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RESEARCH ARTICLE

An interstate highway affects gene flow in a top reptilian predator (*Crotalus atrox*) of the Sonoran Desert

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Abstract Roads can substantially impact the population connectivity of a wide range of terrestrial vertebrates, often resulting in loss of genetic diversity and an increase of spatial genetic structure. We studied the Western Diamond-backed Rattlesnake (*Crotalus atrox*), a large and abundant venomous predator, to test the hypothesis that a large and relatively new roadway in Arizona (Interstate Highway I-10) is a barrier that impacts gene flow and population genetics via habitat fragmentation. Based on 72 *C. atrox* sampled from three specific sampling sites ("subpopulations") on both the west and east corridors of I-10, we used 30 nuclear microsatellite DNA loci and three mitochondrial DNA genes (2615 bp) to assess genetic diversity and structure, estimate effective population size (N_e), and describe patterns of gene flow. We found no evidence for loss of genetic diversity or a decrease in N_e between the three subpopulations. Our microsatellite analysis showed that two subpopulations in close proximity (4 km), but separated by I-10, showed greater levels of genetic differentiation than two subpopulations that were separated by a greater distance (7 km) and not by I-10 or any other obvious barriers. Mitochondrial DNA analyses showed no significant genetic differentiation nor any indication of historically impeded gene flow. Tajima's *D* and mismatch distribution tests revealed that demographic expansion is occurring in the overall population (all three subpopulations). Bayesian clustering and spatial genetic autocorrelation analyses of microsatellite data showed resistance to gene flow at the approximate location of I-10. Simulations that investigated gene flow between the subpopulations (with and without a highway barrier present) were consistent with our molecular results. We conclude that I-10 has reduced gene flow in a population of an important reptilian predator of the Sonoran Desert in southern Arizona and make conservation recommendations for reversing this trend.

Keywords Linear barrier · Road · Gene flow · Genetic diversity · Dispersal · Snakes

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Introduction

The detrimental effects of roads on wildlife are numerous and include depletion (mortality), habitat destruction, and fragmentation of populations, which, in turn, can have long-term and serious genetic consequences (Rosen and Lowe 1994; Shepard et al. 2008a, b; Balkenhol and Waits 2009; Jackson and Fahrig 2011; Brehme et al. 2013). Genetic effects include decreased diversity and increased structure (Hoehn et al. 2007; Balkenhol and Waits 2009; Clark et al. 2010; Munguia-Vega et al. 2013). In simulations successful movements across roads can mitigate the deleterious impacts of disperser mortality only in cases with large populations, moderate to high migration rates, low rates of decline, and when enough time has passed (Jackson and Fahrig 2011).

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Genetic data and associate analytical methods that have been used to investigate the effects of roads on wildlife populations can detect changes in dispersal patterns and genetic structure even at incipient stages (Balkenhol and Waits 2009; Holderegger and Di Giulio 2010; Dileo et al. 2013; Sommer et al. 2013). When large populations undergo substantial division, their effective population size may reduce the effects of inbreeding, loss of genetic diversity, and population differentiation (Manel et al. 2005; Holderegger and Di Giulio 2010; Banks et al. 2013). In such cases, detecting a barrier effect in a large population with only relatively slight genetic changes can be challenging. However, the genetic methods used to infer contemporary migrations across roads (e.g., assignment tests which can identify first-generation migrants) have become sufficiently sophisticated and powerful to detect such small changes, even in large populations. Nonetheless, understanding the ramifications of human-induced habitat fragmentation on dispersal is often problematic owing to lack of genetic data of a population prior to the fragmentation event (Balkenhol and Waits 2009; Beebee 2013).

Several studies on the effects of road barriers in terrestrial vertebrate communities have shown that large roads can impede gene flow in amphibians (Marsh et al. 2008; Richardson 2012) and both small (Ascensão et al. 2016) and large mammals (Riley et al. 2006, 2014; Frantz et al. 2012; Hartmann et al. 2013). Conversely, in some studies, gene flow restrictions appear to be minimal (or nondetectable) in species of amphibians (Prunier et al. 2014) and mammals (Frantz et al. 2012; Anderson et al. 2015; Yokochi et al. 2016). Thus, the effects of roads on genetic population structure are modulated by road size and traffic intensity, as well as individual species ecology. Detecting genetic effects can be demanding due to considerable lag time, as roads are often relatively young and have changing use over time, and species generation times can vary. Lag time is pronounced in species with large effective population size and hence slows genetic drift to new equilibrium.

Many species of snakes commonly traverse both paved and unpaved roads (Shine et al. 2004; Andrews and Gibbons 2005), which can result in substantial levels of mortality (Rosen and Lowe 1994). In southern Arizona, rattlesnakes are abundant on roads during the monsoon season from July to September, the period of mate-seeking (Schuett et al. 2011, 2013a, b; Clark et al. 2014). Rosen and Lowe (1994), for example, estimated that 22.5 snakes are dead-on-the-road (DOR) per kilometer per year in southern Arizona, and the majority of these fatalities are rattlesnakes. Rattlesnakes and other viperid species are particularly vulnerable to road injury and mortality because of their large size and/or slow-moving rectilinear gait (Andrews and Gibbons 2005; Shepard et al. 2008a). In the northeastern part of their range in the United States, Timber Rattlesnakes (*Crotalus horridus*) experience an estimated mortality rate of 80% associated with roads with a traffic volume of 3000 vehicles/day, and with a mortality rate approaching 100% on major roads with traffic volumes of more than 9000 vehicles/day (Andrews and Gibbons 2005; Clark et al. 2010). Individuals of *C. horridus* that occupy communal winter dens that are disjunct and isolated by roads have significantly lower genetic diversity and higher genetic differentiation than individuals which occupy winter dens in habitats that lack roads and where winter dens are contiguous (Clark et al. 2010). The roads studied by Clark et al. (2010) have been barriers for about 80 years, which equals approximately 7–8 snake generations in eastern populations of *C. horridus* (Brown 1993; Clark et al. 2008). Road mortality in the Eastern Massasauga (*Sistrurus catenatus*), an endangered species of small-bodied, swamp-inhabiting rattlesnake, is elevated during the mating season and is male-biased since they are the mate-seeking sex (Duvall et al. 1992; Coupe 2002; Shepard et al. 2008a; Rouse et al. 2011). However, Chiucchi and Gibbs (2010) showed that *S. catenatus* shows high levels of genetic differentiation between populations without signs of recent bottlenecks. This finding suggests that recent habitat fragmentation has had little effect on the population genetics of *S. catenatus* and that these snakes historically existed in small, isolated populations. In a recent study of *Crotalus viridis*, Weyer et al. (2014) were unable to find genetic differentiation in a population fragmented by roads and other anthropogenic features in Canada.

