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5 Phylogenetic relationships of the '*Agkistrodon* complex' based on mitochondrial DNA sequence data

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Synopsis

We investigated phylogenetic relationships among 15 species of pitvipers commonly known as the '*Agkistrodon* complex' or the tribe Agkistrodontini. We sequenced a 505 base-pair region of the mitochondrial 16S ribosomal DNA for all genera of this group, including all three New World species of the genus *Agkistrodon* and four members of the Old World *Agkistrodon*, to test the monophyly of this genus. Results indicate that *Agkistrodon* is paraphyletic, therefore the genus *Gloydius* should be used for the Old World members. The genera *Calloselasma, Deinagkistrodon* and *Hypnale* are separate lineages, thus their status as genera is upheld. The tribe Agkistrodontini is not valid and the name should be suppressed.

Introduction

Snakes of the family Viperidae are united by possession of a solenoglyphous dentition used for venom delivery. These vipers are usually grouped in the Caenophidia or advanced snakes. The family Viperidae has three recognized subfamilies: Azemiopinae, for the monotypic species *Azemiops feae*; Viperinae, for the true vipers and the enigmatic genus *Causus*; and the Crotalinae, for the pitvipers (Liem, Marx & Rabb 1971; Cadle 1988, 1992). The Viperinae are found in Africa and Eurasia, and are argued to be a monophyletic assemblage (Ashe & Marx 1988). The Crotalinae are recognized by most to be monophyletic, although this has recently been questioned by Cadle (1992).

The main synapomorphy of the subfamily Crotalinae is the heat-sensing loreal pit. However, this organ or a functionally similar system has evolved more than once (e.g. in boas and pythons) (Cock Buning 1985). Moreover, Cadle (1992: 44), using immunological data, suggested that *Azemiops* might fall within the subfamily Crotalinae, thus suggesting that the Crotalinae may be paraphyletic. If this is the case, then either a secondary loss of the loreal pit occurred in *Azemiops* or multiple

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evolution of this character is required to explain these relationships. Recently two papers based on sequence data from the mitochondrial (mt) 12S and 16S ribosomal DNA genes (rDNA) suggested that *Azemiops* is the sister group to the Crotalinae, rather than being a member of the Crotalinae (Knight & Mindell 1993; Heise *et al.* 1995). The same DNA sequences for *Azemiops, Vipera* and *Coluber* were used in both studies; the only differences were in alignment, analyses and number of taxa (larger in the Heise *et al.* 1995 study). Thus the phylogenetic position of *Azemiops* is still in question. Here we subscribe to the conservative view that *Azemiops* is not part of the Crotalinae, but could be their sister taxon. In this light we believe the Crotalinae to be a monophyletic group composed of 17–20 genera, depending on taxonomic judgments.

Generic descriptions and systematic problems

Snakes of the genus *Agkistrodon* and the putatively allied genera (*Calloselasma, Deinagkistrodon* and *Hypnale*) were grouped in the subfamily Agkistrodontinae of the family Crotalidae by Burger in his unpublished dissertation (1971). This group was later changed to the tribe Agkistrodontini of the Crotalinae by Hoge & Romano-Hoge (1978/79). This group has been loosely known as the *Agkistrodon* complex (Gloyd & Conant 1990). The Agkistrodontini have recently received much study, both by traditional morphological methods (Gloyd & Conant 1990; Kardong 1990; Malnate 1990) and by molecular techniques (Campbell & Whitmore 1989; Minton 1990; Knight, Densmore & Rael 1992; Porter, Haiduk & de Queiroz 1994). Although these studies have been important, a comprehensive molecular phylogenetic analysis is warranted. Using nucleotide sequence data generated from a region of the mitochondrial 16S rDNA we address the following questions. (1) Is the genus *Agkistrodon* (*sensu stricto*) monophyletic, i.e. what is the status of the genus *Gloydius*? (2) Are the genera *Calloselasma, Deinagkistrodon* and *Hypnale* valid, and if so, what are their relationships to each other and to other pitvipers? (3) What is the relationship of the genus *Trimeresurus* (representing other Old World taxa) to the Agkistrodontini? (4) What is the status of the tribe Agkistrodontini?

The genus *Agkistrodon* (*sensu lato*) presently contains three New World species (*A. bilineatus, A. contortrix* and *A. piscivorus*) while the Old World group contains approximately 10 species (depending on personal preferences): *A. blomhoffii, A. caliginosus, A. halys, A. himalayanus, A. intermedius, A. monticola, A. saxatilis, A. shedaoensis, A. strauchi, A. tsushimaensis, A. ussuriensis* (Gloyd & Conant 1990; Zhao & Adler 1993 and references therein; Isogawa, Moriya & Mitsui 1994). Three taxa which were traditionally placed within this genus have recently been removed and given generic rank: *Calloselasma* (Cope 1860; revived by Chernov 1957), *Deinagkistrodon* (Gloyd 1979), *Hypnale* (Fitzinger 1843; resurrected by Gloyd 1977). *Calloselasma* and *Deinagkistrodon* are monotypic while *Hypnale* contains three species.

The genus *Agkistrodon* Palisot de Beauvois is diagnosed by the following characters (summarized from Gloyd & Conant 1990); (1) deep loreal pit, (2) large

venom glands in temporal region of both sides of the head, (3) maxillary bone bearing large canaliculate fangs, (4) hemipenis deeply bifurcate, (5) choanal process wide, bearing a keel-like process on the dorsal side of the palatine bone, (6) postorbitals in contact or near the frontals, (7) nostril centred between two nasal scales, (8) postoculars and suboculars combined range from 2–5, (9) ventrals range from 127–178, (10) subcaudals range from 26–71, generally paired in the Old World forms whereas they are single in New World forms.

