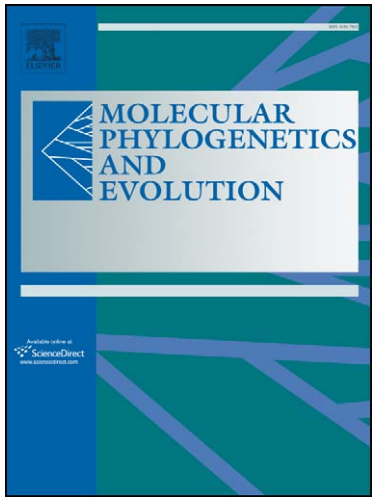

A nesting of vipers: phylogeny and historical biogeography of the Viperidae (Squamata: Serpentes)

Wolfgang Wüster, Lindsay Peppin, Catharine E. Pook, Daniel E. Walker

PII: S1055-7903(08)00421-1
DOI: 10.1016/j.ympev.2008.08.019
Reference: YMPEV 3000

To appear in: *Molecular Phylogenetics and Evolution*

Received Date: 1 November 2007
Revised Date: 1 August 2008
Accepted Date: 28 August 2008

Please cite this article as: Wüster, W., Peppin, L., Pook, C.E., Walker, D.E., A nesting of vipers: phylogeny and historical biogeography of the Viperidae (Squamata: Serpentes), *Molecular Phylogenetics and Evolution* (2008), doi: 10.1016/j.ympev.2008.08.019

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

Wolfgang Wüster*, Lindsay Peppin, Catharine E. Pook, and Daniel E. Walker

School of Biological Sciences, Bangor University, Environment Centre Wales, Bangor, LL57 2UW, UK

Running title: Phylogeny of the Viperidae

*Corresponding author:
Tel. +44 1248 382301
Fax +44 1248 371644
E-mail: w.wuster@bangor.ac.uk

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 russelii* 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 viperines 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 viperines 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* (cyt*b*), 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 @ 25ºC), 20mM $(NH_4)_2SO_4$, 1.5 mM of $MgCl_2$, 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 (Swofford, 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 x 10^6 generations, using four simultaneous independent runs initiated with different random starting trees. Every 500^{th} 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

231 frequency of post-burnin Bayesian trees compatible with the alternative phylogeny.
232 We tested four alternative phylogenetic scenarios:

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

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

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

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

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

248 We compared the constrained MP trees with the original most parsimonious trees
249 using the Wilcoxon signed-ranks test (Templeton, 1983) as implemented in PAUP*.
250 Under the likelihood criterion, we carried out the analogous comparisons using the
251 Shimodaira-Hasegawa test (Shimodaira and Hasegawa, 1999), using the RELL
252 option in PAUP*. In the case of BI, we used PAUP* to filter all trees obtained after
253 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,

278 erroneously early, calibration point from “hijacking” the entire dating analysis.
279 However, given the incompleteness of the fossil record for many groups, this
280 approach is likely to lead many nodes being constrained to an erroneously young
281 age, since some clades may be considerably older than the oldest known fossil.
282 Using maximum age constraints based on geological scenarios that make it much
283 more likely for a cladogenetic event to have occurred after a given time than before,
284 such as the formation of oceanic islands (e.g., Thorpe et al., 1994) or the origin of
285 new land connections between continents (e.g., Wüster et al., 2002, 2005a,b), can
286 potentially provide maximum age constraints. However, constraints based on the
287 origin of land connections require careful evaluation, since they assume a lack of
288 overwater dispersal in the taxon concerned, which may be incorrect (De Queiroz,
289 2005).

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

296 Bayesian dating with “soft” priors, using BEAST v. 4.1.7 (Drummond and Rambaut,
297 2007), allows the likely probability distribution for node ages to be modeled as priors,
298 and uncertainty about the dates of nodes used for calibration, as well as tree
299 uncertainty, to be incorporated into the analysis. In the case of fossil-based
300 calibration points, the actual date of a node is likely to precede the fossil by an
301 unknown amount, but cannot logically postdate it. Lognormal priors using the fossil
302 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

375 (Vidal et al., 2007): the position of *Cerberus rynchops* (Homalopsidae) was adjusted
376 to form the sister group of all non-viperid colubroids, and branches representing the
377 Asiatic Pareatidae and Xenodermatidae were inserted as successively more basal
378 sister groups to all other colubroids. These relationships are robustly supported in the
379 analyses of Vidal et al. (2007).

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

384

385 **3. Results**

386

387 *3.1. Sequence data*

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

396

397 *3.2. Viperid phylogeny*

398 Maximum parsimony analysis of the concatenated data revealed two equally most
399 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

449 discarded the first five million generations of the analysis. All parameters of the
450 analysis had effective sample sizes above 200, in most cases by a large margin. The
451 results of the molecular dating analyses are shown in Figs. 3 and 4 and the details
452 for key nodes on the tree in Table 4.
453
454 *3.5 Dispersal-vicariance analysis*
455 DIVA (Ronquist, 1996) placed the root of the Caenophidian tree in Asia, as expected
456 on the basis of previous phylogenetic analyses of the Caenophidia (Vidal et al.,
457 2007). The distribution of the ancestral node of the Crotalinae + *Azemiops* clade was
458 also identified as Asia. The ancestral node of the Viperinae was placed ambiguously
459 as either Africa or Asia + Africa, and the ancestral node of the Viperidae as Asia or
460 Asia + Africa (Fig. 4).
461
462 **4. Discussion**
463
464 *4.1. Phylogeny of the Viperidae*
465 Our data have shed new light on the phylogeny of the family Viperidae as a whole.
466 First, the Bayesian analyses provide further strong support for the position of
467 *Azemiops* as a sister species to the Crotalines, a position first suggested by Cadle
468 (1992) based on albumin immunological distances and later confirmed by others
469 (Knight and Mindell, 1993; Heise et al., 1995), albeit in all cases from a restricted
470 sampling of viperid taxa. Later workers also found the same relationship in studies of
471 crotaline phylogeny (Parkinson, 1999; Parkinson et al., 2002; Castoe and Parkinson,
472 2006), but these only included a maximum of six species of viperines, and, more
473 importantly, did not include any non-viperid outgroup taxa that could have allowed an

474 unbiased placement of the root of the viperid tree. The fact that our results support
475 the same relationship under a much more comprehensive sampling regime and
476 under inclusion of non-viperid outgroup taxa lends further strength to the hypothesis
477 of *Azemiops* as the sister taxon of the Crotalinae.

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

491 Despite the doubling of the amount of sequence information for the Viperinae in this
492 study compared to Lenk et al. (2001), the relationships between the other major
493 clades of viperine snakes remain largely unresolved. As a general pattern, clades
494 that received moderate support in Lenk et al. (2001) are more strongly supported
495 here, whereas clades lacking statistical support in Lenk et al. (2001) also do so in our
496 results. We have thus recovered strong support for the monophyly of the genera
497 *Atheris*, *Bitis*, *Causus*, *Echis*, and the Eurasian viper clade (*Vipera*, *Macrovipera*,

498 *Montivipera*, *Daboia*, *Eristicophis* and *Pseudocerastes*), but not for the relationships
499 among these groups or the placement of *Cerastes*.

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

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

513 Our Bayesian analysis strongly supports the monophyly of *Bitis*. Within the genus,
514 the relative placement of *B. arietans*, *B. worthingtoni* and the remainder of the genus
515 remains poorly supported. The monophyly of the subgenera *Macrocerastes* and
516 *Calechidna*, resurrected by Lenk et al. (1999), is strongly supported, as is an
517 apparent subdivision of *Calechidna* into a primarily rupicolous clade containing *B.*
518 *atropos*, *B. cornuta*, *B. rubida* and *B. xeropaga*, and an arenicolous clade
519 represented here by *B. caudalis* and *B. peringueyi*. This subdivision by ecology is
520 congruent with the results of Lenk et al. (1999). The definition of subgenera of *Bitis*
521 by Lenk et al. (1999) clearly contributes very effectively to highlighting the
522 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*.

