Shallow phylogeographic structure in the declining Mexican Lance-headed Rattlesnake, *Crotalus polystictus* (Serpentes: Viperidae)

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Abstract

Shallow phylogeographic structure in the declining Mexican Lance-headed Rattlesnake, *Crotalus polystictus* (Serpentes: Viperidae). We investigated matrilineal relationships among populations of the Mexican lance-headed rattlesnake (*Crotalus polystictus*), a pitviper inhabiting high-elevation valleys of the densely populated southern Mexican Plateau. A fragment of the mitochondrial ATPase 8 and 6 genes (589 base pairs) revealed comparatively low levels of genetic diversity, with few nucleotide polymorphisms across the portion of the geographic distribution sampled. The shallow intraspecific sequence divergence (1.0%) in *C. polystictus* ATPase 8 and 6 genes contrasts with deep divergences (~1.0–14.1%) observed within other montane rattlesnake lineages from the Mexican highlands, and is more typical of intraspecific variation observed in lowland rattlesnake species with similar distributional extents (e.g., *C. tigris*). We posit that the low genetic diversity in *C. polystictus* relative to that of other highland rattlesnakes may reflect ecological differences resulting in a different evolutionary response to Pleistocene climatic events. Our finding of apparently low genetic diversity in *C. polystictus* highlights the importance of conservation initiatives to protect high elevation grasslands in central Mexico.

Keywords: ATPase 6, ATPase 8, genetic bottleneck.

Resumen

Análisis preliminar revela bajos niveles de diversidad en la estructura filogeográfica de la serpiente de cascabel Mexicana cabeza de lanza, *Crotalus polystictus* (Serpentes: Viperidae). Se investigaron las relaciones matrilineales entre poblaciones de la serpiente de cascabel Mexicana cabeza de lanza (*Crotalus polystictus*), esta especie se distribuye en valles que presentan elevaciones altas de la meseta del sur de México. Se analizó un fragmento mitocondrial de la ATPasa 8 y 6, los

genes (589 pares de bases) revelaron bajos niveles de diversidad genética, con poco polimorfismo nucleótico entre la muestra geográfica analizada. La poca divergencia (1.0%) intraespecífica encontrada en los genes de la ATPasa 8 y 6 en *C. polystictus* contrastan con los fuertes porcentajes de divergencia (~1.0–14.1%) que han sido observados dentro de otros linajes de serpientes de cascabel mexicanas que habitan elevaciones altas. La variación intraespecífica es observada comúnmente en especies de cascabel que se distribuyen en elevaciones menores y que presentan una distribución amplia (por ejemplo, *C. tigris*). Proponemos que la baja diversidad genética encontrada en *C. polystictus* comparada con la diversidad registrada en otras serpientes de cascabel que habitan elevaciones altas, se debe a diferencias ecológicas que han dado como resultado una respuesta evolutiva diferente en esta especie a los eventos climáticos del Pleistoceno. Nuestros resultados de una aparente baja diversidad genética en *C. polystictus* son un fuerte soporte para hacer un llamado a la importancia de realizar iniciativas de conservación para proteger praderas con elevaciones altas en el centro de México.

Palabras Claves: ATPase 6, ATPase 8, cuello de botella genético.

Resumo

Análise preliminar revela baixos níveis de diversidade na estrutura filogeográfica da cascavel mexicana *Crotalus polystictus* (Serpentes: Viperidae). Foram investigadas as relações matrilineares entre as populações da cascavel mexicana *Crotalus polystictus,* uma espécie que habita vales de altitude do planalto do sul do México. Os genes (589 pares de bases) de um fragmento mitocondrial da ATPase 8 e 6 revelaram níveis relativamente baixos de diversidade genética, com poucos polimorfismos de nucleotídeos entre a amostra geográfica analisada. A baixa divergência da sequência intraespecífica (1.0%) encontrada nos genes da ATPase 8 e 6 de *C. polystictus* contrasta com as fortes divergências (~1.0–14.1%) observadas em outras linhagens de cascavel que habitam grandes altitudes, sendo que a variação intra-específica é observada comumente em espécies de cascavel que estão distribuídas em altitudes mais baixas e apresentam uma ampla distribuição (por exemplo, *C. tigris*). Propomos que a baixa diversidade genética encontrada em *C. polystictus,* comparada com a de outras cascavéis que habitam altitudes elevadas, pode refletir diferenças ecológicas que resultaram em uma resposta evolutiva diferente aos eventos climáticos do Pleistoceno. Nossos resultados de uma baixa diversidade genética aparente em *C. polystictus* destacam a importância de iniciativas de conservação para proteger os campos de altitude da região central do México.