Chernov (1957) resurrected the generic name *Calloselasma* Cope for *Agkistrodon rhodostoma* on the basis of osteological characters. This genus is characterized by the following: (1), (2) and (3) same as above, (4) hemipenis deeply bifurcate, almost entirely covered with enlarged spines on the base of the lobes, and distal two-thirds of the lobes covered with densely packed flounces, (5) choanal process of the palatine very elongate, usually with one tooth, (6) dorsal scales smooth and not keeled, (7) m. retractor pterygoidei inserting at just two sites: anterior pterygoid and medial process of the ectopterygoid, (8) a distinct m. pterygoideus glandulae present.

Gloyd (1979) described the genus *Deinagkistrodon* for the species known as *Agkistrodon acutus* on the basis of the following characters: (1), (2) and (3) are the same as in *Agkistrodon*, (4) hemipenis deeply bifurcate, (5) choanal process of the palatine strongly curved inward and elongate, (6) dorsal scales strongly keeled, (7) tip of snout upturned, formed by greatly enlarged rostral and internasal scales, (8) origin of the m. levator anguli oris lying posterior to that of the m. pseudotemporalis with no overlap of their origins, (9) m. pterygoideus glandulae partially distinct.

The fourth genus, *Hypnale*, was resurrected by Gloyd in 1977 and has the following characteristics: (1), (2) and (3) are the same as in *Agkistrodon*, (4) hemipenis deeply bifurcate, may or may not have proximal spines, closely resembles that of *Calloselasma*, (5) choanal process of the palatine low or absent, (6) dorsal scales weakly keeled, (7) tip of snout upturned in two species and slightly in the third, (8) m. pterygoideus glandulae distinct.

Based on the above morphological characters and some molecular data, numerous phylogenetic hypotheses have been proposed. Gloyd & Conant (1990) in their monograph on the *Agkistrodon* complex did not favour separating Old and New World species; however, they did suggest some relationships between taxa. They listed many characters (Gloyd & Conant 1990: 408) for an affinity between *Calloselasma* and *Hypnale*, although they also stated that there are many differences, especially in the shape of the palatine bone. Brattstrom (1964) did not differentiate the group (*Agkistrodon, sensu lato*) into separate genera, instead classifying all as *Agkistrodon*. He hypothesized that the New World *Agkistrodon* are polyphyletic, having a lineage of *A. bilineatus, A. piscivorus* and the Old World taxon *A. acutus* (*Deinagkistrodon acutus*), while *A. contortrix* is grouped with the rest of the Old World taxa (i.e. all other *Agkistrodon, Calloselasma* and *Hypnale*). Burger (1971) in his doctoral dissertation revised the Crotalinae and placed them as the family Crotalidae. He suggested that all of the Old World species be included in the genus *Hypnale*.

He referred to the New World *Agkistrodon* as a group, while lumping *acutus* and *rhodostoma* into *Calloselasma*. Burger suggested, in a phylogenetic sense, that his *Hypnale* (members of the Old World *Agkistrodon* and *Hypnale*) gave rise to *Calloselasma* and other pitviper genera. *Hypnale* was hypothesized to have crossed the Bering land-bridge, giving rise to the New World *Agkistrodon* and *Porthidium*. Burger, however, never published his dissertation.

Recently, researchers have turned to molecular systematics to help them to understand the questions which have arisen from the morphological hypotheses. Campbell & Whitmore (1989), using epidermal keratins, hypothesized that the agkistrodontines form a monophyletic group. Relationships within the group, however, were uncertain. Their data suggest that New World *Agkistrodon* is polyphyletic; that *Calloselasma* and *Deinagkistrodon* are not closely related; and that Old World *Agkistrodon* may be the sister group of *A. bilineatus*. They did not study *Hypnale*. Knight *et al.* (1992) examined restriction fragment polymorphisms and a partial sequence of the 16S rDNA. Their results indicate monophyly of the New World *Agkistrodon* (*sensu stricto*) and monophyly of the Old World *Agkistrodon* (*sensu stricto*) but not monophyly of the *Agkistrodon* (*sensu lato*) nor a sister-group relationship. They did not, however, suggest that the genus should be split, owing to problems in their data.

The generic status of *Calloselasma* and *Deinagkistrodon* is supported, although *Hypnale* was not included in the Knight *et al.* (1992) study. The validity of *Calloselasma, Deinagkistrodon* and *Hypnale* is supported by immunoelectrophoretic results of Minton (1990), although he does not provide a scenario of relationships among the three genera. Minton states that the New World *Agkistrodon* are closer to each other than to members of the Old World *Agkistrodon*.

Materials and methods

DNA purification, amplification and sequencing

DNA was extracted from red blood cells, soft tissues (i.e., liver, heart), or from shed skins (see specimens examined for voucher data) following procedures found in Knight & Mindell (1993). The 16S ribosomal sequences were amplified with the primers L2510 (5′-CGCCTGTTTATCAAAAACAT-3′) and H3059 (5′-CCGGTCTGAACTCAGATCACGT-3′). The primers are designated L for light strand and H for heavy strand and their positions identified with the 3′ ends corresponding to published human sequences (Anderson *et al.* 1981). A third primer, L2932 (5′-ATCGAAACAAGTTACTCCAG-3′), is an internal primer used only in the cycle sequencing reactions. Polymerase chain reaction (PCR) was accomplished by using *Taq* polymerase and its reaction buffers from Perkin Elmer/Cetus. Final $MgCl_2$ concentration was 3.4–3.9 mM. Thermal cycling was carried out in a Perkin Elmer/Cetus 480 thermal cycler. Thermal cycle parameters were 94°C, 35 s/45°C, 35 s/72°C, 1 min for 35 cycles. Samples were then held at 4°C until removed and frozen.