We studied the Western Diamond-backed Rattlesnake (*C. atrox*) in Southern Arizona. This species is a large, venomous predator and important to the region's ecology (Klauber 1972; Campbell and Lamar 2004; Nowak et al. 2008). We investigated three specific sampling sites ("subpopulations") of a large population of *C. atrox* separated by a relatively new and large Interstate Highway (I-10) to address two main questions: (1) Do subpopulations close to I-10 exhibit a relative decrease in genetic diversity? (2) Does I-10 impede gene flow between subpopulations?

Materials and methods

Study design and sampling

Our study area was located in the Sonoran Desert southeast of Picacho Peak, Pinal County, Arizona, and it is mostly divided (east and west) by I-10. We partitioned our data into three geographic populations identified as I-10 West (I10W), I-10 East (I10E), and Cattle Tank (CT) (Fig. 1). We sampled a total of 72 *C. atrox*: 24 from I-10W, 23 from I-10E, and 25 from CT (Table 1). This species is fairly abundant and can be found throughout the study area.

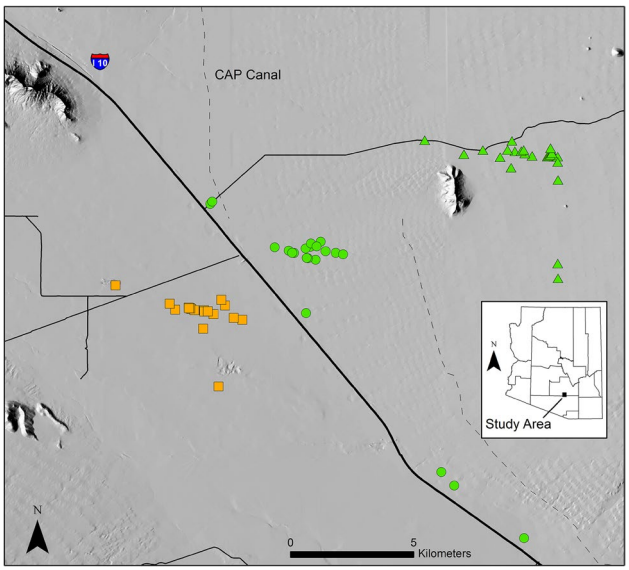

Fig. 1 Map of the study area along Interstate 10 (I-10) at mile post 220 SE of Picacho Peak, Pinal County, Arizona. *Markers* denote sampled *C. atrox* individuals and the three subpopulations: I-10W (*quadrats*), I-10E (*circles*) and Cattle Tank, CT (triangles). Approximate geographic distance between subpopulations I-10W and I-10E is 4 km and between I10E and CT is 7 km

During the seasons of field collecting the snakes are often concentrated in the vicinity of dry washes.

In southern Arizona, *C. atrox* has an estimated generation time of 3.3 years (Schuett et al. 2011, 2013a, b; Clark et al. 2014). Approximately 17 *C. atrox* generations have passed since the initial construction of I-10 at the study site (1956–1958). The dispersal ability of *C. atrox* is moderate, with adults of both sexes capable of movements that exceed 2 km within 24 h, and similar to most pitvipers the home range size tends to be larger in males than in females (Clark et al. 2014).

The straight-line distance between the center of I-10W and I-10E is 4 km, and the distance between I-10E and CT, both east of I-10, is 7 km. Lower Colorado River Desert scrub dominated by Creosote Bush (*Larrea divaricata*) is present at both I-10W and I-10E sites, whereas CT is characterized by a markedly different habitat type located at the ecotone between the former and Arizona Upland Desert scrub or Sonoran bajada which is dominated by Ocotillo (*Fouquieria splendens*), Saguaro (*Cereus giganteus*), and various other cactus species (Brown 1994).

The initial construction of I-10 at our study sites occurred from 1956 to 1958, with subsequent expansion to the present (https://en.wikipedia.org/wiki/Interstate_10_in_Arizona). Additionally, for over 55 years the human population in the region has increased considerably (Pry and Andersen 2011) and has led to dramatically increased traffic volume with road expansions to 4–6 lanes. A 2009 study of I-10 estimated the average annual daily traffic volume at 45,000 vehicles (http://www.adot/ms2soft.com). The study site at I-10 has no large physical barriers, such as fences or walls. Large culverts run underneath I-10 to accommodate water run-off from E to W during the heavy monsoonal rains. DOR rattlesnakes have been observed on I-10 in the wider study area. Owing to high traffic volume and associated safety concerns, we did not collect DOR rattlesnakes on I-10 at the study site.

The Central Arizona Project, a canal that transports water from the Colorado River to the wider Tucson area, runs parallel to I-10 (http://www.cap-az.com). At the study area the canal runs underground for about 2.5 km before it reaches the Red Rock pumping station. The resulting "canal gap" breaks the linear structure and functions as a wildlife corridor between I-10E and CT.

We sampled snakes by way of opportunistic encounter surveys on foot and by vehicle from July through October for three consecutive years (2008–2010). To avoid redundant sampling, individuals were permanently marked with implantable PIT-tags (Gibbons and Andrews 2004). We recorded geographic coordinates, sex, and mass for all subjects encountered alive. Only geographic coordinates and, if possible, sex were recorded for snakes found DOR. We collected blood tissue samples from caudal vessels of live subjects and liver and/or muscle tissue samples from DOR specimens. Tissue samples were stored in lysis buffer or 95% ethanol.

Mitochondrial DNA sequencing and diversity, differentiation, and network analyses

As an independent genetic marker to investigate genetic patterns that might predate the presence of Highway I-10, we PCR amplified and sequenced three mitochondrial DNA loci (ATPase6 and ATPase8 with primers L8331 and H9236, Douglas et al. 2006; ND1 with primers 16Sb and H3518; Lawson et al. 2005; and ND2 with primers L4437b and tRNA-trpR; Wüster et al. 2005). Edited sequences were aligned with MUSCLE (Edgar 2004) implemented in MESQUITE 2.75 (Maddison and Maddison 2011) and concatenated.

DnaSP 5.10 (Librado and Rozas 2009) was used to estimate overall and partition-level parameters and their variances including nucleotide diversity π (Nei 1987), number of haplotypes h, and haplotype diversity Hd (Nei 1987). Tajima's D (Tajima 1989) was calculated to test for possible demographic effects like changes in population size. Recent population expansion can result in an excess of substitutions near the tips of branches (external) leading to negative Tajima's D values (Simonsen et al. 1995). To complement this analysis, we used Ramos-Onsins and Rozas' R_2 (Ramos-Onsins and Rozas 2002), which is based on the

Table 1 Per locus genetic diversity summary including sample size (n), number of alleles (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E), inbreeding coefficient (F_{IS}), deviations from Hardy–Weinberg equilibrium (HWE), and null alleles (NA)

