Historical Biogeography of the Western Rattlesnake (Serpentes: Viperidae: *Crotalus viridis*), Inferred from Mitochondrial DNA Sequence Information

Catharine E. Pook, Wolfgang Wüster, and Roger S. Thorpe

School of Biological Sciences, University of Wales, Bangor, Gwynedd LL57 2UW, Wales, United Kingdom

We infer the phylogeography of the Western Rattlesnake (*Crotalus viridis*) using phylogenetic analysis of mitochondrial DNA sequences from 1345 bp of the genes for cytochrome *b* and NADH dehydrogenase subunit 4. Two main clades are revealed: one includes populations from east and south of the Rocky Mountains (conventionally referred to as *Crotalus viridis viridis* and *C. v. nuntius*), and the other consists of populations west of the Rocky Mountains. Within the western clade, a population from southern Arizona (*C. v. cerberus*) represents the sister taxon to the remaining western populations. The conventional subspecies recognized in this species do not fully correspond to the phylogenetic pattern, and a review of the systematic status of several populations is needed. Our data allow the inferences that small body size evolved twice and that the ability of one population (*C. v. concolor*) to secrete highly lethal toxins related to Mojave toxin arose within the complex. Our phylogeny should represent the basis for further studies on the causes of geographical variation in this complex.   © 2000 Academic Press

Key Words: historical biogeography; *Crotalus;* North America; venom; mitochondrial DNA; phylogenetic analysis; body size; subspecies.

INTRODUCTION

Considerable progress has been made in the past decade in understanding intraspecific population structure and in the analysis of geographic variation and its causes. This progress stems in considerable part from allozyme electrophoresis (see summary in Avise, 1994), followed more recently by the use of mitochondrial DNA as a molecular marker at low taxonomic levels (see Brown *et al.,* 1979; Avise, 1986, 1994; Kocher *et al.,* 1989) and the associated evolution of phylogeographic methodology (Avise *et al.,* 1987; Avise, 1998). Phylogeography seeks to reveal patterns of geographic variation relative to the distributions of extant genealogies, permitting further historical (distant or recent), phylogenetic, and ecogenetic interpretation (Avise, 1994,

1998; Riddle, 1996; Bernatchez and Wilson, 1998). Moreover, this approach overcomes the limitations associated with use of morphological character systems alone, with which it can be difficult to separate ecogenetic adaptation (for example, natural selection for local ecological conditions) from the effects of phylogenesis (for example, the isolation of taxa by vicariance or other historical events) (Daltry *et al.,* 1996; Thorpe *et al.,* 1991, 1994, 1995; Thorpe, 1996).

In addition, the availability of molecular phylogeographical information has greatly reinforced earlier disaffection (e.g., Wilson and Brown, 1953; Thorpe, 1987) with the traditional taxonomic interpretation of geographic variation. Traditionally, geographic variation within widespread continental species has been described through the recognition of subspecies. However, more rigorous morphological and/or phylogeographic studies of populations so divided tend to reveal several evolutionary species, which may not be congruent with the conventional subspecies (e.g., Barker, 1992; Dorcas, 1992; Wüster *et al.,* 1992; Wüster and Thorpe, 1989, 1991; Zamudio and Greene, 1997). In most cases, the subspecies in these complexes were found to be trivially distinct local varieties or separate species or, in some cases, heterogeneous entities comprising populations of several evolutionary lineages.

The recognition of the subspecies category is controversial (Wilson and Brown, 1953), since many applications of the concept have involved the arbitrary subdivision of geographically variable populations, without consideration of congruence between characters or the causes of that variation (Thorpe, 1987; Frost and Hillis, 1990). Furthermore, the use of subspecies rank for differentiated populations is likely to result in a misrepresentation of population phylogeny (Cracraft, 1989).

The need to resolve species systematics using robust molecular phylogenies is therefore paramount, since disregard for species status has serious implications with respect to species conservation (e.g., Bernatchez and Wilson, 1998; McElroy and Douglas, 1995; Avise *et al.,* 1998) and, in the case of the venomous snake

species, toxicological research and the development of efficient antivenoms for the treatment of bites (Wüster and Thorpe, 1991; Wüster *et al.,* 1992; Wüster and McCarthy, 1996).

The Western Rattlesnake (*Crotalus viridis*) is an ideal subject for the study of geographic variation and its causes. This species occurs across a climatically and physiographically diverse range, from southern Canada to northern Mexico and from Iowa to the Pacific coast and is represented in all major vegetation zones (see Brown, 1997; MacMahon, 1997; Whitney, 1998 for descriptions of vegetation types). *C. viridis* exhibits notable geographic variation in morphology (Klauber, 1972; Macartney *et al.,* 1990), habitat preference, and behavior (Klauber, 1972). The observed morphological variation in *C. viridis* (adult color pattern and size) has led to the description of nine subspecies (Fig. 1). Of particular interest is the presence of two dwarfed populations on the Colorado Plateau, currently known as *C. v. concolor* and *C. v. nuntius* (Klauber, 1972). The question of whether small body size arose once or twice independently in *C. viridis* could help shed light on the causes and correlates of body size evolution in snakes.

In addition to the intrinsic interest of the patterns of variation in this species, it is also important to note that *C. viridis* has become a popular study species in fields ranging from movement ecology to foraging behavior and toxicology (e.g., Duvall and Schuett, 1997; Hayes, 1995; Katagiri *et al.,* 1998), and it has in many cases become a model organism representative of the Viperidae in general. Due to its wide range, different studies are often conducted in different parts of the range of the species and on different subspecies. A full understanding of the phylogeny and systematic status of the populations involved is a prerequisite for the interpretation and comparison of the results of these studies.

Variation in venom composition has been noted within and between different populations and age groups of *C. viridis* (Foote and MacMahon, 1977; Glenn and Straight, 1977; Young *et al.,* 1980; Mackessy, 1988; Ownby *et al.,* 1988; Anaya *et al.,* 1992; Mackessy, 1993), and unexpected clinical complications following bites have been noted for some populations (e.g., Gibly *et al.,* 1998). In particular, the venom of one population, currently known as *C. v. concolor,* displays very high lethality in mouse experiments and contains the neurotoxic phospholipase A_2 Concolor toxin (Glenn and Straight, 1977). This toxin belongs to a group of toxins related to Mojave toxin, first isolated from the Mojave Rattlesnake, *Crotalus scutulatus.* The presence of these toxins in rattlesnake venoms is generally associated with extremely high lethality. Populations of a number of other rattlesnake species have venoms containing Mojave toxin-like phospholipases (Glenn and Straight, 1985a) and with correspondingly high lethality. The presence or absence of this toxin often varies among different populations of one species. In *C. viridis* populations other than *C. v. concolor,* these toxins are normally absent, except where possible hybridization with *C. scutulatus* may have resulted in hybrid populations with the relevant genes (Glenn and Straight, 1990). It would clearly be of great interest to test whether this variation is a function of phylogeny, natural selection for diet variation (as appears to be the case in *Calloselasma rhodostoma*—Daltry *et al.,* 1996), or other factors. Despite the potential medical importance of the variability in the presence of Mojave toxin, there has so far been no intraspecific phylogenetic study of a rattlesnake species that includes both Mojave toxin-secreting and nonsecreting populations.