Twenty-five microlitres of the PCR products from amplification were purified by electrophoresis in 2.0% low-melting agarose (Sea Plaque) and visualized by ethidium bromide staining. The bands of interest were cut out with a sterile razor blade and

purified by using Wizard PCR Prep kits (Promega) following the manufacturer's instructions.

Direct sequencing of the PCR product was carried out with the CircumVent thermal cycle sequencing kit (New England BioLabs) using internal incorporation of α ^{35}S nucleotides. After cycle sequencing, 2.5 µl of the products were separated at *c.* 1800 V in 6% poly-acrylamide, 7 M urea, 50 cm × 21 cm 0.4 mm gels (Jordan Scientific). They were dried and autoradiographs were exposed for 2–4 days.

Data alignment and sequence statistics

DNA alignment is tedious and difficult to accomplish with a non-coding gene owing to the fact that one can not use the protein sequence as the template. The 16S region was aligned by using the multiple sequence alignment program CLUSTAL V with the default settings (Higgins & Sharp 1989; Higgins, Bleasby & Fuchs 1992). After alignment, changes were made by eye to minimize gaps and transversions using ESEE (Cabot & Breckenback 1989). Nucleotide composition, number of supposed transitions (ts), supposed transversions (tv), both pairwise (p distance) and corrected pairwise distances (both Jukes and Cantor and Kimura's 2 parameter) of the L-strand were calculated by using MEGA (Kumar, Tumara & Nei 1993). We plotted the transition/transversion ratio against both uncorrected and corrected pairwise distances to ascertain the role of substitutions in our data set.

Phylogenetic analyses

The aligned data were subjected to maximum parsimony (MP) analyses using PAUP 3.1 (Swofford 1993). Numerous weighting schemes have been developed for MP analyses of rDNA sequences. These range from weighting transversions greater than transitions (Brown *et al.* 1982; Knight & Mindell 1993, 1994), weighting stems differently than loops (Wheeler & Honeycutt 1988; Hillis & Dixon 1991; Dixon & Hillis 1993), using the transversions only (Cracraft & Helm-Bychowski 1991), and not weighting at all (Nedbal, Allard & Honeycutt 1994; Heise *et al.* 1995). For this study we chose to analyse all the data unweighted and by weighting the transversions twice as much as the transitions (tv 2 : ts 1). Gaps were either excluded, or added as a fifth character, or coded at the end of the sequence matrix as a non-additive binary matrix (Swofford 1993). A heuristic search with additive tree bisection (10 replications) was performed on the different data sets (three gap methods and weighted data tv 2 : ts 1). Bootstrap (Felsenstein 1985) resampling (500 replications) was also performed to test node support on the weighted data, again using the heuristic option with additive tree bisection (10 replications).

Results

The aligned data are shown in Fig. 1. A total of 138 sites were variable out of a total of 505 bases. Of these 68 sites were informative under the criteria of parsimony. The largest gap was two sites. The region between sites 300–338 is highly variable: 17 of 138 variable sites occur here. Base composition is: adenine (A) ranged from 33.9–36.3,

$\bar{x}$ = 35.2; thymine (T) 22.9–27.1, $\bar{x}$ = 25.2; cytosine (C) 20.2–22.2, $\bar{x}$ = 21.5; guanine (G) 17.3–18.8, $\bar{x}$ = 18.1. The number of supposed transitions ranged from 10 to 28; transversions from four to 36. The highest values were between the outgroups and certain members of the ingroup. The lowest values were between the two species of *Crotalus*. The transition: transversion ratio generally decreases as divergence between taxa increases. This follows the Brown *et al.* (1982) theory that transition saturation increases as divergence increases.

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                                                                                                  6
                                                                                                  0
Bitis                ATATTAAAGG  CAACGCTGCC  CAGTGAACAA  TTAAACGGCC  GCGGTATCCT  AACCGTGCAA
Echis                G......G..  ..........  .......T..  ..........  ..........  ..........
C. adamanteus        G.........  ..........  .......A..  ..T.......  ......C...  ..........
C. tigris            G.........  ..........  .......A..  ..T.......  ......C...  ..........
S. catenatus         G.........  ..........  .......A..  ..T.......  ......C...  ..........
B. asper             G.........  .G........  .......A..  ..........  ..........  ..........
A. contortrix        G.........  .TT.......  .......A..  ..........  ..........  ..........
A. bilineatus        G.........  .G........  .......A..  ..........  ..........  ..........
A. piscivorus        G.........  .G........  .......A..  ..........  ..........  ..........
A. intermedius       G.........  ..........  .......T..  ..........  ..........  ..........
A. shedaoensis       G.........  ..........  .......T..  ..........  ..........  ..........
A. strauchi          G.........  ..........  .......T..  ..........  ..........  ..........
A. ussuriensis       G.........  ..........  .......T..  ..........  ..........  ..........
Deinagkistrodon      G.........  .G........  .......T..  ..........  ......C...  ..........
H. hypnale           G.........  .G........  .......T..  ..........  ..........  ..........
Calloselasma         G.........  .G........  .......T..  ..........  ......C...  ..........
T. flavoviridis      G.........  .G........  ........T.  ..........  ..........  ..........