Locus	n	N_A	H_O	H_E	F_{IS}	HWE_{I10W}	HWE_{I10E}	HWE_{CT}	NA (%)
Ca1_14	72	29	0.76	0.95	0.17	Sig	Ns	Sig	8.9
Ca1_20	67	18	0.75	0.90	0.14	Sig	Sig	Sig	9.2
Ca1_22	71	9	0.55	0.78	0.28	Sig	Sig	Sig	16.5
Ca1_31	64	30	0.72	0.94	0.21	Sig	Sig	Sig	10.6
Ca1_39	72	8	0.47	0.58	0.17	Sig	Sig	Ns	7.4
Ca1_43	70	23	0.58	0.93	0.36	Sig	Sig	Sig	18.6
Ca2_23	72	14	0.80	0.88	0.07	Ns	Ns	Sig	4.0
Ca2_27	71	29	0.56	0.95	0.39	Sig	Sig	Sig	20.3
Ca2_38	71	13	0.79	0.79	-0.02	Ns	Sig	Ns	2.4
Ca2_64	72	16	0.93	0.91	-0.05	Ns	Ns	Ns	1.4
Ca2_71	72	10	0.40	0.74	0.44	Sig	Sig	Sig	18.7
Ca2_74	72	12	0.86	0.85	-0.04	Ns	Ns	Ns	0.8
Ca2_81	72	18	0.77	0.91	0.13	Sig	Sig	Ns	7.2
Ca2_90	71	25	0.90	0.93	0.01	Ns	Ns	Ns	0.8
Crti09	72	28	0.78	0.96	0.17	Sig	Sig	Sig	8.7
Crti10	72	27	0.93	0.93	-0.02	Ns	Ns	Ns	0.0
Crti12	72	15	0.75	0.81	0.05	Ns	Ns	Sig	7.0
Crti14	72	10	0.75	0.68	-0.12	Ns	Ns	Ns	0.0
Crti23	70	16	0.58	0.88	0.32	Sig	Sig	Sig	15.3
Crti32	72	18	0.94	0.91	-0.05	Ns	Ns	Ns	0.6
Crti37	71	9	0.44	0.59	0.24	Ns	Sig	Sig	7.9
Crti47	72	14	0.85	0.88	0.02	Ns	Ns	Ns	3.1
Crti95	72	11	0.88	0.82	-0.08	Ns	Ns	Ns	1.2
CwA14	71	12	0.54	0.85	0.35	Sig	Sig	Sig	15.7
CwA29	70	10	0.69	0.74	0.05	Ns	Ns	Ns	7.2
CwB6	72	10	0.58	0.62	0.04	Ns	Ns	Sig	2.7
MFR15	72	11	0.47	0.46	-0.04	Ns	Ns	Ns	0.0
MFR23	72	18	0.83	0.87	0.03	Ns	Sig	Ns	3.7
Scu05	72	34	0.89	0.95	0.04	Ns	Ns	Sig	3.3
Scu07	72	19	0.43	0.92	0.52	Sig	Sig	Sig	25.9
Mean	71	17.2	0.71	0.83	0.13				7.6

Sig = significant deviation from HWE with $P < 0.05$ after Bonferroni correction

Ns not significant

difference between the number of singleton mutations and the average number of nucleotide differences. R_2 is robust even when sample sizes are small and has high power to detect population growth (Sano and Tachida 2005). Low significant values are interpreted as evidence for recent severe population expansion. With DnaSP we constructed a mismatch distribution plot based on the overall data and examined the fit of our results against a constant population size equilibrium distribution model and a sudden expansion model. We used Arlequin 3.5 (Excoffier and Lischer 2010) to calculate pairwise F_{ST} and its statistical significance after 1000 permutations, and R 3.1.2 (R Core Team 2013) to estimate pairwise D_{est} for the entire alignment length and its statistical significance after 1000 permutations using the method of Pennings et al. (2011). To visualize haplotype relationships we used the median-joining network method (Bandelt et al. 1999) in NETWORK 4.6 (http://www.fluxus-engineering.com).

Microsatellite genotyping, genetic diversity, and differentiation analysis

We extracted genomic DNA and genotyped all samples at 30 polymorphic microsatellite loci (Pozarowski et al. 2012). All PCRs, DNA sequencing, fragment analyses, and allele scoring was carried out as described in Pozarowski et al. (2012). We used FSTAT 2.9 (Goudet 1995) and GENEPOP (Raymond and Rousset 1995) to estimate deviations from Hardy-Weinberg equilibrium per locus and test linkage equilibrium among pairs of loci, respectively. For multiple comparisons at $\alpha = 0.05$ we used a sequential Bonferroni test to account for false positives. To detect and adjust

null alleles we used the software package Microchecker 2.2 (van Oosterhout et al. 2004). Data adjusted for null alleles were used to calculate expected and observed heterozygosities, number of alleles, F_{ST}, G''_{ST}, and D_{est} with GenAlEx 6.5 (Peakall and Smouse 2006, 2012).

We estimated G''_{ST} as the corrected version for small numbers of sampled (sub)populations and pairwise comparisons of the related G'_{ST}, which is suited for markers with high numbers of alleles and is standardized to a 0–1 scale (Meirmans and Hedrick 2011). G'_{ST} has been shown to have a lag time for a signal of a new barrier of only 1–15 generations whereas F_{ST} required approximately 200 generations to reach 50% of its equilibrium maximum (Landguth et al. 2010). This constitutes a relatively short time for new barriers to become detectable. Lag time to barrier detection is even smaller when dispersal ability is large: approximately 1/5th dispersal ability distance of the total study area distance when simulated (Landguth et al. 2010). As past landscape barrier effects are lost within 15 generations if organisms possess relatively large dispersal abilities, the legacy of historical barriers may not be a problem using this parameter.

Bayesian assignment clustering and spatial genetic autocorrelation

We used STRUCTURE 2.3.1 (Pritchard et al. 2000; Hubisz et al. 2009) to infer the number of genetic clusters in our overall sample and to test our hypotheses of genetic differentiation between subpopulation pairs. Bayesian clustering methods as implemented in STRUCTURE are among the most powerful for detecting linear barriers to gene flow within 20 generations or less (Blair et al. 2012). For STRUCTURE analyses we used both a population and no population prior for our three subpopulations, and performed 10 independent STRUCTURE runs for each value of K including all samples from all three study partitions. We ran 500,000 repetitions each with $K=1$ to 5 of which 250,000 were discarded as burn-in. We assumed admixture and correlated allele frequencies (Falush et al. 2003; François and Durand 2010). We used the ΔK method (Evanno et al. 2005) implemented in STRUCTURE HARVESTER (Earl and von Holdt 2012) to find the true K. To test genetic structure between subpopulation pairs, we ran STRUCTURE as described above with data set pairs for I-10W/I-10E, and I-10E/CT with K set to 2. As the ΔK method is unable to find the true K when $K=1$, we compared the average of the probability (mean of Ln probability) of $K=1$ and $K=2$ given the data and selected the larger value as more likely (Mungia-Vega et al. 2013). We used CLUMPP 1.1 (Jakobsson and Rosenberg 2007) to summarize the independent runs, and DISTRUCT 1.1 (Rosenberg 2004) to generate bar graphs from CLUMPP outputs.

