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Genetic diversity and structure of *Crotalus triseriatus*, a rattlesnake of central Mexico

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Abstract. The isolated and fragmented populations are highly susceptible to stochastic events, increasing the extinction risk because of the decline in putative adaptive potential and individual fitness. The population has high heterozygosity values and a moderate allelic diversity, the heterozygosity values are higher than in most other *Crotalus* species and snake studies. Possibly these high levels of genetic diversity can be related to a large founder size, high effective population size, multiple paternity and overlapping generations. We did not find the genetic structuring but the effective number of alleles (N_e) was 138.1. We found evidence of bottlenecks and the majority of rattlesnakes were unrelated, despite the small sample size, endemic status, the isolated and fragmented habitat. The genetic information provided in this study can be useful as a first approach to try to make informed conservation efforts for this species and also, important to preserve the habitat of this species; the endangered *Abies–Pinus* forest of the Nevado the Toluca Volcano.

Keywords. population genetics; conservation; microsatellites; endemic species; Nevado de Toluca; Mexico.

Introduction

Over the one-fifth of the land on planet is converted by small patches of forest, agriculture fields, urbanization, or use of land by human activity (Hoekstra *et al.* 2005; Ribeiro *et al.* 2009). Creating small habitat patches using small populations, isolated from conspecifics, reduce the gene flow and genetic diversity (Kim *et al.* 1998). This fragmentation and the reduced gene flow could increase the loss of genetic variability through genetic drift and increase the chance of inbreeding (O'Brien 1994; Frankham *et al.* 2005; Rueda Zozaya *et al.* 2016) and the extinction risk (Newman and Tallmon 2001; Johansson *et al.* 2006). This change in usage of land has been considered the most general threat to terrestrial biodiversity and ecosystems worldwide (Vandergast *et al.* 2007; Ribeiro *et al.* 2009), given that it affects directly the distribution of the fauna and metapopulation dynamics of the species (Marsh and Trenham 2001; Jehle *et al.* 2005; Cushman 2006). Mexico is one of the mega-diverse countries (Mittermeier and Goettsch de Mittermeier 1992; Challenger 1998), ranked

second worldwide in its number of reptile species, next to Australia (Uetz and Hosek 2015), with 31 rattlesnake species, has been positioned first worldwide (Beaman and Hayes 2008). Unfortunately, in recent decades, the human activities have led to the fragmentation and loss of habitat (Sarukhán *et al.* 2009). This habitat change affects the populations of amphibians and reptiles more than other vertebrate taxa (Jäggi and Baur 1999; Woinarski and Ash 2002; Anadón *et al.* 2006; Castellano and Valone 2006; Ribeiro *et al.* 2009; Sunny *et al.* 2015) because of their small home ranges, high philopatry and low vagility (Huey 1982; Ribeiro *et al.* 2009). Therefore, the threats that are contributing to the decline in rattlesnake populations are the urban expansion, and habitat loss and fragmentation (Campbell and Lamar 2004; Monroy-Vilchis *et al.* 2008). *Crotalus triseriatus* is an endemic pigmy rattlesnake from the highlands of central Mexico, especially along the Trans-Mexican Volcanic Belt (TMVB, figure 1). This area is the most important in the country, considering the number of endemic reptile species (Flores-Villela and Canseco-Márquez 2004) and also have a strong
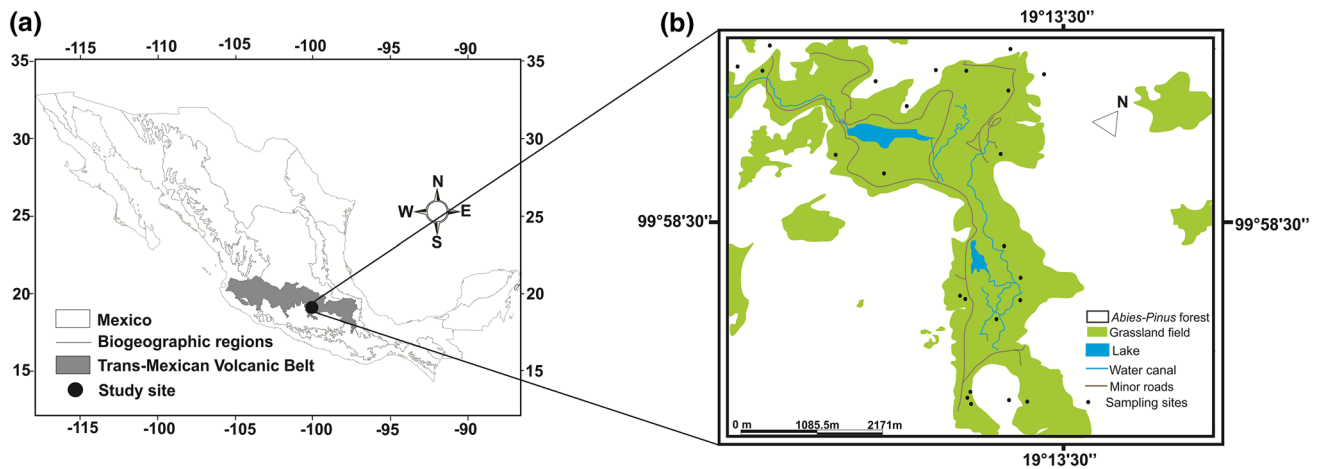
Figure 1. (a) Map of Mexico showing the Trans-Mexican Volcanic Belt, the State of Mexico and Mexico City. (b) The site for the population genetic study.