                                                                                                  1
                                                                                                  2
                                                                                                  0
Bitis                AGGTAGCATA  ATCATTTGTC  TATTAATTGT  AGACCCGTAT  GAAAGGCAAT  ATGAGAGCCT
Echis                ..........  ..........  ..........  .....T....  .........CC  ..........
C. adamanteus        ..........  ..........  ..........  .....A....  .........A  .........C
C. tigris            ..........  ..........  ..........  .....A....  A........A  .........C
S. catenatus         ..........  ..........  ..........  .....A....  .........A  .........C
B. asper             ........C.  ..........  ..........  .....T....  .........A  .........C
A. contortrix        ..........  ..........  ..........  .....A....  .........A  .........C
A. bilineatus        ..........  ..........  ....CC....  .....T....  .........A  .......T.C
A. piscivorus        ..........  ........A.  ..........  .....T....  .........A  .......T.C
A. intermedius       ..........  ..........  ..........  .....T....  .........A  .........C
A. shedaoensis       ..........  ..........  ..........  ..T..T....  .........A  .........C
A. strauchi          ..........  ..........  ..........  .....T....  .........A  .........C
A. ussuriensis       ..........  ..........  ..........  .....T....  .........A  .........C
Deinagkistrodon      ..........  ..........  ..........  .....T....  .........A  .....G..T.
H. hypnale           ..........  ..........  ..........  .....T....  .........A  .....G.T.C
Calloselasma         ........C.  ....C.....  ..........  .....T....  .........A  ....A....C
T. flavoviridis      ..........  ..........  ..........  .....T....  .........A  .........C

                                                                                                  1
                                                                                                  8
                                                                                                  0
Bitis                AACTGTCTCT  TATAACAAAT  CAATTAAACT  GATCTCCCAG  TACAAAAGCT  GGGATACCCA
Echis                ..........  ..........  ..........  .......T..  ..........  A.A..TA.AC
C. adamanteus        ..........  .....T....  ..........  .......T..  ..........  A.A....TA.
C. tigris            ..........  .....T....  ..........  .......T..  ..........  A.A....TA.
S. catenatus         G.........  .....T....  ..........  .......T..  ..........  AA...TTTAT
B. asper             ..........  ..........  ..........  .......T..  ..........  A.A....TA.
A. contortrix        ..........  ..........  ..........  .......T..  ..........  A.T...TTAT
A. bilineatus        ..........  ..........  ..........  .......T..  ..........  A.A...TTA.
A. piscivorus        ..........  ..........  ..........  .......T..  ..........  A.A...TTA.
A. intermedius       ..........  ..........  ..........  .......T..  .C........  A.A....TA.
A. shedaoensis       ..........  ..........  ..........  .......T..  .C........  A.A....TA.
A. strauchi          ..........  ..........  G.........  .....T.T..  .C........  A.A.....A.
A. ussuriensis       ..........  ..........  ..........  .....T.T..  .C........  A.A...TTT.
Deinagkistrodon      ..........  ...GG.....  ..........  .......T..  ..........  A.A.....A.
H. hypnale           ..........  ..........  ..........  .......T..  ..........  A.A.....A.
Calloselasma         G.........  ..........  ....A.....  .......T..  ..........  A.A..GTTA.
T. flavoviridis      ..........  ..........  ..........  .......T..  ..........  A.A....TA.
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                                                                                                  2
                                                                                                  4
                                                                                                  0
Bitis                CATAAGACCA  GAAGACCCTG  TGAAGCTTTA  ACTAACCTAT  TAAACCTCAT  AATAGCTAGT
Echis                ..........  ..........  ..........  .T...A....  ......C...  ....A...A.
C. adamanteus        T.........  ..........  ..........  ..........  .......AG.  ....A...C.
C. tigris            ..........  ..........  ..........  .T...A....  .......A..  ....AT..C.
S. catenatus         ..........  .TT.......  ..........  ..........  ......CT..  ....A...C.
B. asper             ..........  ..........  ..........  .........C  ......CA..  ........C.
A. contortrix        ..........  ..........  ..........  ..........  ...C...AT.  ....A...C.
A. bilineatus        ..........  ..........  ..........  ..........  ......AA..  ........C.
A. piscivorus        .........T  ..........  ..........  .....A....  ......AAG.  ........CC
A. intermedius       ..........  ..........  ..........  ..........  ......CA..  ....A...C.
A. shedaoensis       ..........  ..........  ..........  ..........  ......CA..  ....A...C.
A. strauchi          ..........  ..........  ..........  .....A....  ......CA..  ........C.
A. ussuriensis       ..........  ..........  ..........  ..........  .......A..  ....A...C.
Deinagkistrodon      ..........  ..........  ..........  .AA.......  ....T.AA..  ....A...C.
H. hypnale           ..........  ..........  ..........  ..A.......  ......AA..  ........C.
Calloselasma         ..........  ..........  ..........  .TA.......  ....T.AA..  ....A..TT.
T. flavoviridis      ..........  ..........  ..........  ..A...T...  ......CA..  ....AT..C.

                                                                                                  3
                                                                                                  0
                                                                                                  0
Bitis                TTCGGTTGGG  GCGACCTTGG  AATAAAAAAG  AACTTCCAAA  AAACATGACC  TTCCTCATAT
Echis                ..T.......  ..........  ..-...G..A  .........-  -.CA.GC...  .--...C.GC
C. adamanteus        ..........  ..........  ..........  .........C  --.T....A-  ..-......A
C. tigris            ..A.......  ..........  ..........  .........T  --.T.....-  ..A......A
S. catenatus         ..........  ..........  ..C.......  .........T  --.TG....-  ........TA
B. asper             ..A.......  ..........  ..C.......  .........C  --CT.....-  .C.A.....A
A. contortrix        ..........  ..........  ..........  .........T  --.T.....-  .........A
A. bilineatus        ..........  ..........  ..........  .........T  --.T.....-  .........A
A. piscivorus        ..T.......  ..........  ..........  .........T  --.T.....-  .........A
A. intermedius       ..........  ..........  ..........  .........T  --.T.....-  -........G
A. shedaoensis       ..........  ..........  ..........  .........T  --.T.....-  -........A
A. strauchi          ..........  ..........  ........GA  .........-  C-.T.....-  -........A
A. ussuriensis       ..........  ..........  ..........  .........T  C-.T.....-  -.T......A
Deinagkistrodon      ..........  ..........  ..C.......  .........C  --.......-  ........TA
H. hypnale           ..A.......  ..........  ..........  .........C  ..TT.....-  -........A
Calloselasma         ..T.......  ..A.......  ..........  .........C  ..-T.....-  -........A
T. flavoviridis      ..........  ..........  ..........  .........C  --.T.....-  ....A....A