Palavras-chave: ATPase 6, ATPase 8, gargalo genético.

Introduction

The Mexican Lance-headed Rattlesnake, *Crotalus polystictus* (Cope, 1865), is a strikingly patterned species endemic to the southern Mexican Plateau and southeastern Sierra Madre Occidental regions of central Mexico (Campbell and Lamar 2004). Throughout most of its distribution, *C. polystictus* inhabits mid- to high-elevation valleys and tablelands, and usually is found in grasslands, mesquite scrublands, and meadows associated with pine-oak forests between 1450 and 2739 m (Armstrong and Murphy 1978, Setser *et al.* 2009). The southern Mexican Plateau is among the most agriculturally productive regions of Mexico and is the most densely populated by humans (Flores-Villela and Gerez 1994). As a result, the historic habitat of *C. polystictus* has been greatly fragmented by croplands and urban encroachment. Many populations of *C. polystictus* have been extirpated or are declining (Campbell and Lamar 2004); however, the species has persisted, and may be locally abundant, in some areas where development and cultivation remain less intensive (Bryson *et al.* 2003; Figure 1). Long-term
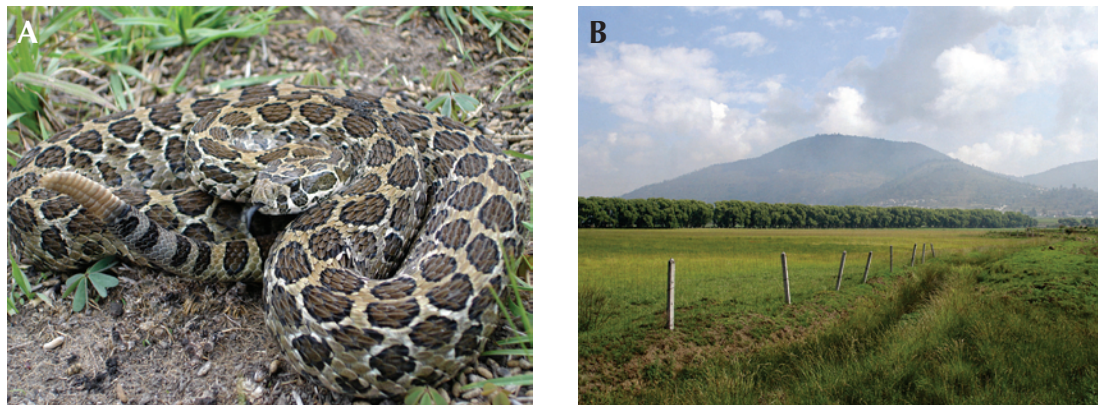
Figure 1. Photograph of *Crotalus polystictus* in life, Toluca Valley, Estado de México, Mexico (**A**), and valley pasture habitat of *C. polystictus,* Estado de México, Mexico (**B**).

survivorship in these agrarian regions may be enhanced by habitat structures such as rodent burrows and rock walls, which afford important refugia for rattlesnakes (Bryson *et al.* 2003, Meik *et al.* 2007).

