelihood [lnP(D)]. Each run was performed using 1,000,000 burn-in periods and 1,000,000 MCMC iterations with correlated allele frequencies (Falush et al.2003). A Dirichlet parameter was used for the degree of admixture, assuming that for several generations following population subdivision, the evolution of allele frequencies in each genetic group was correlated with the allele frequencies of an ancestral population, and without prior information on population origin. Therefore, we determined the most probable number of clusters to best represent our data, and we considered the change of ΔK (i.e. the ad hoc quantity related to the second order rate of change of the log probability of data) with respect to the number of clusters, using the maximum value of ΔK, following the method in Evanno et al. (2005), using the software STRUCTURE HARVESTER 0.6.92 (Earl and von Holdt 2011). The genetic variance distribution was calculated between and within populations and we used an analysis of molecular variance (AMOVA) based on F_{ST} as implemented by GENALEX 6 (Peakall and Smouse 2006). Significance was calculated using a Wilcoxon test with 30,000 permutations in order to detect the degree of similarity of the populations based on the populations' genotypes in GENALEX 6. In the software GENEPOP 4 (Raymond and Rousset 1995) F_{ST} was calculated with 10,000 dememorization steps and 1000 batches of 10,000 iterations per batch, in order to test the divergence between populations. We estimated Nei's genetic distance (Nei 1972) between sampling localities using the software GENALEX 6.

Genetic diversity

Genetic diversity indices were calculated for observed (A) and effective (A_e) number of alleles, number of genotypes, observed (Ho) and expected (He) heterozygosity, using the software GENALEX 6. All loci and departures from Hardy–Weinberg equilibrium (HWE) were tested with a Fisher's exact test and linkage disequilibrium (LD), which was assessed by a log-likelihood ratio statistic (G-test) using the software GENEPOP 4.0. Allelic frequencies and F_{IS} statistics were estimated to evaluate heterozygote deficiency or excess, derived from analysis of variance according to Weir and Cockerham (1984) (W&C), using GENEPOP 4.0, and significance tests were done using the Markov chain method implemented in GENEPOP 4.0, using 10,000 dememorization steps, 1000 batches and 10,000 iterations per batch. We used a False Discovery Rate (FDR) approach according to Benjamini and Hochberg (1995) using QVALUE software (Storey 2002) for R (version 3.0.1; R Development Core Team, 2013) to correct for multiple testing.

Migration, genetic bottlenecks, effective population size and relatedness

Gene flow between demes was estimated using the Bayesian inference implemented in MIGRATE-N 3.0 (Beerli 2008). Brownian motion was used with five independent runs using four long chains with a run of 10,000,000 genealogies sampled every 1000 steps and a burn in of 1,000,000. Four hot chains were used with temperatures: T1 = 1.0, T2 = 1.5, T3 = 3.0 and T4 = 1,000,000. Default values were applied for the remaining parameters. To estimate the number of migrants per generation (N_{em}), M was multiplied by θ (Beerli 2009, 2012). The θ values used in this calculation were: Cluster 1, θ = 0.01554; Cluster 2, θ = 0.04751; and Cluster 3, θ = 0.03271.

Using MSVAR 0.4.1 (Beaumont 1999) to implement coalescent simulations, we explored hypotheses about the historical signal of demographic expansion or contraction in a closed population. Each hypothesis was evaluated and the parameters were estimated via Bayesian inference. We estimated the rate of change (r) of the effective population size, defined as N_{crnt}/N_{stbl} (where N_{crnt} was the current inbreeding effective population size and N_{stbl} was the ancestral stable inbreeding effective population size). The r ratio was expressed in log10. Therefore, the population declined if r was negative, stable if r = 0 and, and expanded if r was positive (Gasca-Pineda et al. 2013). BOTTLENECK 5.1.26 software (Cournet and Luikart 1996; Piry et al. 1999) was used to test for a genetic signature of recent bottlenecks. Observed and expected heterozygosity were estimated under the infinite alleles model (IAM; in this mutational model every mutation event created a new allele, independent from the progenitor allele), step mutation model (SMM; adds or subtracts one or more repeat units from the string of repeats at some constant rate to mimic the process of mutations introduced during) and the two-phase model (TPM; allows a certain proportion of multistep mutations to involve a greater number of repeat units. TPM is an intermediate model of evolution that is considered more appropriate for microsatellites), with settings of 90 % step-wise mutation model, 10 % infinite allele model, and 10 % variance; and used default values (70 % step-wise 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 in the software BOTTLENECK 5.1.26. Relatedness among individuals was evaluated using the software ML-RELATE (Kalinowski et al. 2006), which is based on maximum-likelihood tests and uses genealogical relationships between individuals (represented mathematically) as probabilities that genotypes share zero, one or two alleles identical by descent. In addition, to explore demographic information of *C. triseriatus*, actual effective population size (Ne) was estimated by examining LD. Ne was estimated using the molecular co-ancestry method of Nomura (2008), as implemented in the software NEESTIMATOR 2 (Do et al. 2014).

