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A nesting of vipers: phylogeny and historical biogeography of the Viperidae (Squamata: Serpentes)

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Running title: Phylogeny of the Viperidae

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Abstract

Despite their medical interest, the phylogeny of the snake family Viperidae remains inadequately understood. Previous studies have generally focused either on the pitvipers (Crotalinae) or on the Old World vipers (Viperinae), but there has been no comprehensive molecular study of the Viperidae as a whole, leaving the affinities of key taxa unresolved. Here, we infer the phylogenetic relationships among the extant genera of the Viperidae from the sequences of four mitochondrial genes (cytochrome b, NADH subunit 4, 16S and 12S rRNA). The results confirm Azemiops as the sister group of the Crotalinae, whereas Causus is nested within the Viperinae, and thus not a basal viperid or viperine. Relationships among the major clades of Viperinae remain poorly resolved despite increased sequence information compared to previous studies. Bayesian molecular dating in conjunction with dispersal-vicariance analysis suggests an early Tertiary origin in Asia for the crown group Viperidae, and rejects suggestions of a relatively recent, early to mid-Tertiary origin of the Caenophidia.

Key Words

Viperidae; mitochondrial DNA; phylogeny; molecular dating; Bayesian inference
1. Introduction

The vipers (family Viperidae) comprise approximately 270 species of venomous snakes (David and Ineich, 1999; McDiarmid et al., 1999; Mallow et al., 2003). They are characterized by the possession of the mechanically most sophisticated venom apparatus among all snakes, consisting of fangs positioned on a mobile maxillary bone. As a result, the fangs are rotated back to lie along the roof of the mouth when the mouth of the snake is closed. This mechanism has allowed vipers to evolve particularly long fangs, and represents the most sophisticated venom delivery system known in snakes, which is associated with their life history as predominantly sedentary ambush predators feeding on relatively large prey (Pough and Groves, 1983; Greene, 1992). The same sophisticated venom delivery system has also resulted in vipers being the most medically important group of venomous snakes, with a few species (e.g., Bitis arietans, Echis spp., Daboia russelli and D. siamensis, Bothrops spp.) being responsible for the overwhelming majority of snakebite mortality and morbidity in their respective distributions (Warrell, 1995a,b, 2004).

A robust phylogenetic hypothesis is a fundamental requirement for research into venom composition and the improvement of antivenom therapy for snakebite. Variation in venom composition occurs at all taxonomic levels (Chippaux et al., 1991), but the elucidation of causes of this variation requires a phylogenetic framework (Daltry et al., 1996). This is of applied importance, since variability in venom composition can influence the effectiveness of antivenoms and the treatment of snakebite victims (Warrell, 1995a; Theakston et al., 1995; Harrison et al., 2003; Fry et al., 2003a).
Within the Viperidae, four main clades are usually recognized: two putatively basal taxa, *Azemiops* and *Causus*, and the bulk of the Viperidae. The latter are divided into two major subfamilies, the Viperinae (Old World or pitless vipers) and the Crotalinae (pitvipers).

*Azemiops*, a viper with many primitive traits, had long been regarded as a basal viperid (Liem et al., 1971; Groombridge, 1986), and often assigned to its own subfamily, the Azemiopinae. However, a number of recent studies have provided evidence that *Azemiops* may be the sister taxon of the Crotalinae (Cadle, 1992; Heise et al., 1995), but always with limited sampling of either viperines, or both vipers and crotalines.

*Causus* is also generally regarded as a primitive viper, and has usually been placed either at the base of the Viperinae (Herrmann and Joger, 1995, 1997) or even as the sister group to all other viperids bar *Azemiops* (Groombridge, 1986). Moreover, it has often been classified in a separate subfamily, the Causinae (e.g., McDiarmid et al., 1999). Several studies have taken the basal position of *Causus* for granted and used the genus as an outgroup for analyses of the Viperinae (e.g., Lenk et al., 2001), or even the Viperidae as a whole (Castoe and Parkinson, 2006). However, others have found the evidence for viperine monophyly to the exclusion of *Causus* more ambiguous (Ashe and Marx, 1988; Cadle, 1992). In an analysis of nuclear and mitochondrial DNA sequences, Nagy et al. (2005) recovered this genus nested within the Viperinae, as the sister genus of *Atheris*, and viperine monophyly to the exclusion of *Causus* was contradicted by one strongly supported node. However, their analysis only included three viperines other than *Causus* and no crotalines, making the assessment of the relationships between these groups problematic.
Both the Viperinae and the Crotalinae have traditionally been assumed to be monophyletic, despite weak morphological support in the case of the former (Cadle, 1992), and both have been the subject of multiple phylogenetic analyses. In the Crotalinae, a number of studies have revealed considerable consensus (Gutberlet et al., 2002; Kraus et al., 1996; Parkinson, 1999, Parkinson et al., 2002; Malhotra and Thorpe, 2004; Castoe and Parkinson, 2006), whereas the phylogenetic relationships among the Viperinae remain less clearly resolved, with extensive incongruence between morphological and molecular data and lack of resolution of basal nodes (Groombridge, 1980; Ashe and Marx, 1988; Herrmann and Joger, 1995, 1997; Herrmann et al., 1999; Lenk et al., 2001).

Despite the attention lavished on the Crotalinae and the Viperinae individually, however, there have been few comprehensive studies of viperid phylogeny as a whole (e.g., Cadle, 1992), where both vipers and crotalines, as well as Causus and Azemiops, were equally or comprehensively represented. As a result, the overall phylogeny of the Viperidae remains inadequately investigated, which is reflected in the number of different subfamily arrangements proposed by different authors. There is therefore an obvious need for a modern and comprehensive molecular study with multiple outgroups.

Although vipers comprise only approximately 9% of the total diversity of colubroid snakes, they are widespread across all continents except Australia and Antarctica, display tremendous morphological diversity, occupy a wide variety of niches (Greene, 1992, 1997), and are among the dominant snakes in many ecosystems (e.g., Roman, 1980; Chippaux, 1986; Dixon and Soini, 1986). They have become model organisms in the study of morphological, behavioral and life history evolution (e.g., Madsen and Shine, 1994; Olsson et al., 1997; Madsen et al., 1999; Martins et al.,
2001; Wüster et al., 2004; Araújo and Martins, 2006; Ineich et al., 2006) as well as the evolution of venom and counteradaptations in prey (Poran et al., 1987; Daltry et al., 1996; Creer et al., 2003, Chijiwa et al., 2003; Biardi et al., 2006; Sanz et al., 2006). However, the current uncertainty surrounding the phylogenetic relationships among the major clades impedes the elucidation of the evolution of life history traits in the family (Greene, 1992).

