PHYLOGENY OF THE RATTLESNAKES (*CROTALUS* AND *SISTRURUS*) INFERRED FROM SEQUENCES OF FIVE MITOCHONDRIAL DNA GENES

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ABSTRACT: An investigation of the genealogical relationships among 30 species of rattlesnakes, including all three species of *Sistrurus* and 27 species of *Crotalus*, used sequences of the mitochondrial DNA cytochrome *b*, ND5, 12S RNA, $tRNA^{Val}$, and 16S RNA genes. Two species of *Agkistrodon* were used as outgroup taxa. The data were analyzed using maximum parsimony methods. Significant character covariance was located using nodal permutation tail probabilities. Support for clades was evaluated using jackknife monophyly index, bootstrapping, decay index, and successive approximations. Analysis of the total combined unweighted data yielded two most-parsimonious trees. A preferred tree, which was not the most parsimonious explanation of the sequence data, was obtained by weighting transversions three times transitions, and using *S. catenatus* and *S. miliarius* as a functional outgroup. Our analysis failed to find support for any species group as currently defined. Evidence for the paraphyly of *Sistrurus* was strong and *S. ravus* is considered to be a species of *Crotalus*. The *C. triseriatus* group, composed of largely central and southern Mexican montane species, was paraphyletic and contained two groups of taxa, one of which includes the Sidewinder, *C. cerastes*. The *C. viridis* group consisted of *C. scutulatus*, *C. viridis* and *C. horridus*, the first two clearly sister species. The Baja California Rattlesnake, *C. enyo*, was the sister species of the Neotropical Rattlesnake, *C. durissus*, and immediate derivatives.

INTRODUCTION

Rattlesnakes are New World pitvipers (Viperidae: Crotalinae) that are distributed among two genera, *Sistrurus* (with three species) and *Crotalus* with at least 29 species (and probably more). Gloyd (1940) defined four species groups. These include largely montane, relatively small species (*C. triseriatus* group), and large-bodied lowland species including the *C. atrox* group, the *C. durissus* group, and the *C. viridis* group. Klauber (1972) did not recognize species groups because of the inability to apply formal names to them without splitting the genus *Crotalus* into multiple genera. Nevertheless, Klauber's phylogeny largely corresponds to Gloyd's species groups (Fig. 1). Monophyly of the rattlesnakes has never been doubted, as affirmed by the presence of a rattle.

Phylogenetic relationships among the taxa are controversial. At the generic level, Brattstrom (1964), Foote and MacMahon (1977), Stille (1987), McCranie (1988), and Parkinson (1999) have questioned the validity and desirability of recognizing the genus *Sistrurus*. This genus differs from *Crotalus* by configuration of the vertebrae (Holman, 1964), shape of the rattle (Zimmerman and Pope, 1948), nature of the chromosomes (Zimmerman and Kilpatrick, 1973), size and shape of the rostral scale (Dorcas, 1992), and distance between the lymphapophyses (Burger, 1971). Hemipenal structure and dorsal head scale pattern (Gloyd, 1940; Klauber 1956, 1972) have been the primary characters used to separate the two genera. However, these characters have been interpreted as being plesiotypic (Gloyd, 1940; Klauber, 1956, 1972; Johnson, 1956; Brattstrom, 1964; Burger, 1971), and hence not indicative of monophyly. In some cases, species were erroneously assumed to have a given anatomy and concomitant plesiotypic state (McCranie, 1988). Relationships among species groups are bounded in controversy, as detailed later.

Most previous genealogical hypotheses were based on singular, subjectively weighted anatomical features, and intuition. Unfortunately, anatomical attributes among rattlesnakes, such as the number of ventral scales and body blotches, tend to vary significantly within taxa to the extent that there is considerable overlap among taxa (Klauber, 1972). Morphometric and frequency data in general may be unreliable estimators of phylogeny because of unrealistic constraints on how these characters must evolve to be reasonably informative; at cladogenic events, one lineage must stop evolving and all change in the other lineage must be unidirectional, either increasing or decreasing (Murphy and Doyle, 1998). Previous anatomically based phylogenetic studies have failed to identify a sufficient number of unambiguously informative characters to fully resolve relationships based on cladistic methodology (e.g., Stille, 1987). Consequently, molecular data form an attractive alternative to the more traditional anatomical characters (McCranie, 1988).

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Knight et al. (1993) and Parkinson (1999) used mitochondrial DNA sequence data to investigate the phylogenetic relationships of pitvipers, including a few representative species of rattlesnakes. Both studies suggested that recognition of *Sistrurus* was not justified. In the former case, the authors found it necessary to propose a weighting scheme in order to obtain some level of congruence with anatomical data. However, most nodes were not supported by significantly covaried data, i.e., a randomization of their data produced shorter tree lengths than those obtained by a maximum parsimony analysis (Fu and Murphy, 1999). Surprisingly, Parkinson (1999) discovered that *Sistrurus* branched off from within *Crotalus*. This drastic departure from previous thought united *Sistrurus* with *C. adamanteus* and *C. tigris*, and, in turn, *C. molossus* and *C. atrox* was their sister group. As with Knight et al. (1993), this arrangement was not supported by significantly covaried data. Parkinson also associated *C. adamanteus* with *C. tigris* and not *C. atrox* as all other studies have suggested (Gloyd, 1940; Klauber 1956, 1972; Brattstrom, 1964; Foote and MacMahon, 1977). Consequently, Parkinson's conclusion about paraphyly in *Crotalus* was suspect. Parkinson et al. (this volume) used a larger data set and resolved both *Crotalus* and *Sistrurus* as monophyletic genera. Yet, the two species of diamondbacked rattlesnakes (*C. adamanteus* and *C. atrox*) were not resolved as sister taxa.

Pook et al. (2000) performed a detailed evaluation of variation within a supposed species of *Crotalus*. They found rampant paraphyly among the nine subspecies of the Western Rattlesnake (*C. viridis*), or that some subspecies were merely local variants within larger clades. They suggested that *C. viridis* might be split into two species—east and west of the Continental Divide—once additional data are gathered from anatomy and nuclear gene sequences (for a proposed resolution to this group, see Douglas et al., this volume). The analysis of Wüster et al. (this volume) points to similar problems within the *C. durissus* complex.

We investigated the phylogenetic relationships of rattlesnakes based on homologous sequences for 2,945 base pairs from the mitochondrial DNA genes 12S rRNA, 16S rRNA, $tRNA^{Val}$, cytochrome *b* (cyt-*b*), and ND5. We evaluated 27 of 29 (up to 32) species of *Crotalus* and all three species of *Sistrurus*. The North American Copperhead (*Agkistrodon contortrix)*, Cottonmouth (*A. piscivorus)* and an Asian mamushi (*Gloydius ussuriensis*) were used as outgroups (Parkinson, 1999). Finally, the mix of slowly evolving (12S and 16S rRNA genes) and rapidly evolving genes (cyt-*b*, ND5, $tRNA^{Val}$) makes a particularly valuable combination for investigating divergence among closely related species (Hillis et al., 1996b; Hedges et al., 1991; Helm-Bychowski and Cracraft, 1993; Martin and Palumbi, 1993).

MATERIALS AND METHODS

Specimens Examined

Our survey includes all long-recognized species of rattlesnakes except for *C. lannomi* and *C. stejnegeri* (Table 1). We recognize *C. aquilus* as a species distinct from *C. triseriatus* (Dorcas, 1992) and maintain recognition of *C. vegrandis* and *C. unicolor* (*sensu* Klauber,1972), as opposed to Campbell and Lamar (1989), as working hypotheses. Recently, Grismer (1999) elevated four insular subspecies of rattlesnakes in the Gulf of California to species status (*C. mitchellii angelensis*,*C. mitchellii muertensis*, *C. molossus estebanensis*, and *C. ruber lorenzoensis*). Murphy and Aguirre (2002 b) suggest that recognition of *C. muertensis* is unjustified. Regardless, the absence of these Baja Californian species in our study does not pose a problem because their relationships are without question. Below we use *C. exsul* in quotes; this insular species was synonymized with *C. ruber* (Grismer et al., 1994; Murphy et al., 1995) and the latter has been given priority by the International Commission of Zoological Nomenclature.

Experimental Protocol

A large continuous fragment from the 12S and 16S rRNA mitochondrial genes were sequenced, including 502 base pairs (bp) of the 12S gene, 71 bp of transfer RNA ($tRNA^{Val}$) and 1,330 nucleotide sites from 16S rRNA. We also obtained up to 565 bp from cyt-*b*, and for most species, up to 477 bp from NADH dehydrogenase (cyt-*c* reductase) subunit 5 (ND5) protein-encoding genes.

Standard phenol/chloroform methods were used to extract DNA from muscle or liver tissues, or shed skin from *C. transversus* (Hillis et al., 1996a; Palumbi, 1996). Polymerase Chain Reaction (PCR; Saiki et al., 1988) was used for amplifying the DNA sample, and performed on a DNA Engine, PT200 (MJ Research Inc.); parameters and settings followed Palumbi (1996). Primers used in PCR and sequencing are listed in Table 2. The fragment between primers L2510 and H3060 was sequenced using Autoload Solid Phase Sequencing Kit (Pharmacia) and an ALF automated

Table 1. Species, localities, and tissue voucher numbers for specimens of rattlesnakes and outgroup taxa used in this study. Common names for Mexican species are from Liner (1994).