Results

Population sampling

Ventral scales were collected from 98 rattlesnakes for DNA extraction and DNA of sufficient quality for genotyping was obtained from 85 samples.

Microhabitat PCA and cluster analysis

The PCA found that the first component had an eigenvalue of 1.24E + 11 and explained 96.97 % of the variation. The second component had an eigenvalue of 3.88E + 09 and explained 3.03 % of the variation; therefore, we restricted our analyses to the first component (Table A1). The cover types that distinguished the three microhabitat clusters from each other were: A, G (positive value), PWA and U (negative value) (Table A2 and Fig. 3), indicating that the land composition differed drastically between microhabitat clusters. Microhabitat clusters A and G were positive because they occupied large extensions while PWA and U were negative because there were few protected and urbanized areas and had small extensions (Table A3). A Wards dendrogram was constructed to examine the relationships within and between clusters, this analysis found more similarity between microhabitat Clusters 1 and 3 (Fig. 3), because they had a greater percentage of A (70.9 and 49.1 %, respectively), lower percentages of PWA (0 and 0 %, respectively), and lower percentages of U (2.3 and 2 %, respectively; Table A3).

Population genetic results

Identification of duplicate genotypes and potential scoring errors

We did not find evidence that the same rattlesnake was accidentally sampled twice when DNA samples were analyzed. Null alleles were observed for 2 loci (CRTI05 and CRTI10). After FDR correction, we found departures from HWE in CRTI10 (F_{IS} W&C = 0.164, $p = 0$), CRTI08 (F_{IS} W&C = 0.065, $p = 0.006$) and CRTI05 (F_{IS} W&C = 0.190, $p = 0$), due to heterozygote deficiency. If loci were not in HWE and had null alleles it is not appropriate to use them for inferring neutral population genetic structure (Falush et al. 2003) and genetic diversity indices (Shaw et al. 1999; Van Oosterhout et al. 2004). Therefore, we discarded these three loci for further analysis. We did not find LD between the loci in any population after FDR correction.