The wide and discontinuous range of the vipers adds considerable interest to a historical biogeographical analysis of the group. The historical biogeography of the pitvipers has received considerable attention, with the evidence now strongly favoring an Asiatic origin, with a single dispersal event into North America, followed by multiple exchanges between North, Central and South America (Parkinson, 1999; Parkinson et al., 2002; Wüster et al., 2002). By contrast, in the case of the viperines, the lack of phylogenetic resolution has so far hindered a clear reconstruction of their biogeographic history, and the unresolved phylogenetic status of the putatively basal taxa Azemiops and especially Causus precludes reconstruction of the biogeographical history of the entire family.

In addition to the systematic and biogeographical interest of these snakes, an understanding of the phylogeny and especially the age of the extant viper radiation may provide important insights into the early evolution of the venomous function and venom apparatus in snakes. Anatomical (e.g., Jackson, 2003) and toxin-based studies (Fry et al., 2003; Fry and Wüster, 2004) have shown that venom evolved once at the base of the colubroid snake radiation, or even earlier (Fry et al., 2006), and was subsequently lost in some lineages, whereas others evolved more sophisticated venom delivery systems (Vidal, 2002).
Among snakes, only the Colubroidea (here defined sensu Lawson et al., 2005, as all Caenophidia except *Acrochordus*, rather than in the more restricted sense of Vidal et al., 2007) have evolved extensive dentitional adaptations related to venom injection. The paradox of the vipers is that, despite their sophisticated venom apparatus, these snakes have consistently been recovered as the sister group of most other colubroids (Vidal and Hedges, 2002; Kelly et al., 2003; Lawson et al., 2005; Vidal et al., 2007). Dating the age of the crown clade vipers relative to the split between the vipers and their sister group could provide new evidence on the role of venom in the biology of early colubroid snakes (Fig. 1). An early divergence of the crown clade vipers would suggest that venom may have been important in the earliest colubroids, and that the early origin of solenoglyphy capitalized on the recent origin of a differentiated maxillary dentition in snakes (Vidal, 2002), or even of tubular fangs early in the colubroid radiation (Jackson, 2007) (Fig. 1A). On the other hand, a relatively young viperid crown clade would suggest that the solenoglyphous dentition might have arisen at any time between the split of the vipers from their sister group and the origin of the crown clade vipers (Fig. 1B), and thus not allow inferences about the importance of venom among the earliest colubroid snakes or about the origin of tubular fangs in snakes.

The aim of this study is thus to use mitochondrial DNA sequences to infer the phylogeny of the Viperidae, with the specific objectives of (i) resolving the phylogenetic position of the enigmatic taxa *Azemiops* and *Causus*, (ii) improving our understanding of the phylogeny of the Viperinae, (iii) reconstructing the biogeographic history of the family, and (iv) contributing to the investigation of the role of venom in the early evolution of the advanced snakes.
2. Materials and methods

2.1. Taxon sampling

This study is based on a combination of novel sequences and published sequences obtained from GenBank. The bulk of the latter are those of Castoe and Parkinson (2006) for the Crotalinae, *Azemiops* and *Causus*, and the cytochrome b and 16S rRNA sequences of Lenk et al. (2001) for the Viperinae. Our aim was to include a cross section of viperine and crotaline species with approximately equal sampling density for the two groups, in order to avoid artifacts due to differing sampling densities and branch lengths. As far as possible, we sought to include sequences of at least two species for all but the smallest genera, and representatives of all major clades in the larger genera. In addition, we included a number of other colubroid taxa to provide suitable nodes for the calibration of molecular dating analyses, and to reduce the problem of isolated long branches in the clades containing these calibration points. In the case of taxa sequenced de novo for this paper, we obtained tissue samples (ventral scale clippings), blood samples taken through caudal venepuncture or cardiac puncture (caudal venepuncture is usually straightforward in crotalines, but can be much more difficult in viperines; cardiac puncture was used in such cases) or shed skins. Samples, vouchers and GenBank accession numbers are given in Table 1.

2.2. Laboratory protocols

Total DNA was extracted using the GenElute™ Mammalian Genomic Miniprep kit (Sigma-Aldrich). Four mitochondrial genes, 1100 base pairs (bp) of cytochrome b (cytb), 900 bp of NADH dehydrogenase subunit 4 (NADH4), 455 bp of 12S rRNA
(12S) and 507 bp 16S rRNA (16S) were amplified by polymerase chain reaction (PCR) using the primers given in Table 2.

The PCR protocol involved 20 µl reactions that were carried out using 18µl of 1.1X ReddyMix™ PCR Mastermix (Abgene™, catalogue no. AB-0575-LD/A), consisting of 1.25 units of Thermoprime Plus DNA polymerase, 75mM Tris-HCL (pH 8.8 @ 25°C), 20mM (NH₄)₂SO₄, 1.5 mM of MgCl₂, 0.01% (v/v) Tween® 20, 0.2 mM of each dNTP and precipitant red dye for electrophoresis. Primers were added to a final concentration of 0.4 µM and approximately 4 ng of template DNA.

The amplification protocol involved 3 minutes (m) denaturation at 95°C, then 35 cycles of 30 seconds (s) denaturation at 95°C, 45 s annealing at 43°C (cyt b, 12S, 16S) or 50°C (NADH4), 1.5 m extension at 72° C, and a final extension of 5 m at 72°C. Sequencing was carried out using the same forward primers used in the PCRs by Macrogen (Seoul, S. Korea—http://dna.macrogen.com).

2.3. Sequence and phylogenetic analysis

The sequences obtained as part of this study were aligned with published sequences. Protein-coding gene sequences were aligned by eye, since alignment is trivial in the absence of frame shifts and indels. For the rRNA genes, we used ClustalW (Thompson et al., 1994) to align the 12S and 16S rRNA genes, with additional adjustments by eye.

For phylogenetic analysis, we used maximum parsimony (MP) and Bayesian inference (BI) methods. MP analysis was carried out using the software PAUP* 4.0b10 (Swoford, 2002), and involved an unweighted analysis, using heuristic searching, TBR branch-swapping, and 1000 random addition sequence replicates.
Internal support for different nodes was estimated using non-parametric bootstrap searching (Felsenstein, 1985), using 1000 bootstrap replicates with five random addition sequence replicates each and SPR branch swapping.