anthropogenic transformation of the ecosystems, ∼ 44.7% of this region is used for agricultural purposes, the 3.4% for urban settlements and just 1.1% of *Abies* forest and 5.4% of *Pinus* forest remain (Sunny *et al.* 2017). These types of forest are the most important habitats of *C. triseriatus* (Bryson *et al.* 2014; Sunny *et al.* 2015). This anthropogenic transformation of the ecosystem could increase the extinction risk of *C. triseriatus* due to the loss and fragmentation of their habitat. Therefore, the objective of this study was to describe the genetic variability and structure, inbreeding, its effective population size, and the presence of bottlenecks in a grassland field surrounded by *Abies–Pinus* forest. These results will provide valuable information for management decisions to help preserve this endemic species.

Materials and methods

Study area and population sampling

The sampling was conducted in a grassland area surrounded by *Abies–Pinus* forest in the protected area of the Nevado de Toluca volcano (19°12′24″N; 99°56′36″W, with an altitude of 2865 meters above sea level; figure 1). The intervening matrix is extremely urbanized and populated by the metropolitan valley of Toluca city, the greatest changes in land use and vegetation cover occurred during the period from 1964 to 1990 (FAO 2001 The state of world's forests (http://www.fao.org/docrep/003/y0900e/y0900e00.htm); Rodríguez-Soto *et al.* 2011; Domínguez-Vega *et al.* 2012), therefore, this population is isolated in a sky island system. Tissue samples (ventral scale) were obtained and placed in 90% ethanol. All rattlesnakes were immediately released at the point of capture. The manipulation of rattlesnakes was conducted with the approval of the ethics committee of the Autonomous University of the

State of Mexico and the authors declare that they have no conflict of interest.

Genetic analysis

DNA was extracted from ventral scales following the instructions that accompany the GF-1 Nucleic Acid extraction kit (Vivantis). Briefly, 60 μL DTT (5%) was added, 3x of proteinase K, and lysis enhancer, and the eluted DNA was used directly as a template for polymerase chain reaction (PCR). Eleven fluorescently labelled 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. PEAKSCANNER 1.0 (Applied Biosystems) software was used to obtain allele sizes. TANDEM 1.08 (Matschiner and Salzburger 2009) software was used to measure and bin the fragment lengths. Negative controls were included in at least two runs to guarantee reproducibility.

Statistical analyses

Identification of duplicate genotypes and potential scoring errors: We thoroughly checked so that we did not have recaptured rattlesnakes to avoid mistakes in interpreting the population's genetic variability using GIMLET 1.3.2 software (Valiére 2002). MICROCHECKER 2.2.3 software (Van Oosterhout *et al.* 2004) was used to test the presence of null alleles and other typing errors. In addition, using POPPR 2.4.1 (Kamvar *et al.* 2014) for the R

software (ver. 3.4.0; R Development Core Team 2017), we made an analysis to create a genotype accumulation curve, useful for determining the minimum number of loci necessary to discriminate between individuals in a population (Kamvar *et al.* 2014). This function will randomly sample loci without replacement and count the number of multi-locus genotypes observed (Kamvar *et al.* 2017).

