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Phylogeographic structure and historical demography of the western diamondback rattlesnake (*Crotalus atrox*): A perspective on North American desert biogeography

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Abstract

The western diamondback rattlesnake (*Crotalus atrox*) is a prominent member of North American desert and semi-arid ecosystems, and its importance extends from its impact on the region's ecology and imagery, to its medical relevance as a large deadly venomous snake. We used mtDNA sequences to identify population genetic structure and historical demographic patterns across the range of this species, and relate these to broader patterns of historical biogeography of desert and semi-arid regions of the southwestern USA and adjacent Mexico. We inferred a Late Pliocene divergence between peninsular and continental lineages of *Crotalus*, followed by an Early Mid Pleistocene divergence across the continental divide within *C. atrox*. Within desert regions (Sonoran and Chihuahuan Deserts, Southern Plains, and Tamaulipan Plain) we observed population structure indicating isolation of populations in multiple Pleistocene refugia on either side of the continental divide, which we attempt to identify. Evidence of post-glacial population growth and range expansion was inferred, particularly in populations east of the continental divide. We observed clear evidence of (probably recent) gene flow across the continental divide and secondary contact of haplotype lineages. This recent gene flow appears to be particularly strong in the West-to-East direction. Our results also suggest that *Crotalus tortugensis* (Tortuga Island rattlesnake) and a population of '*C. atrox*' inhabiting Santa Cruz Island (in the Gulf of California) previously suggested to be an unnamed species, are in fact deeply phylogenetically nested within continental lineages of *C. atrox*. Accordingly, we suggest *C. tortugensis* and '*C. atrox*' from Santa Cruz Island be placed in the synonymy of *C. atrox*.

Keywords: *Crotalus atrox*; *Crotalus tortugensis*; Desert biogeography; mtDNA; Phylogeography; Pleistocene refugia; Western diamondback rattlesnake

1. Introduction

The western diamondback rattlesnake, *Crotalus atrox*, is a prominent member of North American desert and semi-arid communities. The importance of this species is manyfold, with a strong impact on this region's ecology as well as its folklore and imagery. The western diamondback rattlesnake is also the most medically important species of North American snakes (Campbell and Lamar, 2004). Its expansive geographic range, readiness to defend itself, large size, and relatively potent venom result in this species being responsible for more human deaths from envenomation than any other snake within its range (Campbell and Lamar, 2004).

Despite the importance, abundance, and expansive range of this species, its ecology, systematics, and biogeography remain poorly known (Campbell and Greene, 1992; Fitch and Pisani, 1993). Unlike most wide-ranging species of rattlesnakes (e.g., *C. durissus*, *C. mitchelli*, *C. oreganus*, *C. ruber*, *C. scutulatus*, *C. viridis*) there are no recognized subspecies within *C. atrox*. Several authors have, however, demonstrated geographic morphological variation within

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C. atrox (Beaupre et al., 1998; Fitch and Pisani, 1993; Greene, 1997; Klauber, 1930, 1972; Spencer, in press).

Klauber (1972) hypothesized that *C. atrox* was most closely related to the Tortuga Island rattlesnake (*C. tortugensis*) and the red diamond rattlesnake (*C. ruber*). Brattstrom (1964) included the eastern diamondback rattlesnake (*C. adamanteus*) in this grouping, based on osteological and morphological characters and Minton's (1956) data on venom composition. Based on a large sampling of mitochondrial gene sequences for almost all rattlesnake species, Murphy et al. (2002a) redefined the "*atrox* species group" to include the three mainland species: *C. atrox*, *C. adamanteus*, and *C. ruber*, and four island forms (*C. catalinensis*, *C. lorenzoensis*, *C. tortugensis*, and '*C. atrox*' from Santa Cruz Island in the Gulf of California). Within this group, they identified a clade containing *C. atrox* (from Texas, USA) as the sister taxon to *C. tortugensis* plus '*C. atrox*' from Santa Cruz Island. Based on their results, Murphy et al. (2002a) suggested that the population currently assigned to *C. atrox* on Santa Cruz Island likely represents an unnamed species (although they did not formally name this population).

Based on the results of Murphy et al. (2002a; see also Castoe and Parkinson, 2006), the "*atrox* species group" is composed of two clades: a continental "*atrox* clade" including *C. atrox*, *C. sp. nov.* from Santa Cruz Island, and *C. tortugensis*, and a peninsular "*ruber*" clade including *C. ruber* and *C. catalinensis* (*C. lorenzoensis* has never been included in a molecular phylogenetic study). These two groupings also are consistent with other studies estimating the relative affinities of island forms with either *C. atrox* or *C. ruber* (Klauber, 1972; Murphy et al., 1995).

In the USA, *C. atrox* occurs from western Arkansas through Oklahoma and Texas, westward into the deserts of central and southern New Mexico and Arizona, reaching the southern point of Nevada and the extreme southeast of California (Campbell and Lamar, 2004; Klauber, 1972; Fig. 1). In Mexico, *C. atrox* occurs from Nuevo León westward to the northeastern corner of Baja California del Norte, on several islands in the Gulf of California, and south along the Gulf of California on the Mexican mainland to northern Sinaloa, and then eastward across Chihuahua to northern Veracruz, along the Gulf of Mexico (Campbell and Lamar, 2004; Klauber, 1972; Fig. 1). Also, "relictual populations" (Campbell and Lamar, 2004; Klauber, 1972) are known from central Hidalgo, and further south, in Tehauntepec, Oaxaca (Fig. 1).

Across its range, *C. atrox* generally inhabits a wide array of lower elevation xeric or seasonally dry habitats and a variety of vegetation types including mesquite-grassland, desert, and pine-oak forests, tropical deciduous forest and thorn forest (Campbell and Lamar, 2004). Overall, the range of this species spans several major arid and semi-arid biogeographic regions that include: the Sonoran Desert,

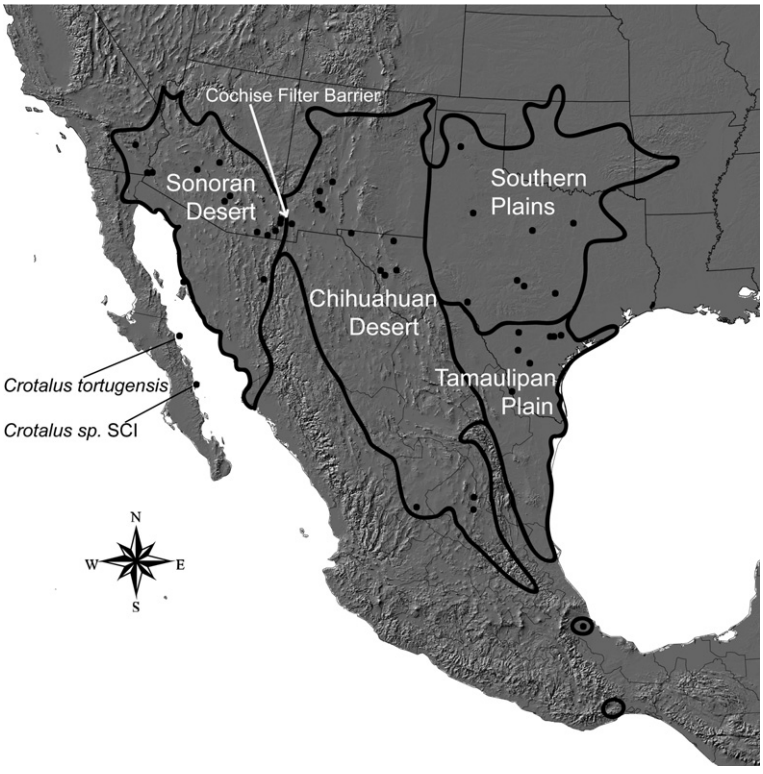

Fig. 1. Geographic range of *C. atrox* across United States and Mexico, showing four biogeographic regions discussed in the text: Sonoran Desert, Chihuahuan Desert, Tamaulipan Plain, and Southern Plains. Black dots indicate collecting localities sampled for molecular analyses in this study. The ranges of two island forms, *C. tortugensis* and *Crotalus sp.* Santa Cruz Island (SCI) are also shown. The location of the "Cochise Filter Barrier", which corresponds to the Continental Divide in southeastern Arizona–southwestern New Mexico, is indicated. Ranges are redrawn from Campbell and Lamar (2004) and Spencer (in press).

Chihuahuan Desert, Tamaulipan Plain, and Southern Plains regions (Fig. 1; based on regional designations from Blair, 1949; MacMahon, 1985; Tennant, 1998).

The major continental desert and semi-arid regions of the southwestern USA and northern Mexico are bisected by the continental divide (Fig. 1). Extending down the Rocky Mountains, through the Colorado Plateau, and down the Sierra Madre Occidental of Mexico, the continental divide separates the Sonoran Desert to the West from the Chihuahuan Desert and other semi-arid regions in the East. The initial separation of these desert regions on either side of the continental divide was formed through uplifting of the Colorado Plateau and Sierra Madre Occidental during the late Miocene–early Pliocene, followed by subsequent intensification of the barrier between these regions during the Pleistocene (Morafka, 1977). These continental desert regions appear to have been isolated from desert regions associated with the Baja Peninsula by the Bouse embayment, a northward extension of the Sea of Cortés, which began 5.5 MYA and climaxed approximately 3 MYA (Morafka, 1977). Evidence of this historical separation between peninsular and continental arid regions has been observed across various taxa (Riddle et al., 2000c; Riddle and Hafner, 2006), including mice (e.g., Riddle et al., 2000a,b), birds (Zink et al., 2001), spiders (Crews and Hedin, 2006), fishes (Riginos, 2005), snakes (Mulcahy, unpublished data), lizards (Hedges et al., 1991; Orange et al., 1999), toads (Jaeger et al., 2005), and tortoises (Lamb et al., 1989).

The Sonoran and Chihuahuan Deserts are widely separated by the Sierra Madre Occidental throughout Mexico, and only in the USA (near the border of Arizona and New Mexico) do these two deserts nearly meet. This interface, called the Deming Plains, or Cochise filter barrier (Morafka, 1977), is a lower elevation arid-grassland/desert-scrub region, spanning 100–200 miles across. Morafka (1977) suggested that the last corridor of continuous desert habitat connecting the Sonoran and Chihuahuan Deserts (across the Deming Plains) was transformed by climatic cooling that began roughly 3 MYA, and continued through the Pleistocene. Evidence from packrat middens suggests that this region was woodland (*Pinus*, *Juniperus*, and *Quercus* spp.) during the last glacial period (~18 thousand years ago; KYA), and only transformed into arid-grassland and desert as recently as 4 KYA (Van Devender, 1990; Van Devender et al., 1984). Collectively, available evidence suggests a scenario where the biogeography of North American deserts has been heavily impacted during the Neogene (Miocene through Pliocene) by major tectonic events, and later impacted strongly by the effects of Pleistocene climatic cycling and the onset of Holocene climate patterns.