548

549 *4.2 Molecular dating and historical biogeography of the Viperidae*

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

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

569 Within the Viperidae, *Azemiops* diverged from the Crotalinae approximately 37 Mya,
570 an estimated 10 My after their separation from the Viperinae, followed by the basal
571 branching among the Crotalinae 31 Mya, the branching off of the *Deinagkistrodon-*
572 *Garthius-Tropidolaemus* clade 30 Mya, the arboreal *Trimeresurus* group

573 approximately 26 Mya, and a clade consisting of the genus *Protobothrops* and
574 *Ovophis monticola* approximately 25 Mya. The New World pitvipers diverged from
575 their Asiatic sister group approximately 24 Mya, and the first radiation of the New
576 World clade occurred approximately 22 Mya. The most recent common ancestor of
577 the Viperinae is estimated to have lived approx 40 Mya, and the genera *Atheris*, *Bitis*,
578 *Causus*, *Cerastes*, *Echis* and *Proatheris* had separated from each other and the
579 Eurasian clade by approximately 33 Mya.

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

596 The origin of the Crotalinae (and *Azemiops*) is firmly placed in Asia, as is the entire
597 history of the Crotalinae until the single invasion of the New World, as inferred from

598 previous analyses (Parkinson, 1999). The colonization of the New World, 24-22 Mya,
599 was followed by further rapid cladogenesis, the *Bothrops* + *Bothrocophias*,
600 *Bothriechis*, *Porthidium* + *Atropoides* + *Cerrophidion*, *Ophryacus*, *Lachesis,*
601 rattlesnakes and *Agkistrodon* clades having separated by 18 Mya, although the
602 crown clades within these genera are often considerably younger, lying towards the
603 lower end of the age range suggested by Zamudio and Greene (1997) and Wüster et
604 al. (2002) in the case of *Lachesis* and *Bothrops* (Table 4).

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

621 The colonization of the New World was followed by rapid adaptive radiation into
622 diverse ecological and morphological niches (Martins et al., 2001; Wüster et al.,

623 2002), and gave rise to a total of 117 currently recognized species (Campbell and
624 Lamar, 2004), giving a Speciation Interval (age of most recent common ancestor of a
625 clade divided by the natural logarithm of the number of species – Coyne and Orr,
626 2004) of 4.64, compared to 8.46 for the Viperidae as a whole and 9.04 for the
627 Caenophidia. The speciation interval in New World pitvipers is thus shorter than in
628 many other squamate clades (Sanders and Lee, 2008), suggesting relatively rapid
629 adaptive radiation in this clade following colonization of the New World.

630

631 *4.3 Implications for venom evolution in snakes*

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

647

Acknowledgements

Numerous individuals helped with the provision of tissue samples, laboratory work or data analysis. For this, we thank Markos Alexandrou, Marco Buegel, Maik Dobiey, Matthew Harris, Tomáš Mazuch, Abraham Mijares-Urrutia, Mark O'Shea, Tony Phelps, Harold van der Ploeg, Wolfgang Schneyer, Peter Schilperoord, Zoran Tadić, Sylvain Ursenbacher, Freek Vonk, José Luís Yrausquin, Fundación Herpetológica Gustavo Orcés (Jean-Marc Touzet, María Elena Barragán), Latoxan (Yvon Doljansky, Franck Principaud) and Liverpool School of Tropical Medicine (David Theakston, Paul Rowley, Gavin Laing, Robert Harrison). This study was funded by Leverhulme Trust grant F/00 174/I to WW.

References

Araújo, M., Martins, M., 2006. Defensive behaviour in pitvipers of the genus *Bothrops* (Serpentes, Viperidae). Herpetol. J. 16, 297-303.

Arévalo, E., Davis, S.K., Sites, J.W., 1994. Mitochondrial DNA sequence divergence and phylogenetic relationships among eight chromosome races of the *Sceloporus grammicus* complex (Phrynosomatidae) in Central Mexico. Syst. Biol. 43, 387-418.

Ashe, J.S., Marx, H., 1988. Phylogeny of the viperine snakes (Viperinae): Part II. Cladistic analysis and major lineages. Fieldiana Zool. New Ser. 52, 1-23.

Biardi, J.E., Chien, D.C., Coss, R.G., 2006. California ground squirrel (*Spermophilus beecheyi*) defenses against rattlesnake venom digestive and hemostatic toxins. J. Chem. Ecol. 32, 137-154.

Bromham, L., Penny, D., 2003. The modern molecular clock. Nature Rev. Genet. 4: 216-224.

673 Burbrink, F.T., Lawson, R., 2007. How and when did Old World ratsnakes disperse
674 into the New World? Mol. Phylogenet. Evol. 43, 173-189.
675 Burbrink, F.T., Lawson, R., Slowinski, J.B., 2000. Mitochondrial DNA phylogeography
676 of the polytypic North American Rat Snake (*Elaphe obsoleta*): a critique of the
677 subspecies concept. Evolution 54, 2107–2118.
678 Burbrink, F.T., Pyron, R.A., 2008. The taming of the skew: estimating proper
679 confidence intervals for divergence dates. Syst. Biol. 57, 317-328.
680 Cadle, J.E. 1992. Phylogenetic relationships among vipers: Immunological evidence.
681 In: Campbell, J.A., Brodie Jr., E.D. (Eds), Biology of the Pitvipers. Selva, Tyler,
682 Texas, pp. 41-48.
683 Campbell, J.A., Lamar, W.W., 2004. The Venomous Reptiles of the Western
684 Hemisphere. Comstock, Ithaca and London.
685 Castoe, T.A., Parkinson, C.L., 2006. Bayesian mixed models and the phylogeny of
686 pitvipers (Viperidae: Serpentes) Mol. Phylogenet. Evol., 39, 91-110.
687 Castoe, T.C., Sasa, M., Parkinson, C.L., 2005. Modeling nucleotide evolution at the
688 mesoscale: The phylogeny of the Neotropical pitvipers of the *Porthidium* group
689 (Viperidae: Crotalinae). Mol. Phylogenet. Evol. 37, 881–898.
690 Chijiwa, T., Yamaguchi, Y., Ogawa, T., Deshimaru, M., Nobuhisa, I, Nakashima, K.,
691 Oda-Ueda, N., Fukumaki, Y, Hattori, S., Ohno, M., 2003. Interisland evolution of
692 *Trimeresurus flavoviridis v*enom phospholipase A2 isozymes. J. Mol. Evol. 56, 286–
693 293.
694 Chippaux, J.-P., 1986. Les Serpents de la Guyane Française. Éditions ORSTOM,
695 Paris.
696 Chippaux, J.-P., Williams, V. and White, J., 1991. Snake venom variability: methods
697 of study, results and interpretation. Toxicon, 29, 1279-1303.

698 Coyne, J.A., Orr, H.A., 2004. Speciation. Sinauer Associates, Sunderland,
699 Massachusetts.
700 Creer, S., Malhotra, A., Thorpe, R.S., Stöcklin, R., Favreau, P., Chou, W.-H., 2003.
701 Genetic and ecological correlates of intraspecific variation in pitviper venom
702 composition detected using matrix assisted laser desorption time-of-flight mass
703 spectrometry (MALDI-TOF-MS) and isoelectric focusing. J. Mol. Evol. 56, 317-
704 329.
705 Crother, B.I., Campbell, J.A., Hillis, D.M., 1992. Phylogeny and historical
706 biogeography of the palm-pitvipers, genus *Bothriechis*: biochemical and
707 morphological evidence. In: Campbell, J.A., Brodie Jr., E.D. (Eds.), Biology of the
708 Pitvipers, Selva, Tyler, pp. 1-19.
709 Daltry, J.C., Wüster, W., Thorpe, R.S., 1996. Diet and snake venom evolution.
710 Nature, 379, 537-540.
711 David, P., Ineich, I., 1999. Les serpents venimeux du monde: systématique et
712 repartition. Dumerilia 3, 3-499.
713 De Queiroz, A., Lawson, R., Lemos-Espinal, J. A., 2002. Phylogenetic relationships
714 of NorthAmerican garter snakes: how much DNA is enough? *Mol. Phylogenet. Evol*.
715 22, 315–329.
716 De Queiroz, A., 2005. The resurrection of oceanic dispersal in historical
717 biogeography. Trends Ecol. Evol. 20, 68-73.
718 Dixon, J.R., Soini, P., 1986. The Reptiles of the Upper Amazon Basin, Iquitos
719 Region, Peru. Milwaukee Public Museum, Milwaukee.
720 Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by
721 sampling trees. BMC Evol. Biol. 7: 214

