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When continents collide: Phylogeny, historical biogeography and systematics of the medically important viper genus *Echis* (Squamata: Serpentes: Viperidae)

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ABSTRACT

We analyze the phylogeny of the medically important and taxonomically unresolved viper genus *Echis* using four mitochondrial gene fragments. The results show that the populations of the genus fall into four main clades: the *Echis carinatus*, *E. coloratus*, *E. ocellatus* and *E. pyramidum* groups. The *E. pyramidum* and *E. coloratus* groups are sister taxa but the interrelationships of this clade and the *E. ocellatus* and *E. carinatus* groups are unresolved. The initial divergence of the genus appears to coincide with the collision between Afro-Arabia and Eurasia, and that between the *E. coloratus* and *E. pyramidum* clades appears to be associated with the opening of the Red Sea. Later land connections between Africa and Arabia may have contributed to shaping the distribution of the *E. pyramidum* complex. The present distribution of *E. carinatus* may be the result of range expansion from southern India. Taxonomically, our results provide molecular evidence for the validity of *Echis omanensis*, *E. khosatzkii*, *E. borkini* and *E. jogeri*, for the presence of unsuspected genetic diversity within the *E. pyramidum* complex in eastern Africa, and for the conspecificity of *E. carinatus* and *E. multisquamatus*. The status of *E. leucogaster* remains to be confirmed.

1. Introduction

Historically, much debate in biogeography has centered on the relative roles of vicariance and dispersal in causing disjunct or multicontinental distributions (de Queiroz, 2005). Much of this discussion was stimulated by the observation of disjunct distributions of organisms across the southern continents, coupled with the acceptance of plate tectonics and the realization that these southern land masses once formed the single supercontinent Gondwana, leading to vicariance as a plausible explanation for disjunct southern distributions. Vicariance thus became the standard assumption for disjunct distribution patterns in organisms, except in the case of isolated oceanic islands. However, more recently, an increasing number of molecular phylogenetic studies, often incorporating estimates of times of cladogenesis, have cast doubt on many putative instances of vicariance-caused disjunct distribution patterns (e.g., Raxworthy et al., 2002; de Queiroz, 2005).

The frequent emphasis on vicariance as a key mechanism in causing disjunct distribution patterns has also contributed to attention in historical biogeography being focused on scenarios where landmasses have become separated by tectonic events, as opposed to those instances where they became joined. Prominent examples of the latter include North and South America, which became joined by the Isthmus of Panamá approximately 3.5 Mya (Coates and Obando, 1996), and Africa and Asia, which became joined through the "*Gomphotherium* landbridge" approximately 18 Mya (Rögl, 1998, 1999).

Both these events had the effect of allowing free(er) biotic interchanges between the newly connected landmasses, profoundly impacting on their subsequent faunas. However, one important observation is that the impact and timing of these faunal exchanges appear to have differed sharply between different groups of organisms. The joining of North and South America resulted in the Great American Biotic Interchange among mammals, a sudden and recent mass interchange of faunal elements that played a decisive role in shaping the diversity of the faunas of both continents, including at high taxonomic levels (Stehli and Webb, 1985; Webb and Rancy, 1996; but see Koepfli et al., 2007). In contrast, the effect was less drastic among ectothermic tetrapods: many of the shared faunal groups had already achieved an amphi-Isthmian distribution long before the emergence of the Isthmus (Estes and Báez, 1985; Wüster et al., 2002). Post-Isthmian dispersal events primarily affected the distributions of single species or species complexes (e.g., Wüster et al., 2005b; Venegas-Anaya et al., 2008).

The establishment of the Afro-Arabia – Eurasia landbridge allowed increasing mammalian interchanges, starting in the early

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Miocene, approximately 22–21 Mya (Harzhauser et al., 2007). However, its effect on ectothermic vertebrates has been less well studied. There are indications that some taxa may owe their current distribution across both continents to that initial collision, but in others, the distribution appears to predate any land connection. The oldest fossils of the elapid genus *Naja* showing the synapomorphies of the (monophyletic) Asian cobra clade date back to approximately 16 Mya (Szyndlar and Rage, 1990), consistent with differentiation after the formation of the land connection (Wüster et al., 2007). However, Amer and Kumazawa (2005) estimated an age of 29–25 Mya for the divergence between Eurasian and Afro-Arabian *Uromastyx* lizards, and Wüster et al. (2008) an age of 58–38 Mya for the split between the ancestrally Asian Crotalinae and the principally African Viperinae, i.e., both long before the *Gomphotherium* bridge.

In addition to the Afro-Arabian – Eurasian collision, another major tectonic development took place in the same region approximately simultaneously: the beginning of the African Rift and the opening of the Red Sea. Rifting at the southern end of the Red Sea is believed to have started approximately 27.5–23 Mya (Hughes et al., 1991; Chorowicz, 2005), and to have spread northward to reach the present northern end of the Red Sea by the early Miocene (Bosworth et al., 2005). This interaction between continental collision and rifting makes the biogeography of the African–Arabian–Asian crossroads a particularly interesting research topic.