Genetic structure

We searched for the genetic structure using several algorithms. First, we used a Bayesian algorithm in combination with STRUCTURE 2.3.4 software (Pritchard *et al.* 2000; Falush *et al.* 2003; Hubisz *et al.* 2009). Due to the lack of genetic structure found in previous studies and based on exploratory analyses of these data and the Delta K Evanno plot (not shown), we decided to explore the values of K from 1 to 10, by running the analysis 10 times per K value to determine the maximum value of posterior likelihood [lnP(D)]. The parameters we chose were correlated allele frequencies with 1,000,000 burn-in periods and 1,000,000 MCMC iterations (Falush *et al.* 2003). In addition, using ADEGENET 2.0.1 (Jombart *et al.* 2016) and ADE4 1.7-6 (Dray and Siberchicot 2017) packages for the R software, we performed the principal components analysis; this analysis represented the genetic structure in two complementary ways: by the distances, further away, more genetically different and by the different gray scales, more different the shade of gray, the more they were genetically different (Jombart *et al.* 2016). We tested the existence of population structure computing. Further, we estimated Nei's genetic distance (Nei 1972) among individuals applying 1000 bootstraps in POPPR 2.4.1 and MAGRITTR 1.5 (Bache and Wickham 2016) for the R software.

Genetic diversity

Using GENALEX (Peakall and Smouse 2006) and STRATAG 2.0.2 (Archer *et al.* 2017) for the R software, we calculated observed (H_o) and expected (H_e) heterozygosity, the number of alleles (N_a), the effective number of alleles, the number of genotypes (N_e), and the number of heterozygote and homozygote genotypes and also the evenness indices in POPPR. Departures from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) between pairs of microsatellite loci and within each sample and locus were evaluated using a Markov chain approximation considering 10,000 dememorizations, 1000 batches, and 10,000 iterations per batch. The allelic frequencies and F_{IS} statistics were estimated to evaluate heterozygote deficit or excess, derived from analysis of variance according to Weir and Cockerham (1984) implemented in the software GENEPOP 4.0 (Raymond and Rousset 1995). To correct the *P* values, we used a false discovery rate (FDR) approach as described by Benjamini and Hochberg (1995). This was implemented using R and QVALUE software (Storey 2002).

Demography history

***Genetic bottlenecks, relatedness and effective population size*:** Historical signals of demographic fluctuations were explored by applying a Bayesian algorithm, which was implemented using Markov-swtiching vector autoregression (MSVAR) 0.4.1 (Beaumont 1999; Girod *et al.* 2011) software. We estimated the rate of change (*r*) of the effective population size, defined as N_{crnt}/N_{stbl} (where N_{crnt} is the current inbreeding effective population size and N_{stbl}, the ancestral stable inbreeding effective population size). The *r* ratio was expressed in log10. Thus, the population declined if we had a negative *r* value, stable if *r* was equal to zero and, the population is expanded if the *r* value was positive (Gasca-Pineda *et al.* 2013; Sunny *et al.* 2015). We used a *t*-test to assess the existence of significant differences between subpopulations. To test for a genetic signature of recent bottlenecks, we used the BOTTLENECK 5.1.26 (Cournet and Luikart 1996; Piry *et al.* 1999) software. We estimated the observed and expected heterozygosity under three different mutation models (i) the infinite alleles model (IAM), (ii) the step mutation model (SMM) and (iii) the two-phase model (TPM). Also, because the TPM is an intermediate model of evolution considered more appropriate for microsatellites, we interpreted the result based just on this model and considering the distribution of the mode shifts. The settings applied were for a 90% step-wise mutation model, 10% infinite allele model, and 10% variance. The default values used were those appropriate for a 70% step-wise mutation model, 30% infinite allele model, and 10% variance. Both settings were run using 10,000 replicates. Excess heterozygosity was tested using a Wilcoxon test in the software BOTTLENECK 5.1.26. Relatedness among individuals were evaluated using the software ML-RELATE (Kalinowski *et al.* 2006), in addition, we obtained the F_{IS} value in GENEALEX as an indicator of total inbreeding in the population. Finally, to explore the actual effective population size (N_e), we used the molecular coancestry method of Nomura (2008), as implemented in the software NEESTIMATOR 2 (Do *et al.* 2014).