722 Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the
723 bootstrap. Evolution 39, 783-791.
724 Fry, B.G., Winkel, K.D., Wickramaratna, J.C., Hodgson, W.C., Wüster, W., 2003.
725 Effectiveness of snake antivenom: species and regional venom variation and its
726 clinical impact. J. Toxicol. -Toxin Rev. 22, 23-34.
727 Fry, B.G., Wüster, W., 2004. Assembling an arsenal: Origin and evolution of the
728 snake venom proteome inferred from phylogenetic analysis of toxin sequences.
729 Mol. Biol. Evol. 21, 870-883.
730 Fry, B.G., Wüster, W., Ramjan, S.F.R., Jackson, T., Martelli, P., Kini, R.M., 2003.
731 Analysis of Colubroidea snake venoms by liquid chromatography with mass
732 spectrometry: evolutionary and toxinological implications. Rapid Comm. Mass
733 Spectrom. 17, 2047-2062.
734 Fry, B.G., Vidal, N., Norman, J.A., Vonk, F.J., Scheib, H., Ramjan, S.F.R., Kuruppu,
735 S., Fung, K., Hedges, S.B., Richardson, M.K., Hodgson, W.C., Ignjatovic, V.,
736 Summerhayes, R., Kochva, E., 2006. Early evolution of the venom system in lizards
737 and snakes. Nature 439, 584-588.
738 Greene, H.W. 1992. The ecological and behavioral context of pitviper evolution. In:
739 Campbell, J.A., Brodie Jr., E.D. (Eds), Biology of the Pitvipers. Selva, Tyler, Texas,
740 pp. 107-118.
741 Greene H.W., 1997. Snakes, The Evolution of Mystery in Nature. University of
742 California Press, Berkeley, CA.
743 Groombridge, B., 1980. A phyletic analysis of viperine snakes. Unpublished PhD
744 thesis. City of London Polytechnic, London.

745 Groombridge, B., 1986. Phyletic relationships among viperine snakes. In: Roček, Z.
746 (Ed.), Studies in Herpetology. Charles University / Societas Europaea
747 Herpetologica, Prague, pp. 219-222.
748 Guo, P., Malhotra, A., Li, P.P., Pook, C.E., Creer, S., 2007. New evidence on the
749 phylogenetic position of the poorly known Asian pitviper *Protobothrops kaulbacki*
750 (Serpentes: Viperidae: Crotalinae) with a redescription of the species and a revision
751 of the genus *Protobothrops*. Herpetol. J. 17, 237-246.
752 Gutberlet Jr., R.L., Harvey, M.B., 2002. Phylogenetic relationships of New World
753 pitvipers as inferred from anatomical evidence. In: Schuett, G.W., Höggren, M.,
754 Douglas, M.E., Greene, H.W. (Eds.), Biology of the Vipers. Eagle Mountain
755 Publishing, Eagle Mountain, Utah, pp. 51–68.
756 Gutberlet Jr., R.L., Harvey, M.B., 2004. The evolution of New World venomous
757 snakes. In: Campbell, J.A., Lamar, W.W. (Eds.), The Venomous Reptiles of the
758 Western Hemisphere. Comstock, Ithaca, New York, pp. 634-682.
759 Hallam, A., 1994. An Outline of Phanerozoic Biogeography. Oxford Univ. Press,
760 Oxford.
761 Harrison, R.A., Wüster, W., Theakston, R.D.G., 2003. The conserved structure of
762 snake venom toxins confers extensive immunological cross-reactivity to toxin-
763 specific antibody. Toxicon 41, 441-449.
764 Head, J.J., Holroyd, P.A., Hutchison, J.H., Ciochon, R.L., 2005. First report of snakes
765 (Serpentes) from the Late Middle Eocene of the Ponduang Formation, Myanmar. J.
766 Vert. Paleontol. 25, 246–250.
767 Heise, P.J., Maxson, L.R., Dowling, H.G., Hedges, S.B., 1995. Higher level snake
768 phylogeny inferred from mitochondrial DNA sequences of 12S rRNA and 16S rRNA
769 genes. Mol. Biol. Evol. 12, 259–265.

770 Herrmann, H.W., Joger, U., 1995. Molecular data on the phylogeny of African
771 viperids – preliminary results. In Llorente, A., Montori, A., Santos, X., Carretero,
772 M.A. (Eds.), Scientia Herpetologica. Associación Herpetológica Española,
773 Salamanca, pp. 92-97.
774 Herrmann, H.W., Joger, U., 1997. Evolution of viperine snakes. In Thorpe, R.S.,
775 Wüster, W., Malhotra, A. (Eds.), Venomous Snakes: Ecology, Evolution and
776 Snakebite. Symposia of the Zoological Society of London, No. 70. Clarendon
777 Press, Oxford, pp. 43-61.
778 Herrmann, H.W., Lenk, P., Joger, U., Wink, M., 1999. Morphological and molecular
779 phylogenies of Viperinae: conflicting evidence? Kaupia, 8, 21-30.
780 Hugall, A.F., Foster, R., Lee, M.S.Y., 2007. Calibration choice, rate smoothing, and
781 the pattern of tetrapod diversification according to the long nuclear gene RAG-1.
782 Syst. Biol. 56, 543-563.
783 Ineich, I., Bonnet, X., Shine, R., Brischoux, F., Lebreton, M., Chirio, L., 2006. What, if
784 anything, is a 'typical' viper? Biological attributes of basal viperid snakes (genus
785 *Causus* Wagler, 1830). Biol. J. Linn. Soc., 89, 575-588.
786 Jackson, K. 2003. The evolution of venom-delivery systems in snakes. Zool. J. Linn.
787 Soc. 137, 337-354.
788 Jackson, K., 2007. The evolution of venom-conducting fangs: Insights from
789 developmental biology. Toxicon 49, 975-981.
790 Joger, U., 2005. *Montivipera* Nilson, Tuniyev, Andrén, Orlov, Joger und Herrmann,
791 1999. In: Joger, U., Stümpel, N. (Eds.), Handbuch der Reptilien und Amphibein
792 Europas. Schlangen (Serpentes) III. Aula-Verlag, Wiesbaden, pp. 61-62.
793 Joger, U., Courage, K., 1999. Are palaearctic "rattlesnakes" (*Echis* and *Cerastes*)
794 monophyletic? Kaupia 8, 65–81.

795 Kelly, C.M.R., Barker N.P., Villet, M.H., 2003. Phylogenetics of advanced snakes
796 (Caenophidia) based on four mitochondrial genes. Syst. Biol., 52, 439-459.
797 Knight, A., Mindell, D.P., 1993. Substitution bias, weighting of DNA sequence
798 evolution, and the phylogenetic position of Fea's viper. Syst. Biol. 42, 18-31.
799 Kraus, F., Mink, D.G., Brown, W.M., 1996. Crotaline intergeneric relationships based
800 on mitochondrial DNA sequence data. Copeia, 1996, 763-773.
801 Kuch, U., Müller, J., Mödden, C., Mebs, D., 2006. Snake fangs from the Lower
802 Miocene of Germany: evolutionary stability of perfect weapons.
803 Naturwissenschaften 93, 84-87.
804 Kumazawa, Y., Ota, H., Nishida, M., Ozawa, T., 1998. The complete nucleotide
805 sequence of a snake (*Dinodon semicarinatus*) mitochondrial genome with two
806 identical control regions. Genetics 150, 313-329.
807 Lawson, R., Slowinski, J.B., Crother, B.I., Brubrink, F.T., 2005. Phylogeny of the
808 Colubroidea (Serpentes): new evidence from mitochondrial and nuclear genes. Mol.
809 Phylogenet. Evol. 37, 581-601.
810 Lenk, P., Herrmann, H.-W., Joger, U., Wink, M., 1999. Phylogeny and taxonomic
811 subdivision of *Bitis* (Reptilia: Viperidae) based on molecular evidence. Kaupia, 8,
812 31-38.
813 Lenk, P., Kalayabina, S., Wink, M., Joger, U., 2001. Evolutionary relationships
814 among the true vipers (Reptilia: Viperidae) inferred from mitochondrial DNA
815 sequences. Mol. Phylogenet. Evol. 19, 94-104.
816 Liem, K.F., Marx, H., Rabb, G.B., 1971. The viperid snake *Azemiops*: its comparative
817 cephalic anatomy and phylogenetic position in relation to Viperinae and Crotalinae.
818 Fieldiana Zool. 59, 65-126.