Species	Common name	Museum number[1]	Locality
Crotalus adamanteus	Eastern Diamond-backed Rattlesnake	ROM 18130, 18131, ROM-FC 345	Commercially purchased
Crotalus aquilus	Queretaran Dusky Rattlesnake	ROM 18117:	SLP: Mexico, San Luis Potosi
Crotalus atrox	Western Diamond-backed Rattlesnake	ROM 18144:	California, Riverside Co.
		ROM 18149:	Texas, Val Verde Co.
		ROM 18148:	Texas, Terrel Co.
		ROM 18224:	Baja California, Isla Santa Cruz
Crotalus basiliscus	Mexican West Coast Rattlesnake	ROM 18188:	Mexico, Nayarit
Crotalus catalinensis	Rattleless Rattlesnake	ROM 18250, BYU 34641-42:	Mexico, Baja California Sur, Isla Santa Catalina
Crotalus cerastes	Sidewinder	ROM-FC 2099:	(no collecting data)
		ROM 19745:	California, Riverside Co.
Crotalus enyo	Baja California Rattlesnake	ROM-FC 441, ROM 13648:	Mexico, Baja California Sur
Crotalus durissus	Neotropical Rattlesnake	ROM 18138	Venezuela
Crotalus "exsul"	Cedros Island Diamond Rattlesnake	BYU 34753-54:	Baja California, Isla de Cedros
Crotalus horridus	Timber Rattlesnake	ROM 18132-33: UTA R-14697:	New York; Arkansas
Crotalus intermedius	Mexican Smallhead Rattlesnake	ROM-FC 223: ROM 18164:	Mexico, Veracruz
Crotalus lepidus klauberi	Banded Rock Rattlesnake	ROM 18128:	Mexico, Chihuahua
Crotalus mitchellii	Speckled Rattlesnake	ROM 18178:	California, Imperial Co.
Crotalus molossus	Black-tailed Rattlesnake	ROM 18141-42:	Mexico, Veracruz
Crotalus polystictus	Mexican Lancehead Rattlesnake	ROM-FC 263, ROM 18139:	Mexico, D. F.
Crotalus pricei	Twin-spotted Rattlesnake	ROM-FC 2144, ROM 18158:	Mexico, Nuevo Leon
Crotalus pusillus	Tancitaran Dusky Rattlesnake	ROM-FC 271:	Mexico, Michoacan (voucher sent to Mexico)
Crotalus ruber	Red Diamond Rattlesnake	ROM 18197-98, 18207:	California, Riverside Co.
Crotalus scutulatus	Mojave Rattlesnake	ROM 18210, 18218:	Arizona, Mojave Co.
Crotalus tigris	Tiger Rattlesnake	ROM 18167-68, 18171:	Mexico, Sonora
Crotalus tortugensis	Tortuga Island Rattlesnake	ROM 18192, 18195:	Mexico, Baja California Sur, Isla Tortuga
Crotalus transversus	Cross-banded Mountain Rattlesnake	KZ-shed skin:	Mexico, specific locality unknown
Crotalus triseriatus	Mexican Dusky Rattlesnake	LG: ROM 18114:	Mexico, D. F., Llano Grande
		Xo: ROM 18120:	Mexico, D. F., Xochomiko
		To: ROM 18121:	Mexico, D. F., Toluca
Crotalus unicolor	Aruba Island Rattlesnake	ROM 18150:	Aruba Island (captive born)
Crotalus vegrandis	Urocoan Rattlesnake	ROM 18261:	Venezuela (purchased from Brazil)
Crotalus viridis	Western Rattlesnake	ROM 19656:	California, Los Angeles Co.
Crotalus willardi	Ridge-nosed Rattlesnake	ROM 18183, 18185,	Mexico, Sonora
		ROM-FC 363: KZ 413:	Arizona, Santa Cruz Co.
		HWG 2575:	Arizona, Cochise Co.
Sistrurus catenatus	Massasauga	ROM-FC 243, 245:	Canada, Ontario Province
Sistrurus miliarius	Pygmy Rattlesnake	ROM 18232, 19834, ROM-FC 2032:	Florida, (commercially purchased)
Sistrurus ravus	Mexican Pygmy Rattlesnake	ROM 18242:	Mexico, D. F.
Agkistrodon contortrix	Copperhead	ROM 18230, 2172-3 ROM-FC 252:	(commercially purchased)
Agkistrodon piscivorus	Cottonmouth	ROM-FC 5599:	(commercially purchased)
Gloydius ussuriensis	Mamushi	ROM 20459:	P. R. China, Jilin Prov.

[1]Museum voucher specimens are deposited either in the preserved herpetological collection of the Royal Ontario Museum (ROM), ROM frozen tissue collections (ROM-FC), or Brigham Young University (BYU). KZ and HWG numbers refer to the tissue collection of Kelly Zamudio and Harry Greene (Cornell University). Precise locality data are available from the respective institutions. For ROM-FC tissue collections listed below, the voucher specimens were lost in shipment.

Table 2. Primers sequences used in this study for amplifying mitochondrial gene sequences from rattlesnakes.

Human position[1]	Gene	Sequence	Reference
L1091	12S rRNA	5' CAA ACT GGG ATT AGA TAC CCC ACT AT 3'	Kocher et al. 1989
H1478	12S rRNA	5' AGG GTG ACG GGC GGT GTG T 3'	Kocher et al. 1989
H1497	12S rRNA	5' ACA CAC CGC CCG TCA CCC TC 3'	This study[2]
L1921	16S rRNA	5' CCC GAA ACC AAA CGA GCA A 3'	This study
H1990	16S rRNA	5' CCA GCT ATC ACC AAG TTC GGT AGG CTT TTC 3'	This study
L2510	16S rRNA	5' CCG ACT GTT TAC CAA AAA CAT 3'	This study[3]
H2568	16S rRNA	5' CTA CCT TTG CAC GGT TAG GAT ACC GCG GC 3'	This study
H3060	16S rRNA	5' CCG GAT CCC CGG CCG GTC TGA ACT CAG ATC ACG 3'	Palumbi (1996)
L12301	$tRNA^{Leu}$	5' AGG AGC AAT CCG TTG GTC TTA GG 3'	D. Marshall (pers. comm.)
L12321	$tRNA^{Leu}$	5' CGC CAC AAC TCT TGG TGC AA 3'	This study
H12766	ND5	5' GAC ATG ATT CCT ACT CCT TCT CA 3'	D. Marshall (pers. comm.)
L14841	cyt-*b*	5' CCA TCC AAC ATC TCA GCA TGA TGA AA 3'	Kocher et al. 1989
L14838	cyt-*b*	5' GCT TCC ATC CAA CAT CTC AGC ATG ATG 3'	W. Wüster (pers. comm.)
H15149	cyt-*b*	5' GCC CCT CAG AAT GAT ATT TGT CCT CA 3'	Kocher et al. 1989
H15149	cyt-*b*	5' CCC TCA GAA TGA TAT TTG TCC TCA 3'	W. Wüster (pers. comm.)
H15488	cyt-*b*	5' TTG CTG GGG TGA AGT TTT CTG GGT C 3'	Birt et al. (1992)
H15555	cyt-*b*	5' GGC AAA TAG GAA GTA TCA TTC TG 3'	W. Wüster (pers. comm.)
H15407	cyt-*b*	5' TTG TAG GAG TGG TAG GGG TG 3'	This study

[1]H and L designate heavy- and light-strand primers, respectively. Numbers refer to the 3' ends, which correspond to the position in the human mitochondrial genome (Anderson et al., 1981) for convenience. [2]Complementary of H1478. [3]Modified from Palumbi (1996).

DNA sequencer (Pharmacia). Sequencing of other fragments was conducted using ^{33}P terminator cycle sequencing kits (Amersham). Protocols followed Hillis et al. (1996a) and manufacturer's recommendations, with minor modification.

Sequence Analysis

Clustal W (version 1.6, Thompson et al., 1994) was used to initially align the 12S through 16S rRNA gene sequences. The sequences were subsequently optimized by eye using MacClade (Ver. 3.04; Maddison and Maddison, 1992) with maximum parsimony as a criterion for accepting alternative alignments. Parsimony analyses used PAUP* (Ver. 4.02b; Swofford, 2000). Missing data were coded as such. Nucleotide ratios were examined using MacClade.

Only potentially cladistically informative characters were used for all parsimony analyses. Maximum parsimony analyses were performed using the heuristic search algorithm of PAUP*. The data were initially evaluated both including and deleting areas of ambiguous alignment. Ultimately all available data were included because deletion did not affect the branching sequence. All PAUP* analyses used random addition sequence, 50–200 replicates while retaining minimal trees only, tree bisection-reconnection branch swapping with steepest descent, and collapsed zero length branches. All multistate characters were evaluated as nonadditive (unordered). When applied, transversions were weighted more than transitions using Sankoff matrices.

Multi-gene data sets can be evaluated using total evidence (Kluge, 1989, 1998; Ernisse and Kluge, 1993; Kluge and Wolf, 1993), partitioned subsets (e.g., Bull et al., 1993; de Queiroz et al., 1995; Miyamoto and Fitch, 1995), or both (Page, 1996b). Total evidence is the only approach philosophically justified for hypothesizing phylogenetic relationships (Kluge, 1998; Kluge and Wolf, 1993). Nevertheless, we have evaluated our sequence data by gene and gene class—combining all RNA encoding sequences and then all protein-encoding sequences—and by total evidence. The data sets were partitioned to discover whether the different genes or gene classes supported alternative parts of the trees, agreed with each other, or were reflective of different selective pressures, (i.e., yielded different gene tree topologies).

Nodal consistency was assessed for all combined sequences as follows:

(1) Transversion weighting via Sankoff matrices (Sankoff and Cedergren, 1983) using arbitrarily chosen weights.
(2) Functional ingroup-outgroup evaluations (Watrous and Wheeler, 1981; Murphy et al., 1983; Fu and Murphy, 1997).

(3) Nodal-specific permutation tail probabilities for character covariation (Fu and Murphy, 1999). Trials were restricted to four taxon statements in order to reduce the likelihood of Type 1 error (Peres-Neto and Marques, 2000).
(4) Jackknife monophyly index (Lanyon, 1985; Siddall, 1995).
(5) Decay index (Bremer, 1988).
(6) Bootstrapping (Felsenstein, 1985).

Lee (2000) reviewed the assumptions and limitations of most of these methods. Bootstrapping, functional ingroup-outgroup evaluations, nodal-specific permutation tail probabilities, jackknife monophyly index and transversion weighting via Sankoff matrices were performed in PAUP*. Decay analyses were preformed in AutoDecay 4.0.2 Eriksson, 1998), which operates in conjunction with PAUP*. Results were visualized in TreeView (Page, 1996a). Bootstrap proportions evaluations involved 1000 randomizations. Nodal permutation tail probabilities used four-taxon trials with 10000 randomizations and two replicates during heuristic searches; *P*-values were not calculated but rather a node was considered to be supported by significantly covaried data only if the randomizations did not produce an equal or shorter tree length irrespective of the frequency of occurrence. Homoplasy excess ratios (Archie, 1989b; Fu and Murphy, 1999) were calculated from permutation tail probabilities evaluations and used to superficially compare trees and data sets. Statistical comparisons of different trees, for example Wilcoxon ranked-sum tests (Templeton, 1983) were not attempted. The relocation of one taxon on a tree can result in a statistically significant difference in tree length and yet all other nodes remain the same. Thus, statistical tests of tree shape differences are misleading in the same manner as g1 (Hillis, 1991; Huelsenbeck, 1991; Hillis and Huelsenbeck, 1992) and permutation tail probability tests (Archie, 1989a; Faith and Cranston, 1991) are for "structure" in a data set (Källersjö et al., 1992; Carpenter, 1998; Murphy and Doyle, 1998; Slowinski and Crother, 1998; Fu and Murphy, 1999; Wenzel and Siddall, 1999; Lee, 2000). We are concerned only about the relative positions of specific taxa within the frameworks of total evidence and maximum parsimony.

Our data were mapped onto other major hypotheses of relationships using constraint trees designed in MacClade (Maddison and Maddison, 1992). When required, the trees were imported into PAUP* for subsequent analyses. Branch length and alternative tree length determinations, constraint trees, and Sankoff matrices were made using MacClade.

RESULTS

All species were sequenced for the RNA genes, although 16S and $tRNA^{Val}$ could not be sequenced from *C. transversus*. Similarly, ND5 could not be amplified for *C. catalinensis, C. cerastes*, and *C. enyo*. In some species, an internal primer was used for DNA amplification and sequencing, thus omitting a 21 bp region at the beginning of the ND5 fragment. The protein encoding genes could not be amplified for all specimens in some species, particularly *C. horridus* and *C. willardi*. All sequences were deposited in GenBank (12S = AF 259224–259261; $tRNA^{Val}$ = AF 259080–259116; 16S rRNA = AF 259117–259153; cyt-*b* = AF 259154–259191; ND5 = AF 259192–259223). No potentially synapomorphic indels were identified.

