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Molecular Systematics and Biogeographical History of Pitvipers as Determined by Mitochondrial Ribosomal DNA Sequences

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Systematics of the snake subfamily Crotalinae (Serpentes: Viperidae) were investigated by means of mtDNA (12S and 16S rDNA sequences); 45 species, which included all genera, were sequenced and analyzed using both maximum parsimony (MP) and maximum likelihood (ML) as the optimality criteria. Differential a priori weighting methods were employed, because there was a transition bias within the data. All analyses support *Azemiops feae* as the sister to a monophyletic Crotalinae. The New World pitvipers are monophyletic, but the identification of their sister group is unclear. These data suggest a single invasion into North America, a subsequent north temperate and tropical divergence, and at least three invasions of South America. All currently proposed Central American genera (excluding *Crotalus*) are valid. *Crotalus* is paraphyletic with regard to *Sistrurus*, and *Bothrops* is paraphyletic with respect to *Bothriopsis*. Inclusion of *Porthidium melanurum* in *Ophryacus* is supported. The Old World *Protobothrops* is monophyletic and perhaps the most basal crotaline. This, along with New World monophyly, suggests that pitvipers evolved in Eurasia. All proposed Old World genera are monophyletic; thus, 19 genera are currently recognized within the Crotalinae.

CURRENTLY, the snake family Viperidae contains three subfamilies: the monotypic Azemiopinae, Viperinae (including *Causus*), and Crotalinae (Cadle, 1987; McDowell, 1987; Rage, 1987). Phylogenetic relationships among the subfamilies are controversial, immunological results placed *Azemiops* within or sister to the Crotalinae (Cadle, 1992), whereas mitochondrial sequence data placed *Azemiops* as the sister group to the Crotalinae (Knight and Mindell, 1993; Heise et al., 1995). The Crotalinae are characterized by a pair of heat-sensory loreal pits and are thought to be monophyletic (although see above). The subfamily contains approximately 160 species and make up 75–80% of the Viperidae (Hoge and Romano-Hoge, 1981). Most pitvipers are terrestrial, although arboreality (presence of a prehensile tail) has evolved several times, and at least one species is semiaquatic. There are both oviparous and viviparous pitvipers, and some closely related species differ in mode of reproduction. They inhabit temperate and tropical forests, as well as deserts, savannahs, and montane environments in Asia, North America, Central America, and South America.

Despite their ecological and morphological diversity and wide distribution, many systematic and biogeographic questions remain unresolved. Early systematists divided the subfamily into five to 10 genera (Brattstrom, 1964; Burger, 1971); however, in the last two decades 13 additional genera have been diagnosed. Five of the 19 genera currently recognized are monotypic, and seven genera have fewer than five species. Thus, the recent trend has been to define small, morphologically distinct monophyletic groups from within larger species groups (Campbell and Lamar, 1992; Crother, et al., 1992; Werman, 1992).

According to Brattstrom (1964), Burger (1971) and Gloyd, and Conant (1990), the New World pitvipers are polyphyletic, suggesting multiple invasions into the New World via the Bering Land Bridge. Kraus et al. (1996) using mitochondrial DNA (mtDNA) provide the first complete dataset addressing the intergeneric relationships and biogeographical implications of the Crotalinae, they included all proposed genera except *Ophryacus*, which is monotypic and probably did not affect their results. They determined that the New World pitvipers are monophyletic, suggesting a single immigration across the Bering Land Bridge. They also inferred, albeit not confidently, that the sister taxon to the New World pitvipers was *Protobothrops*. Thus, there are two major hypotheses as to the evolution of New World pitvipers, one of monophyly and one of polyphyly.

A region of the mitochondrial 16S ribosomal DNA gene (16S rDNA) and a region of the mitochondrial 12S ribosomal DNA gene (12S rDNA) were studied, as well as the nucleotide base composition and transitional bias of these two gene regions to investigate the intergeneric relationships and the biogeographical history of the viperid subfamily Crotalinae. Specific questions addressed include the following: (1) Is

Azemiops the sister group of the Crotalinae, as proposed by Cadle (1992) and Knight and Mindell (1993)? (2) Are the New World crotalines monophyletic, as suggested by Kraus et al. (1996)? (3) Are the genera Burger (1971) resurrected (*Bothriechis, Bothriopsis, Ophryacus,* and *Porthidium*) and proposed (*Ovophis*) monophyletic? (4) Is *Protobothrops* (Hoge and Romano-Hoge 1983) monophyletic and is it the sister group to a monophyletic New World assemblage as found by Kraus et al. (1996)?