                                                                                                  3
                                                                                                  6
                                                                                                  0
Bitis                AA--AAGGCG  GACAGGCCAC  AATTA-GACC  CAGAACTTCT  GATAAATGAA  CCAAGTTACT
Echis                .CCT.....T  A...A...T.  C.AATA....  ...C.AAG..  ...TG.....  ..........
C. adamanteus        ..TT-....A  A...A....A  C.CC......  ...C..AG..  ..C..T....  A.........
C. tigris            ..T.-....A  ....A....A  C.CC......  ...C.TAG..  .....T....  A.........
S. catenatus         ..C.-.....  .........A  T.CA.....A  ...C..AG..  .....T....  AT........
B. asper             .........A  A...A....A  C.C.......  ...C..AG..  ..C..TC...  A.........
A. contortrix        .-.C.....A  A...A....A  T.........  ...C.TAG..  .....T....  AT........
A. bilineatus        ..TC.....A  A...A....A  T.........  ...T.AAG..  .....TC...  AT........
A. piscivorus        .-CC......  A...A....A  T.........  ...T.TAA..  .....TC...  AT........
A. intermedius       G.C..-...A  A...A.....  .C-.......  ...T.T.A..  .....T....  AT........
A. shedaoensis       G.C..-...A  A...A.....  .C-.......  ...T.T.A..  .....T....  AT........
A. strauchi          ..C......A  ....A.....  .C-A......  ...T.T.A..  .....T....  AT........
A. ussuriensis       ..T......A  A...A.....  .C-.......  ...T.T.A..  ...G.T....  AT........
Deinagkistrodon      ..T.-....A  A..GA....A  C.CCT...T.  ...T..AA..  ..........  A.........
H. hypnale           ..CT.....A  A...C.....  ..AC......  ...T.TAG..  ..A..T....  A.........
Calloselasma         ..T......A  A...C...G.  ..A..T....  ...C..GG..  ..GT.C....  AT........
T. flavoviridis      ....C....A  T...A...TA  C.C.......  ...C..AG..  .....T....  AT.......A
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                                                                                                  4
                                                                                                  2
                                                                                                  0
Bitis                CCAGGGATAA  CAGCGCTATC  TTCTTTAAGA  GCCCATATCA  AA-AAGAAGG  TTTACGACCT
Echis                ..........  ..........  .....C....  ..........  ..........  ..........
C. adamanteus        ..........  ..........  .....C....  ..........  ..C.......  ..........
C. tigris            ..........  ..........  .....C....  ..........  ..C.......  ..........
S. catenatus         ..........  ..........  .....C....  .T........  ..........  ...C......
B. asper             ..........  ..........  ..........  ..........  ..........  ..........
A. contortrix        ..........  ..........  ..........  ..........  ..........  ..........
A. bilineatus        ..........  ..........  ..........  ..........  ..........  ..........
A. piscivorus        ..........  ..........  ..........  ..........  ..........  ..........
A. intermedius       ..........  ..........  ......T...  ..........  ..........  ..........
A. shedaoensis       ..........  ..........  ......T...  ..........  ..........  ..........
A. strauchi          ..........  ..........  ......T...  ..........  ..........  ..........
A. ussuriensis       ..........  ..........  ......T...  ..........  ..........  ..........
Deinagkistrodon      ..........  ..........  .....C....  ..........  ..........  ..........
H. hypnale           ..........  ..........  .....C....  .T........  ..........  ..........
Calloselasma         ..........  ..........  .....C....  ..........  ..........  ..........
T. flavoviridis      ..........  ......C...  ..........  ..........  ..........  ..........

                                                                                                  4
                                                                                                  8
                                                                                                  0
Bitis                CGATGTTGGA  TCAGGACATC  CTAGCAATGC  AACAGTTACT  AAGGGTTCGT  TTGTTCAACG
Echis                ..........  ..........  ....T.....  ..........  ..A.......  ..........
C. adamanteus        ..........  ..........  .A..T.....  .G.C......  ..A.......  ..........
C. tigris            ..........  .......G..  .A..T.....  .G.C......  ..A.......  ..........
S. catenatus         ..........  ..........  .C..T.....  ...C......  ..A.......  ..........
B. asper             ..........  ........C.  .C..T.G...  ...C.C....  ..A.......  ..........
A. contortrix        ..........  ..........  .C..T.....  ...C......  ..A.......  ..........
A. bilineatus        ..........  ..........  .C..T.....  .G.C......  ..A.......  ..........
A. piscivorus        ..........  ..........  ....T.....  ...C......  ..A..AA...  ..........
A. intermedius       ..........  ..........  .C..T.....  .C.C......  ..A.......  ..........
A. shedaoensis       ..........  ..........  .C..T.....  .C.C......  ..A.......  ..........
A. strauchi          ..........  ..........  .C..T.....  ...C......  ..A.......  ..........
A. ussuriensis       ..........  ..........  .C..T.....  ...C......  ..A.......  ..........
Deinagkistrodon      ..........  ........C.  .C..T.....  .C.C......  ..A.......  ..........
H. hypnale           ..........  ..........  .C..T.....  .G.C......  ..A.......  ..........
Calloselasma         ..........  ........C.  .C..T.....  .G.C......  ..A.......  ..C.......
T. flavoviridis      ..........  ..........  .C..T.....  .G.C......  ..A.......  ..........