In addition to *Crotalus polystictus,* the highlands of central Mexico harbor a diverse assemblage of small, montane rattlesnakes. Recently, phylogenetic relationships within the two major clades of these snakes (*Crotalus triseriatus* Group: *C. aquilus, C. lepidus, C. pusillus, C. ravus, C. triseriatus;* and *Crotalus intermedius* Group: *C. intermedius, C. pricei, C. tancitarensis, C. transversus*) were examined using detailed analyses of mtDNA sequences (Bryson *et al.* 2011a, b). Given the complex geography and geologic history of the Mexican highlands, it is perhaps not surprising that these clades contain cryptic species-level diversity and considerable phylogeographic structuring, as well as evidence of recent connections between currently disjunct populations. Bryson *et al.* (2011a) concluded that major lineages in the *C. triseriatus* Group diverged through orogenic vicariance during the late Neogene, with most phylogeographic structuring within these major lineages attributable to Quaternary glacial-interglacial climatic cycles. A similar pattern

was recovered for the *C. intermedius* Group, although lineage formation was biased toward the Quaternary rather than the Neogene (Bryson *et al.* 2011b). Because *C. polystictus* occurs at high elevations, but does not inhabit montane forests, it may have responded differently to Quaternary climatic events than have progenitors of the *C. triseriatus* and *C. intermedius* groups, the members of which primarily inhabit pine-oak forests. Given the threatened grassland habitats and declining populations of *C. polystictus,* we present a preliminary survey of genetic variation at the mtDNA ATPase 8 and 6 loci, and an analysis of phylogeographic structuring. We further compare within-species divergence and nucleotide diversity at these loci among various species of North American pitvipers to assess general patterns of genetic diversity.

Materials and Methods

We investigated relationships among populations of *Crotalus polystictus* by sampling 16 individuals from 10 localities in the Mexican states of Jalisco, Querétaro, Michoacán, México, and Distrito Federal (Table 1). Liver or blood samples were obtained during 2007–2009 and stored in 96% ethanol. We extracted liver from a

road-killed specimen found near Tapalpa, Jalisco (Museo de Zoología, Facultad de Ciencias [MZFC], Universidad Autónoma de México, uncatalogued specimen); otherwise we used syringes to obtain approximately 1 ml of blood from the caudal vein of live snakes. All live snakes were later released at site of capture. Tissue samples from all localities are housed at the University of Texas at Arlington Amphibian and Reptile Diversity Research Center, and are available upon request.

Prior to DNA extraction, blood samples were spun in a centrifuge at 8000 rpm for 1 min and supernatant ethanol was removed with a pipette. The remaining blood pellet was then washed with 180 µl of Buffer ATL (Qiagen®, Valencia, California, USA) and spun again at 8000 rpm for 1 min. After the supernatant Buffer ATL from this step was removed, we extracted genomic DNA using a standard protocol from the DNeasy kit (Qiagen). PCR amplification was conducted with primers we designed from *Crotalus* ATPase 8 and 6 sequences used in a previous study (Douglas *et al.* 2006). Our design resulted in forward primer CmitchF (5' ATG CCA CAA CTA GAT ATT GTT 3') and reverse primer

CMR2 (5' CGG TGA TGT TGG CTG TAA GT 3'). The thermal cycling profile for this primer set had the following steps: (1) an initial denaturation of 2 min at 94˚C, (2) 25 cycles of 30 sec denaturation at 94˚C, 30 sec annealing at 46˚C, 1 min elongation at 72˚C, and (3) a final 7 min elongation at 72˚C. All DNA extractions and PCR products were visualized on agarose gels (1X TAE, 1% agarose). PCR products were cleaned using ExoSap-IT (USB Corporation, Cleveland, Ohio, USA). Sequencing was conducted at the Genomics Core Facility at the University of Texas at Arlington (Arlington, Texas, USA; http://gcf.uta.edu).

Sanger sequencing resulted in a 589 base pair (bp) fragment of ATPase 8 and 6. This fragment contains almost the entire ATPase 8 gene (153 bp) and a section of the ATPase 6 gene (442 bp). Our complete ATPase alignment of *Crotalus polystictus* corresponds to bases 8723–9317 of the mitochondrial genome of *Agkistrodon piscivorus* (NC 009768, Jiang *et al.* 2007). Chromatograms were compared and preliminary alignments were made using Sequencher 4.1 (Gene Codes Corporation, Ann Arbor Michigan, USA). Alignments were improved by eye using

Table 1. Geographic origins, haplotype designations, and GenBank accession numbers for *Crotalus polystictus* samples sequenced for this study.