A thorough review of the systematics and population phylogeny of *C. viridis* is clearly needed for a fuller understanding of the geographic variation in this species complex. This paper examines the phylogenetic relationships of the *C. viridis* complex based on mitochondrial cytochrome *b* and ND4 gene sequence data, and we attempt to elucidate the intraspecific taxonomic associations based on an historical interpretation of the results. This robust phylogenetic hypothesis for the various populations of *C. viridis* will serve as a basis for future work on this complex.

MATERIALS AND METHODS

Preparation of DNA Template

Tissue or blood samples were obtained from 68 specimens from 33 localities representing most of the range of *C. viridis* (Fig. 2, Appendix 1) and for two outgroups, *Crotalus scutulatus* and *Crotalus durissus.* Samples were taken from living rattlesnakes by caudal venepuncture or obtained from institutional tissue
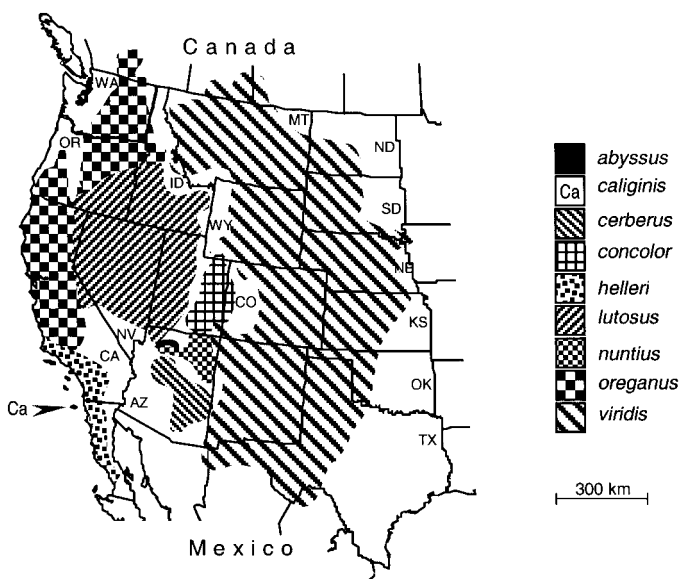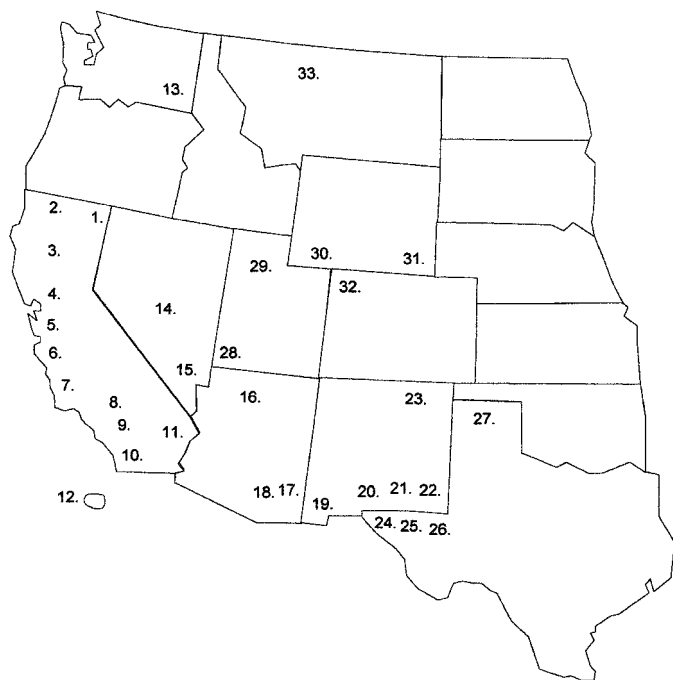

FIG. 1. The distribution of the currently recognized subspecies of *Crotalus viridis* (after Klauber, 1972).

FIG. 2. Sampling localities for *C. viridis.* UNITED STATES California: 1, Modoc County; 2, Siskiyou County; 3, Tehama County; 4, Alameda County; 5, Stanislaus County; 6, Santa Cruz County; 7, San Luis Obispo County; 8, Los Angeles County; 9, San Bernardino County; 10, San Diego County; 11, Riverside County; Washington: 13, Whitman County; Nevada: 14, Nye County; 15, Clark County; Arizona: 16, Coconino County; 17, Graham County; 18, Pima County; New Mexico: 19, Hidalgo County; 20, Dona Ana County; 21, Otero County; 22, Eddy County; 23, Colfax County; Texas: 24, El Paso County; 25, Hudspeth County; 26, Culberson County; 27, Sherman County; Utah: 28, Washington County; 29, Salt Lake County; Wyoming: 30, Sweetwater County; 31, Laramie County; Colorado: 32, Moffat County; Montana: 33, Choteau County. MEXICO: 12, South Coronado Island.

collections. Blood was stored in a buffer (2.0% SDS; 100 mM Tris, pH 8.0; 0.1 M EDTA, pH 8.0), and other body tissues were stored in 80% ethanol.

Template DNA was extracted using a standard proteinase K protocol (Miller *et al.,* 1988; Sambrook *et al.,* 1989) without phenol/chloroform. Ice-cold 5 M ammonium acetate and centrifugation was used to precipitate and then pellet the proteins. DNA was then precipitated from the resulting supernatant with ice-cold isopropanol and centrifuged to form a pellet, which was washed with 70% ethanol and air-dried (Buffone, 1985).

PCR

Double-stranded PCR (Saiki *et al.,* 1988) was used to amplify the mitochondrial DNA fragments of cytochrome *b* (758 bp) and ND4 (900 bp). The cytochrome *b* primers were 5′-TCA AAC ATC TCA ACC TGA TGA AA-3′ (703Bot, L-strand; modified from Kocher *et al.,* 1989) and 5′-GGC AAA TAG GAA GTA TCA TTC TG-3′ (H-strand; modified version of primer MVZ 16 of Moritz

et al., 1992). The 5′ ends of these primers correspond to positions 14977 and 15735 of the total mtDNA sequence of *Dinodon semicarinatus* (Kumazawa *et al.,* 1998), whereas the ND4 primers were primers ND4 and Leu of Arévalo *et al.* (1994); 50-μl reactions were used, containing 50 ng template, 0.52 μM primers, 20 mM Tris–HCl, 50 mM KCl, 0.5 mM MgCl$_2$, 0.4 μM dNTP, 2 units *Taq* DNA polymerase, 0.5% DMSO. Amplification conditions involved an initial denaturation step of 4 min at 94°C, 35 cycles of denaturation for 1 min at 94°C, primer annealing for 1 min at 50°C, extension for 2 min at 72°C, ending with an extra extension step for 3 min at 72°C, and cooling to 4°C. PCR products were concentrated by pooling three products per sample and purified using the GenElute (Supelco) nucleic acid purification kit.

Single-Stranded Automated Sequencing

Single-stranded sequencing was carried out using an automated method; 10-μl reactions containing approximately 50 ng PCR template, 0.16 pmol 5′ primer (703Bot for Cyt *b* reactions; ND4 for ND4 reactions), and BigDye Terminator Ready Reaction Mix (ABI) were cycled in a PE-ABI 9700 Thermal Cycler for 30 s at 94°C, 50 cycles of 10 s at 96°C, 5 s at 50°C, 4 min at 60°C, and then cooled to 4°C. The samples were precipitated using 2 M sodium acetate and 100% ethanol, and the resulting pellet was washed in 70% ethanol and air-dried. The samples were suspended in 4 μl of a dextran blue EDTA/formamide loading buffer, and 1 μl of this mixture was loaded onto a 5% Longranger gel (Flowgen) in 1× TBE running buffer. The samples were analyzed on an ABI 377 DNA Sequencer, generating a chromatogram and a text sequence.