RNA Gene Sequences

Sequence variability.—RNA nucleotide base pair composition is summarized in Table 3. The pairwise transition to transversion ratio averaged 7.9:1 (range 3.3:1–13.0:1) indicating that the data were not saturated with transitional changes.

Parsimony evaluation.—For the 502 aligned sites of 12S, 157 (31%) sites were variable, of which 98 (20%) were potentially informative. Analysis of the potentially informative sites yielded five most-parsimonious trees (Table 4). We did not attempt a separate phylogenetic analysis of $tRNA^{Val}$ because there were too few potentially phylogenetically informative sites for a meaningful analysis. 16S yielded 427 (32%) variable sites of which 274 (21%) were potentially phylogenetically informative. Thirteen most-parsimonious trees were resolved (Table 4). The topologies resulting from the two genes are largely the same indicating the absence of conflict. Thus, a combined analysis of all RNA gene sequence data was performed for a single analysis.

The combined RNA gene sequence data resulted in 382 potentially phylogenetically informative characters. Analysis of these data yielded one most-parsimonious tree (Fig. 2). The tree differed from the traditional classification in several ways. First, and most surprising, *C. willardi* and *C. horridus* were resolved as sister species. The former species is small and traditionally grouped in the *C. triseriatus* group, while *C. horridus* is large and usually grouped with *C. durissus*. The alliance of *C. enyo* with *C. cerastes* and *C. polystictus* was unanticipated,

Table 3. Summary of nucleotide variability from mitochondrial DNA gene sequences of rattlesnakes used in this study. Included are the number of base pairs resolved and their relative frequency.

	Base pairs					Percent base pairs			
Gene	A	G	T	C	Total	A	G	T	C
12S	6717	3306	3867	4642	18532	36.2	17.8	20.9	25.0
$tRNA^{Val}$	1080	312	655	475	2522	42.8	12.4	26.0	18.8
16S	18431	7582	10152	11769	47934	38.4	15.8	21.2	24.6
All RNA gene data	26228	11200	14674	16886	68988	38.0	16.2	21.3	24.5
Cyt-*b*	5730	2708	5792	6408	20638	27.8	13.1	28.1	31.0
ND5	5256	1387	4083	4054	18780	35.6	9.3	37.6	27.4
All protein gene data	10986	4095	9875	10462	35418	31.0	11.6	27.9	29.5
All sequence data	37214	15295	24549	27348	104406	35.7	14.6	23.5	26.2

especially as Klauber (1972) summarized data supporting the inclusion of *C. enyo* in the *C. durissus* group (Fig. 1). Placement of *S. ravus* well within *Crotalus*, as opposed to rooting at the base of the tree, was counter to a substantial body of anatomical information (McCranie, 1988; Knight et al., 1993). The *C. atrox* group of diamond-backed rattlesnakes was not resolved as monophyletic.

Assessing Nodal Stability.—By arbitrarily conservatively weighting transversions two times more than transitions, six most-parsimonious trees were obtained. They differed from the unweighted tree mostly in basal relationships. *Crotalus triseriatus* and its sister species moved to the base of the tree, followed by the clade *C. intermedius*-*C. pricei*, then *C. enyo* with *C. cerastes* and *C. polystictus*, and finally by the large-bodied rattlesnakes. The clade of *C. willardi*-*C. horridus* was resolved as sister group of the *C. durissus* group, and relationships within the *C. viridis* clade became largely unresolved. Increased transversion weights of three through five chose one tree from among the six.

Eleven nodes received support from nodal permutation tail probabilities, and most of these were restricted to multiple samples of supposed species. All of these nodes had bootstrap proportions of 99% or higher and jackknife monophyly indices of 100% except for one node at the base of the clade containing *C. triseriatus*. The problematic associations of *C. willardi* and *C. horridus*, and the placement of *S. ravus* within *Crotalus* were not well supported.

Protein Encoding Mitochondrial DNA Partial Gene Sequences

Sequence variability.—We sequenced 274 nucleotides from mtDNA cyt-*b* gene from 49 individual pitvipers representing 28 species of rattlesnakes plus four specimens from two outgroup taxa, *Agkistrodon contortrix* and *Gloydius ussuriensis* (Kovac, 1994). For these sequences all species of rattlesnakes were represented by at least two specimens with the following exceptions: *C. basiliscus*, *C. cerastes*, *C. intermedius*, *C. lepidus*, *C. mitchellii*, *C. pricei*, *C. polystictus*, *C. scutulatus*, *C. tortugensis*, *C. unicolor* and *C. viridis*. Intraspecific variation was observed in *C. molossus*, *C. tigris*, *C. willardi* and *A. contortrix*, and each variant was included in our initial analyses. Because the species with intraspecific variability always formed monophyletic clades, we generally assumed one specimen to be representative of a given species at this level of universality. An additional 291 bp from cyt-*b* were sequenced from all species of rattlesnakes. We did not attempt to obtain the additional sequences from *G. ussuriensis* because of its relatively high divergence, and concomitant increased homoplasy in these data (Kovac, 1994).

We sequenced 477 bp of ND5 from 28 species of rattlesnakes; no sequences were obtained for *C. catalinensis*, *C. cerastes* or *C. enyo*, and for only one representative of *C. horridus* and *C. willardi*. A summary of nucleotide ratios for the individual and combined protein encoding genes is given in Table 3. Overall, the data are thymine (28%) and cytosine (30%) rich, slightly over represented in adenine (31%), and noticeably meager in guanine (12%). For the protein encoding sequences, the majority of potentially phylogenetically informative transformations occurred in the third position (115, or 59%). There were 50 (26%) and 30 (15%) transformations in the first and second position, respectively. The relatively high proportion of changes in the first and second codon positions, and a homoplasy excess ratio (Archie, 1989b) of 0.62, suggested that these data had high levels of homoplasy.

Table 4. Summary of variation in the mitochondrial DNA genes sequenced from rattlesnakes and the outgroup taxa, and their unweighted parsimony evaluation. NT = total number of taxa (individuals) analyzed; NS = total number of homologous sites resolved; NVS = number of variable sites; NPPIS = number of potentially phylogenetically informative sites; NMPTs = number of most-parsimonious trees resolved; LMPTs = Length of most-parsimonious solution; CI = consistency index; RI = retention index; PTP = permutation tail probability level of significance; HER = homoplasy excess ratio (Archie, 1989b; Fu and Murphy, 1999). Trees for the $tRNA^{Val}$ gene were not calculated (n/a) owing to too few characters (10) available to resolve nodes among the 29 ingroup species in the analysis.

Gene	NT	NS	NVS	NPPIS	NMPTs	LMPTs	CI	RI	PTP	HER
12S rRNA	38	502	157	98	5	303	0.45	0.62	< 0.001	0.56
$tRNA^{Val}$	37	71	23	10	n/a	n/a	n/a	n/a	n/a	n/a
16S rRNA	37	1330	427	274	13	994	0.41	0.57	< 0.001	0.58
Cyt-*b*	38	565	285	219	26	1017	0.33	0.52	< 0.001	0.67
ND5	32	477	276	195	6	771	0.38	0.62	< 0.001	0.53
All RNAs	38	1903	607	382	1	1342	0.42	0.58	< 0.001	0.56
All protein	38	1042	556	414	2	1870	0.34	0.53	< 0.001	0.62
All genes	38	2945	1168	796	2	3270	0.36	0.54	< 0.001	0.60

Parsimony evaluation.—Although we initially evaluated the two protein encoding genes separately, owing to similar levels of divergence, missing sequences for ND5 in many species, nucleotide composition and resulting trees, we combined the cyt-*b* and ND5 data sets. All taxa were included, although more than half the data were missing for some. Our initial parsimony analysis obtained two most-parsimonious trees, owing to an uncertain placement of *C. cerastes*. Figure 3 summarizes the trees showing the alternative placements of *C. cerastes*.

Assessing Nodal Stability.—When transversions were weighted two times transitions, two most-parsimonious trees were resolved, variation owing to placement of the clade of *C. viridis*-*C. mitchellii*. In both trees, *C. cerastes* clustered with *C. intermedius* and *C. transversus*.

Because *S. catenatus* and *S. miliarius* were consistently resolved at the base of the cladogram, we deleted the other outgroups (*Gloydius* and two species of *Agkistrodon*) and used instead the two North American species of *Sistrurus* as an outgroup. Two most-parsimonious trees were resolved. In these trees, *S. ravus* was recovered as the sister group of all *Crotalus*, and *C. cerastes* became the sister group of *C. polystictus*. This finding suggested that divergence in *Agkistrodon* formed multiple, incorrect character state polarizations.

The RNA and protein encoding data sets gave different associations of some taxa, some in strong conflict. For example, whereas the RNA gene data associated *C. enyo* with *C. polystictus* and *C. cerastes* at the base of the tree, the protein nucleotides placed *C. enyo* in the *C. durissus* group. The RNA gene data supported an association of *C. mitchellii*, *C. tigris* and *C. adamanteus* in the *C. viridis* clade. However, the protein encoding sequence data placed *C. adamanteus* as the sister taxon to other members of the *C. atrox* clade, and the data did not unite *C. mitchellii* and *C. tigris* with *C. viridis* and *C. scutulatus*.

Combined Sequence Data Sets

Sequence variability.—Our combined gene segments resulted in 2,945 homologous nucleotide positions. Among these, 1168 sites were variable, with nearly 800, or 27% being potentially phylogenetically informative. The protein-encoding sequences were slightly less variable than the RNA gene sequences (Table 4) but they contained a greater number of potentially phylogenetically informative sites.

Parsimony evaluation.—An unweighted parsimony analysis of the combined data yielded two most-parsimonious trees (3270 steps, CI = 0.36, RI = 0.54). The strict consensus tree (Fig. 4) was a blend of strongly supported associations from Figures 2 and 3. For example, *C. enyo* moved from being a sister species of *C. polystictus* with the RNA gene data (Fig. 2) to the *C. durissus* group as depicted in the protein encoding data (Fig. 3). The association of *C. scutulatus, C. horridus*, and *C. viridis* with the *C. intermedius* clade (Fig. 4) was surprising, and likely reflected the attraction of *C. willardi* and *C. horridus*. Because *S. catenatus* and *S. miliarius* always appeared at the base of the tree, and rooting with *Agkistrodon* yielded trees that were contrary to anatomical evidence, we performed combined gene analyses using the North American *Sistrurus* as our functional outgroup; this analysis resolved a single tree (Fig. 5) that resolved *S. ravus* as being the sister group to *Crotalus*.