Materials and Methods

DNA isolation, PCR, and sequencing.—*Bitis arietans* and *Causus defilippii* were used as outgroup taxa for all analyses, whereas the ingroup consisted of 45 crotaline taxa. Voucher data for taxa are listed under Materials Examined or can be obtained from the author's Web site (http://sunflower.bio.indiana.edu:/~cparkins). Total DNA isolation and PCR of the 16S rDNA region was accomplished as described in Parkinson et al. (1997). PCR of the 12S rDNA region was accomplished as in Knight and Mindell (1993). Direct sequencing of the 12S PCR product was carried out using the CircumVent thermal cycle sequencing kit (New England BioLabs, Beverly, MA) or the Applied Biosystems FS automated cycle sequencing kit (Applied Biosystems Intl., Foster City, CA). The products of the CircumVent sequencing reactions were separated at approximately 1800 V in 6% polyacrylamide, 7 M urea, 50 cm × 21 cm × 0.4 mm gels (Jordan Scientific, Bloomington, IN). The gels were dried and autoradiography was performed for 2–4 days. The products from the Applied Biosystems reactions were separated on an Applied Biosystems 377 automated sequencer (Applied Biosystems Intl., Foster City, CA). Certain PCR products (*Azemiops feae* 16S, *Bothrops insularis* 16S, *Ophryacus undulatus* 16S, *Sistrurus ravus* 16S, *Bothrops cotiara* 12S, *Crotalus atrox* 12S, *Ovophis okinavensis* 12S) could not be sequenced directly. These PCR products were cloned via the pCR-TRAP cloning kit (Genhunter Corp., Nashville, TN) or by the TA cloning kit (Invitrogen, Palo Alto, CA) following the manufacturer's directions. The clones were either sequenced using the Sequenase version Plasmid Sequencing kit (USB Corp., Cleveland, OH) or directly sequenced using the Applied Biosystems FS automated cycle sequencing kit.

Alignment and sequence evolution.—Gutell's (1994) proposed secondary structures of mitochondrial 12S and 16S rDNA sequences from *Bos taurus* were used as guides for alignment following the protocol of Kjer (1995); Kjer's nomenclature is also used throughout the paper. The 12S rDNA alignment was also improved by comparing it to the general structural model proposed in Hickson et al. (1996). Using MEGA version 1.01(S. Kumar, K. Tamura, and M. Nei, MEGA: Molecular Evolutionary Genetics Analysis, Pennsylvania State Univ., University Park, 1993, unpubl.), transitional bias, nucleotide base composition, and mutational class types were calculated. The transition:transversion (ti:tv) ratios were estimated and plotted against divergence values. Absolute numbers of transitions and transversions were calculated using MEGA; these were then standardized for sequence length variation and plotted against divergence values.

Phylogenetic analysis.—The data were subjected to both maximum-parsimony (MP) analyses and maximum-likelihood (ML) analyses using PAUP* version 4d59 (D. L. Swofford, PAUP: Phylogenetic Analysis Using Parsimony, Sinauer Associates, Sunderland, MA, 1997, unpubl.) and fastDNAml version 1.06 (Olsen et al., 1994). The MP searches were conducted using the heuristic search option, random addition sequences with 100–1000 replications, and tree bisection reconstruction (TBR) branch swapping. The ML analyses employed the F84 model, input order was randomized (using the jumble option), and global swapping across all nodes was implemented (using the global option). The ti:tv ratio was input into the fastDNAml file using the T-option; it was estimated from the data using the HKY model in the PUZZLE program (Strimmer and von Haeseler, 1996). While determining positional homologies, certain positions could not be aligned based on the proposed secondary structures; thus, these were deemed unalignable and excluded from all analyses. Characters were also excluded if they had 10 or more changes/steps on preliminary trees, because these were deemed excessively homoplasious. This was tested by excluding each individual character and performing a phylogenetic analysis, measuring the increase in the consistency index (CI). At nine steps, the increase in CI was not measurable; thus, all characters that had 10 or more changes were excluded.

Various a priori weighting schemes have been suggested for rDNA data. Most of these involve down-weighting transitions (Brown et al., 1982; Knight and Mindell, 1993, 1994); some advocate excluding transitions totally (Cracraft and Helm-Bychowski, 1991), whereas others use differential weighting of stems versus loops in rRNA (Wheeler and Honeycutt, 1988; Hillis and

Dixon, 1991; Dixon and Hillis, 1993). For this study, three different weighting schemes were used: equal weighting; weighting transversions twice that of transitions (ti:tv = 1:2); and weighting transversions four times that of transitions (ti:tv = 1:4). When more than a single tree was found, a strict consensus tree was generated. A Kishino-Hasegawa test (K-H test, Kishino and Hasegawa, 1989), as implemented in PAUP*, was also used to try to determine whether there were significant statistical differences between topologies. The base frequencies, ti:tv ratio, proportion of invariable sites and gamma shape parameter were estimated from the data during optimization for each topology during the K-H test.

To assess the stability of internal nodes, I employed bootstrapping (Felsenstein, 1985; Hillis and Bull, 1993). A bootstrap analysis based on 100 replications was conducted using PAUP* on all datasets using parsimony. For bootstrapping via maximum likelihood, the bootstrapped datasets were generated using the SEQBOOT program in PHYLIP version 3.5c.(J. Felsenstein, PHYLIP, Univ. of Washington, Seattle, 1993, unpubl.). These were then analyzed using the "P4" code, which distributes the computational task over 63 nodes in parallel on an Intel Paragon supercomputer. The bootstrap values were then calculated using the CONSENSE program of PHYLIP version 3.5c. The bootstrap proportions were not interpreted as probabilities of the node being true but as a relative ranking of the degree of support for a node (Reeder, 1995). An a posteriori weighting method, successive character approximation, was also applied (Farris, 1969; Carpenter, 1988) to test the stability of internal nodes based on the equally weighted data. This method was implemented in PAUP* by reweighting the characters based on their maximum rescaled consistency index (Farris, 1989).