Within the Viperinae, the genus *Echis* (saw-scaled vipers) stands out as having a distribution highly likely to have been shaped by these tectonic events. The distribution of these small to medium-sized vipers extends across open and xeric formations from Africa North of the Equator (from Morocco and Guinea to Egypt and Kenya), to the Arabian Peninsula, and south and east to Sri Lanka and West Bengal. Wüster et al. (2008) dated the basal cladogenic split within *Echis* at 18.5 Mya, i.e., at approximately the same time as the *Gomphotherium* bridge. This genus therefore represents an ideal model for the study of the effects of the Eurasian–African collision on organismal distribution.

Apart from their biogeographical interest, the phylogeny of the genus *Echis* is also of considerable interest for the resolution of a problematic taxonomy, which furthermore impinges on the treatment of snakebite. *Echis* has a long history of taxonomic confusion and controversy (David and Ineich, 1999; Mallow et al., 2003). Until the 1970s, only two species were recognized, *E. coloratus* from the Middle East and the Arabian Peninsula, and *E. carinatus* from the vast remainder of the range of the genus. A number of taxa were described as subspecies of *E. carinatus* or as separate species from the 1960s to the 1980s (Stemmler, 1969, 1970; Stemmler and Sochurek, 1969; Roman, 1972, 1975; Cherlin, 1981, 1983, 1984), but despite progress in defining the limits of some species or species complexes (Hughes, 1976; Arnold, 1980; Joger, 1984), no consensus on species limits and relationships emerged. A genus-wide revision grouped the populations of the genus into four subgenera, and recognized 12 species with 20 subspecies, including nominal forms (Cherlin, 1990). However, some of Cherlin's conclusions were later questioned (Auffenberg and Rehman, 1991; Schätti and Gasperetti, 1994; Trape and Mané, 2006), and Babocsay (2003, 2004) described an additional species and a subspecies within the *Echis coloratus* group. Recent compendia of venomous snake diversity (Spawls and Branch, 1995; David and Ineich, 1999; McDiarmid et al., 1999; Mallow et al., 2003; Dobiey and Vogel, 2007) have accepted some but not all aspects of Cherlin's conclusions, underscoring the need for a rigorous revision of the complex. Some of the alternative interpretations of the systematics of *Echis* proposed in the recent literature are summarized in Table 1.

All previous work on the systematics of *Echis* has been based on morphological data, with occasional recourse to advanced multivariate approaches that seek to reveal patterns of morphological variation across multiple characters simultaneously (Babocsay,

Table 1
Interpretations of the taxonomy of the genus *Echis* in key references, and results of this study.

Klemmer (1963)	Cherlin (1990)	David and Ineich (1999)	This study
Echis carinatus • *E. c. carinatus*	*Echis carinatus* • *E. c. carinatus* • *E. c. sinhaleyus*	*E. carinatus* • *E. c. carinatus* • *E. c. astolae*	*E. carinatus*
• *E. c. pyramidum*	*Echis (Turanechis) multisquamatus*	• *E. c. multisquamatus*	
	Echis (Turanechis) sochureki • *E. s. sochureki* • *E. s. astolae*	• *E. c. sinhaleyus* • *E. c. sochureki*	
	Echis (Toxicoa) pyramidum • *E. p. pyramidum* • *E. p. lucidus* • *E. p. leakeyi*	*E. pyramidum* • *E. p. pyramidum* • *E. p. aliaborri* • *E. p. leakeyi*	*E. pyramidum*
	Echis (Toxicoa) varia • *E. v. aliaborri* • *E. v. darevskii* • *E. v. varia* • *E. v. borkini*		? *E. varius* *E. borkini*
	Echis (Toxicoa) khosatzkii		*E. khosatzkii*
	Echis (Toxicoa) arenicola • *E. a. arenicola* • *E. a. leucogaster*	*E. leucogaster*	*E. leucogaster*
	Echis (Toxicoa) megalocephalus	*E. megalocephalus*	?
	Echis (Toxicoa) ocellatus	*E. ocellatus*	*E. ocellatus*
	Echis (Toxicoa) jogeri	*E. jogeri*	*E. jogeri*
	Echis (Toxicoa) hughesi	*E. hughesi*	?
E. coloratus	*Echis (Turanechis) "froenatus" (= coloratus)*	*E. coloratus* Babocsay (2003, 2004): *E. coloratus coloratus* *E. coloratus terraesanctae* *E. omanensis*	*E. coloratus* *E. omanensis*

2003, 2004). As yet, there has been no comprehensive attempt to address the phylogeny and systematics of the complex using molecular markers.