The earliest fossil remains assigned to *C. atrox* ("*Crotalus* c.f. *atrox*") occur in the Middle Pliocene (Blancan III, approx. 3.7–3.2 MYA) deposits of Scurry County, Texas (Holman, 2000; Rogers, 1976) in the Southern Plains region of Texas. It is difficult to determine, however, if these remains indeed represent an exclusive ancestor of *C. atrox* or a more inclusive taxon. Several additional fossils allocated to *C. atrox* occur in the Mid Pleistocene, as well as the early portions of the Late Pleistocene of Texas (reviewed by Holman, 1995, 2000). No fossil remains of *C. atrox* are known west of the continental divide until the Late Pleistocene, when they appear in the fossil record of Arizona, California, and Nevada (in addition to sites east of the divide in New Mexico and Texas from this later period). All of the fossil sites west of the continental divide from which *C. atrox* fossils are known have been radiocarbon dated, and none are estimated to be older than approximately 13,000 years (Holman, 2000). The fossil data suggest that *C. atrox* (or a more inclusive taxon) has inhabited at least the eastern portions of the southwestern USA and adjacent areas of Mexico as early as the middle Pliocene. These data also suggest that *C. atrox* has occupied eastern regions relatively continuously since that time. Fossil evidence, however, does not provide much detail relative to the history of western populations of *C. atrox* except that they can be placed there during the Late Pleistocene.

We designed this study to address several questions relative to the genetic structure and historical patterns of gene flow within the western diamondback rattlesnake, and to interpret these taxon-specific patterns in the broader context of the historical biogeography of North American deserts. Our goal was to extract as much historical information as possible from our genetic sampling of *C. atrox* including relationships among individuals and populations, historical population-size changes, directional patterns of gene flow, historical population genetic structure, and timing of major population events. To accomplish this, we used an array of analytical approaches including: (1) phylogenetic analyses, (2) Nested Cladistic Phylogeographical Analysis (Templeton, 1998), (3) population genetic analyses (classical population genetic statistics, mismatch distributions, molecular analyses of variance), and (4) fitting of the isolation-with-migration model of Hey and Nielsen (2004). Using these overlapping methods of inference, we formulate a cohesive hypothesis for the historical population-level patterns detectable within *C. atrox*, and relate these to potential biogeographical patterns and historical processes that may have generally impacted North American desert biota. In addition to broader questions regarding biogeography, we use our data to address the taxonomic status of *C. tortugensis* and of the rattlesnake population inhabiting Santa Cruz Island that is currently assigned to *C. atrox*.

2. Materials and methods

2.1. Geographic and taxonomic sampling

Specimens of *C. atrox* from across nearly the entire range in the USA and Mexico were sampled ($n = 48$; Table 1). The sampling design was constructed to include essentially all major and, in many cases, minor putative geographic regions inhabited by this species. Our

Table 1
Data for individuals sampled including taxon, unique specimen code (corresponding to labels on subsequent figures), collector or museum data, geographical origin, and GenBank Accession

Taxon	Specimen code	Voucher	Country	State	County	GenBank Accession
Crotalus tigris	*Crotalus tigris*	CLP169	USA	AZ	Pima	AF156574
Crotalus molossus	*Crotalus molossus*	CLP66	USA	TX	El Paso	AY223695
Crotalus ruber	*Crotalus ruber*	RWV2001-08	USA	CA	Riverside	DQ679838
Crotalus tortugensis	*Crotalus tortugensis*	ROM18192	Mexico	Baja California Sur	(Isla Tortuga)	DQ679839
Crotalus sp.	*Crotalus sp.* SCI	ROM18244	Mexico	Baja California Sur	(Santa Cruz Is.)	DQ679840
Crotalus atrox	MEX SanLuisPotosi C31	ENS10537	Mexico	San Luis Potosi		DQ679842
	MEX SanLuisPotosi C35	ENS10536	Mexico	San Luis Potosi		DQ679841
	MEX Sonora C75	TWR1249	Mexico	Sonora		DQ679843
	MEX Sonora C79	LSUMZ H-5548	Mexico	Sonora		DQ679844
	MEX Veracruz C32	ENS10538	Mexico	Veracruz		DQ679845
	MEX Zacatecas C33	ENS10539	Mexico	Zacatecas		DQ679846
	USA AZ Cochise C6	CLS474	USA	AZ	Cochise	DQ679848
	USA AZ Cochise C9	CLS494	USA	AZ	Cochise	DQ679849
	USA AZ Cochise C38	CWP2	USA	AZ	Cochise	DQ679847
	USA AZ Maricopa C44	ENT2	USA	AZ	Maricopa	DQ679850
	USA AZ Maricopa C46	ENT11	USA	AZ	Maricopa	DQ679851
	USA AZ Maricopa C48	ENT7	USA	AZ	Maricopa	DQ679852
	USA AZ Pima C49	LACM150957	USA	AZ	Pima	DQ679853
	USA AZ Pinal C42	ENTA21	USA	AZ	Pinal	DQ679854
	USA AZ Pinal C43	ENTA49	USA	AZ	Pinal	DQ679855
	USA CA Imperial C12	RNF2595	USA	CA	Imperial	DQ679856
	USA CA Imperial C13	RNF2596	USA	CA	Imperial	DQ679857
	USA CA Riverside C81	ROM2398	USA	CA	Riverside	DQ679858
	USA CA Riverside C82	ROM18144	USA	CA	Riverside	DQ679859
	USA NM Dona Ana C22	RWV2001-14	USA	NM	Dona Ana	DQ679864
	USA NM Grant C7	CLS482	USA	NM	Grant	DQ679861
	USA NM Hidalgo C5	CLS471	USA	NM	Hidalgo	DQ679863
	USA NM Hidalgo C47	ENT12	USA	NM	Hidalgo	DQ679862
	USA NM Sierra C21	RWV2001-13	USA	NM	Sierra	DQ679860
	USA NM Sierra C39	BLC27	USA	NM	Sierra	DQ679865
	USA NM Sierra C41	BLC	USA	NM	Sierra	DQ679866
	USA NM Socorro C40	BLC	USA	NM	Socorro	DQ679867
	USA TX Culbertson C73	JJ	USA	TX	Culberson	DQ679868
	USA TX Dallas C72	JJ	USA	TX	Dallas	DQ679869
	USA TX Duval C57	TJL566	USA	TX	Duval	DQ679870
	USA TX ElPaso C51	CLP60	USA	TX	El Paso	DQ679871
	USA TX Garza C74	CLS576	USA	TX	Garza	DQ679872
	USA TX Goliad C58	TJL588	USA	TX	Goliad	DQ679873
	USA TX Jeff Davis C18	RWV2001-09	USA	TX	Jeff Davis	DQ679874
	USA TX Jeff Davis C28	RWV2001-22	USA	TX	Jeff Davis	DQ679875
	USA TX Jeff Davis C52	CLP64	USA	TX	Jeff Davis	DQ679876
	USA TX Karnes C60	TJL593	USA	TX	Karnes	DQ679877
	USA TX Karnes C61	TJL719	USA	TX	Karnes	DQ679878
	USA TX LaSalle C20	RWV2001-12	USA	TX	LaSalle	DQ679879
	USA TX LaSalle C62	TJL527	USA	TX	LaSalle	DQ679880
	USA TX Llano C63	TJL601	USA	TX	Llano	DQ679881
	USA TX Mason C64	TJL	USA	TX	Mason	DQ679882
	USA TX Potter C65	TJL868	USA	TX	Potter	DQ679883
	USA TX Stephens C53	CLP199	USA	TX	Stephens	DQ679884
	USA TX Travis C66	TJL718	USA	TX	Travis	DQ679885
	USA TX ValVerde C68	TJL347	USA	TX	Val Verde	DQ679886
	USA TX ValVerde C69	TJL348	USA	TX	Val Verde	DQ679887
	USA TX Zapata C71	TJL775	USA	TX	Zapata	DQ679888

Acronyms for vouchers (collectors or museums) are as follows: BLC, Bruce Christman; CLP, Christopher Parkinson; CLS, Carol Spencer; CWP, Charles Painter; ENS, Eric Smith; ENT, Emily Taylor; JJ, Jim Jones; LACM, Los Angeles County Museum; LSUMZ, Louisiana State University Museum of Zoology; RNF, Robert Fisher; ROM, Royal Ontario Museum; RWV, R. Wayne Van Devender; TJL, Travis LaDuc; TWR, Tod Reeder.

geographic sampling of the USA excluded only peripheral regions of the range of *C. atrox* (e.g., Nevada, Oklahoma, Arkansas), although our sampling of the Mexican range of this species was limited to a smaller number of sampling localities (with substantial sampling gaps in the Mexican portions of the Chihuahuan desert, Tamaulipan Plain, and Sonoran Desert). To assess the monophyly of *C. atrox*, two species thought to be close relatives of *C. atrox* (*C. tortugensis*, and *C. ruber*) were sequenced. Additionally, we included a sample from the Santa Cruz Island population

of '*C. atrox*' (referred to hereafter as "*Crotalus sp.* SCI") suggested to be a distinct but unnamed species by Murphy et al. (2002a). Previously published sequences for *C. molossus* and *C. tigris* (Parkinson et al., 2002) were used as outgroups for rooting phylogenies. Voucher specimens and geographic localities corresponding to tissues used in this study are detailed in Table 1.

2.2. Laboratory methods

Genomic DNA was isolated from tissue samples (shed skins or liver, heart, or scale fragments preserved in ethanol) using the Qiagen DNeasy extraction kit and protocol (Qiagen). A fragment of the mitochondrial gene encoding the fourth subunit of NADH dehydrogenase (plus downstream Serine, Histidine, and Leucine tRNAs; hereafter collectively referred to as ND4) was amplified via PCR using the primers ND4 and Leu as described in Arévalo et al. (1994). Positive PCR products were purified using the GeneCleanIII kit (BIO101). Purified PCR products were sequenced with the amplification primers and an additional internal primer HIS (Arévalo et al., 1994). Samples that could not be sufficiently sequenced directly (e.g., if direct sequencing of PCR products did not produce acceptable quality sequence) were cloned using the Topo TA cloning kit (Invitrogen) according to the manufacturer's protocols except with 1/3 total reaction volume. Positive clones were grown in liquid culture and plasmids were isolated from multiple clones per individual using the Qiaquick spin miniprep kit (Qiagen). Plasmids were sequenced using M13 primers (provided by Topo TA kit, Invitrogen) and the internal HIS primer. Purified PCR products and plasmids were sequenced using the CEQ Dye Terminator Cycle Sequencing Quick Start Kit (Beckman-Coulter) and run on a Beckman CEQ8000 automated sequencer according to the manufacturers' protocols (except reactions cut to 1/3 volume).