Selection of Haplotypes

Sequence chromatograms were viewed in Chromas 1.51 (Technelysium Pty. Ltd., 1998) and checked for discrepancies against the corresponding text sequences, which were aligned by eye. The analysis of sequences from 68 individuals from 33 localities (Fig. 2) revealed 37 unique haplotypes, which were used as the basis for all Cyt *b* analyses. For the purposes of outgroup rooting, sequences from *C. scutulatus* and *C. durissus terrificus* were also included.

After phylogenetic analysis of the cytochrome *b* sequences, 19 specimens of *C. viridis,* representing the principal groupings observed, were selected for sequencing of the ND4 gene. Those ND4 sequences were used to make a combined cyt *b*–ND4 data set, given that a larger number of characters should provide a more robust estimate of the matrilinear phylogeny of the species (Wiens and Reeder, 1997).

Assessment of Phylogenetic Signal and Saturation

Pairwise sequence comparisons were made for the independent cytochrome *b* and ND4 data sets using

MEGA 1.02 (Kumar *et al.,* 1993) to determine the number, nature, distance, and distribution of base substitutions. Skewness (g_1) statistics were calculated from 10^6 randomly generated trees in PAUP* 4.0b2 (Swofford, 1998) to evaluate the probability of phylogenetic signal (Hillis and Huelsenbeck, 1992; Hillis, 1991) in the combined data set. Levels of saturation at first, second, and third codon positions were assessed from isometric plots of uncorrected pairwise sequence divergences against Tamura–Nei (Tamura and Nei, 1993) pairwise divergences for transitions and transversions, in which deviations from the isometric lines represent a qualitative measure of degree of saturation (Zamudio *et al.,* 1997 and references therein). There was no deviation from the isometric line in plots for first and second position transitions and transversions and third position transversions. A more marked deviation was noted for third position transitions among the ingroup taxa (Fig. 3a), suggesting some saturation for these positions. As expected, this deviation increased with inclusion of the outgroups (Fig. 3b).

Parsimony Analysis

The cytochrome *b* and combined data sets were subjected to unweighted and weighted parsimony analyses in PAUP* 4.0b2. Branch-and-bound searches were used with the combined set, whereas the cyt *b* set was limited by the number of taxa being compared to heuristic searches (specifying random addition of sequences for 500 replications). Three assumptions were applied to the combined set independently in separate parsimony analyses to test the effects of saturation and transition–transversion bias on tree topology, as follows: (1) all substitutions weighted equally; (2) codon positions weighted first 4, second 10, third 1, calculated according to Zamudio *et al.* (1997) and references therein; and (3) transition and transversion ratio weighted 1:6. Bootstrapping (Felsenstein, 1985) for 100 replications was conducted for all parsimony analyses. Branch support was also assessed from decay indices

FIG. 3. Isometric plots of the uncorrected pairwise sequence differences (% difference) against Tamura–Nei indices of pairwise divergences illustrating levels of saturation at 3rd codon position transitions in mitochondrial DNA sequences of *Crotalus viridis* (a) exclusive of outgroups and (b) inclusive of outgroups *C. scutulatus* and *C. durissus.*

calculated for all internal branches of the tree. This method suggests that a branch present in one of the most-parsimonious trees is more strongly supported by the data if a large increase in length of additional trees is required before that branch is lost (Bremer, 1994). Decay indices were calculated from consensus trees generated in PAUP* 4.0b2 using branch-and-bound searches. Trees, one step longer in each successive search, were retained using the "keep" option, starting with a tree length one step longer than the optimal tree and repeating the process until all internal structure had been lost. The decay index for a particular branch was calculated as the number of steps required to collapse that branch.

Testing Alternative Hypotheses

We used two-tailed Wilcoxon signed-ranks tests (Templeton, 1983) to test whether the cladograms predicted by alternative phylogenetic hypotheses were significantly different from the most-parsimonious tree obtained in our analyses or whether differences in topology were likely to have arisen as a result of chance alone. To do this, we ran heuristic searches (random addition, 500 replications) on the unweighted, combined cyt *b* and ND4 dataset, constraining the analysis to retain only the most-parsimonious trees compatible with the alternative phylogenetic hypothesis to be tested. Differences in tree length between each of the constraint trees and the most-parsimonious trees obtained from the unconstrained analysis were tested for significance using the Wilcoxon signed-ranks test, as implemented by PAUP* 4.0b2.

The following hypotheses were tested (see Appendix 2 for trees): (i) all conventional subspecies of *C. viridis* are monophyletic; (ii) the presence of concolor toxin was inherited from the ancestor of all *C. viridis;* to test this, the analysis was constrained to retain only trees in which *C. v. concolor* represents the sister group to all other populations of *C. viridis;* (iii) small body size arose only once in *C. viridis;* for this, the analysis was constrained to retain only the most-parsimonious tree in which *C. v. concolor* and *C. v. nuntius* are sister taxa; and (iv) codistributed populations of *C. viridis* and *Phrynosoma douglassii* have congruent biogeographic histories; to test this, the analysis was constrained to make the area cladogram of *C. viridis* congruent with that of *P. douglassii* (Zamudio *et al.,* 1997); populations of *C. viridis* occurring outside the range of *P. douglassii* (South Coronado Island, coastal California) were excluded from this analysis.

Maximum-Likelihood Analysis

Maximum-likelihood analysis was carried out in PAUP* 4.0b2 following the procedure of Wiens *et al.* (1999), in which likelihood scores were calculated for all trees resulting from the unweighted parsimony analysis using six nested likelihood models of increasing
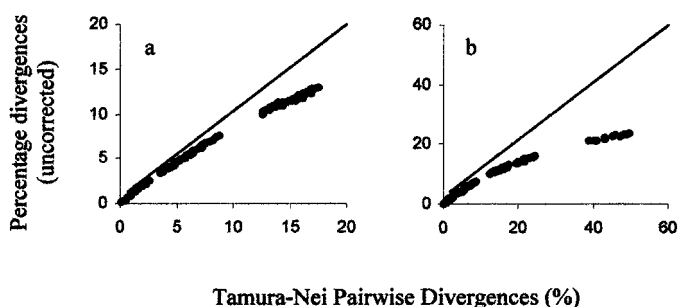
complexity. A heuristic search under the likelihood optimality criterion, imposing the parameters for the tree with the best likelihood score, was used to recalculate the maximum-likelihood scores for all possible trees and thereby to be certain that there were no potentially "better" trees.