Locality	State	GPS (WGS 84 datum)	Haplotype (Figure 2)	Genbank accession numbers
San Felipe	México	19.745 N 99.894 W	F	JF748396, JF748402
Ixtlahuaca	México	19.644 N 99.894 W	F	JF748395, JF748405
Atlacomulco	México	19.768 N 99.856 W	F	JF748403, JF748404
Acambay	México	19.857 N 99.808 W	C	JF748407, JF748408
Xochimilco	Distrito Federal	19.283 N 99.101 W	B	JF748406, JF748401
Joyitas	Michoacán	19.702 N 101.358 W	D	JF748400
Tacicuaro	Michoacán	19.664 N, 101.353 W	D	JF748399
Balvanera	Queretaro	20.541 N 100.473 W	D	JF748398
San Ignacio	Queretaro	20.301 N 100.313 W	E	JF748397
Tapalpa	Jalisco	19.982 N 103.776 W	A	JF748394, JF748393

MacClade (Maddison and Maddison 2002) after inferring the appropriate reading frames. Minimum spanning (parsimony/haplotype) networks were constructed using TCS 1.21 (Clement *et al.* 2000).

During the past decade, ATPase 8 and 6 have become the most common mitochondrial markers used in phylogeographic studies of North American pitvipers (e.g., Douglas *et al.* 2006, Bryson *et al.* 2011a, b, Gibbs *et al.* 2011); therefore, we could compare patterns of genetic diversity in *Crotalus polystictus* with 21 other species from three genera (Appendix I). To obtain descriptive statistics on maximum within-species sequence divergence (SD), we calculated pairwise genetic distances (uncorrected "p" distances) using MEGA 5.0 (Tamura *et al.* 2007). We used DNASP 4.9 (Rozas *et al.* 2003) to calculate per site nucleotide diversity ($^\wedge\pi$) within species (Table 2), and to estimate haplotype (*h*) diversity for *C. polystictus.* For comparisons of genetic diversity with other species, we combined species or separated subspecies depending on whether the original studies found taxa to be paraphyletic or polyphyletic (e.g., *C. transversus* is paraphyletic with respect to *C. tancitarensis,* so we refer to this clade as *C. transversus* + *C. tancitarensis*).

Results

Levels of genetic divergence within *Crotalus polystictus* are low; only six haplotypes were obtained among 16 specimens and 10 sampling localities (Figure 2). Despite the low genetic diversity, there is evidence of regional segregation of haplotypes among eastern populations (Estado de México and Distrito Federal), central populations (Querétaro and Michoacán), and a population from the western periphery of *C. polystictus* distribution (Tapalpa, Jalisco). The Jalisco population is the most divergent, and also is the most distant locality from the other sampling localities; therefore, any apparent structure may reflect isolation by distance rather than the phylogenetic signature of a particular geologic or climatic event.

Table 2. Maximum levels of percent sequence divergence (uncorrected "p") for ATPase 8 and 6, and per site nucleotide diversity ($^\wedge\pi$), for intraspecific comparisons of various North American pitvipers. *Includes *Crotalus lepidus morulus;* **includes *Crotalus tancitarensis.*

Species	Uncorrected "p"	Per site nucleotide diversity
Agkistrodon contortrix	0.042	0.020
A. piscivorous	0.065	0.025
*Crotalus aquilus**	0.094	0.042
C. cerastes	0.048	0.032
C. intermedius	0.017	0.019
C. lepidus	0.087	0.049
C. mitchellii	0.073	0.025
C. oreganus	0.051	0.030
C. poystictus	0.010	0.004
C. pricei	0.043	0.039
C. pusillus	0.034	0.021
C. ravus	0.071	0.035
C. ruber	0.010	0.006
C. stephensi	0.029	0.013
C. tigris	0.017	0.008
*C. transversus***	0.006	0.005
C. triseriatus armstrongi	0.141	0.067
C. triseriatus triseriatus	0.124	0.036
C. viridis	0.012	0.008
Sistrurus catenatus	0.134	0.068
S. miliarius	0.010	0.006