For BI, we used MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). Since complex models of sequence evolution can extract additional phylogenetic signal from data, especially where saturation of base pair substitutions is commonplace (Castoe et al., 2005; Castoe and Parkinson, 2006), we partitioned our data into eight biologically relevant subsets. Each protein coding gene was treated separately, and the first, second and third codon positions, which are known to display different patterns of sequence evolution, were treated as separate partitions. The genes for 12S and 16S rRNA were treated as two additional separate partitions. The eight separate data partitions were therefore 12S rRNA and 16S rRNA, and the first, second and third codon positions separately for cyt b and NADH4. To identify the most appropriate models of sequence evolution for each data partition, we used MrModeltest 2.2 (Nylander, 2004), and selected the model favored under the Akaike Information Criterion (AIC). In all phylogenetic analyses, Acrochordus granulatus was specified as the sole outgroup. We ran the analysis for $5 \times 10^6$ generations, using four simultaneous independent runs initiated with different random starting trees. Every 500th tree was sampled. Plots of ln(L) against generation were inspected to determine the burnin period, and trees generated prior to the completion of burnin were discarded, with a generous “safety margin”.

2.4. Testing alternative phylogenetic hypotheses

In order to determine whether our data rejected alternative phylogenetic scenarios with statistical significance, we used tree topology tests and the analysis of the
frequency of post-burnin Bayesian trees compatible with the alternative phylogeny. We tested four alternative phylogenetic scenarios:

1. Monophyly of Viperinae + Crotalinae to the exclusion of both *Azemiops* and *Causus* (e.g., Groombridge, 1986)

2. *Causus* as the sister group of all other viperids (Castoe and Parkinson, 2006)

3. *Azemiops* as sister group of all other viperids (Groombridge, 1986)

4. Monophyly of the Viperinae to the exclusion of *Causus*

Trees representing the alternative topology were generated in PAUP* by constraining the analysis to retain only the optimal trees consistent with the alternative topology. We used both MP and maximum likelihood (ML) searches. For MP, we used the same search algorithm as used for the initial unconstrained search. For ML, we first used MrModeltest to identify the best model of sequence evolution for the unpartitioned dataset under the AIC. An ML search using the model and parameters identified by MrModeltest was then run in PAUP* to generate the optimum unconstrained ML tree, using a neighbor-joining starting tree and SPR branch swapping. Using the same model of sequence evolution, we then carried out additional ML searches under implementation of the aforementioned constraints.

We compared the constrained MP trees with the original most parsimonious trees using the Wilcoxon signed-ranks test (Templeton, 1983) as implemented in PAUP*. Under the likelihood criterion, we carried out the analogous comparisons using the Shimodaira-Hasegawa test (Shimodaira and Hasegawa, 1999), using the RELL option in PAUP*. In the case of BI, we used PAUP* to filter all trees obtained after completion of the burnin phase to retain only those consistent with the alternative
constraint trees. The alternative hypothesis was rejected if supported by fewer than 5% of Bayesian trees.

2.5. Molecular dating

The use of molecular data to estimate the divergence time of clades has been a topic of intense research interest in recent years (Bromham and Penny, 2003; Rutschmann, 2006; Yang and Rannala, 2006). From a long-standing reliance on a priori assumptions of fixed rates (Zuckerhandl and Pauling, 1965), molecular dating has progressed to increasingly realistic models of sequence evolution, relaxed molecular clocks that allow among-lineage variation in rates of sequence, the use of multiple fossil calibrations within a single tree, and the use of increasingly sophisticated likelihood and Bayesian Markov chain-Monte Carlo (MCMC) methods.

A critical part of any molecular dating analysis is the choice and use of calibration points. The use and interpretation of fossil evidence is often problematic, in particular due to the incompleteness of the fossil record, as well as uncertainty in the dating of the fossils and their placement on the tree. Logically, fossil evidence can provide an approximate minimum age for the existence of a clade, but it cannot provide a maximum age, since absence of evidence cannot be interpreted as evidence of absence.

With regard to molecular dating, reliance on minimum age constraints alone within the clade of interest, with a maximum age constraint provided solely by the root of the tree, is likely to result in an overestimate of the age of the internal nodes (Hugall et al., 2007). Treating calibration points as fixed may avoid overestimates of node ages due to the lack of maximum age constraints, and also prevent a single,
erroneously early, calibration point from “hijacking” the entire dating analysis. However, given the incompleteness of the fossil record for many groups, this approach is likely to lead many nodes being constrained to an erroneously young age, since some clades may be considerably older than the oldest known fossil. Using maximum age constraints based on geological scenarios that make it much more likely for a cladogenetic event to have occurred after a given time than before, such as the formation of oceanic islands (e.g., Thorpe et al., 1994) or the origin of new land connections between continents (e.g., Wüster et al., 2002, 2005a,b), can potentially provide maximum age constraints. However, constraints based on the origin of land connections require careful evaluation, since they assume a lack of overwater dispersal in the taxon concerned, which may be incorrect (De Queiroz, 2005).

A recent development that avoids some of these pitfalls is the development of algorithms employing priors with “soft” boundaries, which allow for uncertainty in the dating of fossils and the tree (Yang and Rannala, 2006). This approach has been shown to be superior to the use of hard boundaries in resolving conflict between calibrations, as well as reducing the effect of erroneous calibration points (Sanders and Lee, 2007).

Bayesian dating with “soft” priors, using BEAST v. 4.1.7 (Drummond and Rambaut, 2007), allows the likely probability distribution for node ages to be modeled as priors, and uncertainty about the dates of nodes used for calibration, as well as tree uncertainty, to be incorporated into the analysis. In the case of fossil-based calibration points, the actual date of a node is likely to precede the fossil by an unknown amount, but cannot logically postdate it. Lognormal priors using the fossil calibration point as a zero offset (a hard boundary), and specifying a mode somewhat
older than the fossil, model the likelihood of the actual dates of the node. On the other hand, in the case of biogeographical calibrations, a normal prior is likely to be more reflective of the uncertainty of the timing of the node around the estimated age of the event.

The Caenophidia in general and the Viperidae in particular represent a good example of a clade likely to benefit from the use of soft boundary calibrations. The clade has a poor pre-Miocene fossil record: most fossils date back no further than the Miocene, and most of those known from the early Miocene onward belong to extant genera (e.g., Szyndlar and Rage, 1990, 1999; but see Scanlon et al., 2003), suggesting a plethora of extinct earlier Caenophidia that are unrepresented in the fossil record. Consequently, the oldest fossils of any particular clade are likely to represent underestimates of the age of the clade.