Molecular Clock Calibration

Assuming a provisional molecular clock can make a useful guide for estimating species duration (Walker and Avise, 1998), for timing divergence (e.g., Thorpe *et al.,* 1994), and for estimating population distribution range shifts (Riddle, 1995). However, clock assumptions must be treated cautiously since the differences in mtDNA evolution in higher vertebrate groups have not yet been fully identified, and many studies have shown considerable rate heterogeneity (Hasegawa and Kishino, 1989; Martin *et al.,* 1992; Rand, 1994; Hillis *et al.,* 1996; Mindell *et al.,* 1996). Nevertheless, the use of clock assumptions for closely related taxa is generally considered to be more reliable than for distantly related taxa (Caccone *et al.,* 1997), which stems from the premise that rates of evolution of a particular gene are likely to be stable in closely related taxonomic groups, with similar life histories, metabolic rates, and generation times. In this respect, the estimation of "local" rates for closely related taxa may be preferable over a "universal" rate (Hillis *et al.,* 1996). Furthermore, estimating a molecular clock can be complicated by the fact that different parts of the mitochondrial DNA molecule evolve at different rates. Rates of overall sequence divergence estimated for the entire molecule (e.g., from RFLP studies) may thus be misleading for studies using sequences from specific genes.

So far, there have been no specific estimates for the rate of sequence evolution of the cyt *b* and ND4 genes in squamate reptiles. Zamudio and Greene (1997) provided a "ballpark" estimate of 0.47–1.32% my^{-1} for overall mtDNA divergence rates for small to medium-sized ectotherms. Their estimated rates of cytochrome *b* and ND4 sequence differentiation together with the distribution of mtDNA haplotypes in bushmasters (*Lachesis*) appear to coincide well with specific vicariant geologic events, the uplifting of the Andes (14–11 Mya) and the Cordillera de Talamanca (8–5 Mya). Accepting these events as causing the split between Central and South American bushmasters (8.44% sequence divergence) and between *Lachesis melanocephala* and *L. stenophrys* (5.30% sequence divergence) leads to estimates of divergence rates of 0.60–0.76% my^{-1} and 0.66–1.06% my^{-1}, respectively. Since this appears to be the only available calibration for the rate of sequence divergence in the cyt *b* and ND4 genes in squamates, we will base our estimate of the divergence times of the *C. viridis* lineages in subsequent sections of this paper on these rates. Clearly, further evidence on the rate of evolution of these genes in squamates would be desir-

able, especially in view of their considerable importance in squamate phylogenetics in recent years.

RESULTS

Sequence Variation

A total of 37 unique haplotypes from 68 samples of *C. viridis* was obtained for 678 bp of cyt *b* sequence, corresponding to the segment between positions 15045 and 15720 of the total mtDNA sequence of *Dinodon semicarinatus* (Kumazawa *et al.,* 1998). In addition, 669 bp of ND4 sequence were obtained, corresponding to the segment between positions 11743 and 12396 of *Dinodon,* for 19 selected individuals of *C. viridis* and the two outgroup taxa. For the combined analysis, 1345 bp were aligned, of which 165 sites (12.27%) were variable among the ingroup taxa (22.45% including outgroups) and 122 sites (9%) were parsimony informative (12.27% including outgroups). Uncorrected (p) sequence divergence ranged between 0.2 and 6.1% among ingroup taxa, between 7.1 and 7.8% between *C. viridis* and *C. scutulatus,* and between 12.7 and 13.6% between *C. viridis* and *C. durissus.* The level of similarity in number and distribution of transitions and transversions in the two mitochondrial fragments, as well as the absence of stop codons, provides satisfactory evidence that both fragments were mitochondrial and not nuclear (Lopez *et al.,* 1994; Arctander, 1995). Further support is found in the similarity in genetic distance in taxa for both cyt *b* and ND4 and congruence between clade composition and order. Tree length distribution, determined from random sampling of 10^6 unweighted trees, was significantly skewed to the left (g$_1$ = −1.179), suggesting strong phylogenetic signal in the data (*P* < 0.01; Hillis, 1991; Hillis and Huelsenbeck, 1992).

Phylogenetic Relationships

The unweighted heuristic analysis of the 37 unique cyt *b* sequences resulted in 24 equally most-parsimonious trees (length = 202; CI = 0.649; RI = 0.877), the bootstrap consensus of which is shown in Fig. 4. *C. v. cerberus* is represented as the sister group of the remainder of the *C. viridis* complex. Within the remainder of the complex, a clade consisting of *C. v. viridis* and *C. v. nuntius* (east and south of the Rocky Mountains) constitutes the sister clade of the populations west of the Rocky Mountains. However, bootstrap support for these groupings is weak.

The results of the parsimony analysis of the combined cyt *b*–ND4 data set are summarized in Table 1. As expected, the combined data set provided a sufficient number of informative characters to produce a better resolved tree. The unweighted branch-and-bound analysis generated a total of 10 equally most-parsimonious trees (440 steps; CI = 0.747; RI = 0.793) (Fig. 5). The main clades in the consensus receive high bootstrap
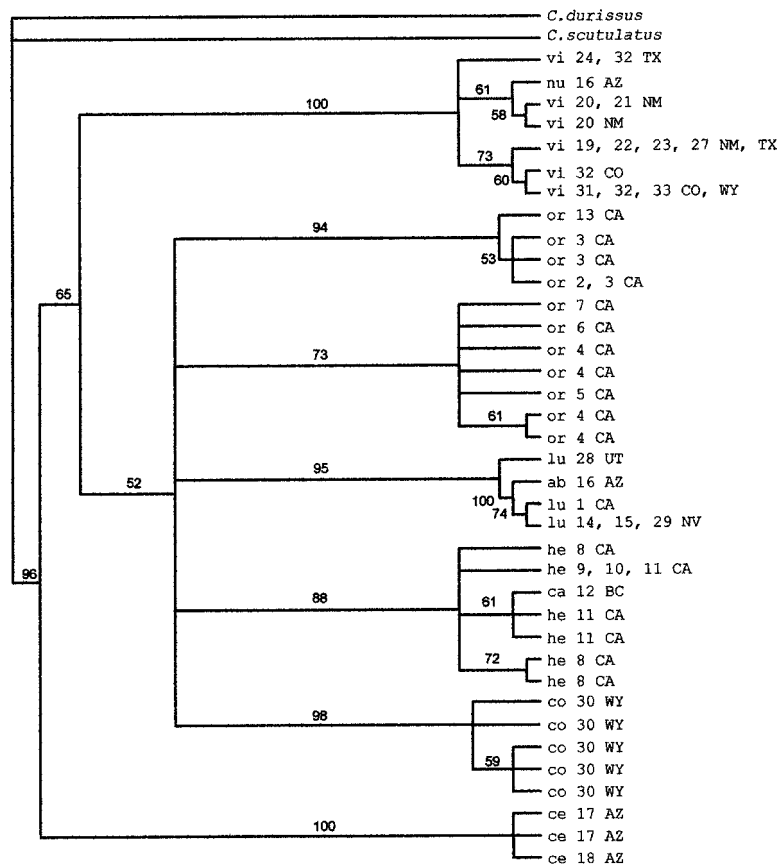
FIG. 4. Consensus of 24 most-parsimonious trees from an unweighted heuristic search for the 678-bp mitochondrial cytochrome *b* sequence data. Numbers on branches represent percentage bootstrap support for 100 iterations. Lowercase abbreviations refer to the conventional subspecies to which the relevant populations are normally assigned: ab, *abyssus;* ca, *caliginis;* ce, *cerberus;* co, *concolor;* he, *helleri;* nu, *nuntius;* lu, *lutosus;* or, *oreganus;* vi, *viridis.* Terminal numbers refer to localities (see Fig. 2), and uppercase abbreviations refer to United States states (BC, Baja California, México).

and Bremer support (4–23), although some resolution is lost within them.