Assessing Nodal Stability.—Transversion weights were arbitrarily increased. Weighting transversions

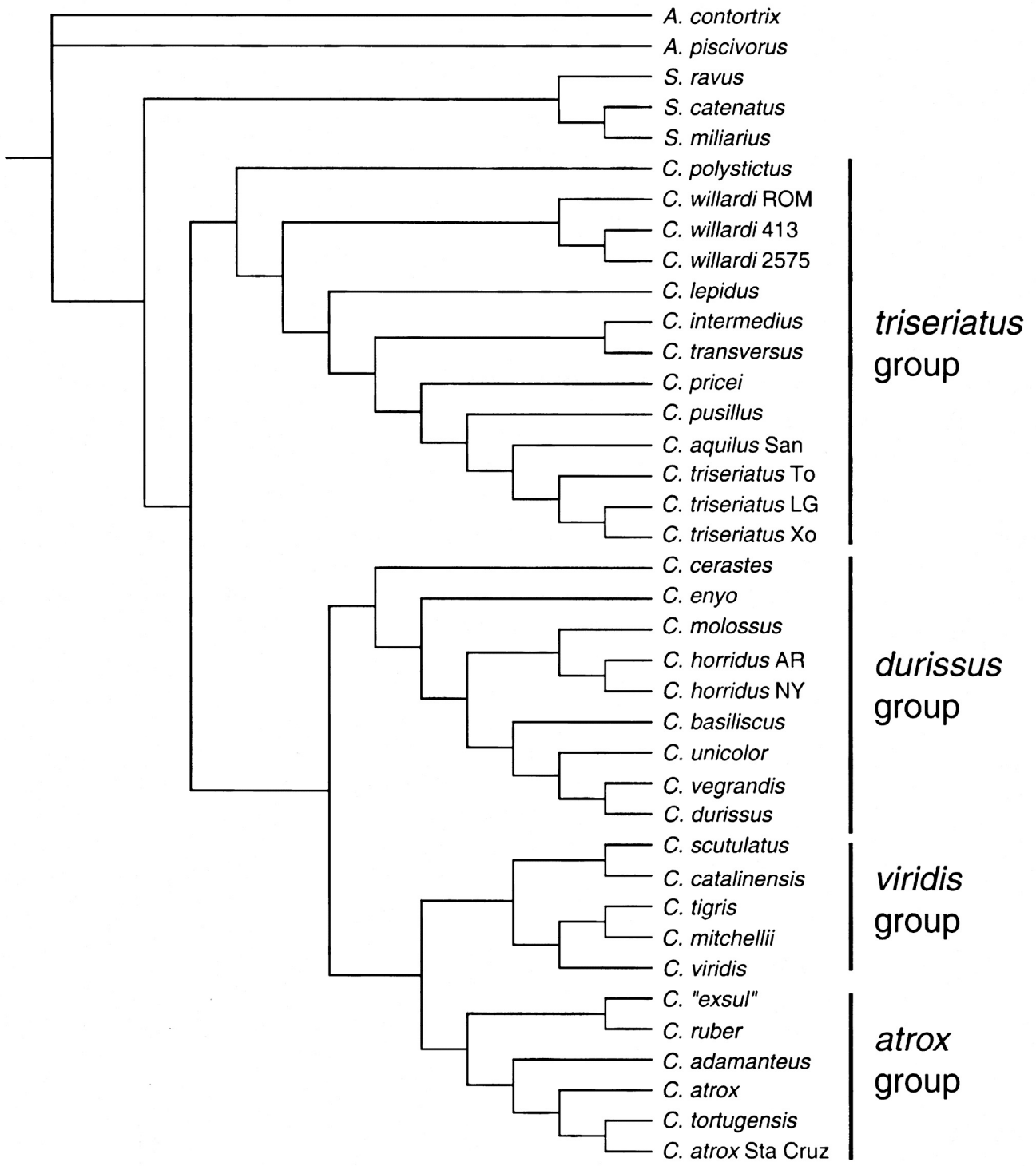

Fig. 1. The phylogenetic relationships of the rattlesnakes, genera *Crotalus* and *Sistrurus*, as hypothesized by Klauber (1972) with species groups as defined by Gloyd (1940). For *C. triseriatus* locality abbreviations, refer to Table 1.

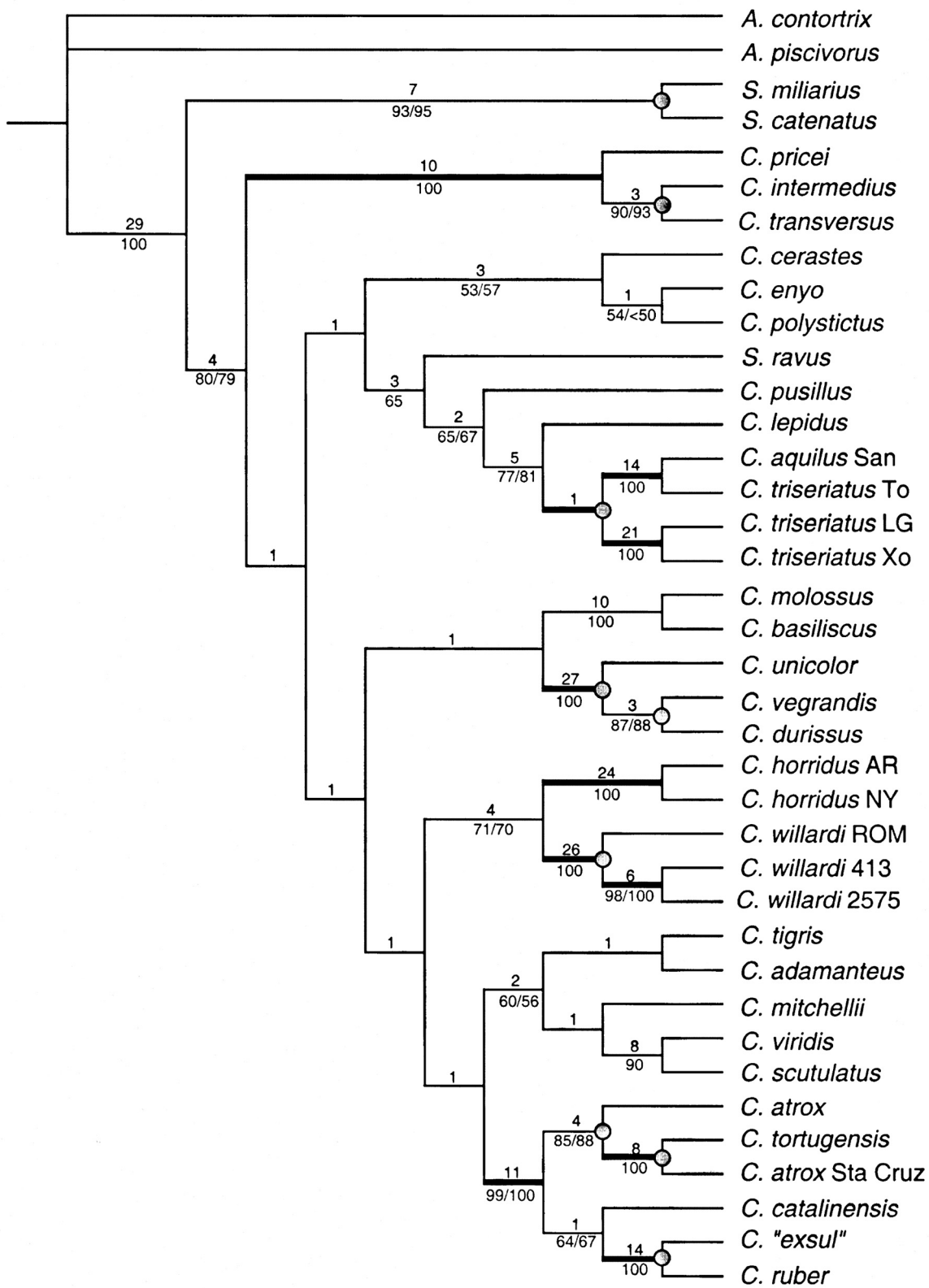

Fig. 2. Single most-parsimonious tree for the rattlesnakes, genera *Crotalus* and *Sistrurus*, derived from the RNA sequences of the 12S, $tRNA^{Val}$, and 16S mtDNA genes. Numbers above nodes are decay values and those below are given as "jackknife monophyly index/bootstrap proportion"; when equal, only one value is given, and values lower than 50% are not given. Thick lines indicate nodes supported by significant nodal permutation tail probabilities (Fu et al., 1997; Fu and Murphy, 1999). Large gray dots at nodes indicate correspondence to Klauber's (1972) hypothesis (Fig. 1). For *C. triseriatus*, *C. willardi*, and *C. horridus* locality abbreviations, refer to Table 1.

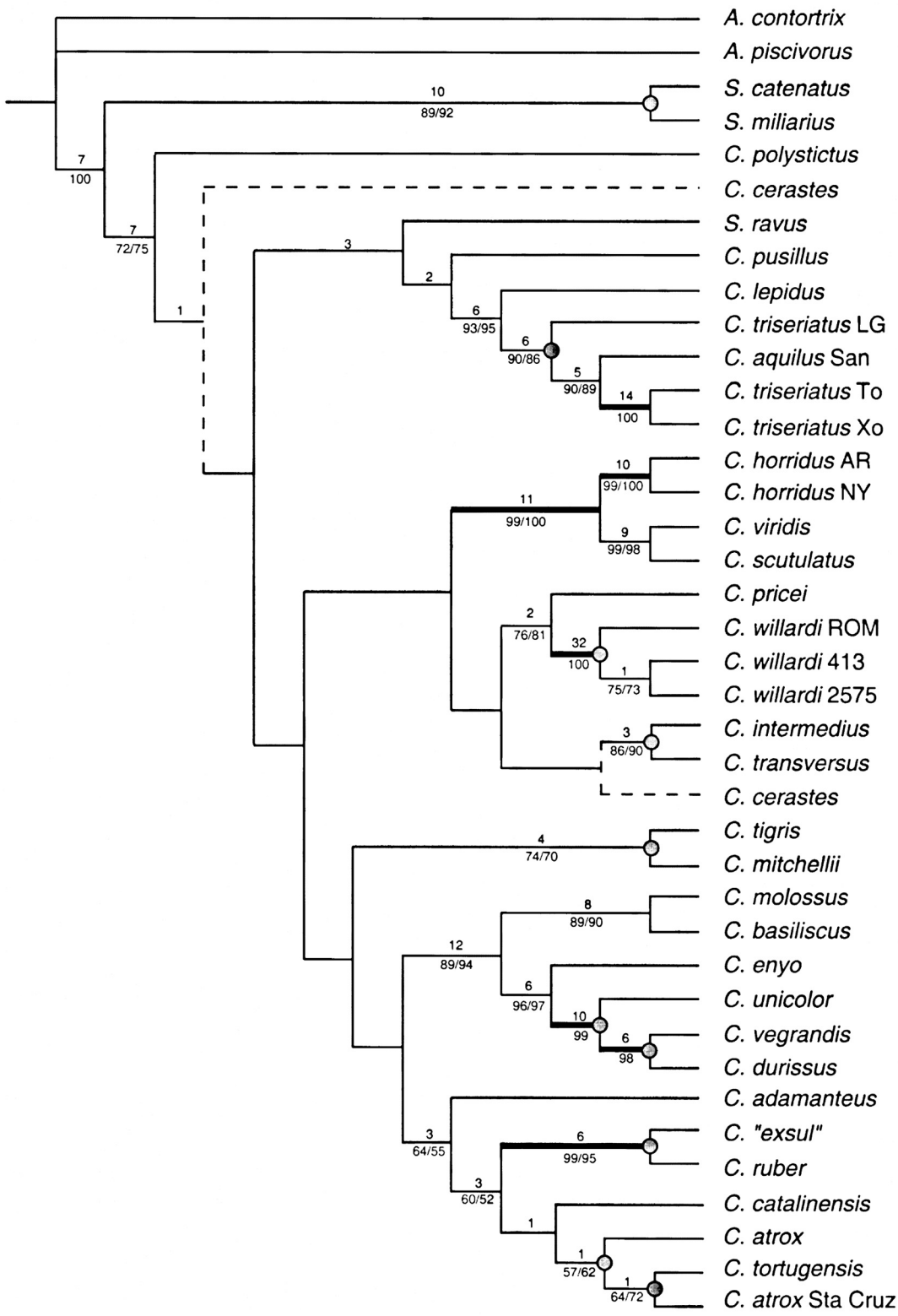

Fig. 3. Phylogenetic relationships for the rattlesnakes, genera *Crotalus* and *Sistrurus*, derived from the combined protein encoding sequences of the cytochrome *b* and ND5 mtDNA genes. Dashed branches indicate alternative placements for *C. cerastes* on the two most-parsimonious trees. Numbers above nodes are decay values and those below are given as "jackknife monophyly index/bootstrap proportion"; when equal, only one value is given, and values lower than 50% are not given. Thick lines indicate nodes supported by significant nodal permutation tail probabilities (Fu et al., 1997; Fu and Murphy, 1999). Large gray dots at nodes indicate correspondence to Klauber's (1972) hypothesis (Fig. 1). For *C. triseriatus*, *C. willardi*, and *C. horridus* locality abbreviations, refer to Table 1.