819 Madsen, T., Shine, R., Olsson, M., Wittzell, H. 1999. Conservation biology -
820 Restoration of an inbred adder population. Nature 402, 34-35.
821 Madsen, T., Shine, R. 1994. Costs of reproduction influence the evolution of sexual
822 size dimorphism in snakes. Evolution 48, 1389-1397.
823 Malhotra, A., Thorpe, R.S., 2004. A phylogeny of four mitochondrial gene regions
824 suggests a revised taxonomy for Asian pitvipers (*Trimeresurus* and *Ovophis*). Mol.
825 Phylogenet. Evol. 32, 83-100.
826 Mallow, D., Ludwig, D., Nilson, G., 2003. True Vipers. Natural History and Toxinology
827 of Old World Vipers. Krieger Publishing Company, Malabar, Florida.
828 Martins, M., Araújo, M.S., Sawaya, R.J., Nunes, R., 2001. Diversity and evolution of
829 macrohabitat use, body size and morphology in a monophyletic group of
830 Neotropical pitvipers (*Bothrops*). J. Zool. Lond. 254, 529-538.
831 McDiarmid, R.W., Campbell, J.A., Touré, T.A., 1999. Snake Species of the World: A
832 Taxonomic and Geographical Reference, Vol. 1. The Herpetologists' League,
833 Washington, D.C.
834 Nagy, Z.T., Joger, U., Wink, M., Glaw, F., Vences, M., 2003. Multiple colonization of
835 Madagascar and Socotra by colubrid snakes: evidence from nuclear and
836 mitochondrial gene phylogenies. Proc. R. Soc. Lond. B 270, 2613–2621.
837 Nagy, Z.T., Vidal, N., Vences, M., Branch, W.R., Pauwels, O.S.G., Wink, M., Joger,
838 U., 2005. Molecular systematics of African Colubroidea (Squamata: Serpentes) In:
839 Huber, B.A., Sinclair, B.J., Lampe, K.H. (Eds.), African Biodiversity: Molecules,
840 Organisms, Ecosystsms. Proc. 5th Intern. Symp. Trop. Biol. Museum Koenig, Bonn,
841 pp. 221-228
842 Nilson, G., Tuniyev, B., Andrén, C., Orlov, N., Joger, U., Herrmann, H.-W., 1999.
843 Taxonomic position of the *Vipera xanthina* complex. Kaupia 8, 99–102.

844 Nylander, J.A.A., 2004. MrModeltest v2. Program distributed by the author.
845 Evolutionary Biology Centre, Uppsala Univ..
846 Olsson, M., Madsen, T., Shine, R., 1997. Is sperm really so cheap? Costs of
847 reproduction in male adders, *Vipera berus*. Proc. R. Soc. Lond. B 264, 455-459.
848 Palumbi, S.R., 1996. Nucleic acids II: the polymerase chain reaction. In: Hillis, D.M.,
849 Moritz, C., Mable, B.K. (Eds.), Molecular Systematics. Second Edition. Sinauer,
850 Sunderland, Massachusetts, pp. 205-247.
851 Parkinson, C.L., 1999. Molecular systematics and biogeographical history of pitvipers
852 as determined by mitochondrial ribosomal DNA sequences. Copeia, 1999, 576-586.
853 Parkinson, C.L., Campbell, J.A., Chippindale, P.T., 2002. Multigene phylogenetic
854 analysis of pitvipers, with comments on their biogeography. In: Schuett, G.W.,
855 Höggren, M., Douglas, M.E., Greene, H.W. (Eds.), Biology of the Vipers. Eagle
856 Mountain Publishing, Eagle Mountain, Utah, pp.93-110.
857 Parmley, D., Holman, J.A., 2007. Earliest fossil record of a pigmy rattlesnake
858 (Viperidae: *Sistrurus* Garman). J. Herpetol. 41, 141-144.
859 Poran, N.S., Coss, R.G., Benjamini, E., 1987. Resistance of California ground
860 squirrels (*Spermophilus beecheyi*) to the venom of the northern Pacific rattlesnake
861 (*Crotalus viridis oreganus*): a study of adaptive variation. Toxicon 25, 767-777.
862 Pough, F.H., Groves, J.D., 1983. Specializations of the body form and food habits of
863 snakes. Am. Zool. 23, 443-454.
864 Rage, J.-C., Bajpai, S., Thewissen, J.G.M., Tiwari, B.N., 2003. Early Eocene snakes
865 from Kutch, Western India, with a review of the Palaeophiidae. Geodiversitas 25,
866 695-716.

867 Rage J.-C., Werner C., 1999. Mid-Cretaceous (Cenomanian) snakes from Wadi Abu
868 Hashim, Sudan: the earliest snake assemblage. Palaeontologia Africana 35, 85-
869 110.
870 Rambaut, A, Drummond, A.J., 2007 Tracer v1.4. Available from
871 http://beast.bio.ed.ac.uk/Tracer
872 Roman, B., 1980. Serpents de Haute-Volta. C.N.R.S.T., Ouagadougou.
873 Ronquist, F., 1996. DIVA version 1.1. Computer program and manual available by
874 anonymous FTP from Uppsala University (ftp.uu.se or ftp.systbot.uu.se).
875 Ronquist, F. 1997. Dispersal-vicariance analysis: A new approach to the
876 quantification of historical biogeography. Syst. Biol. 46, 195-203.
877 Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference
878 under mixed models. Bioinformatics 19, 1572–1574.
879 Rutschmann, F., 2006. Molecular dating of phylogenetic trees: A brief review of current
880 methods that estimate divergence times. Diversity Distrib. 12, 35–48.
881 Sanders, K.L., Lee, M.S.Y., 2007. Evaluating molecular clock calibrations using
882 Bayesian analyses with soft and hard bounds. Biol. Lett. 3, 275-279.
883 Sanders, K.L., Lee, M.S.Y., 2008. Molecular evidence for a rapid late-Miocene radiation
884 of Australasian venomous snakes (Elapidae, Colubroidea). Mol. Phylogenet. Evol. 46,
885 1180-1188.
886 Sanmartín, I., Enghoff, H., Ronquist, F., 2001. Patterns of animal dispersal, vicariance
887 and diversification in the Holarctic. Biol. J. Linn. Soc. 73, 345-390.
888 Sanz, L., Gibbs, H.L., Mackessy, S.P., Calvete, J.J., 2006. Venom proteomes of
889 closely related *Sistrurus* rattlesnakes with divergent diets. J. Proteome Res. 5,
890 2098-2112.

891 Scanlon, J.D., Lee, M.S.Y., Archer, M., 2003. Mid-Tertiary elapid snakes (Squamata,
892 Colubroidea) from Riversleigh, northern Australia: early steps in a continent-wide
893 adaptive radiation. Geobios 36, 573-601.
894 Scotese, C.R., 2004. A continental drift flipbook. J. Geol. 112: 729-741.
895 Shimodaira, H. Hasegawa, M., 1999. Multiple comparisons of log-likelihoods with
896 applications to phylogenetic inference. Mol. Biol. Evol. 16, 1114-1116.
897 Swofford, D.L., 2002. PAUP* - Phylogenetic Analysis Using Parsimony (*and Other
898 Methods). Beta version 4.0b10. Sinauer, Sunderland.
899 Szyndlar, Z., Rage, J.-C., 1990. West Palearctic cobras of the genus *Naja*
900 (Serpentes: Elapidae): interrelationships among extinct and extant species.
901 Amphibia-Reptilia 11, 385-400.
902 Szyndlar, Z., Rage, J.-C., 1999. Oldest fossil vipers (Serpentes: Viperidae) from the
903 Old World. Kaupia 8, 9-20.
904 Templeton, A.R., 1983. Phylogenetic inference from restriction endonuclease
905 cleavage sites maps with particular reference to the evolution of humans and the
906 apes. Evolution 37, 221–244.
907 Theakston, R.D.G., Laing, G.D., Fielding, C.M., Freire Lascano, A., Touzet, J.-M.,
908 Vallejo, F., Guderian, R.H., Nelson, S.J., Wüster, W., Richards, A.M., Rumbea
909 Guzmán, J., Warrell, D.A., 1995. Treatment of snake bites by *Bothrops* species and
910 *Lachesis muta* in Ecuador: laboratory screening of candidate antivenoms. Trans. R.
911 Soc. Trop. Med. Hyg., 89, 550-554.
912 Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the
913 sensitivity of progressive multiple sequence alignment through sequence weighting,
914 positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22,
915 4673-4680.