A nearly identical topology was achieved from weighting the codon positions 4:10:1, which generated two equally most-parsimonious trees (969 steps; CI = 0.565; RI = 0.771). The only structural difference was improved resolution in the most terminal group of *C. v. viridis.* Weighting transversions to transitions 6:1 resulted in a single most-parsimonious tree (length = 846; CI = 0.8085; RI = 0.8087). Marginally improved resolution was noted in the *C. v. viridis–C. v. nuntius* clade

TABLE 1

Summary of Results for Unweighted and Weighted Branch-and-Bound Parsimony Analyses for the Combined cyt *b*–ND4 Data Sets

Model	No. trees	Tree length	CI	RI
Unweighted	10	440	0.747	0.793
Codons weighted 4:10:1	2	969	0.565	0.771
TS:TV ratio 6:1	1	846	0.808	0.809

and resolution was lost in the *C. v. helleri–C. v. caliginis* clade. In both instances, overall bootstrap support was high despite minor differences in the values on some branches and reduced support for monophyly of the Pacific coast clade within the western clade.

The highest maximum-likelihood score (ln L = −3960.90) was obtained using the general time-reversible model with rate heterogeneity (GTRr: Lanave *et al.* 1984; Rodriquez *et al.* 1990). A χ^2 test showed that the GTRr model generated a significantly better likelihood result ($P < 0.005$) than the other models used. The topology of the "best tree" was identical to that in Fig. 5.

The consistency of the results of the combined cyt *b*–ND4 dataset, irrespective of weighting regime, supports a single phylogenetic hypothesis for *C. viridis.* Two major clades are apparent within the *C. viridis* complex. The first includes the populations of *C. v. viridis* and *C. v. nuntius,* with a distribution south and east of the Rocky Mountains, and the second includes all remaining taxa west of the Rocky Mountains. The western group comprises three main divisions, *C. v.*
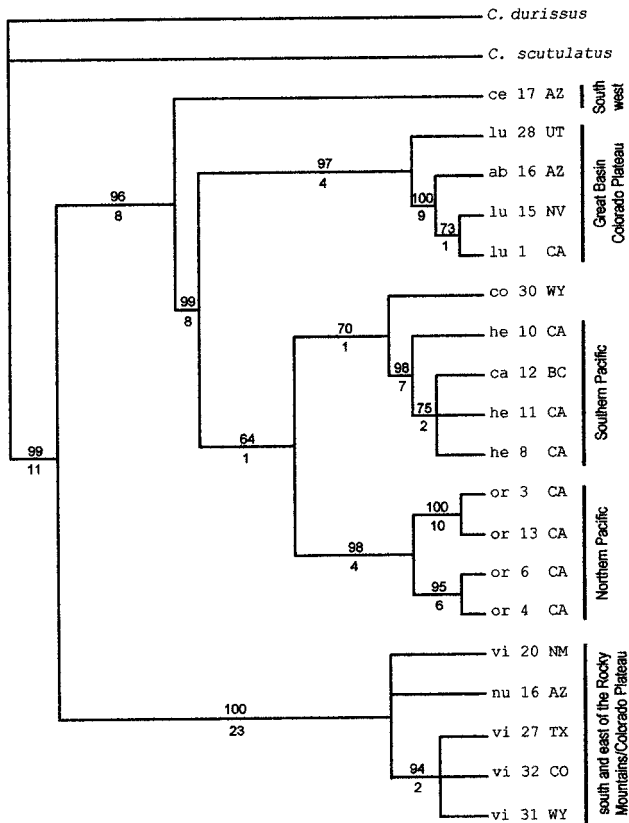
FIG. 5. The consensus of 10 most-parsimonious trees from an unweighted branch-and-bound search for the 1345-bp combined cyt *b*-ND4 sequence data, showing the regional distribution of the mitochondrial haplotype clades. Branch numbers represent bootstrap support for 100 iterations (above) and Bremer support values (below). See Fig. 4. for interpretation of labels.

cerberus in the southwest, a group corresponding to the Great Basin region (*lutosus–abyssus*), and a Pacific regional group (*caliginis–helleri–oreganus–concolor*). *C. v. cerberus* appears as the sister taxon to the remaining western populations in all trees in which this relationship is resolved. The only geographical incongruence is the grouping of our sample of *C. v. concolor* (a Colorado Plateau population) within the Pacific group.

Bootstrap and Bremer support are high for most groups, except for the monophyly of the Pacific regional group and some relationships therein, as well as internal resolution within the *C. v. viridis* and *C. v. nuntius* clade. The close relationship between *C. v. viridis* and *C. v. nuntius* is also supported by the low levels (under 1%) of sequence divergence within this clade, which is considered to be low when distances are compared among and between other ingroup taxa. The position of *C. v. cerberus* as a sister group to all other *C. viridis* populations in the cyt *b* tree is not concordant with the results of the combined data sets. However, bootstrap and Bremer support for the sister group position of *C. v. cerberus* in the analysis of the cyt *b* sequences is weak, whereas support for the position of *C. v. cerberus* as a

sister taxon to the other western populations is high in the combined analyses. In any case, *C. v. cerberus* still forms a third distinct clade, separate from the other western populations.

Phylogenetic Hypotheses (Wilcoxon Signed-Ranks Tests)

The results of the Wilcoxon signed-ranks tests are summarized in Table 2. All results were found to be statistically significant, suggesting that in each case the data reject the alternative hypothesis in favor of the most parsimonious interpretation. We can therefore reject the hypotheses that the various subspecies of *C. viridis* are monophyletic, that concolor toxin was inherited from the common ancestor of all *C. viridis,* that small body size evolved only once in *C. viridis,* and that *C. viridis* and *P. douglassii* share a common biogeographic history.

Molecular clock estimates (Table 3) date the *C. viridis* lineage back as far as the mid-Miocene or earlier. It would seem that differentiation into eastern and western forms, closely followed by separation of *C. v. cerberus* from the remaining western taxa, probably took place early in the history of the lineage, late Miocene to early Pliocene.

DISCUSSION

Mitochondrial DNA Phylogeny and Systematics of the C. viridis Complex

The results of this study reveal a well-resolved phylogeny and identify a number of haplotype clades which, judging from their levels of sequence divergence, represent long-separated lineages. As is often the case in studies of this kind, the distribution of the mitochondrial haplotypes is not fully congruent with the subspecies recognized by Klauber (1972). Whereas the populations of some subspecies form distinct clades, others are rooted deep within clades of other subspe-

TABLE 2

Results of the Wilcoxon Signed-Ranks Tests (Templeton, 1983) for Three Alternative Phylogenetic Hypotheses

	N_1	Z_1	P_1	N_2	Z_2	P_2
Subspecies monophyly	14	−3.207	0.0013	25	−2.268	0.0233
Ancestral origin of Concolor toxin	23	−3.128	0.0018	29	−2.537	0.0112
Single evolution of small size	71	−7.4019	<0.0001	76	−7.040	<0.0001
Biogeography congruent with *Phrynosoma douglassii*	67	−7.759	<0.0001			

Note. The highest (N_1) and lowest (N_2) values are shown of the range of values obtained. A significant result ($P < 0.05$) indicates that the data reject each alternative hypothesis in favor of the most-parsimonious trees.

