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The phylogeny of cobras inferred from mitochondrial DNA sequences: Evolution of venom spitting and the phylogeography of the African spitting cobras (Serpentes: Elapidae: *Naja nigricollis* complex)

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Abstract

We use phylogenetic analysis of 1333 bp of mitochondrial DNA sequence to investigate the phylogeny and historical biogeography of the cobra-like elapid snakes, with special reference to the evolution of spitting and the phylogeography of the African spitting cobras, a radiation widespread in open vegetational formations throughout sub-Saharan Africa. Our results suggest that spitting adaptations appear to have evolved three times in cobras, but alternative scenarios cannot be rejected. The Asiatic *Naja* are monophyletic and originate from a single colonization of Asia from Africa. The radiation of the African spitting *Naja* appears to date back to the early Miocene and many speciation events in the group predate the Pliocene expansion of grasslands and the radiation of large grazing mammals in Africa. The cladogenic events in this complex appear to have been triggered by both ecological changes and tectonic events associated with the formation and expansion of the African Rift Valley. Taxonomically, our data confirm the inclusion of *Boulengerina* and *Paranaja* within *Naja*, and reveal a clade of African rainforest cobras including *N. melanoleuca*, *Paranaja multifasciata* and *Boulengerina* that constitutes the sister clade of the African open-formation non-spitting cobras. *Naja nigricollis* is polyphyletic, and we therefore recognize *N. nigricincta* as a separate species, more closely related to *N. ashei* and *N. mossambica* than to *N. nigricollis*.

Keywords: Phylogeography; Phylogeny; Africa; Miocene; Rift Valley; Serpentes; *Naja*; Spitting cobra

1. Introduction

The advent of mitochondrial DNA based analyses has led to a plethora of phylogeographic studies of more or less widespread groups of organisms, in which the relationship between mtDNA haplotype phylogeny and distribution has been used to infer the history of that distribution, as well as the demographic processes involved (Avise, 2000; Templeton et al., 1995; Templeton and Sing, 1993). Additionally, these studies have been used extensively in taxonomic revisions and for the delimitation of species boundaries (e.g., Wiens and Penkrot, 2002), as well as in the investigation of the evolution of organismal traits.

Phylogeographic patterns of co-distributed organisms have been particularly useful in unraveling the history of biota and regions (Hewitt, 2004), and elsewhere, phylogeographic studies have been used to make inferences about past habitat fragmentation or connections (e.g., Wüster et al., 2005a,b). However, studies of this nature have been heavily biased towards organisms living in northern temperate zones, with a relative paucity of studies from

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tropical regions, especially Asia and Africa. Moreover, in the tropical world and particularly in Africa, they have tended to be biased towards mammals and birds rather than ectothermic vertebrates. However, a wider taxonomic coverage is of fundamental importance for a broader picture of the biogeographical history of any area. It has become increasingly clear that even when faced with severe ecological or geological disruption, the ranges of different species composing present-day biota change individually and independently of each other (Hewitt, 2004). Moreover, there are also trends for different taxonomic groups to display different biogeographical patterns. For instance, whereas large-scale exchanges of mammalian species between North and South America (Stehli and Webb, 1985; Webb and Rancy, 1996), and subsequent adaptive radiations, followed the uplift of the Isthmus of Panamá above sea level, the same is not true of reptiles and amphibians, in which many faunal exchanges and radiations predate the formation of the Isthmus by a considerable margin (Estes and Báez, 1985; Wüster et al., 2002, 2005b). Consequently, studies of a wide range of taxonomic groups are essential to obtain a comprehensive understanding of the biogeographical history of any given region.

The African continent provides a good example of the taxonomic bias of phylogeographic studies, especially at a wider geographic level. Whereas there have been numerous phylogeographic studies of widespread African mammals (e.g., Arctander et al., 1999; Eggert et al., 2002), much less attention has been paid to ectothermic vertebrates, especially reptiles. There have been no phylogeographic studies of species or species complexes widespread throughout sub-Saharan Africa, although there have been published phylogenies of higher taxonomic groups (Lenk et al., 1999), as well as regional studies of taxa restricted to southern Africa (Matthee and Flemming, 2002; Scott et al., 2004; Tolley et al., 2006), the Eastern Arc Mountains (Gravlund, 2002) or the Saharan and Middle Eastern regions (Amer and Kumazawa, 2005).

For convenience, and as far as the distributions of wide ranging species are concerned, sub-Saharan African can be divided into two major vegetation zones: the tropical forests of central and western Africa, together with the coastal forests of eastern Africa, and the open formations separating these forest habitats from each other and from the Sahara to the north. Many widespread sub-Saharan reptile species or species complexes occupy either one or the other of these two major formations (Hughes, 1983; Spawls and Branch, 1995). A number of sources of evidence, including both paleontological and palynological evidence (de Menocal, 2004) as well as phylogenetic and phylogeographic studies of extant mammal groups (Arctander et al., 1999), have identified the Miocene–Pliocene boundary as a key event in the spread of open grassland formations in Africa. This is evidenced especially from the evolution of the present-day grassland mammal faunas so characteristic of these formations (Eggert et al., 2002). However, we know little or nothing of the origin of the equally characteristic reptilian taxa that occupy these formations. This begs the question of whether the evolution and radiation of the savanna herpetofaunas of Africa have been affected in the same way by Mio-Pliocene vegetational and climatic changes as the mammals, or whether the origin of the grassland herpetofauna extends further back in time.

The African spitting cobras represent an ideal model organism for phylogeographic studies on the open areas of sub-Saharan Africa: they are widespread throughout the drier parts of the African continent, occurring from southern Egypt in the north to the Western Cape and KwaZulu-Natal in South Africa, and from Senegal in the west to Somalia in the east. They were long regarded as a single, highly variable species, *Naja nigricollis*, but several revisions over the last 40 years have led to the recognition of six species (Broadley, 1968, 1974; Wüster and Broadley, 2003, 2007). However, the presence of considerable variation within *N. nigricollis* sensu stricto has been noted, and the presence of additional species suspected (Spawls and Branch, 1995; Spawls et al., 2004).

The monophyly of the complex has traditionally been taken for granted by most authors, given that all forms used to be considered conspecific, and is supported by the morphological synapomorphy of twin preocular scales (however, this condition is variable in *N. pallida* and *N. nubiae*—see Wüster and Broadley, 2003 for illustrations). However, this has never been tested rigorously, and other authors have suggested that the presence of both spitting and non-spitting cobras in Asia may be due to separate colonizations of Asia by two different African stocks (Minton, 1986; Ineich, 1995). This implies that the African spitting cobras may be paraphyletic with respect to the Asian spitting cobras. The spitting behavior of cobras has been the topic of several recent functional studies (Rasmussen et al., 1995; Young et al., 2004; Westhoff et al., 2005) as well as speculation on the ecological context of its evolution (Barbour, 1922; Bogert, 1943). However, a robust and comprehensive phylogenetic framework for the cobras and related elapid snakes is required to resolve the question of the evolution of spitting adaptations. Several phylogenetic studies have identified a monophyletic ''core cobra group'' (Slowinski and Keogh, 2000) within which all spitting elapids are nested, consisting of the genera *Naja*, *Boulengerina*, *Paranaja*, *Aspidelaps*, *Walterinnesia* and *Hemachatus* (Slowinski et al., 1997; Slowinski and Keogh, 2000). However, limited sampling of this clade in previous studies has not allowed the resolution of phylogenetic relationships within this group, although several authors have found *Naja* to be non-monophyletic due to the exclusion of *Boulengerina* and *Paranaja* (Slowinski et al., 1997; Slowinski and Keogh, 2000; Nagy et al., 2005).

In addition to contributing to the investigation of the biogeography functional anatomy and behavioral biology of these snakes, a resolution of their systematics and phylogeny is also of importance for medical reasons: spitting cobras are important causes of snakebite accidents in

sub-Saharan Africa (Warrell et al., 1976; Warrell and Ormerod, 1976; Tilbury, 1982; Warrell, 1995), and a robust phylogenetic and systematic framework is essential for research into venom composition, both for academic and applied purposes (e.g., Daltry et al., 1996; Fry et al., 2003).

In this paper, we analyze sequences of two mitochondrial genes with the aim of reconstructing the phylogeny of the cobra group, the evolution of spitting among these elapids, and the phylogeography of the African spitting *Naja*.

