

Molecular Systematics of the Western Rattlesnake, *Crotalus viridis* (Viperidae), with Comments on the Utility of the D-Loop in Phylogenetic Studies of Snakes

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***Crotalus viridis*, the western rattlesnake, ranges throughout western North America and has been divided into at least eight subspecies. However, the validity of and relationships among these subspecies and the monophyly of *C. viridis* as a whole are questionable. We used mitochondrial DNA sequence data from the D-loop region and ND2 gene to examine the relationships among 26 populations of *C. viridis* and to test the monophyly of this species. These data were analyzed separately and combined using maximum-likelihood and maximum-parsimony. The *C. viridis* group was monophyletic in all combined analyses, consisting of two strongly divergent clades. We recommend that these clades be recognized as two distinct evolutionary species: *C. viridis* and *C. oreganus*. *Crotalus viridis* should be restricted to the subspecies *viridis* and *nuntius* and the remaining subspecies be assigned to the species *C. oreganus*. Our data do not allow strong evaluation of the validity of the subspecies. We found that the ND2 gene had greater sequence divergences among closely related individuals than the D-loop region, but this relationship reversed at higher levels of divergence. This pattern is apparently due to: (1) ND2 third positions evolving faster than the D-loop but becoming saturated at higher levels of divergence, and (2) the D-loop evolving faster than ND2 second (and possibly first) positions. Our results suggest that the ND2 gene is preferable for examining intraspecific relationships and the D-loop may better resolve relationships between species of snakes. The latter result is contrary to the common perception of the phylogenetic utility of the D-loop. Another unusual result is that the 145 bp spacer region, adjacent to the 5' end of the light strand of the D-loop, provides greater phylogenetic resolution than the 1030 bp D-loop.** © 2001

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INTRODUCTION

Studying the relationships among populations of a species has been useful for several reasons. First, such studies have revealed that many species show structuring among populations similar to that observed among species (Avice *et al.*, 1987; Avice, 1989, 1994, 2000). Second, coupled with knowledge of the geographic distribution of lineages, these studies facilitate inferences about the historical biogeography of a species (Avice, 1994, 2000). Third, studies of interpopulation relationships can provide a framework for other evolutionary inquiries about a species, as well as provide the means to identify phylogenetically independent data points for comparative analyses. Fourth, such analyses may reveal distinct lineages that deserve recognition as species (e.g., Zamudio and Greene, 1997; Burbrink *et al.*, 2000; Rodriguez-Robles and De Jesus-Escobar, 2000).

Crotalus viridis, the western rattlesnake, is a polytypic species that ranges from southern Canada to northern Mexico and from the Pacific coast to the midwestern United States (Fig. 1; Klauber, 1972; Stebbins, 1985). Additionally, *C. viridis* currently inhabits three islands (Ashton, 2000), and one of these island populations has previously received subspecific recognition (Klauber, 1949). Western rattlesnakes occur in a variety of habitats including grasslands, montane forests, shrublands, and deserts (Stebbins, 1985). Populations differ widely in color pattern, scutellation, body size, life history, and behavior (Klauber, 1972; Macartney *et al.*, 1990; Ashton and Patton, 2001). In fact, much of Klauber's (1972) classic two-volume work on rattlesnakes focuses on variation in *C. viridis*. Such morphological, ecological, and behavioral diversity has led to the recognition of nine subspecies (Klauber, 1972). Research concerning the variability among populations of *C. viridis* has also served as the foundation for understanding several aspects of snake behavior, ecology, and evolution (e.g., Fitch, 1949; Klauber, 1972; Aldridge, 1979; King and Duvall, 1990; Duvall and

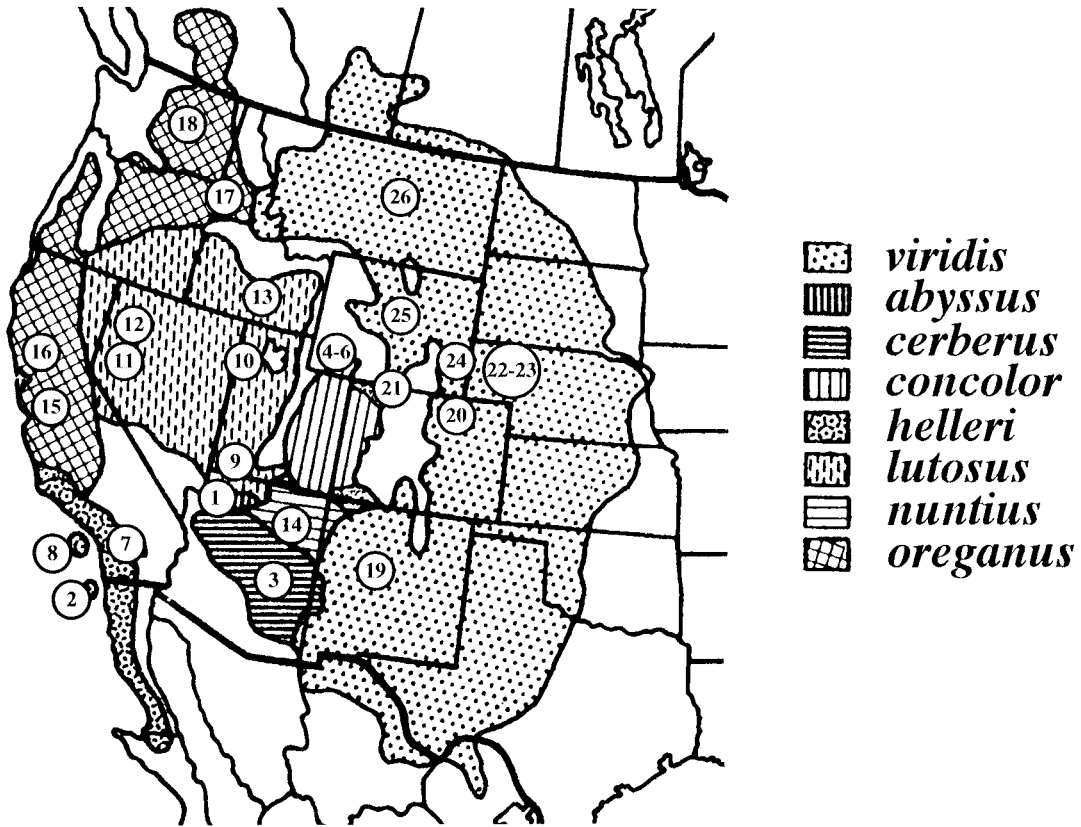


FIG. 1. The current distribution of subspecies of *C. viridis* (from Stebbins, 1985). Numbers correspond to localities of samples used in this study (Table 1).

Schuett, 1997; Duvall and Beaupre, 1998; Fitch, 1998). Despite this interest, several aspects of the evolutionary history and systematics of this group remain to be addressed. In particular, questions about the monophyly of the species as a whole, relationships among populations of this species, and the possibility that *C. viridis* actually consists of several distinct species remain to be answered.

The monophyly of *C. viridis* is supported by a unique characteristic, the presence of more than two internal scales (Klauber, 1972). Most authors have presented *C. viridis* as monophyletic (Gloyd, 1940; Brattstrom, 1964; Klauber, 1972; Foote and MacMahon, 1977), however, Quinn's (1987) morphological phylogenetic analysis suggests that *C. viridis* may be paraphyletic with respect to *C. scutulatus*, the Mojave rattlesnake. A recent molecular phylogenetic analysis found *C. viridis* to be monophyletic (Pook *et al.*, 2000). However, *C. scutulatus* was one of only two outgroups included, the second of which may be too distantly related to provide appropriate character polarization.