Results

Alignment.—Sequences used in this paper have been deposited in Genbank under the accession numbers AF057185–AF057278. Structurally aligned data are available on the author's Web site. Of 925 characters, 405 sites were variable. Of those, 256 sites were informative under the criterion of parsimony. The largest gap was seven positions, in the 12S rDNA gene of *Deinagkistrodon acutus*, and this indel resulted in the loss of a partial loop structure in helix 28. There are five four-position gaps within the dataset. Two are located in the 12S rDNA gene within helix 42 of *Bothriechis lateralis* and *B. nigroviridis*,

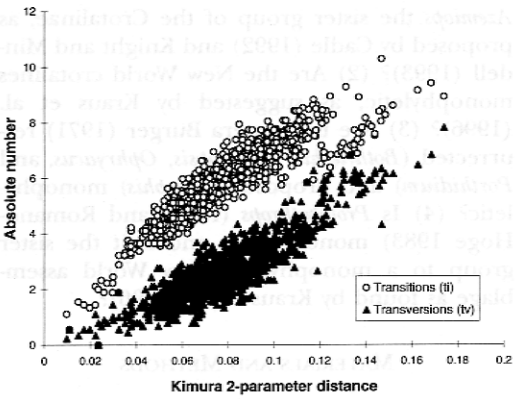

Fig. 1. Standardized absolute numbers of transitions and transversions, plotted against Kimura 2-parameter distance values. Data were standardized by dividing the absolute number of transitions and tranversions by their respective sequence length, excluding gaps, and then multiplying by 100.

creating a synapomorphy. The third occurs in *Azemiops feae* and the last two in outgroup taxa within the 16S rDNA region. In *Causus*, the gap occurs between the proposed helices 84 and 88. In *Bitis* and *Azemiops*, the gap occurs between the proposed helices 83 (which is questionable) and 84. Characters 283, 316, 700, 701, and 724–727 were removed from all phylogenetic analyses because determination of positional homology was tenuous. Characters (25, 57, 142, 210, 214, 220, 252, 258, 314, 595, 596, 645, 713, 723, 746, 756, 758, 760, 873, and 907), which had more than 10 steps, were also removed from the analyses by the method described above. In total, 28 of 925 sites were excluded from analyses.

Sequence evolution.—Percent base composition for sequenced regions of the L-strand are as follows: adenine (A) ranged from 34.6–36.5, $\bar{x}$ = 35.4; thymine (T) 20.6–23.8, $\bar{x}$ = 22.8; cytosine (C) 22.6–24.0, $\bar{x}$ = 23.9; guanine (G) 17.1–18.2, $\bar{x}$ = 17.8. Thus, base composition of the L-strand is somewhat A-rich. This bias has also been reported for certain fishes (Alves-Gomes et al., 1995) and lizards (Reeder, 1995).

Six classes of base substitution were tabulated from the data. There seems to be a transition bias in these data as seen from plotting absolute numbers of transitions and transversions versus percent divergence (Fig. 1). This is largely due to the abundance of T↔C ($\bar{x}$ = 34) and A↔G ($\bar{x}$ = 18) changes. In all of the comparisons, A↔T ($\bar{x}$ = 9.6) and A↔C ($\bar{x}$ = 10) transversions were more abundant than T↔G ($\bar{x}$ = 1) or C↔G ($\bar{x}$ = 2.1) transversions. Transition:transversion ratios for the aligned dataset were plot-

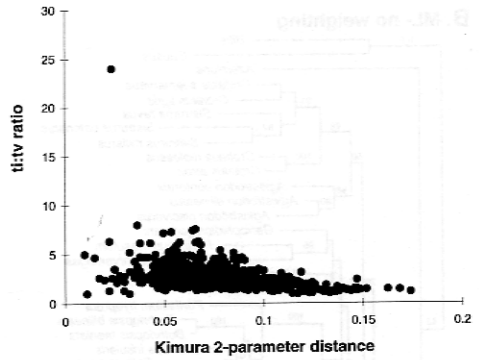

Fig. 2. Plot of transition:transversion ratio against Kimura 2-parameter distances, for all taxa.

ted against corrected divergence values (Fig. 2). The lowest ti:tv ratio (0.946) was between *Causus* and *Sistrurus catenatus*, and the highest was between the two species of *Atropoides* (24.0). The largest divergence was between *Causus* and certain members of the ingroup (~17%), whereas the lowest was between two species within *Bothriopsis* (~1%).

Unweighted analyses of the total dataset.—An initial MP heuristic analysis with 100 random-addition searches of the equally weighted characters (excluding sites mentioned above and gaps) yielded six equally parsimonious trees (1176 TL, CI = 0.44, RC = 0.23, RI = 0.52), and a strict consensus is shown in Figure 3A. Using a K-H test, it was determined that tree number one was the best topology (−ln likelihood = 7151.9: range 7151.9–7157.6). The topology is the same as Figure 3B; however, there were no significant statistical differences between any of the MP topologies. An ML analysis utilizing global swapping across all branches, jumbled input order, and a ti:tv ratio of 3.61 yields a single tree with a −ln-likelihood score of 7848.513 (Fig. 4B).