2. Materials and methods

2.1. Laboratory methods

We obtained tissue (ventral scale clippings) or blood samples, or shed skins, from specimens covering most of the range of the African spitting cobra complex (Fig. 1), as well as from representatives of other Asian and African cobra species complexes and related species. Total DNA was extracted by standard methods (Sambrook et al., 1989). Two regions of the mitochondrial DNA molecule were amplified using the polymerase chain reaction (PCR): we used primers ND4 and Leu (Arévalo et al., 1994) to amplify a section of the ND4 gene and adjoining tRNAs, and for cytochrome *b*, we used the primers Gludg (5′-TGACTTGAARAACCAYCGTTG-3′) (Palumbi, 1996) and H16064 (5′-CTTTGGTTTACAAGAACAATGCTT TA-3′) (Burbrink et al., 2000). PCRs were set up with 10× PCR buffer, 3–3.5 mM $MgCl_2$, 0.4 μM each primer, 0.8 μM total dNTPs, 1 U of *Taq* (Invitrogen, product code 10342-020) and made up to of 25 μl with ultrapure water.

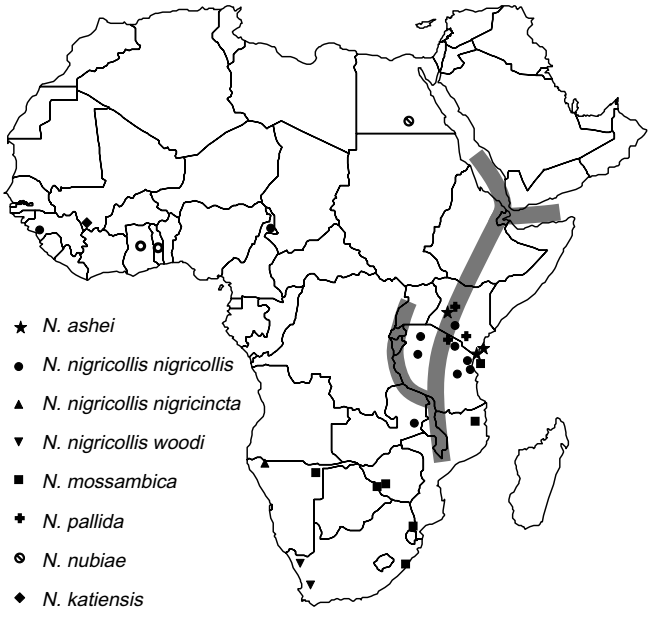

Fig. 1. Sampling localities. Hollow symbols indicate approximate localities, and grey shading indicates the Great Rift Valley. For maps of the distributions of the different species, see Spawls and Branch (1995) and Wüster and Broadley (2003, 2007).

Typical amplification conditions involved initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 47 °C (cyt*b*) or 60 °C (ND4) for 30 s, then 72 °C for 2 m, followed by a final extension step of 72 °C for 5 m. Sequencing was carried out using the same primers by Macrogen (Seoul, S. Korea—http://dna.macrogen.com).

We included samples of a number of species of the core cobra group from outside the African spitting cobra complex in order to test the monophyly of the African spitting cobras and investigate the origin of spitting in cobras. These included representatives of each of the four classically recognized African non-spitting cobra complexes (*Naja annulifera*, *N. haje*, *N. melanoleuca*, *N. nivea*), and four Asiatic cobras, *N. naja* (non-spitting), *N. kaouthia* (occasional spitter), *N. siamensis* and *N. sputatrix* (both frequent spitters—Wüster and Thorpe, 1992). Other elapids of the cobra clade (Slowinski and Keogh, 2000) included here are *Boulengerina annulata*, *Paranaja multifasciata*, *Walterinnesia aegyptia*, *Aspidelaps scutatus* and *Hemachatus haemachatus*. The latter is of particular interest in view of the likely independent origin of spitting in this taxon. In addition, the sampling of additional taxa allowed us to test the monophyly of *Naja* and the hypothesis of multiple dispersal events to Asia (Minton, 1986; Ineich, 1995).

Molecular dating requires calibration points in the tree, based on either paleontological evidence or geological events. In order to estimate the timing of different branching events in the spitting cobra phylogeny, we included a number of suitable additional snake taxa that provide suitable calibration points: South American *Porthidium* (Wüster et al., 2002), Eurasian vipers (Szyndlar and Rage, 1999), the sea krait *Laticauda colubrina* and a representative of its Australasian elapid sister clade, *Oxyuranus scutellatus* (Scanlon et al., 2003), and four species of *Hemorrhois* (Nagy et al., 2003). Since adequate sampling is a crucial factor in molecular dating analyses (Linder et al., 2005), we obtained a number of additional sequences in order to break up long branches between the different snake families: we included two more basal viperines and crotalines (*Bitis nasicornis* and *Calloselasma rhodostoma,* respectively) and two further viperids frequently regarded as basal to the Crotalinae and Viperinae, respectively (*Causus rhombeatus* and *Azemiops feae*), the additional colubrines *Pantherophis guttatus*, *Boiga dendrophila* and *Dinodon semicarinatus*, the xenodontines *Alsophis portoricensis* and *Hypsiglena torquata*, the natricines *Nerodia erythrogaster* and *Afronatrix anoscopus* (Natricinae), the homalopsine *Cerberus rhynchops*, the atractaspidid *Atractaspis bibronii* and the elapids *Ophiophagus hannah* and *Elapsoidea nigra*. The basal caenophidian *Acrochordus granulatus*, a representative of the Acrochordidae, the sister group of the Colubroidea, represented the outgroup taxon.

2.2. Phylogenetic analysis

For phylogenetic analysis, we used maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference

(BI) methods. MP analysis was carried out using the software PAUP* 4.0b10 (Swofford, 2002). For MP analysis, we carried out an unweighted analysis, using heuristic searching, TBR branch-swapping, and 10,000 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 TBR branch swapping. For ML analysis, we estimated the best model of sequence evolution across all sites under the Akaike Information Criterion (AIC) using MrModeltest 2.2 (Nylander, 2004), and carried out a heuristic search in PAUP*4.0 using a Neighbor-Joining starting tree and TBR branch swapping.

For phylogenetic analysis using BI, we used MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). There is increasing evidence that complex models of sequence evolution can extract additional phylogenetic signal from data, especially where saturation of base pair substitutions is commonplace (Castoe et al., 2004, 2005; Castoe and Parkinson, 2006). Therefore, we used different models of sequence evolution for biologically relevant partitions of our data. In the case of protein coding mitochondrial genes, the most relevant partitions are first, second and third codon positions, which are known to display different patterns of sequence evolution. We therefore partitioned our data into six separate data partitions, namely first, second and third codon positions separately for cytochrome *b* and ND4. To identify the most appropriate models of sequence evolution for each data partition, we used MrModeltest 2.2, and selected the model favored under the Akaike Information Criterion for each category in our Bayesian analysis. In all phylogenetic analyses, *Acrochordus granulatus* was specified as the sole outgroup. We ran the analysis for 5×10^6 generations using four simultaneous independent runs initiated with different random starting trees. Plots of $\ln L$ against generation were inspected to determine the burn-in period, and trees generated prior to the completion of burn-in were discarded, with a fivefold "safety margin".

2.3. Testing alternative phylogenetic hypotheses

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

1. Monophyly of the genus *Naja*.
2. Monophyly of all African and Asian spitting *Naja* as implied by the hypothesis of two invasions of Asia.
3. Monophyly of all species of spitting elapid (African and Asian spitting *Naja* and *Hemachatus*).

ML trees representing the alternative topology were generated in PAUP* by constraining the analysis to only retain the optimal trees consistent with the alternative topology, and retaining the same search parameters as used for the initial ML tree. We then compared the constrained ML trees with the original two ML trees using the RELL option of the Shimodaira–Hasegawa test (Shimodaira and Hasegawa, 1999), as implemented in PAUP*.

In the case of Bayesian inference analysis, we used PAUP* to filter all trees obtained after completion of the burnin phase to retain only those consistent with the alternative constraint trees, and considered the alternative hypothesis as rejected if it was supported by less than 5% of Bayesian trees.

2.4. Molecular timing

Bayesian molecular dating was used to estimate the time of cladogenic events within the spitting cobra clade. Our procedure followed the protocol of Rutschmann (2004): evolutionary model parameters, maximum likelihood branch lengths and a variance–covariance matrix were estimated for the rooted tree resulting from Bayesian phylogenetic analysis, using the PAML software (Yang, 1997); a Bayesian MCMC analysis of 50,000 cycles (sampled every 100 cycles) following a burn-in of 100,000 cycles was then performed using Multidivtime (Thorne and Kishino, 2002) in order to approximate the posterior distribution of substitution rates and divergence times (Thorne et al., 1998; Kishino et al., 2001; Thorne and Kishino, 2002).