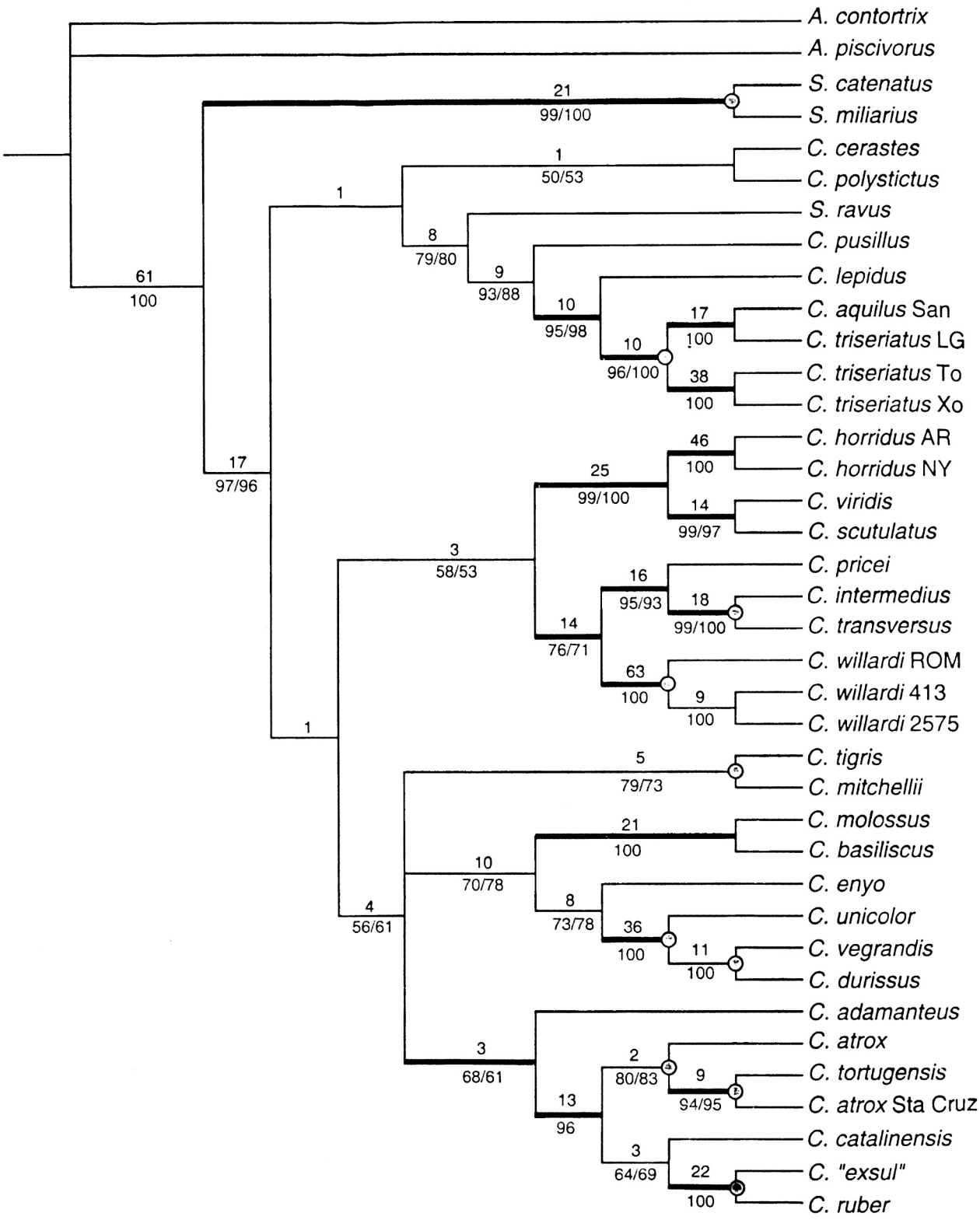

Fig. 4. A strict consensus tree for the rattlesnakes, genera *Crotalus* and *Sistrurus*, derived from the two most-parsimonious solutions of the combined protein and RNA encoding mtDNA sequences. Numbers above nodes are decay values and those below are given as "jackknife monophyly index/bootstrap proportion"; when equal, only one value is given, and values lower than 50% are not given. Thick lines indicate nodes supported by significant nodal permutation tail probabilities (Fu et al., 1997; Fu and Murphy, 1999). Large gray dots at nodes indicate correspondence to Klauber's (1972) hypothesis (Fig. 1). For *C. triseriatus*, *C. willardi*, and *C. horridus* locality abbreviations, refer to Table 1.

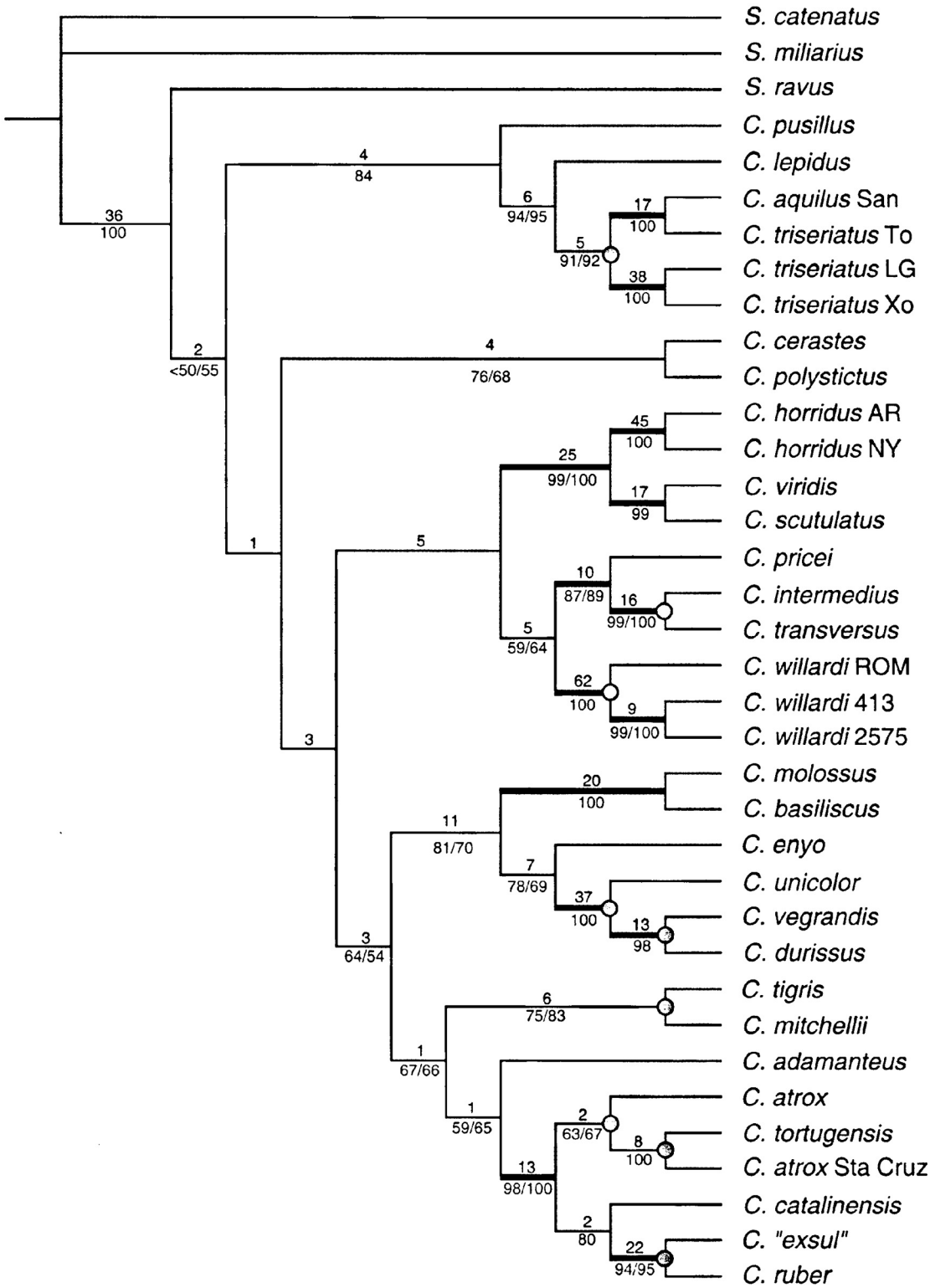

Fig. 5. The single most-parsimonious tree for the rattlesnakes using *Sistrurus catenatus* and *S. miliarius* as the functional outgroup (see Fig. 4) derived from the combined protein and RNA encoding mtDNA sequences. Numbers above nodes are decay values and those below are given as "jackknife monophyly index/bootstrap proportion"; when equal, only one value is given, and values lower than 50% are not given. Thick lines indicate nodes supported by significant character covariation as determined using nodal permutation tail probabilities (Fu et al., 1997; Fu and Murphy, 1999). Large gray dots at nodes indicate correspondence to Klauber's (1972) hypothesis (Fig. 1). For *C. triseriatus*, *C. willardi*, and *C. horridus* locality abbreviations, refer to Table 1.