Mantel tests, though frequently used, have proven inappropriate to detect small scale genetic structure (Legendre and Fortin 2010; Guillot and Rousset 2012). Instead, we used genetic spatial autocorrelation (Smouse and Peakall 1999), which is sufficiently sensitive to detect spatial genetic structure over small spatial scales (Epperson 2010; Banks and Peakall 2012). Unlike traditional spatial autocorrelation, the method implemented in GenAlEx 6.5 uses a multivariate approach, which strengthens the spatial signal and reduces noise to estimate genetic versus geographic connectivity. GenAlEx calculates an autocorrelation coefficient (r) that provides a measurement of the pairwise genetic similarity of individuals whose geographic distance falls within a specified distance bin. We specified distance bins of 0.6 km for 11 runs so that the first distance interval would calculate r based on all pairwise comparisons within a distance of 0–0.6 km, the second analysis for 0–1.2 km, and continued in increments of 0.6 km added until the last run (i.e., 0–6.6 km) was completed. Additional distance bins at 0.35, 1.0, and 1.6 km were examined with similar results. However, a distance bin size of 0.6 km offered the best resolution of spatial genetic structure within local groups and positive spatial autocorrelation indicating distances within which gene flow occurs. The statistical significance of r was determined using 9999 permutation and bootstrap replicates, with randomized genotypes among distance bins. We inferred statistically significant spatial genetic structure ($r \neq 0$) when the observed r-value fell outside of the 95% confidence interval (CI) of the r-values generated through permutation and corresponding to the null hypothesis of no spatial genetic structure ($r=0$). Within a specific distance bin, r was statistically significant when the 95% bootstrap CI did not intersect $r=0$. Genetic spatial autocorrelation was performed for all individuals simultaneously ($n=72$) and for the subpopulation pairs I10W/I10E ($n=47$) and I10E/CT ($n=48$). Specifically, we tested whether significant genetic spatial autocorrelation was present when a potential road barrier is present (I-10W vs. I-10E) or absent (I-10E vs. CT).

Effective population sizes and migration

We calculated contemporary N_e sizes and confidence intervals using the Linkage Disequilibrium Estimator with a P_{Crit} of 0.05 for rare alleles in NeEstimator 2.0 (Do et al. 2014). This was followed by simulations of multiple genotype datasets under different migration scenarios in EASYPOP 2.0.1 (Balloux 2001) to determine the best-fit model for the observed G''_{ST} values assuming a conservative census size of $N=2N_e$ (Frankham 1995) for each subpopulation. Gene flow simulations were performed between adjacent subpopulations (I10W/I10E and I10E/CT) for 100 generations considering a 1:1 proportion of males and

females, a polygynous mating system, a proportion of matings by subordinate males of 0.35 (Duvall et al. 1992; Clark et al. 2014; Schuett, unpubl. data), 30 loci, 30 alleles per locus, and a stepwise mutation rate of $\mu = 5 \times 10^{-4}$ locus/indvidual/generation (Garza and Williamson 2001) with a proportion of double step mutations of 0.05 (Piry et al. 1999). For the first 83 generations we assumed a proportion of male migration (PMM) of 0.5 and a proportion of female migration (PFM) of 0.1 based on migration data from radio-telemetry studies in *Crotalus horridus* and *C. atrox* (Clark et al. 2014; Schuett, unpubl. data). For the last 17 generations (the estimated time since the construction of I-10), we considered six scenarios with varying degrees of PMM/PFM: 0.50/0.10, 0.40/0.08, 0.30/0.06, 0.20/0.04, 0.10/0.02, and 0.00/0.00. Each migration scenario consisted of 100 iterations (200 simulated subpopulations). Pairwise G''_{ST} values were calculated in GenAlEx 6.5.

Results

Nuclear and mtDNA genetic diversity

All 72 sampled individuals were successfully genotyped. We found no evidence for departure from linkage disequilibrium (435 pairwise comparisons, adjusted $P < 0.0001$). Fifteen of the 90 per locus and subpopulation Hardy–Weinberg Equilibrium (HWE) tests showed significant deviation from HWE. We did not observe any consistent pattern of HWE deviation among loci or sample partitions. Null alleles with frequencies of 20% and higher in at least one subpopulation were found in loci Ca1_43, Ca2_27, Ca2_71, Crti23, CwA14, and Scu07. Null alleles are commonly found in population genetics studies and are likely to be encountered when effective population sizes are large and mutation rates are high (Chapuis and Estoup 2007). Assessments of the effects of null alleles on analyses using simulation studies indicate that *F*-statistics can be improved with correction methods (e.g., Microchecker; Chapuis and Estoup 2007). Simulations show that null alleles in assignment tests cause only a slight reduction of power to correctly assign individuals (Carlsson 2008; Marsh et al. 2008). Data adjusted for null alleles with Microchecker were used for *F*-statistics analyses. Overall, observed and expected heterozygosities for the subpopulations were similar (Table 2), with means of 0.74 ± 0.02 and 0.82 ± 0.01, respectively, as was the average number of alleles per subpopulation (mean 12.5 ± 0.54).

The three mitochondrial DNA loci produced a concatenated alignment of 2615 bp total length after sites with missing data were eliminated. Individuals of the CT subpopulation showed slightly higher nucleotide diversity, although the number of haplotypes was highest in I10W (Table 3). The haplotype diversity increased from E to W (with overlapping SE for subpopulation pairs). Tajima's *D* was negative for all partitions and significant ($P < 0.05$) for the overall sample with all individuals included. Ramos-Onsins and Rozas' R_2 was close to zero in the overall sample and in I10W with the latter being significant ($P < 0.05$). Mismatch distribution supports a sudden population expansion model (Fig. 2; Rogers and Harpending 1992).

Genetic differentiation

Population differentiation between subpopulation pairs (I10W/I10E and I10E/CT) showed significant differentiation ($P < 0.05$) for the nuclear microsatellite markers

Table 2 Genetic diversity of 30 microsatellite loci for each of the three subpopulations sampled including sample size (*n*), number of alleles (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E) with standard error (±SE)

Population	*n*	N_A (±SE)	H_O (±SE)	H_E (±SE)	N_e
I-10W	24	12.0 (±0.87)	0.75 (±0.03)	0.81 (±0.02)	58–112
I-10E	23	12.5 (±0.95)	0.72 (±0.03)	0.82 (±0.02)	70–192
CT	25	12.9 (±1.00)	0.76 (±0.02)	0.82 (±0.02)	78–170
Mean	24	12.5 (±0.54)	0.74 (±0.02)	0.82 (±0.01)	69–158

N_e shows the 95% CI of the effective population size

CT cattle tank

Table 3 Genetic diversity of three concatenated mitochondrial DNA loci (2615 bp) for each of the three subpopulations including sample size (*n*), nucleotide diversity (π; Nei 1987), number of haplotypes (*h*), haplotype diversity (*Hd*; Nei 1987), Tajima's *D* (Tajima 1989), and Ramos-Onsins and Rozas R_2 (Ramos-Onsins and Rozas 2002)