Results

Population sampling

We found 50 live adult rattlesnakes (mean = 56 cm) and four live juvenile snakes (mean = 20 cm), but we just obtained sufficient quality of DNA for genotyping the 45 tissue samples.
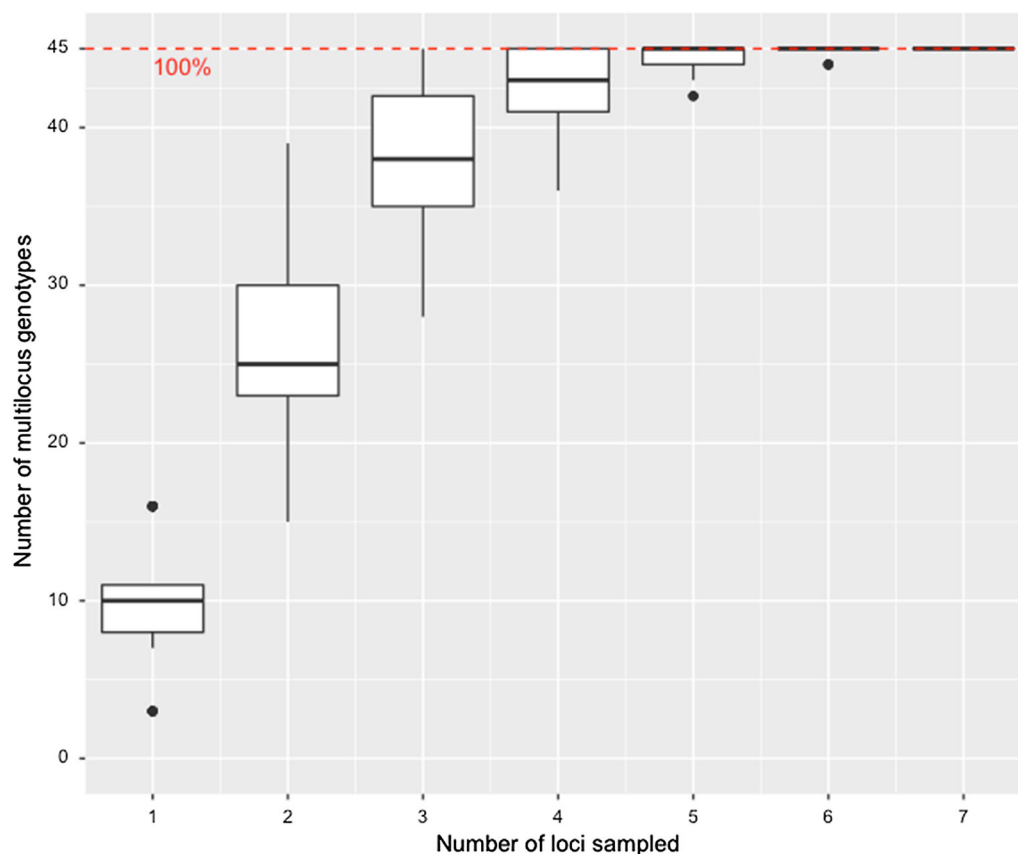
Figure 2. Genotype accumulation curve to determine the minimum number of loci necessary to discriminate between individuals in the population.

Population genetic

Identification of duplicate genotypes and potential scoring errors: We did not find confirmation of recaptured rattlesnake when DNA samples were analysed. We did not find null alleles or another typing error in any loci and finally, the FDR correction did not find LD between the loci. The genotype accumulation curve found that the minimum number of loci necessary to discriminate between individuals in a population was six (figure 2). Therefore, we conclude that our study has enough microsatellite primers ($n = 9$).

Genetic structure

The maximum log likelihood assumed by STRUCTURE was detected when K = 1 (LnPr (k = 1) = −1097.6) also neighbour-joining (N–J) tree. The PCA of pairwise F_{ST} and the Goudet's G values show low values of genetic structuring. (figures 3 and 4).