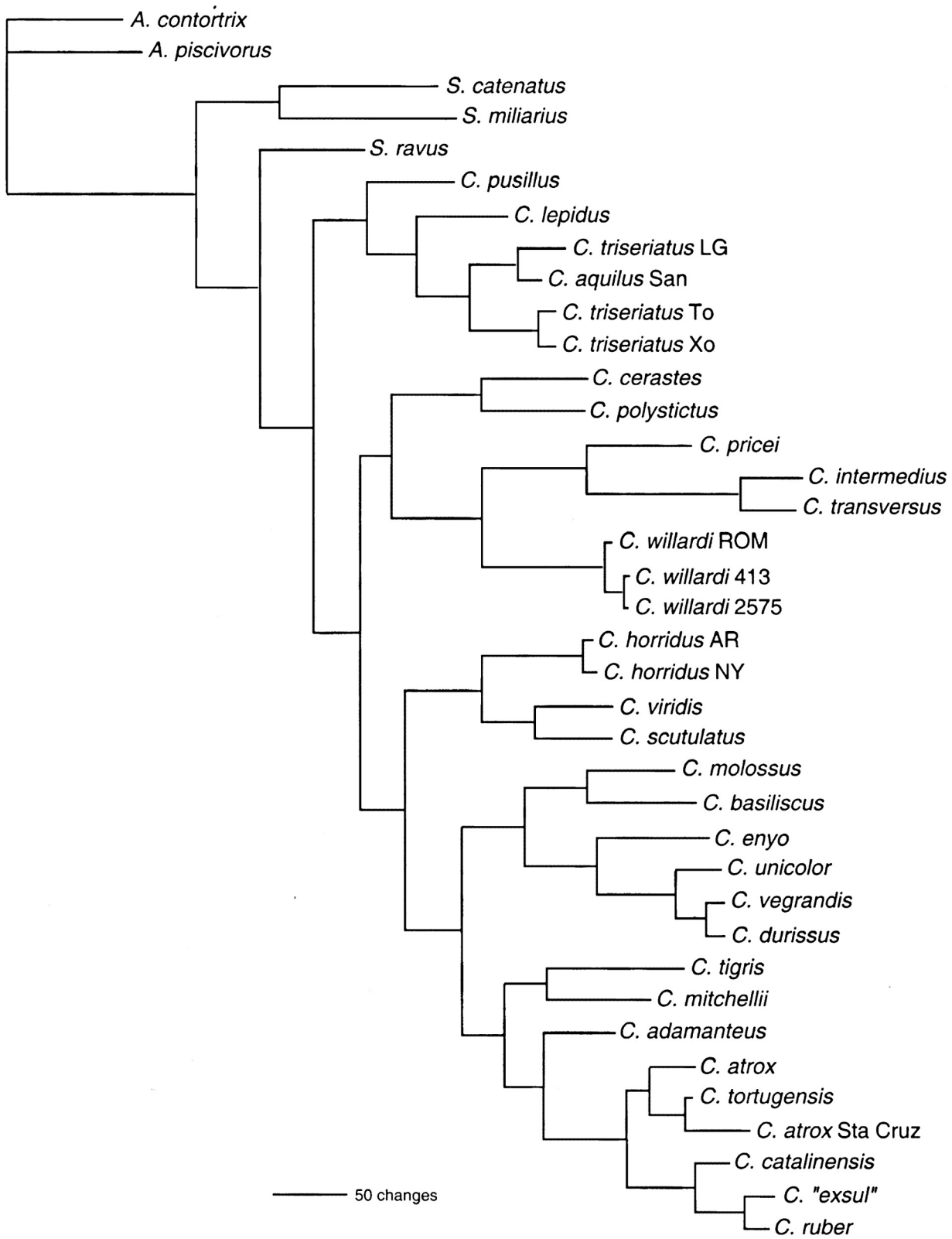

Fig. 6. Our preferred phylogenetic hypothesis for the rattlesnakes, genera *Crotalus* and *Sistrurus*, derived from mtDNA gene sequences using *S. catenatus* and *S. miliarius* as the functional outgroup and weighting transversions three times transitions. Branch lengths are proportional to the number of unambiguous changes mapped on the cladogram, assuming delayed transformation of the characters. Measures of nodal support are not provided because of their ambiguous meaning in weighted analyses, but values from unweighted analyses are given in Figs. 4-5. For *C. triseriatus*, *C. willardi*, and *C. horridus* locality abbreviations, refer to Table 1.

three times transitions resulted in one most-parsimonious tree (Fig. 6). It differed from the unweighted tree in several respects: *C. horridus* and *C. willardi* were no longer closely associated with one another; the clade of *C. cerastes* and *C. polystictus* moved up the tree and was recovered as the sister group of *C. intermedius*, *C. pricei*, *C. transversus*, and *C. willardi*; and the clade of *C. horridus*, *C. scutulatus* and *C. viridis* was recovered as the sister group of all the large rattlesnakes. The transversion-weighted tree (Fig. 6) was more similar to traditional insight and anatomical evidence (Fig. 1) than the unweighted tree (Fig. 5).

Functional ingroup-outgroup evaluations revealed instability in some associations. The position of the clade containing *C. horridus*, *C. scutulatus*, and *C. viridis* was unstable owing to weak support. It was either the sister group to all other large-bodied rattlesnakes, or it formed the sister group of the *C. atrox* clade. The latter association occurred when *C. horridus* was deleted from the data set. Among the species of the *C. triseriatus* clade, two to three species groups are recovered. Among them, either *S. ravus* was the sister group of all *Crotalus*, or it fell out within the *C. triseriatus* clade. Although the association of *C. cerastes* and *C. polystictus* did not vary, their relationship to other clades was unstable. *Crotalus intermedius*, *C. pricei*, and *C. transversus* always clustered together, as did *C. aquilus*, *C. lepidus*, C. *pusillus* and *C. triseriatus*.

Nodal permutation tail probabilities, jackknife monophyly index, bootstrap proportions and decay index values are shown in Figure 5. The nodal permutation tail probability evaluations revealed that all of the unstable associations were not supported by significantly covaried character states; unsupported nodes included species that moved during the transversion weighting and functional ingroup-outgroup trials, including the problematic *C. willardi-C. horridus* clade. Jackknife monophyly index values were highly correlated with bootstrap proportions, and both of these measures were correlated with nodal permutation tail probabilities, although much variation was observed. As expected, some levels of nodal support increased significantly when we used *Sistrurus catenatus* and *S. miliarius* as the outgroup (Fig. 5).

DISCUSSION

The Preferred Tree (Figure 6)

Our examination of tree node reliability using various weighting schemes and functional outgroups revealed considerable stability among most relationships. Nevertheless, two associations in our most-parsimonious tree (Fig. 4) remained problematic, particularly because they were counter to a wealth of anatomical information. When the genus *Agkistrodon* was used as the outgroup, *S. ravus* was located within one clade of the *C. triseriatus* complex of *Crotalus*. However, deletion of *Agkistrodon* and the use of *S. miliarius* and *S. catenatus* as a functional outgroup unambiguously placed *S. ravus* as the sister taxon to all species of *Crotalus* (Fig. 5), even though the arrangement still left the genus *Sistrurus* paraphyletic. The basal tree position of *S. ravus* was preferable for it did not conflict with the extensive anatomical data, including among others, head scale morphology, hemipenal structure, and rattle shape. Thus, the extent of mitochondrial DNA divergence in the species of *Agkistrodon* appeared to yield at least one misinformative association among rattlesnakes.

The association of *C. willardi* and related species with *C. horridus* in the *C. viridis* clade (Fig. 5) was rejected based on morphological evidence summarized by Klauber (1972) and Knight et al. (1993), and our nodal permutation probability trials. Given that our data contained a significant amount of homoplasy (homoplasy excess ratio = 0.60), we arbitrarily progressively weighted transversions until the association of these two problematic groups was no longer maintained. The single most-parsimonious tree obtained on weighting transversions three times transitions (Fig. 6) was our preferred hypothesis of genealogical relationships. We included the anatomical data traditionally used to define *Sistrurus*. Inclusion of these data did not change our most-parsimonious tree (Figs. 4–5) unless they were weighted three times more than any nucleotide site. Once weighted, our preferred tree was resolved.

Monophyly of the Genera and Species Groups

The best, most-parsimonious explanations of our data (Fig. 5), and our preferred, weighted tree (Fig. 6) revealed several departures from previously suggested kinships.

Sistrurus.—The cladistic validity and desirability of recognizing both *Sistrurus* and *Crotalus* has been questioned (McCranie, 1988; Brattstrom, 1964; Stille, 1987; Foote and MacMahon, 1977; Knight et al., 1993; Parkinson, 1999). The sister species relationship of *S. catenatus* and *S. miliarius* seems certain as supported by anatomical (Gloyd, 1940; Klauber, 1972; McCranie, 1988) and mtDNA sequence data

(Knight et al., 1993; Fu and Murphy, 1999; Parkinson, 1999; Parkinson et al., this volume; this study). However, the relationships of *S. ravus* are uncertain because this rattlesnake exhibits a suite of both plesiomorphic and apomorphic anatomical conditions found in both *Sistrurus* and *Crotalus*.

Knight et al. (1993) used 35 potentially phylogenetically informative RNA sites to investigate the monophyly of *Sistrurus* and the generic status of rattlesnakes. Unfortunately, Fu and Murphy (1999) found that only one of the five ingroup nodes was supported by significant character covariation—the association of *S. catenatus* and *S. miliarius*. Randomizations of their sequence data frequently produced a more parsimonious explanation than non-randomized data. A nodal permutation tailed probability evaluation of Parkinson's (1999) larger data yielded an identical conclusion; the data were not adequate to answer the question. The more extensive analyses of Parkinson et al. (this volume) were concordant with our findings.

Our unweighted sequence data associated *S. ravus* with *C. triseriatus* and its sister taxa, a finding generally concordant with hemipenal morphology (McCranie, 1988). Our preferred weighted tree places *S. ravus* as the sister group to the genus *Crotalus*. Keeping *Sistrurus* monophyletic requires 14 additional steps on the preferred tree. Locating *S. ravus* at the base of all *Crotalus* does not conflict with either the retention of plesiotypic attributes observed in *Sistrurus*, or synapomorphies shared with *Crotalus*. Biogeographically, the cladistic placement of *S. ravus* at the base of the tree associates it near other small species in southern and central Mexico. Nevertheless, because this placement leaves *Sistrurus* a paraphyletic genus, it is necessary to either (1) include *S. ravus* in the genus *Crotalus*, (2) designate a new genus for *S. ravus*, or (3) synonymize the genus *Sistrurus* into *Crotalus*. A substantial body of literature recognizes the genus *Sistrurus*, including popular, ecological, and medical titles. The literature is particularly affluent with respect to *S. catenatus* and *S. miliarius*. Therefore, in the interest of nomenclatorial stability, we prefer retaining *Sistrurus* for the northern species and consider only *S. ravus* to be a member of the genus *Crotalus,* hereafter referred to as *Crotalus ravus* Cope 1865.

Crotalus.—Within *Crotalus*, none of our most-parsimonious trees was consistent with group membership as defined by Gloyd (1940), applied by Brattstrom (1964) and Klauber (1956, 1972), and assumed by Knight et al. (1993) (Table 5; Fig. 1). Our analyses consistently render the *C. triseriatus* group polyphyletic. They re-circumscribe the *C. durissus* group especially in aligning *C. enyo* with *C. durissus* and *C. unicolor*, and exclude *C. cerastes* and *C. horridus*. The cladogram (Fig. 6) redefines the *C. viridis* group as consisting only of *C. horridus*, *C. viridis*, and *C. scutulatus*.