Weighted analyses of the total dataset.—A priori weighting of the transversions twice that of transitions (ti:tv = 1:2) under a random addition heuristic search (100 times) yielded a single MP tree (TL = 1520; Fig. 3B); weighting the transversions four times that of transitions (ti:tv = 1:4) under a MP random addition heuristic search (1000 times) yielded 24 trees (TL =

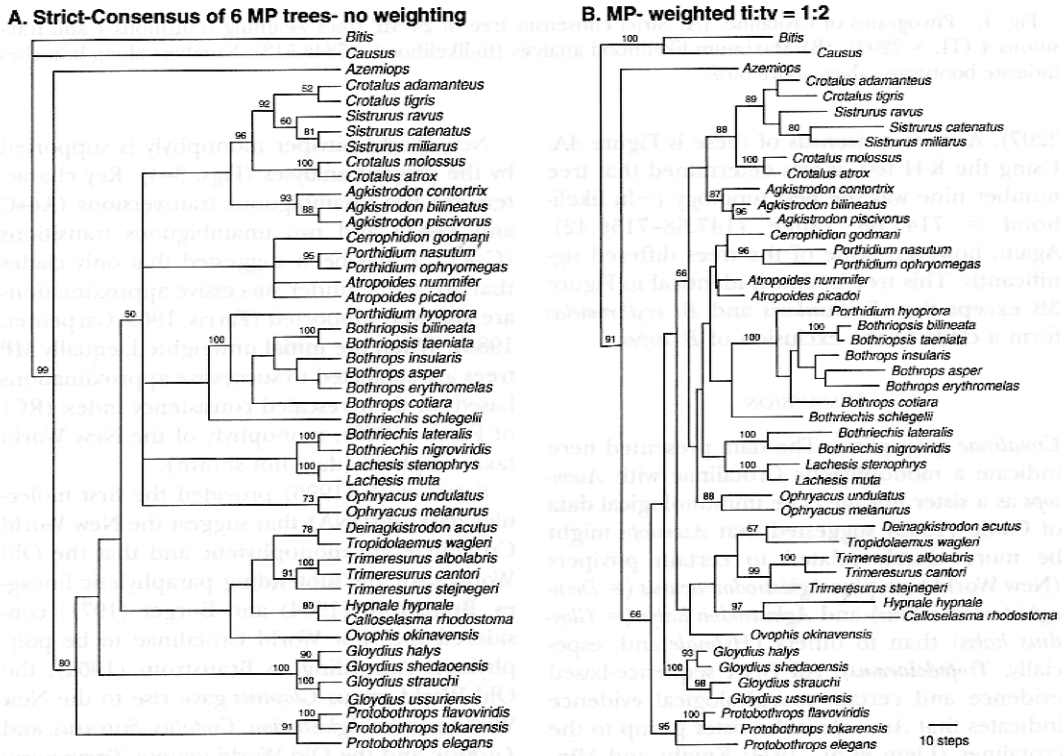

Fig. 3. Phylograms of Crotalinae (A) Strict consensus tree of 6 MP trees, all characters equally weighted. (B) Maximum-parsimony analysis weighting transitions (ti) 1 and transversions (tv) 2 (TL = 1520). Numbers along branches indicate bootstrap values above 50%.

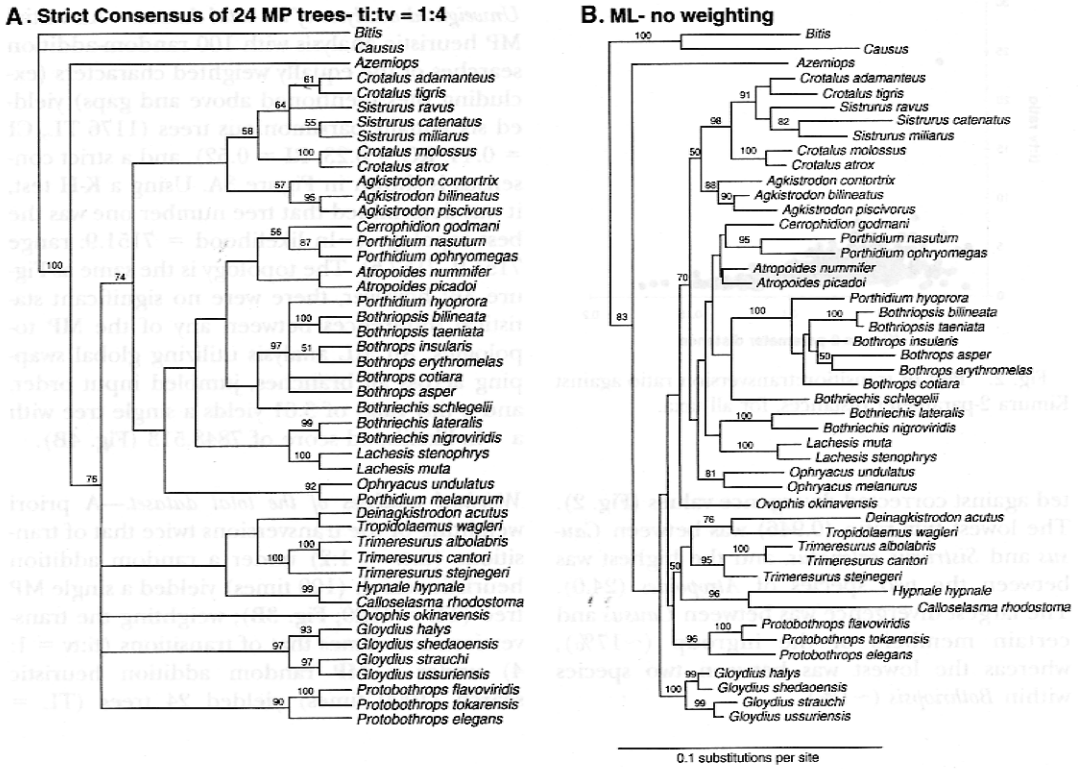

Fig. 4. Phyograms of Crotaline. (A) Strict consensus tree of 24 MP trees weighing transitions 1 and transitions 4 (TL = 2207). (B) Maximum likelihood analysis (ln-likelihood −7848.513). Numbers along branches indicate bootstrap values above 50%.