2.3. Sequence homology and alignment

Raw sequence chromatographs were edited and initially aligned using Sequencher 4.1 (Gene Codes Corp.). In cases where gene fragments were cloned, chromatographs from multiple clones as well as partial sequences from directly sequenced PCR products were combined and edited together per specimen. These alignments were rechecked based on inferred amino acid sequence homology (protein coding region) and secondary structure models (for tRNA regions; Parkinson, unpublished) using Genedoc (Nicholas and Nicholas, 1997). Alignment was unambiguous and no indels were inferred among *C. atrox* samples (see results for other details). This final alignment consisted of a total of 876 aligned positions (bases), three of which were inferred as gaps (relative to outgroup sequences) occurring in the loop structures of tRNAs in *C. atrox*. Sequences were deposited in GenBank under the accession numbers listed in Table 1.

2.4. Phylogenetic analyses

Phylogenetic relationships among haplotypes were estimated using maximum parsimony (MP) analyses implemented in PAUP* 4.0b10 (Swofford, 2002), and Bayesian Markov-chain Monte Carlo phylogenetic analyses (MCMC) implemented in MrBayes version 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003).

Maximum parsimony searches were conducted with all characters equally weighted and employed the heuristic search option, tree bisection reconnection (TBR) branch swapping, and 10,000 random taxon-addition sequence replicates. Gaps were treated as "missing" for all analyses. Nodal support was assessed using non-parametric bootstrapping (Felsenstein, 1985) with 10,000 full-heuristic pseudo-replicate and 100 random taxon-addition sequence replicate per bootstrap pseudo-replicate. Trees representing alternative hypotheses (1: *C. atrox* is monophyletic [with respect to *C. tortugensis* and *Crotalus sp.* SCI] and, 2: haplotypes sampled from east and west of the continental divide form reciprocally monophyletic groups) were constructed and tested for significance using the Wilcoxan signed ranks test (or Templeton test; Templeton, 1983), implemented in PAUP* 4.0b10, and interpreted in light of the suggestions of Goldman et al. (2000).

ModelTest 3.0 (Posada and Crandall, 1998, 2001) was used to select a best-fit model of evolution, based on Akaike Information Criteria (AIC; for justification, see Posada and Buckley, 2004; Posada and Crandall, 2001). The selected model (general time reversible model with gamma-distributed among-site rate variation; GTR + γ) was implemented in MCMC analyses conducted in MrBayes version 3.1. The program's default priors for parameters of the MCMC analyses were used for all analyses. Four independent MCMC runs were initiated (each with two tandem runs, as per the program's default) with random starting trees and were each run for 4×10^6 generations with trees (and parameters) sampled every 100 generations. Stationarity was assessed by examining the relative stabilization of cold chain likelihood scores and parameter estimates using Tracer (Rambout and Drummond, 2003), and by confirming convergence by comparing the variance across chains within a search to the chain variance among searches using Rubin and Gelman's "*r*" statistic (Gelman et al., 1995).

Apparent stationarity of MCMC runs was reached at no later than 100,000 generations. Conservatively, the initial 1×10^6 generations were discarded as "burn-in" from each independent run. A 50% majority-rule consensus phylogram was constructed from the posterior distribution of MCMC trees for each run independently and compared (along with parameter and chain likelihood values) across independent runs to confirm convergence. A 50% majority-rule consensus tree was estimated based on all 24×10^6 post-burn-in generations (from all four independent runs combined) with bipartition frequency representing the

posterior probabilities of clades derived from the sum of these pooled post-burn-in estimates. This strategy of pooling post-burn-in data from multiple independent MCMC runs has been shown to produce essentially equivalent nodal posterior probability estimates compared with MCMC runs employing very large numbers of generations (i.e., tens of millions of generations; Castoe et al., 2004).

2.5. Nested cladistic phylogeographical analysis

To infer population history, we used Nested Cladistic Phylogeographical Analysis (or nested contingency analysis of geographical associations; NCPA; Templeton, 2004; Templeton et al., 1995). A 95% plausible parsimony network connecting the ND4 haplotypes was inferred using statistical parsimony (Templeton, 1998; Templeton et al., 1992) as implemented in the program TCS version 1.13 (Clement et al., 2000). The nesting design was constructed by hand based on the haplotype network following the guidelines of Templeton et al. (1992), Crandall and Templeton (1996), and Templeton (1998). This nested clade design was used, along with geographic location designated by unprojected latitude and longitude for each sampled haplotype (decimal degrees, WGS84 datum), to analyze geographic associations among hierarchically nested clades. The program GeoDis 2.0 (Posada et al., 2000) was used to estimate NCPA distance measures that we then interpreted based on the inference key given by Templeton (2004). Statistical significance was calculated by comparison with a null distribution generated from 100,000 random permutations of clades against sampling localities.

2.6. Estimating mutation rates and generation time

Ideally, estimates of mutation rates are obtained based on known historical events that may be used as calibration points for a taxon-specific mutation-rate estimate (i.e., local molecular clock rates). In our case, no such events were available, so we were forced to rely on estimates from other studies. Early estimates of rates of mtDNA evolution in reptiles (based mostly on RFLP data) implied a very slow relative rate of mitochondrial gene evolution (e.g., Lamb et al., 1989; Hedges et al., 1991). These early estimates led subsequent researchers to infer similarly slow rates for snake mtDNA (e.g., Zamudio and Greene, 1997). Recent evidence for the relatively rapid rate of snake mtDNA evolution (e.g., Dong and Kumazawa, 2005) instead supports a relatively high rate of mitochondrial gene evolution in snakes. A previous study on Neotropical species of pitvipers (Wüster et al., 2002) used a well-known geologic event (formation of the Panamanian isthmus) to estimate rates of evolution of mtDNA (including the ND4 gene region used here) at approximately 1.4% per MY between lineages. This estimate is more congruent with contemporary views of relatively rapid rates of mtDNA evolution in snakes. We apply this estimate of evolutionary rate to our dataset. Given this rate, we qualify this application by examining the congruence of fossil and biogeographic data available with the estimates of timing of historical events that this rate provides.

The application of a single rate of evolution across a tree implies the existence of clock-like evolution. To test this assumption, we used maximum likelihood heuristic searches (conducted in PAUP* with the substitution model suggested by AIC in Modeltest) to estimate the likelihood with and without the molecular clock constraint. For these analyses, we excluded the outgroup sequences (*C. molossus* and *C. tigris*). We compared these models using a likelihood ratio test (Felsenstein, 1981; Huelsenbeck and Crandall, 1997), and evaluated the significance by comparing twice the likelihood ratio to the one-tailed chi-squared distribution with S-2 degrees of freedom (where S equals the number of sequences).

We estimated the generation time of *C. atrox* to be 3.3 years, based on 78 female adult *C. atrox* museum specimens that were examined for age at reproduction (Spencer, unpublished data). Reproductive status was determined by examining the ovaries and oviducts for the presence of embryos, enlarged follicles or ovulation scars (corpora lutea) in females with complete rattle strings. The youngest females reproduced at 1.5 years of age (3 rattle segments) and, on average, females reproduced when they were 3.3 years of age (6.67 rattle segments, $n = 78$). This latter number was used for average generation time (3.3 years).

2.7. Estimates of genetic diversity, genetic structure, and historical demography

We used Arlequin version 3.000 (Schneider et al., 2000) to estimate an array of population genetic statistics. For comparisons between groups, we split the *C. atrox* dataset (including *C. tortugensis* and *C. sp.* SCI) into the following geographically defined populations: populations east of the continental divide ("East Population"), subdivided into the Chihuahuan Desert, Tamaulipan Plain, and Southern Plains populations, and populations west of the continental divide ("West Population", the Sonoran Desert and immediately adjacent regions; Fig. 1). Where applicable, we also estimated statistics to describe and to compare major clades recovered from our phylogenetic and nested cladistic phylogeographical analyses. Genetic diversity and demographic patterns were examined using nucleotide diversity (π), the mean number of pairwise differences (k), and Fu's Fs (Fu, 1997). We estimated the hierarchical partitioning of genetic variation using an analysis of molecular variance (AMOVA; Excoffier et al., 1992). Demographic patterns were examined using mismatch distributions comparing observed versus expected distributions of pairwise nucleotide differences between haplotypes to evaluate the hypothesis of recent population growth. The fit of the observed data to the expected data (under the sudden growth model) was compared using the sum of squares deviations between observed and expected data estimated from 1000 parametric bootstrap

replicate, and Harpending's raggedness index (Harpending, 1994).

2.8. Estimation of parameters of the isolation-with-migration model

We used the program IM (Hey and Nielsen, 2004) to estimate the marginal posterior probability density of parameters of the isolation-with-migration model (see Hey and Nielsen, 2004, for details and justification of the model). This model is designed to estimate several population patterns or phenomena when a single ancestral population splits into two populations, including the time since the two populations diverged, the sizes of the ancestral and descendent populations, and directional gene flow between descendent populations (Hey and Nielsen, 2004).

We used IM to estimate parameters of the isolation-with-migration model between populations East and West of the continental divide, assuming that these split from a single ancestral population. These parameters included: θ_{West} (effective female population size of the western population), θ_{East} (effective female population size of the eastern population), θ_A (effective female population size of the ancestral population at the time of population divergence), t (time of divergence), m_{West} (effective number of western female migrants to the eastern population per generation), and m_{East} (effective number of eastern female migrants to the western population per generation). These parameters were converted (rescaled) to population parameters (e.g., $\theta_{West} \rightarrow N_{ef\text{-}West}$; $m_{West} \rightarrow M_{West}$ [migrants per generation]) using the mutation-rate estimate (1.4% per site, per MY, between lineages) and the estimate of generation time (3.3 years), as described in Hey and Nielsen (2004).

All IM runs were conducted with a burn-in period of 5×10^6 generations, followed by 15×10^6 post-burn-in generations. The length of the burn-in period was determined based on results of early trial runs, and the number of post-burn-in generations was chosen based on the effective sample size (ESS) values exceeding 100 for all parameters for runs of this length in early trials. Maximum values for the range of parameter priors of the IM model used for final analyses were as follows: q1 (θ_{East}) = 250, q2 (θ_{West}) = 250, qA (θ_A) = 1000, t (t) = 100, m1 (m_{East}) = 5, m2 (m_{West}) = 5. These priors were set based on results of early runs with various broad-ranged priors. Parameter estimates from IM were rescaled post-analyses using the mutation-rate and generation-time estimates. Convergence of parameters from individual IM runs was confirmed by running a total of four independent runs.