Population partition	*n*	π (±SE)	*h*	*Hd* (±SE)	Tajima's *D*	R_2
I-10W	22	0.0008 (±0.0001)	12	0.91 (±0.04)	−1.58	**0.07**
I-10E	22	0.0007 (±0.0001)	7	0.86 (±0.04)	−0.48	0.15
CT	23	0.0010 (±0.0003)	8	0.78 (±0.07)	−1.71	0.13
Overall	67	0.0009 (±0.0001)	16	0.88 (±0.02)	**−1.99**	0.06

Values with $P < 0.05$ are bold

CT cattle tank

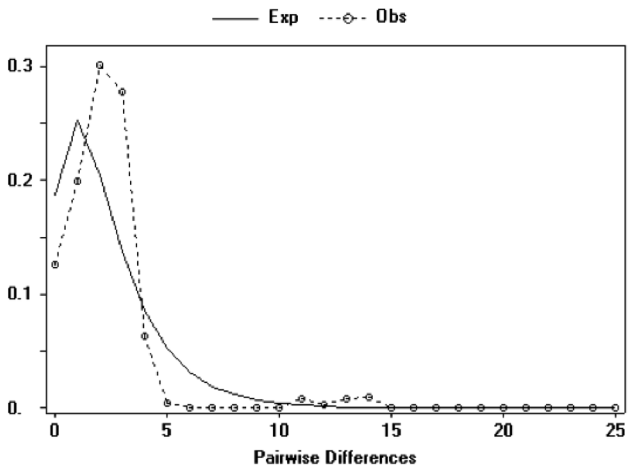

Fig. 2 Mismatch distribution of pairwise differences for three concatenated mtDNA loci with a total length of 2615 bp ($n=72$) fitted against a single expansion growth model. The observed values show a relatively good fit to the expected distribution. $\theta_{initial} = 3.372$, $\theta_{final} = 1.000$

(Table 4). Differentiation based on the microsatellite data was greater ($F_{ST} = 0.022$, $G''_{ST} = 0.111$, $D_{est} = 0.094$) in the subpopulation pair I10W/I10E in close geographic proximity (4 km), but separated by I-10, than in subpopulation pair I10E/CT, which has a greater geographic separation (7 km), but has a smaller genetic differentiation ($F_{ST} = 0.016$, $G''_{ST} = 0.036$, $D_{est} = 0.030$).

Population differentiation based on mitochondrial DNA between the subpopulation pairs I10W/I10E and I10E/CT was not significant ($P > 0.05$) for F_{ST} and D_{est} (Table 3). The median joining haplotype network (Fig. 3) includes 16 different haplotypes from the total sample and shows no clear pattern of haplotype-subpopulation separation on either side of I-10. Instead, the network pattern is consistent with other test results indicating one overall population in expansion.

Table 4 Genetic differentiation between the three subpopulations calculated based on 30 microsatellite loci corrected for null alleles using Microchecker (van Oosterhout et al. 2004)

Population partitions	Distance (km)	F_{ST}	G''_{ST}	D_{est}	F_{ST}^{mtDNA}	D_{est}^{mtDNA}
I-10W/E	4	0.022*	0.111*	0.094*	0.017	0.136
I-10E/CT	7	0.016*	0.036*	0.030*	0.012	0.057

Genetic differentiation for microsatellites was calculated with GenAlEx 6.5 (Peakall and Smouse 2012) with inbreeding coefficient within subpopulations (F_{ST}), Hedrick's corrected G_{ST} for small number of subpopulations (G''_{ST}; Meirmans and Hedrick 2011; Hedrick 2005), and Jost's estimate for differentiation D_{est} (Jost 2008). F_{ST}^{mtDNA} is based on three concatenated mitochondrial DNA loci with a total of 2615 nucleotides and was calculated using Arlequin 3.5 (Excoffier and Lischer 2010). D_{est}^{mtDNA} are calculated with in R (R Core Team 2013) using the method of Pennings et al. (2011)

CT cattle tank

* $P < 0.05$

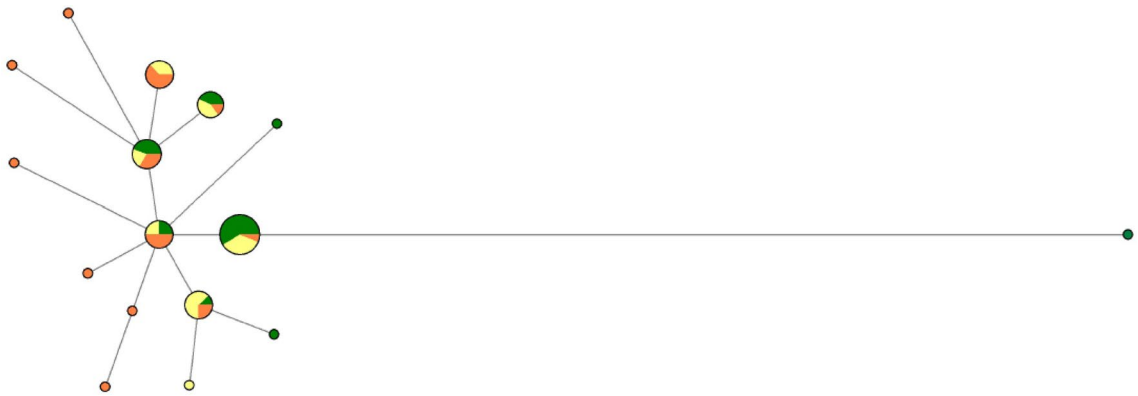

Fig. 3 Median joining haplotype network for three concatenated mtDNA loci with a total length of 2615 bp. *Nodes* represent haplotypes, *node size* represents haplotype frequency with *smallest nodes* representing one individual. *Orange* denotes I-10W subjects, *yellow* I-10E subjects, and *green* CT subjects. The shortest distance between two nodes represents one substitution. (Color figure online)

Bayesian assignment clustering and spatial genetic autocorrelation

Bayesian assignment clustering analyses (STRUCTURE) with and without population priors did not differ markedly and supported the same true *K* values. Only the results of analyses with population priors are presented here (Hubisz et al. 2009). HARVESTER identified three genetic clusters ($K = 1$–5) (Fig. 4, mean Ln probability −9,955). CT shows very little admixture, yet I10W and I10E showed considerable amounts of admixture with a greater amount in I10E. However, when *K* is restricted to 2 clusters, I10E and CT constitute one uniform genetic cluster, whereas I10W represents a second admixed group with only 33% of the individuals with more than 50% genetic identity with the I10E and CT cluster (mean Ln probability −10,008). When we tested our hypothesis of I-10 being a barrier to gene flow and restricted *K* to 1 and 2 in pairwise comparisons of subpopulations, our previous results are supported. For the I10W/I10E pair, a higher mean Ln probability