Klauber (1972) was the first to propose an intraspecific phylogeny for the nine subspecies of *C. viridis* (Fig. 2a). These relationships were based on morphological characters and were constructed using an unspecified phylogenetic reconstruction method. Foote and Mac-

Mahon (1977) proposed relationships among most subspecies of *C. viridis* from numerical taxonomic analysis of venom protein bands (Fig. 2b), but the use of this kind of venom data to estimate phylogenetic relationships has been questioned (e.g., Daltry *et al.*, 1996). Additionally, the authors did not specify how they reconstructed relationships, although it is likely that they used a distance method that assumes clock-like evolution, and such methods have been shown to be inferior to alternative methods when reconstructing phylogenies (Wiley, 1981; Swofford *et al.*, 1996). Quinn (1987) used morphology, isozymes, and restriction fragments to investigate relationships among populations throughout the range of *C. viridis*. His analyses separated *C. viridis* into two main clades (one of primarily eastern populations, the other of western populations) but are not well resolved further. A recent study by Pook *et al.* (2000) using mtDNA (cyt *b* and ND4) provides the most convincing estimate of relationships within *C. viridis*. Pook *et al.*'s (2000) analyses support the monophyly of *C. viridis*, recover the same two divergent clades as did Quinn (1987), and are generally well resolved (Fig. 2c).

Here we present results of analyses of the relationships among populations of *C. viridis* based on DNA sequences of two mitochondrial regions, the displace-

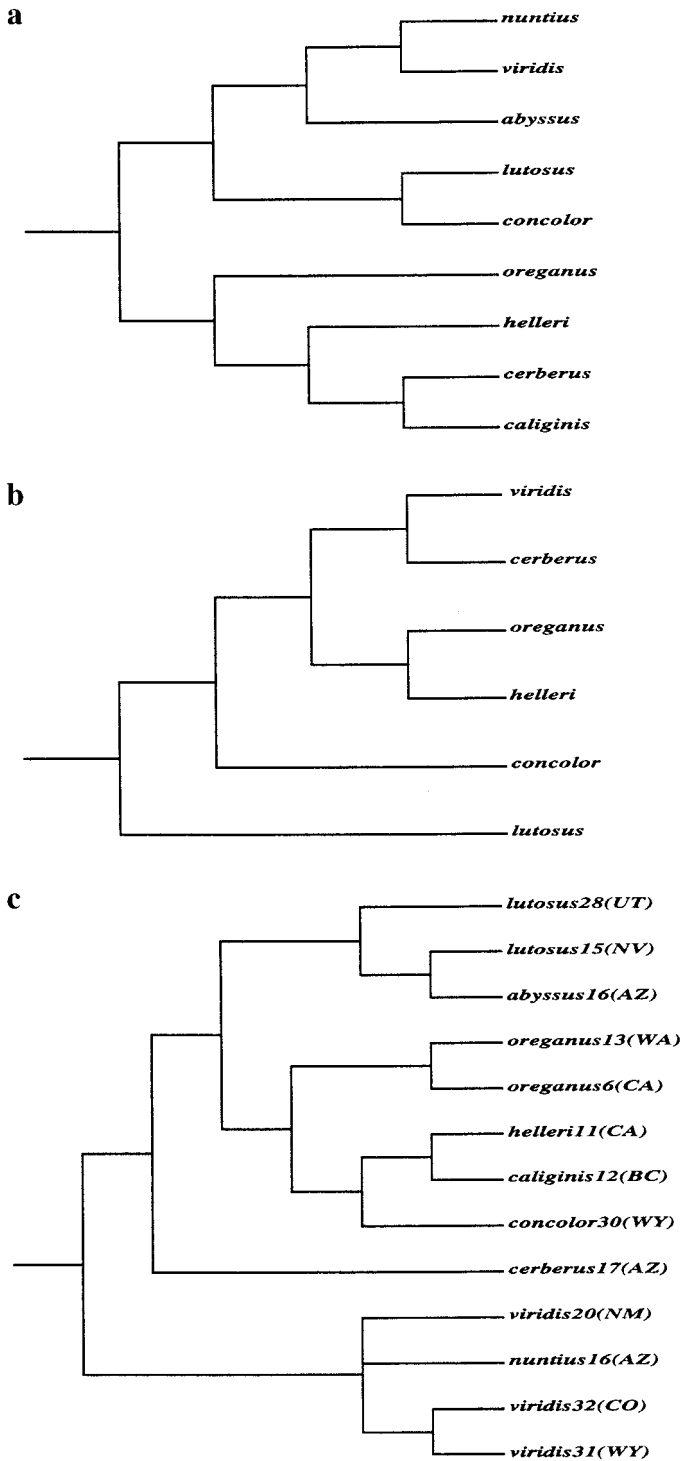


FIG. 2. Previous phylogenetic hypotheses for subspecies of *C. viridis*: (a) based on morphological analysis (Klauber, 1972); (b) based on distance analysis of whole venom profiles (Foote and MacMahon, 1977); (c) based on MP analysis of *cyt b* and ND4 (Pook *et al.*, 2000) and trimmed for direct comparison with our samples (see text). Subspecies names followed by sample number and two-letter abbreviation of state of collection (see Pook *et al.*, 2000).

ment loop (D-loop) and the NADH 2 gene (ND2). We chose the D-loop because, as a non-protein coding region, it is more variable than other regions of the mitochondrial genome (Saccone *et al.*, 1987; Baker and Marshall, 1997; Palumbi, 1997), and hence is especially useful for reconstructing relationships among closely related populations (e.g., Hoelzel and Dover, 1991; Brown *et al.*, 1993; Yang *et al.*, 1994; Vila *et al.*, 1997). We chose the ND2 gene because it evolves at a high rate, yet is typically less variable than the D-loop (Otto *et al.*, 1996) and has been effective in discerning relationships among closely related species (e.g., Duvernell and Turner, 1998; Macey *et al.*, 1998). We expected these genes used in conjunction to allow resolution of close as well as more distant relationships within *C. viridis* because of their different rates of evolution.

We present both separate and combined analyses of the two mitochondrial regions. These analyses show that both regions are phylogenetically informative, but the expected difference in utility of the two regions for resolving relationships is not seen. To gain insight into this unexpected result, we evaluate rates of sequence divergence for the two regions. We also present separate phylogenetic analyses of a short, highly variable spacer region found adjacent to the 5' end of the light strand of the D-loop in *Crotalus* (Kumazawa *et al.*, 1996) for direct comparison with the two larger DNA regions. We compare our phylogenetic estimates to previous work on *C. viridis*, make taxonomic recommendations, comment on the historical biogeography of this group, and discuss the implications of our comparisons of ND2 and the D-loop for phylogenetic studies of snakes.

MATERIALS AND METHODS

Taxon Sampling

We obtained DNA sequence data for several individuals from most of the named subspecies and one individual from each of the current island populations of *C. viridis* (Table 1; Fig. 1). Additionally, we gathered samples for two individuals of *C. scutulatus* because it may be nested within the *C. viridis* group (Quinn, 1987), and for one individual from each of five different out-group species representing close as well as more distant relatives: *C. atrox*, *C. cerastes*, *C. enyo*, *C. mitchelli*, and *C. tigris* (Brattstrom, 1964; Klauber, 1972; Foote and MacMahon, 1977).