3. Results

3.1. Results of phylogenetic analyses

Within *C. atrox*, a total of 32 haplotypes were identified (and two additional haplotypes from *C. tortugensis* and *C. sp.* SCI). Uncorrected pairwise percent sequence divergence among *C. atrox* haplotypes ranged from 0% to 2.48%. Uncorrected percent divergence between *C. tortugensis* and *C. atrox* haplotypes ranged from 0.2% to 2.2%, from 0.2% to 2.2% between *Crotalus sp.* SCI and*C. atrox*, and from 4.1% to 5.1% between *C. ruber* and *C. atrox*. Of the total 876 positions analyzed, 705 character were invariant and 70 variable characters were parsimony informative (considering all ingroup and outgroup sequences).

The heuristic MP search identified 16 equally parsimonious trees of 218 steps, and tree statistics as follows: consistency index = 0.872, retention index = 0.890, rescaled consistency index = 0.775, and homoplasy index = 0.128. The strict consensus of these 16 trees is provided with bootstrap support for nodes indicated (Fig. 2).

The four independent runs of Bayesian phylogenetic analyses (MCMC) all converged on similar estimates of MCMC chain likelihood scores after similar numbers of post-burn-in generations. Means and standard deviations (in parentheses) calculated over all MCMC runs for estimated parameters were as follows: −ln likelihood = 2447.87 (9.413), rAC = 4.39 (2.18), rAG = 50.62 (18.22), rAT = 5.95 (2.85), rCG = 3.72 (2.3), rCT = 31.79 (12.83), rGT = 1.00 (0), γ = 0.27 (0.11). A consensus phylogram of the four independent MCMC runs was nearly topologically identical to the MP consensus tree (Fig. 2). Bipartition posterior probabilities derived from each individual MCMC analysis were all very similar. The posterior probabilities for nodes estimated from the combination of all MCMC runs (excluding generations discarded as burn-in) are provided (Fig. 2). *Crotalus ruber* (the red diamond rattlesnake) is strongly supported as the sister lineage to the clade including *C. atrox*, *C. tortugensis*, and *Crotalus sp.* SCI by both phylogenetic methods. Overall, both methods provided very similar estimates for relationships among haplotypes, as can be seen by the juxtaposition of MP and MCMC trees (Fig. 2). All methods strongly support the paraphyly of *C. atrox* with respect to *C. tortugensis* and *Crotalus sp.* SCI. A Templeton test rejected the hypothesis that *C. atrox* is monophyletic with respect to *C. tortugensis* and *Crotalus sp.* SCI ($P < 0.034$). Phylogenetic estimates suggest that haplotypes of *Crotalus sp.* SCI and *C. torgugensis* are most closely related to haplotypes of *C. atrox* from the adjacent mainland of Mexico ("MEX Sonora C75" and "MEX Sonora C79") and other Sonoran Desert-associated haplotypes.

Within the clade that includes all *C. atrox*, *C. tortugensis*, and *Crotalus sp.* SCI haplotypes, there is a strongly supported (posterior probability = 95, bootstrap = 100) primary phylogenetic split that generally separates haplotypes to the East and West of the continental divide (hereafter, these clades are referred to as the "Eastern Clade" and "Western Clade"; Fig. 2). Both major haplotype clades, however, do not represent monophyletic groups of populations strictly from one side of the continental divide. The null hypothesis that haplotypes formed two exclusive monophyletic groups across the axis of the continental divide was rejected by a Templeton test ($P < 0.0001$). In addition to the discordance between

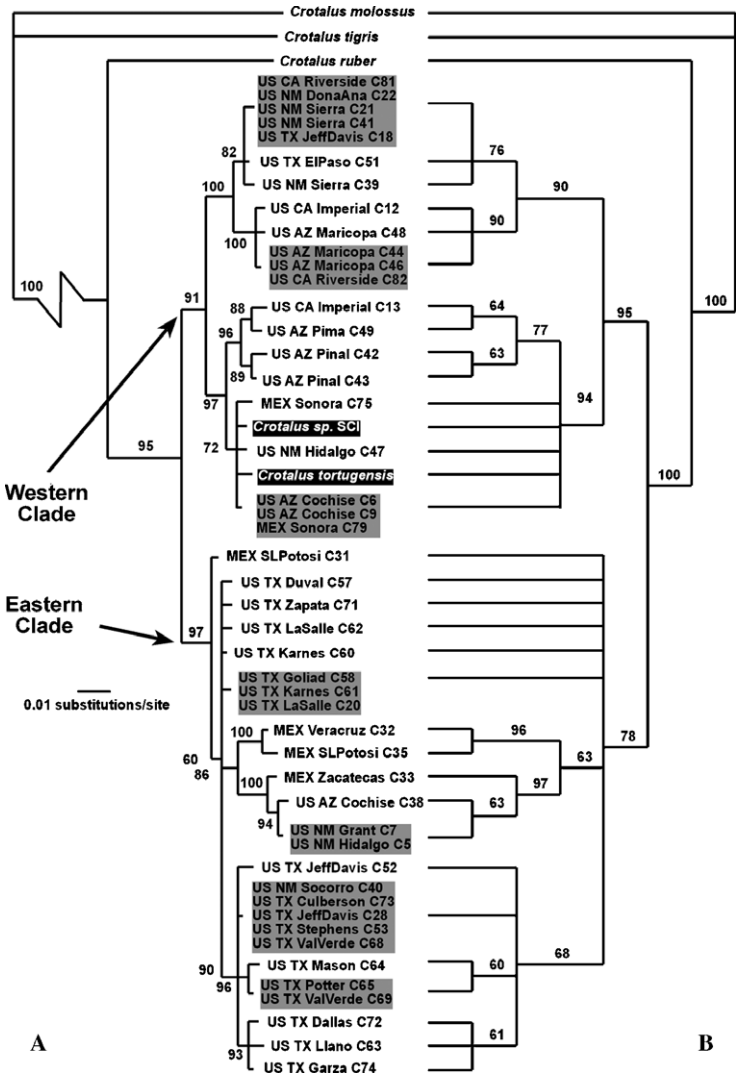

Fig. 2. Inferred phylogenetic relationships among halplotypes of *C. atrox* and *C. tortugensis*, along with outgroups included in this study. Haplotypes are assumed to be *C. atrox* unless specifically labeled. Gray-shaded boxes indicate a single haplotype shared among samples named within each shaded rectangle. Black boxes denote haplotypes of specimens suggested to represent distinct species from *C. atrox* by various authors (see text for details). Names of *C. atrox* haplotypes are based on the naming scheme in Table 1. (A) Fifty percentage majority rule consensus phylogram estimated using Bayesian phylogenetic methods. Bipartition posterior probabilities are given adjacent to respective nodes. (B) Strict consensus of 12 equally parsimonious trees from maximum parsimony analysis. Bipartition bootstrap support is given adjacent to respective nodes.

major clades and the continental divide, less inclusive haplotype clades are not strictly from geographically adjacent populations.

The null hypothesis of clock-like evolution of ingroup sequences was not rejected by a likelihood ratio test comparing unconstrained and molecular-clock-constrained models of evolution (twice the likelihood ratio = 36.5; degrees of freedom = 32; $P > 0.05$). Since a molecular clock was not rejected for ingroup sequences, we used measures of sequence divergence and the estimate of the evolutionary rate to estimate divergence times between the Eastern and Western Clades (including *C. atrox*, *C. tortugensis*, and *C. sp.* SCI haplotypes), and the divergence between *C. ruber* and *C. atrox* (including *C. atrox*, *C. tortugensis*, and *C. sp.* SCI haplotypes). We used the uncorrected average between-group divergence (Dxy = 1.87%; Nei and Tajima, 1983) to estimate the divergence time between Eastern and Western Clades (Fig. 2) at 1.36 MYA (Table 2). Similarly, based on the mean uncorrected sequence divergence between *C. atrox* and *C. ruber*, we estimated the divergence between these species at 3.29 MYA (Table 2).

Table 2
Estimates of divergence times based on mean uncorrected sequence divergence between groups or species

	Sequence divergence (%)	Divergence time (MYA)
Divergence between "East Clade" and "West Clade" of *C. atrox*	1.87	1.36
Divergence between *C. atrox* clade and *C. ruber*	4.61	3.29

3.2. Haplotype network and nested cladistic phylogeographical analysis

Evaluation of the 95% confidence limit of validity of parsimony with the program TCS suggested that a maximum of 12 parsimonious steps could be considered under the 95% connection limit. The inferred haplotype network with the hierarchical nested clade design is shown in Fig. 3. In addition to the 34 haplotypes observed, 37 missing (intermediate) haplotypes were inferred. A total of 20 1-step clades were constructed, of which 7 contained multiple extant sampled haplotypes (leaving 13 degenerate 1-step clades comprising a single haplotype). The entire nested cladogram was accommodated by a 5-step clade that included all haplotypes of *C. atrox*, *C. tortugensis*, and *C. sp*. SCI (Fig. 3).

The haplotype network (and nominal nested clades; Fig. 3) corresponds well with phylogenetic estimates. Nested clades 4-1 and 4-2 correspond exactly to the Eastern and Western Clades identified from phylogenetic reconstructions (Fig. 2). The geographic distributions of the two 4-step and six 3-step clades are plotted geographically on a map of the region (Fig. 4). Haplotypes belonging to the Eastern Clade (4-2) are almost completely confined to localities east of the continental divide, whereas Western Clade (4-1) haplotypes occur throughout localities west of the continental divide as well as regions of southern New Mexico and Texas (Figs. 3 and 4). Haplotypes belonging to clade 3-3 are particularly widespread and extend across the northern sampling localities from California through Texas.

Among haplotypes in the Eastern Clade, clade 3-4, occurring only in southern Texas and in San Luis Potosi, Mexico, has a more restricted distribution (Fig. 4). The other eastern 3-step clades show much broader distributions with clade 3-6 occurring throughout much of northern and central Texas and New Mexico, and 3-5 ranging from the Arizona–New Mexico border, through eastern Mexico, and into southern Texas (overlapping with clade 3-4). Three-step clades nested within the Western Clade have nearly non-overlapping distributions. Clade 3-2 extends from the Arizona–New Mexico border south through the Sonoran Desert of Mexico, and includes haplotypes of *C. tortugensis* and *C. sp.* SCI (Figs. 3 and 4).