A robust systematic and phylogenetic framework is essential for toxinological research and the treatment of snake bites. Variation in venom composition is a ubiquitous phenomenon at all taxonomic levels in snakes (Chippaux et al., 1991), and can greatly affect the ability of antivenoms to neutralize different venoms (Fry et al., 2003; Harrison et al., 2003). Understanding the causes and correlates of variation in venom composition requires first and foremost a sound understanding of the systematics of the snakes involved. Due to the extensive documented variation in diet and venom composition in *Echis*, this genus constitutes a model system for the study of the causes of variation in venom composition (Barlow et al., 2009).

Moreover, *Echis* epitomizes the issues surrounding snake taxonomy and antivenom treatment. This genus accounts for more deaths or severe morbidity in West Africa than any other venomous snake genus (Warrell and Arnett, 1976). It is probably responsible for the majority of the estimated 20,000 snakebite deaths per annum for the whole of Africa (Chippaux, 1998, 2006), and one of the major causes of snakebite mortality and morbidity in India (Bhat, 1974; Kochar et al., 2007).

Despite similar clinical signs and symptoms (consisting of local and systemic hemorrhage, incoagulable blood and local necrosis), venom composition varies considerably between different populations of *Echis*, resulting in antivenoms raised against one venom being ineffective in neutralizing another venom (Mebs and Kornalik, 1981; Gillissen et al., 1994). For instance, antivenom raised against Asian *E. carinatus* failed in the treatment of bites by *E. ocellatus* in West Africa, and its use led to greatly increased fatality rates (Warrell and Arnett, 1976; Hughes, 1976), a tragedy now being repeated in parts of West Africa due to the unaffordable price of specific antivenoms and the unscrupulous selling of Asian antivenoms on the African market (Visser et al., 2008; Warrell, 2008). A sound understanding of the phylogeny of these snakes could provide a background to the failure of certain antivenoms (Fry et al., 2003).

In this study, we use mitochondrial DNA sequences to infer the phylogeny of the genus *Echis* with the aims of elucidating their biogeographical history and providing a phylogeographic framework for the identification of species limits within this complex. We also analyze partially co-distributed species groups of elapids and viperids to test for the generality of the timing of divergence events across geological barriers, such as the Red Sea, and the timing of isolation of West African savanna clades.

2. Materials and methods

2.1. Taxon sampling

Samples of *Echis* (ventral scale clippings, shed skins, or blood samples taken through caudal venepuncture or cardiac puncture) were obtained from institutional live collections, herpetoculturists, colleagues and through fieldwork. To test for shared historical biogeographical patterns with co-distributed taxa, we also included samples of three species groups sharing biogeographically interesting parts of their range with *Echis*: the Egyptian cobra (Elapidae: *Naja haje*) complex and the puff adder (Viperidae: *Bitis arietans*) both occur across open formations in much of Africa and allopatrically in the southern Arabian Peninsula; and among spitting cobras (Elapidae: *Naja nigricollis* complex), *N. katiensis* shares with *Echis ocellatus* a somewhat isolated distribution in the West African savannas (Wüster et al., 2007). In addition, as in Wüster et al. (2008), we included a number of other Caenophidian 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. Sampling localities are shown in Fig. 1 and sample details and GenBank Accession numbers are given in Appendix I.

2.2. Laboratory protocols

Genomic DNA was extracted using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma–Aldrich). Four mitochondrial gene fragments, 1100 base pairs (bp) of cytochrome *b* (CYTB), 900 bp of NADH dehydrogenase subunit 4 (NADH4), 455 bp of the