Laboratory Methods

DNA was extracted from liver, blood, and skin samples using the QIAamp Tissue Kit (Qiagen Inc., Santa Clarita, CA). The entire D-loop region plus adjacent tRNA genes and a spacer region, and the entire ND2 gene with portions of adjacent tRNA genes were am-

TABLE 1

Information for Specimens from which Sequences Were Obtained for This Study

Taxon	Sample no.	GenBank accession no.	Voucher no. or collector	Locality
<i>Crotalus atrox</i>	—	AY016049, AY016239	D. Chiszar	Exact locality unknown
<i>C. cerastes</i>	—	AY016245	MVZ 137597	California: Imperial Co.
<i>C. enyo</i>	—	AY016246	MVZ 175915	Mexico: Baja California
<i>C. mitchelli</i>	—	AY016051, AY016241	MVZ161441	Mexico: Baja California
<i>C. scutulatus</i> (1)	—	AY016048, AY016238	MVZ 137600	California: Kern Co.
<i>C. scutulatus</i> (2)	—	AY016047, AY016237	MVZ 164852	Exact locality unknown
<i>C. tigris</i>	—	AY016050, AY016240	MVZ150244	Arizona: Pima Co.
<i>C. v. abyssus</i>	1	AY016037, AY016227	T. Moisi	Arizona: Coconino Co.
<i>C. v. caliginis</i>	2	AY016038, AY016228	T. Moisi	Mexico: Baja California, Isla Coronado Sur
<i>C. v. cerberus</i>	3	AY016035, AY016225	UCM 60231	Arizona: Coconino Co.
<i>C. v. concolor</i>	4	AY016043, AY016233	UCM 60074	Wyoming: Sweetwater Co.
<i>C. v. concolor</i>	5	AY016041, AY016231	UCM 60075	Wyoming: Sweetwater Co.
<i>C. v. concolor</i>	6	AY016042, AY016232	K. Ashton	Wyoming: Sweetwater Co.
<i>C. v. helleri</i>	7	AY016032, AY016222	CAS 200713	California: Riverside Co.
<i>C. v. helleri</i>	8	AY016039, AY016229	K. Ashton	California: Los Angeles Co., Santa Catalina Island
<i>C. v. lutosus</i>	9	AY016036, AY016226	UCM 60230	Arizona: Mohave Co.
<i>C. v. lutosus</i>	10	AY016026, AY016215	CAS 170415	Utah: Salt Lake Co.
<i>C. v. lutosus</i>	11	AY016033, AY016223	CAS 202965	Nevada: Washoe Co.
<i>C. v. lutosus</i>	12	AY016040, AY016230	K. Ashton	Nevada: Washoe Co., Anaho Island
<i>C. v. lutosus</i>	13	AY016244	D. Carpenter	Idaho: Butte Co.
<i>C. v. nuntius</i>	14	AY016028, AY016218	CAS 170444	Arizona: Navajo Co.
<i>C. v. oreganus</i>	15	AY016034, AY016224	CAS 202983	California: Monterey Co.
<i>C. v. oreganus</i>	16	D86120 (Kumazawa <i>et al.</i> , 1996)	MVZ 137602	California: Alameda Co.
<i>C. v. oreganus</i>	17	AY016031, AY016221	CAS 170492	Idaho: Nez Perce Co.
<i>C. v. oreganus</i>	18	AY016027, AY016217	CAS 170431	Washington: Kittitas Co.
<i>C. v. viridis</i>	19	AY016025, AY016216	CAS 170419	New Mexico: Bernalillo Co.
<i>C. v. viridis</i>	20	AY016243	CAS 170445	Colorado: Weld Co.
<i>C. v. viridis</i>	21	AY016029, AY016219	CAS 170453	Colorado: Moffat Co.
<i>C. v. viridis</i>	22	AY016045, AY016235	UCM 59107	Nebraska: Scotts Bluff Co.
<i>C. v. viridis</i>	23	AY016044, AY016234	UCM 59108	Nebraska: Scotts Bluff Co.
<i>C. v. viridis</i>	24	AY016046, AY016236	K. Ashton	Wyoming: Laramie Co.
<i>C. v. viridis</i>	25	AY016242	CAS 170470	Wyoming: Fremont Co.
<i>C. v. viridis</i>	26	AY016030, AY016220	CAS 170472	Montana: Wheatland Co.

plified using the polymerase chain reaction (Saiki *et al.*, 1988; Palumbi 1997). To amplify the D-loop we used primers H690 (Kumazawa *et al.*, 1996) and L16047 ([5'-AAC AAA ATC TCA GGT CTC ACC TGC-3'] (this study)). The 3' ends of these primers correspond to positions 111 and 16047 of the light strand of *Dinodon semicarinatus* (Kumazawa *et al.*, 1998). These primers are located in 12S rRNA and tRNA^{Thr} and thus amplified the D-loop region that is positionally homologous with that of other vertebrates, rather than the duplicated region (Kumazawa *et al.*, 1996, 1998). The D-loop region amplification (1332 total bp) included 1030 bp of the D-loop, 27 bp of 12S rRNA, 65 bp of tRNA^{Phe}, 58 bp of tRNA^{Thr}, 145 bp of a spacer region, and 7 bp of noncoding and nonfunctional sequence found at the 3' end of the light strand of the D-loop (Kumazawa *et al.*, 1996). To amplify the ND2 gene we used primers L4437b (Kumazawa *et al.*, 1996) and tRNA-trpR ([5'-GGC TTT GAA GGC TMC TAG TTT-3'] (R. Lawson, unpubl.)). The 3' ends of these primers correspond to positions 4801 and 5877 of the light strand of *D. semicarinatus* (Kumazawa *et al.*, 1998).

The ND2 amplification (1076 total bp) included 1030 bp of ND2, 11 bp of tRNA^{Met}, and 6 bp of tRNA^{Trp}. Amplification conditions consisted of an initial denaturation for 1 min at 95°C followed by 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 50°C for D-loop, and 55°C for ND2, and extension for 1 min at 72°C, ending with a final extension of 3 min at 72°C. PCR products were purified using the QIAquick PCR purification Kit (Qiagen Inc.). Cycle sequencing was performed with the original primers using Big Dye (Perkin-Elmer, Norwalk, CT) reaction premix for 28 cycles of 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. The nucleotide sequences were visualized on an ABI Prism model 377 automated DNA sequencer (Applied Biosystems, Norwalk, CT).

Phylogenetic Analyses

We aligned sequences using the "align automatically" command in the program Sequencher (version 3.0; Gene Codes Corp., Ann Arbor, MI). To compare the substitution rates of ND2, D-loop, and the spacer region we plotted uncorrected percent sequence diver-

gences for the three regions. To assess saturation, we compared the uncorrected percent sequence divergences against Tamura-Nei corrected distances (Tamura and Nei, 1993) for transitions and transversions at each codon position for the ND2 gene, and for transitions and transversions for the D-loop region. We used the Tamura-Nei model because it is a frequently used method for evaluating saturation (e.g., Zamudio and Greene, 1997; Pook *et al.*, 2000; Rodriguez-Robles and de Jesus-Escobar, 2000). All percent sequence divergences were calculated using PAUP*4.0b3a (Swofford, 1999).

We used two methods of phylogenetic reconstruction implemented by PAUP* 4.0b3a: maximum-parsimony (Camin and Sokal, 1965; Swofford *et al.*, 1996) and maximum-likelihood (Felsenstein, 1981; Huelsenbeck and Crandall, 1997). These two tree reconstruction methods were chosen because they often perform best in reconstructing phylogenetic relationships based on DNA sequence data (e.g., Hillis *et al.*, 1994; Kuhner and Felsenstein, 1994; Swofford *et al.*, 1996). Phylogenetic analyses were first performed using each DNA region separately. For the separate analyses we only used sequences from the D-loop and ND2, not any of the adjacent regions, in order to compare the utility of the two regions. We also performed separate analyses of the 145 bp spacer region because it was shown to contain a relatively large proportion of phylogenetically informative characters. We used all sequence data for the combined analyses and tested for incongruence among the data sets using the partition homogeneity test (Farris *et al.*, 1994) as implemented by PAUP*. Trees were rooted by outgroup comparisons (Watrous and Wheeler, 1981; Maddison *et al.*, 1984), using all five outgroup species simultaneously. The status of *C. scutulatus* was not constrained in the analyses because its relationship to individuals of *C. viridis* was uncertain (Quinn, 1987).

Equal weighting of all characters was used for the D-loop because no saturation was evident for transitions or transversions, and the ti:tv ratio was 1.3. The ND2 gene showed evidence of saturation of transitions, and transitions were more common than expected (ti:tv = 4.2), therefore we used two weighting schemes in parsimony analyses of ND2 and for the ND2 gene in the combined analyses: 1:1 and 1:5 (ti:tv). Differential weighting of characters in phylogenetic analyses is controversial (e.g., Reeder, 1995; Milinkovitch *et al.*, 1996; Vidal and Lecointre, 1998; Voelker and Edwards, 1998; Savolainen *et al.*, 2000), however, we included such analyses to examine whether differential weighting influenced our results.