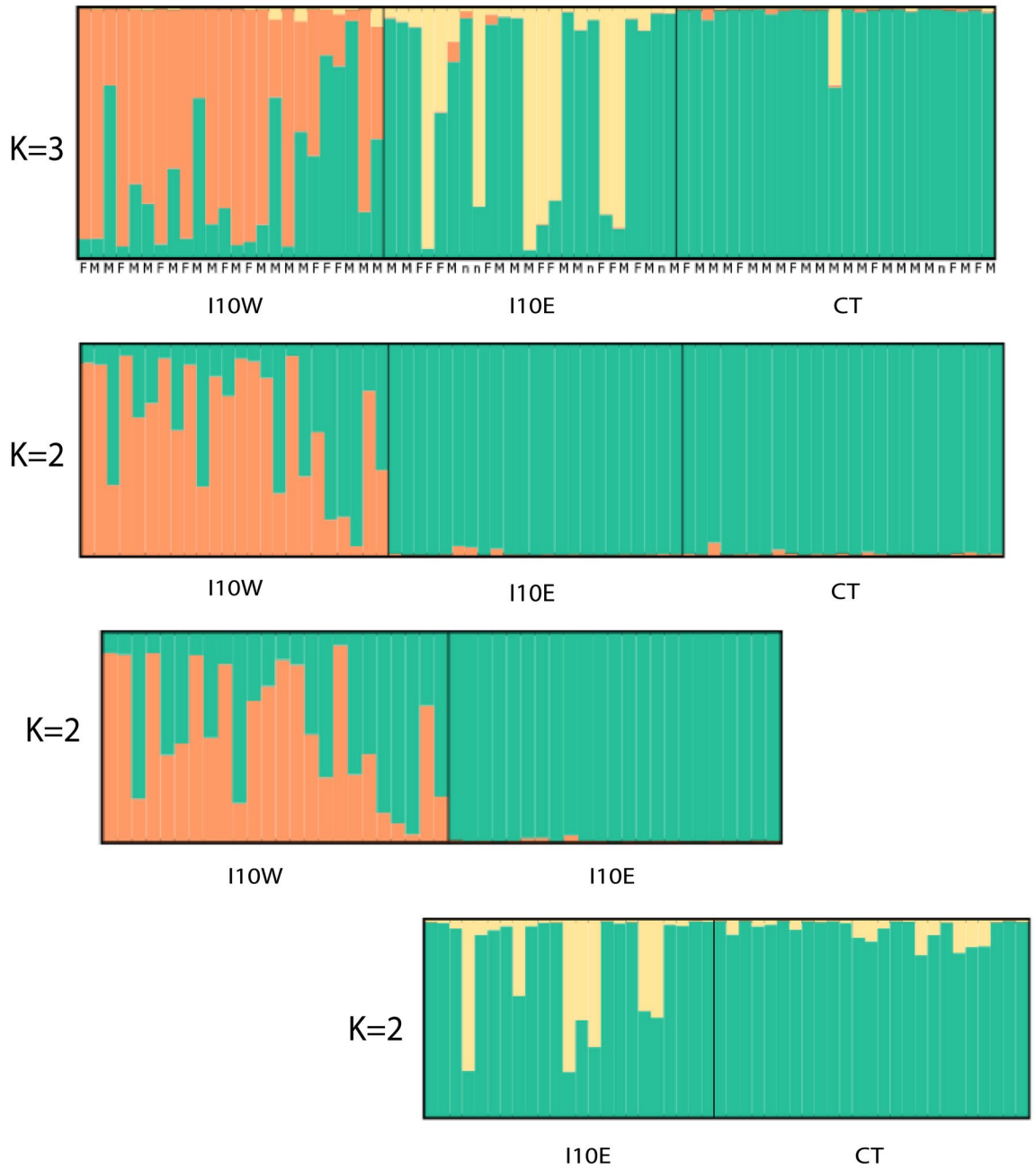

Fig. 4 Bayesian assignments of individuals for all three and pairwise subpopulations ($K = 2$) comparisons representing the average probability of population membership over 10 independent runs each (STRUCTURE 2.3.4). *M* male, *F* female, *n* neonate

indicated two distinct genetic clusters (mean Ln probability −6,464 for $K=2$ vs. −6,482 for $K=1$), with 46% of the sampled individuals from I10W showing greater than 50% genetic identity with the I10E subpopulation. The I10E/CT partition had a higher mean Ln probability of $K=1$ versus $K=2$, which indicates a single genetic cluster (mean Ln probability −6,633 for $K=1$ vs. −6,655 for $K=2$), with 78% of the individuals sampled from I10E showing greater than 50% genetic identity with the CT partition.

Correlograms of the spatial autocorrelation analysis are presented in Fig. 5. The correlogram for all 72 samples (Fig. 5a) shows a positive autocorrelation for the first two distance groups (0.6 and 1.2 km, $P<0.001$) with an x axis intercept at 3.2 km. If only samples from the I10W/I10E subpopulations are included in the analysis, the correlogram (Fig. 5b, n = 47) showed positive autocorrelation at the same first two distance classes 0.6 and 1.2 km ($P<0.001$) with an intercept at 2.2 km. The I10E/CT correlogram (Fig. 5c, n = 48) showed no significant positive autocorrelation (all $P>0.001$) at any distance bin. Significant positive autocorrelation indicates spatial genetic structure within the respective distance bin and x axis intercepts indicate that genetic distances were significantly correlated with geographic distances up to a distance of 1.2 km and possibly caused by resistance to gene flow at a distance of approximately 2 km between I10W and I10E.

Effective population sizes and migration

Recent effective subpopulation sizes calculated in NeEstimator are I10W = 78, I10E = 104, and CT = 108. All subpopulations show overlapping 95% CIs (Table 2).

Simulated genotypes in EASYPOP suggest that current levels of genetic differentiation in I10E/CT (G''_{ST} = 0.033; Table 3) are consistent with a PMM/PFM of 0.30/0.06 (95% CI 0.027–0.044; Fig. 6). In contrast, the genetic differentiation observed in I10W/I10E (G''_{ST} = 0.103; Table 3) is consistent with a scenario where the PMM/PFM between these subpopulations is more restricted: 0.10/0.02 (95% CI 0.086–0.130; Fig. 6).