Genetic diversity

Across the eight loci, 3–12 alleles were identified (mean length = 7.5) with a total of 60 alleles (table 1;
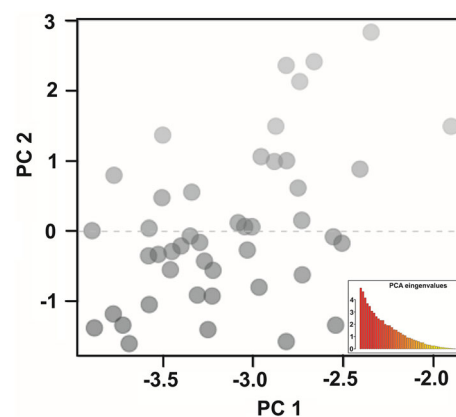

Figure 3. Scatterplot showing the relationships among the two populations based on a principal components analysis of eight microsatellite genotypes, this analysis represented the genetic structure in two complementary ways: by the distances, further away, more genetically different and by the different gray scales, more different the shade of gray, the more genetically different.

figure 5). We found 54 heterozygous genotypes and 28 homozygous genotypes with a total of 82 genotypes. The population showed medium values of $H_o = 0.722 \pm 0.044$, $H_e = 0.693 \pm 0.043$ (table 1).
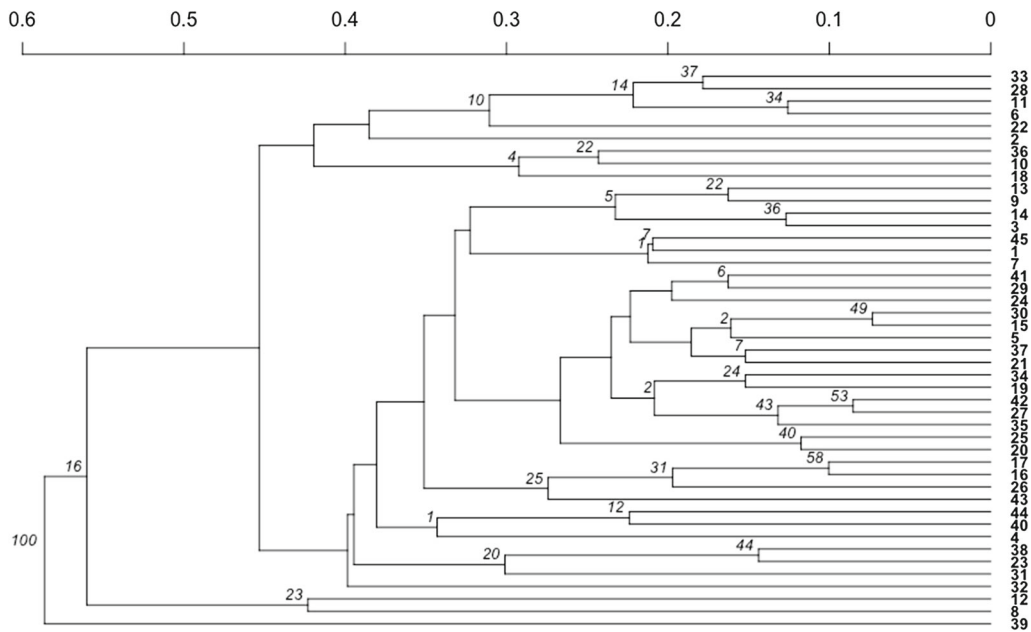
Figure 4. Tree constructed by the NJ method using the estimated standardized genetic distances Nei DS (Nei 1972) using a bootstrap of 1000.

Genetic bottlenecks, effective population size and relatedness

MSVAR showed a bottleneck, $r = -3.067$. The MSVAR r estimate ranged from -3.941 to -1.221 and the BOTTLE-NECK results yielded a normal L-shaped distribution, suggesting no recent genetic bottleneck. The data suggested a recent genetic bottleneck under the infinite alleles model (I.A.M.) $P = 0.034$ (table 1). The actual N_e estimated from LD with 0.05 allele frequency was $N_e = 138.1$. Further, there was no evidence of inbreeding signs in the relatedness analysis: unrelated (83.7%), half-siblings (9.1%), full siblings (6.7%) and parent/offspring (0.6%). In addition, inbreeding coefficient values were negative and low in the population ($F_{IS} = -0.053$; table 1).