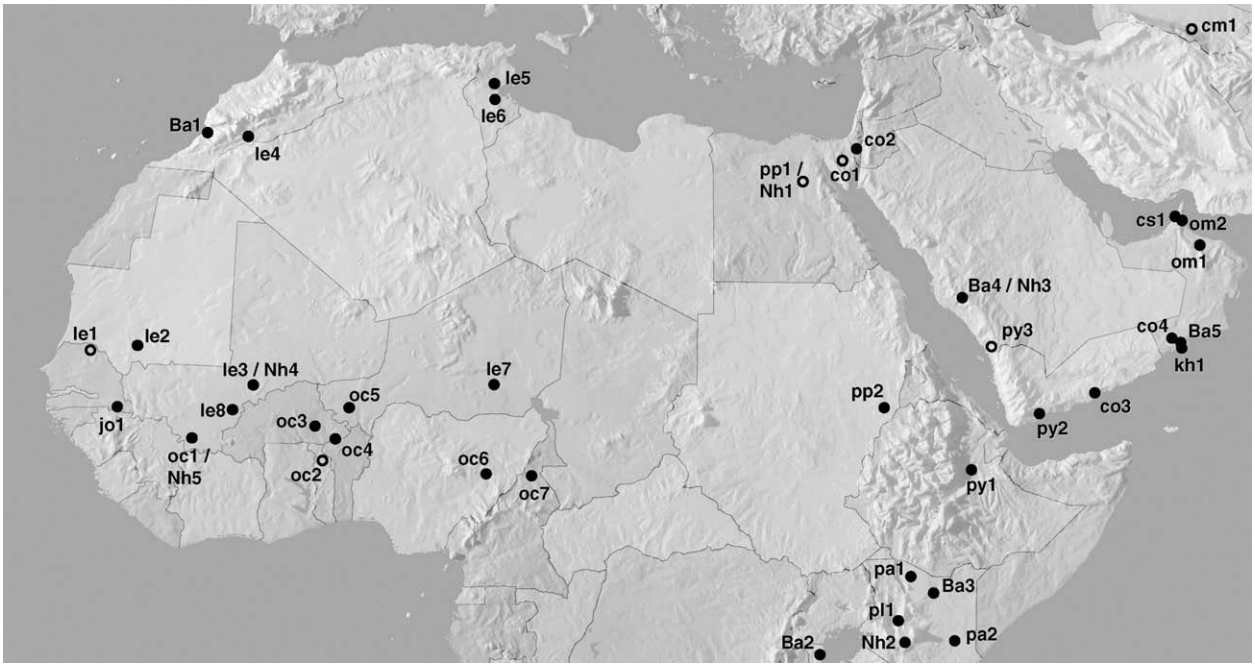

Fig. 1. Sampling localities of *Echis* in Africa and the Middle East. Labels refer to taxon: co, *E. coloratus*; cs, *E. carinatus sochureki*; cm, *E. carinatus multisquamatus*; le, *E. leucogaster*; jo, *E. jogeri*; om, *E. omanensis*; pp, *E. pyramidum pyramidum*; pl, *E. pyramidum leakeyi*; pa, *E. pyramidum aliaborri*; py, *E. pyramidum* group, affinities uncertain; kh, *E. khosatzkii*, Ba, *Bitis arietans;* Nh, *Naja haje*. Hollow symbols indicate imprecise localities.

Table 2
Primers used in the amplification of mitochondrial genes. [a]Wüster et al. (2008); [b]Arévalo et al. (1994); [c]S. Ursenbacher (personal communication); [d]Knight and Mindell (1993); [e]Palumbi (1996); position refers to the position in the mitochondrial genome of *Dinodon semicarinatus* (Kumazawa et al., 1998) at which the 5′ end of the primer aligns.

Primer name	Position	Primer
CYTB		
GludgMod2[a]	14902	CTGAAAAACCACCGTTGT
EchR[a]	15787	GCTCCDCCBAGTTTRTT
NADH4		
NADH4[b]	11677	CACCTATGACTACCAAAAGCTCATGTAGAAGC
HIS12763V[c]	12594	TTCTATCACTTGGATTTGCACCA
12S		
L1091[d]	478	AAACTGGGATTAGATACCCC ACTAT
H1557[d]	980	GTACACTTACCTTGTTACGACTT
16S		
L2510[e]	1828	CGCCTGTTTATCAAAAACAT
H3059[e]	2376	CCGGTCTGAACTCAGATCACGT

small subunit of 12SrRNA (12S), and 507 bp of the small subunit of 16SrRNA (16S) were amplified by polymerase chain reaction (PCR) (Table 2). 20 μl volume PCRs were made up of 18 μl Abgene 1.1× ReddyMix™ (1.25 U Thermoprime Plus DNA polymerase; 75 mM Tris–HCl, pH 8.8; 20 mM $(NH_4)_2SO_4$; 1.5 mM $MgCl_2$; 0.01% (v/v) Tween® 20; 0.2 mM each dNTP; precipitant red dye for electrophoresis), 0.4 μM of each primer (Table 2) and 1–5 ng of template DNA. The amplification conditions were: denature at 95 °C for 3 minutes (min), then 35 cycles of 95 °C denature for 30 seconds (s), 43 °C (12S, 16S) or 50 °C (CYTB, NADH4) anneal for 45 s, extension 72 °C for 1 min, followed by one final extension step of 72 °C for 5 min. Prior to sequencing, PCR products were incubated at 37 °C for 50 min with the enzymes Exonuclease I and Shrimp Alkaline Phosphatase (Werle et al., 1994), which degrade extraneous single stranded PCR product and primers, and hydrolyze unwanted dNTPs. Direct DNA sequencing was carried out by Macrogen Inc. (Korea).

Sequences were checked in Chromas (Technylysium) and aligned using ClustalW (Thompson et al., 1994) via Mega 4.0 (Tamura et al., 2007). The placement of gaps in the RNA genes was not reliable using this method and further adjustments were made by eye. The hypervariable regions in the 12S and 16S sequences were impossible to align and were excluded from the phylogenetic analysis. Protein-coding genes were translated into amino acid sequences in MEGA (Tamura et al., 2007) to check for the presence of indels or unexpected stop codons, which would indicate nuclear pseudogene sequences (Zhang and Hewitt, 1996).