The *Crotalus triseriatus* group.—Species membership in this group was largely unchallenged until this study. Keeping the group monophyletic *sensu* Klauber (1972) required 13 additional steps on our preferred tree, although this reduced to six steps if *C. cerastes* was included in the group, as suggested by Foote and MacMahon (1977). Thus, the *C. triseriatus* group of Klauber was likely a paraphyletic assemblage of species defined primarily by anatomical characters plesiomorphic for *Crotalus*.

Relationships among members of the *C. triseriatus* group have been very unstable. Brattstrom (1964) removed *C. polystictus* making it an "intermediate" associate of *C. stejnegeri*; these two species were considered to be transitory between small and large rattlesnakes. The sister species relationship of *C. lepidus* and *C. triseriatus* (including *C. aquilus*) has received wide support (Smith, 1946; Klauber, 1952, 1956, 1972; Armstrong and Murphy, 1979; our study). However, Campbell and Lamar (1989) and Dorcas (1992) believed that *C. aquilus* was more closely related to *C. lepidus* than to *C. triseriatus*. Uniting *C. aquilus* and *C. lepidus* as sister species was unlikely as it requires 35 additional steps on our preferred tree. Furthermore, *C. triseriatus* did not appear to be a monophyletic species because it required 24 additional steps, if *C. aquilus* was recognized. The substantial amount of sequence divergence among relatively close localities indicates that multiple cryptic species are contained in this complex, especially considering that *C. triseriatus armstrongi*, which was not included in our study, occurs in relatively distant Jalisco (Campbell, 1979; Campbell and Lamar, 1989). Unfortunately, apparently neither Campbell and Lamar (1989) nor Dorcas (1992) evaluated their taxonomic samples by locality, but rather by predefined subspecies.

Brattstrom (1964) argued that *C. lepidus* shared a most recent common ancestor with *C. willardi*, a problematic association requiring 52 more steps. Foote and MacMahon (1977) considered *C. cerastes* and *C. pricei* to be sister taxa, followed basally by *C. willardi* and *C. ravus*. Klauber (1952) believed *C. pricei* and *C.*

Table 5. History of taxonomic groupings of rattlesnakes in the genus *Crotalus* in groups defined by Gloyd (1940). The relationships from Klauber (1972) are taken from his "phylogenetic tree" and do not necessarily correspond with text discussions. Dashes indicate species not surveyed. The phylogeny of Stille (1987) cannot be mapped on this table. See text for discussion.

Species group	Gloyd 1940	Klauber 1956	Brattstrom 1964	Klauber 1972	Foote and MacMahon 1977
C. triseriatus					*cerastes*
					S. ravus
	t. omiltemanus	*intermedius*	*intermedius*	*intermedius*	—
	t. lepidus	*lepidus*	*lepidus*	*lepidus*	*lepidus*
		polystictus	*polystictus*	*polystictus*	—
	t. pricei	*pricei*	*pricei*	*pricei*	*pricei*
		pusillus	*pusillus*	*pusillus*	—
		stejnegeri	*stejnegeri*	*stejnegeri*	—
		transversus	*transversus*	*transversus*	—
	t. triseriatus	*triseriatus*	*triseriatus*	*triseriatus*	*triseriatus*
		willardi	*willardi*	*willardi*	*willardi*
	—	—	—	*lannomi*	—
C. durissus	*basiliscus*	*basiliscus*	*basiliscus*	*basiliscus*	—
		cerastes		*cerastes*	
	durissus	*durissus*	*durissus*	*durissus*	*durissus*
		enyo		*enyo*	—
	horridus	*horridus*	*horridus*	*horridus*	*horridus*
	molossus	*molossus*	*molossus*	*molossus*	*molossus*
	unicolor	*unicolor*	*unicolor*	*unicolor*	—
	—	—	—	*vegrandis*	—
					mitchellii
					tigris
					scutulatus
"annectant"	*scutulatus*				
C. atrox	*adamanteus*	*adamanteus*	*adamanteus*	*adamanteus*	*adamanteus*
	atrox	*atrox*	*atrox*	*atrox*	*atrox*
	—	*catalinensis*	*catalinensis*		—
	exsul	*exsul*	*exsul*	*exsul*	—
	ruber	*ruber*	*ruber*	*ruber*	*ruber*
	tortugensis	*tortugensis*	*tortugensis*	*tortugensis*	—
C. viridis	—	—	—	*catalinensis*	—
			cerastes		
			enyo		
	mitchellii	*mitchellii*	*mitchellii*	*mitchellii*	
		scutulatus	*scutulatus*	*scutulatus*	
		tigris	*tigris*	*tigris*	
	viridis	*viridis*	*viridis*	*viridis*	*viridis*
"uncertain"	*cerastes*				
	enyo				
	polystictus				
	stejnegeri				
	tigris				
	willardi				

intermedius were sister taxa, whereas Klauber (1956, 1972) and Brattstrom (1964) united *C. intermedius* and *C. transversus*, as we resolved. Klauber and Brattstrom also placed these species as the sister group of *C. pricei*, *C. pusillus*, and *C. triseriatus* (and *C. aquilus*), an arrangement that was very different from ours.

We resolved a sister species relationship for *C. cerastes* and *C. polystictus*; however, these two species were associated either with the *C. triseriatus* group (Fig. 4), or as the sister group of all other rattlesnakes except for *Sistrurus* and the *C. triseriatus* group (Fig. 5). The relationships of the clade of *C. cerastes* and *C. polystictus* remained uncertain; their association with other groups was never supported by significant character covariation (Figs. 2–5). *Crotalus intermedius* was always associated with *C. transversus* and usually with *C. pricei* (Figs. 2–4), and with *C. willardi* upon either deletion of *C. horridus* or weighting transversions. Little support occurred for any node at the base of the tree as reflected in insignificant nodal-specific permutation tail probabilities and relatively small jackknife monophyly indices (Fig. 5). Consequently, basal associations remained tentative, as reflected in our rejection of the most-parsimonious solution evaluating all data and taxa (Fig. 4). Although the relationships at the base of the tree may change with additional data, it is unlikely that the *C. triseriatus* group will stand as a monophyletic entity.

The *Crotalus viridis* group.—Phylogenetic relationships and membership of the broadly distributed *C. viridis* group have been especially problematic. Some workers included the Sidewinder, *C. cerastes*, and *C. enyo* as sister taxa, within this group (Amaral, 1929; Klauber, 1931; Minton, 1956; Brattstrom, 1964). Others contended that *C. cerastes* and *C. enyo* belonged to the *C. durissus* group (Klauber, 1956, 1972), and still others argued that *C. cerastes* is a member of the *C. triseriatus* group (Foote and MacMahon, 1977).

Our sequence data did not support inclusion of *C. cerastes* in the *C. viridis* group. Although a *C. enyo*-*C. cerastes* association was resolved in our RNA gene data (along with *C. polystictus*; Fig. 2), both were placed within the group of small, mostly montane "*C. triseriatus* group" rattlesnakes and not in the *C. viridis* group. The association of these two species was not maintained in our total evidence analysis.

Many authors have proposed a sister species relationship for *C. mitchellii* and *C. tigris* (e.g., Amaral, 1929; Klauber 1931, 1952, 1956, 1972; Brattstrom, 1964; Shaw and Campbell, 1974; this study). However, *C. tigris* was aligned with *C. enyo* by Cope (1900). Foote and MacMahon (1977) moved *C. mitchellii*, *C. tigris*, and *C. scutulatus* to the *C. durissus* group and retained only *C. viridis* in the *C. viridis* group.

Association of the Mojave Rattlesnake (*C. scutulatus*), with the *C. viridis* group has been somewhat unstable. Whereas Gloyd (1940) placed *C. scutulatus* as the sister group to both the *C. viridis* and *C. atrox* groups, Foote and MacMahon (1977) moved it to the *C. durissus* group. A new hypothesis, our data strongly supported a *C. scutulatus*-*C. viridis* sister species relationship. But it is possible that the mtDNA genome of one species has been acquired through introgressive hybridization from the other as these two species hybridize (Murphy and Crabtree, 1988). This possibility could be tested using nuclear gene markers.

We did not find strong support for a speciose *C. viridis* group. Maintaining membership of the *C. viridis* group as defined by Klauber (1972), but allowing for a more parsimonious arrangement of the species, required 75 additional steps on our most-parsimonious tree.

Owing to the rejected association of *C. horridus* and *C. willardi* in the RNA encoding data, we alternatively deleted these species from the data set. These trials and the functional ingroup-outgroup analyses most frequently united *C. viridis* and *C. scutulatus*, and associated them with *C. horridus*. Upon deletion of *C. horridus*, *C. viridis*, and *C. scutulatus* formed a species group association with *C. tigris* and *C. mitchellii*. However, the latter two were not always resolved as sister species, and the association was not supported by significantly covaried data. Inclusion of *C. horridus* in the data set forced the *C. mitchellii*-*C. tigris* clade to be the sister group of the *C. atrox* group.

Klauber (1972) considered *C. catalinensis* to be a sister species of *C. scutulatus*, and thus a member of the *C. viridis* group. This unlikely arrangement required 69 additional steps on our preferred tree.

Brattstrom (1964) placed *C. cerastes* and *C. enyo* in the *C. viridis* group as sister taxa. Moving only *C. cerastes* to the clades of *C. scutulatus* and *C. viridis*, or *C. mitchellii* and *C. tigris* while maintaining other relationships (Fig. 5) required at least 20 additional steps. Including *C. enyo* required a minimum of 29 additional steps. Placement of *C. cerastes* and *C. enyo* as sister taxa within the *C. viridis* group required 29 additional steps. Thus, Brattstrom's (1964) arrangement was very unlikely.

The *Crotalus durissus* group.—Membership within the *C. durissus* group must be changed. Klauber (1956, 1972) included both *C. cerastes* and *C. enyo* in the *C. durissus* group, but Gloyd (1940), Brattstrom (1964), and Foote and MacMahon (1977) did not (Table 5). Foote and MacMahon (1977) added *C. mitchellii*, *C. scutulatus* and *C. tigris*. The sister relationship of ((*C. durissus*, *C. vegrandis*) *C. unicolor*) has never been challenged. Our finding that the Baja California Rattlesnake (*C. enyo*) is the sister taxon of the Neotropical Rattlesnake clade, (*C. unicolor*, *C. durissus*, and *C. vegrandis*) was a distinct departure from other hypotheses. Gloyd (1940) considered *C. enyo* to be a "species of uncertain relationships," whereas Klauber (1956, 1972) and Brattstrom (1964) considered it to be the sister species of *C. molossus*, *C. basiliscus*, and *C. horridus*, except for *C. cerastes* (Fig. 1). Regarding the close association of these four *durissus*-like species (Figs. 3–6), *C. enyo* appeared to have been isolated from its shared common ancestor with the Neotropical rattlesnakes during the formation of the Gulf of California and the insular Cape Region (Murphy, 1983a). Its diminutive size relative to *C. durissus* was likely a reflection of insular dwarfism in snakes, a trend noted for the region by Soulé and Sloan (1966) and Case (1978).