TABLE 3

Inferred Times of Divergence within the *C. viridis* Complex Estimated from Mitochondrial cyt *b* and ND4 Nucleotide Sequence Data, Using a "Reptile" Rate of 0.60–1.06% my^{-1} Calculated Using Information from Zamudio and Greene (1997)

Evolutionary divergence	Sequence divergence (%)	mtDNA clock (% my^{-1})	
		0.60	1.06
East from west	5.6	9.34	5.28
C. v. cerberus from west	3.5	5.84	3.30

cies. Thus, *C. v. caliginis* and *C. v. abyssus* are rooted within *C. v. helleri* and *C. v. lutosus,* respectively, making these paraphyletic. Similarly, *C. v. nuntius* may be rooted within *C. v. viridis.* Thus, of the nine conventional subspecies, six are thus either paraphyletic or insignificant local variants rooted deep within other clades.

A highly significant Wilcoxon signed-ranks test result further rejects the hypothesis of monophyly of the subspecies in favor of the topology in which some of the subspecies groupings are violated. The basal split of *C. viridis* into an eastern and a western lineage agrees with the results of Quinn (1987). The same is true of the close association of *C. v. viridis* and *C. v. nuntius* in the present phylogeny, which supports the idea that these populations may share recent common ancestry. Within the western group, our evidence of a close association between populations conventionally assigned to *C. v. abyssus* and those assigned to *C. v. lutosus* agree with evidence from morphological similarity (Klauber, 1972) and venom protein profiles (Young *et al.,* 1980). This result suggests either recent common ancestry or continued gene flow between these two populations where their ranges meet at the north rim of the Grand Canyon. Opportunity for gene flow at the north rim may be enhanced due to the easier natural access to the Grand Canyon provided by tributary canyons (Young *et al.,* 1980). Further investigation is required to clarify the relationships of these populations.

The recognition of the nine conventional subspecies of *C. viridis* as categories of equal rank masks and confounds the strong phylogenetic pattern present. Clearly, the subspecies cannot be regarded as categories of equal rank, since some appear to be local varieties without independent phylogenetic histories, whereas others appear to constitute separate lineages with a long history of independent evolution.

The clear separation of populations east and west of the Rocky Mountains and the presence of *C. v. cerberus* as an isolated southwestern population suggest that these populations may represent separate evolutionary species. However, further evidence from other data sets (Thorpe, 1996; Cannatella *et al.,* 1998), such as nuclear

markers and/or morphology, should be sought before definitive changes can be recommended. This additional evidence serves to test for potential effects of lineage sorting (Moore, 1995) and to test various causal hypotheses of geographic variation, which may not necessarily be a reflection of population phylogeny (e.g., Thorpe *et al.,* 1991, 1995; Daltry *et al.,* 1996).

Evolution of Small Body Size

Small adult body size evolved twice independently in *C. viridis,* once in the eastern clade (*C. v. nuntius*) and once in the Pacific coast clade (*C. v. concolor*), a hypothesis that is strongly supported by a highly significant result from the Wilcoxon signed-ranks test. This convergence offers excellent opportunities for the testing of the causes, correlates, and consequences of the evolution of small body size in snakes.

Evolution of Venom Composition

The populations of *C. v. concolor,* with highly lethal venoms containing Concolor toxin, are rooted within the Pacific coast clade. The ability to secrete this particular component appears to have arisen *de novo* within this part of the complex. This hypothesis is strongly supported by statistically significant results of the Wilcoxon signed-ranks tests, which show that the data support the most-parsimonious tree more strongly than the alternative phylogenetic hypotheses. It is interesting to note that the other dwarfed population of the complex, *C. v. nuntius,* is not known to secrete Mojave toxin-like venom components.

This is the first analysis in which the phylogenetic position of populations secreting Mojave toxin-like phospholipases A_2 relative to nonsecreting populations is assessed. Variation in this characteristic is known in other rattlesnake species (e.g., Glenn and Straight, 1978, 1985a,b, 1990; Gutierrez *et al.,* 1991) and similar analyses in these other taxa may help to elucidate the causes and correlates of the ability to secrete this highly toxic venom component. This issue is also of the utmost importance with respect to improving the treatment of snake bite.

Although the rooting of *C. v. concolor* with the Pacific coast rattlesnakes is difficult to comprehend from a geographical point of view, it is congruent with previous results on venom composition. Despite the presence of Concolor toxin and its associated properties in *C. v. concolor,* Foote and MacMahon (1977) noted greater similarity in the venom profiles of *C. v. concolor* and southern Pacific rattlesnakes (*C. v. helleri*) than between *C. v. concolor* and those from the Great Basin (*C. v. lutosus*). This result is consistent with the phylogenetic position of these taxa as presented here. Furthermore, Young *et al.* (1980) note that the venom profiles of the subspecies of *C. viridis* examined (*abyssus, nuntius, lutosus,* and *concolor*) showed only small differences, which are consistent with the minor morphological

differences noted by Klauber (1972). Another interesting observation from venom studies is the greater similarity of *C. v. cerberus* venom to *C. v. viridis* venom than to venom of one of the western forms, *C. v. lutosus* (Foote and MacMahon, 1977), despite the fact that *C. v. cerberus* is the sister taxon to the remaining western populations.

Biogeography

The main clades show strong concordance with the major geographic areas of the western United States (Figs. 5 and 6). This regional structuring closely resembles that described by Zamudio *et al.* (1997) for short-horned lizards (*P. douglassii*), but there are statistically significant differences. The *C. viridis* phylogeny, for example, is rooted across the Rocky Mountains, whereas *P. douglassii* is rooted in the Pacific northwest. The *C. viridis* group shows a more distinct partitioning of the Colorado Plateau populations: some are associated with the populations south and east of the Rocky Mountains (*C. v. viridis, C. v. nuntius* populations), others with the Great Basin (*C. v. lutosus*), and, surprisingly, the populations of *C. v. concolor* (also from the Colorado Plateau) with the Pacific coast clade. Finally, *P. douglassii* is not represented by populations corresponding geographically with the west coast populations
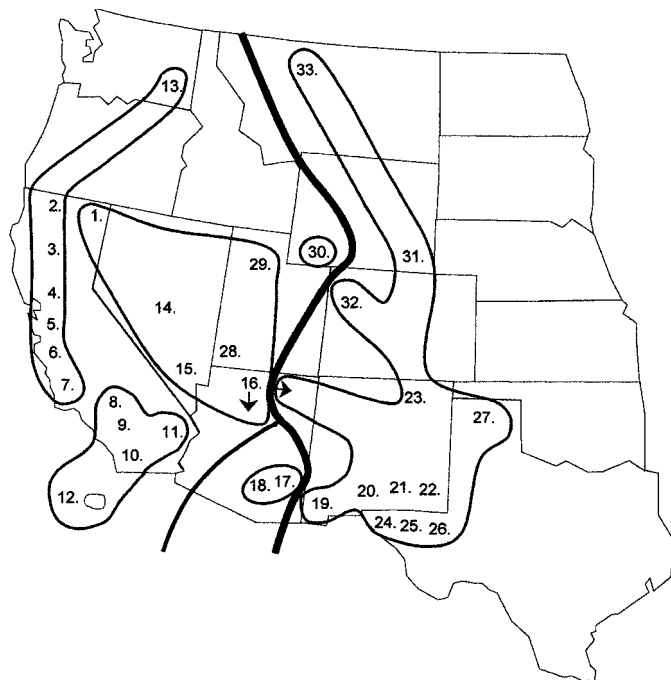

FIG. 6. Distribution of the major mitochondrial clades in *Crotalus viridis.* Numbers refer to the sample localities listed in Fig. 2. Locality no. 16 is represented in two different clades; however, there is no actual sympatry between the two haplotype clades, since locality 16 represents a large county including the Grand Canyon as well as part of the Colorado Plateau. The very bold line represents the basal split between the eastern/southern clade and the bold line the split between *C. v. cerberus* and the remaining western clades.

tions of *C. viridis.* This disparity in the geographic distributions of haplotypes is supported by the highly significant results obtained from the Wilcoxon signed-ranks tests, which reject the hypothesis that codistributed populations of *C. viridis* and *P. douglassii* might have congruent biogeographic histories.