2.3. Phylogenetic analysis

Phylogenetic resolution of heterogeneous data sets and a reduction in the effects of saturation can be improved when the best evolutionary models are used for individual data partitions (Wilgenbusch and de Queiroz, 2000; Brandley et al., 2005; Castoe and Parkinson, 2006). Logical gene partitions within the dataset were defined according to the nature of evolution of each gene (Nylander, 2004). The protein coding genes were partitioned by codon position, because different codon positions show different substitution rates, and the two rRNA genes were treated as two additional separate partitions. The software MrModeltest 2.2 was used to identify the optimal model of sequence evolution for each data partition under the Akaike Information Criterion.

Bayesian inference was carried out using Markov Chain Monte Carlo (MCMC) randomization in MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). Three parallel runs of four chains (3 heated and 1 cold) were performed for 8 million generations and the runs were sampled every 200th generation. The point of convergence (burnin) was estimated in Tracer v1.4 (Rambaut and Drummond, 2007) to occur well before completion of the first 2 million generations, but we conservatively discarded these 25% of generations as burnin and calculated the consensus tree from the remaining 75% of the posterior distribution. Sample sizes for all parameters were estimated using Tracer.

In all analyses, *Acrochordus granulatus*, representing the putative sister group of the Colubroides (Zaher et al., 2009), was used as the outgroup.

2.4. Molecular dating

Times of cladogenesis within *Echis* were estimated using a Bayesian MCMC approach in BEAST v1.4.7 (Drummond and Rambaut, 2007). Representatives of all major viperid, elapid and "colubrid" clades were included to furnish additional fossil or geology-based calibration points, with additional taxa to break up overlong branches. We implemented a Yule branching process with lognormal priors and no topological constraints, which assumes a constant speciation rate per lineage and which represents the average net rate of lineage birth. 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. Partitioning of the data and models of sequence evolution were as above. Each MCMC chain was run for 7 million generations following a 10% pre-burnin, with ten independent runs.

Calibration of the molecular dating analysis was as described in Wüster et al. (2008). We used lognormal priors for fossil-based calibration points, and normal priors for calibration points based on new land connections between land masses, and the same calibration points:

1. "*Porthidium*": the initial divergence of the South American populations of this genus 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 (SD) of 0.51 My, providing a 95% confidence interval of 2.5–4.5 Mya.
2. "Eurasian vipers": fossil evidence indicates that the divergence of the Eurasian viper clade (excluding *Pseudocerastes* and *Eristicophis*) had begun by 20 Mya (Szyndlar and Rage, 1999). This was modeled with a lognormal prior with a 20 Mya zero offset, a default lognormal mean of 1 and the default lognormal SD of 1.
3. "*Naja*": the split between the Asian *Naja* clade and its African sister clade dates back to a minimum age of 16 My (Szyndlar and Rage, 1990; Wüster et al., 2007). A lognormal prior was used with a 16 Mya zero offset, lognormal mean of 1 and a lognormal SD of 1.
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). A normal prior was used with a mean of 18 Mya and a SD of 2.04 My, creating a 95% CI of 14–22 My.
5. "Rattlesnakes": the divergence between *Crotalus* and *Sistrurus* occurred before 9 Mya (Parmley and Holman, 2007). A lognormal prior was used with a zero offset of 9 Mya, a lognormal mean of 1 and a lognormal SD of 1.
6. "Colubroidea": Divergence between vipers and their sister group is assumed to have taken place long before the Eocene, but probably after the Cenomanian (see Wüster et al., 2008). A lognormal prior was used with a zero offset of 40 Mya, a lognormal mean of 2 and a lognormal SD 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. Based on the assumption that the initial divergence of the Cae-

nophidia is logically older than that of the Colubroidea, and quite possibly considerably older, a lognormal prior was implemented with a zero offset of 45 Mya, a lognormal mean of 2.5, and a lognormal SD of 1.25.

The resulting BEAST log files were viewed in Tracer v1.4 (Rambaut and Drummond, 2007) to estimate the point of convergence. Post-burnin trees from all ten runs were then combined using the software LogCombiner v. 1.4.8 (Drummond and Rambaut, 2007). Effective sample size of all parameters was checked in Tracer. The resulting file of combined trees was entered into TreeAnnotator v. 1.4.8 (Drummond and Rambaut, 2007), and the ages of nodes estimated from the tree resulting from the initial Bayesian phylogenetic analysis, which was defined as the user target tree. The final dating tree together with confidence limits was visualized in FigTree v1.1.2 (Rambaut, 2008, http://tree.bio.ed.ac.uk/software/figtree/).