We performed maximum-parsimony (MP) analyses separately for ND2, D-loop, and the spacer region, and a combined analysis including only samples for which we had sequences from both DNA regions. We did not have data for both DNA regions for the following sam-

ples: *C. viridis* #13, 16, 20, 25, *C. cerastes*, and *C. enyo* (Table 1). In all cases we used the branch and bound search option in PAUP* 4.0b3a. Support for individual clades was evaluated using nonparametric bootstrapping (Felsenstein, 1985) with nodes represented in $\geq 70\%$ of bootstrap replicates considered strongly supported (Hillis and Bull, 1993).

Maximum-likelihood (ML) analyses were performed separately for each DNA region using PAUP* 4.0b3a. We also performed a combined analysis using only samples with data from both DNA regions. For each ML analysis all of the most-parsimonious trees, for that dataset, were used as the starting trees. We estimated the parameters for each model (e. g., base frequencies, ti:tv ratios, gamma distribution shape parameter, number of invariable sites) from these starting trees. We tested six nested likelihood models (JC69, F81, HKY85, HKY85+I, HKY85+I+ Γ , GTR+I+ Γ ; see Wiens *et al.* (1999) for details of the models) with likelihood ratio tests (Huelsenbeck and Crandall, 1997) to assess which model best explained the data for each region and combined datasets. In comparisons between models, the more complex model was considered to better explain the data only if its likelihood score was significantly better than that of the simpler model, using a likelihood ratio test. The best-fitting model for each DNA region under this criterion was then used in a heuristic search to find the tree that conferred the highest likelihood on that data set. Support for individual clades was evaluated using nonparametric bootstrapping (Felsenstein, 1985) obtained from the fast-search option in PAUP*4.0b3a.

Comparisons to Previous Hypotheses

We used the Shimodaira-Hasegawa test (Shimodaira and Hasegawa, 1999) implemented by PAUP* to test our phylogenetic hypotheses against those previously proposed (Fig. 2) because this test is preferred over other tests of topology (Goldman *et al.*, 2000). We trimmed taxa from our best estimate of relationships (Fig. 5) to compare directly with subspecies relationships presented by previous authors (Figs. 2a,b; Klauber, 1972; Foote and MacMahon, 1977). For comparison with the results of Pook *et al.* (2000), we only included samples that were collected from the same general locality for both studies. This analysis included 13 of the 19 samples from Fig. 5 of Pook *et al.* (2000) (see Fig. 2c for comparison).

RESULTS

Sequence Data

We recovered between 1264 and 1330 bp of the D-loop and adjacent regions for 23 individuals; we recovered shorter sequences for the following samples: #10 (665 bp), #3 (1213 bp), *C. atrox* (1204 bp), *C. mitchelli*

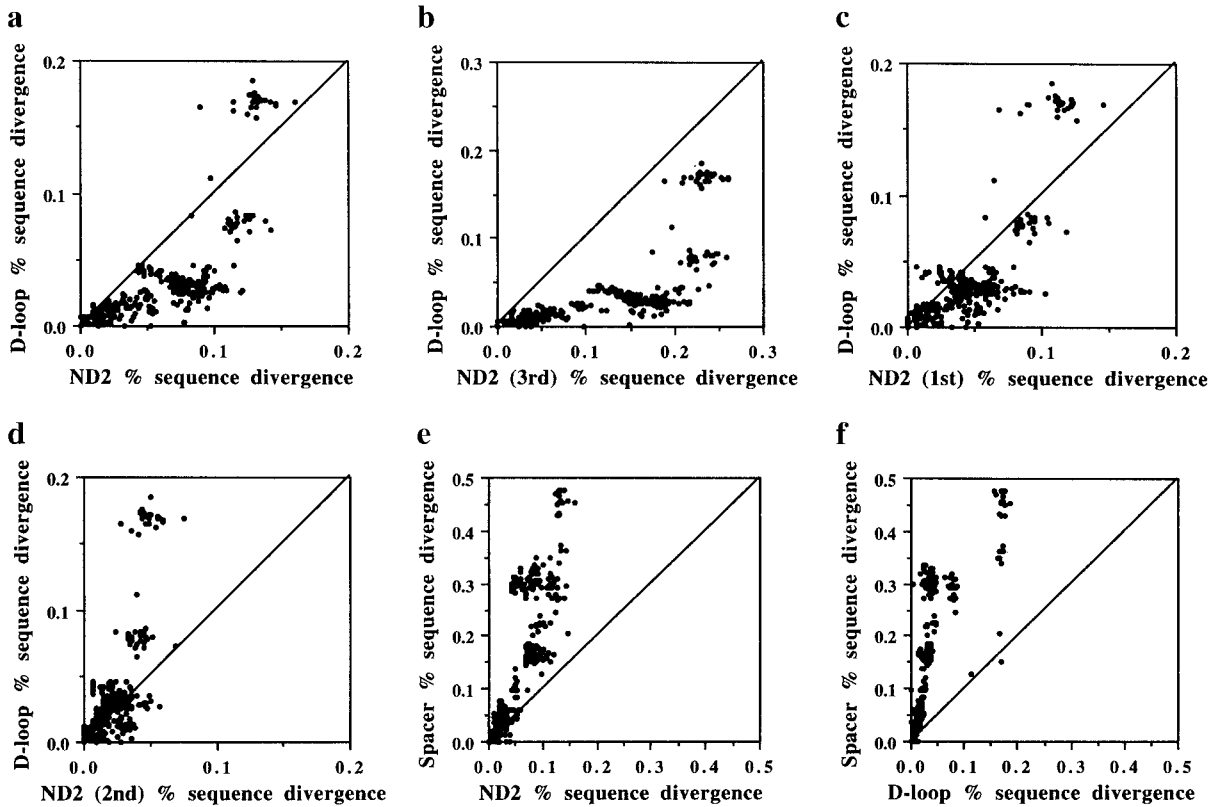


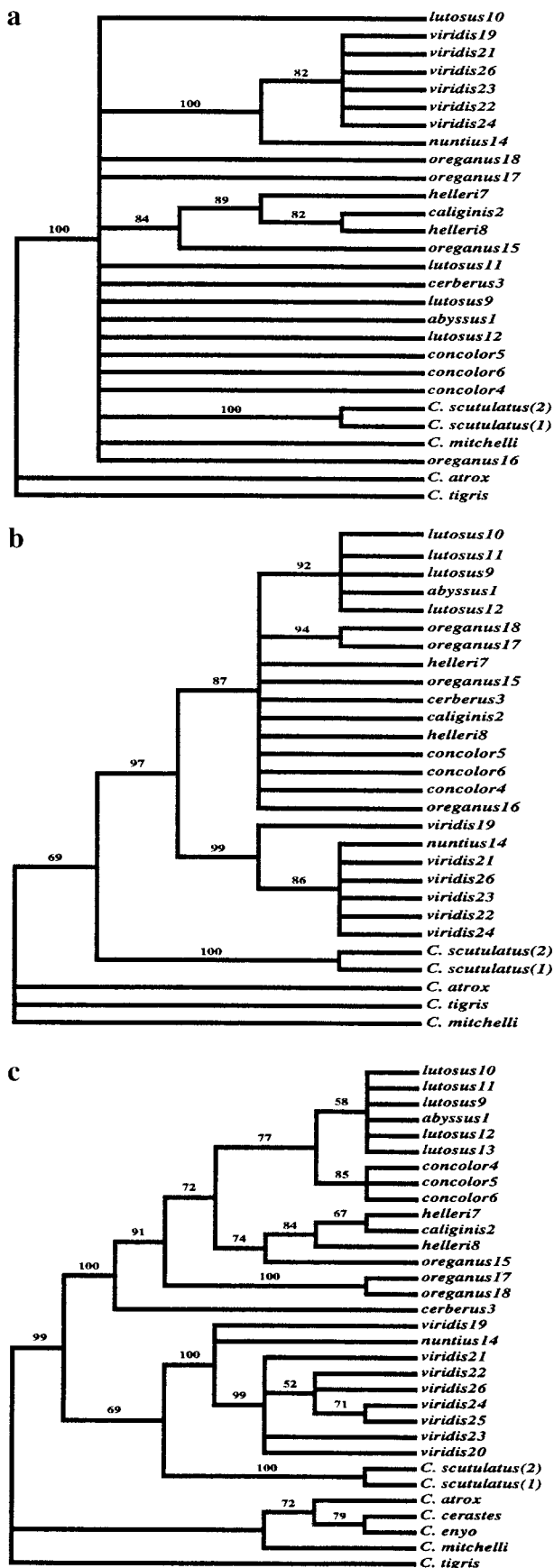
FIG. 3. Uncorrected percent sequence divergences of the D-loop region vs the overall uncorrected percent sequence divergences for the ND2 gene, and separately for each codon position of the ND2 gene. Comparisons are also made between the spacer region and ND2 and D-loop.