As calibration points, we included sequences 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, approx. 3.5 Mya (see Wüster et al., 2002; for details), and constrained their maximum age accordingly. The split between the Asian *Naja* clade and its African sister clade was constrained to a minimum age of 16 Mya based on the presence of characteristic apomorphies of the Asian clade at that time (Szyndlar and Rage, 1990). The divergence of *Laticauda* and its sister lineage was constrained to a minimum age of 24 Mya based on the fossil evidence of Scanlon et al. (2003). We also included the Eurasian vipers *Vipera ammodytes*, *V. berus*, *Montivipera albizona* and *Macrovipera lebetina*, based on the appearance of the different genera of European vipers in the fossil record (Szyndlar and Rage, 1999), and constrained the initial divergence of this clade to a minimum age of 20 Mya. We constrained the divergence of four species of the colubrine genus *Hemorrhois*, *H. algirus*, *H. hippocrepis*, *H. nummifer* and *H. ravergieri* to a maximum age of 16 Mya, based on the likely cladogenesis between eastern and western species after Asia and Africa became joined approximately 16–18 Mya (Nagy et al., 2003). Finally, the age of the basal divergence of the Colubroidea was constrained to a maximum of 95 Mya, based on earliest colubroid fossils from the Cenomanian (Rage et al., 2003).

3. Results

3.1. Sequence data

We aligned a total of 1333 bp, 606 for ND4 and 727 for cytochrome *b*. From 52 ingroup specimens, we identified 35 distinct haplotypes. The sequences were deposited with GenBank (Accession Numbers in Appendix A). Translation of the DNA sequences into amino acid sequences using MEGA 2.1 (Kumar et al., 2001) revealed no indels, frame shifts or unexpected stop codons, leading us to conclude that our sequences represented mitochondrial DNA rather than nuclear insertions (Zhang and Hewitt, 1996). Of the 1333 bp, 755 were variable and 666 parsimony informative across all taxa.

Pairwise *p*-distances among haplotypes found in the African spitting cobras ranged from 0% to 11.01%, and the highest average interspecific distance was 10.91% between *N. katiensis* and *N. nubiae*. Average *p*-distances between species or species groups and associated standard errors are shown in Table 1. The 100,000 random trees generated in PAUP* produced a g1 statistic of −0.325556, suggesting that the data contain significant phylogenetic signal ($p < 0.01$) (Hillis and Huelsenbeck, 1992).

3.2. Phylogenetic analysis

Unweighted parsimony analysis of the sequence data yielded 58 equally most parsimonious trees of 5772 steps (consistency index: 0.2372, retention index: 0.5520) distributed across nine "islands" of equally most parsimonious trees. One or other of these was found in 5633 out of 10,000 replicates. An additional 185 islands of less optimal trees of 5773–5804 steps were identified during the other replicates. Bootstrap support for nodes is shown in Figs. 2 and 3.

The GTR + I + Γ model was identified as the optimal model of sequence evolution under the Akaike Information Criterion. The parameter values used in this study are given in Table 2. The maximum likelihood analysis returned two equally most likely trees ($-\ln L = 24021.82131$), which differed only in that one tree grouped haplotypes *pallida* Kenya 1 and *pallida* Tanzania as monophyletic, whereas in the other, the four *N. pallida* haplotypes other than *pallida* Baringo were recovered as unresolved.

For Bayesian analysis, the models of sequence evolution identified as optimal by MrModeltest for the six data partitions used in this study are shown in Table 3. These were implemented for the six data partitions. Burn-in was achieved after approximately 200,000 generations, but we conservatively discarded all trees produced in the first 10^6 generations. The tree of the cobras resulting from the Bayesian analysis is shown in Fig. 2, and the branching order of other taxa in Fig. 3. The branching order of the Bayesian tree was identical to that of the first ML tree mentioned above.

Table 1
Matrix of *p*-distances between different species and clades of cobra included in the study

	nigricol.	*moss.*	*ashei*	*nigricin.*	*katien.*	*pallida*	*nubiae*	Asian	*haje–nivea*	*melan.*	*Boule.*	*Para.*	*Hema.*	*Walter.*	*Aspid.*	*Ophio.*
nigricollis		0.0055	0.0052	0.0048	0.0067	0.0070	0.0071	0.0072	0.0082	0.0087	0.0089	0.0093	0.0095	0.0090	0.0097	0.0094
mossambica	0.0505		0.0046	0.0043	0.0077	0.0072	0.0081	0.0074	0.0089	0.0090	0.0094	0.0095	0.0102	0.0093	0.0099	0.0099
ashei	0.0473	0.0359		0.0043	0.0071	0.0071	0.0080	0.0074	0.0088	0.0091	0.0090	0.0093	0.0093	0.0093	0.0097	0.0091
nigricincta	0.0492	0.0363	0.0349		0.0067	0.0069	0.0078	0.0072	0.0080	0.0090	0.0090	0.0095	0.0097	0.0091	0.0098	0.0092
katiensis	0.0808	0.0779	0.0745	0.0739		0.0081	0.0084	0.0079	0.0082	0.0093	0.0098	0.0098	0.0101	0.0092	0.0112	0.0092
pallida	0.0877	0.0924	0.0911	0.0947	0.1036		0.0070	0.0076	0.0084	0.0089	0.0091	0.0097	0.0099	0.0091	0.0095	0.0088
nubiae	0.0830	0.0936	0.0926	0.0956	0.1091	0.0785		0.0082	0.0091	0.0092	0.0095	0.0101	0.0101	0.0097	0.0108	0.0102
Asian *Naja*	0.1263	0.1208	0.1193	0.1174	0.1371	0.1259	0.1357		0.0081	0.0081	0.0081	0.0081	0.0087	0.0082	0.0087	0.0087
haje-nivea	0.1354	0.1353	0.1318	0.1261	0.1341	0.1436	0.1423	0.1387		0.0082	0.0088	0.0083	0.0094	0.0088	0.0096	0.0091
melanoleuca	0.1089	0.1105	0.1147	0.1135	0.1213	0.1143	0.1236	0.1233	0.1245		0.0080	0.0086	0.0100	0.0091	0.0104	0.0096
Boulengerina	0.1350	0.1302	0.1274	0.1298	0.1420	0.1405	0.1382	0.1373	0.1451	0.1014		0.0082	0.0101	0.0089	0.0109	0.0101
Paranaja	0.1373	0.1380	0.1321	0.1321	0.1409	0.1431	0.1481	0.1389	0.1404	0.1067	0.1245		0.0099	0.0097	0.0104	0.0096
Hemachatus	0.1424	0.1477	0.1341	0.1407	0.1599	0.1550	0.1512	0.1548	0.1585	0.1450	0.1614	0.1584		0.0098	0.0097	0.0105
Walterinnesia	0.1507	0.1503	0.1473	0.1501	0.1549	0.1489	0.1591	0.1532	0.1539	0.1555	0.1673	0.1635	0.1629		0.0102	0.0092
Aspidelaps	0.1669	0.1619	0.1569	0.1625	0.1791	0.1631	0.1682	0.1637	0.1786	0.1675	0.1755	0.1815	0.1644	0.1748		0.0102
Ophiophagus	0.1494	0.1461	0.1430	0.1452	0.1538	0.1537	0.1629	0.1616	0.1614	0.1488	0.1590	0.1620	0.1674	0.1688	0.1845	

Below the diagonal, between-group distances; above the diagonal, standard errors calculated from 1000 bootstrap replicates.