In this analysis, we used the following calibration constraints:

1. “Porthidium”: the initial divergence of three South American populations of the Neotropical pitviper genus *Porthidium*, which almost certainly invaded South America and diverged there after the uplift of the Isthmus of Panamá, approximately 3.5 Mya (Wüster et al., 2002) was modeled with a normal distribution with a mean of 3.5 My and a standard deviation of 0.51 My, providing a 95% confidence interval of 2.5-4.5 Mya.

2. “Eurasian vipers”: fossil evidence suggests that the initial divergence of the Eurasian viper clade (excluding *Pseudocerastes* and *Eristicophis*) had begun by 20 Mya (Szyndlar and Rage, 1999). We used a lognormal prior of 20 Mya as zero offset, the default lognormal mean of 1 and the default lognormal standard deviation of 1 to constrain this node.
3. “Naja”: the split between the Asian Naja clade and its African sister clade dates back to a minimum age of 16 My based on the presence of characteristic apomorphies of the Asian clade in the fossil record (Szyndlar and Rage, 1990; Wüster et al., 2007). We used a lognormal prior with a 16 Mya zero offset, lognormal mean of 1 and a lognormal standard deviation of 1 for this node.

4. “Hemorrhois”: the likely cladogenesis between eastern and western species occurred after Asia and Africa became joined approximately 16-18 Mya (Nagy et al., 2003). We used a normal prior with a mean of 18 Mya and a standard deviation of 2.04 My, creating a 95% CI of 14-22 My.

5. “Rattlesnakes”: the divergence between Crotalus and Sistrurus occurred before 9 Mya, based on the age of a fossil vertebra of Sistrurus (Parmley and Holman, 2007). We used a lognormal prior with a zero offset of 9 Mya, a lognormal mean of 1 and a lognormal standard deviation of 1.

6. “Colubroidea”: the age of the basal divergence of the Colubroidea (i.e., between the vipers and their sister clade in the context of this study) remains subject to considerable debate. The youngest unambiguous colubroid fossils date back approximately 40 My to the Eocene of Asia (Head et al., 2005). However, Rage and Werner (1999) and Rage et al. (2003) described putative colubroid fossils from the Cenomanian (approximately 95 Mya), although these remain contentious (Head et al., 2005; Hugall et al., 2007). To reflect the fact that the divergence between vipers and their sister group may have taken place long before the Eocene, but probably after the Cenomanian, we applied a lognormal prior with a zero offset of 40 Mya, a lognormal mean of 2 and a lognormal standard deviation of 1.2, giving a 95% CI of 40-95 My, spanning the likely range of potential divergence dates.
7. Tree root height: the problem of the root height of the tree ties in with the issue of
the age of the Colubroidea and Caenophidia. Since the initial divergence of the Caenophidia is logically older that that of the Colubroidea, and quite possibly considerably older, we implemented a lognormal prior with a zero offset of 45 Mya, a lognormal mean of 2.5, and a lognormal standard deviation of 1.25.

The analysis was run for 20 million generations, of which the first 25% were discarded as burn-in. The posterior probability density of divergence times was estimated with a UCLN relaxed clock, which does not assume spatial autocorrelation in rates of sequence evolution. The sequence data were partitioned by gene and codon position as described above, and the appropriate models of sequence evolution, as determined via MrModeltest, specified for each partition. We implemented a Yule branching process with lognormal priors. Topological constraints were not employed. We used the program Tracer v. 1.4 (Rambaut and Drummond, 2007) to verify the completion of burnin and to estimate effective sample sizes for all parameters.

2.6 Dispersal-vicariance analysis

We used dispersal-vicariance analysis (Ronquist, 1997), implemented using the software DIVA 1.1 (Ronquist, 1996), to infer the ancestral distribution of the vipers and important nodes within the Viperidae. We used the tree generated by the BI analysis as a basis for DIVA, with modifications to (i) avoid a situation where the basal node of the Viperidae is situated near the root of the tree, causing the analysis to assume an unrealistically wide distribution of the common ancestor and (ii) better reflect our improved understanding of the phylogeny of the Caenophidia as a whole.
(Vidal et al., 2007): the position of *Cerberus rynchops* (Homalopsidae) was adjusted to form the sister group of all non-viperid colubroids, and branches representing the Asiatic Pareatidae and Xenodermatidae were inserted as successively more basal sister groups to all other colubroids. These relationships are robustly supported in the analyses of Vidal et al. (2007).

All taxa included in the analysis were assigned to one or more of three continental landmasses (Africa, Eurasia and the Americas). Given the size and separation between landmasses, ancestral distributions were restricted to a maximum of two landmasses.

### 3. Results

#### 3.1. Sequence data

We aligned 2148 bp of mtDNA sequence from all viperid genera except *Adenorhinos* and *Montatheris*. After removal of a 29 bp ambiguously aligned segment of the 16S rRNA gene, the final alignment contained 2119 usable base pairs (cyt*bd*: 660 bp; NADH4: 648 bp; 12S rRNA: 409 bp; 16S rRNA: 402 bp). Of these, 1287 bp were variable, and 1084 parsimony-informative. Alignment of the protein coding genes revealed no indels, frameshifts or nonsense codons, which would have indicated the presence of nuclear pseudogenes (Zhang and Hewitt, 1996). NADH4 could not be PCR-amplified in *Pseudocerastes fieldi* and *Macrovipera schweizeri*.

#### 3.2. Viperid phylogeny

Maximum parsimony analysis of the concatenated data revealed two equally most parsimonious trees of 16597 steps (consistency index: 0.1443; excluding
uninformative characters: 0.1326; retention index: 0.4502; rescaled consistency index: 0.0650).

For BI, MrModeltest 2.2 identified the following models of sequence evolution for the data partitions: GTR+I+Γ for cyt*b codon position 1, NADH4 codon positions 1 and 2, 12S rRNA and 16S rRNA, GTR+Γ for cyt*b and NADH4 codon position 3; and HKY+I+Γ for cyt*b codon position 2. Visual inspection of the plot of tree ln(L) versus generation number indicated that burn-in was completed after approximately 60,000 generations. However, we discarded the first $10^6$ generations as an additional safety margin.

The MP and BI trees show a number of areas of agreement, but also some areas of disagreement (Fig. 2):

1. MP and BI analyses strongly support the monophyly of the Viperidae as a whole and the reciprocal monophyly of the clades Viperinae + *Causus* and Crotalinae + *Azemiops*

2. The status of *Azemiops* as the sister taxon to all Crotalinae is supported with high levels of branch support in the BI tree, but not in the MP analysis, which recovers *Azemiops* within the Crotalinae, as the sister taxon of *Deinagkistrodon*, albeit without support.