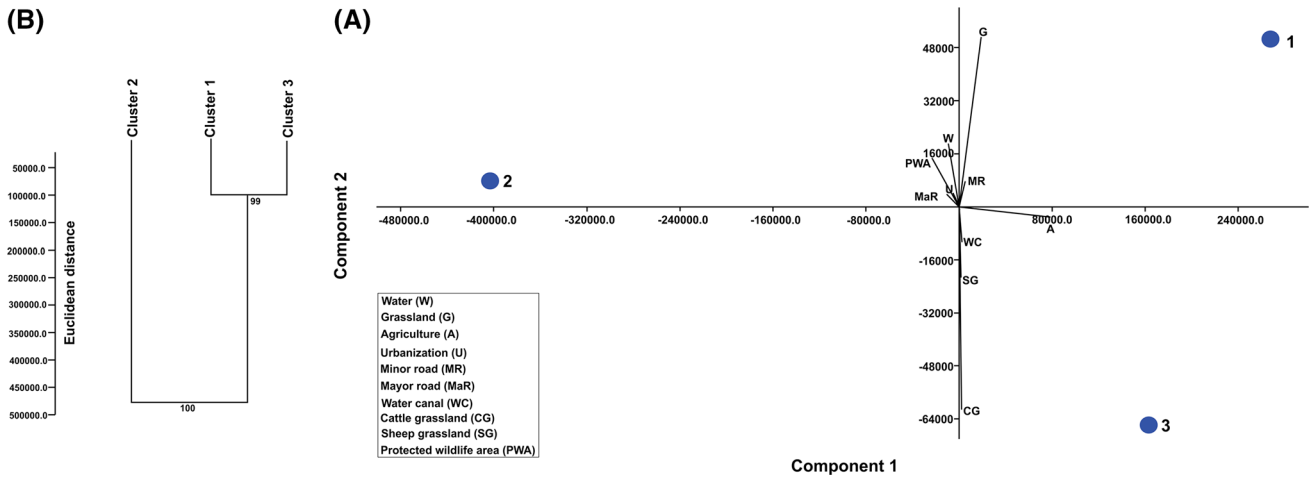

Fig. 3 *a* PCA. The first component explained 96.97 % of the variation and the second component explained 3.03 % of the variation. The most important cover types were: area covered by agriculture field (A), area covered by grassland (G), area covered by protected wildlife area (PWA) and area covered by urbanization (U). *b* Dendogram (using the Ward's algorithm with a Euclidean similarity index) showing three different types of microhabitats (Clusters 1, 2 and 3)

Genetic structure

The best log likelihood given by STRUCTURE was observed when K = 3 (LnPr (k = 3) = −1520.7) and ΔK chose the best model considering three populations (Cluster 1, N = 25; Cluster 2, N = 30; and Cluster 3, N = 30), rattlesnakes were systematically distributed in the same clusters (Fig. 2 and Fig. A1). Other methodologies showed little differentiation, and AMOVA results revealed that the majority of genetic variation resided within clusters (91 %; $p = 0.001$), followed by among clusters (9 %; $p = 0.001$) (Table A4). The F_{ST} and Nei's genetic structuring was low among clusters (Table 1); Clusters 1 and 2 had a pairwise $F_{ST} = 0.046$, Nei = 0.123 and the same was observed for Clusters 1 and 3 (pairwise $F_{ST} = 0.048$, Nei = 0.135). A greater difference was observed between the Clusters 2 and 3 ($F_{ST} = 0.053$, Nei = 0.161), although the values were not significantly different between clusters.

Genetic diversity

With regards to genetic diversity values, 32 alleles were identified across the eight loci in all clusters, with a range of 3–7 (average 3.79) alleles per locus: Cluster 1 had 3–4 alleles (mean = 3.5) with a total of 28 alleles, Cluster 2 had 3–6 alleles (mean = 3.85) with a total of 31 alleles, and Cluster 3 had 3–7 alleles (mean = 4) with a total of 32 alleles (Table 2; Fig. 4). Cluster 3 was the only sampling locality that had private alleles (Table 2).

We found 57 genotypes for all clusters (Table A5), with a range of 3–12 (average 7.12) genotypes per locus. Cluster 1 had 22 heterozygous genotypes and 17 homozygous genotypes with a total of 39 genotypes, Cluster 2 had 23 heterozygous genotypes and 23 homozygous genotypes with a total of 46 genotypes and Cluster 3 had 28 heterozygous genotypes and 23 homozygous genotypes with a total of 51 genotypes. Expected and observed heterozygosity in each cluster showed medium values: Cluster 1: $H_o = 0.590$, $H_e = 0.527$; Cluster 2: $H_o = 0.633$, $H_e = 0.594$; and Cluster 3: $H_o = 0.558$, $H_e = 0.568$ (Table 2).

Table 1 Genetic differentiation in each cluster

	Cluster 1	Cluster 2	Cluster 3
Cluster 1	–	0.123	0.135
Cluster 2	0.046	–	0.161
Cluster 3	0.048	0.053	–

Below the diagonal F_{ST}, above the diagonal Nei's genetic distance

Migration, genetic bottlenecks, effective population size and relatedness

All migration models had M values greater than one, indicating that migration (and not mutation) was the main factor contributing to genetic variation in these groups. Estimates of θ for the three demes were: Cluster 1 = 0.01554, Cluster 2 = 0.04751 and Cluster 3 = 0.03271. Migration rates per generation between all clusters were determined. Between Cluster 1 to Cluster 2 = 1.4, between Cluster 1 to Cluster 3 = 3.7, between Cluster 2 to Cluster 1 = 1.2, between Cluster 2 to Cluster 3 = 2.6, between Cluster 3 to Cluster 1 = 3.6 and between Cluster 3 to Cluster 2 = 1.6. Bayesian analysis of recent bottlenecks implemented in MSVAR indicated that all populations of *C. triseriatus* had evidence of population decline. Cluster 1: r = −3.474, Cluster 2: r = −3.570 and Cluster 3: r = −3.487. The MSVAR r estimate ranged from −1.129 for Cluster 2 to −3.623 for Cluster 1, indicating a historical bottleneck. BOTTLENECK results suggested a recent genetic bottleneck for the three clusters, with a variance of 30 % and probability of 70 % under the IAM model (Cluster 1, $p = 0.006$; Cluster 2, $p = 0.002$ and Cluster 3, $p = 0.014$) and with the TPM model (Cluster 2, $p = 0.009$ and Cluster 3, $p = 0.038$) and with a variance of 10 % and probability of 90 % under the IAM model (Cluster 1, $p = 0.006$; Cluster 2, $p = 0.002$ and Cluster 3, $p = 0.014$; (Table A6). The actual Ne estimated from LD with 0.05 allele frequency was Ne = 31.7 for Cluster 1, Ne = 33.4, for Cluster 2 and Ne = 29.4 for Cluster 3.