We sequenced two individuals from six localities, and in each case, the haplotype pairs are identical. The greatest distance between samples that shared haplotypes was 140 km (straight line) over complex topography (Balvanera, Querétaro, and Tacicuaro, Michoacán). Our most dense sampling is from northern Estado de México, where we sampled six individuals from three sites in the Toluca Valley (San Felipe, Atlacomulco, and Ixtlahuaca), and two individuals from a small montane valley (Acambay) about 20 km northeast of Toluca
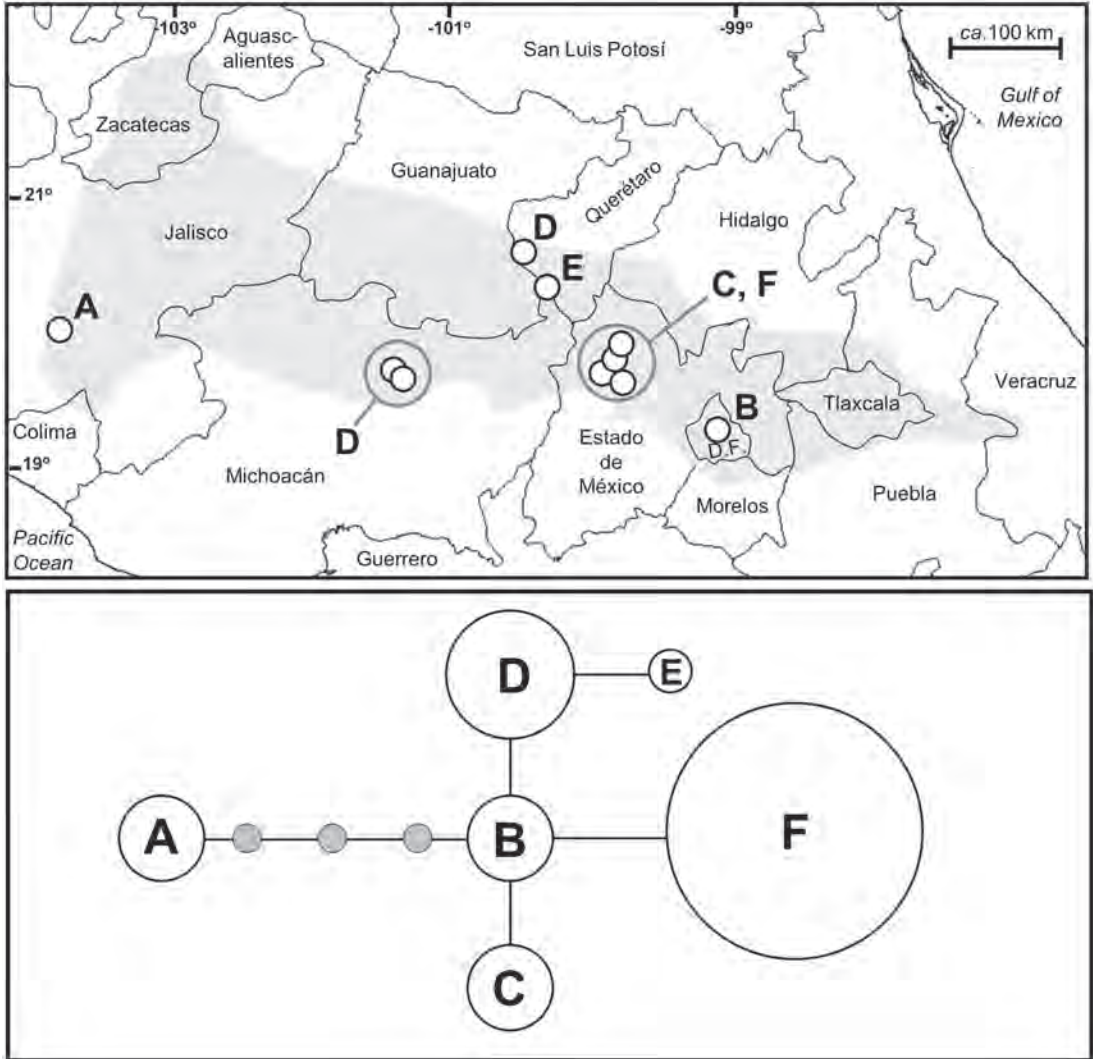
Figure 2. Geographic sampling of *Crotalus polystictus* in central Mexico (top). Shaded area indicates range modified from Campbell and Lamar (2004). Haplotype network for 16 individuals sequenced for ATPase 8 and 6 (bottom). Shaded dots indicate hypothetical mutational steps. Circle size corresponds to number of individuals with a particular haplotype (Table 1). Letters adjacent to locality markers in the map correspond to letters from the haplotype network.