                                                   5
                                                   0
                                                   5
Bitis                ATTAATAGTC  CTACGTGATC  TGAGT
Echis                ..........  ......A...  .....
C. adamanteus        .....C....  ..........  .....
C. tigris            .....C....  ......T...  .....
S. catenatus         .....C....  .C........  .C...
B. asper             ..........  ..........  .....
A. contortrix        .....C....  ..........  .....
A. bilineatus        .....C....  ..........  .....
A. piscivorus        .....C....  ..........  .....
A. intermedius       ..........  ..........  .....
A. shedaoensis       ..........  ....T.....  .CT.A
A. strauchi          .....C....  ..........  .C...
A. ussuriensis       ..........  ..........  .T...
Deinagkistrodon      ..........  ..........  .....
H. hypnale           .....C....  ..........  .....
Calloselasma         ..........  ..........  .....
T. flavoviridis      .....C....  ..........  .T...
```

Fig. 1. Aligned mitochondrial 16S rDNA sequences (L-strand) of *Bitis arietans, Echis ocellatus, Crotalus adamanteus, C. tigris, Sistrurus catenatus, Bothrops asper, Agkistrodon contortrix, A. bilineatus, A. piscivorus, A. intermedius, A. shedaoensis, A. strauchi, A. ussuriensis, Deinagkistrodon acutus, Hypnale hypnale, Calloselasma rhodostoma* and *Trimeresurus flavoviridis*. A dot(.) indicates identity with *Bitis*. Dashes (–) indicate gaps inserted to maintain alignment. Sequences will be deposited in GENBANK.

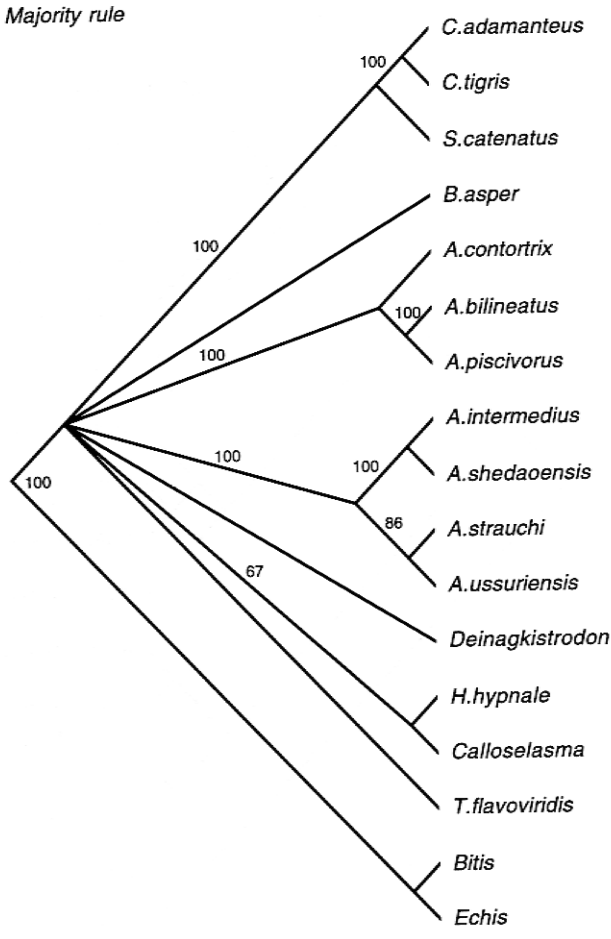

Fig. 2. Majority rules consensus tree of the 21 most parsimonious trees found treating gaps as: (1) missing = seven trees; (2) new-state = four trees; (3) nonadditive binary matrix = 10 trees. Nodal values indicate the percentage of times that node was represented out of 21 trees. *Bitis* and *Echis* were used as outgroups.

Comparing heuristic trees with no weighting for the three different gap methods reveals no clear-cut differences. Figure 2 is a 50% majority rules consensus tree of the 21 most parsimonious (MP) trees generated from the unweighted heuristic searches. Gaps were treated as: (1) missing; seven MP trees, length 280; (2) new-state; four MP trees, length 309; (3) nonadditive binary; 10 MP trees, length 296. For the weighted parsimony analyses we did not include gaps in the analyses. By weighting ts:tv 1:2 and using a heuristic search with additive tree bisection (10 replications) a single most parsimonious tree was found (Fig. 3) having a length of 400. A bootstrap analysis of the weighted data resulted in Fig. 4. The bootstrap values are low on certain nodes but groupings are consistent with the other trees presented here.

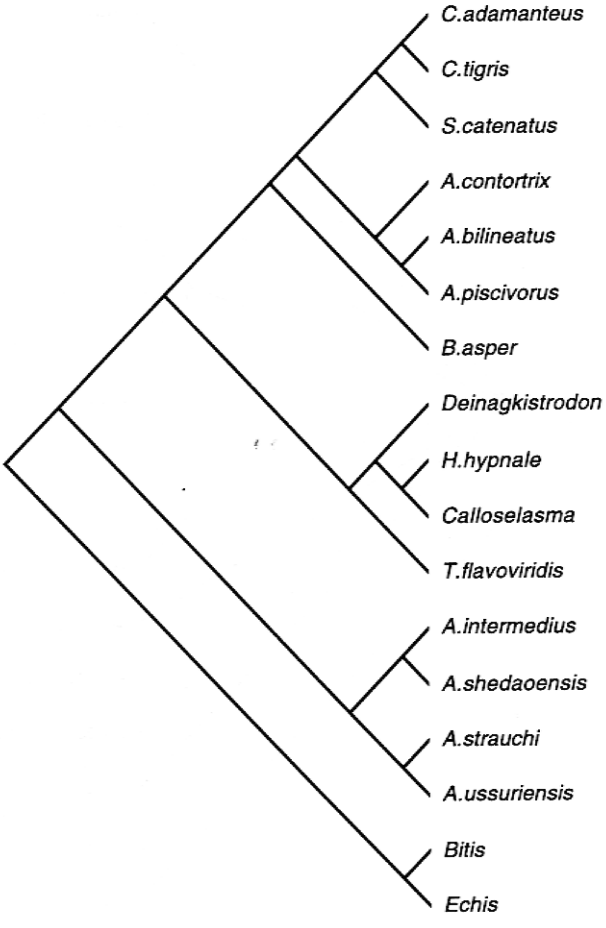

Fig. 3. Heuristic search, using random addition sequence and 10 replications, of the weighted data (tv 2 : ts 1) with *Bitis* and *Echis* as outgroups. Total length = 400.

Discussion

Comparing the three different trees we see that the main difference is in the placement of *Trimeresurus flavoviridis* and *Deinagkistrodon acutus*. The placement of *T. flavoviridis* (representing other Old World taxa) is not certain. In the weighted analysis it falls as the sister group of *Calloselasma*, *Deinagkistrodon* and *Hypnale*, but in the consensus trees its placement is in the basal polytomy of the pitvipers. *Deinagkistrodon* follows the same pattern: in the weighted analysis it is the sister group to the *Calloselasma/Hypnale* clade and in the consensus trees it falls within the basal polytomy of the pitvipers.