2207). A strict consensus of these is Figure 4A. Using the K-H test, it was determined that tree number nine was the best topology (−ln likelihood = 7147.68, range 7147.68–7156.42). Again, however, none of the trees differed significantly. This tree is almost identical to Figure 3B except that *B. insularis* and *B. erythromelas* form a clade to the exclusion of *B. asper.*

Discussion

Crotalinae systematics.—The data presented here indicate a monophyletic Crotalinae with *Azemiops* as a sister lineage. The immunological data of Cadle (1992) suggested that *Azemiops* might be more closely related to certain pitvipers (New World taxa plus *Agkistrodon acutus* (= *Deinagkistrodon acutus*) and *Agkistrodon halys* (= *Gloydius halys*) than to others (*Hypnale* and, especially, *Tropidolaemus*). All DNA sequence-based evidence and certain morphological evidence indicates that *Azemiops* is the sister group to the crotalines (Liem et al., 1971; Knight and Mindell, 1993; Heise et al., 1995); thus the immunological data are inconsistent with the sequence data.

New World pitviper monophyly is supported by the present analyses (Figs. 3–4). Key characters are two unambiguous transversions (A↔C and A↔T) and two unambiguous transitions (C↔T). It has been suggested that only clades that are stable under successive approximations are also well supported (Farris, 1969; Carpenter, 1988). When the initial unweighted, equally MP trees are subjected to successive approximations based on their rescaled consistency index (RC) of Farris (1989), monophyly of the New World taxa is upheld (data not shown).

Kraus et al. (1996) provided the first molecular data (mtDNA) that suggest the New World Crotalinae are monophyletic and that the Old World taxa are subtending paraphyletic lineages. Brattstrom (1964) and Burger (1971) considered the New World Crotalinae to be polyphyletic. According to Brattstrom (1964), the Old World genus *Gloydius* gave rise to the New World genera *Agkistrodon, Crotalus, Sistrurus,* and *Lachesis*; and the Old World genera *Trimeresurus* (including *Tropidolaemus*) gave rise to the bothropoid genera (*Atropoides, Bothriechis, Bothriopsis, Bothrops, Cerrophidion, Ophryacus,* and *Porthi-*

dium). According to Burger (1971), there were two lineages with New World connections: one gave rise to *Agkistrodon, Sistrurus, Crotalus, Porthidium,* and *Bothriechis* and the Old World *Hypnale* and *Calloselasma,* whereas the second gave rise to *Ophryacus, Bothriopsis, Bothrops,* probably *Lachesis,* and the Old World *Ovophis,* and *Trimeresurus* (including *Tropidolaemus*).

Although two studies using mtDNA sequences have found that the New World pitvipers are monophyletic, compelling evidence for their sister group is lacking. The genera *Trimeresurus, Deinagkistrodon, Tropidolaemus, Hypnale, Calloselasma,* and *Ovophis* are found as their sister group in the equally weighted and ti:tv 1:2 weighted MP analyses (Fig. 3A–B). Resolution is lost in the strict consensus tree for the ti:tv 1:4 analysis (Fig. 4A), but in the "best" topology, as determined using the K-H test, this relationship is supported.

Crotalus and Sistrurus (rattlesnakes) relationships.—All analyses support a monophyletic *Agkistrodon* which is sister to the rattlesnakes (*Crotalus* and *Sistrurus*). Rattlesnakes form a monophyletic group supported by fairly high bootstrap values and by 11 synapomorphies plus the unique rattle on the tip of their tail. However, *Crotalus* are paraphyletic with regard to a monophyletic *Sistrurus* in all analyses.

Monophyly of *Sistrurus* was questioned with regard to *S. ravus* (McCranie, 1988; Stille, 1987; Knight et al., 1993). The genus is based on nine large symmetrical head scales versus small, fragmented scales in *Crotalus.* The head plate condition of *Sistrurus* is present in many colubrids, elapids, and viperids; thus, it is considered the pliesiomorphic condition in pitvipers (Klauber, 1972; Campbell and Solorzano, 1992). Furthermore, there is wide variability (i.e., whole or fragmented) in the nine head plates (as defined by Klauber, 1972, including internasals, prefrontals, frontals, supraoculars, and parietals) within and between pitviper taxa. Nine head plates are found in *Agkistrodon, Calloselasma, Dienagkistrodon, Gloydius,* and *Sistrurus,* whereas other pitviper genera have various levels of fragmentation. *Trimeresurus macrolepis* has large head plates, but most members of this genus posses highly fragmented head scales (Smith, 1943).

Knight et al. (1993), concluded that *Crotalus* and *Sistrurus* are monophyletic sister taxa; however, they also presented a topology based on MP using equally weighted characters (1993: 364, Fig. 3A.), where *Crotalus* is paraphyletic in respect to *Sistrurus.* They dismissed *Crotalus* paraphyly based on morphology, "because this scenario would require loss and subsequent reacquisition of an identical arrangement of nine large head scales" (p. 363). The present analyses do not support their conclusion (that *Crotalus* and *Sistrurus* are sister taxa) but indicate that *Crotalus* is paraphyletic with regard to *Sistrurus.* However, both datasets do support the monophyly of *Sistrurus.* Therefore, these data imply that either *Sistrurus* reevolved the primitive head scutellation or multiple lineages of *Crotalus* have independently evolved small head scales.