Valley (Figure 1, Table 1). All individuals from the Toluca Valley share the same haplotype, but differ at two nucleotide positions from the haplotype shared by both individuals from Acambay.

Although our sample size is relatively low, it is similar to most previous intraspecific sample sizes for studies that have used ATPase 8 and 6 to examine phylogenetic relationships within and among rattlesnakes (e.g., Douglas *et al.* 2006,

Bryson *et al.* 2011a, b); therefore, comparisons to those datasets are warranted. Maximum within-species SD and per site nucleotide diversity at ATPase 8 and 6 is low in *Crotalus polystictus* when compared with other North American pitvipers in general, and with other Mexican montane rattlesnakes in particular (Table 2). Of the 22 species for which we could access sequence data, *C. polystictus, C. ruber, C. transversus + C. tancitarensis, C. viridis,* and *Sistrurus miliarius* have the lowest maximum within-species SD (1.0% or lower), followed by *C. intermedius, C. tigris,* and *C. viridis* (<2%). With the exception of *C. intermedius* and *C. transversus + C. tancitarensis,* these are predominately lowland species with larger geographic distributions than that of *C. polystictus* (Campbell and Lamar 2004).

Discussion

Our preliminary data for *Crotalus polystictus* indicate relatively shallow phylogeographic structure within the species, and the addition of comparative data indicates that mitochondrial genetic diversity in *C. polystictus* is likely in the lower 25% of North American pitvipers. Male rattlesnakes tend to disperse farther than females (Clark *et al.* 2008, Glaudas and Rodríguez-Robles 2011); thus, estimates of phylogeographic structure based on mitochondrial markers likely will underestimate actual gene flow between populations. Our geographic sampling is limited, given the absence of samples from the northwestern portion of the distribution of *C. polystictus.* However, we think it unlikely that more extensive structure would be detected using our mitochondrial marker because the samples from Tapalpa, Jalisco, are partially separated from other samples by the lower elevations of the Río Santiago Basin in central Jalisco, which is one of the most likely filter barriers separating extant populations. With respect to our comparative dataset, we note that most North American pitvipers with high levels of within-species SD traditionally have been recognized as widespread polytypic taxa, and multiple lines of evidence suggest that many are species complexes (e.g., Douglas *et al.* 2006, Bryson *et al.* 2011a); thus, as taxonomy more accurately reflects species boundaries, we presume that the high SD exhibited by some taxa will be reduced.

When compared with Mexican montane rattlesnakes of the *Crotalus triseriatus* Group, *C. polystictus* has the lowest maximum within-species SD (1.0% versus 3.4–14.1%). With the exception of *C. transversus + C. tancitarensis,* which is highly restricted in distribution, *C. polystictus* also has lower maximum within-species SD than do species of the *C. intermedius* Group. The large-scale genetic diversity and diversification in the *C. triseriatus* and *C. intermedius* groups is spatially and temporally concordant with events that occurred during the dynamic geologic history of central Mexico, most notably the Late Neogene uplift of the Transvolcanic Axis and later Quaternary climatic cycling (Bryson *et al.* 2011a, b). During Quaternary glacial periods, Mexican pine-oak forests expanded and were displaced to lower elevations, linking populations of species dependent on these habitats. During the warmer interglacial periods, these forests were fragmented as they retracted to higher elevations (Martin and Harrell 1957, McDonald 1993). *Crotalus polystictus* is associated with mid-elevation grasslands, which presumably expanded and replaced pine-oak forests during interglacial periods, and retracted during colder glacial periods as forests advanced to lower elevations (Lozano-Garcia and Ortega-Guerrero 1994). The shallow phylogeographic structure in *C. polystictus* compared with the *C. triseriatus* Group and most species of the *C. intermedius* Group may reflect prolonged, cyclical bottlenecks in *C. polystictus* during glacial periods when grasslands were reduced and likely fragmented, followed by rapid expansion of distribution and population size during interglacials. Douglas *et al.* (2006) also invoked Pleistocene bottlenecks to explain genetic diversity in *C. tigris,* a species with phylogeographic structure and distributional