3. Results

3.1. Sequence analysis and phylogeny

The final alignment consisted of 2342 base pairs (789 bp CYTB; 644 NADH4; 414 12S; 495 16S). No indels or stop codons were found in the coding genes, which suggests that the sequences were mitochondrial and not pseudogenes (Zhang and Hewitt, 1996). The best evolutionary models for each partition, as determined by MrModeltest 2.2 (Nylander, 2004), were defined as: GTR+I+Γ for CYTB POS1 and POS2, NADH4 POS1 and POS2, 12S and 16S; GTR+Γ for CYTB POS3 and NADH4 POS3 (GTR = General Time Reversible model (Tavaré, 1986; Yang, 1994); I = proportion of invariable sites; Γ = shape of the gamma distribution; POS1, POS2, POS3 = 1st, 2nd and 3rd codon position).

The Bayesian phylogeny (Fig. 2) showed a significantly supported sister-group relationship between *Echis* and *Cerastes*, and revealed four highly distinct and well supported haplotype clades within *Echis*, corresponding to four major species complexes, here termed the *Echis ocellatus*, *E. pyramidum*, *E. carinatus* and *E. coloratus* groups.

The *E. carinatus* group includes haplotypes from India (*E. c. carinatus*), Pakistan and the northeastern Arabian Peninsula (*E. c. sochureki*), and *E. multisquamatus* from Central Asia. Haplotypes from the northern part of the range (Arabia, Pakistan, Central Asia, northwestern India) form a tightly knit cluster without clear phylogeographic structure. Successively more basal sister haplotypes originate from Maharashtra (western India) and Tamil Nadu, southern India. The haplotype representing *E. multisquamatus*, from Central Asia, is found nested among the other northern haplotypes.

The *E. coloratus* group consists of two distinct clades, representing *E. omanensis* in the northeast of the Arabian Peninsula and *E. coloratus* from southern and western Arabia, Israel and Egypt. Within the latter, a haplotype from southern Oman formed the sister group to a clade consisting of Yemeni, Israeli and Egyptian haplotypes.

The *E. ocellatus* group consists of four deeper haplotype clades. Haplotypes from the western end of the range (Senegal) form the highly divergent sister group to the remainder of the group, within which there are three distinct haplotype clades: (i) a single haplotype from southern Mali; (ii) a clade of haplotypes from Togo, Benin, eastern Burkina Faso and Niger; and (iii) a clade of haplotypes from northeastern Nigeria and northern Cameroon.

The *E. pyramidum* group contains four highly distinct haplotype clades: (i) the main *E. pyramidum* group, containing haplotypes from most of Africa north of the Equator, including populations conventionally assigned to *E. p. pyramidum*, *E. p. leakeyi*, *E. p. aliaborri* and *E. leucogaster*; (ii) a single, highly divergent haplotype from the Afar Depression, eastern Ethiopia; (iii) several haplotypes from southern Yemen and southwestern Saudi Arabia, normally assigned to *E. pyramidum*; (iv) several haplotypes from southern Oman, corresponding to populations sometimes assigned to *E. khosatzkii*. Within the main *E. pyramidum* clade, haplotypes of *E. leucogaster* form the sister clade to haplotypes of *E. p. pyramidum*, *E. p. aliaborri* and *E. p. leakeyi*, but divergence levels are low. The *E. leucogaster* haplotypes are in turn subdivided into western (Mauritania, Senegal, Morocco, western Mali) and eastern (Tunisia, Niger, eastern Mali) clades. In *E. pyramidum*, Egyptian and Sudanese haplotypes cluster separately from Kenyan haplotypes, again with low divergences.

The phylogeny showed significant posterior probability support for a sister-group relationship between the *E. pyramidum* and *E. coloratus* groups. The interrelationships between this clade and the *E. ocellatus* and *E. carinatus* groups were largely unresolved: the analysis placed the latter two groups as sister taxa, but without significant support.

3.2. Molecular dating

Analysis in Tracer showed that the BEAST runs converged after between 1.5 and 2.5 million generations. We conservatively discarded the first 3 million generations from each of the ten runs and combined the remaining 4 million generations of each run, sampling every 4000th generation, in LogCombiner. Rate heterogeneity among branch lengths was confirmed from the fact that the standard deviation of the uncorrelated lognormal relaxed clock was greater than 0, and the histogram frequency curve for the coefficient of variation was not aligned with zero.

The resulting dating tree (Figs. 3 and 4) placed the split of the genus *Echis* from its sister group at approximately 30 Mya. The basal cladogenesis in *Echis* is estimated to have taken place approximately 22 Mya, followed rapidly by the split between *E. ocellatus* and *E. carinatus*, and slightly later (19.4 Mya) by that between the *E. pyramidum* and *E. coloratus* groups. The basal splits within the four species groups took place an estimated between 3.8 and 9.2 Mya. Within the *E. pyramidum* group, the separation between the Arabian clade and African *E. pyramidum* took place approximately 8.1 Mya.