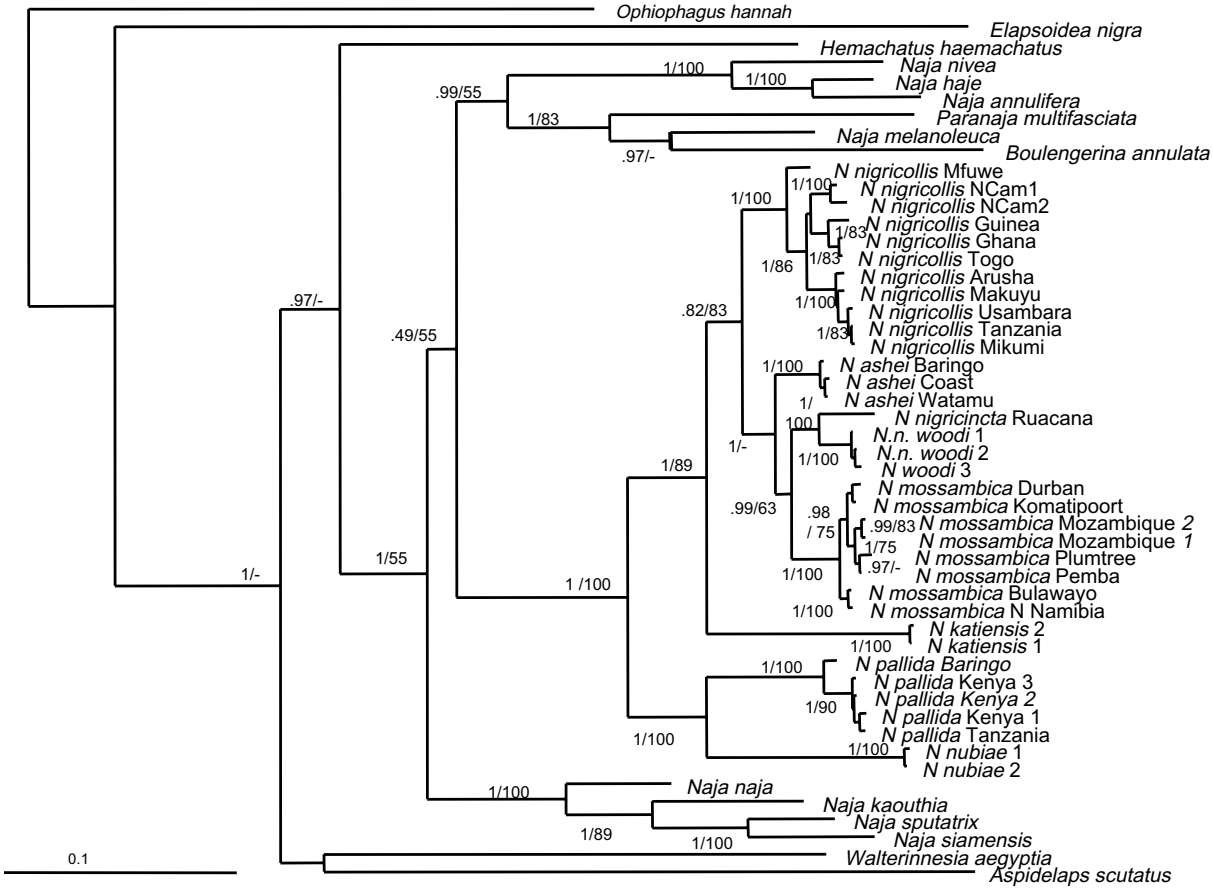

Fig. 2. Bayesian tree of the included species of *Naja*. Tip labels include species identity, haplotype name (see Appendix A), and the country of origin where this is not part of the haplotype name. Support values on nodes are Bayesian posterior probabilities and Maximum Parsimony bootstrap values. Dashes indicate bootstrap support values of less that 50%. Relationships among taxa outside the *Naja* clade and corresponding support values are shown in Fig. 3.

Although Bayesian and parsimony analyses differed on the placement of some of the non-cobra taxa, both methods produced trees of identical topology for the African spitting cobras, supported by high bootstrap support and/or Bayesian posterior probabilities. Noteworthy features of the trees include the following:

- The monophyly of the core cobra clade (Slowinski and Keogh, 2000), including the genera *Aspidelaps, Boulengerina, Hemachatus, Naja, Paranaja* and *Walterinnesia*, is strongly supported, whereas there is no support for the inclusion of the king cobra (*Ophiophagus*) in this clade.
- *Aspidelaps* and *Walterinnesia* form the two basal lineages relative to the other members of the cobra clade; the relationships between them and the remainder of the clade are not clearly resolved.
- *Hemachatus* forms the sister taxon of a large clade consisting of *Boulengerina*, *Naja* and *Paranaja*.
- The latter consists of three clades of unclear interrelationships: a strongly supported Asian *Naja* clade, an African non-spitting clade including the African non-spitting *Naja* as well as *Boulengerina* and *Paranaja*, and the African spitting *Naja*, which are strongly supported as monophyletic. *Boulengerina*, *Paranaja* and *N. melanoleuca* form a strongly supported clade within the genus *Naja*, rendering the latter paraphyletic.
- The Asiatic *Naja* form a strongly supported clade, with the spitting species being in turn monophyletic to the exclusion of *N. naja*.
- Among the African spitting *Naja*, *N. nubiae* and *N. pallida* form the sister clade to all the other African spitting cobras, and *N. katiensis* is the sister taxon of the remaining African spitting cobras. Within the latter, a basal dichotomy separates all populations of *N. nigricollis nigricollis* from a clade containing *N. mossambica*, *N. nigricollis nigricincta*, *N. n. woodi* and *N.. ashei*, rendering *N. nigricollis* polyphyletic.

3.3. Alternative phylogenetic hypotheses

The Shimodaira–Hasegawa tests comparing likelihood trees constrained to be consistent with alternative phylogenetic hypotheses with the optimal ML tree rejected the hypotheses of a monophyletic genus *Naja* (d[ln *L*] =

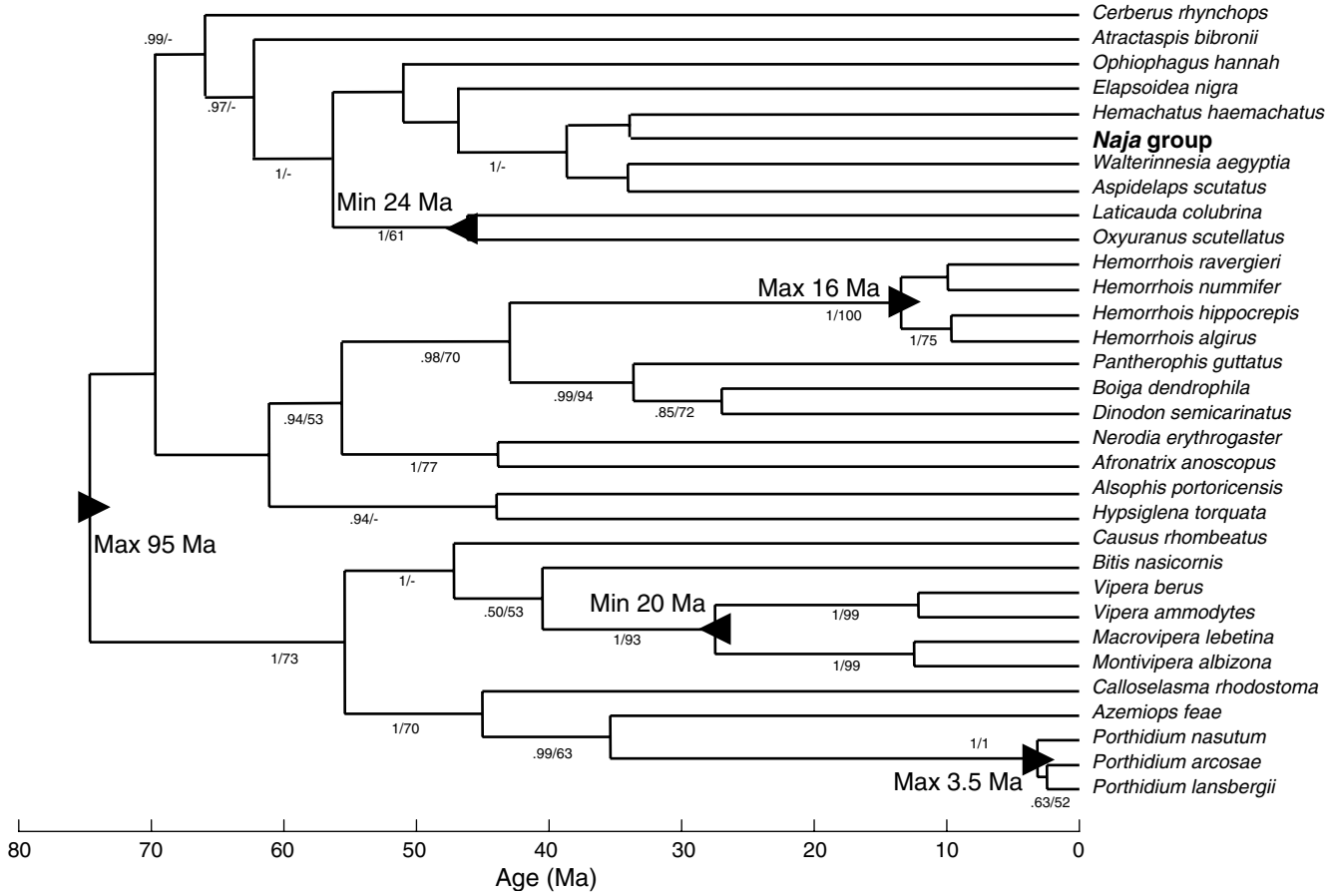

Fig. 3. Linearized tree showing relationships among taxa lying outside the African cobra clade, with estimated dates of divergence. Arrows along branches with age indications indicate age constraints imposed during the analysis. Branch support values are as in Fig. 2. The African *Naja* are shown separately in Fig. 4.