(403 bp), and *C. tigris* (1209 bp). The total D-loop data set (D-loop and adjacent regions) had 344 variable sites of which 151 were parsimony informative. The D-loop itself had 255 variable (25%) and 83 parsimony informative (8%) sites. A large portion of the variability in the D-loop data set was found in the 145 bp noncoding spacer region. This spacer region had 69 variable sites (48%), with 57 of them being parsimony informative (39%).

We recovered between 985 and 1046 bp of the ND2 gene and adjacent regions for 27 individuals, and recovered less for the following: #11 (679 bp), #24 (653 bp), *C. scutulatus* (1) (503 bp), *C. scutulatus* (2) (635 bp), and *C. cerastes* (702 bp). The total ND2 data set had 378 variable sites (37%), with 193 parsimony informative (19%). All of the variable characters were found in the ND2 gene—none were in the adjacent tRNAs.

For the combined analyses, using only samples for which we had both ND2 and D-loop sequences ($n = 27$), there were 314 parsimony informative characters. Uncorrected percent sequence divergences, combining all the sequence data, ranged from 0 to 7.6% for in-group taxa, 5.9 to 7.3% between *C. viridis* and *C. scutulatus*, and 9.0 to 17.1% between *C. viridis* and other outgroup taxa.

To test the relative rates of evolution of the two regions we plotted uncorrected percent sequence divergence of the ND2 gene vs the D-loop region (Fig. 3a). We found that the ND2 gene showed greater divergences among closely related individuals (i. e., members of the ingroup), whereas the D-loop region showed greater divergences among recognized species (Fig. 3a). This unusual pattern could result if some kinds of changes in ND2 occur more frequently and others less frequently than most changes in the D-loop, with only the “fast” changes in ND2 showing considerable saturation in comparisons among relatively highly divergent taxa. This conjecture is supported by further analyses. Plots of pairwise sequence divergence for the D-loop against each of the three codon positions in ND2 indicate that ND2 third positions show greater sequence differences than the D-loop for all levels of divergences (Fig. 3b), first positions show greater sequence differences at lower divergences only (Fig. 3c), and second positions have lower sequence differences than the D-loop (Fig. 3d). The plot of D-loop vs ND2 third positions also indicates saturation of the latter; ND2 third positions show much higher divergences than the D-loop for close pairs of taxa, but the divergences for the two regions become more similar for more distant pairs (Fig. 3b). In fact, saturation plots do



show that only ND2 third positions exhibit saturation (Ashton, 2001).

We also compared the sequence divergences of ND2 and D-loop with the spacer region found adjacent to the D-loop because of the high variability in this region. As expected, sequence differences in the spacer region were much higher than for either ND2 or D-loop at all levels of divergences (Figs. 3e, f). The spacer region shows strong evidence of saturation of transitions, but not transversions (not shown).

Separate Analyses

The MP analysis of the D-loop using equal weighting resulted in 7296 most parsimonious trees, the strict consensus of which is largely unresolved (Fig. 4a; tree length = 358; CI = 0.61—all reported CI values exclude uninformative characters; RI = 0.79). However, this analysis does support a monophyletic eastern clade (*C. v. viridis* and *C. v. nuntius*) and a clade of *C. v. oreganus* #15 (Monterey Co., CA; Table 1) + *C. v. helleri* + *C. v. caliginis* (Fig. 4a). The most complex model for ML analyses, GTR + I + Γ , had the highest likelihood ($-\ln L = 3298.89$) and the likelihood ratio test showed this model to be significantly better than the next most complex model ($P < 0.001$). The D-loop ML analysis recovered five trees with the same likelihood (not shown). The strict consensus of the five ML topologies does not support the monophyly of *C. viridis*, because *C. scutulatus* is the sister taxon to the eastern populations (*C. v. viridis* and *C. v. nuntius*) and *C. mitchelli* is deeply nested within *C. viridis*. Similar to the MP analysis, the ML analysis recovered a clade of the eastern populations (*C. v. viridis* and *C. v. nuntius*) and of *C. v. oreganus* #15 + *C. v. helleri* + *C. v. caliginis*. Unlike the largely unresolved MP analysis (Fig. 4a), the ML analysis recovered a clade of the western populations (*C. v. abyssus*, *C. v. caliginis*, *C. v. cerberus*, *C. v. concolor*, *C. v. helleri*, *C. v. lutosus*, and *C. v. oreganus*) with *C. v. cerberus* as the sister taxon to the remaining taxa, although as mentioned above, *C. mitchelli* is included in this group. The placement of *C. mitchelli* may be because of the relatively small amount of sequence available for this taxon. *Crotalus v. abyssus* and *C. mitchelli* are nested within *C. v. lutosus*, *C. v. caliginis* is nested within *C. v. helleri*, and *C. v. oreganus* is polyphyletic.

Maximum-parsimony analysis of the spacer region resulted in 516 most parsimonious trees, the strict

FIG. 4. Parsimony analyses of separate DNA regions: (a) Strict consensus of 7296 most parsimonious trees from equal weighting of all characters of D-loop. Bootstrap values based on 100 replicates; (b) Strict consensus of 516 most parsimonious trees from equal weighting of all characters for the spacer region. Bootstrap values based on 100 replicates; (c) Strict consensus of 480 most parsimonious trees from equal weighting of all characters for ND2. Bootstrap values based on 1000 replicates.

consensus of which is shown (Fig. 4b; tree length = 117; CI = 0.73; RI = 0.89). *Crotalus scutulatus* is weakly supported as the sister taxon to the *C. viridis* group, and an eastern and western clade are resolved within the *C. viridis* group. A clade of *C. v. nuntius* plus *C. v. viridis* is strongly supported, with *C. v. viridis* #19 (Bernalillo Co., New Mexico) as the sister taxon to an unresolved clade of *C. v. nuntius* and the remaining *C. v. viridis*. Relationships within the western clade are largely unresolved, with the exception of a clade of *C. v. lutosus* plus *C. v. abyssus*, and *C. v. oreganus* #17 and #18 as sister taxa. The HKY85 + I model was significantly better than the HKY85 model ($-\ln L = 721.19$ vs 726.30 , respectively; $P < 0.001$), but not significantly worse than the HKY85 + I + Γ model ($-\ln L = 720.15$; $P > 0.05$), for the spacer region. ML analysis of the spacer region resulted in one topology (not shown) with the only difference from the MP analysis being better resolution within the western clade.