Discussion

Genetic diversity

The population had high heterozygosity values and a moderate allelic diversity, the heterozygosity values were higher than in most other *Crotalus* species and snake studies (H_o: 0.722 and $N_a = 7.500$; Holycross *et al.* 2002; Goldberg *et al.* 2003; Oyler-McCance *et al.* 2005; Anderson 2006; Holycross and Douglas 2007; Clark *et al.* 2008; Munguia-Vega *et al.* 2009; Oyler-McCance and Parker 2010; Ávila-Cervantes 2011). Possibly these high levels of genetic diversity can be related to a large founder size, high effective population size, multiple paternity and overlapping generations (Sunny *et al.* 2014a). For these reasons it is important to preserve the habitat of this species; the endangered *Abies–Pinus* forest of the Nevado

the Toluca Volcano and the TMVB (Figueroa-Rangel *et al.* 2010; Vargas-Rodríguez *et al.* 2010; Ponce-Reyes *et al.* 2012; Bryson *et al.* 2014; Heredia-Bobadilla *et al.* 2016, 2017), this is because the TMVB has the great extent of the *Abies* forest (91.143%) and a great percentage of *Pinus* forest (29.657%) in the country, but these types of forest are scarce in the TMVB just 1.1% of *Abies* forest and 5.4% of *Pinus* forest covers the biogeographic zone (Sunny *et al.* 2017). Unfortunately, government laws have recently changed the protection status of the Nevado de Toluca Volcano (Diario Oficial de la Federación Mexicana 2013). This change in protection status could lead to logging and changes in land use (Mastretta-Yanes *et al.* 2014), affecting *C. triseriatus* population and the populations of many other species of amphibians and reptiles of the TMVB (González-Fernández *et al.* 2018).

Genetic structure

We did not find the genetic structure in our population which could be related to the fact that in our study site there were no anthropogenic barriers like roads and urbanization which are two of the most difficult features to avoid in snakes (Andrews and Gibbons 2005; Keyghobadi 2007; Belkenhol and Waits 2009; Clark *et al.* 2010; Souza *et al.* 2015), possibly the migrations in mating season is favouring that the population is not structured, some studies have shown that snakes can migrate long distances of 7–14.99 km during mating season (Beck 1995; Clark *et al.* 2008), but as this is a small and fragmented population with a small population size this could lead to a deleterious genetic effects associated with inbreeding and reductions
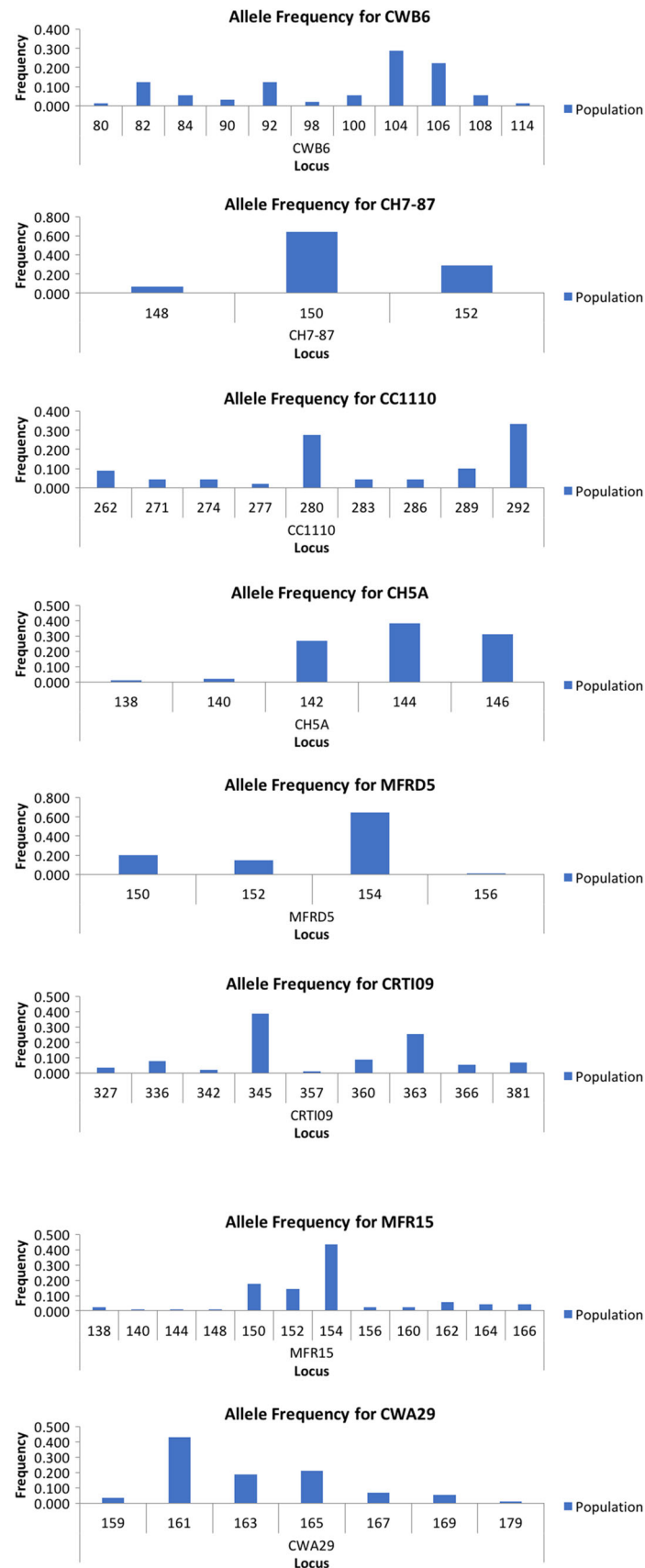
Figure 5. *C. triseriatus* allelic frequencies of loci in the population.