916 Thorpe, R.S., McGregor, D.P., Cumming, A.M., Jordan, W.C., 1994. DNA evolution
917 and colonization sequence of island lizards in relation to geological history: mtDNA
918 RFLP, cytochrome b, cytochrome oxidase, 12S rRNA sequence, and nuclear
919 RAPD analysis. Evolution. 48, 230-240.
920 Thorpe, R.S., Pook, C.E., Malhotra, A., 2007. Phylogeography of the Russell's viper
921 (*Daboia russelii*) complex in relation to variation in the colour pattern and symptoms
922 of envenoming. Herpetol. J.,17, 209-218.
923 Vidal, N. 2002. Colubroid systematics: evidence for an early appearance of the
924 venom apparatus followed by extensive evolutionary tinkering. J. Toxicol.—Toxin
925 Rev. 21, 21–41.
926 Vidal, N., Hedges, S.B., 2002. Higher-level relationships of caenophidian snakes
927 inferred from four nuclear and mitochondrial genes. C. R. Biologies 325, 987–995.
928 Vidal, N., Lecointre, G., 1998. Weighting and congruence: a case study based on
929 three mitochondrial genes in pitvipers. Mol. Phylogenet. Evol., 9, 366-374.
930 Vidal, N., Delmas, A.-S., David, P., Cruaud, C., Couloux, A., Hedges, S.B., 2007. The
931 phylogeny and classification of caenophidian snakes inferred from seven nuclear
932 protein-coding genes. C.R. Biologies 330, 182-187.
933 Warrell, D.A., 1995a. Clinical toxicology of snakebite in Africa and the Middle
934 East/Arabian Peninsula. In: Meier, J., White, J. (Eds.), Handbook of Clinical
935 Toxicology of Animal Venoms and Poisons. CRC Press, Boca Raton, Florida, pp.
936 433-492.
937 Warrell, D.A., 1995b. Clinical toxicology of snakebite in Asia. In: Meier, J., White J.
938 (Eds.), Handbook of Clinical Toxicology of Animal Venoms and Poisons. CRC
939 Press, Boca Raton, Florida, pp. 493-594.

940 Warrell, D.A., 2004. Snakebites in Central and South America: epidemiology, clinical
941 features, and clinical management. In: Campbell, J.A., Lamar, W.W. (Eds.), The
942 Venomous Reptiles of the Western Hemisphere. Comstock, Ithaca, New York, pp.
943 709-761.
944 Wüster, W. Allum, C.S.E., Bjargardóttir, I.B., Bailey, K.L., Dawson, K.J., Guenioui, J.,
945 Lewis, J., Mcgurk, J., Moore, A.G., Niskanen, M., Pollard, C.P., 2004. Do
946 aposematism and Batesian mimicry require bright colours? A test, using European
947 viper markings. Proc. R. Soc. Lond. Biol. Sci. 271, 2495-2499.
948 Wüster, W., Crookes, S., Ineich, I., Mané, Y., Pook, C.E., Trape, J.-F., Broadley,
949 D.G., 2007. The phylogeny of cobras inferred from mitochondrial DNA sequences :
950 evolution of venom spitting and the phylogeography of the African spitting cobras
951 (Serpentes: Elapidae: *Naja nigricollis* complex). Mol. Phylogenet. Evol.,
952 doi:10.1016/j.ympev.2007.07.021.
953 Wüster, W., Dumbrell, A.J., Hay, C., Pook, C.E., Williams, D.J., Fry, B.G., 2005a.
954 Snakes across the Strait: Trans-Torresian phylogeographic relationships in three
955 genera of Australasian snakes (Serpentes: Elapidae: *Acanthophis*, *Oxyuranus* and
956 *Pseudechis*). Mol. Phylogenet. Evol. 34, 1-14.
957 Wüster, W., Ferguson, J.E. Quijada-Mascareñas, J.A., Pook, C.E., Salomão, M.G.,
958 Thorpe, R.S., 2005b. Tracing an invasion: landbridges, refugia and the
959 phylogeography of the Neotropical rattlesnake (Serpentes: Viperidae: *Crotalus*
960 *durissus*). Mol. Ecol. 14, 1095-1108.
961 Wüster, W., Otsuka, S., Malhotra, A., Thorpe, R.S., 1992. Population systematics of
962 Russell's viper: a multivariate study. Biol. J. Linn. Soc. 47, 97-113.
963 Wüster, W., Salomão, M.G., Quijada-Mascareñas, J.A., Thorpe, R.S., B.B.B.S.P.,
964 2002. Origin and evolution of the South American pitviper fauna: evidence from

965 mitochondrial DNA sequence analysis. In: Schuett, G.W., Höggren, M., Douglas,
966 M.E., Greene, H.W. (Eds.), Biology of the Vipers. Eagle Mountain Publishing, Eagle
967 Mountain, Utah, pp. 111-128.
968 Yang, Z., Rannala, B., 2006. Bayesian estimation of species divergence times under
969 a molecular clock using multipe fossil calibrations with soft bounds. Mol. Biol. Evol.
970 23, 212–226.
971 Zamudio, K.R., Greene, H.W., 1997. Phylogeography of the bushmaster (*Lachesis*
972 *muta*: Viperidae): implications for neotropical biogeography, systematics, and
973 conservation. Biol. J. Linn. Soc. 62, 421-442.
974 Zhang, D.-X., Hewitt, G.M., 1996. Nuclear integrations: challenges for mitochondrial
975 DNA markers. Trends Ecol. Evol. 11, 247-251.
976 Zuckerkandl, E., Pauling, L., 1965. Evolutionary divergence and convergence in
977 proteins. In: Bryson, V., Vogel, H.J. (Eds.), Evolving Genes and Proteins. Academic
978 Press, New York, pp. 97–166.

979 Figure legends

980

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

990

991 Fig. 2. Bayesian inference tree of the vipers and their sister clade. Branch support
992 measures are Bayesian posterior probabilities/MP bootstrap support (only where >
993 50%). Branch support indices are not given for most intrageneric nodes to preserve
994 clarity.

995

996 Fig. 3. BEAST maximum credibility ultrametric tree showing the timing of the
997 evolution of the Colubroidea and the origin of the vipers. Grey bars indicate 95%
998 confidence intervals for selected nodes. Calibration points are indicated by numbered
999 circles on nodes; numbering corresponds to that used in Materials and Methods. The
1000 timing of the evolution of the Viperidae is shown in Fig. 4.

1001

1002 Fig. 4. BEAST maximum credibility ultrametric tree showing the timing of the
1003 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.

Taxon	Locality	Voucher(s) / sample(s)	Genbank accession number (NADH4, cyt*b*, 16s, 12s). New sequences generated for this study are marked with an asterisk*
Acrochordus granulatus	unknown	NUM-Az0375	**NC007400** for all
Alsophis portoricensis	British Virgin Islands (NADH4) / Puerto Rico (others)	FK 2440 (NADH4) / CAS 200813 (cyt*b*) / SBH 160062 (16s, 12s)	**U49308**, **AF471085**, **AF158517**, **AF158448**
Cerberus rynchops	Sabah (NADH4) / Myanmar (cyt*b*) / Polillo (16s, 12s)	FMNH 251594 (NADH4) / CAS 206574 (cyt*b*) / USNM 497590 (16s, 12s)	**U49327**, **AF471092**, **AF499303**, **AF499289**
Contia tenuis	California (NADH4, cyt*b*) / n.a. (16s, 12s)	CAS 207044 (NADH4) / CAS 202582 (cyt*b*) / n.a. (16s, 12s)	**AF402656**, **AF471095**, **AY577030**, **AY577021**
Coronella	Morocco	MVZ 178073	**AY487066**, **AF471088**,

girondica	(NADH4, cyt*b*, 16s) / n.a. (12s)	(NADH4, cyt*b*) / E512.20 (16s) / unknown (12s)	**AY643353**, **AY122835**
Diadophis punctatus	California (NADH4) / Florida (cyt*b*) / n.a. (16s, 12s)	SDSNH 68893 (NADH4) / CAS 184351 (cyt*b*) / n.a. (16s, 12s)	**DQ364667**, **AF471094**, **AY577023**, **AY577051**
Dinodon semicarinatus	n.a.	n.a.	**AB008539** for all
Eirenis modestus	Turkey(NADH4, cyt*b*) / n.a. (16s) / n.a. (12s)	HLMD J159 / HLMD J159 / n.a. / n.a.	**AY487072**, **AY486933**, **AY376780**, **AY039143**
Elaphe sauromates	European Turkey(NADH4, cyt*b*) / n.a. (16s) / n.a. (12s)	LSUMZ 40626 (NADH4, cyt*b*) / n.a. (16s) / SH972 (12s)	**AY487067**, **AY486931**, **AF215267**, **AY122795**
Farancia abacura	Florida/ n.a. / n.a. / Georgia	UMMZ 205023 / n.a. / n.a. / RH 53660	**U49307**, **U69832**, **AY577025**, **Z46467**
Hemorrhois algirus	Morocco (NADH4, cyt*b*) / Tunisia (16s) / n.a. (12s)	HLMD RA 1187 (NADH4, cyt*b*) / E1110.1 (16s)/ MHNG2415.6	**AY487037**, **AY486911**, **AY643349**, **AY039149**