Table 2
Parameter values for the GTR + I + G model estimated for maximum likelihood analysis

Base frequencies	A	0.37830
	C	0.35660
	G	0.05330
	T	0.21180
Rates	AC	0.2996
	AG	6.9473
	AT	0.5531
	CG	0.1119
	CT	5.3872
	GT	1.0000
Proportion of invariable sites		0.3751
Gamma shape parameter		0.6104

39.86288, $p = 0.001$), of monophyly of the spitting *Naja* (d[ln L] = 33.97512, $p = 0.006$), and of the monophyly of all spitting Elapids (d[ln L] = 49.95125, $p < 0.001$). Similarly, none of the 32,004 post-burnin trees recovered from the Bayesian analysis supported any of these three alternative phylogenetic hypotheses. We therefore consider these alternative phylogenetic scenarios strongly rejected.

Table 3
Models of sequence evolution identified as optimal for the six data partitions used in Bayesian Inference Analysis, using MrModeltest v. 2.2 (Nylander, 2004)

Gene	Codon position	Model
cyt*b*	1	GTR + I + Γ
	2	GTR + I + Γ
	3	GTR + Γ
ND4	1	GTR + I + Γ
	2	GTR + I + Γ
	3	GTR + Γ

3.4. Molecular timing

The result of the molecular timing analysis is shown in Figs. 3 and 4. Although our 95% confidence limits are wide, our analyses place the basal cladogenic event among the African spitting cobras in the early Miocene (although with confidence limits ranging from the late Eocene to the late Miocene). Later major cladogenic events dated at approximately 13–14 Mya separate *N. katiensis* from the *nigricollis–mossambica* group and *N. nubiae* from *N. pallida*. Later interspecific cladogenic events between widely recognized

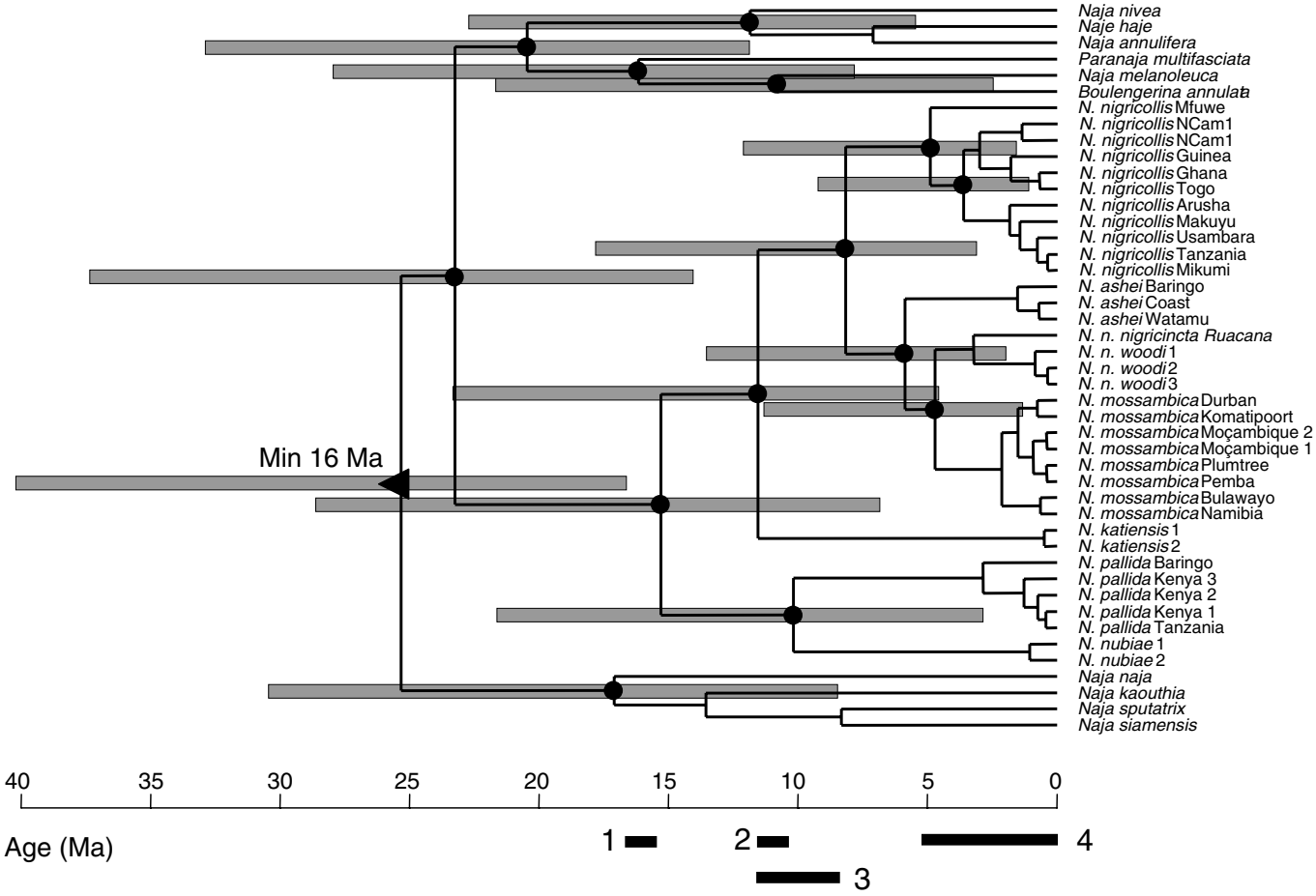

Fig. 4. Bayesian dating of the *Naja* clade, which estimated dates of divergence. Grey bars indicate 95% confidence intervals for selected nodes discussed in the text. Black bars under the time axis indicate geological/ecological events: 1, earliest evidence of savanna grasslands in Africa; 2, beginning of rifting in the Ethiopian Rift; 3, beginning of rifting along the western branch of the Great Rift; 4, presence of extensive C4 grasslands and large grazing mammal herds.

species within the *nigricollis–mossambica* group are dated at 5–10 Mya.

4. Discussion

Our data reveal a generally well-supported phylogenetic hypothesis for the cobra-like elapids in general and the African spitting cobras in particular. This provides the raw material for a discussion of long-standing taxonomic issues, the evolution of spitting in cobras, the biogeographical history of the cobra clade in general and the phylogeography of the African spitting cobras in particular.

4.1. Broad phylogenetic and biogeographical patterns

Our results provide strong support for the monophyly of the core cobra group sensu Slowinski and Keogh (2000), of which *Ophiophagus* does not appear to be part. The group is entirely African except for the Middle Eastern *Walterinnesia* and the Asiatic species of *Naja*, strongly suggesting an African origin for the clade. *Hemachatus*, *Walterinnesia* and *Aspidelaps* represent the most basal taxa within the group. The monophyly of the large clade consisting of *Naja*, *Boulengerina* and *Paranaja* is strongly supported. It is subdivided into three robustly supported major subclades of unresolved interrelationships: the Asian species of *Naja*, the African spitting *Naja*, and a clade consisting of the African non-spitting *Naja*, *Boulengerina* and *Paranaja*.

Our data strongly confirm the monophyly of the African spitting cobras to the exclusion of all other cobras. Similarly, the monophyly of the Asian cobras, spitting and non-spitting, is also strongly supported. The reciprocal monophyly of these two groups strongly rejects the hypothesis of Minton (1986) and Ineich (1995) that the presence of both spitting and non-spitting cobras in Asia could be the result of two separate ancestral stocks entering Asia from Africa. Instead, our molecular results confirm the morphological data of Szyndlar and Rage (1990), who found evidence for the monophyly of the Asiatic cobras.