Furthermore, there was no evidence of inbreeding in the relatedness analysis. In Cluster 1, relatedness analyses showed the following results: unrelated (74.3 %), half-siblings (9.7 %), full siblings (9 %) and parent/offspring (7 %). The percentage of relatedness in each cluster was similar (Table A7). In addition, inbreeding coefficient values were low in all clusters: Cluster 1 (F_{IS}; from −0.149), Cluster 2 (F_{IS}; from −0.0.86) and Cluster 3 (F_{IS}; from 0.003), with a total F_{IS} value of −0.077 (Table 2).

Discussion

This was the first study to investigate *C. triseriatus* genetic variability and genetic structure from eight microsatellite loci.

Microhabitat

Cover types that differentiated between microhabitat cluster types were agriculture (A), grassland (G), protected wildlife area (PWA) and urbanization (U). Rattlesnakes require certain habitat conditions to survive, grow, and reproduce.

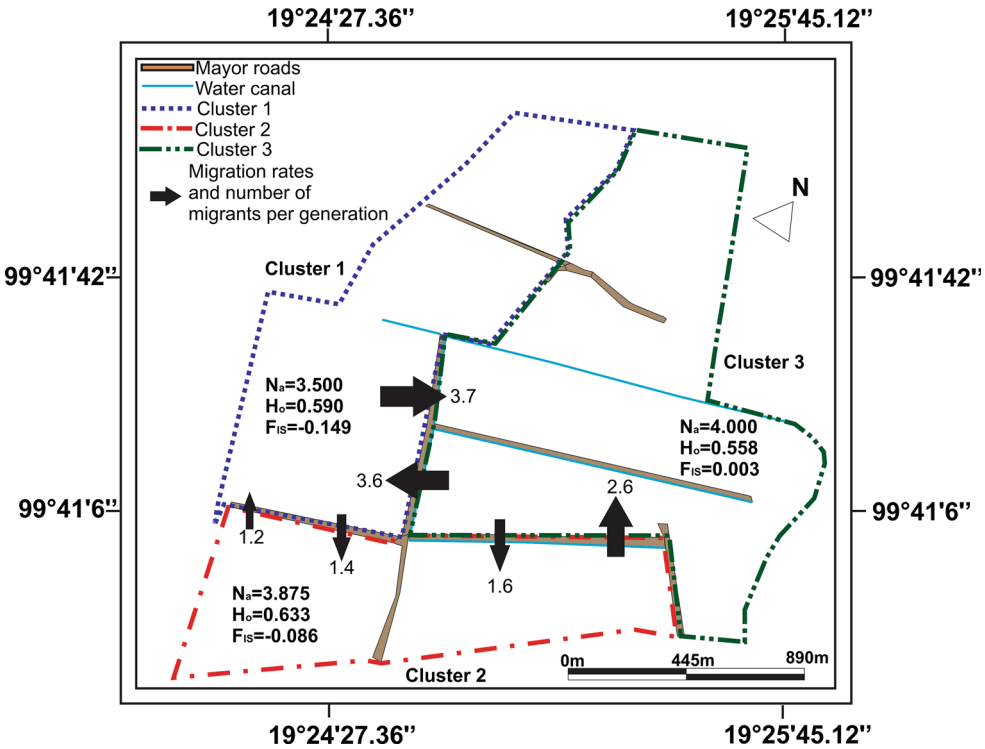

Fig. 4 Study site showing migration dynamics between clusters and genetic diversity in each cluster

Table 2 *C. triseriatus* genetic diversity values in each cluster and the entire cluster

	N	Na	Ne	Np	Ho	He	F_{IS}
Cluster 1	25	3.500	2.312	0	0.590	0.527	−0.149
Cluster 2	30	3.875	2.617	0	0.633	0.594	−0.086
Cluster 3	30	4.000	2.780	1	0.558	0.568	0.003
Total mean	28.333	3.792	2.570	0.333	0.594	0.563	−0.077