Vicariance events associated with the uplifting of the North American western Cordillera throughout the mid-Miocene to late-Pliocene (17 and 1.64 Mya), and possibly extending into the early Pleistocene, explain most effectively the major separations of the *C. viridis* complex. In particular, the Colorado Plateau appears to represent an important center of phylogeographic partitioning within this species. It has to be assumed, however, that ancestral *C. viridis* must have already been reasonably widespread throughout the west at this time.

From the estimated times of divergence (Table 3), it would seem that the eastern lineages may have separated from the western lineages during the mid-Miocene to early Pliocene (5–9 Mya). The tectonic activity responsible for the uplifting of the Rocky Mountains had virtually ended; however, there continued an extended period of significant volcanism in the southwest associated with the formation of the plateaux now associated with the Grand Canyon and, in particular, the Colorado Plateau (Hunt, 1967; Whitney, 1996). Extensive lava flows and deposition of volcanic ash (throughout the late Tertiary and Quaternary periods) have played a significant role in sculpturing the Colorado Plateau landscape, even into more recent times in the Pleistocene (Hunt, 1974).

The geological activity associated with the Colorado Plateau and surrounding area, together with the shift in vegetational distribution, appears to coincide with the estimated time of basal split between *C. viridis* east and south of the Rocky Mountains (*C. v. viridis* and *C. v. nuntius*) and the remainder of the complex. The most southwesterly extremity of the current range of the *C. v. viridis*–*C. v. nuntius* clade corresponds to the mountainous zone of the Canyon and Datil Section of the Colorado Plateau, which roughly defines the Plateau's southern rim. The Grand Canyon may present a barrier to further westerly spread of this clade. This argument is consistent with the hypothesis that extreme volcanic activity taking place around the southern edge of the Colorado Plateau and around the Grand Canyon throughout the mid-Miocene to mid-Pliocene must have presented a major biogeographic barrier that prevented further south-southwesterly movement of the eastern *C. viridis* lineage, with the result that these populations became gradually separated from the western forms.

During the same time period (3–6 Mya), a significant climatic change occurred resulting from the Sierra Nevada–Cascadian orogeny, which initiated the process of aridification in the west (Potts and Behrens-

meyer, 1992; Whitney, 1996; Brown, 1997). Increasing aridity, together with the rain-shadow effect of the Rocky Mountains (Axelrod, 1985; Potts and Behrensmeyer, 1992), caused a gradual replacement of largely semi-open sclerophyllous woodlands by shrubsteppe and open and desert grasslands (Axelrod, 1985; Keeley and Keeley, 1989; Sims, 1989; Brown, 1997; Zamudio *et al.,* 1997), which, east of the Rocky Mountains, marked the start of the expansion of the Great Plains (Axelrod, 1985). Further differentiation of the east and southeast Rocky Mountain populations involved adaptation in concert with the gradually expanding grassland and desert shrub habitat associated with climatic change.

The present distribution of *C. v. cerberus* within the southern limits of the Basin and Range Province and delimited by the southern rim of the Colorado Plateau and the Central Highlands is consistent with the sister group relationship with the western as opposed to the eastern forms found in our phylogeny. The separation and isolation of the *C. v. cerberus* lineage from the remaining western populations also falls within the late Miocene to early Pliocene but appears to have followed between 2 and 4 Mya after the divergence of the eastern lineage. Continued volcanic disturbance spreading into the Basin and Range Province (Hunt, 1974), together with a rapid expansion of regional deserts associated with the aridification process (Riddle, 1995), restricted the forests to cooler, moister, upland areas (Potts and Behrensmeyer, 1992) with large expanses of inhospitable terrain in between (Whitney, 1996). Consequently, in the late Miocene to mid-Pliocene, populations of *C. viridis* became isolated in relict montane forests in Arizona, prevented from redispersal. These isolated populations gave rise to modern *C. v. cerberus,* which is found only in woodland habitats and for which, at present, there is no reliable evidence of contact with other members of the species group.

Interpretation of the remaining western clades corresponding to the Pacific and Great Basin regions is more difficult. Our results would suggest more recent divergence of these lineages, and it is possible that gene flow continued throughout the Pacific and Great Basin regions during the formation of the Sierra Nevada and Cascade Mountains. These mountain chains now form firm barriers between the two regions with the exception of a pass in the Sierra Nevada Mountains in Northeast California through which there may be limited gene flow between *C. v. oreganus* (northern Pacific) and *C. v. lutosus* (Great Basin). Similarly, a contact zone exists between *C. v. oreganus* and *C. v. helleri* where the ranges of these populations meet (Klauber, 1972). The colonization of South Coronado Island (off the northwest coast of Mexico), traditionally recognized as *C. v. caliginis,* is clearly a recent event, and the only haplotype from this island is contained within the clade of the southern coastal populations (*C. v. helleri*). It is likely that the island was occupied

during the repeated cycles of eustatic lowering of sea levels coincident with Pleistocene glaciations. The status of *C. v. caliginis* as a subspecies must be regarded as open to question.

The sister group relationship between the widely separated southern Pacific coast populations (*C. v. helleri*) and those from southwestern Wyoming (*C. v. concolor*) appears geographically incongruent. However, Bremer support for this relationship is weak. If substantiated by further studies, it may be explicable as vicariance due to the continued aridification of the southwestern United States during the latter part of the Pliocene to early Pleistocene (Hunt, 1974). This hypothesis would suggest the original existence of a continuous population extending from southern California to the Colorado River Valley. The final stages of aridification and habitat change (Webb, 1977) around the mid- to late- Pliocene (2–3 Mya) would have led to the extinctions of populations in the lower and middle Colorado River Valley and the existence of relict populations on the Colorado Plateau and in the mesic parts of southern California. The middle course of the Colorado River, especially the Grand Canyon, was later occupied by populations originating from the Great Basin (*C. v. lutosus, C. v. abyssus*), which may now be contiguous with *C. v. concolor.* A phylogeographic pattern uniting populations from southern California and the upper Colorado River might also be found in other wide-ranging, non-hot-desert species of similar age in the southwestern United States.