The origin of spitting cannot be definitively resolved based on our data, at least under a criterion of naïve parsimony: a hypothesis of three independent origins of spitting in *Hemachatus*, Asian spitting *Naja* and African spitting *Naja* requires the same number of evolutionary

transitions as a single origin at the base of the *Hemachatus–Naja–Boulengerina–Paranaja* clade, followed by two losses in the African non-spitters and the Asian non-spitting *N. naja*. However, the variable degree of morphological specialization among the Asian *Naja* (Wüster and Thorpe, 1992) and their more stereotypical spitting behavior compared to African spitting *Naja* (Rasmussen et al., 1995) is more consistent with a separate origin in that clade. Further in-depth studies of anatomical and behavioral specializations are required to test whether these specializations are likely to be homologous or analogous.

4.2. Taxonomic implications

Our data represent the first reasonably complete phylogenetic analysis of the cobra clade of elapid snakes, and allow a number of taxonomic conclusions to be reached and/or confirmed.

At the generic level, our data confirm the inclusion of *Boulengerina annulata* within *Naja* as the sister taxon of *N. melanoleuca*, a result previously recovered by Nagy et al. (2005), but with limited sampling of the elapids involved. The phylogenies recovered by Slowinski et al. (1997) using gene tree parsimony analysis of toxin sequences also recovered *Boulengerina* (in that case *B. christyi*) within *Naja*, although not in particular association with *N. melanoleuca*.

Our results also recover *Paranaja multifasciata* as the sister taxon of *B. annulata* and *N. melanoleuca*, the three constituting a clade of primarily rainforest inhabiting cobras. Taxonomically, these results would require either the splitting of *Naja* to retain the genera *Paranaja* and *Boulengerina*, or the synonymization of the latter two genera with *Naja*. The former would emphasize the recognition of distinct lineages within the genus *Naja*, but would also require the assignment of all African *Naja* to different genera, destabilizing the nomenclature of a number of species of considerable medical and social importance. On the other hand, synonymizing *Paranaja* and *Boulengerina* with *Naja* would emphasize the monophyly of the majority of the cobra-like elapids and the close relationship between specialized and generalist species of the group, and, moreover, would only involve returning three relatively obscure species (*Boulengerina annulata*, *B. christyi*, *Paranaja multifasciata*) to the genus in which they were first described. For reasons of nomenclatural stability, we therefore follow the approach taken by Nagy et al. (2005) and Branch (2005) in the case of *Boulengerina*, and consider both that genus and *Paranaja* as synonyms of *Naja*.

At the species level, the African spitting cobras have a long history of taxonomic instability. All African spitting cobras were regarded as part of the single species *Naja nigricollis* Reinhardt, 1843, until Broadley (1968) recognized *N. mossambica* as distinct at the species level, and considered the taxa *katiensis*, *nigricincta*, *pallida* and *woodi* as subspecies of *N. mossambica*. Later, based on sympatry between *nigricincta* and *mossambica* in parts of Angola and Namibia, *nigricincta* and *woodi* were transferred back to the status of subspecies of *N. nigricollis* (Broadley, 1974), an arrangement followed by the majority of recent workers (e.g., Spawls and Branch, 1995; David and Ineich, 1999). However, Haagner et al. (2000) treated *nigricincta* as a separate species, *N. nigricincta*, and Bauer and Branch (2001) treated *woodi* as a separate species, *N. woodi*, but in both cases without providing new evidence. *Naja katiensis* was recognized as a separate species from *N. mossambica* by Roman (1968, 1969), while Branch (1979) and Hughes (1983) elevated *pallida* to full species status. This was later confirmed by Wüster and Broadley (2003), who also described populations previously assigned to *N. pallida* from northern and northeastern Africa as a new species, *N. nubiae*. Finally, Wüster and Broadley (2007) described the large brown spitting cobra of eastern and northeastern Africa as a new species, *N. ashei*.

Sites and Crandall (1997) emphasized the importance of treating the delimitation of species as a hypothesis-testing operation, with predefined criteria for considering different populations as separate species. Like de Queiroz (1998), we consider species to be in effect independent organismal lineages. A number of operational criteria have been proposed to identify and delimit such lineages (reviewed by Sites and Marshall, 2004). Here, in the absence of data from nuclear markers, we consider as separate species sets of populations that contain exclusive clades of mitochondrial haplotypes (Wiens and Penkrot, 2002), and differ clearly in their morphology from other sets of populations. We regard congruence of morphology and mtDNA clades as particularly important, since mtDNA, due to its non-recombining nature, cannot represent intergrade zones (Puorto et al., 2001). Moreover, profound phylogeographic breaks can form and be maintained even in the presence of extensive gene flow under some conditions (Irwin, 2002; Thorpe and Richard, 2001; Ogden and Thorpe, 2002), and thus be unrepresentative of limits between organismal lineages. Morphological differences between sets of populations bearing differentiated haplotype clades thus constitute evidence that haplotype clades represent divergent organismal lineages, just as possession of separate exclusive haplotype clades shows that morphologically differentiated sets of populations constitute independent lineages rather than the result of ecogenesis (e.g., Keogh et al., 2005). Nuclear markers would be required to test whether populations differentiated by either morphology or mtDNA haplotype clades, but not both, represent evolutionary lineages.

Our data provide a number of new insights on the taxonomy of the African spitting cobras. Both their phylogenetic position and high levels of sequence divergence confirm the status of the morphologically distinct (Broadley, 1968; Roman, 1969; Wüster and Broadley, 2003, 2007) *N. ashei*, *N. pallida*, *N. nubiae* and *N. katiensis* as distinct species from both *N. nigricollis* and *N. mossambica* as well as each other.

Our results also reveal that the taxa *nigricincta* and *woodi*, traditionally treated as subspecies of *N. nigricollis*,

nest with *N. mossambica* and *N. ashei*, not other *N. nigricollis*. This clade approaches the distribution of *N. nigricollis* in Angola. However, they differ profoundly from the geographically closest southern populations of *N. nigricollis* in most scale counts, as well as in pattern. The congruence of mitochondrial DNA and morphological data strongly suggests that the *nigricincta–woodi* clade constitutes an independent evolutionary lineage from *N. nigricollis*. We recognize the taxon *nigricincta* as a separate evolutionary species, *N. nigricincta*. We retain *woodi* as a subspecies of *N. nigricincta*for the time being: intergrades between the two have been noted along the Kuiseb River in Namibia, and resolution of the status of these two forms will require additional and more fine-grained analysis of mitochondrial DNA and morphological variation through the range of these snakes. Within *N. nigricollis sensu stricto*, there is considerable genetic variation, including a distinct Zambian haplotype, and an indication of differentiation between western and eastern African populations. Since there is also considerable variation in scalation and pattern even within this restricted definition of *N. nigricollis*, it is possible that there may be additional taxa included within this species, but wider sampling will be required to resolve this question.

4.3. Historical biogeography

The combination of molecular phylogeographic data and dating methods, coupled with palaeoecological and tectonic data, allow the observed phylogeographic patterns to be related to past geological or ecological events, and can help to identify the causal factors promoting diversity in a given clade. Since the African spitting cobras are largely restricted to open vegetational formations, they provide an excellent model for the study of the history of this group of organisms against the backdrop of geological and ecological change.

Most recent studies of the history of climate and ecological change in Africa have emphasized the expansion of grasslands at the expense of forests and the increased dominance of grassland faunas in the late Miocene or Pliocene (e.g., Bobe and Behrensmeyer, 2004) as a key event in determining the faunal composition of African open formations, as well as on other continents (Cerling et al., 1997). Phylogeographic studies of a number of open-formation mammalian species have shown that many of these are relatively recent radiations that date back to the Pliocene (e.g., Arctander et al., 1999). However, palaeoecological data suggest the presence of savanna grasslands in different parts of Africa as long ago as the Early to Middle Miocene (Potts and Behrensmeyer, 1993), approximately 16 Mya (Jacobs, 2004). However, these early African grassland formations may have been localized and found as habitat mosaics with forested habitats (Bobe and Behrensmeyer, 2004).