N sample size, *Na* number of alleles, *Ne* number of effective alleles, *Np* number of private alleles, *Ho* observed heterozygosity, *He* expected heterozygosity, F_{IS} fixation index

We believe that an agricultural habitat was a trade-off for rattlesnakes. Many small mammals live in are found in agriculture, which is favorable for rattlesnakes because they eat small mammals (Doonan and Slade 1995; Hoss et al. 2010). On the other hand, agricultural habitats provide insufficient cover for large-bodied rattlesnakes (Hoss et al. 2010) and may have unfavorably high ambient and substrate temperatures due to a lack of trees (Hoss et al. 2010). On large-scale agriculture, rattlesnakes are usually found in narrow rows of *Pinus, Abies* and grassland bushes that separate agriculture fields (the second important cover type in our analysis). Grassland bushes allow rattlesnakes to exploit the thermal gradients (Blouin-Demers and Weatherhead 2001b) and the high density of prey at the edge of crops (Martin et al. 2000; but see Blouin-Demers and Weatherhead 2001a). However, agriculture fields are also exposed to human activities, where practices such as agricultural burning or roller chopping may eliminate the advantages provided by agriculture fields and surrounding areas (Hoss et al. 2010). Therefore rattlesnakes might thrive in some seasons in agricultural fields, but in others they may suffer increased mortality above a threshold of reproductive sustainability (Hoss et al. 2010). Only the microhabitat of Cluster 2 had a protected area (PWA) surrounded by a fence to preserve flora and fauna. However, the fence or the extinction of other clusters could have led to inbreeding due to a disturbance in the natural dynamics of the metapopulation. Finally, although there was little urbanization (U) in the three clusters, urbanization and major roads (MaR) are increasing and this may lead to isolation of clusters and an increase in human-rattlesnake encounters.

Genetic diversity

In this small and highly fragmented population, we observed moderate heterozygosity values and allelic diversity that could have been caused by large founder size, large effective population size in the past, multiple paternity and

overlapping generations (Sunny et al. 2014a). Medium genetic diversity was found for the clusters obtained by STRUCTURE in contrast with what would be expected for small (<100 rattlesnakes) and isolated populations (Frankham 1998). These levels of genetic diversity were similar to that of *Crotalus* species and reptilian populations that suffered recent habitat fragmentation, population size reductions and genetic bottlenecks associated with founder effects (Prosser et al. 2000; Holycross and Douglas 2007; Clark et al. 2008; Jansen et al. 2008; Clark et al. 2010; Ávila Cervantes 2011; Vázquez-Domínguez et al. 2012). However, when the average number of alleles was considered, the clusters of *C. triseriatus* had fewer alleles than in most other *Crotalus* species and snakes (Na = 3.792; Table 2). Nevertheless, these results should be interpreted with caution because they are relative and factors such as number and type of loci, number of individuals and population characteristics all influence genetic diversity and allelic richness (Vázquez-Domínguez et al. 2013). It is possible that the low number of alleles found in the clusters is a sign that genetic diversity and allelic richness is declining as a result of habitat fragmentation, anthropogenic activities and isolation.

Genetic structure

We have three clusters (k = 3), therefore we may be detecting a relatively recent genetic structure. It is important to consider the biological and life history features that characterize *Crotalus* species and serpents in general, like the fine genetic structure over very short distances (1–2 km) is common in *Crotalus* species and serpents because of female philopatry (Gibbs et al. 1997; Bushar et al. 1998; Lougheed et al. 1999; Clark et al. 2008) and anthropogenic barriers (Keyghobadi 2007; Belkenhol and Waits 2009). Roads can have a strong impact on gene flow and population structure (Andrews and Gibbons 2005; Clark et al. 2010; Souza et al. 2015). In the study site there were several minor roads (MR) and major roads (MaR) (Fig. 2) that may have delimited the populations by limiting gene flow; snakes avoid roads because they don't like open areas and the high mortality suffered when they try to cross them (Rosen and Lowe 1994; Shine et al. 2004; Andrews and Gibbons 2005). Most of the road mortality occurred between Cluster 2 and Cluster 3 and Cluster 1. The causes of road mortality included being run over by cars (N = 15) or killed by people (N = 13) based on how their bodies looked on the road. Moreover, there were many water canals and fences in the study site that may acted as barriers to gene flow. Water canals are deadly mainly for juveniles; we found many juvenile bodies that appeared to have died from desiccation or drowning (N = 28), mainly belonging to Clusters 1 and 3. Cluster 2 was fenced and the rattlesnake population in this cluster had high heterozygosity values, likely because this area was protected. Despite these barriers, clusters behaved as metapopulations rather than isolated populations because gene flow still occurred between them, especially during mating season when males were more likely to move to other clusters to mate (Beck 1995; Clark et al. 2008).