Branch and bound parsimony analysis of the ND2 sequences, with equal weighting of all characters, resulted in 480 most parsimonious trees (Fig. 4c; tree length = 641; CI = 0.60; RI = 0.82). *Crotalus scutulatus* is weakly supported as the sister taxon to a clade of only *C. v. viridis* and *C. v. nuntius*. A monophyletic clade of *C. v. viridis* and *C. v. nuntius* is strongly supported, with the relationship of *C. v. nuntius* to *C. v. viridis* #19 (Bernalillo Co., NM) unresolved. The western clade is strongly supported as monophyletic, with *C. v. cerberus* as the sister taxon to the others. Monophyletic clades of *C. v. helleri* plus *C. v. caliginis*, *C. v. concolor*, and *C. v. lutosus* plus *C. v. abyssus* are recovered, although the latter is not strongly supported. Again, *C. v. oreganus* #15 (Monterey Co., CA) is part of the *C. v. helleri* clade. The relationships among these members of the western clade are strongly supported, with *C. v. oreganus* (minus #15) as the sister taxon to the others, then *C. v. helleri* + *C. v. caliginis* + *C. v. oreganus* (#15) as the sister taxon to *C. v. lutosus* + *C. v. abyssus* and *C. v. concolor*.

Thirty-six most parsimonious trees were found when we analyzed the ND2 data with transversions weighted five times as heavily as transitions (tree length = 1478; CI = 0.64; RI = 0.82). The strict consensus MP tree (not shown) differed from the equal weighting MP analysis in several important respects. First, *C. scutulatus* is the sister taxon to the entire *C. viridis* group, rather than just the *C. v. viridis* + *C. v. nuntius* clade. Thus, in this analysis, the *C. viridis* group is monophyletic. Also, the *C. v. lutosus* clade is paraphyletic, *C. v. concolor* is polyphyletic, and *C. v. oreganus* (#15) is not found in the *C. v. helleri* + *C. v. caliginis* clade. However, none of these differences were strongly supported.

The most complex model for ML analyses, GTR + I + Γ , had the highest likelihood ($-\ln L = 4699.21$) and the likelihood ratio test showed this model to be signifi-

cantly better than the next most complex model for the ND2 data ($P < 0.001$). The ML analysis recovered two trees (not shown) with the same relationships among the major clades as did the MP analysis with equal weighting. Both the *C. v. viridis* and *C. v. lutosus* clades were better resolved in the ML analysis.

Combined Analyses

The partition homogeneity test (500 replicates) failed to detect significant incongruence between the two data sets ($P = 0.10$), suggesting the two mtDNA regions could be combined. The combined, equally weighted, MP analysis resulted in 57 most parsimonious trees (Fig. 5a; tree length = 1009; CI = 0.65; RI = 0.84). The MP analysis of the combined data with transversions weighted five times as heavily as transitions for ND2 recovered the same strict consensus topology (12 most parsimonious trees; tree length = 1689; CI = 0.65; RI = 0.82). *Crotalus scutulatus* is strongly supported as the sister taxon to a monophyletic *C. viridis* group. Two divergent clades are present in the *C. viridis* group: a strongly supported *C. v. viridis* + *C. v. nuntius*, and a western clade of the remaining subspecies. *Crotalus v. nuntius* is weakly supported as the sister taxon to *C. v. viridis*. *Crotalus v. cerberus* is the sister taxon to the other members of the western clade. Monophyletic groups of *C. v. abyssus* nested within *C. v. lutosus*, *C. v. concolor*, and *C. v. helleri* with *C. v. caliginis* nested within the latter are strongly supported. *Crotalus v. oreganus* is paraphyletic based on #15 (Monterey Co., CA).

The ML GTR + I + Γ model best explained the combined data ($-\ln L = 8688.81$; $P < 0.001$) and the topology of the single most likely tree (Fig. 5b) was nearly identical to the MP tree. The only difference between the two trees was that in the ML analysis *C. v. viridis* #22 and #23 (both from Scotts Bluff Co., NE) are sister taxa, whereas in the MP analysis their relationship to other *C. v. viridis* is unresolved.

Comparisons to Previous Hypotheses

The phylogenetic hypothesis that we consider our best estimate of the true relationships is based on the combined analyses (Fig. 5). Shimodaira-Hasegawa tests indicate that our phylogenetic hypothesis is significantly different from Klauber's (1972) morphology-based tree ($-\ln L = 5183.48$ vs 5217.29 , respectively; $P < 0.01$) and Pook *et al.*'s (2000) mitochondrial DNA tree ($-\ln L = 5465.78$ vs 5500.21 , respectively; $P < 0.001$). The comparison of our tree with Foote and MacMahon's (1977) tree is suggestive of a difference as well ($-\ln L = 4825.59$ vs 4829.26 , respectively; $P = 0.14$).

DISCUSSION

The analyses that resulted in the best resolution, and were based on the greatest amount of data, were

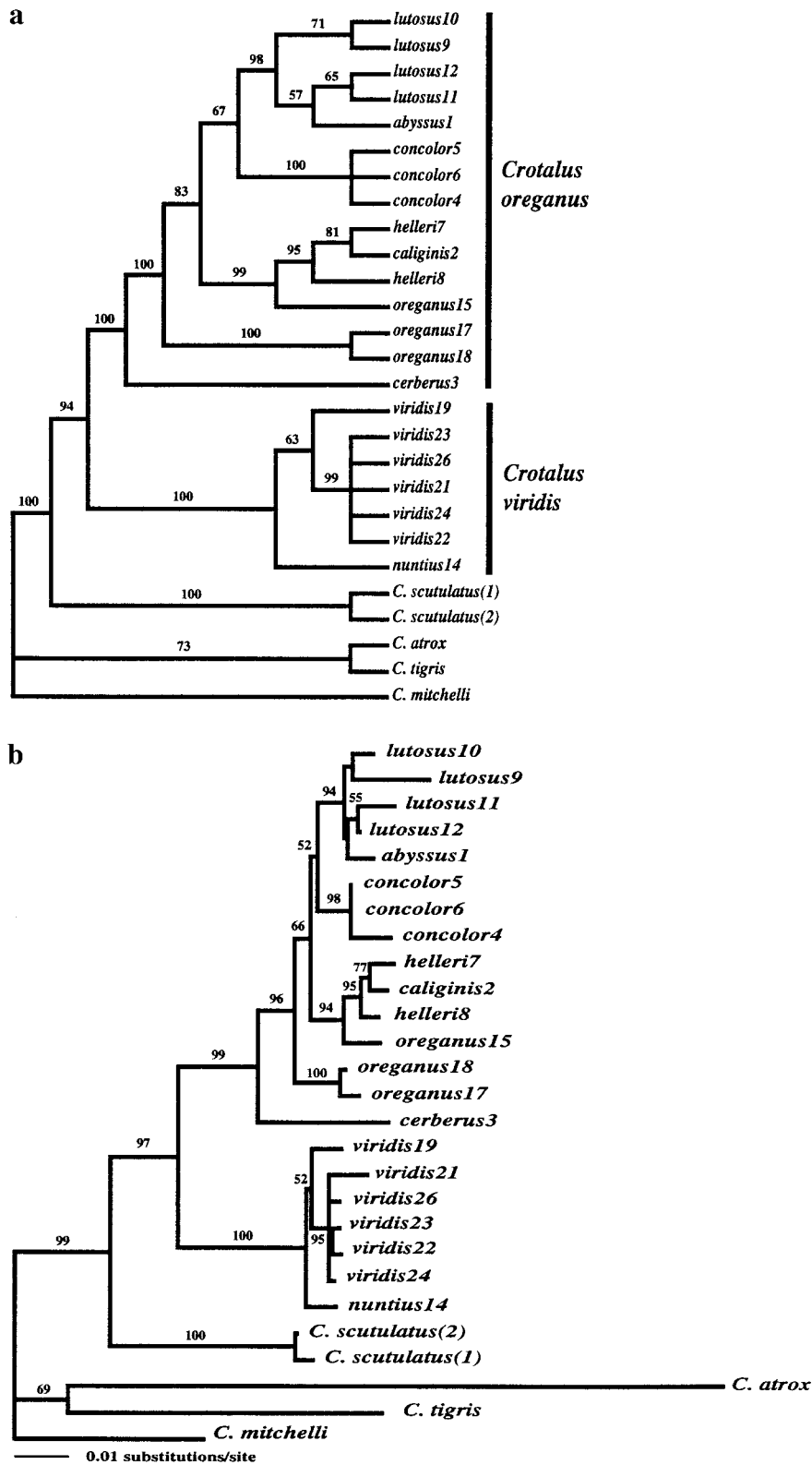


FIG. 5. Phylogenetic analyses of the combined data for only taxa with data from both DNA regions: (a) Strict consensus of 57 most parsimonious trees based on equal weighting of all characters. Bootstrap values based on 1000 replicates. Proposed taxonomy indicated; (b) ML phylogram using the best-fitting model (GTR + I + Γ). Branch lengths are proportional to amounts of change. Bootstrap values based on 500 replicates.