Table 1. *C. triseriatus* genetic diversity values.

Locus	N_a	N_e	A	H_o	H_e	Evenness	F_{IS}
CWB6	11	5.753	0.244	0.756	0.826	0.76	0.085
CH7-87	3	1.987	0.066	0.711	0.497	0.77	− 0.431
CC1110	9	4.661	0.200	0.778	0.785	0.72	0.010
CH5A	5	3.127	0.111	0.822	0.680	0.89	− 0.209
MFRD5	4	2.100	0.088	0.422	0.524	0.71	0.194
CRTI09	9	4.171	0.200	0.778	0.760	0.69	− 0.023
MFR15	12	4.014	0.266	0.733	0.751	0.60	0.023
CWA29	7	3.613	0.155	0.778	0.723	0.74	− 0.075
Mean	7.5	3.678	0.166	0.722	0.693	0.74	− 0.053
Standard error	1.165	0.449	0.022	0.044	0.043	0.025	0.068

N_a, number of alleles; N_e, number of effective alleles; A, allelic richness; H_o, observed heterozygosity; H_e, expected heterozygosity; evenness, evenness index; F_{IS}, fixation index.

in genetic variability, increasing the risk of extinction because of the decline in putative adaptive potential and individual fitness (Bijlsma *et al.* 2000; Mitrovski *et al.* 2007; Castañeda-Rico *et al.* 2011). The low number of alleles detected could be indicative of allelic fixation, associated with a genetic and demographic deterioration associated with isolation and decrease in an effective population size (Castañeda-Rico *et al.* 2011).

Effective population size

The N_e found was moderate ($N_e = 138.1$), despite this estimate being sensitive to sample size and could be underestimated (England *et al.* 2005), it gives a good approximation of the real population numbers (Sunny *et al.* 2014a). N_e is a measure directly related to the rate of genetic diversity loss and the increase of inbreeding in a population, and some studies report that a $N_e > 50$ is necessary to avoid the loss of genetic diversity and inbreeding effects (Allendorf and Ryman 2002). Therefore, this value is sufficient to avoid inbreeding effects and may be this population has a stable *C. triseriatus* population of thousands of individuals. Hence, to retain adaptive genetic variation, a minimum of 500 individuals is required (Allendorf and Ryman 2002). In addition, this estimate is affected by population size fluctuations which is important to consider, since in the study area each year the pasture where rattlesnakes inhabit are burnt and occasionally the forest authorities make roller chopping reforestation, causing bottlenecks to the rattlesnake population, therefore, multiple negative consequences can be associated with a small population size, both at the demographic and genetic levels (Frankham 1998; Eldridge *et al.* 1999).

Historical demography

We did not find signatures of bottleneck with BOTTLENECK software, however, these results have to

be interpreted with caution since for this software to achieve reasonably high statistical power (0.80), it is necessary for at least 10 polymorphic loci and the standardized differences test is recommended using ∼20 or more polymorphic loci (Cournet and Luikart 1996). Therefore, we complemented our bottleneck analyses with the one implemented in MSVAR (Girod *et al.* 2011), and found the signatures of a bottleneck, these bottlenecks could be related (i) due to pasture burning and the roller chopping reforestation practices and (ii) the local people kill the rattlesnakes for fear or beliefs that rattlesnake consumption will cure cancer and diabetes (Monroy-Vilchis *et al.* 2008; Sunny *et al.* 2015). Further, it will be necessary to provide support to environmental education programmes and increase awareness to authorities, farmers and ranchers, to avoid further killing of rattlesnakes for fear or bad reforestation practices.