3. *Causus* is consistently nested within the Viperinae, but its exact position varies between analyses, and the branch support for nodes placing *Causus* within the Viperinae rather than as their sister group is low in both the MP and BI trees.

4. Relationships among the major lineages within the Viperinae are generally poorly supported. Novel and/or noteworthy relationships that are strongly supported include (i) very strong support for the Eurasian clade consisting of *Daboia*, *Eristicophis*,
Macrovipera, Pseudocerastes and Vipera; (ii) a strongly supported sister group relationship between a clade consisting of Daboia siamensis and D. mauritanica on one hand, and the Vipera and Pelias groups of the Eurasian vipers of the genus Vipera on the other.

5. Our data strongly support the individual monophyly of the larger viperine genera Atheris, Bitis, Causus and Echis.

3.3. Testing alternative hypotheses

None of the alternative phylogenetic hypotheses were convincingly rejected by Wilcoxon signed-ranks tests under the parsimony criterion (Table 3), although the monophyly of Viperinae + Crotalinae to the exclusion of Causus and Azemiops was rejected by a few of the pairwise tests comparing optimal and constrained trees. Under the likelihood criterion, the monophyly of Viperinae + Crotalinae to the exclusion of Causus and Azemiops was rejected, whereas the position of either Causus or Azemiops alone as basal viperids was not rejected (Table 3). However, in Bayesian analyses, none of the 32004 trees obtained after the completion of burnin supported the monophyly of Viperinae + Crotalinae or the position of either Azemiops or Causus as the sister taxon of the remaining Viperidae, signaling strong rejection of the alternative hypotheses. Only 540 out of 32004 trees (1.69%) supported the monophyly of the Viperinae to the exclusion of Causus, causing us to reject this alternative phylogenetic scenario.

3.4. Molecular dating

Tracer v. 1.4 (Rambaut and Drummond, 2007) revealed that burnin had occurred after approximately one million generations for all parameters, but we conservatively
discarded the first five million generations of the analysis. All parameters of the analysis had effective sample sizes above 200, in most cases by a large margin. The results of the molecular dating analyses are shown in Figs. 3 and 4 and the details for key nodes on the tree in Table 4.

3.5 Dispersal-vicariance analysis

DIVA (Ronquist, 1996) placed the root of the Caenophidian tree in Asia, as expected on the basis of previous phylogenetic analyses of the Caenophidia (Vidal et al., 2007). The distribution of the ancestral node of the Crotalinae + Azemiops clade was also identified as Asia. The ancestral node of the Viperinae was placed ambiguously as either Africa or Asia + Africa, and the ancestral node of the Viperidae as Asia or Asia + Africa (Fig. 4).

4. Discussion

4.1. Phylogeny of the Viperidae

Our data have shed new light on the phylogeny of the family Viperidae as a whole. First, the Bayesian analyses provide further strong support for the position of Azemiops as a sister species to the Crotalines, a position first suggested by Cadle (1992) based on albumin immunological distances and later confirmed by others (Knight and Mindell, 1993; Heise et al., 1995), albeit in all cases from a restricted sampling of viperid taxa. Later workers also found the same relationship in studies of crotaline phylogeny (Parkinson, 1999; Parkinson et al., 2002; Castoe and Parkinson, 2006), but these only included a maximum of six species of vipers, and, more importantly, did not include any non-viperid outgroup taxa that could have allowed an
unbiased placement of the root of the viperid tree. The fact that our results support
the same relationship under a much more comprehensive sampling regime and
under inclusion of non-viperid outgroup taxa lends further strength to the hypothesis
of *Azemiops* as the sister taxon of the Crotalinae.

Our data also shed new light on the position of the enigmatic night adders by
providing new evidence that *Causus* is not a basal viperid. In all the analyses, the
three species of *Causus* consistently nested not only in association with the
Viperinae, but within that clade. Our Bayesian analyses provide significant support
for the nesting of *Causus* within the Viperinae, although their precise placement
remains unresolved. These results highlight the pitfalls surrounding the choice of
outgroup in analyses of viperid snake phylogeny: clearly, the use of *Causus* as an
outgroup for phylogenetic studies of all other vipers is inappropriate, and the *a priori*
use of *Causus* as an outgroup for studies of the phylogeny of the Viperinae will
preclude elucidation of the true phylogenetic position of this taxon. Similarly,
ecological and natural history characteristics of *Causus* (e.g., Ineich et al., 2006) do
not necessarily represent the basal viperid (or viperine) condition, but may instead
constitute autapomorphies of the genus.

Despite the doubling of the amount of sequence information for the Viperinae in this
study compared to Lenk et al. (2001), the relationships between the other major
clades of viperine snakes remain largely unresolved. As a general pattern, clades
that received moderate support in Lenk et al. (2001) are more strongly supported
here, whereas clades lacking statistical support in Lenk et al. (2001) also do so in our
results. We have thus recovered strong support for the monophyly of the genera
*Atheris*, *Bitis*, *Causus*, *Echis*, and the Eurasian viper clade (*Vipera*, *Macrovipera,*
Montivipera, Daboia, Eristicophis and Pseudocerastes), but not for the relationships among these groups or the placement of Cerastes.

The monophyly of Atheris needs to be interpreted in the context of the results of Lenk et al. (2001), who found strong support for the inclusion of Adenorhinos barbouri within Atheris, and proposed the synonymy of Adenorhinos with Atheris. We were unable to include Adenorhinos in our analysis, but in view of the unambiguous results of Lenk et al. (2001), we consider the case for inclusion of Adenorhinos within Atheris to have been adequately supported. Interestingly, we found no support for a sister-group relationship between Atheris and Proatheris, the affinities of the latter being entirely unresolved. Unfortunately, we were unable to include the enigmatic Kenyan mountain endemic Montivipera hindii, which may represent another basal lineage.

Our trees support the monophyly of the “Palearctic rattlesnakes” (Joger and Courage, 1999) Cerastes and Echis, but not with significant support. A sister group relationship between Atheris and Bitis is supported by a near-significant Bayesian posterior probability, but not by MP analysis.