Unweighted heuristic searches of the data yielded 21 most parsimonious trees, depending on how gaps were considered. Seven MP trees resulted when gaps were treated as missing; four MP trees when gaps were added as a fifth character state; and 10 MP trees resulted when gaps were coded as non-additive binary characters. When these trees were compared, most of the discordance came from certain taxa

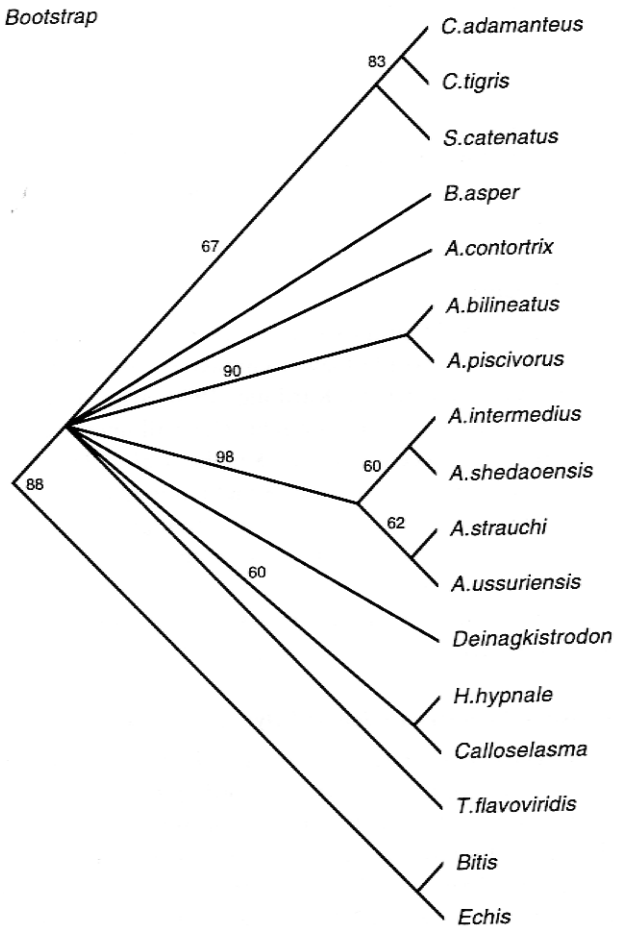

Fig. 4. Bootstrap analysis (using heuristic search, random addition sequence, and 10 replications per resampling) of the weighted data (tv 2 : ts 1) with *Bitis* and *Echis* as outgroups. Values at a node indicate percentage of times the node is represented out of 500 bootstrap replications.

(i.e. *Trimeresurus flavoviridis*, *Deinagkistrodon acutus*), but differences also arose from the placement of monophyletic lineages. By testing the three different gap modes for our data and building a consensus tree (Fig. 4) a better understanding of species groups is attained. Owing to the nature of consensus trees, resolution is lost when polytomies are formed. Thus, Figs 2 and 4 do not yield much information on higher phylogenetic relationships, with the exceptions of the *Crotalus/Sistrurus* and *Calloselasma/Hypnale* clades.

Many authors have used different weighting schemes for transversions and transitions (Brown *et al.* 1982; Knight & Mindell 1993; Alves-Gomes *et al.* 1995). The weighted tree gave greater resolution for deeper branches than the unweighted consensus trees. The possibility of near-transition saturation is evident by looking at the transition:transversion ratios. Thus by weighting the transversion higher, a more robust phylogeny is attained.

In traditional classifications the crotalids form a monophyletic group, and all *Agkistrodon* (*sensu lato*) are monophyletic. However, our data indicate (Fig. 3) that the New and Old World *Agkistrodon* do not form a monophyletic group nor are they sister groups. The New World pitvipers form a monophyletic group in relation to the Old World taxa. The data thus suggest that there was only a single immigration into the New World, but since all crotaline genera were not examined, they do not demonstrate it unequivocally.

Status of the genus *Gloydius*

Hoge & Romano-Hoge (1978/79) proposed a new generic name *Gloydius* for the Old World *Agkistrodon* based on morphological characters which they felt separated them from their New World congeners. Kardong (1990) argued that the characters which were used to diagnose the new genus might reflect allometric differences rather than taxonomic differences. A recent study by Knight *et al.* (1992), however, found taxonomic differences on a molecular level. Knight nonetheless stated that more conclusive data are needed to make the taxonomic change, thus did not side with Hoge & Romano-Hoge (1978/79). The data presented here and by Knight *et al.* (1992) are conclusive evidence of the validity of the genus *Gloydius*; we therefore support the change proposed by Hoge & Romano-Hoge (1978/79) of the generic name for the Old World *Agkistrodon* (*sensu stricto*) to *Gloydius*.

Validity of *Calloselasma*, *Deinagkistrodon* and *Hypnale*

In all analyses *Calloselasma*, *Deinagkistrodon* and *Hypnale* form separate evolutionary lineages, thus their validity as genera, according to these data, is supported. An interesting point to note is that in all analyses *Calloselasma* and *Hypnale* formed a clade. These data and the morphological characters presented by Groombridge (1986), Gloyd & Conant (1990), and Malnate (1990) suggest that *Calloselasma* is the sister taxon to *Hypnale*. This is very peculiar biogeographically as their ranges do not overlap at all, and indeed the two are separated by many miles and various mountain ranges. *Calloselasma* is found on the Malayan peninsula while *Hypnale* is found in the Western Ghats