Bothrops (sensu Burger).—The New World *Bothrops* has been divided into seven genera, (*Atropoides, Bothrops, Bothriopsis, Bothrechis, Cerrophidion, Ophryacus, Porthidium*), and I refer to them as the "bothropoid" genera. Although considerable confusion exists regarding this large and ecologically diverse assemblage, several tentative suggestions can be made from the molecular data.

The genus *Bothrops* (sensu stricto) consists of approximately 31 species found both in Central and South America. I examined one Central American species and three South American species. Because of this limited representation, it is inappropriate to comment on interspecies relationships. *Bothrops,* however, appears paraphyletic with regard to *Bothriopsis.* The two species of *Bothriopsis* (*bilineata* and *taeniata*) cluster together and fall within the genus *Bothrops.* The problem remains whether *Bothriopsis* should be given generic status.

Bothriopsis, the forest pitviper, contains seven or eight species, most of which are arboreal and are primarily found in South America (Campbell and Lamar, 1989, 1992), although *Bothriopsis punctata* ranges into southern Panama. On the basis of distribution and certain morphological characters, Burger (1971) proposed that it be maintained as a separate genus. This arrangement has been accepted by subsequent authors (Pérez-Higareda et al., 1985; Campbell and Lamar, 1989, 1992). The validity of this genus has been questioned numerous times (Schätti et al., 1990; Salomão et al., 1997; Vidal et al., 1997). *Bothriopsis* was synonymized into *Bothriechis* by Schätti et al. (1990) because they found no evidence to warrant separation. Campbell and Lamar (1992) refuted this arrangement and cited characters delimiting the genera. In none of the phylogenies presented here does *Bothriopsis* group with *Bothriechis;* thus, claims by Schätti et al. (1990) and Schätti and Kramer (1991) are not substantiated.

Werman (1992) indicated that *B. taeniatus* (*taeniata*) fell within the South American *Bothrops* group but suggested that more evidence is

needed before taxonomic changes could be justified. Using immunological distances, Cadle (1992) showed that *B. taeniata* was closer to *Bothrops atrox* than was *Bothrops alternatus*. Kraus et al. (1996) found that *Bothriopsis bilineata* is sister to the two *Bothrops* in one analysis but lies within *Bothrops* in a reweighted analysis. Because they used only three taxa to represent this large genus, their results may be due to taxon sampling. *Bothrops* and *Bothriopsis* were also found to be sister taxa by Vidal et al. (1997); however, only a single species of each was used. Salomão et al. (1997) found *Bothrops* to be paraphyletic with regard to *B. taeniata* using 595 bp of cytochrome *b* sequence data, and they synonymized *Bothriopsis* with *Bothrops* citing numerous reasons. My data support Salomão et al. (1997), that is, a monophyletic *Bothriopsis* within a paraphyletic *Bothrops*. *Bothriopsis* is valid based on both morphological (Burger, 1971; Campbell and Lamar, 1989) and molecular data. Because the genus *Bothrops* is large and contains many ecologically diverse taxa, other monophyletic lineages within this group may be illuminated upon further investigation. More studies on the genus *Bothrops*, both molecular and morphological, are needed before more taxonomic changes are proposed.

The Central American members of the genus *Porthidium* form a clade in all analyses, with *Cerrophidion godmani* as their sister taxon. Werman (1992) described *Atropoides*, the jumping vipers, based on allozymes and morphology. Kraus et al. (1996) did not find this genus to be monophyletic; however, the data presented here support Werman's findings. Interestingly, the only member of the genus *Porthidium* to be found east of the Andes is *P. hyoprora*, which is restricted to the Amazon basin (Campbell and Lamar, 1989). This taxon groups with members of the genus *Bothrops* in all analyses, and its placement is highly supported. Kraus et al. (1996) found this same result and hypothesized that the upturned snout morphology may be due to convergence.

Werman (1992) examined allozymes, isozymes, and morphology, whereas Kraus et al. (1996) used DNA sequence data from the mitochondrial ND4 gene to investigate bothropoid phylogeny. Kraus et al. (1996) included all proposed genera within the Crotalinae except *Ophryacus*, whereas Werman (1992) included only New World taxa and divided his analyses into two parts, one for the Central American forms and one for the South American forms. Thus, Werman (1992) did not explicitly test the monophyly of the bothropoid genera but stated "*Bothrops* (sensu lato) is most likely monophyletic. . ." (p. 33). Kraus et al.'s (1996) analyses do not support a monophyletic bothropoid group, and my data concur with this finding. This is due to the disconcerting observation that *Lachesis* seems to be the sister group of *Bothriechis*. Previously, *Lachesis* was assumed to be a basal New World crotalid.