Our Bayesian analysis strongly supports the monophyly of Bitis. Within the genus, the relative placement of B. arietans, B. worthingtoni and the remainder of the genus remains poorly supported. The monophyly of the subgenera Macrocerastes and Calechidna, resurrected by Lenk et al. (1999), is strongly supported, as is an apparent subdivision of Calechidna into a primarily rupicolous clade containing B. atropos, B. cornuta, B. rubida and B. xeropaga, and an arenicolous clade represented here by B. caudalis and B. peringueyi. This subdivision by ecology is congruent with the results of Lenk et al. (1999). The definition of subgenera of Bitis by Lenk et al. (1999) clearly contributes very effectively to highlighting the phylogenetic structure within the genus. However, in view of the now strongly
supported monophyly of *Bitis*, we feel strongly that recognition of these subgenera as full genera, and the consequent splitting of the monophyletic genus *Bitis*, would only serve to confuse the nomenclature of a hitherto stable group without significantly enhancing our understanding of its evolution (see Wüster et al., 2002, for comments on a similar situation in South American pitvipers).

Within the Eurasian vipers, we have found greatly enhanced support for a number of relationships previously recovered with limited support by Lenk et al. (2001). Our data confirm a strongly supported *Pseudocerastes* + *Eristicophis* clade as the sister clade to the robustly monophyletic remaining Eurasian vipers. Like Lenk et al. (2001), we recovered the taxon *mauritanica* as the sister taxon of *Daboia siamensis* (see Wüster et al., 1992, and Thorpe et al., 2007, for the taxonomic status of this species), not of *Macrovipera lebetina*, with increased branch support. This therefore supports Lenk et al.’s (2001) assignment of *mauritanica*, together with *deserti* and *palaestinae*, to the genus *Daboia*. Similarly, the sister group relationship between the *xanthina* group and *Macrovipera lebetina* and *schweizeri* is strongly supported, which supports recognition of *Montivipera* as the genus containing the species of the *xanthina* group (Nilson et al., 1999; Joger, 2005). More surprisingly, our data provide strong support for a sister group relationship between *Daboia* (*siamensis* and *mauritanica*) and the small European vipers of the genus *Vipera sensu stricto*, which are in turn strongly supported as monophyletic (contrary to Groombridge, 1986).

The relationships recovered within the Crotalinae are as reported by Castoe and Parkinson (2006), including as principal conclusions the monophyly of the New World pitvipers (indicating a single colonization of the New World from an Asian origin), and the basal position of the smaller Asian genera *Calloselasma*, *Hypnale*, *Deinagkistrodon*, *Tropidolaemus* and *Garthius*. 
4.2 Molecular dating and historical biogeography of the Viperidae

The molecular dating analysis estimated the divergence between *Acrochordus* and the Colubroidea, and that between the vipers and their sister group, to have taken place approximately 70 Mya and 61 Mya, respectively, i.e., around the Cretaceous-Tertiary boundary. The radiation of the Caenophidia in the Tertiary may thus be linked to the K/T event. The origin of the crown clade vipers is estimated to have occurred approximately 47 Mya, in the early Cenozoic, i.e., approximately 14 million years after their divergence from the remaining Caenophidia.

These estimates suggest that the origin of the Caenophidia predates the oldest definite fossils of this clade, dated at approximately 37 Mya (Head et al., 2005), most likely by a considerable margin: the confidence intervals for the split between vipers and the remaining Colubroidea suggest an early Lutetian divergence of the Colubroidea at the latest, and the age of the most recent common ancestor of the Caenophidia is estimated as no younger than 50 Mya. This is consistent with the results of Burbrink and Pyron (2008), although they arrive at a somewhat younger estimate for the same node, and their confidence intervals do not extend beyond 75 Mya. This suggests that use of the fossils reported by Head et al. (2005) as calibration points in molecular dating studies (e.g., Sanders and Lee, 2008) may lead to an underestimate of dependent divergence times unless the distribution of priors is left sufficiently wide to accommodate earlier divergence times.

Within the Viperidae, *Azemiops* diverged from the Crotalinae approximately 37 Mya, an estimated 10 My after their separation from the Viperinae, followed by the basal branching among the Crotalinae 31 Mya, the branching off of the *Deinagkistrodon-Garthius-Tropidolaemus* clade 30 Mya, the arboreal *Trimeresurus* group
approximately 26 Mya, and a clade consisting of the genus *Protobothrops* and *Ovophis monticola* approximately 25 Mya. The New World pitvipers diverged from their Asiatic sister group approximately 24 Mya, and the first radiation of the New World clade occurred approximately 22 Mya. The most recent common ancestor of the Viperinae is estimated to have lived approx 40 Mya, and the genera *Atheris*, *Bitis*, *Causus*, *Cerastes*, *Echis* and *Proatheris* had separated from each other and the Eurasian clade by approximately 33 Mya.

In terms of the historical biogeography of the Viperidae, the dispersal-vicariance analysis (Ronquist, 1997) suggested either an Asian or an Asian + African origin for the Viperidae as equally parsimonious solutions, and an African or African + Asian origin for the Viperinae as equally parsimonious. However, the molecular dating results provide further insight into the more likely origin of both clades: Asia and Africa remained widely separated by the Tethys Sea throughout the late Mesozoic and through to the middle Miocene (Hallam, 1994; Scotese, 2004). The story of Asian/African distributions in the early and middle Tertiary is thus one of Africa and Asia coming together, rather than one of vicariance. The estimated dates of origin of the crown clade Viperidae (47 Mya) and the Viperinae (40 Mya) suggests that a bicontinental origin followed by later vicariance is an implausible scenario, as Africa and Asia remained widely separated by the Tethys Sea for another 20-30 My. Consequently, a monocontinental origin in Asia for the Viperidae and in Africa for the Viperinae is a much more plausible scenario, although the lack of resolution of the basal nodes within the Viperinae suggests caution in the interpretation of this part of the analysis.

The origin of the Crotalinae (and *Azemiops*) is firmly placed in Asia, as is the entire history of the Crotalinae until the single invasion of the New World, as inferred from
previous analyses (Parkinson, 1999). The colonization of the New World, 24-22 Mya, was followed by further rapid cladogenesis, the *Bothrops* + *Bothrocophias*, *Bothriechis*, *Porthidium* + *Atropoides* + *Cerrophidion*, *Ophryacus*, *Lachesis*, rattlesnakes and *Agkistrodon* clades having separated by 18 Mya, although the crown clades within these genera are often considerably younger, lying towards the lower end of the age range suggested by Zamudio and Greene (1997) and Wüster et al. (2002) in the case of *Lachesis* and *Bothrops* (Table 4).