		(12s)	
Hemorrhois hippocrepis	Spain (NADH4, cyt*b*) / Morocco (16s) / n.a. (12s)	MNCN 11988 (NADH4, cyt*b*) / E2509.2 (16s) / MHNG2415.94 (12s)	**AY487045**, **AY486916**, **AY643350**, **AY039158**
Hemorrhois nummifer	Armenia (NADH4, cyt*b*, 16s) / n.a. (12s)	ZISP 27709(NADH4, cyt*b*, 16s) / SH548 (12s)	**AY487049**, **AY376742**, **AY376771**, **AY039163**
Heterodon platirhinos	North Carolina	MVZ 175928	**AF402659**, n/a, n/a, n/a (spliced with *H. simus* sequences for analysis)
Heterodon simus	Florida (cyt*b*) / n.a. (16s, 12s)	CAS 195598 (cyt*b*) / n.a. (16s, 12s)	n/a, **AF217840**, **AY577029**, **AY577020**
Lamprophis fuliginosus	Burundi (NADH4) / Tanzania (cyt*b*, 16s)/ n.a. (12s)	n.a. (NADH4) / CAS 168909 (cyt*b*, 16s) / SH1210 (12s)	**AF544664**, **AF471060**, **AY188079**, **AY122681**
Leioheterodon madagascariensis	Madagascar	RAN 42543 (NADH4) / MRSN-FAZC 10621 (cyt*b*, 16s) / n.a. (12s)	**U49318**, **AY188022**, **AY188061**, **AF544768**

Macroprotodon brevis	Spain	MVZ 186073 (NADH4, cyt*b*) / E608.6 (16s, 12s)	**AY487064**, **AF471087**, **AY643321**, **AY643280**
Malpolon monspessulanus	Spain (NADH4) / Greece (cyt*b*, 16s) / Morocco (12s)	MVZ 186256 (NADH4)/ HLMD RA-2606 (cyt*b*,16s) / E2509.18 (12s)	**AY058989**, **AY188029**, **AY188068**, **DQ451927**
Mimophis mahfalensis	Madagascar	MZUSP 12188 (NADH4, 12s) / HLMD J68 (cyt*b*, 16s)	**AF544662**, **AY188032**, **AY188071**, **AF544771**
Natrix natrix	France (all)	n.a. (all)	**AY873736**, **AY866537**, **AF158530**, **AF158461**
Psammophis condanarus	Myanmar (NADH4, cyt*b*) / Thailand (16s, 12s)	CAS 205003 (NADH4, cyt*b*) / RH 5601 (16s, 12s)	**AY058987**, **AF471075**, **Z46479**, **Z46450**
Bungarus fasciatus	Brunei (NADH4) / Myanmar (cyt*b*) / unknown (16s, 12s)	UMMZ 201916 (NADH4) / CAS 207988 (cyt*b*) / RH 63881 (16s, 12s)	**U49297**, **AF217830**, **Z46501**, **Z46466**

Naja kaouthia	Chumphon Province, Thailand	WW585 (all)	**EU624209***, **EU624298***, **EU624269***, **EU624235***
Naja naja	Nepal	WW595 (all)	**AY713378**, **EU624299***, **EU624270***, **EU624236***
Naja nigricollis	Lara, Kaélé, northern Cameroon	Latoxan, live collection number N. ni. ssp. 9735002 (all)	**AY713377**, **EU624300***, **EU624271***, **EU624237***
Naja nivea	unknown (NADH4, cyt*b* / South Africa (16s, 12s)	n.a. (NADH4, cyt*b*) WW1295 (16s, 12s)	**AY058983**, **AF217827**, **EU624272***, **EU624238***
Ophiophagus hannah	Myanmar (NADH4, cytb) / unknown (16s, 12s)	CAS 206601 (NADH4, cyt*b*) / RH 6081 (16s, 12s)	**AY058984**, **AF217842**, **Z46480**, **U96803**
Agkistrodon contortrix	South Carolina (NADH4) / Ohio (others)	UMMZ 199957 (NADH4) / Moody 338 (others)	**U41868**, **AY223612**, **AF057277**, **AF057229**
Agkistrodon piscivorus	Florida (NADH4) / South Carolina (others)	CLP 74 (NADH4) / CLP 30 (others)	**U41870**, **AY223615**, **AF057278**, **AF156588**

Atropoides olmec	Veracruz, Mexico	JAC 16021 (all)	**AY223632**, **AY223585**, **AY223669**, **AY223656**
Atropoides picadoi	Mexico (12s) / Costa Rica (others)	UMMZ 177000 (NADH4) / CLP 45 (cyt*b*, 16s) / ENS-10515 (12s)	**U41872**, **AY223583**, **AF057255**, **DQ305422**
Bothriechis aurifer	Guatemala	UTA R-35031 (all)	**DQ305483**, **DQ305466**, **DQ305448**, **DQ305425**
Bothriechis nigroviridis	Costa Rica / Costa Rica	MZUCR 11151 (all)	**AY223635**, **AY223589**, **AF057259**, **AF057212**
Bothriechis schlegelii	Costa Rica	MZUCR 11149 (all)	**AY223636**, **AY223590**, **AF057260**, **AF057213**
Bothrocophias hyoprora	Leticia, Colombia	n.a. (all)	**U41886**, **AY223593**, **AF057253**, **AF057206**
Bothrops alternatus	unknown	DPL 2879 (all)	**AY223642**, **AY223601**, **AY223673**, **AY223660**
Bothrops asper	Siquirres, Limón, Costa Rica	WW1318 (all)	**EU624210***, **EU624301***, **EU624273***, **EU624239***
Bothrops diporus	Argentina	PT 3404 (all)	**DQ305489**, **DQ305472**, **DQ305454**, **DQ305431**
Bothrops taeniatus	Suriname (all)	n.a. (all)	**AY223637**, **AY223592**, **AF057262**, **AF057215**
Calloselasma rhodostoma	unknown	UMMZ 184314 (NADH4)/ UTA R-22247	**U41878**, **AY223562**, **AF057237**, **AF057190**

		(others)	
Cerrophidion godmani	Costa Rica	UMMZ 177001 (NADH4), MZUCR 11153 (others)	**U41879**, **AY223578**, **AF057250**, **AF057203**
Crotalus adamanteus	Florida (all)	UMMZ 188768 (NADH4), CLP 4 (others)	**U41880**, **AY223605**, **AF057269**, **AF057222**
Crotalus ravus	Puebla, Mexico	UTA live coll. (all)	**AY223647**, **AY223609**, **AF057273**, **AF057226**
Crotalus simus	Guanacaste Province, Costa Rica	WW 1097 (NADH4, cyt*b*) / WW1312 (16s, 12s)	**AY704885**, **EU624302***, **EU624274***, **EU624240***
Crotalus tigris	Arizona	CLP 169 (all)	**AF156574**, **AY223606**, **AF057270**, **AF057223**
Cryptelytrops insularis	E. Java	AM A109 (all)	**AY352883**, **AY352767**, **AY352738**, **AY352799**
Cryptelytrops macrops	Bangkok, Thailand	AM B27 (all)	**AF517219**, **AF517184**, **AF517176**, **AF517163**
Deinagkistrodon acutus	China	CLP 28 (all)	**U41883**, **AY223560**, **AF057235**, **AF057188**
Garthius chaseni	Sabah	AM B306 (all)	**AY352825**, **AY352760**, **AY352729**, **AY352791**
Gloydius shedaoensis	Liaoning, China	ROM 20468 (all)	**AY223623**, **AY223566**, **AF057241.** **AF057194**
Gloydius strauchi	Sichuan, China	ROM 20473 (all)	**AY223620**, **AY223563**,