4. Discussion

4.1. Phylogeny and systematics of Echis

This study represents the first comprehensive molecular investigation of the phylogeny of the genus *Echis*. Given the history of taxonomic confusion surrounding the genus, these results represent an important phylogenetic scaffold for the interpretation of previous systematic hypotheses. Although much remains to be done to arrive at a full understanding of this complex genus, we believe that our data contain sufficient novel information to warrant a revision of the taxonomy of *Echis*.

Our analyses provide significant support for a sister-group relationship between *Echis* and *Cerastes*, previously suggested by Joger and Courage (1999) and recovered by Wüster et al. (2008), but not by Lenk et al. (2001). Lenk et al. may have been confounded by their choice of *Causus* as an outgroup: this genus is probably nested within the Viperinae (Nagy et al., 2005; Wüster et al., 2008). Reanalysis of the data of Lenk et al. (2001) with a more appropriate crotaline outgroup also resulted in a sister-group relationship between *Echis* and *Cerastes* (Joger and Lenk, unpublished data). Since none of the recent analyses supported a close relationship between *Atheris* and the *Echis-Cerastes* clade, the serrated 'rattle scales' of

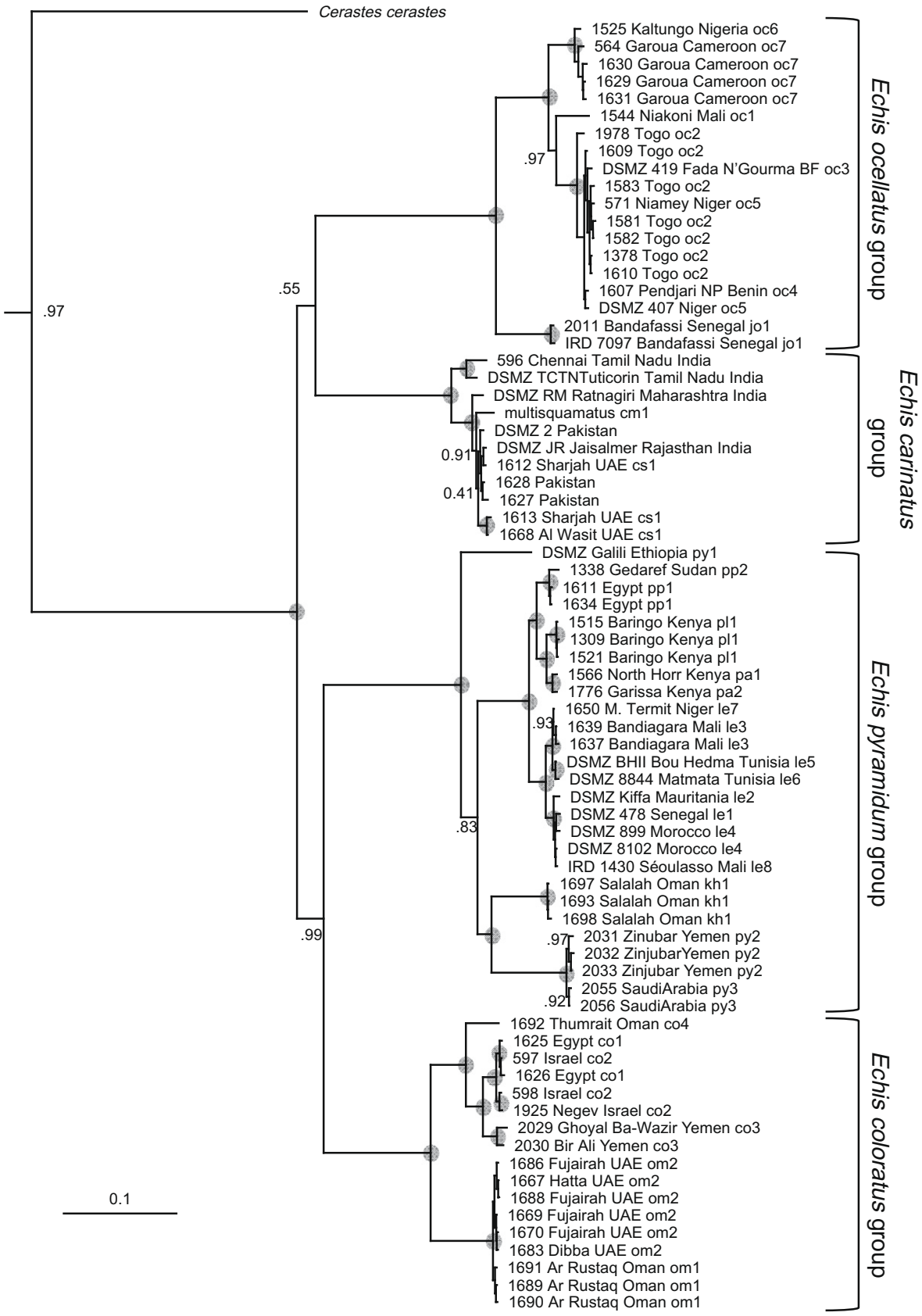

Fig. 2. Bayesian inference tree of the genus *Echis*. Phylogenetic relationships among other Caenophidian taxa included primarily for the purposes of providing calibration points for molecular dating are shown in Fig. 3. Nodes with grey circles received a Bayesian posterior probability (bpp) of 1.00; to preserve clarity, bpp values for distal nodes within haplotype clades are not shown, unless the node is discussed in the text. Locality indications refer to Fig. 1.