The genus *Ophryacus* was monotypic until Gutberlet (1998) transferred *Porthidium melanurum* into it based on considerable morphological evidence. The present analyses support this finding. Werman (1992) placed *B. undulatus* (*O. undulatus*) as the sister group of *Bothriechis* but stated that more data are needed to fully understand the position of this species. *Ophryacus* was used as the outgroup to polarize character states by Crother et al. (1992) in a study of *Bothriechis*. Gutberlet (1998), using only morphology, found this relationship as well. This relationship is not supported by the present analyses. *Ophryacus undulatus* and *O. melanurus* occur as the sister group to the bothropoid genera plus *Lachesis*. The generic status of *Ophryacus* is upheld, and the inclusion of *Porthidium melanurum* into *Ophryacus* is supported. However, the phylogenetic position of the genus is still in question.

The three members of the genus *Bothriechis* do not form a monophyletic group. Two species (*B. lateralis* and *B. nigroviridis*) consistently group together, but the third, *B. schlegelii*, groups as a basal member of the genus *Bothrops*. When one resamples the dataset for bootstrap analysis, the three taxa group together between 18% and 33% of the time. These bootstrap values are low, and they indicate that there is homoplasy within the dataset even after removing certain characters. Crother et al. (1992) found that *Bothriechis* is indeed monophyletic and consists of seven species, *B. schlegelii* being the most basal member of the group.

Trimeresurines.—This is a complex of Old World forms for which the present data enable me to comment on three possible relationships. The genus *Trimeresurus* contains approximately 30 species. I have only included three members, and they form a clade that is well supported with high bootstrap values. This may be misleading, and until better taxon sampling is done, generic monophyly cannot be addressed.

The genus *Protobothrops* was proposed by Hoge and Romano-Hoge (1983) for a group of long, slender snakes previously assigned to *Trimeresurus* (sensu lato). They erected the genus based on scale microstructure and the shape of the palatine, maxillary, ectopterygoid, and prefrontal bones. This genus had not been recog-

nized in the past, although Kraus et al. (1996) found evidence for its validity, as do the analyses presented herein. Hoge and Romano-Hoge (1983) placed *T. flavoviridis, T. jerdoni,* and *T. mucrosquamatus* into *Protobothrops.* Kraus et al. (1996) found that *T. elegans* should also be included. Support for this arrangement is found in my analyses as is the inclusion of *T. tokarensis.*

Burger (1971) proposed the genus *Ovophis* for four species of stout, terrestrial, egg-laying members of the genus *Trimeresurus* (sensu lato). In my study, material was only available from *O. okinavensis,* which appeared as the sister taxon to a group containing *Trimeresurus, Tropidolaemus, Deinagkistrodon, Hypnale,* and *Calloselasma* in the MP analyses. However in the ML analysis, *Ovophis* was the sister group to the New World Crotalinae. These results indicate that more data are needed before a reliable phylogenetic placement of *Ovophis* may be determined.

The monotypic genus *Tropidolaemus* was considered a divergent pitviper (Brattstrom, 1964; Burger, 1971). This arboreal species is found on the Asian mainland and on many islands of southeastern Asia. It is highly variable geographically and sexually dimorphic. In all analyses, *T. wagleri* groups with *Deinagkistrodon acutus,* the hundred-pace viper of China and Taiwan. This relationship seems problematic, because these two taxa are very morphologically distinct. However, this relationship was also found in the ND4 analyses of Kraus et al. (1996).

The "Agkistrodon complex."—Kraus et al. (1996), Parkinson et al. (1997), Vidal et al. (1997), and the present study do not support Gloyd and Conant's (1990) "*Agkistrodon* complex." A large body of data supports separating the New World and Old World *Agkistrodon* into two genera (*Agkistrodon* for the New World and *Gloydius* for the Old World). Kraus et al. (1996) found *Gloydius* to be the sister group of a clade containing *Ovophis, Calloselasma, Hypnale, Trimeresurus.* The equally weighted and ti:tv 1:2 weighted MP analyses indicate that *Gloydius* is the second most basal lineage of all pitvipers (excluding *Protobothrops*), whereas the ML analysis indicates that *Gloydius* is the most basal pitviper. More data are required to fully resolve this issue.

Gloyd and Conant (1990) suggested that *Calloselasma rhodostoma* is the sister taxon to *Hypnale* based on many morphological characters. Kraus et al. (1996), Parkinson et al. (1997), and this study support this conclusion. The *Calloselasma–Hypnale* clade is sister to the *Trimersurus* clade in all phylogenies presented by Kraus et al. (1996), whereas my data place them as the sister group to ((*Deinagkistrodon, Tropidolaemus*) *Trimeresurus*). Five synapomorphies, including two transversions and three transitions, unite these two groups.

Biogeography.—The goal in phylogenetic and biogeographical studies is to elucidate the evolutionary history, both in terms of species relatedness and present-day distributional patterns. Pitviper distribution spans three continents; their proposed center of origin is Eurasia. Thus, one or more migration events across the Bering Land Bridge have been hypothesized followed by subsequent north temperate divergence and migration into tropical Middle and South America (Brattstrom, 1964; Burger, 1971; Kraus et al., 1996). A monophyletic relationship for New World pitvipers was proposed by Kraus et al. (1996), whereas Brattstrom (1964) and Burger (1971) hypothesized multiple dispersal events into the New World. In my analyses, the New World Crotalinae form a monophyletic group; the node is supported by four synapomorphies, two transversions, and two transitions. Monophyly is also supported by a successive approximations analysis (data not shown); thus, I corroborate Kraus et al.'s (1996) conclusions. If the New World crotalines are monophyletic, the most-parsimonious hypothesis indicates a single emigration into the New World without reinvasions back into the Old World.