The fossil record reveals that *C. viridis* was widespread by the start of the Pleistocene, with a distribution not dissimilar from that of the present day (Holman, 1995); judging from the scenario outlined above, the various *C. viridis* clades may already have been well defined. This distribution is thought to have remained fairly stable throughout the Pleistocene (Holman, 1995), despite some changes resulting from the fluctuating climate. Despite these changes, evidence is lacking to support the once-popular hypothesis that most of the present-day Great Basin desert reptiles have extended their ranges into the Basin through post-Pleistocene range expansions from Mexican refugia (Holman, 1995). This is rejected by our sequence analyses: the Great Basin is occupied by a distinctive clade of populations (conventionally assigned to *C. v. lutosus*) which is not found elsewhere. The average sequence divergence of 2.63% from its sister clade, the Pacific coast populations, suggests that these populations have occupied at least parts of the Great Basin continuously for the last 2.5–4.5 million years. Instead, *C. viridis* could easily have survived in woodland habitats during cooler periods.

This study represents an important contribution toward our knowledge of the systematics of the western rattlesnake and should provide a broader phylogenetic

context for further studies of the population systematics and evolutionary biology of the *Crotalus viridis* complex.

APPENDIX 1

Specimens for Which Sequence Data Were Obtained

Crotalus durissus terrificus: Brazil: São Paulo: Pindamonhangaba WW 136 *AF147877; AF194168* (IB 55600-1).

Crotalus scutulatus scutulatus: USA: New Mexico: Dona Ana Co. 050 *AF147876; AF194167* (UTEP(CRH) 153).

Crotalus viridis abyssus: USA: Arizona: Coconino Co. [16]: 010 *AF147857; AF194148* (TM).

Crotalus viridis caliginis: México: Baja California Norte: Isla Coronado Sur [12]: 014 *AF147858; AF194149* (TM).

Crotalus viridis cerberus: USA: Arizona: Graham Co. [17]: 016; *AF147859; AF194150* (TM), 117 (WCS); Pima Co. [18]: 047 (ASDM 91076).

Crotalus viridis concolor: USA: Wyoming: Sweetwater Co. [30]: 065 *AF147860; AF194151,* 066, 068-071 (KA).

Crotalus viridis helleri: USA: California: Los Angeles Co. [8]: 096 *AF147861; AF194152* (RM), 097, (ROM 19656), 098 (RM1967); Riverside Co. [11]: 007 (WH H65), 008 (WH H63), 009 *AF147862; AF194153* (WH H91); San Bernardino Co. [9]: 022 (MC 84070401), 039 (WH H18); San Diego Co. [10]: 079 *AF147863; AF194154* (UTEP17519).

Crotalus viridis lutosus: USA: California: Modoc Co. [1]: 020 *AF147871; AF194162* (MC 87083001); Utah: Washington Co. [28]: 021 *AF147865; AF194156* (MC); Salt Lake Co. [29]: 099 (ROM19760), 100 (ROM 19761). Nevada: Clark County [15]: 011 *AF147864; AF194155* (TM); Nye Co. [14]: 101 (ROM1).

Crotalus viridis nuntius: USA: Arizona: Coconino Co. [16]: 012 *AF147875; AF194166* (TM).

Crotalus viridis oreganus: USA: California: Alameda Co. [4]: 102 (ROM 19764), 104 (MVZ 150247), 105 (MVZ 150245), 106 *AF147874; AF194165* (MVZ 150248), 108 (MVZ 128187), 109 (MVZ 128188), 111 (MVZ 128193); San Luis Obispo Co. [7]: 017 (TM), 023 (MC 97052501); Santa Cruz Co. [6]: 026 *AF147873; AF194164* (WH O03); Tehama Co. [3]: 001 *AF147870; AF194161,* 002-004 (LS), 089 CA (LS); Siskiyou Co. [2]: 088, 090 (LS); Stanislaus Co [5]: 114 (MVZ 150250). Washington: Whitman Co. [13]: 006 (WH O07); 025 (WH O13) *AF147872; AF194163.*

Crotalus viridis viridis: USA: Colorado: Moffat Co. [32]: 055 *AF147867; AF194158,* 056-057 (KA). Montana: Choteau Co. [33]: 093-095 (ROM 16109, RM 2053, RM 2058). New Mexico: Colfax Co. [23]: 076 (UTEP 14754); Dona Ana Co. [20]: 048 *AF147866; AF194157* (UTEP 17625); 074 (UTEP 17518); 085 (UTEP 17714); 086 (UTEP 17715); Eddy Co. [22]: 044 (ASDM 92290); Hidalgo Co. [19]: 015 (TM); Otero Co. [21]: 073 (UTEP 15871); Texas: El Paso [24]: 046 (ASDM 92302); Hud-speth Co. [25]: 075 (UTEP 14147), 078 (UTEP 15469); Sherman Co. [27]: 081 *AF147869; AF194160* (UTEP 15872) Wyoming: Laramie Co. [31]: 059, 060 *AF147868; AF194159,* 063 (KA).

Note. The first number refers to the sample number of C. Pook; abbreviations and numbers in brackets represent institution voucher number/captivity reference code or personal credit (UTEP, University of Texas El Paso; ASDM, Arizona-Sonora Desert Museum; TM, Tom Moisi; WH, Bill Hayes; KA, Kyle Ashton; LS, Lee Simons; MC, Mike Cardwell; MVZ, Museum of Vertebrate Zoology, Berkeley; RM, Ross MacCulloch; ROM, Royal Ontario Museum, Toronto; IB, Instituto Butantan, São Paulo; WCS, Wade Sherbrooke; WW, W. Wüster). Where applicable, GenBank accession numbers (italics) follow sample numbers of C. Pook reference. Numbers (in square brackets) are the localities codes shown in Fig. 2.

APPENDIX 2

Constraint Trees Used to Test Alternative Phylogenetic Hypotheses Using the Wilcoxon Signed-Ranks Test (Templeton, 1983)

(1) Monophyly of subspecies categories: (sc23,du,(ab16,ca12,ce17,co30,nu16,(he11,he10,he8),-(lu15,lu28,lu1)(vi23,vi32, vi31,vi27),(or3,or13,or6,or4))).

(2) Evolution of Concolor toxin *de novo* within the *Crotalus viridis* lineage *C. v. concolor* as a sister group to all the other *C. viridis,* which represent a single monophyletic group: (du,sc23,(co30,(ab16,ca12,ce17,-he11,he10,he8,lu15,lu28,vi23,vi32,vi31,ce17,or3,lu1,or 13,or6,or4,nu16))).

(3) Evolution of small body size within *C. viridis:* (du,sc23,((co30,nu16),ab16,ca12,ce17,he11,he10,he8,-lu15,lu28,vi23,vi32,vi31,or 3,lu1,or13,or6,or4)).

(4) A hypothetical phylogeny based on the area cladogram for *P. douglassii* (Zamudio *et al.,* 1997) to test whether codistributed populations of *C. viridis* and *P. douglassii* have had congruent biogeographic histories:
(sc23,du,((ab16,co30,lu15,lu28,vi32,lu1,nu16),(ce17,-vi23,vi31,vi27)),(or3,or13)).

Note. Abbreviations refer to taxon (du, *Crotalus durissus;* sc, *Crotalus scutulatus; Crotalus viridis:* ab, *abyssus;* ca, *caliginis;* ce, *cerberus;* co, *concolor;* he, *helleri;* nu, *nuntius;* lu, *lutosus;* or, *oreganus;* vi, *viridis*); numbers refer to the localities listed in Fig. 2.

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