Discussion

Our study of gene flow in populations of the Western Diamond-backed Rattlesnake (*C. atrox*) near a major highway

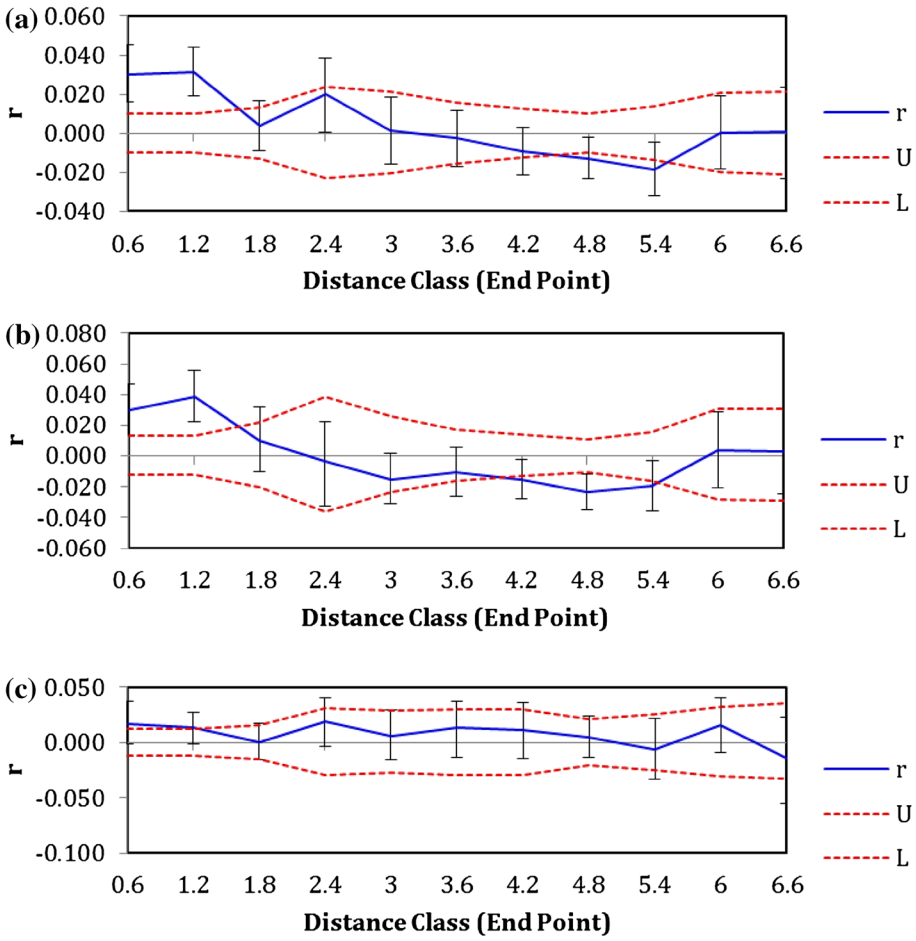

Fig. 5 Spatial autocorrelation correlograms for all individuals sampled (**a**; $n=72$), for I-10W and I-10E subpopulations (**b**; $n=47$), and for I-10E and CT subpopulations (**c**; $n=48$). Distances are in km, permutated 95% CI upper and lower boundaries are *dashed lines, error bars* are 95% confidence bootstrap values, r represents the autocorrelation coefficient. Significant positive autocorrelation implies nonrandom genetic similarity. All correlograms are $P<0.01$

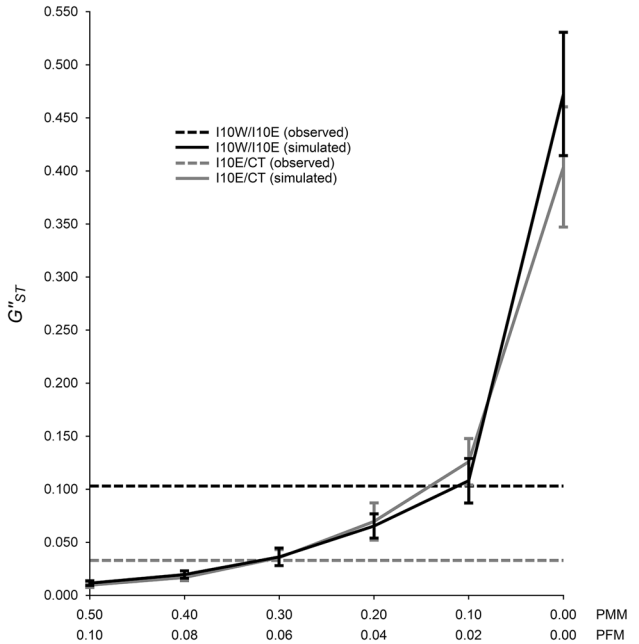

Fig. 6 Gene flow simulations and changes in G''_{ST} between adjacent subpopulations I-10W/E (*solid black lines*) and I-10E/CT (*solid grey line*) for 100 generations considering a 1:1 proportion of males and females, a polygynous mating system, a proportion of matings by subordinate males of 0.35, 30 loci, 30 alleles per locus, and a stepwise mutation rate of $\mu = 5\times10^{-4}$ locus/individual/generation with a proportion of double step mutations of 0.05. For the first 83 generations we assumed a proportion of male migration (PMM) of 0.5 and a proportion of female migration (PFM) of 0.1. For the last 17 generations (the estimated time since the construction of I-10) we considered six scenarios with varying degrees of PMM/PFM: 0.50/0.10, 0.40/0.08, 0.30/0.06, 0.20/0.04, 0.10/0.02, and 0.00/0.00. Each migration scenario consisted of 100 iterations (200 pairwise comparisons). Vertical lines on solid lines represent 95% CI for each gene flow scenario. Dashed black and grey lines represent observed G''_{ST} values for I-10W/E and I-10E/CT, respectively, for empirical nuclear microsatellite data (Table 3)

yielded two major conclusions: (1) genetic diversity is not markedly decreased in the populations sampled close to a major highway (I-10), and (2) gene flow between individuals on opposite corridors of I-10 was reduced relative to gene flow between individuals at the two sites that were sampled east of I-10. Furthermore, our analyses of nuclear microsatellites loci support the view that gene flow is unidirectional and occurs from E to W based on Bayesian assignment tests. We found no evidence that decreased gene flow between the populations now separated by I-10 is historical and existed before the construction of I-10.

Clark et al. (2010), who investigated Timber Rattlesnakes (*C. horridus*) from New York, showed a clear reduction of genetic diversity in populations separated by old roads. In our study, the individuals of *C. atrox* we sampled came from areas separated by a more recent road barrier, and we did not observe a relative reduction in the number of alleles or haplotypes, or reduction of nucleotide and haplotype diversity in these samples. Moreover, we found no evidence of a genetic bottleneck based on heterozygosity deficiency or excess. The estimated recent effective population size (N_e) of *C. atrox* at our study sites, based on microsatellite data, showed overlapping 95% CIs. Tajima's *D* and mismatch distribution tests, as well as the median joining network for the mitochondrial DNA data all indicate that the regional *C. atrox* population at the study site has a large effective population size and shows a good fit to the demographic expansion model. At our study site in the Sonoran Desert, *C. atrox* and other rattlesnake species are abundant and widely distributed. Unlike conditions for *C. horridus* in New York and other parts of its range in the northeastern United States, large (>25) aggregations of *C. atrox* occupying communal dens are rare, and individuals are more or less evenly distributed across the landscape (Repp and Schuett 2008; Herrmann, pers. obs.). Communal dening was not evident at our study sites (Herrmann, pers. obs.). Although the geographic area studied by Clark et al. (2010) was not considerably different from the area that we studied, the roads were considerably older (80–90 years-old vs. 55 years-old) in their study. Estimated generation time for *C. horridus* is 10 years (Brown 1991), while generation time is approximately three times shorter (3.3 years) for *C. atrox* in southern Arizona (Schuett et al. 2011, 2013a, b; Clark et al. 2014). This is an important distinction and means that genetic change ocurs more rapidly in the *C. atrox* populations. We predicted approximately 17 generations of *C. atrox* since the initial construction of I-10 in southern Arizona, and that latter generations have experienced much stronger resistance than earlier ones due to increasing traffic and a wider roadway.