My data support a monophyletic North American group (*Agkistrodon, Crotalus,* and *Sistrurus*) and a monophyletic Neotropical group (bothropoid genera + *Lachesis*). Building on the most-parsimonious hypothesis, at least three invasions into South America have taken place: (1) *Crotalus durissus;* (2) proto-*Lachesis;* and (3) proto-*Bothrops.* The phylogenetic relationships suggest that *Crotalus durissus* migrated into South America from the north and that its present distribution is a retraction of a more widespread distribution during the time when drier savanna habitat predominated (Hoogmoed, 1982; Cadle, 1987). *Lachesis* is attributed to a South American faunal assemblage using vicariance biogeography. Zamudio and Greene (1997) point out that Central American *Lachesis* could be members of the initial tropical assemblage that colonized from the north; however, more data are needed to test this hypothesis. Two other invasions may have occurred—one for the two species of *Porthidium* (*lansbergii* and *nasutum*) presently distributed in South America and another for *Bothriechis schlegelii,* which is found in northern South America. However, due to the lack of data, I am unable to comment on these hypotheses.

This scenario explains current large-scale dis-

tributional patterns but fails to account for the high species diversity and the large ecological and morphological differences exhibited by this subfamily. Such understanding will be gained as more species of pitvipers are added to the phylogeny.

Materials Examined

Institutional abbreviations are listed in Leviton et al. (1985). Personal abbreviations are as follows: Cadle (John Cadle, DNA number); CLP (Chris Parkinson, field tag); FK (Fred Kraus, field tag); MOODY (Scott Moody, field tag); RG (Nelson da Silva, Xingó Hydroelectric Project field tag).

Agkistrodon bilineatus, Tamaulipas, Mexico: CLP 140. *Agkistrodon contortrix*, Fox Lake Dam, Waterloo Tsp., Athens Co., OH, US: MOODY 338.

Agkistrodon piscivorus, SC., US: CLP 30. *Atropoides nummifer*, Costa Rica: CLP 168. *Atropoides picadoi*, Varablanca, Costa Rica: MZUCR 11156.

Azemiops feae, China: CLP 157.

Bitis arietans, Togo, West Africa: no number.

Bothrops asper, Costa Rica: MZUCR 11152. *Bothrops cotiara*, Brazil: no number. *Bothrops erythromelas*, Piranhas, Alagóas, Brasil: RG 829. *Bothrops insularis*, Isla Queimada Grande, Sao Paulo State, Brazil: no number.

Bothriechis lateralis, Acosta, Costa Rica: MZUCR 11155. *Bothriechis nigroviridis*, San Gerondo de Dota, Costa Rica: MZUCR 11151. *Bothriechis schlegelli*, Cariblanco de Sarapique, Costa Rica: MZUCR 11149. *Bothriopsis bilineata smaragdina*, Leticia, Amozonas, Columbia: No number. *Bothriopsis taeniata*, Suriname: No number.

Calloselasma rhodostoma, UTA-R 22247.

Causus defilippii, Africa: CLP 154.

Cerrophidion godmani, Las Numbres de Coronado, Costa Rica: MZUCR 11153.

Crotalus adamanteus, Flagler Estates, St. Johns Co., FL, US: CLP 4. *Crotalus atrox*, Boy Scout Ranch rd., Jeff Davis Co., TX, US: CLP 64. *Crotalus molossus*, El Paso Co., TX, US: CLP 66. *Crotalus tigris*, Pima Co., AZ, US: CLP169.

Deinagkistrodon acutus, China, CLP 28.

Gloydius halys caraganus, Kazakhstan: no number. *Gloydius shedaoensis*, Snake Is., Liaoning, PRC: ROM 20468. *Gloydius strauchi*, Waqie Sichuan, PRC: ROM 20473. *Gloydius ussuriensis*, Kouqian, Jilin, PRC: ROM 20452.

Hypnale hypnale, near Columbo, Sri Lanka: CLP 164.

Lachesis stenophrys, Limon, Costa Rica: No number. *Lachesis muta*, Peru: Cadle 135.

Ophryacus melanurus, Captive born, UTA R-34605. *Ophryacus undulatus*, Mexico: CLP 73.

Ovophis okinavensis, Okanawa, Japan: CLP 162.

Porthidium nasutum, Costa Rica: MZUCR 11150. *Porthidium hyoprora*. Letícia, Columbia: No number. *Porthidium ophryomegas*, Guanacaste, Costa Rica: UMMZ 210276.

Protobothrops elegans, Ishigaki Is., Ryukyu Is., Japan: UMMZ 199970. *Protobothrops flavoviridis*, Tokunoshima, Ryukyu Is., Japan: UMMZ 199973. *Protobothrops tokarensis*, Takarajima, Ryukyu Is., Japan: FK 1997.

Sistrurus catenatus, near Stanford Lake, Haskell Co., TX. US: MOODY 502. *Sistrurus miliarus*, Ft. Meyers, FL., US: UTA live collection. *Sistrurus ravus*, Zapotitlán, Puebla, Mexico: UTA live collection.

Trimeresurus albolabris, Yim Tin Tsi., Port Shelter Is., Hong Kong: MCZ R177966. *Trimeresurus cantori*, Kamurta, Nicobar Is., India: No number. *Trimersurus stejnegeri*, Yanmingshan National Park, Taipei, Taiwan: UMMZ 190532.

Tropidolaemus wagleri, West Kalimantan, Borneo; CLP 141.

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