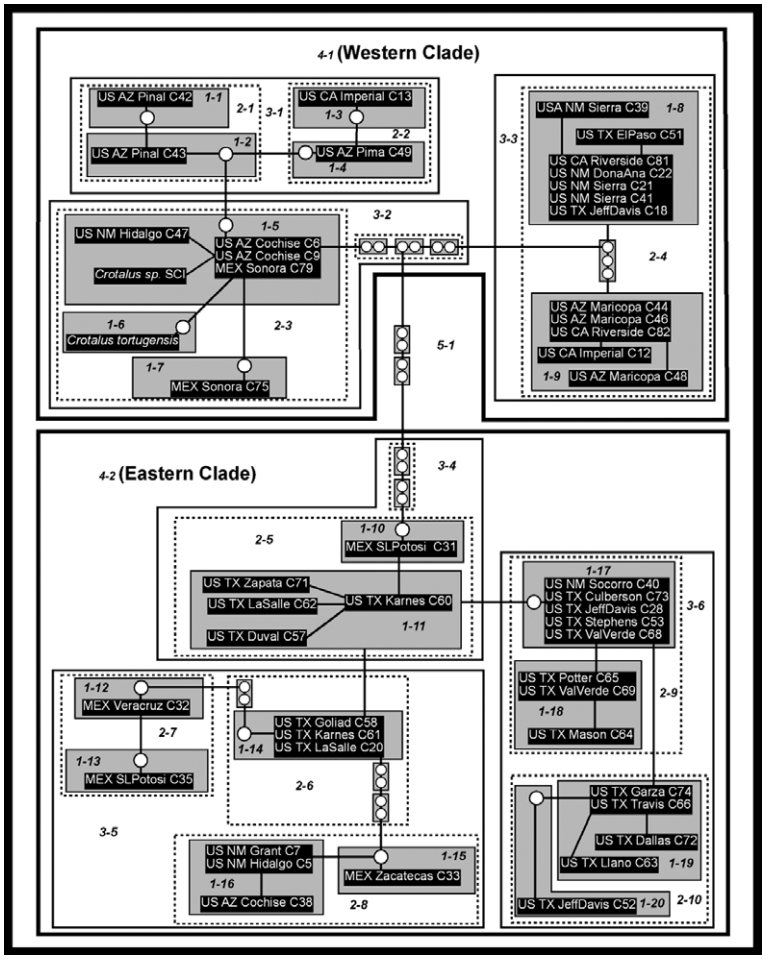

Fig. 3. Haplotype network estimated using statistical parsimony (95% connection limit) connecting haplotypes of *C. atrox*, as well as haplotypes of *C. tortugensis* and *C. sp. SCI* (from Santa Cruz Island). Hierarchically nested clades, for use in nested cladistic phylogeographical analysis (NCPA) are indicated. Haplotypes are represented with a black rectangle, and multiple samples within a single black rectangle share a common haplotype.

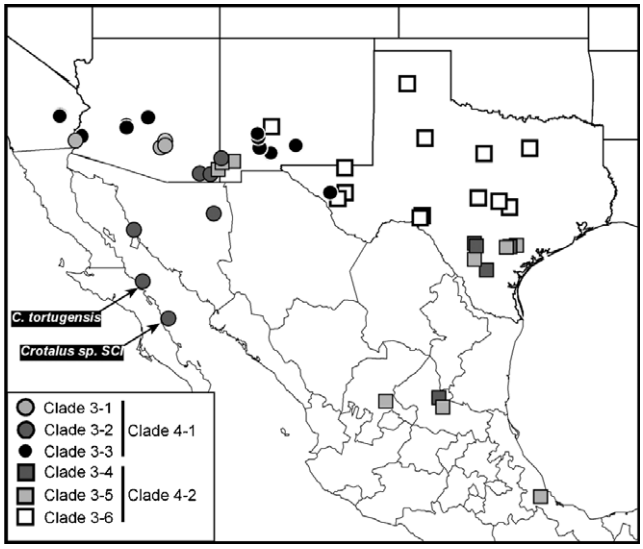

Fig. 4. Geographic distribution of 3-step and 4-step clades of haplotypes from *Crotalus atrox*, *C. tortugensis*, and *C. sp.* SCI. Clade assignment is based on names in Fig. 3.

Three nested clades resulted in significant inferences under NCPA (Table 3). Geographical haplotype distributions associated with clade 3-5 were inferred to represent contiguous range expansion of a central clade (2-6, including samples from southern Texas; Table 3, Fig. 4). Restricted gene flow with isolation by distance was inferred to explain the distribution of haplotype clades nested within the Western Clade (4-1). Range expansion (with inconclusive detailed inference) was inferred for clade 4-2 to explain distributions of clades nested within the Eastern Clade (Table 3).

3.3. Population genetic analyses

Nucleotide diversity (π) across the entire haplotype dataset, including *C. atrox*, *C. tortugensis*, and *C. sp.* SCI haplotypes, was 0.12 and the mean number of pairwise differences (k) was 10.36 (Table 4). Fu's Fs statistic was significantly negative for the entire sample, supporting the general inference of recent population expansion. The West Population showed higher nucleotide diversity and a higher value for k than did the East Population (Table 4). Recent population expansion was inferred by the significantly negative value of Fs for the East Population. Within the East Population, both π and k were highest for the Chihuahuan population (Table 4), which is consistent with the Chihuahuan population containing haplotypes from several clades (e.g., Fig. 4). Comparing population genetic statistics among nominal clades, the Western Clade had the highest values of π and k, while the Eastern Clade had a significantly negative value for Fs (Table 5). Fu's Fs was significantly negative for subclades 3-2, 3-4, and 3-6, suggesting recent population growth (Table 5).

Plots of the observed pairwise differences between haplotypes (mismatch distributions) were multimodal or bimodal in most cases (Table 5, Fig. 5), although none of the comparisons between the observed distributions and the simulated distributions under the sudden expansion model significantly rejected the expansion model (all P-values > 0.1 based on sum of squares distances [SSD], and Harpending's raggedness index [HRI]; Table 5, Fig. 5). Despite the good fit of non-unimodal models to the sudden expansion models, typically multimodal (including bimodal) distributions indicate either structured populations or populations that are stable or shrinking in size (Excoffier and Schnieder, 1999; Rogers and Harpending, 1992; Rogers et al., 1996); thus, good fit of the sudden expansion model to multimodal mismatch distributions is not taken here as strong evidence of expansion. However, an expanding population that receives substantial migration, is subdivided, and/or has undergone historical contraction also may lead to a multimodal mismatch distribution (Bertorelle and Slatkin, 1995; Marjoram and Donnelly, 1994; Ray et al., 2003). Observed pairwise difference distributions for three clades (3-2, 3-4, and 3-6) were unimodal, fit the sudden expansion model particularly well, and had significantly negative Fs values, providing strong support that these clades have undergone sudden expansion (Table 5). In the case of the three clades that did show a unimodal distribution of differences between haplotypes, we estimated the years since sudden expansion based on the estimate of tao from the mismatch distribution. All three of these clades (3-2, 3-4, and 3-6) were estimated to have undergone sudden expansion 1.3–1.5 MYA, although the range of estimates based on the confidence intervals of tao provide much broader estimates ranging from 0 to ~2.7 MYA (Table 5).

Table 3
Significant results ($P < 0.05$) and corresponding historical inferences based on Nested Cladistic Phylogeographical Analysis

Clades	Significant results	D_c	D_n	Chain of inference	Historical process inferred
3-5	2-6	(S) $P = 0.024$	(S) $P = 0.024$	1-2-11-12-No	Contiguous range expansion
	I-T		(S) $P = 0.024$		
4-1	3-1	(S) $P = 0.017$		1-2-3-4-No	Restricted gene flow with isolation by distance
4-2	3-4	(S) $P = 0.030$		1-2-11(Yes)-12-17-No	Range expansion/inconclusive outcome
	3-5	(L) $P = 0.006$	(L) $P = 0.004$		
	3-6	(S) $P = 0.005$	(S) $P = 0.027$		

Results of within clade (D_c) and within nested clade (D_n) values that were significantly large are denoted with an "L" and those that were significantly small with an "S". "Chain of inference" refers to the path through the inference key of Templeton (2004).

Table 4
Population genetic statistics for populations (based on biogeographic regions) and clades

	n	No. Hap.	Nucleotide diversity (π)	Mean number of pairwise differences (k)
Overall	50	32	0.012 ± 0.006	10.36 ± 4.81
East Population	30	20	0.008 ± 0.004	7.32 ± 3.52
Chihuahuan	14	10	0.012 ± 0.006	10.12 ± 4.95
Tamaulipan Plain	8	6	0.002 ± 0.002	2.54 ± 1.52
Southern Plains	9	6	0.002 ± 0.002	1.94 ± 1.21
West Population	20	15	0.011 ± 0.005	9.47 ± 4.53
Eastern Clade (= Clade 4-2)	27	18	0.006 ± 0.003	5.08 ± 2.54
Clade 3-4	5	5	0.002 ± 0.002	1.60 ± 1.13
Clade 3-5	9	6	0.006 ± 0.004	5.06 ± 2.97
Clade 3-6	13	7	0.002 ± 0.001	1.77 ± 1.09
Western Clade (= Clade 4-2)	23	15	0.008 ± 0.004	7.21 ± 3.51
Clade 3-1	4	4	0.004 ± 0.003	3.83 ± 2.43
Clade 3-2	7	5	0.002 ± 0.001	1.71 ± 1.13
Clade 3-3	12	6	0.003 ± 0.002	2.79 ± 1.58

Table 5
Results of historical demographic analyses

	n	Fu's Fs	Observed modality of mismatch distribution	SSD	HRI	Tao	Time since expansion
Overall	50	−10.795*	Multimodal				
Eastern Clade (= Clade 4-2)	27	−7.705*	Bimodal	0.006	0.012		
Clade 3-4	5	−3.578*	Unimodal	0.143	0.560	2.013 (0.00–3.49)	1.48 MYA (0–2.49 MYA)
Clade 3-5	9	0.252	Multimodal	0.080	0.215		
Clade 3-6	13	−2.687*	Unimodal	0.001	0.049	1.835 (0.00–3.48)	1.31 MYA (0–2.49 MYA)
Western Clade (= Clade 4-2)	23	−3.133	Multimodal	0.011	0.018		
Clade 3-1	4	−0.884	Bimodal	0.030	0.111		
Clade 3-2	7	−1.889*	Unimodal	0.006	0.066	1.917 (0.00–3.75)	1.37 MYA (0–2.68 MYA)
Clade 3-3	12	−0.479	Bimodal	0.069	0.137		

P-values < 0.05 for Fu's Fs statistic are indicated with "*". *P*-values for the sum of squares deviation (SSD) and HarDQ6798's Raggedness index (HRI) were all greater than 0.05. For unimodal mismatch distributions, estimates of tao (with 95% confidence intervals in parentheses) and estimates of the number of years since sudden expansion is given (calculated based on $\text{tao} = 2ut$).

We conducted a molecular analysis of variance (AMOVA) to identify the components of variance among haplotypes that could be explained based on geographic distribution at two hierarchical levels: variation based on areas east versus west of the continental divide (among group variance), and variation within biogeographic/physiographic regions (Chihuahuan Desert, Tamaulipan Plain, Southern Plains; among populations within group variance). The AMOVA was significant at $P < 0.001$ and indicated that >24% of variation could be explained based on East versus West geographic groups and >17% of the variance could be explained based on biogeographic regions (Table 6). The largest component of variation (>58%) existed within populations (Table 6).