Effective population size

The Ne observed for each cluster was 29.4–33.4, however, this estimate was sensitive to sample size and could have been underestimated (England et al. 2005). To avoid loss of genetic diversity and inbreeding a Ne > 50 is needed to minimize consanguinity effects, while a Ne > 500 is needed to retain adaptive genetic variation (Allendorf and Ryman 2002). Therefore, Ne values of 29.4–33.4 indicates that cluster populations likely suffered from multiple negative consequences associated with small population size, at both the demographic and genetic level (Frankham 1998; Eldridge et al. 2004).

Historical demography

We found bottlenecks in Clusters 2 and 3 under the TPM model when variance and probability were set to 30 and 70 %, respectively (Table A6). We also detected ancestral bottlenecks in all clusters, which could have been associated with two factors: 1) a founder effect when the population was separated from a larger ancestral population and 2) locals who kill rattlesnakes out of fear or beliefs that rattlesnake consumption will cure cancer and diabetes (Monroy-Vilchis et al. 2008). To solve all these environmental problems, it will be necessary to provide support to environmental education programs and increase awareness to farmers and ranchers, to avoid killing rattlesnakes.

Inbreeding and relatedness

Despite the small sample size and the characteristics of this species (endemic, restricted and isolated), our results show that the majority of rattlesnakes in a population are unrelated (74.3 %), indicating a low probability of inbreeding. The low relatedness may be due to inherent characteristics of the mating behavior and reproductive biology of *Crotalus* species members such as differential male dispersal and high juvenile mortality, which result in few relatives coexisting in a colony (Dixon 2011). Other reproductive strategies (like multiple paternity, sperm competition, long-term sperm storage, facultative parthenogenetic and kin recognition) that avoid inbreeding have also been observed in other *Crotalus* species (Schuett 1992; Sever and Hamlett 2001; Aldridge and Duvall 2002; Greene et al. 2002; Uller and Olsson 2008; Booth and Schuett 2011; Clark et al. 2014).

Conservation implications

To maintain the *C. triseriatus* population, it will be necessary to preserve highly endangered remnant patches of *Abies-Pinus* forest (Figueroa-Rangel et al. 2010; Vargas-Rodríguez et al. 2010; Ponce-Reyes et al. 2012; Bryson et al. 2014). As well, it will be necessary to preserve the connectivity between populations that sustain the natural dynamics of *C. triseriatus* populations and another species populations including *C. tlaloci*, *Barisia imbricata*, *Phrynosoma orbiculare*, *Plestiodon copei*, *Sceloporus torquatus*, *S. grammicus* and amphibians like: *Hyla eximia*, *H. plicata*, *Pseudoeurycea leprosa* and several species of the Genus *Ambystoma*. All these species are threatened by loss and habitat fragmentation. The TMVB has undergone habitat fragmentation and urbanization (Bryson et al. 2014; Galicia and García-Romero 2007). The TMVB is 44.7 % agriculture, 25.3 % roads, 3.4 % urban and only 1.1 % *Abies* forest and 5.4 % *Pinus* forest. Moreover, land-use change has increased in recent years; this will exacerbate *C. triseriatus* isolation and may potentially lead to its extinction (Neuwald 2010; Sunny et al. 2014b). In order to increase gene flow between fragmented populations, it will be necessary build corridors to increase migration between *C. triseriatus*. Several studies have indicated that corridors may reduce road mortality and promote gene flow in reptiles and amphibians (Yanes et al. 1995; Aresco 2005). To accomplish these conservation efforts, the genetic information provided in this study can be used as a basis for future research and conservation planning. Furthermore, *C. triseriatus* may be used as a proxy for other species in the region and reptiles in fragmented environments

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Compliance with ethical standards

Conflict of interest and ethical approval The authors declare that they have no conflict of interest. The manipulation of rattlesnakes was conducted with the approval of the ethics committee of Universidad Autónoma del Estado de México.

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