			AF057239, **AF057192**
Himalayophis tibetanus	Nepal	ZMB 65641 (all)	**AY352810**, **AY352749**, **AY322715**, **AY352776**
Hypnale hypnale	Sri Lanka	CLP 164 (all)	**U41884**, **AY223561**, **AF057268**, **AF057189**
Lachesis muta	Peru	Cadle 135 (all)	**AY223644**, **AY223604**, **AF057268**, **AF057221**
Lachesis stenophrys	Costa Rica (all)	UMMZ 176987 (NADH4) / n.a. (others)	**U41885**, **AY223603**, **AF057267**, **AF057220**
Ophryacus melanurus	Mexico (all)	UTA R 34605 (NADH4, cyt*b*), CLP 73 (16s, 12s)	**AY223634**, **AY223587**, **AF057257**, **AF057210**
Ovophis monticola	Yunnan, China	CAS 215050 (all)	**DQ305480**, **DQ305462**, **DQ305439**, **DQ305416**
Ovophis okinavensis	Ryukyu islands, Jaoan	UMMZ 199980 / CLP 162 (cyt*b*, 16s) / n.a.	**U41895**, **AY223573**, **AF057246**, **DQ305418**
Parias flavomaculatus	Luzon (NADH4, 16s, 12s), Mindanao (cyt*b*)	AM B3 (NADH4, 16s, 12s) / AM B4 (cyt*b*)	**AY059584**, **AY352764**, **AY059551**, **AY059535**
Parias hageni	Thailand	AM B33 (all)	**AY059585**, **AF171911**, **AY059552**, **AY059536**
Popeia popeiorum	Laos	FMNH 258950 (all)	**AY059590**, **AY059571**, **AY059554**, **AY059538**

Porthidium arcosae	Salango, Manabí, Ecuador	WW 750 (all)	**AF292613**, **AF292575**, **EU624275***, **EU624241***
Porthidium lansbergii rozei	San Antonio, Falcón, Venezuela	WW 787	**AF393623**, **AY713375**, **EU624276***, **EU624242***
Porthidium nasutum (Costa Rica)	Costa Rica (all)	UMMZ 176992 (NADH4) / MZUCR 11150 (others)	**U41887**, **AY223579** , **AF057251**, **AF057204**
Porthidium nasutum (Ecuador)	Zapallo Grande, Río Cayapas, Esmeraldas, Ecuador	WW 751 (NADH4, cyt*b*), WW 1010 (NADH4, cyt*b*)	**AF292612**, **AF292574**, **EU624277***, **EU624243***
Porthidium ophryomegas	Costa Rica	UMMZ 210276 (all)	**U41888**, **AY223580**, **AF057252**, **AF057205**
Protobothrops cornutus	Vietnam	ZFMK 75067	**AY294262**, **AY294272**, **AY294267**, **AY294276**
Protobothrops flavoviridis	Tokunoshima, Japan	UMMZ 199973 (all)	**U41894**, **AY223574**, **AF057247**, **AF057200**
*Protobothrops mangshanensis***	Hunan, China	AM B300 (all)	**AY352821**, **AY352758.** **AY352726**, **AY352787**
Protobothrops mucrosquamatus	Vietnam	ROM 25717 (all)	**AY223629**, **AY223577**, **AY223666**, **AY223653**
*Protobothrops sieversorum***	Vietnam	ZFMK 75066 (all)	**DQ305478**, **DQ305460**, **DQ305437**, **DQ305414**
Sistrurus	Texas	Moody 502 (all)	**AY223648**, **AY223610**,

catenatus			**AF057274**, **AF057227**
Sistrurus miliarius	Florida (all)	UMMZ 175538 (NADH4), UTA-live (others)	**U41889**, **AY223611**, **AF057275**, **AF057228**
Trimeresurus borneensis	Sabah	AM B301 (all)	**AY352817**, **AY352754**, **AY352722**, **AY352783**
Trimeresurus gracilis	Taiwan	NTNUB 200515 (all)	**DQ305479**, **DQ305461**, **DQ305438**, **DQ305415**
Trimeresurus trigonocephalus	Sri Lanka	AM A58	**AY059597**, **AF171890**, **AY059565**, **AY059549**
Tropidolaemus wagleri	Brunei (NADH4) / West Kalimantan (others)	UMMZ 201917 (NADH4) / CLP 141 (others)	**U41896**, **AY223571**, **AF057245**, **AY352788**
Viridovipera medoensis	Myanmar	CAS 221528 (all)	**AY352831**, **AY352765**, **AY352735**, **AY352797**
Viridovipera stejnegeri	Taiwan	UMMZ 190532 (all)	**U41892**, **AY223570**, **AF057244**, **AF057197**
Azemiops feae	China (all)	UTA R-32069 (NADH4) / CLP157 (others)	**U41865**, **AY223559**, **AF057234**, **AF057187**
Atheris ceratophora	unknown	n.a. (all)	**DQ305474**, **DQ305456**, **DQ305433**, **DQ305410**
Atheris chlorechis	Togo (cyt*b*), unknown (others)	HLMD RA-2892 (cyt*b*), WW1579 (others)	**EU624211***, **AJ275679**, **EU624278***, **EU624244***
Atheris nitschei	Tanzania	n.a. (all)	**AY223618**, **AY223557**,

			AY223663, **AY223650**
Atheris squamigera	DRC (12s), unknown (others)	n.a. (12s), WW1314 (others)	**EU624212***, **EU624303***, **EU624279***, **AF544762**
Bitis arietans	Agadir, Morocco	T. Mazuch, private collection (all)	**EU624213***, **EU624304***, **EU624280***, **EU624245***
Bitis atropos	Swartburg, South Africa (cyt*b*), Bettys Bay, Western Cape, South Africa (others)	PEM (no number – cyt*b*), WW1446 (others)	**EU624214***, **AJ275691**, **EU624281***, **EU624246***
Bitis caudalis	Swakopmund (Namibia) (cyt*b*), Springbok, Northern Cape, South Africa (others)	ZFMK 65212 (cyt*b*), WW1555 (others)	**EU624215***, **AJ275693**, **EU624282***, **EU624247***
Bitis cornuta	near Springbok, Northern Cape, South Africa	WW 1554 (NADH4, 12s), WW 1589 (cyt*b*, 16s)	**EU624216***, **EU624305***, **EU624283***, **EU624248***
Bitis gabonica	Kivu, DRC (cyt*b*) / St. Lucia, KwaZulu	ZFMK 64335 (cyt*b*) / WW1330 (others)	**EU624217***, **AJ275695**, **EU624284***, **EU624249***

	Natal, South Africa (others)		
Bitis nasicornis	Bioko, Equatorial Guinea	CAS 207874	**DQ305475**, **DQ305457**, **DQ305434**, **DQ305411**
Bitis peringueyi	Swakopmund, Namibia	CAS 193863	**DQ305476**, **DQ305458**, **DQ305435**, **DQ305412**
Bitis rhinoceros	Togo (cyt*b*) / Ghana (others)	HLMD RA-2909 (cyt*b*) / Liverpool School of Tropical Medicine, live coll.	**EU624218***, **AJ275696**, **EU624285***, **EU624250***
Bitis rubida	80km N Ceres, S. Africa	WW1397 (all)	**EU624219***, **EU624306***, **EU624286***, **EU624251***
Bitis worthingtoni	Kenya	WW1369 (NADH4, 12s),	**EU624220***, **AJ275692**, **AJ275745**, **EU624252***
Bitis xeropaga	unknown	WW1380 (all)	**EU624221***, **EU624307***, **EU624287***, **EU624253***
Causus defilippii	Tanzania	CLP 154 (all)	**AY223617**, **AY223556**, **AF057233**, **AF057186**
Causus resimus	unknown	Moody 515 (all)	**AY223616**, **AY223555**, **AY223662**, **AY223649**
Causus rhombeatus	unknown	n.a. (all)	**DQ305473**, **DQ305455**, **DQ305432**, **DQ305409**
Cerastes cerastes	Egypt	Latoxan, live collection 0504-	**EU624222***, **EU624308***, **EU624288***, **EU624254***