3.4. Results from the isolation-with-migration model analyses

Results from the four independent runs of the isolation-with-migration model analysis provided essentially identical results for marginal posterior probability densities of parameter estimates. All runs had ESS values for parameters that minimally exceeded 200, indicating sufficient MCMC mixing across parameter space for reasonable estimates of parameters (Hey and Nielsen, 2004). For convenience, plots of the marginal posterior probability densities of parameters from one run (the one with the highest ESS values) are shown (Fig. 6). Estimates of θ_{East}, θ_{West}, m_{East}, and m_{West} were associated with moderate-strongly defined peaks in posterior density, indicating ample evidence within the data to estimate these parameters. The estimate of t showed a weakly defined peak, with a generally flat upper-end tail. The estimate of θ_A (the estimate of the ancestral *C. atrox* population size) was essentially flat, suggesting insufficient data to obtain an estimate for this parameter. The diffuse densities of parameter estimates for t and θ_A suggest that the model used may be overly parameter rich for the size of the dataset used. For this reason, we also conducted alternative analyses in IM where we constrained either migration parameters ($m_{East} = m_{West}$) or theta values ($\theta_{East} = \theta_{West}$). Results of these alternative analyses are not shown or discussed for several reasons: (1) estimates of θ_A and t were quantitatively similar in these; (2) the diffuse nature of the distributions of θ_A and t were not substantially changed in the constrained models; (3) evidence from

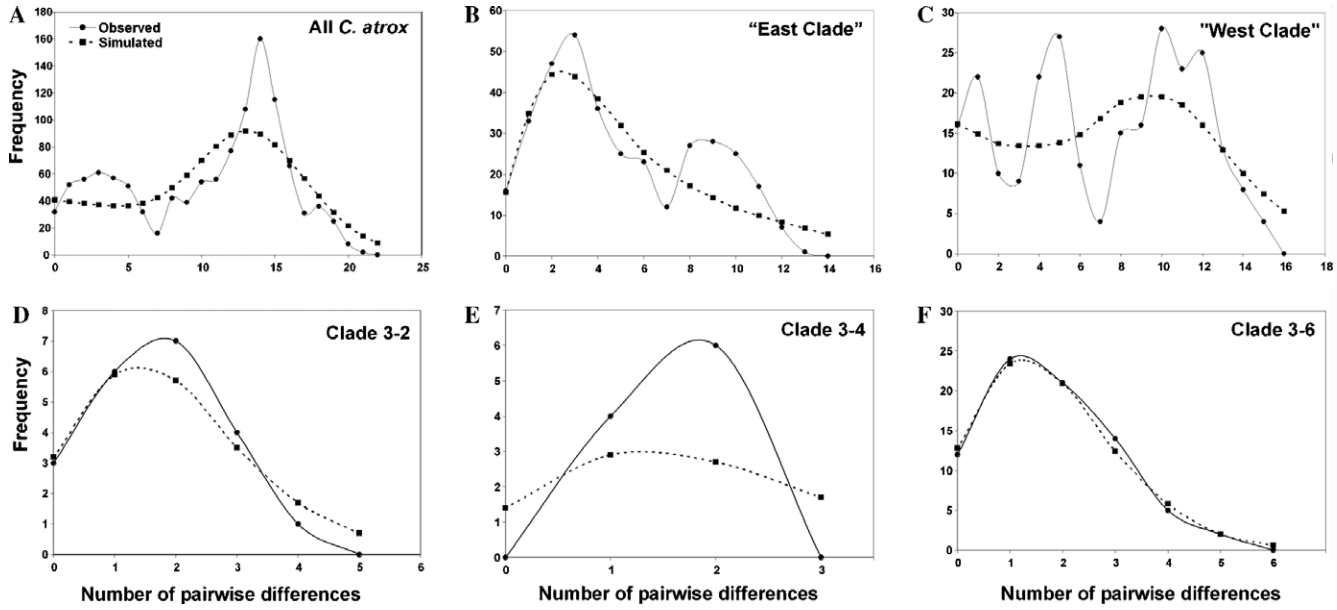

Fig. 5. Selected mismatch distributions comparing observed distributions of differences among haplotypes to distributions predicted under a model of sudden expansion. Mismatch distributions for the following groups of haplotypes are shown: (A) all *C. atrox* samples (including *C. tortugensis* and *C. sp.* SCI), (B) all haplotypes of the East Clade (clade 4-2), (C) all haplotypes of the West Clade (clade 4-1), (D) haplotypes belonging to clade 3-2, (E) haplotypes belonging to clade 3-4, (F) haplotypes belonging to clade 3-6. Details of parameter estimates from these comparisons are provided in Table 5.

Table 6
Molecular analyses of variance (AMOVA) hierarchically examining differentiation on either side of the continental divide and differentiation among major biogeographic regions occurring on either side of the divide

	d.f.	Percentage of variation (%)
Among groups	1	24.20
Among populations within groups	2	17.64
Within populations	46	58.17

Groups are defined as "East population" and "West population" and populations include: Chihuahuan Desert, Tamaulipan Plain, and Southern Plains (all within the East population). The AMOVA was significant at $P < 0.001$.

unconstrained models was strong for quantitative differences between θ_{East} and θ_{West}, as well as m_{East} and m_{West}, and equating these parameters in restricted models may result in inaccurate estimates of θ_A and t.

Estimates of the rescaled population parameters from IM (based on the non-restricted isolation-with-migration model) inferred current effective female population sizes (N_{ef}) to be relatively similar between East and West populations (Fig. 5; Table 7), with high points of approximately ~593,000 and ~521,000 individuals, respectively. As discussed above, the estimation of the ancestral effective female population size ($N_{ef\text{-}Ancestral}$) appeared to suffer from a lack of sufficient data, as the posterior probability density for this parameter was essentially flat (i.e., the data were insufficient to substantially modify the priors). For this reason, the plot of the density of this parameter is not shown, although we report summary statistics of this distribution (Table 7). Estimates of the directional migration rates (M; migrants per generation) inferred nearly negligible or low values of migration from East → West (high point = 0.0599; Fig. 6; Table 7), compared to higher values that were broadly/diffusely distributed for West → East migration (M_{West} high point = 1.7359; Fig. 6; Table 7).

4. Discussion

4.1. Status of Crotalus tortugensis and Crotalus sp. from Santa Cruz island

The taxonomic status of the rattlesnake population occurring on Tortuga Island (Isla Tortuga), *C. tortugensis*, has been the subject of a long historical debate. Several authors have questioned the validity of this taxon, suggesting that it may instead be a junior synonym or subspecies of *C. atrox*, with which it shares an extremely similar morphology (Amaral, 1929; Grismer, 1994; Stejneger and Barbour, 1933). The results of all analyses conducted in this study agree with this conclusion of synonymy with *C. atrox* based on three points: (1) recognition of *C. tortugensis* sampled in this study renders *C. atrox* paraphyletic, (2) the haplotype of *C. tortugensis* is extremely closely related to *C. atrox* from the adjacent Mexican mainland, differing by as little as two nucleotide substitutions (~0.2% genetic divergence), and (3) the genetic distance within *C. atrox* across its range greatly exceeds the genetic differentiation between *C. tortugensis* and *C. atrox* from adjacent mainland Mexican (and even USA) populations by more than fivefold. Based on these considerations, we regard *C. tortugensis* as a junior synonym of *C. atrox* and hereafter both are referred to as *C. atrox*.

In addition to Tortuga Island, *C. atrox* is known to inhabit three other islands in the Gulf of California: San

Table 7
Summary of rescaled parameter estimates from the isolation with migration model (conducted in IM; Hey and Nielsen, 2004)

Parameter	High point	95% Confidence interval	
		Lower 95%	Upper 95%
$N_{ef\text{-}East}$	592,984	341,415	1,167,99
$N_{ef\text{-}West}$	521,108	269,539	3,791,503
$N_{ef\text{-}Ancestral}$	—[b]	736,738	>35,000,000[a]
M_{East} (East → West migrants/generation)	0.0599	0.034	37.140
M_{West} (West → East migrants/generation)	1.7359	0.136	331.071
Time since population divergence (years before present)	1,033,835	780,484	>8,000,000[a]

The "High Point" reported is the parameter estimate associated with the highest marginalized likelihood (posterior density) after the likelihood density distribution was smoothened.

[a] These two parameter estimates did not contain complete upper-bound tails, indicating that the upper 95% confidence interval may be larger than the indicated values in the table (see text).

[b] We do not provide an estimate of the high point for this distribution because the distribution was essentially flat and a high point on this distribution is not meaningful.

Pedro Mártir, Santa Cruz, and Tiburón (Campbell and Lamar, 2004; Grismer, 2002; Murphy and Aguirre-Léon, 2002b). *Crotalus atrox* also may occur on the following islands: Dátil (Grismer, 2002; Murphy and Aguirre-Léon, 2002b), Santa María and Turner (Campbell and Lamar, 2004). Grismer states that Santa María Island does not exist in the Sea of Cortés, but this island is listed by Murphy et al. (2002b) and Campbell and Lamar (2004) as part of Sinaloa. In this study, we have included an individual from one of these additional islands, Santa Cruz (*Crotalus sp.* SCI). The haplotype of the Santa Cruz Island specimen differs from the nearest Mexican mainland specimen sampled ("MEX Sonora C79") by a single nucleotide substitution (~0.1% sequence divergence), suggesting that this population colonized Santa Cruz Island recently. Based on the same reasoning outlined for the synonymy of *C. tortugensis* and *C. atrox*, we suggest that the rattlesnake population on Santa Cruz Island be assigned to *C. atrox* (and we follow this synonymy hereafter), contrary to suggestions that it be recognized as a species distinct from *C. atrox* (Murphy et al., 2002a). The conclusion of Murphy et al. (2002a) that the rattlesnake population on Santa Cruz Island represented an undescribed species was essentially based on two points, that the population was allopatric (inhabiting an island), and that the mitochondrial haplotype of their sample was quite divergent from the other sample of *C. atrox* (from Texas) included in their study. With our broad sampling of *C. atrox*, it is now clear that the large difference they observed represents the difference between the two major clades of *C. atrox*, corresponding generally to populations east and west of the continental divide, rather than a substantial genetic differentiation between mainland and island populations (e.g., Figs. 2 and 3).