of India and on the island of Sri Lanka. Many other organisms have been found to share this distributional pattern (Smith 1931; Moody 1980; Nussbaum & Gans 1980). Hora (1949, 1953) proposed the 'Satpura hypothesis' as an explanation of these enigmatic distributions: migrating fauna followed the Satpura trend of mountains through peninsular India down into the Western Ghats region of India and onto the island of Sri Lanka during the Pleistocene. However, there are problems with his hypothesis, the major one being the Garo–Rajmahal Gap, in which two great rivers flow. Hora (1953) countered this assertion by stating that during the many glaciation events uplifting occurred, generating a bridge. The data presented here lend credence to the Satpura hypothesis, but more testing is needed.

Relationships of *Trimeresurus* to the Agkistrodontini and the status of the tribe Agkistrodontini

We used *Trimeresurus flavoviridis* to represent Old World crotalids not considered part of the '*Agkistrodon* complex'. This is very risky as we are using only one species to represent many taxa, thus these results lack stringency. In the weighted analysis (Fig. 3), *T. flavoviridis* forms the sister taxon to ((*Calloselasma, Hypnale) Deinagkistrodon*); in the consensus trees it falls within the basal polytomy. We suggest that the New World taxa are monophyletic with respect to the Old World taxa, and the tribe Agkistrodontini is invalid. If *Calloselasma, Deinagkistrodon* and *Hypnale* are the sister group to the other Old World genera (*Ovophis, Protobothrops, Trimeresurus, Tropidolaemus*) rather than to the genus *Gloydius*, the Agkistrodontini of Hoge & Romano-Hoge (1978/79) is paraphyletic. Additional data (C. L. Parkinson unpubl.) support these findings. F. Kraus (pers. comm.) is also in agreement with these results, supporting both the validity of the genus *Gloydius* and the paraphyly of the tribe Agkistrodontini.

The genus *Agkistrodon* formerly (Brattstrom 1964), included *Calloselasma, Deinagkistrodon, Hypnale* and *Gloydius* (Burger 1971; Chernov 1957; Gloyd 1977; and Hoge & Romano-Hoge 1978/79). It was diagnosed on the basis of a pitviper which has nine large symmetrical head shields and lacks a rattle. We suggest that these characters are pleisiomorphic at the generic level, and therefore new characters and character systems are needed. Molecular sequence data are just one system, which may be used as long as it is in conjunction with morphology. Many more data are needed in order to ascertain the phylogenetic relationships within this group of crotalids.

Specimens examined

Institutional abbreviations are listed in Leviton *et al.* (1985). Personal abbreviations are: CLP (Chris Parkinson, field tag), MOODY (Scott Moody, field tag).

Agkistrodon bilineatus — CLP 140. Mexico: Tamaulipas.
Agkistrodon contortrix — MOODY 338. US: OH, Athens Co., Waterloo Tsp.
Agkistrodon piscivorus — CLP 30. US: SC.
Bitis arietans — West Africa; Togo.
Bothrops asper — CLP 50. Costa Rica.
Calloselasma rhodostoma — UTA-R 22247. No locality data.
Crotalus adamanteus — CLP 4. US: FL, St. Johns Co., Flagler Estates.
Crotalus tigris — CLP169. US: AZ, Pima Co.
Deinagkistrodon acutus — CLP 28. China.
Echis ocellatus — MVZ 174468. West Africa: Ghana, Upper West Region.
Gloydius intermedius — UTA-R 16966. No locality data.
Gloydius shedaoensis — ROM 20468. PRC: Liaoning, Snake Is.
Gloydius strauchi — ROM 20473. PRC: Sichuan, Waqie.
Gloydius ussuriensis — ROM 20452. PRC: Jilin, Kouqian.

Hypnale hypnale — CLP 164. Sri Lanka: near Colombo.
Sistrurus catenatus — MOODY 502. US: TX, Haskell Co. near Stanford Lake.
Trimeresurus flavoviridis — UMMZ 199973. Japan: Ryukyu Is., Tokunoshima.

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