		2 (all)	
Daboia mauritanica	Morocco	HLMD-RA1182 (cyt*b*) / Latoxan live coll. 0415-3 (others)	**EU624229***, **EU624313***, **EU624295***, **EU624261***
Daboia siamensis	Mandalay Division, Myanmar	CAS205253 (all)	**DQ305477**, **DQ305459**, **DQ305436**, **DQ305413**
Echis carinatus sochureki	Pakistan	Latoxan, live coll. 0012-74 (all)	**EU624223***, **EU624309***, **EU624289***, **EU624255***
Echis coloratus	Israel	WW597 (all)	**EU624224***, **EU624310***, **EU624290***, **EU624256***
Echis ocellatus	Togo	WW1378 (all)	**EU624225***, **EU624311***, **EU624291***, **EU624257***
Echis pyramidum	Egypt	WW1611	**EU624226***, **EU624312***, **EU624292***, **EU624258***
Eristicophis macmahonii	Pakistan (cyt*b*) / unknown (others)	HLMD RA-2890 (cyt*b*) / WW1360 (others)	**EU624227***, **AJ275711**, **EU624293***, **EU624259***
Macrovipera lebetina	Kopet Dagh, Turkmeinstan (cut*b*) / Nuratau, Uzbekistan (others)	G. Nilson (private coll. - cyt*b*) / Latoxan live coll. 0413-2 (others)	**EU624228***, **AJ275713**, **EU624294***, **EU624260***
Macrovipera	Milos, Greece	G. Nilson	n/a, **AJ275715**,

schweizeri		(private coll. – cyt*b*, 16s) / Latoxan live coll. 0411-3 (12s)	**AJ275768**, **EU624262***
Montivipera albizona	unknown	unknown (cyt*b*, 16s) / WW1377 (NADH4, 12s)	**EU624231***, **AJ275727**, **AJ275780**, **EU624265***
Montivipera xanthina	Turkey (cyt*b*, 16s) / unknown (NADH4, 12s)	G. Nilson (private coll. – cyt*b*, 16s) / Zoran Tadić, private coll. (NADH4, 12s)	**EU624234***, **AJ275724**, **AJ275777**, **EU624268***
Proatheris superciliaris	Malawi (cyt*b*) / unknown (others)	HLMD RA-2880 (cyt*b*) / WW1578 (others)	**EU624230***, **AJ275685**, **EU624296***, **EU624263***
Pseudocerastes fieldi	Israel (cyt*b*, 16s) / unknown (12s)	HLMD RA-1182 (cyt*b*, 16s) / WW1365 (12s)	n/a, **AJ275716**, **AJ275769**, **EU624264***
Vipera ammodytes	unknown	Liverpool School of Tropical Medicine, live coll., Va1	**EU624232***, **EU624314***, **EU624297***, **EU624266***

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: [1]Palumbi (1996); [2]Pook (this paper); [3]de Queiroz et al (2002); [4]Burbrink et al (2000); [5]Arévalo (1994); [6]S. Ursenbacher, pers. comm; [7]Knight and Mindell (1993). *internal primers.

Primer name	Position	Sequence
CYT B		
Gludg[1]	14889	TGACTTGAARAACCAYCGTTG
GludgMod[2]	14902	CTGCGGCCTGAAAAACCACCGTTG
H15787*	15787	GCTCCDCCBAGTTTRTT
L14929[2]*	14929	GTCCCACCATCACACTCTCAAAC
H15440[2]*	15440	CTAATTTGTTTGGGATTGATC
L14910[3]	14833	GACCTGTGATMTGAAAAACCAYCGTTGT
H16064[4]	16090	CTTTGGTTTACAAGAACAATGCTTTA
NADH4		
ND4[5]	11677	CACCTATGACTACCAAAAGCTCATGTAGAAGC
Leu[5]	12594	CATTACTTTTACTTGGATTTGCACCA
HIS12763V[6]	12594	TTCTATCACTTGGATTTGCACCA
12S		
L1091[7]	478	AAACTGGGATTAGATACCCCACTAT

H1557[7]	980	GTACACTTACCTTGTTACGACTT
16S		
L2510[1]	1828	CGCCTGTTTATCAAAAACAT
H3059[1]	2376	CCGGTCTGAACTCAGATCACGT

Table 3

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.

	Maximum Parsimony					Maximum Likelihood				Bayesian Inference
	Length of optimal tree(s)	Length of constraint tree(s)	N	-z	*P*	-ln(L) of optimal tree	-ln(L) of constrained tree	δln(L)	*P*	Proportion of Bayesian trees consistent with alternative hypothesis
Azemiops as basal viperid	16597	16617	291-362	0.7684 - 1.1015	0.2707 – 0.4422	67076.62	67093.73	17.11	0.381	0
Causus as basal viperid	16597	16625	211-304	1.4077 – 1.8108	0.07 – 0.1592	67076.62	67107.52	30.90	0.158	0
Monophyly of Crotalinae + Viperinae to the exclusion of *Causus* and *Azemiops*	16597	16637	288-310	1.8202 – 1.9671	0.0492 – 0.0703	67076.62	67150.79	74.17	0.006	0

Monophyly of Viperinae exclusive of *Causus*	n/a					n/a				0.0169 (540 out of 32004)

Table 4. Estimated dates (and 95% confidence intervals) of important nodes and clades mentioned in the text. MRCA = most recent common ancestor

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

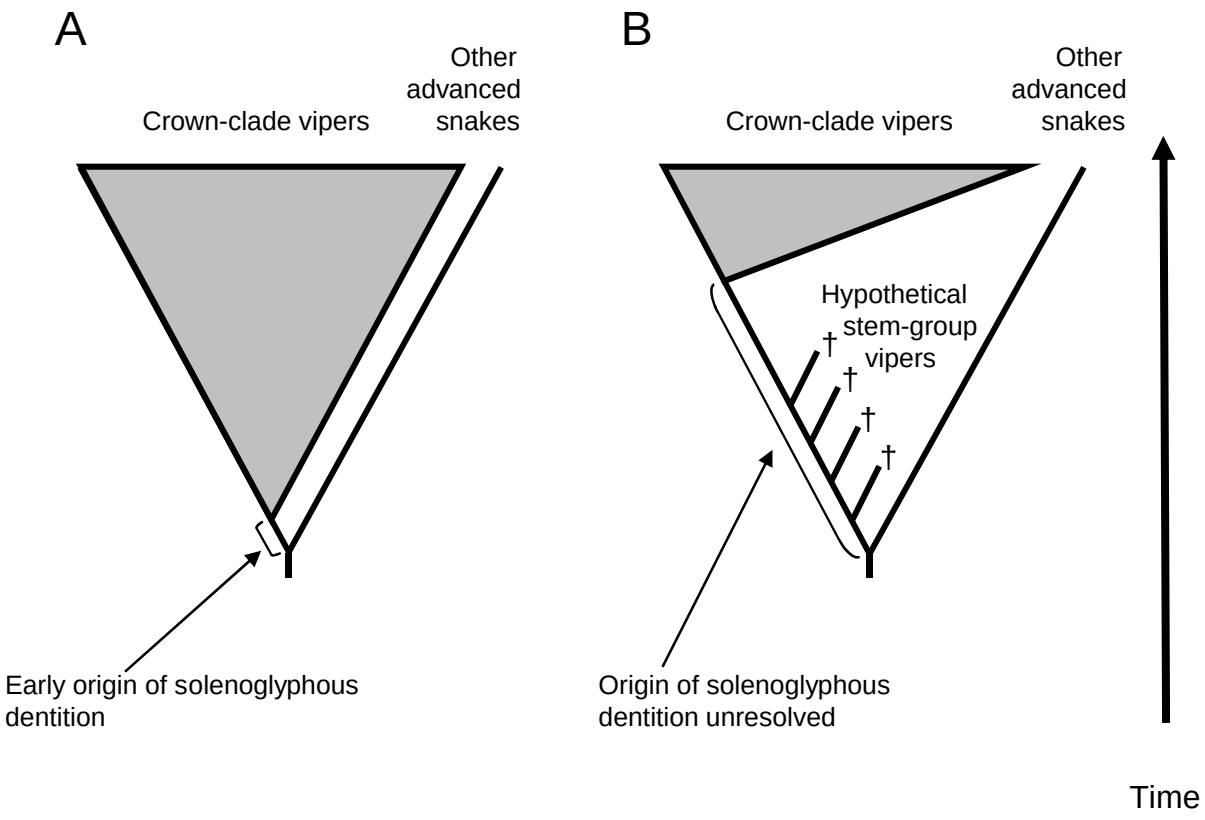

Figure 2