Campbell and Lamar (1989) included *C. vegrandis* and *C. unicolor* in *C. durissus* in order to avoid paraphyly. Wüster et al. (this volume) evaluated most nominate subspecies of *C. durissus* and thus expand on our study. All South American populations were found to be monophyletic and hypothesized to have been formed in the late Pleistocene, only 1–2 mya. The Mexican and Central American taxa were also monophyletic but only one of three subspecies, *C. durissus tzabcan*, formed the sister group of the South American clade. The three northern subspecies exhibited substantial divergence in their mitochondrial DNA sequences, and the recognition of multiple species may be warranted. In terms of divergence within the South American clade, substantial morphological change in both body size and color pattern must have occurred very rapidly given the young ages of *C. vegrandis* and *C. unicolor* (Wüster et al., this volume).

The presence of black tails and similarity of body pattern led most investigators to include *C. horridus* in the *C. durissus* group, associating it with *C. molossus*. Moving *C. horridus* to the *C. durissus* clade required 43 additional steps, making it the sister taxon to *C. molossus* required 58 additional steps (leaving all other relationships intact), but making it the sister group to the *C. molossus*-*C. basiliscus* clade required only six additional steps. Thus, a sister species relationship between *C. horridus* and *C. molossus* appeared highly unlikely. In this case, as in others (e.g., Upton and Murphy, 1997), nuances of color pattern similarity may not be indicative of phylogeny (see Douglas et al., this volume).

The *Crotalus atrox* group.—Although most researchers placed the Rattleless Rattlesnake (*C. catalinensis*) as the sister taxon of *C. ruber* (e.g., Cliff, 1954; Klauber, 1956; Brattstrom, 1964; Armstrong and Murphy, 1979; Murphy and Crabtree, 1985), others associated it with *C. scutulatus* (Klauber, 1972; Radcliffe and Maslin, 1975; Harris and Simmons, 1977), a member of the *C. viridis* group. Our data unambiguously placed *C. catalinensis* with the *C. atrox* group, and particularly with the *C.* "*exsul*"-*C. ruber* clade in the *C. atrox* group.

The relationships among the western species of the *C. atrox* group remain tenuous. A reconstruction of the paleostratigraphy in the Gulf of California region suggests that peninsular Baja California was isolated from the remainder of North America by the San Gorgonio Barrier (Murphy, 1983a), a narrow, relatively mesic strip of land or, more likely a seaway at the head of the Gulf of California (Riddle et al., 2000; Murphy and Aguirre, 2002a). If the formation of *C. atrox* and *C.* "*exsul*"-*C. ruber* was facilitated by the formation of this barrier (Murphy, 1983a), and if most Gulf insular occurrences reflect previous continental land connections rather than over water dispersion (Murphy, 1983b), then peninsular *C. ruber* should be the sister taxon of most insular Gulf populations. Up to four insular populations have been accorded species status: *C. catalinensis, C.* "*exsul,*" *C. lorenzoensis*, and *C. tortugensis*.

The sister relationship of *C. atrox* and *C. tortugensis* appeared to be particularly close (Klauber, 1930, 1956, 1972; Cliff, 1954; Brattstrom, 1964; Harris and Simmons, 1977; Campbell and Lamar, 1989). Apparently, because of their overall similarity, Stejneger and Barbour (1933) synonymized *C. atrox* and *C. tortugensis* in their checklist, although in subsequent editions they recognized species status for both. Unlike most other authorities, Gloyd (1940) and Case (1983) believed *C. tortugensis* was more closely related to *C. ruber*-*C.* "*exsul*". Considering the Holocene age of Isla Tortuga (Gastil et al., 1983), little differentiation might be expected between island colonizers and their mainland sister taxa, and a very

Table 6. Composition of species groups of rattlesnakes of the genus *Crotalus* based on our preferred tree (Fig. 6).

Species groups							
C. ravus	*C. triseriatus*	*C. polystictus*	*C. durissus*	*C. viridis*	*C. mitchellii*	*C. atrox*	*incertae sedis*
C. ravus	*C. triseriatus*	*C. polystictus*	*C. durissus*	*C. viridis*	*C. mitchellii*	*C. atrox*	*C. lannomi*
	C. aquilus	*C. cerastes*	*C. vegrandis*	*C. scutulatus*	*C. angelensis*[2]	*C. adamanteus*	*C. stejnegeri*
	C. lepidus	*C. willardi*	*C. unicolor*	*C. horridus*	*C. tigris*	*C. catalinensis*	
	C. pusillus	*C. pricei*	*C. enyo*			*C. exsul*	
		C. intermedius	*C. basiliscus*			*C. lorenzoensis*[3]	
		C. transversus	*C. estebanensis*[1]			*C. ruber*	
			C. molossus			*C. tortugensis*	

[1]Not sequenced but presumably a sister species of *C. molossus.* [2]Not sequenced but presumably a sister species of *C. mitchellii.* [3]Not sequenced but presumably a sister species of *C. ruber.*

strong sister species relationship should be demonstrable from both anatomical and molecular perspectives. Isla Tortuga is geographically much closer to the Baja Californian peninsula than to mainland Mexico.

Our sequence data suggested that *C. atrox* from Santa Cruz Island and *C. tortugensis* were sister species (Figs. 2–6). As with *C. ruber* and *C.* "*exsul*", the association was supported by significantly covaried data. In contrast, the association of C. *catalinensis* and *C. ruber* was not supported by significantly covaried characters, and neither was mainland *C. atrox* with either *C. tortugensis* or *C. atrox* from Isla Santa Cruz. Only two additional steps were required to unite the insular "*C. atrox*" from Baja California with peninsular *C. ruber*. Thus, the associations should be considered tenuous.

The instability in relationships of "*C. atrox*" likely was not a spurious observation but rather reflected relative ages of isolation. Isla Tortuga is an oceanic, volcanic island apparently formed during the Holocene (Gastil et al., 1983). If *C. tortugensis* and the Isla Santa Cruz population of *C. atrox* are sister taxa (Figs. 2–6) and of Holocene origin, and if *C. tortugensis* was derived from mainland *C. atrox*, then we would expect to observe an unambiguous association of all three species of "*atrox*" as has been observed for other reptiles on recently formed islands. Examples include *C.* "*exsul*"-*C. ruber* (Figs. 2–6; Murphy et al., 1995), Side-blotched Lizards (*Uta*; Upton and Murphy, 1997), Whiptails (*Cnemidophorus*; Radtkey et al., 1997), and Chuckwallas (*Sauromalus*; Petren and Case, 1997; for a summarization of data see Murphy and Aguirre, 2002a). Thus, it appears that either (1) *C. atrox* and *C. tortugensis* from Isla Santa Cruz are much older than the most recent volcanic rocks on Isla Tortuga, (2) they are sister species of the *C. ruber*-*C.* "*exsul*" group and all species in this clade had a Holocene divergence, (3) they have undergone extremely rapid DNA divergence, or (4) we have been mislead by unknown geographic variability in either *C. atrox* or *C. ruber*. Although more data are required for certainty, an older origin seems most likely. Notwithstanding, the rattlesnake on Isla Santa Cruz is both genetically (Figs. 2–6) and anatomically (R. Murphy, unpublished) distinctive. Species recognition is warranted, and such also removes paraphyly in the taxonomy of the *C. atrox* group. Paraphyly is acceptable in cases of peripheral isolation (Graybeal, 1995), but only if the sequence of multiple isolation events is faithfully reflected in the taxonomy (i.e., relatively recent peripheral isolates cannot be taxonomically recognized without also recognizing relatively older isolates).

Recognition of Species Groups

Non-monophyly of the *C. triseriatus* group is apparent, and the relationships among these groups of small, largely montane species are tenuous. Instability also occurs in the association of the *C. mitchellii*-*C. tigris* clade. Two additional taxa, *C. lannomi* and *C. stejnegeri*, cannot be confidently placed within any species group because we have no fresh tissue samples from either species. Notwithstanding, Brattstrom (1964) associated *C. stejnegeri* with *C. polystictus*, and thus it may also be closely associated with *C. cerastes*. The relationships of *C. lannomi* remain unknown.

If our preferred tree is correct, then we must recognize seven species groups (Table 6) to maintain monophyly of group membership in the rattlesnakes. Our study makes numerous departures from previous hypotheses. Changes in membership of *C. atrox* and *C. durissus* groups are unlikely. However, the *C. viridis* group might also contain *C. mitchellii* and *C. tigris*, as was observed when *C. horridus* was removed from the data set. Regardless of these concerns, the revised groupings serve as a working hypothesis.

Table 7. Comparison of various phylogenetic hypotheses for the rattlesnakes, including our global parsimony analysis using two species of *Agkistrodon* as the outgroup. Unresolved nodes (e.g., Stille, 1987) are allowed to have resolved relationships within groups for the shortest tree length possible. NT = number of rattlesnake species assigned to a given group; NTC = number of taxa in common with our study; TL = tree length required to explain our data for sets of taxa; MPT = length of most-parsimonious tree for NTC; CI = consistency index; RI = retention index; RC = rescaled consistency index; PTL = percent tree length increase required to explain the data; RR = relative rank based on PTL.

Hypothesis	NT	NTC	TL	MPT	CI	RI	RC	PTL	RR
Unweighted parsimony (this study)	33	33	3282	3270	0.36	0.54	0.19	0.4	1
Gloyd (1940)	23+	21[1]	2539	2396	0.39	0.50	0.19	8.2	4
Klauber (1956)	34	32[2]	3576	3215	0.33	0.45	0.15	11.2	6
Brattstrom (1964)	34	32[2]	3558	3189	0.33	0.43	0.14	11.6	7
Klauber (1972)	35	33	3706	3270	0.32	0.44	0.14	13.3	8
Foote and MacMahon (1977)	18	18[2]	2778	2505	0.36	0.43	0.16	10.9	5
Stille (1987)	30	31[3]	3463	3215	0.35	0.48	0.16	7.7	3
Knight et al. (1993)[4]	7	7	1051	1004	0.57	0.56	0.32	4.7	2

[1]Excludes taxa of uncertain relationships, and *C. atrox* from Isla Santa Cruz. [2]Excludes *C. atrox* from Isla Santa Cruz. [3]Includes both *C. triseriatus* and *C. aquillus*, and excludes *C. atrox* from Isla Santa Cruz. [4]Dynamic weighted parsimony tree used for comparison.

Comparison of Hypotheses

We ranked other hypotheses of relationships (Table 7) based on the percent tree length change required to explain our data because of variance in the number of taxa in common, and the effect that varying numbers of taxa and characters have on indices of consistency. For the evaluation, those taxa listed as "uncertain relationships" by Gloyd (1940) were not included in the compared trees. Where ambiguity in interpreting phylogenies occurred, we always chose arrangements that most parsimoniously explained our data.

Among the previous hypotheses of rattlesnake relationships, Klauber (1972) fared worst against our analyses. His genealogical hypothesis required 436 additional steps, the largest single portion (60 steps) attributable to his placement of *C. catalinensis* as the sister species of *C. scutulatus*. His earlier phylogeny (Klauber, 1956) provided a far better estimate, but still required 361 additional steps. Other major increases in tree length were attributable to the close association of *C. cerastes* and *C. enyo*. Brattstrom's (1964) phylogeny was similar to Klauber's (1956, 1972) as reflected in a similar increase of 369 steps. Clearly, these scenarios do not provide an adequate explanation for the patterns of variability in our sequence data.

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