The age estimates provided here are more recent than some other hypotheses on the origin of the pitvipers and in particular the invasion of the New World. Several authors (Crother et al., 1992; Vidal and Lecointre, 1998; Parkinson et al., 2002; reviewed by Gutberlet and Harvey, 2004) have suggested a late Cretaceous/early Tertiary colonization of the New World by pitvipers. The data presented here suggest a much more recent origin of the New World clade, most likely during the late Oligocene or early Miocene, corresponding very closely to the estimated date of the invasion of the New World by lampropeltine colubrids (Burbrink and Lawson, 2007). This occurred at a time when eastern North America and Eurasia were already widely separated across the Atlantic, whereas northeastern Asia and Alaska remained connected via the Bering land bridge. Coupled with the absence of pitvipers in Europe, this strongly suggests a Beringian dispersal event. In the late Oligocene/early Miocene, Beringia was covered by a mixed hardwood and deciduous forest (Sanmartín et al., 2001), a habitat type still inhabited by some North American (*Crotalus*, *Agkistrodon*) and East Asian (*Gloydius*) pitvipers, the latter being part of or close to the sister clade of the New World pitvipers (Castoe and Parkinson, 2006).

The colonization of the New World was followed by rapid adaptive radiation into diverse ecological and morphological niches (Martins et al., 2001; Wüster et al.,
2002), and gave rise to a total of 117 currently recognized species (Campbell and Lamar, 2004), giving a Speciation Interval (age of most recent common ancestor of a clade divided by the natural logarithm of the number of species – Coyne and Orr, 2004) of 4.64, compared to 8.46 for the Viperidae as a whole and 9.04 for the Caenophidia. The speciation interval in New World pitvipers is thus shorter than in many other squamate clades (Sanders and Lee, 2008), suggesting relatively rapid adaptive radiation in this clade following colonization of the New World.

4.3 Implications for venom evolution in snakes

Our molecular dating analyses indicate a time delay of approximately 14 My between the split between the vipers and their sister clade and the origin of the crown clade vipers. Our results are thus closer to Fig. 1B than Fig. 1A: there is a considerable window of evolutionary opportunity during which a solenoglyphous dentition may have evolved. Consequently, our results do not provide evidence that the last common ancestor of vipers and other colubroids necessarily had a particularly advanced venom apparatus or even tubular fangs, as suggested by Jackson (2007), and thus shed little light on the importance of venom in early colubroids. However, the inferred dating of colubroid diversification places the discovery of Lower Miocene viperid and elapid fangs (Kuch et al., 2006) in context: since the fangs of vipers and elapids are structurally conserved across their respective clades, they presumably evolved at or before the beginning of the radiation of these respective crown clades in the early Tertiary. The similarity of the Miocene fangs to present-day viperid and elapid fangs, described by Kuch et al. (2006), is to be expected, and not an indication of the presence of tubular fangs in ancestral colubroids.
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References


Evolutionary Biology Centre, Uppsala Univ..


Figure legends

Fig. 1. Schematic representation of possible phylogenetic scenarios for the vipers and their impact on our understanding of the evolution of the colubroid venom apparatus. A: an early origin of the crown clade vipers implies that the solenoglyphous dentition arose soon after the split between the vipers and their sister clade, and thus an important role of venom and possibly tubular fangs in the common ancestor of vipers and other colubroids. B: a recent viper crown clade suggests the existence of a number of extinct stem-group viper lineages and leaves the timing of the evolution of the solenoglyphous dentition unresolved, providing little evidence on the venom apparatus of early colubroids.

Fig. 2. Bayesian inference tree of the vipers and their sister clade. Branch support measures are Bayesian posterior probabilities/MP bootstrap support (only where > 50%). Branch support indices are not given for most intrageneric nodes to preserve clarity.

Fig. 3. BEAST maximum credibility ultrametric tree showing the timing of the evolution of the Colubroidea and the origin of the vipers. Grey bars indicate 95% confidence intervals for selected nodes. Calibration points are indicated by numbered circles on nodes; numbering corresponds to that used in Materials and Methods. The timing of the evolution of the Viperidae is shown in Fig. 4.

Fig. 4. BEAST maximum credibility ultrametric tree showing the timing of the evolution of the Viperidae. Legend as for Fig. 3. Continental locations indicate
distribution of common ancestor at nodes, and black circles indicate major dispersal events between Eurasia, Africa and the New World, elucidated by dispersal-vicariance analysis. Species occurring on two continents (*Vipera latastei* in Iberia and N. Africa; *Cerastes* and *Echis coloratus* in Africa and Arabia) have not been considered. Dispersal events between North, Central and South America have been discussed elsewhere (Parkinson et al., 2002; Wüster et al., 2002) and are not indicated here.
Table 1. Details of samples and sequences used in this study. Locality and voucher information are not provided for previously published sequences. **See Guo et al. (2007) for comments on the generic affinities of these species.