While the radiations of large grassland mammals appear to have been triggered largely by the increase in C4 grasslands in the late Miocene/Early Pliocene (Cerling et al., 1997), there is little information currently available on reptiles. Since the African spitting cobras are largely animals of grassland formations (Spawls and Branch, 1995), it might have been predicted that their radiation would also have been associated with this expansion of open formations. However, although the molecular dating results in this study come with wide confidence intervals, they strongly suggest that the cladogenesis and radiation of the spitting cobras predates the end-Miocene/Pliocene transition to grasslands, most probably by a considerable margin. Instead, the basal divergences within the group, dated at approximately 15 Mya, are more consistent with the earliest evidence of increases in grassland coverage of parts of Africa in the Early to Middle Miocene (Potts and Behrensmeyer, 1993; Jacobs, 2004), although an even earlier origin of the group cannot be excluded. This pattern, which differs significantly from that observed in many groups of large mammals, illustrates the pitfalls of generalizing conclusions on faunal changes from single groups of animals: just as reptilian faunal exchanges between Central and South America differ from the predominant mammalian pattern (Stehli and Webb, 1985; Estes and Báez, 1985; Wüster et al., 2002), the radiation of the spitting cobras presents a notably different pattern from that of many large African mammals.

The early origin of the African spitting *Naja* also suggests that spitting in this clade did not evolve as a mechanism to avoid trampling by large herds of ungulates, as was suggested by Barbour (1922), since the evolution of spitting adaptations appears to predate the appearance of the great African grasslands and the large mammal herds grazing them. In any case, Barbour's hypothesis would not be applicable to the Asian spitting *Naja*, which live in forested parts of south-east Asia, and would not have been subject to the risk of trampling by large herds.

The phylogeographic pattern observed in the spitting cobras may have been caused by a combination of ecological and geological forces. Within the northeastern clade, *N. pallida* is found to the east of the African Rift Valley, whereas *N. nubiae* is found to the west. The estimated date of the divergence of these two species, approximately 10 Mya, corresponds broadly to the beginning of rifting along the main Ethiopian rift, approximately 11 Mya (Wolfenden et al., 2004; Chorowicz, 2005), with repeated episodes of intense volcanism (Chorowicz, 2005), although again confidence intervals are wide. *Naja pallida* currently occupies the Ethiopian Rift Valley itself, but has not been recorded to its north, nor north of Djibouti on the Red Sea coast, whereas only *N. nubiae* has been recorded from the Eritrean coast and north of the Ethiopian Plateau (Wüster and Broadley, 2003).

Naja katiensis occupies an isolated phylogenetic position, and an equally distinctive biogeographical position in the West African savannas. It occurs in sympatry with *N. nigricollis*in much of its range, including the type locality, Kati in Mali. *Naja katiensis* is also morphologically dis-

tinctive among the African spitting cobras due to its low number of ventral and subcaudal scales. The causes and origins of regional endemism in the West African savannas remain insufficiently understood. A number of other squamate species exhibit similar distributions (Hughes, 1983; Böhme, 1985; Chippaux, 2006), and in at least some cases occupy similarly isolated phylogenetic positions (e.g., *Echis ocellatus*—Pook et al., unpublished data).

Among the remaining spitting cobras, the basal divergence, dated at approximately 8 Mya, separates *N. nigricollis*, with a distribution around the periphery of the western and central African rainforests, from a clade consisting of *N. mossambica, N. ashei* and *N. nigricincta*. The latter clade is found along the eastern and southern edges of the distribution of *N. nigricollis*, albeit with considerable overlap in parts of eastern Africa. The estimated date of the divergence between *N. nigricollis* and its sister clade corresponds approximately to the beginning of volcanic activity in the western Branch of the East African Rift System (Chorowicz, 2005). It is possible that the divergence between *N. nigricollis* and its sister clade may initially have been due to volcanism in the Rift Valley, followed by later eastward dispersal (to the Indian Ocean coast in parts of Tanzania) by *N. nigricollis*, resulting in sympatry with *N. mossambica* and *N. ashei* in parts of East Africa, although the wide confidence interval around that dating estimate suggests a cautious interpretation.

The *ashei–mossambica–nigricincta* clade, dated as approximately 6 Mya old, may represent another instance of clades confined to occurring primarily in relatively arid areas in both northeastern and southwestern Africa (van Zinderen Bakker, 1969). Although this clade occupies a considerable diversity of habitats, it occurs in generally drier areas than *N. nigricollis*, which inhabits generally less xeric habitats around the rainforests, and even enters anthropogenically impacted rainforest areas (Luiselli, 2001, 2002). Past arid corridors connecting East and southwest African arid areas (De Winter, 1971) have been invoked as potential causal factors behind the distribution of a number of arid-adapted African organisms, such as bathyergid mole rats (Honeycutt et al., 1987; Ingram et al., 2004) and ground squirrels (Herron et al., 2005). The distribution of the *ashei–mossambica–nigricincta* clade, from the Horn of Africa to arid southwestern Africa, may thus represent a remnant of past arid corridors from northeastern to southwestern Africa.

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Appendix A

Samples, haplotypes and GenBank accession numbers of sequences used

Taxon	Locality	Voucher(s)/sample(s)	Haplotype label (Figs. 2–4)	Genbank Accession Nos. (ND4, cyt*b*)
Naja nigricollis	Lara, Kaélé, Cameroon	Latoxan, live collection number N. ni. ssp. 9735002, 9735004/ WW1074, WW1076	*nigricollis* NCam1	DQ897693, DQ897736
Naja nigricollis	Lara, Kaélé, Cameroon	Latoxan, live collection number N. ni. ssp. 9735003/WW1075	*nigricollis* NCam2	DQ897694, DQ897737
Naja nigricollis	Ghana	LSTM, live coll./WW842	*nigricollis* Ghana	DQ897695, DQ897738
Naja nigricollis	Togo	LSTM, live coll./WW1062	*nigricollis* Togo	DQ897697, DQ897740
Naja nigricollis	Foulaya, Guinea	TR 568, collection J.-F. Trape, IRD Dakar, Senegal	*nigricollis* Guinea	DQ897696, DQ897739

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Appendix A (*continued*)

Taxon	Locality	Voucher(s)/sample(s)	Haplotype label (Figs. 2–4)	Genbank Accession Nos. (ND4, cyt*b*)
Naja nigricollis	Arusha, Tanzania	WW296	*nigricollis* Arusha	DQ897698, DQ897741
Naja nigricollis	Kigosi Camp, Tanzania	WW297	*nigricollis* Tanzania	DQ897699, DQ897742
Naja nigricollis	Mbwewe, Tanzania	JB, live coll./WW1404, WW1405	*nigricollis* Tanzania	DQ897699, DQ897742
Naja nigricollis	Bombani, Amani, Tanzania	JB, live coll./WW1403, WW1406, WW1407	*nigricollis* Usambara	DQ897703, DQ897746
Naja nigricollis	Bulyanhulu, Tanzania	WW1614	*nigricollis* Tanzania	DQ897699, DQ897742
Naja nigricollis	Makuyu, Kenya	Bio-Ken live coll. BK10221, BK 10246/ WW1271, WW1272	*nigricollis* Makuyu	DQ897700, DQ897743
Naja nigricollis	Mfuwe, Chipata District, Zambia	WW1198, WW1393	*nigricollis* Mfuwe	DQ897701, DQ897744
Naja nigricollis	Mikumi NP, Tanzania	WW1415	*nigricollis* Mikumi	DQ897702, DQ897745
Naja ashei	Watamu, Kenya - HOLOTYPE	NMK S-3993/WW1430	*ashei* Watamu	DQ897706, DQ897749
Naja ashei	Watamu, Kenya	Bio-Ken live coll. BK10022, BK10177/ WW1267, WW1268	*ashei* Coast	DQ897704, DQ897747
Naja ashei	Diani, Kenya	Bio-Ken live coll. BK10248/WW1270	*ashei* Coast	DQ897704, DQ897747
Naja ashei	Baringo, Kenya	BMNH 2005.1604	*ashei* Baringo	DQ897705, DQ897748
Naja katiensis	Doussoudiana, Mali	TR 913, collection J.-F. Trape, IRD Dakar, Senegal	*katiensis*1	DQ897707, DQ897750
Naja katiensis	Doussoudiana, Mali	TR 1234, collection J.-F. Trape, IRD Dakar, Senegal	*katiensis*2	DQ897708, DQ897751
Naja katiensis	Laminina, Mali	TR 924, collection J.-F. Trape, IRD Dakar, Senegal	*katiensis*2	DQ897708, DQ897751
Naja nigricincta nigricincta	Ruacana, Namibia	WW879	*nigricincta*	DQ897709, DQ897752
Naja nigricincta woodi	Goegap, Northern Cape, South Africa	WW1563, WW1564	*woodi*1	DQ897710, DQ897753
Naja nigricincta woodi	Goegap, Northern Cape, South Africa	WW1395	*woodi*2	DQ897711, DQ897754
Naja nigricincta woodi	Paardeberg, Paarl, Western Cape, South Africa (prob. accidental introduction)	WW1396	*woodi*2	DQ897711, DQ897754
Naja nigricincta woodi	Cedarberg, Western Cape, South Africa	WW1588	*woodi*3	DQ897712, DQ897755
Naja pallida	Kenya	ZSL, live coll.; WW834, WW835	*pallida* Kenya1	DQ897713, DQ897756
Naja pallida	Tanzania	Ophiofarm, live coll./ WW1080	*pallida* Tanzania	DQ897714, DQ897757
Naja pallida	Kenya	Latoxan N.pa.90007, N.pa.90009/WW1081, WW1083	*pallida* Kenya2	DQ897715, DQ897758