Inbreeding and relatedness

The data indicate that the majority of rattlesnakes are unrelated, despite the small sample size, the endemic status and the isolated and fragmented habitat. Different mechanism of mating behaviour and reproductive biology of *Crotalus* species could help to prevent endogamy like: high juvenile mortality decreasing the percentage of close relatives coexisting in the same colony (Dixon 2011), male dispersal, multiple paternity, sperm competition, long-term sperm storage, facultative parthenogenetic and kin recognition (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

Conservation actions should aim to maintain enough suitable habitat for *Abies–Pinus* forest and landscape connectivity between patches of forest to sustain the natural

dynamics of this species, and all other amphibian and reptile species of this habitat (Figueroa-Rangel *et al.* 2010; Vargas-Rodríguez *et al.* 2010; Ponce-Reyes *et al.* 2012; Bryson *et al.* 2014), to preserve genetic diversity and survival over the long term (Cushman 2006). The TMVB is a region highly fragmented due to changes in land use. This habitat fragmentation and the limited vagility of the snakes (a few tens of hectares per year) and movement rates of some tens of meters per day (Macartey *et al.* 1988; Sasa *et al.* 2009; Hoss *et al.* 2010) could have long-term consequences, such as a decrease in genetic diversity and increased chance of inbreeding (Espindola *et al.* 2014; Sunny *et al.* 2014a, b). This reduced genetic variability could reduce fitness and increase the susceptibility of disease (Frankham *et al.* 2005; Townsend *et al.* 2009; Rulon *et al.* 2011) such as the proliferation of fungal pathogens (Jacobson *et al.* 2000; Paré *et al.* 2003; Rajeev *et al.* 2009; Allender *et al.* 2011, 2015; Guthrie *et al.* 2015; Tetzlaff *et al.* 2015; Glorioso *et al.* 2016), including some that cause widespread mortality in free-ranging rattlesnake populations (Cheatwood *et al.* 2003; Allender *et al.* 2011, 2015; Guthrie *et al.* 2015; Tetzlaff *et al.* 2015; Glorioso *et al.* 2016). This process increases extinction risk (Newman and Tallmon 2001). Also, *C. triseriatus* are facing the potential effects of climate change. This species of serpent and *Thamnophis scalaris* are the snakes that inhabit the highest areas of the American continent (Campbell and Lamar 2004; González-Fernández *et al.* 2018). As climate change takes hold, this montane species, which prefers cooler temperatures associated with the highest elevations, will be limited in its ability to shift distribution upslope, increasing the possibility of becoming extinct (González-Fernández *et al.* 2018). However, over the short term, it is urgent to implement environmental education to avoid further rattlesnake killing due to fear or reasons related to uses in the context of traditional medicine (Monroy-Vilchis *et al.* 2008). We must also prevent the introduction or substitution of species and pursue reforestation practices that do not involve roller chopping or agricultural burning (Mullin and Seigel 2009). Although some *Crotalus* species are quite tolerant or can even be beneficial to the small patches of crops, probably due to the small preys that live there (Bastos *et al.* 2005; Mociño-Deloya *et al.* 2009; Reinert *et al.* 2011; Wittenberg 2012; Mociño-Deloya *et al.* 2014), only the crops close to *Abies–Pinus* forests seems to be suitable habitats for *C. triseriatus* (Mociño-Deloya *et al.* 2014). Therefore, it is important to preserve the *Abies–Pinus* forest for several reasons, one of them is the possibility that the rattlesnakes could find a different kind of food (e.g. rodents, amphibians, lizards, birds, eggs; Mociño-Deloya *et al.* 2014), in anthropized areas, the large rattlesnakes have insufficient refuges and they do not have enough cover type and variety of vegetation to allow exploitation of the thermal gradients (Blouin-Demers and Weatherhead 2001; Hoss *et al.* 2010; Sunny *et al.* 2015), the lack of different types of vegetation limits the growth of

snakes and therefore only medium-sized rattlesnakes were found. To achieve these conservation strategies, the genetic information provided in this study can be used as a first approach to try to make inform conservation efforts, but also, we suggest to make more population genetic studies in other small patches to get more accurate data and a better understanding of the population genetic status of this species.

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