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<td><em>Montivipera xanthina</em></td>
<td>Turkey (cyt b, 16s) / unknown (NADH4, 12s)</td>
<td><strong>EU624234</strong>*, <strong>AJ275724</strong>, <strong>AJ275777</strong>, <strong>EU624268</strong>*</td>
<td></td>
</tr>
<tr>
<td><em>Proatheris superciliaris</em></td>
<td>Malawi (cyt b) / unknown (others)</td>
<td><strong>EU624230</strong>*, <strong>AJ275685</strong>, <strong>EU624296</strong>, <strong>EU624263</strong>*</td>
<td></td>
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<tr>
<td><em>Pseudocerastes fieldi</em></td>
<td>Israel (cyt b, 16s) / unknown (12s)</td>
<td>n/a, <strong>AJ275716</strong>, <strong>AJ275769</strong>, <strong>EU624264</strong>*</td>
<td></td>
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<tr>
<td><em>Vipera ammodytes</em></td>
<td>Liverpool School of Tropical Medicine, live coll., Va1</td>
<td><strong>EU624232</strong>*, <strong>EU624314</strong>*, <strong>EU624297</strong>, <strong>EU624266</strong>*</td>
<td></td>
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</tbody>
</table>
Table 2. Primers used in the sequencing of mitochondrial genes in viperid snakes. The position corresponds to point at which the 5’ end of the primer aligns against the mitochondrial sequence of *Dinodon semicarinatus* (Kumazawa et al, 1998). References are: ¹Palumbi (1996); ²Pook (this paper); ³de Queiroz et al (2002); ⁴Burbrink et al (2000); ⁵Arévalo (1994); ⁶S. Ursenbacher, pers. comm; ⁷Knight and Mindell (1993). *internal primers.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Position</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td><strong>CYT B</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gludg¹</td>
<td>14889</td>
<td>TGACTTGAAARACCAYCGTTG</td>
</tr>
<tr>
<td>GludgMod²</td>
<td>14902</td>
<td>CTGCGGCCCTGAAACCACCGTTG</td>
</tr>
<tr>
<td>H15787*</td>
<td>15787</td>
<td>GCTCCDCCBAGTTTRTT</td>
</tr>
<tr>
<td>L14929²*</td>
<td>14929</td>
<td>GTCCCACCATCACACTCTCAAAC</td>
</tr>
<tr>
<td>H15440²*</td>
<td>15440</td>
<td>CTAATTTGGTTTGGGATTGATC</td>
</tr>
<tr>
<td>L14910³</td>
<td>14833</td>
<td>GCTCTGATGATGAAAAACCGTTTG</td>
</tr>
<tr>
<td>H16064⁴</td>
<td>16090</td>
<td>CTTTGTTTACAAGAACATGCTTTA</td>
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<tr>
<td><strong>NADH4</strong></td>
<td></td>
<td></td>
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<tr>
<td>ND4⁴*</td>
<td>11677</td>
<td>CACCTATGACTACAAAGCTCATGAGAGC</td>
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<tr>
<td>Leu⁵</td>
<td>12594</td>
<td>CATTACTTTACTTGATTTGCACCA</td>
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<tr>
<td>HIS12763V⁶</td>
<td>12594</td>
<td>TTCTATCACTTGATTTGCACCA</td>
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<tr>
<td><strong>12S</strong></td>
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<td>L1091⁷</td>
<td>478</td>
<td>AAACTTGGGATTAGATACCCCACTAT</td>
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<td>Accession</td>
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<td>Sequence</td>
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<tr>
<td>H1557^7</td>
<td>980</td>
<td>GTACACTTACCTTGTTACGACTT</td>
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<td>16S</td>
<td>1628</td>
<td>CGCCTGTGTTATCAAAAAACAT</td>
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<td>L2510^1</td>
<td>1828</td>
<td>CGCCTGTGTTATCAAAAAACAT</td>
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<tr>
<td>H3059^1</td>
<td>2376</td>
<td>CCGGTCTGAACTCAGATCAGT</td>
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</tbody>
</table>
Table 3. Testing alternative phylogenetic scenarios: tree topology tests comparing optimal MP and ML trees with trees constrained to represent alternative phylogenetic scenarios, and proportion of Bayesian trees compatible with alternative scenarios. Monophyly of the Viperinae to the exclusion of *Causus* was not tested in MP and ML, since these tests already failed to reject the hypothesis of *Causus* as a basal viperid.

<table>
<thead>
<tr>
<th></th>
<th>Maximum Parsimony</th>
<th>Maximum Likelihood</th>
<th>Bayesian Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length of optimal tree(s)</td>
<td>Length of constraint tree(s)</td>
<td>N</td>
</tr>
<tr>
<td><em>Azemiops</em> as basal viperid</td>
<td>16597</td>
<td>16617</td>
<td>291-362</td>
</tr>
<tr>
<td><em>Causus</em> as basal viperid</td>
<td>16597</td>
<td>16625</td>
<td>211-304</td>
</tr>
<tr>
<td>Monophyly of Crotalinae + Viperinae to the exclusion of <em>Causus</em> and <em>Azemiops</em></td>
<td>16597</td>
<td>16637</td>
<td>288-310</td>
</tr>
<tr>
<td>Monophyly of Viperinae exclusive of <em>Causus</em></td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>
Table 4. Estimated dates (and 95% confidence intervals) of important nodes and clades mentioned in the text. MRCA = most recent common ancestor

<table>
<thead>
<tr>
<th>Node</th>
<th>Estimated date (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root of Tree (<em>Acrochordus</em> vs. <em>Colubroidea</em>)</td>
<td>70.2 (51.1-91.2)</td>
</tr>
<tr>
<td>Split between Viperidae and other Caenophidia</td>
<td>61.5 (48.5-75.1)</td>
</tr>
<tr>
<td>MRCA of Viperida</td>
<td>47.4 (38.1-57.4)</td>
</tr>
<tr>
<td>Split between <em>Azemiops</em> and <em>Crotalinae</em></td>
<td>37.4 (29.7-45.6)</td>
</tr>
<tr>
<td>MRCA of <em>Crotalinae</em></td>
<td>31.2 (25.4-37.8)</td>
</tr>
<tr>
<td>Split between <em>Tropidolaemus</em>, <em>Deinagkistrodon</em> and <em>Garthius</em> and remaining <em>Crotalinae</em></td>
<td>30.3 (24.6-36.7)</td>
</tr>
<tr>
<td>Split between <em>Trimeresurus</em> and rest of <em>Crotalinae</em></td>
<td>26.5 (21.2-31.8)</td>
</tr>
<tr>
<td>Split between <em>Protobothrops</em> + <em>Ovophis</em> and remaining <em>Crotalinae</em></td>
<td>25.2 (20.6-30.8)</td>
</tr>
<tr>
<td>New World <em>Crotalinae</em> vs. sister group</td>
<td>24.3 (20.1-29.1)</td>
</tr>
<tr>
<td>MRCA of New World <em>Crotalinae</em></td>
<td>22.1 (17.9-26.9)</td>
</tr>
<tr>
<td>MRCA of <em>Bothrops</em>+<em>Bothrocophias</em> clade</td>
<td>13.7 (10.2-17.4)</td>
</tr>
<tr>
<td>MRCA of <em>Lachesis</em></td>
<td>6.5 (3.5-9.8)</td>
</tr>
<tr>
<td>MRCA of Viperinae</td>
<td>39.7 (31.9-48.1)</td>
</tr>
<tr>
<td>Divergence between <em>Causus</em> + <em>Cerastes</em> + <em>Echis</em> and <em>Atheris</em> + <em>Bitis</em></td>
<td>38.5 (31.0-46.8)</td>
</tr>
<tr>
<td>Split between <em>Causus</em> and <em>Echis</em> + <em>Cerastes</em></td>
<td>35.9 (28.4-43.8)</td>
</tr>
<tr>
<td>Split between <em>Cerastes</em> and <em>Echis</em></td>
<td>33.0 (25.9-41.1)</td>
</tr>
<tr>
<td>Split between <em>Atheris</em> and <em>Bitis</em></td>
<td>35.2 (28.1-43.5)</td>
</tr>
<tr>
<td>Split between <em>Proatheris</em> and Eurasian clade</td>
<td>36.1 (28.1-44.6)</td>
</tr>
</tbody>
</table>
Figure 1

A

Crown-clade vipers

Other advanced snakes

Early origin of solenoglyphous dentition

B

Crown-clade vipers

Other advanced snakes

Hypothetical stem-group vipers

† † †

Origin of solenoglyphous dentition unresolved

Time