Appendix A (*continued*)

Taxon	Locality	Voucher(s)/sample(s)	Haplotype label (Figs. 2–4)	Genbank Accession Nos. (ND4, cyt*b*)
Naja pallida	Kenya	Latoxan N.pa.90008/ WW1082	*pallida* Kenya3	DQ897716, DQ897759
Naja pallida	Tsavo East N.P., Kenya	Bio-Ken live coll. BK-10054/WW1273	*pallida* Kenya2	DQ897715, DQ897758
Naja pallida	Lake Baringo, Kenya	Bio-Ken live coll. BK10659/ WW1431	*pallida* Baringo	DQ897717, DQ897760
Naja nubiae	Unknown	ZSL live coll./WW836	*nubiae*1	DQ897718, DQ897761
Naja nubiae	Unknown	ZSL live coll./WW837	*nubiae*2	DQ897719, DQ897762
Naja mossambica	Durban, KwaZulu-Natal, South Africa	WW590, gift H.-W. Herrmann	*mossambica* Durban	DQ897720, DQ897763
Naja mossambica	Namagure Village, Moçambique	PEM R 13244/WW190	*mossambica* Moçam1	DQ897722, DQ897765
Naja mossambica	Namagure Village, Moçambique	PEM R 13252/ WW191	*mossambica* Moçam2	DQ897721, DQ897764
Naja mossambica	Bulawayo, Zimbabwe	NMZB 15862/WW882	*mossambica* Bulawayo	DQ897723, DQ897766
Naja mossambica	Shinyungwe, Namibia	WW287	*mossambica* Namibia	DQ897724, DQ897767
Naja mossambica	Plumtree, Zimbabwe	NMZB 16971/WW1289	*mossambica* Plumtree	DQ897725, DQ897768
Naja mossambica	Komatipoort, Mpumalanga, South Africa	WW1300	*mossambica* Komati	DQ897726, DQ897769
Naja mossambica	Pemba Island, Tanzania	KMH26380, KMH26381/ WW1391, WW1392	*mossambica* Pemba	DQ897727, DQ897770
Naja melanoleuca	Nyasoso, Cameroon	WW182	*Naja melanoleuca*	DQ897689, DQ897732
Naja nivea			*Naja nivea*	*AY058983, *AF217827
Naja haje	Egypt	WW893	*Naja haje*	DQ897692, DQ897735
Naja naja	Southwestern Nepal	WW595	*Naja naja*	DQ897690, DQ897733
Naja kaouthia		CAS 206602	*Naja kaouthia*	*AY058982, *AF217835
Naja sputatrix	West Java	WW584	*Naja sputatrix*	DQ897691, DQ897734
Walterinnesia aegyptia			*Walterinnesia aegyptia*	*AY058988, *AF217838
Boulengerina annulata			*Boulengerina annulata*	*AY058970, ***AY188010**
Paranaja multifasciata			*Paranaja multifasciata*	*AY058985, *AF217837
Hemachatus haemachatus			*Hemachatus haemachatus*	J.S.Keogh, pers. comm, *AF217821
Aspidelaps scutatus			*Aspidelaps scutatus*	*AY058969, *AF217828
Laticauda colubrina			*Laticauda colubrina*	*AY058977, *AF217834
Ophiophagus hannah		CAS 206601	*Ophiophagus hannah*	*AY058984, *AF217842
Hemorrhois ravergieri		ZISP 27733	*Hemorrhois ravergieri*	*AY487050, *AY486920
Hemorrhois nummifer		ZISP 27709	*Hemorrhois nummifer*	*AY487049, *AY376742
Hemorrhois hippocrepis		MNCN 11988	*Hemorrhois hippocrepis*	*AY487045, *AY486916

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Appendix A (*continued*)

Taxon	Locality	Voucher(s)/sample(s)	Haplotype label (Figs. 2–4)	Genbank Accession Nos. (ND4, cyt*b*)
Hemorrhois algirus		HLMD RA-1187	*Hemorrhois algirus*	*AY487037, *AY486911
Pantherophis guttatus			*Pantherophis guttatus*	*AF138756, *AF337173
Boiga dendrophila			*Boiga dendrophila*	*U49303, *AF471089
Vipera berus	Anglesey, UK/	WW199 (ND4)/Lenk et al. (1999) (cyt*b*)	*Vipera berus*	DQ897728, *AJ275719
Macrovipera lebetina	Nura Tau, Uzbekistan/ Lenk et al. (1999)	Latoxan 0413-2/WW1641 (ND4); Lenk et al. (1999) (cyt*b*)	*Macrovipera lebetina*	DQ897729 *AJ275713
Vipera ammodytes	unknown	LSTM live coll. Va1/ WW1312	*Vipera ammodytes*	DQ897730, DQ897771
Montivipera albizona	Unknown/Lenk et al. (1999)		*Montivipera albizona*	DQ897731, *AJ275727
Causus rhombeatus			*Causus rhombeatus*	*U41866, *DQ305455
Porthidium arcosae	Salango, Manabí, Ecuador	FHGO live collection 738/ WW750	*Porthidium arcosae*	AF292613, AF292575
Porthidium nasutum	Zapallo Grande, Río Cayapas, Esmeraldas, Ecuador	FHGO live collection 517/ WW751	*Porthidium nasutum*	AF292612, AF292574
Porthidium lansbergii rozei	San Antonio, Falcón, Venezuela		*Porthidium lansbergii*	AF393623, AY713375
Calloselasma rhodostoma			*Calloselasma rhodostoma*	U41878, AF038882
Azemiops feae			*Azemiops feae*	*U41865, *AF352747
Bitis nasicornis		CAS207874	*Bitis nasicornis*	*DQ305475, *DQ305457
Elapsoidea nigra			*Elapsoidea nigra*	*AY058975, *AF217820
Atractaspis bibronii			*Atractaspis bibroniii*	*U49314, *AY188008
Nerodia erythrogaster			*Nerodia erythrogaster*	*AF420084, *AF420081
Alsophis portoricensis				*U49308, *AF471085
Oxyuranus scutellatus	Cairns, Queensland, Australia	WW1199		AY340788, DQ897772
Cerberus rhynchops				*U49327, *AF471092
Afronatrix anoscopus				*AF420076, *AF420073
Hypsiglena torquata				*U49309, *AF471038
Dinodon semicarinatus				*AB008539, *AB008539
Acrochordus granulatus				*U49296, *AF217841

GenBank accession numbers with asterisks were not generated by the authors. WW, Wolfgang Wüster, personal collection; LSTM, Liverpool School of Tropical Medicine; JB, Joe Beraducci, personal collection, Arusha, Tanzania; NMK, National Museum, Nairobi, Kenya; BMNH, Natural History Museum, London; IRD, Institut de Recherche pour le Dévelopement, Dakar, Senegal; ZSL, Zoological Socoety of London; PEM, Port Elizabeth Museum, South Africa; NMZB, Natural History Museum of Zimbabwe, Bulawayo; KMH, Kim Howell collection, Dar es Salam, Tanzania; CAS, California Academy of Sciences; ZISP, Zoological Institute of the Russian Academy of Sciences, Saint Petersburg; MNCN, Museo Nacional de Ciencias Naturales, Madrid, Spain; HLMD, Hessisches Landesmuseum Damstadt, Germany; FHGO, Fundación Herpetologica Gustavo Orcés, Quito, Ecuador.

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