The phylogenetic affinities and biogeographic histories of several Gulf of California island populations of rattlesnakes remain poorly known. The two included in this study, '*C. tortugensis*' and *C. atrox* from Santa Cruz Island appear to represent *C. atrox*, as opposed to the suggestion of some authors that they be recognized as distinct species (e.g., Campbell and Lamar, 2004; Grismer, 2002; Murphy et al., 2002a). Although the establishment of much of the herpetofauna of insular Baja California has been attributed to vicariant causes (Murphy, 1983; Murphy and Aguirre-Léon, 2002a; Murphy et al., 2002a), most authors attribute the occurrence of *C. atrox* and their relatives on these islands to over-water colonization (Grismer, 1994; Murphy, 1983; Murphy and Aguirre-Léon, 2002a). Our data agree with these authors and suggest that recent colonization may best account for the founding of these island populations of *C. atrox*. Although these islands are a considerable distance off the mainland Mexican coast (e.g., Fig. 1), rattlesnakes are able to swim (Klauber, 1972), and multiple invasions have been recorded for other Gulf of California insular herpetofauna (e.g., Grismer, 1994; Radtkey et al., 1997). Other possible island colonization scenarios (aside from active over-water movement) include the possible rafting of snakes on debris (e.g., following major storms), or even translocation via human introduction, such as accidental introduction via the fishing industry (as suggested for *Peromyscus* on Isla Tortuga; Hafner et al., 2001).

The mitochondrial sequence data in this study strongly support the synonymy of *C. tortugensis* and the rattlesnake population from Santa Cruz Island with *C. atrox* because of the phylogenetic nesting of haplotypes from these populations deep within continental lineages of *C. atrox*, and the very shallow sequence divergence between mainland and island haplotypes. These mitochondrial data are at odds with the hypothesis that these island populations are distinct evolutionary units (that may justify taxonomic recognition for the island populations). It is possible that introgression of mitochondrial haplotypes (e.g., from recent mainland—island gene flow) could be masking patterns of deep evolutionary divergence between mainland and island populations associated with a more ancient colonization of the islands. Additional molecular work (e.g., microsatellite or allozyme data) to verify the results from the mitochondrial data would be preferable, although the most appropriate decision at present seems to be the placement of these island populations in synonymy with *C. atrox*, as we have suggested.

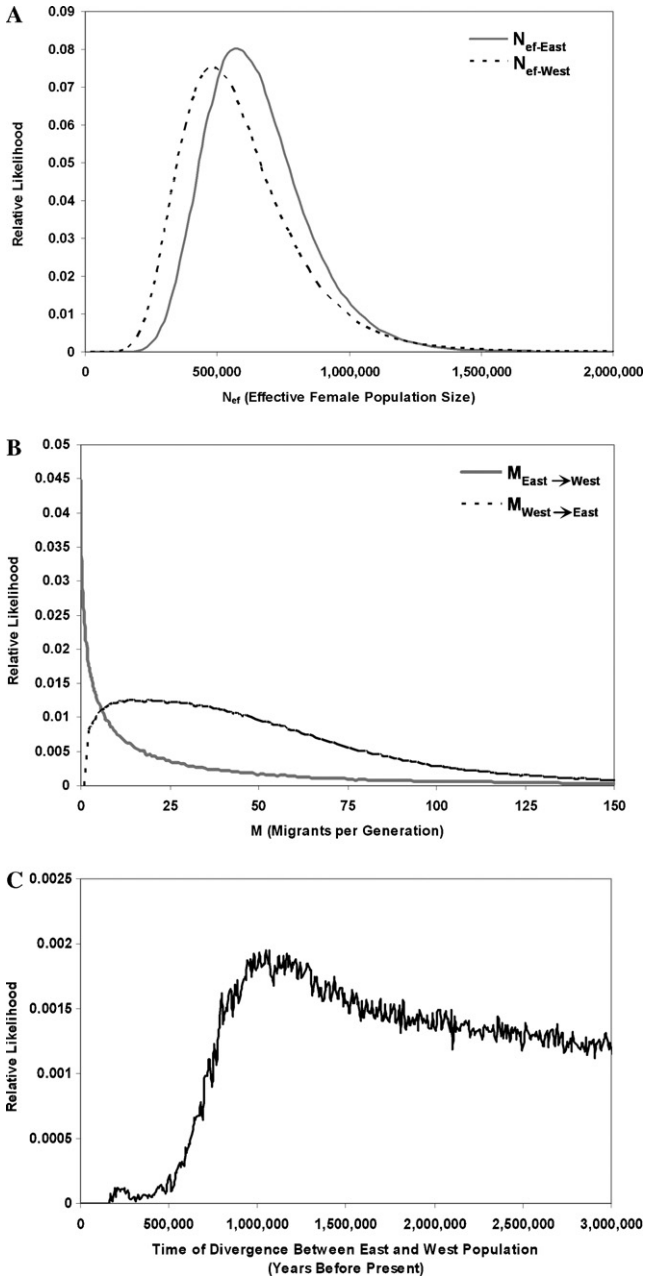

Fig. 6. Plots of the marginal likelihood densities (posterior probability densities) of parameters estimated from the isolation-with-migration model in IM (Hey and Nielsen, 2004). (A) Posterior densities of estimates of the effective female population sizes (N_{ef}) for the East and West populations. (B) Posterior densities of estimates of the directional migration rates (M; migrants per generation) between East and West populations. (C) Posterior density of the estimate of the time of divergence between East and West populations (see comments in text about the incomplete upper tail of this distribution).

Following a similar pattern to that observed for *C. atrox*, the mouse population on Tortuga Island (previously recognized as *Peromyscus dickeyi*) was found to represent a lineage deeply evolutionarily nested within continental lineages of *P. merriami* (particularly *P. merriami* from adjacent mainland Mexico), based on both mitochondrial DNA and allozyme data (Hafner et al., 2001). This example (and examples from other lineages of *Peromyscus* inhabiting islands in the Sea of Cortés) supports our inferences of recent island colonization and shallow evolutionary divergence (and synonymy) of island and mainland populations, rather than a more complex scenario of mitochondrial introgression into already established and diverged island populations.

4.2. Divergence between Crotalus ruber and Crotalus atrox

The sister clade to *C. atrox* includes *C. ruber* and *C. catalinensis*, whose distribution centers on the Baja Peninsula of México and adjacent islands. The support for these western members of the *atrox* species group (Murphy et al., 2002a; this study, in part) forming the sister clade to *C. atrox* implies that a split between the common ancestor of these taxa preceded any subdivisions within *C. atrox*. This implies an early vicariant event that severed gene flow between an ancestral *atrox* group, isolating peninsular populations from continental populations that would later become *C. atrox*. This vicariance is likely to have been the result of the Late Pliocene formation of the Gulf of California (Sea of Cortés). This extended incursion has been cited as a vicariant event for other arid-adapted taxa (e.g., Lamb et al., 1989; Riddle et al., 2000a,c), and was essentially predicted by Klauber (1972) to explain the differentiation of *C. atrox* and *C. ruber*.

The Gulf of California began to form approximately 5.5 MYA as a result of subsidence due to the separation of the Baja Peninsula from the mainland (Longsdale, 1989; Shafiqullah et al., 1980). This process climaxed approximately 3 MYA when the Gulf of California extended far inland into present day California and Arizona (generally following the track of the present day Colorado River), forming the Bouse embayment (Blair, 1978; Buising, 1990; Eberly et al., 1978). Based on the evolutionary rate estimates, we have applied to *C. atrox*, the divergence between *C. ruber* and the *C. atrox* clade is estimated at 3.29 MYA (using the 1.4% per MY between lineage calibration; Table 2). The general congruence between the dates obtained for this split between *C. atrox* and peninsular species, and the known geological history of the region, imply the molecular rate calibration is, at minimum, acceptable for establishing coarse-scale estimates of divergence times.

The concept of Mojavia (Axelrod, 1958)—a contiguous expanse of arid vegetation extending from the Pacific coast across the continental divide of the USA during the Miocene and (at least) the Early Pliocene—is well-supported by paleoclimatic and fossil data (Morafka, 1977; Morafka et al., 1992). This concept implies a large and direct means of broad East–West dispersal for arid-adapted taxa up until the Mid Pliocene, at which time continued uplift of the Colorado Plateau and Sierra Madre Occidental initiated the bisection of this continuum (Morafka, 1977). The established vicariance between *C. atrox* and the "*ruber* clade" implies that the ancestor of *C. atrox* ranged, at least, in areas west of the continental divide during a much earlier time period than that suggested by fossil data. Whereas

fossil evidence establishes what appears to be *C. atrox* in areas east of the continental divide during the Mid Pliocene, no fossils are known from west of the continental divide until the Late Pleistocene. Our phylogenetic data together with biogeographic estimates suggest that this absence from the west of the divide is a collecting artifact (false negative). Collectively, estimates of historical desert habitat in the Pliocene, the fossil data, and the phylogenetic evidence lead to a hypothesis that the ancestral range of *C. atrox* spanned the continental divide prior to the end of the Pliocene (3.7–3.2 MYA based on fossils from Texas; Rogers, 1976), and prior to any major divisions (i.e., East–West bisection) detected within *C. atrox*.

4.3. An early east–west divergence within Crotalus atrox

Beginning in the late Pliocene and extending through Pleistocene glaciopluvial periods, the continental divide has been hypothesized to act as an increasingly effective barrier to connectivity between desert regions to the east and west (Morafka, 1977; Morafka et al., 1992). This East–West division was likely compounded by a southern shift in the range of herpetofaunal communities as a result of Pleistocene climatic cooling (i.e., glaciopluvial periods) that dominated as much as 94% of the Quaternary (Morafka et al., 1992; Van Devender and Burgess, 1985). Estimates of the phylogeny and nested cladogram of *C. atrox* haplotypes provide strong evidence of a primary divergence within *C. atrox* that generally corresponds to an East–West split in the Pleistocene, 1.36 MYA. This is broadly concordant with vicariant phylogeographic patterns reported for many other desert fauna (Riddle and Hafner, 2006), including horned lizards (Leaché and McGuire, 2006; Reeder and Montanucci, 2001), fence lizards (Leaché and Reeder, 2002), western rattlesnakes (Ashton and de Queiroz, 2001; Pook et al., 2000), pocket and deer mice (e.g., Riddle, 1995; Riddle et al., 2000a,b), and scaled quails (Zink and Blackwell, 1998).

The only physiographic portal between the two desert regions, following the Pliocene uplift of the Sierra Madre Occidental and Colorado Plateau, occurs at the Cochise filter barrier, in the valley in between the Chiricahua and Animas mountain ranges, near the eastern border of Arizona (Morafka, 1977; Fig. 1). Since this division within *C. atrox* is inferred to have resulted from the expansion and intensification of a corridor of non-desert habitat, the degree to which this filter barrier acted to prevent gene flow was likely to depend on taxon-specific natural history. The relative dispersal capability and relative habitat specificity of species were probably the most important factors establishing the temporal closure of this portal between major desert regions. *Crotalus atrox* is a large snake species with high dispersal ability, and extant populations display a particularly broad habitat preference that includes mesquite-grassland and desert, in addition to pine-oak, tropical deciduous and thorn forests (Campbell and Lamar, 2004). Thus, the natural history of *C. atrox* likely facilitated gene flow through this portal during periods (e.g., Late Pliocene and Early Pleistocene) when other more desert-specific taxa were excluded by non-desert habitat in this region.