the combined analyses (Fig. 5). These combined analyses had similar RI values, fewer numbers of most parsimonious trees, better resolution, and stronger bootstrap support than the separate analyses (Fig. 4). The equally weighted analyses are preferred over the differentially weighted analyses because differential weighting decreased resolution and support. Thus, we consider the combined ML and MP analyses (Fig. 5) the best hypotheses of relationships among populations of the *C. viridis* group.

Populations of *C. viridis* formed a monophyletic group with *C. scutulatus* as the sister taxon (Fig. 5). There were two strongly divergent clades within *C. viridis*, one consisting of the subspecies *C. v. viridis* and *C. v. nuntius*, and the other of the remaining subspecies. These two clades correspond roughly to the eastern and western populations of *C. viridis* (Fig. 1). In the eastern clade, *C. v. nuntius* is weakly supported as the sister taxon to populations of *C. v. viridis*. Relationships within the western clade are generally well resolved with *C. v. cerberus* as the sister taxon to the remaining groups. The other western groups include *C. v. oregonus*, *C. v. helleri*, *C. v. concolor*, and *C. v. lutosus*. The population on South Coronado Island, previously named *C. v. caliginis* (Klauber, 1949), is nested within the *C. v. helleri* clade. The Grand Canyon rattlesnake, *C. v. abyssus* (Klauber, 1930), is nested within the *C. v. lutosus* clade. *Crotalus v. oregonus* (minus #15) is generally the sister taxon to all remaining groups, *C. v. helleri* + *C. v. caliginis* is the sister group to *C. v. concolor* and *C. v. lutosus* + *C. v. abyssus*, and the latter two are sister groups. *Crotalus v. oregonus* #15 (Monterey Co., CA) consistently clusters with *C. v. helleri* even though morphologically it is identified as *C. v. oregonus*. This individual is from just north of the broad intergrade zone between *C. v. oregonus* and *C. v. helleri* (Schneider, 1986), and therefore probably is further evidence for interbreeding between these two groups.

Our topology fits our data significantly better than that proposed by Klauber (1972). The only relationship that our hypothesis (Fig. 5) shares with that of Klauber (1972) is the recognition of *C. v. viridis* and *C. v. nuntius* as sister taxa (Fig. 2a). Our hypothesis, though it does not fit our data significantly better than that of Foote and MacMahon (1977), shares no relationships with their tree (Fig. 2b). Further, our hypothesis fits our data significantly better than the one presented by Pook *et al.* (2000), the only other hypothesis based on molecular sequence data (Fig. 2c). The main differences between our hypothesis and theirs (Fig. 2c) are the relationships among members of the western clade. Our results have higher levels of bootstrap support (contrast our Fig. 5 with Fig. 5 in Pook *et al.*, 2000) and are more concordant with the geographic distribution and morphological similarity of the clades (see Klauber (1972) and Stebbins (1985)). These differences in our

phylogenetic analyses may be due to differences in rates of evolution of the genes used and the numbers of characters used in each analysis. Our combined dataset contained 314 parsimony informative characters, whereas Pook *et al.*'s (2000) combined analysis was based on 122 parsimony informative characters.

One potential problem with using genes to reconstruct phylogenies is the possible conflict between gene trees and species trees (Avice, 1989, 1994). However, this conflict may not be as widespread as once suggested (Brower *et al.*, 1996). Our analyses and those of Pook *et al.* (2000) are based on mitochondrial genes, which estimate the mitochondrial DNA phylogeny. Obviously it is important for future analyses to use independent data sets, such as nuclear DNA and morphology, to verify that the tree generated by this analysis correctly represents the organismal phylogeny.

Taxonomic Recommendations

An evolutionary species is "a single lineage of ancestral descendant populations of organisms which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate" (Wiley 1978, p. 18). We prefer the evolutionary species concept over other species concepts because it represents the most general and universal notion of a species (de Queiroz, 1998). The evidence that we used to identify species include (1) exclusivity (monophyly of the collection of DNA sequences representing the group), (2) geographic isolation, (3) differentiation of characters other than the mtDNA sequences, and (4) degree of sequence differentiation. However, to be recognized as a species, a group does not have to meet all of these criteria (e.g., sympatric populations that are exclusive and have high levels of character differentiation). We conservatively retain the ranks of subspecies to designate potentially unique entities because our data do not permit full evaluation of their validity.

Regardless of our method of analysis (separate or combined, different weighting schemes, MP or ML), two divergent clades were always strongly supported: *C. v. viridis* + *C. v. nuntius* and the remaining groups (Figs. 4 and 5). That result, combined with observed levels of sequence divergence between groups, suggests the recognition of two evolutionary species from the *C. viridis* group. First, we propose that *C. viridis* only refer to the clade of *C. v. viridis* and *C. v. nuntius* (Fig. 5). Minimum uncorrected percent sequence divergence of this clade from other members of the *C. viridis* group (5.1%) is comparable to that observed between *C. scutulatus* and any member of the *C. viridis* group (5.9%). These sequence divergences are comparable to those observed between recognized species of snakes (de Queiroz and Lawson, 1994; Kraus *et al.*, 1996; Zamudio and Greene, 1997; Parkinson, 1999). When Klauber (1935) first described *C. v. nuntius*, he recognized its similarity to *C. v. viridis* and noted that

nuntius may represent part of a cline in *C. v. viridis*. The main difference between the two is in body size (*nuntius* is smaller), number of scale rows (a character that is correlated with body size), and possibly coloration (Klauber, 1935). Our results generally place *C. v. nuntius* as the sister group to the remaining members of the *C. v. viridis* clade, however, this position is only marginally supported (Figs. 4 and 5), and is not recovered in the ML analysis of ND2 or ML and MP (Fig. 4b) analyses of the spacer region (see Ashton (2001)). More sampling of *C. v. nuntius* and *C. v. viridis* from the southern portion of its range is needed to assess the validity of *C. v. nuntius* as a distinct form.

The second species that we recognize is *Crotalus oreganus*, with the tentative recognition of seven subspecies: *C. o. cerberus*, *C. o. oreganus*, *C. o. helleri*, *C. o. caliginis*, *C. o. concolor*, *C. o. lutosus*, and *C. o. abyssus* (Fig. 5). The name *C. oreganus* is based on the oldest name for any member of this clade (see Klauber, 1949, 1972). The exclusivity of the various subspecific lineages within this species is questionable, based on different analyses of our dataset (Figs. 4 and 5) and inadequate sampling for some subspecies. Thus, at this time, we do not think any deserve recognition as separate evolutionary species. In particular, *C. o. oreganus* and *C. o. helleri* are known to interbreed extensively (Schneider, 1986), and an individual that was designated *C. o. oreganus* (sample #15) based on external morphology was always part of the *C. o. helleri* clade in our analyses (Figs. 4 and 5).