area similar to that of *C. polystictus*. Both species have relatively low nucleotide diversity ($^\wedge\pi$ = 0.004 in *C. polystictus* and $^\wedge\pi$ = 0.008 in *C. tigris*) but moderate haplotype diversity (h = 0.83 ± 0.07 in *C. polystictus* and h = 0.71 ± 0.09 in *C. tigris*), a pattern that may be caused by recent demographic bottlenecks (Grant and Bowen 1998).

Although *Crotalus polystictus* has relatively shallow genetic structure at ATPase 8 and 6 loci, published analyses of rattlesnake phylogeny show a long branch leading to *C. polystictus* (Murphy *et al.* 2002, Castoe and Parkinson 2006), indicating substantial divergence from other species. If the lineage leading to *C. polystictus* experienced late Neogene diversification, as did the *C. triseriatus* and *C. intermedius* groups (Bryson *et al.* 2011a, b), then closely related lineages have either gone extinct or have not been sampled in this study. Low sequence divergence in *C. polystictus* is not exceptional among rattlesnakes and is consistent with Quaternary climatic events, and in itself does not necessarily indicate recent inbreeding depression mediated by human activities. However, the continued fragmentation and extirpation of populations could exacerbate naturally low diversity through the loss of unique regional haplotypes.

Acknowledgments

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Appendix I. GenBank sequences used in this study.

Agkistrodon contortrix (*N* = 33): FJ417848–880, FJ417907–939. *A. piscivorus* (*N* = 23): FJ417884–906, FJ417943–965.

Crotalus aquilus (*N* = 16): HQ257641–642, HQ257652, HQ257662, HQ257665, HQ257682–685, HQ257709–710, HQ257720, HQ257728, HQ257737, HQ257753, HQ257756. *C. aquilus* x *lepidus* (*N* = 3): HQ257705, HQ257715, HQ257717. *C. cerastes* (*N* = 11): DQ493803–813. *C. cerberus* (*N* = 11): AF462360–366, AF462372–375. *C. intermedius* (*N* = 7): JN022801–806, JN022819. *C. lepidus* (*N* = 29): HQ257645–646, HQ257650, HQ257657, HQ257661, HQ257664, HQ25766–680, HQ257692–694, HQ257706–708, HQ257714, HQ257725–726, HQ257730–732, HQ257735, HQ257741, HQ257754. *C. lepidus morulus* (*N* = 13): HQ257647–649, HQ257655, HQ257659, HQ257663, HQ257686–687, HQ257722–724, HQ257742. *Crotalus mitchellii* (*N* = 29): DQ493761–762, DQ493772–798. *C. pricei* (*N* = 39): JN022792–799, JN022807–818, JN022820–823, JN022826–285. *C. pusillus* (*N* = 6): HQ257653–654, HQ257695–696, HQ257719, HQ257758. *Crotalus ravus* (*N* = 12): HQ257691, HQ257697–702, HQ257711, HQ257716, HQ257718, HQ257721, HQ257729. *C. ruber* (*N* = 4): DQ493799–802. *C. stephensi* (*N* = 9): DQ493763–771. *C. tigris* (*N* = 11): DQ493814–824. *C. transversus* + *C. tancitarensis* (*N* = 5): JN022789–791, JN022800, JN022824. *C. triseriatus armstrongi* (*N* = 9): HQ257640, HQ257643–644, HQ257688–690, HQ257713, HQ257734, HQ257743, HQ257745–747. *C. triseriatus triseriatus* (*N* = 14): HQ257703–704, HQ257712, HQ257733, HQ257736, HQ257739–740, HQ257744, HQ257749, HQ257756–757. *Crotalus v.* (*N* = 5): AF462367–371.

Sistrurus catenatus (*N* = 21): FJ659860–880. *S. miliarius* (*N* = 6): FJ659881–886.