Our observation that genetic diversity is unchanged but genetic differentiation is substantial is consistent with genetic diversity being lost at a much lower rate than genetic differentiation increases in previously contiguous panmictic populations that become subdivided (Keyghobadi et al. 2005). Genetic differentiation among subpopulation reflects the contemporary patterns of fragmentation while levels of genetic diversity do not (Keyghobadi et al. 2005). Our results support the view that I-10 acts as a gene flow barrier that governs the observed genetic structure we detected, and that road mortality has not led to marked depletion of genetic diversity (Jackson and Fahrig 2011).

In a study on the effects of anthropogenic features in a fragmented landscape on a *C. viridis* population, Weyer et al. (2014) were unable to detect genetic differentiation. They conclude that long-distance migration may play a role in genetic fragmentation not being detected over the spatial and temporal scale of their study. At the Weyer et al. (2014) study site, *C. viridis* has large communal overwintering aggregations and among the longest known seasonal

migratory displacement distances of any terrestrial snake native to North America. This is markedly different from the population of *C. atrox* we report herein. Furthermore, their *C. viridis* population also showed an immeasurably large effective population size and traffic volumes on roads were lower and fewer microsatellite loci were used (8 vs. 30 loci). These two species have markedly different life- and natural histories, especially a longer generation time in *C. viridis*, and thus may partially explain the different results.

The Bayesian assignment tests suggest genetic structure is present between all three sampling areas of *C. atrox*. However, genetic structure was largest between I-10W and I-10E, despite its geographic proximity and similarity in habitat, which suggests that I-10 may restrict gene flow. This hypothesis is supported by our genetic spatial autocorrelation analysis results. Genetic spatial autocorrelation, when combined with a large number of hypervariable DNA markers, can be particularly powerful (Peakall et al. 2003; Double et al. 2005). In our study, there has been considerable gene flow restriction at approximately 2 km inbetween I-10W/E sampling areas, which coincides with the location of the highway I-10.

Overall, we identified marked genetic structure between adjacent subpopulations of *C. atrox* separated by I-10 after only 17 *C. atrox* generations since the initial construction of I-10. When one considers that *C. atrox* at our study site has a large population with signatures of recent demographic expansion (Castoe et al. 2007; Herrmann, unpubl. data), this result is remarkable. As natural populations are rarely panmictic (Coltman et al. 2003; Repaci et al. 2006), it is likely that rattlesnake genotypes are not evenly distributed across this landscape. Rather they probably form demes at some threshold spatial scale (Clark et al. 2008; Pernetta et al. 2011). It is plausible that the observed genetic structure in our study is caused by resistance to gene flow at the potential barrier I-10 which would add to demic structure. That scenario may over time dilute the barrier effects visible now or genetic drift may cause fixed genetic differences on either side of I-10.

Another interesting result is the apparent directionality of gene flow indicated by our assignment tests where gene flow seems to occur from CT to I-10E to I-10W. Road width (>50 m), heavy traffic load (24 h), and recent structural concrete barriers make it highly improbable for rattlesnakes and other reptiles to successfully cross I-10 at the study site without injury or death. Yet, an alternative route for snakes to traverse/cross I-10 is by large culverts. In both cases, if voluntary travel over (or under) I-10 is possible, bi-directional gene flow appears to be more likely. Voluntary dispersal in rattlesnakes and other snakes is generally driven by males seeking mates (Dubey et al. 2008; Clark et al. 2008, 2010; Anderson 2010; Pernetta et al. 2011) whereas I10W individuals with genotypes of subpopulations East of I-10 are males and females. Therefore, we speculate that individuals cannot successfully or do not voluntarily cross (over or under) I-10. We hypothesize that existing movement (travel) of snakes from E to W is largely non-voluntary and potentially associated with torrential rainfall and flash floods during the monsoon season (i.e. individuals are washed to I-10W), July to September (Mock 1996), a period of increased *C. atrox* activity at our study site and throughout southern Arizona.

Overall, our genetic findings agree with those of other studies which have investigated the effects of major roadways on terrestrial vertebrates. The Red-backed Salamander (*Plethodon cinereus*), which is abundant throughout most of its extensive range, showed increased genetic divergence in populations divided by a large interstate highway, and no genetic divergence in populations divided by smaller-sized roads (Marsh et al. 2008). Simulations of population connectivity in the Spotted Salamander (*Ambystoma maculatum*) and the Wood Frog (*Rana sylvatica*) suggest that road barriers negatively impact population connectivity in these two species, albeit to different degrees. The Wood Frog is more vagile and was more negatively impacted by roads (Richardson 2012). Differences in longevity and generation time between the longer lived *A. maculatum* and the shorter lived *R. sylvatica* likely impact the detectability of certain genetic metrics such as differentiation.

Populations of a widely distributed and abundant small mammal, the Wood Mouse (*Apodemus sylvaticus*) in the Iberian Peninsula, exhibited highway barrier effects due to road avoidance (Ascensão et al. 2016). Even large mammals, such as Red Deer (*Cervus elaphus*; Frantz et al. 2012), and predators, like the European Wildcat (*Felis sylvestris*; Hartmann et al. 2013), Coyote (*Canis latrans*; Riley et al. 2006), Bobcat (*Lynx rufus*; Riley et al. 2006), and Mountain Lion (*Puma concolor*; Riley et al. 2014) showed deleterious genetic effects at least partially owing to road barriers.

Our study provides compelling evidence that a relatively new major highway in southern Arizona that has heavy and continuous traffic had significant impacts on the genetic structure of an abundant large rattlesnake (*C. atrox*). The elevated genetic differentiation we detected indicates that processes such as gene flow can be affected by human-made structures such as roads. In such recently fragmented populations, loss of genetic diversity is likely to occur as equilibrium patterns of genetic differentiation are achieved more rapidly than equilibrium levels of within-population diversity (Kekkonen 2016). Although *C. atrox* is not a species of conservation concern in Arizona, its life-history traits are similar to several other species of viperid snakes which are considered threatened or endangered. If genetic effects of such physical barriers are detectable in a widespread and common species, it is likely that they will be

present in localized and rare species. Accordingly, *C. atrox* can serve as surrogate study system (Levine et al. 2016).

It appears that migration movements by individuals of *C. atrox* across the I-10 highway are mainly unidirectional and possibly passive. To mitigate these problems in *C. atrox* and other terrestrial species, eco-passages could be designed to facilitate safety and re-establish population connectivity. Consequently, it would be important to evaluate the effectiveness of such measures (Lesbarrères and Fahrig 2012; van der Grift et al. 2013). Ultimately, genetic studies like ours can identify road mitigation needs and provide a robust method to monitor the long-term genetic health of populations next to roads.

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