These two species, *C. viridis* and *C. oreganus*, differ in several morphological characteristics. For instance, *C. viridis* have subrectangular dorsal blotches with a white border, whereas the shape of the dorsal blotches of *C. oreganus* are more variable and are only bordered with white as juveniles in some populations (Klauber, 1972). In addition, *C. viridis* have preocular and postocular white stripes that are one scale row wide. In contrast, *C. oreganus* have white preocular stripes that are several scale rows wide and postocular stripes that are either faint or missing (Klauber, 1972).

It is possible that *C. o. cerberus* may be an evolutionary species (see Klauber, 1949 for description of this taxon). This taxon was always positioned as the sister group to all remaining members of the western clade (Figs. 4 and 5), and this relationship is supported by Pook *et al.*'s analysis (2000). Furthermore, *C. o. cerberus* is moderately well diverged from other members of the western clade (3.5% minimum sequence divergence) and there is no evidence of intergradation with any other subspecies.

Subsequent analyses may show that at least some members of *C. oreganus* and *C. viridis* should be elevated to species status and that others do not represent unique entities. However, based on the available evidence, we conservatively recognize two evolutionary

species and retain all subspecies from within what was formerly known as *C. viridis*.

Historical Biogeography

Pook *et al.* (2000) recently reviewed the historical biogeography of the *C. viridis* group. Rather than reiterate all of the details, we will discuss new information based on our analyses, supplement certain explanations, and give detailed treatment of areas of conflict between our studies. We agree with Pook *et al.*'s (2000) historical biogeographical interpretations for the split between eastern and western clades, and of *C. o. cerberus* from other members of the western clade. The results of our phylogenetic analyses, and those of Pook *et al.* (2000), suggest a southern origin for this group, followed by northward dispersal of the eastern clade (*C. viridis*), and westward and northward dispersal of the western clade (*C. oreganus*).

Our results were consistent with the recognition of each of the subspecies in the western clade, with the exception of *C. o. abyssus* and *C. o. caliginis* which may cause paraphyly of *C. o. lutosus* and *C. o. helleri*, respectively. This is not surprising because these subspecies all differ in habitat preference, morphology, and other characteristics—with the exception of the very similar *C. o. oreganus* and *C. o. helleri* clades (Klauber, 1972). These differences among groups probably reflect isolation during a period of increased aridification, and concomitant habitat changes throughout the western United States, as suggested by Pook *et al.* (2000). Our results differ from Pook *et al.* (2000) with respect to the relationships among members of the western clade (compare Fig. 2c with Fig. 5). Based on our analyses, it appears that members of the western clade split from *C. o. cerberus* and dispersed to the Pacific Coast. The ancestors of *C. o. lutosus* + *C. o. abyssus* + *C. o. concolor* then split from the Pacific populations, possibly initiated by the uprising of the Sierra Nevada and Cascade Mountains (late Miocene to early Pliocene; Storer and Usinger, 1963; Potts and Behrensmeyer, 1992; but see House *et al.*, 1998), spread throughout the Great Basin, then gave rise to *C. o. concolor* in the drainage basin of the Green and Colorado Rivers and *C. o. abyssus* in the Grand Canyon. This somewhat dispersalist scenario suggests secondary contact between *C. viridis* and *C. oreganus* in northern Arizona, southwestern and northwestern Colorado, and southeastern Utah. Our results do not allow finer resolution of the historical biogeography of members of the western clade because only a few individuals from each clade were included.

In contrast to the western clade, we found low levels of sequence divergence among populations of the *C. v. viridis* and *C. v. nuntius* clade. It appears that the southern populations, those from Arizona and New Mexico, are sister to the others (Figs. 4 and 5), but further resolution is lacking. Levels of sequence diver-

gence among the northern populations (0.3–1%) are surprisingly low considering their presence in the northern part of the range at least 4.5 million years ago (Holman, 2000). Zamudio *et al.* (1997) found low levels of sequence divergence among populations of short-horned lizards from the same region and suggested that this may reflect recolonization after glacial retreat in the northern part of the range about 18,000 years ago. Another possible explanation for the low levels of sequence divergence in the northern Great Plains region is current gene flow among populations with subsequent extinction of some mitochondrial lineages.

Comparisons of mtDNA Regions

Phylogeographic studies of snakes are rare, and most have used the mitochondrial genes *cyt b* and/or ND4 (Pook *et al.*, 2000; Rodriguez-Robles and De Jesus-Escobar, 2000; Rodriguez-Robles *et al.*, 1999; Zamudio and Greene, 1997). Studies of other vertebrates have often used the D-loop region because of its extreme variability (e.g., Barratt *et al.*, 1999; Doukakis *et al.*, 1999; Nesbo *et al.*, 1999; Steinfartz *et al.*, 2000; Terry *et al.*, 2000; Vila *et al.*, 1997). This study is one of the first to use the D-loop region to construct phylogenetic relationships for snakes.

Unlike in other vertebrate groups, we found that the D-loop region shows less sequence divergence than the protein coding gene, ND2, for recent divergences (Fig. 3a). Yet, the D-loop displayed greater sequence divergence than ND2 for more ancient divergences (Fig. 3a). Saturation plots (Ashton, 2001) and comparisons of the D-loop with the three codon positions in ND2 (Fig. 3) suggest that the above pattern results from ND2 third position transitions occurring much more frequently than changes in the D-loop, while other changes in ND2 occur as or less frequently than changes in the D-loop. Because it is a non-protein coding region, one would expect changes in the D-loop to occur at a rate similar to third position transitions in a protein coding gene. The fact that the D-loop in *Crotalus* evolves much slower suggests either a lower mutation rate than ND2 or some unknown selective constraint on the D-loop. When sequence divergences are compared between ND2, D-loop, and the spacer region, it is shown that the spacer region has greater divergences for all comparisons (Figs. 3e, f). That this relationship is found for the D-loop in comparison to the non-coding, apparently non-functional spacer region (Kumazawa *et al.*, 1996) further suggests that, despite being non-protein coding, the D-loop region is constrained in some manner. Of course, it should be noted that different portions of the D-loop can evolve at different rates (see Burbrink *et al.* (2000) for discussion with respect to snakes).

Contrary to our initial expectations, our phylogenetic analyses suggest that ND2 may be better than the D-loop for intraspecific analyses in snakes. In particular, levels of sequence divergence were lower for

the D-loop relative to ND2 (Fig. 3), the number of parsimony informative characters was less for the D-loop than for ND2 (83 and 193, respectively), and parsimony analyses of the D-loop were less resolved than for ND2 (Figs. 4a, c). These results are probably due to the rapid rate of evolution of third positions of ND2, which provide abundant data to infer intraspecific relationships despite evidence of saturation. To evaluate this possibility further, we ran equally weighted MP analyses of only third positions of the ND2 gene and compared the results with analyses based on first and second positions of the ND2 gene. The third position data had 125 parsimony informative characters (36%) and resulted in 2812 most parsimonious trees with 15 clades resolved. In contrast, only 68 characters (10%) were parsimony informative in the analyses using first and second positions. This analysis resulted in 56,956 most parsimonious trees with 11 clades resolved. The number of parsimony informative characters, clades resolved, and most parsimonious trees all support the hypothesis that most of the phylogenetic information for the ND2 dataset comes from changes at the third position.

In summary, we found that for snakes, the ND2 gene may better resolve intraspecific relationships than the D-loop. The D-loop may be evolving at a rate at all positions that is useful for inferring interspecific relationships, whereas the rates of change, while informative, are lower in the D-loop at small divergences than for the ND2 gene because of the rapid rate of change at ND2 third positions. A surprising amount of the phylogenetic information for our analyses came from the 145 bp spacer region found adjacent to the D-loop, and the very high rate of evolution in this region may make it extremely useful for evaluating relationships among recently diverged populations. However, this spacer region thus far has only been found in *Crotalus* (Kumazawa *et al.*, 1996). Our results are in contrast to most studies that have found the ND2 gene to be a very useful interspecific marker and the D-loop region a highly variable marker with great utility for intraspecific analyses (Johnson and Lanyon, 1999; Omland *et al.*, 1999; Avise, 2000).

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