The onset of Pleistocene climatic cycles would be expected to lead to the contraction, and possible southern-shift of habitats hospitable to *C. atrox*. This in turn would have facilitated the closure of the connection between eastern and western desert regions, as the Cochise filter barrier region has been shown to harbor woodlands during glaciopluvial periods (Morafka et al., 1992; Van Devender, 1990; Van Devender et al., 1984). The divergence between eastern and western clades of *C. atrox* is estimated to have taken place 1.36 MYA, based on the application of the mutation rate to the mean between group sequence divergence (Dxy; Table 2). Similarly, the estimate of the time of divergence of East and West Populations, based on analyses from IM, indicate high posterior probability density for a period in the Mid–Early Pleistocene (~0.9–1.5 MYA; Fig. 6, Table 7).

4.4. Historical population structure and demography of C. atrox

Estimates of the number and location of North American desert Pleistocene refugia vary across studies. While some have found little genetic structure within major desert regions east and west of the continental divide (e.g., Klicka and Zink, 1997) consistent with a single eastern and single western Pleistocene refugium, others have shown higher levels of genetic structure more consistent with multiple refugia per region (e.g., Jaeger et al., 2005; Riddle et al., 2000b; Zamudio et al., 1997). Our data together with fossil evidence provide an opportunity to examine general hypotheses for the number and potential localization of refugia, although our sparse sampling in potential refugial areas in Mexico limits precise estimates.

In the West, two well-resolved clades of western haplotypes were found (Fig. 2; corresponding to clades [3-1 + 3-2] and 3-3 in Fig. 3), supporting two distinct Pleistocene refugia. The first clade (3-1 + 3-2) contains haplotypes that occur in southern portions of the West (in the central and southern Sonoran desert) and likely represent the signature of a Pleistocene refugium that occurred within the Sonoran desert (Morafka et al., 1992). The second western clade (3-3) contains haplotypes that occur across the northern portions of the West, and also into eastern regions. The distribution of this clade supports previous hypotheses of a Pleistocene refugium associated with the lower Colorado River valley (along the California–Arizona border). This region appears to have maintained more desert-like conditions during the last glacial period (Bentacourt et al., 1990; Thompson and Anderson, 2000), and has been proposed as a possible Pleistocene refugium based on the genetic structure of toads (Jaeger et al., 2005) and polyploid races of creosote bushes (Hunter et al., 2001). The Western Clade does not show strong evidence of population expansion, and NCPA inferred restricted gene flow with isolation by

distance for this clade; both results imply that Pleistocene refugia may not have drastically restricted populations in the West compared to their current distribution and size. Only clade 3-2 shows substantial evidence of population expansion (based on the mismatch distribution and Fs; Table 5; Fig. 5).

The current area occupied by the range of *C. atrox* in the East is substantially larger than that in the West (approximately fivefold; Fig. 1), yet estimates of effective female population sizes show only slightly larger values in the East, relative to the West (Fig. 6; Table 7). This may indicate that the large eastern range of *C. atrox* was dramatically smaller during the Pleistocene, nearly equal to the western range in size. Eastern Clade haplotypes show less defined genetic structure, compared to the Western clade (Fig. 2; also see Fig. 5B versus C). The deep phylogenetic position of the Mexican haplotype "MEX SLPotosi C31" (Figs. 2 and 3) compared to the terminal positions of more northern haplotypes supports the existence of a southern Pleistocene refugium in the Mapimian subregion of the Chihuahuan desert, as has been suggested by multiple studies (Morafka, 1977; Orange et al., 1999; Riddle et al., 2000a; Riddle and Hafner, 2006; Tanner and Banta, 1977). The resolution of two other eastern haplotype clades, 3-5 and 3-6 (Fig. 3; see also Fig. 2), may indicate additional eastern refugia. The distribution of haplotypes in clade 3-5 generally overlaps with that of 3-4, both supporting a refugium or multiple refugia in the Chihuahuan region. It is possible that clades 3-4 and 3-5 share a common Pleistocene refugium in the Mapimian subregion of the Chihuahuan Desert (i.e., Morafka, 1977), or that one may represent a second refugium within the Gulf Coast/Tamaulipan Plain of Mexico (based on their current distributions). The probable existence of a large Pleistocene pluvial lake in the northern Chihuahuan Desert (Axtell, 1977; Sullivan, 1994) excludes this region as a likely refugium, and bolsters the probability of a refugium in the Tamaulipan region, thought to have been less affected by cool mesic Pleistocene episodes (Morafka, 1989; Morafka et al., 1992; see also Riddle and Honeycutt, 1990). Our sparse sampling in the Chihuahuan Desert and Tamaulipan Plain, areas likely to have been Pleistocene refugia, substantially limits our ability to identify precisely how many refugia may have existed in the region, and where these may have been located.

Fossil remains of *C. atrox* have been documented throughout the Mid Pleistocene and early portions of the Late Pleistocene in the Edwards Plateau region of central Texas: Travis County (900–400 KYA; Holman, 1995; Holman and Winkler, 1987), Bexar County ("Middle Pleistocene"; Brattstrom, 1954), and Llano County (150–25 KYA; Holman, 1966, 1995). This evidence from the fossil record provides tentative evidence counter to the hypothesis that eastern populations of *C. atrox* were restricted to a single southern refugium (e.g., within the Mapimian region of the Chihuahuan Desert) throughout a majority of the Pleistocene, although it is possible that these fossils represent interglacial periods where *C. atrox* dispersed northward from more southern refugia. If a northern refugia did exist in the East (in southern-central Texas), the eastern clade 3-6 distributed across northern portions of Texas (including the Southern Plains, Edwards Plateau, and New Mexico; Fig. 4) may represent the genetic signature of a northern Pleistocene refugium.

Pleistocene climatic effects are predicted to have produced a greater number of isolated refugia in the East, relative to desert regions west of the continental divide, due to the less uniform topography and physiography of the East (Morafka et al., 1992). During glacial cycles, arid-adapted taxa in the East would have been fragmented in highly isolated rocky basins and disjunct ranges, whereas western populations would have retained a more continuous distribution along sandy coastal plains (Morafka, 1990; Morafka et al., 1992). As mentioned, comparisons of the estimates of effective female population sizes (N_{ef}; Fig. 6; Table 7) imply a dramatic Pleistocene contraction of *C. atrox* populations (relative to the current range) in the East, compared to the West. Accordingly, the Eastern Clade shows stronger evidence of post-refugial range expansion and population growth. Results of NCPA suggest range expansion for the entire Eastern Clade (Table 3). The two wide-ranging eastern subclades (3-5 and 3-6; Fig. 4) also show strong evidence of recent population growth based on mismatch distributions and neutrality statistics (Fig. 5; Table 5), and contiguous range expansion (via NCPA for 3-5; Table 3). Collectively, all methods of inference imply a more dramatic pattern of Pleistocene contraction and subsequent expansion (in northern and western directions) in the East, as compared with the West.

Within major regions, as well as across the continental divide, we found strong evidence for the secondary contact and overlapping ranges of haplotype groups (e.g., Fig. 4). The period of time since the last glacial maximum surely has lead to the range expansion and secondary contact of haplotype lineages, but this secondary contact may have begun earlier, during Pleistocene interglacial periods. Fossil remains from southeastern New Mexico (near the border with Texas) suggest that interglacial periods may have contributed to dispersal across the Cochise filter barrier, allowing gene flow from West to East. At this site in New Mexico, desert tortoise remains (*Xerobates* [*Geochelone*]*agassizi*) dating to the Late Pleistocene have been found far east of the current range of this species in the Sonoran and Mojave Deserts (Holman, 1995). The distribution of haplotypes of *C. atrox* across the continental divide demonstrates a strong pattern of West → East dispersal (e.g., clade 3-3). Similarly, the asymmetric migration rate estimates (from IM) suggest a strong West → East migration bias (Fig. 6; Table 6). This Late Pleistocene–Holocene directional migration tendency across the continental divide is observed in other taxa (Riddle and Hafner, 2006), including leopard lizards (Orange et al., 1999), mice (Riddle et al., 2000a,b), quail (Zink and Blackwell, 1998), and creosote bushes (Hunter et al., 2001). Strong evidence for post-refugial expansion in the East, yet strong West → East migration across the continental divide may suggest that

eastern refugia were located distantly from the divide (as we have suggested, in southern Mexico, and possibly the Gulf-coastal plain and central Texas), compared to western refugial populations (in the Sonoran Desert and possibly the Colorado River valley), which show only weak evidence of expansion yet elevated rates of migration across the divide. It is interesting to note that no Western Clade haplotypes were observed southeast of the Rio Concho and Rio Grande, which form the northwestern border of the area of the Chihuahuan desert that has been viewed as the "core" of this region (e.g., Morafka, 1977; Riddle and Hafner, 2006; see also Riddle et al., 2000a). Our limited sampling within this potential core of the Chihuahuan desert constrains our ability to precisely infer the role of this region in the context of historical refugia and local demographics in this biogeographic province, and represents an important target for future studies that may add substantial clarification to our conclusions.

The outgrowth of phylogeographic and population genetic data available has facilitated a dramatic shift in the methodology for deciphering historical biogeographic events from information about species and their geographic distributions. Many authors have stressed the importance of using some type of evolutionarily relevant units, rather than biogeographic/physiographic provinces (i.e., areas) or nominal species distributions, as units for identifying broad historical patterns of vicariance and biogeography (e.g., Avise, 2000; Riddle and Hafner, 1999). Our results for *C. atrox* strongly support this contention, demonstrating the impact that historical processes, like Pleistocene refugium size and location, may have on determining patterns of genetic diversity and population structure, in addition to the fact that many complex patterns of genetic diversity exist below the level of nominal species. Our comparisons of biogeographic regions relative to genetic population structure show that each region considered contained haplotypes of mixed ancestry (e.g., Fig. 4), and that biogeographic regions explained only 17% of the genetic diversity (Table 6). These results clearly illustrate the pitfalls of using biogeographic regions as units in deciphering historical processes and patterns, largely because such regions may not represent meaningful and cohesive evolutionary units.

As phylogeographic studies of North American arid-adapted taxa have accumulated, a number of common patterns have begun to emerge. While many studies show differential patterns of gene flow towards the periphery of major desert regions, many also show common patterns of genetic structure, particularly regarding the relationships among the internal cores of biogeographic regions or provinces. With the continued accumulation of data from different co-distributed taxa, it is becoming increasingly feasible to reconcile these cumulative phylogeographic patterns on the basis of recognizing core and peripheral biogeographic regions (e.g., Riddle and Hafner, 2006) to finally investigate and identify overreaching patterns of historical biogeography that have shaped the regional flora and fauna. Only with this type of multi-taxon approach to identifying core phylogeographic regions will it meaningful to shift again to an area-based biogeographic approach.

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