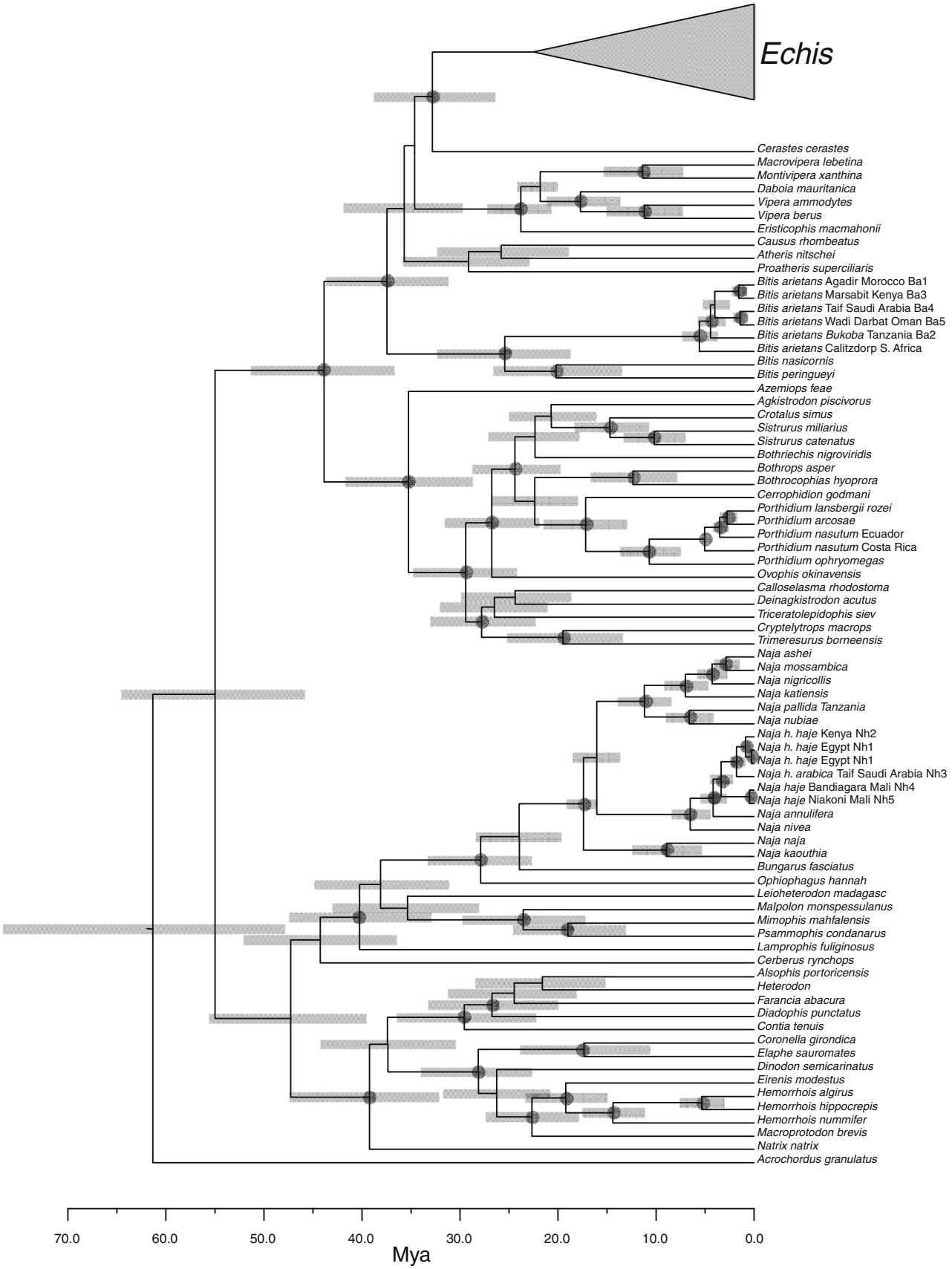

Fig. 3. BEAST maximum credibility ultrametric tree showing the timing of the evolution of the Colubroidea and the origin of the vipers. Grey bars indicate 95% confidence intervals, and dark grey circles on nodes indicates >0.95 Bayesian posterior probability support.

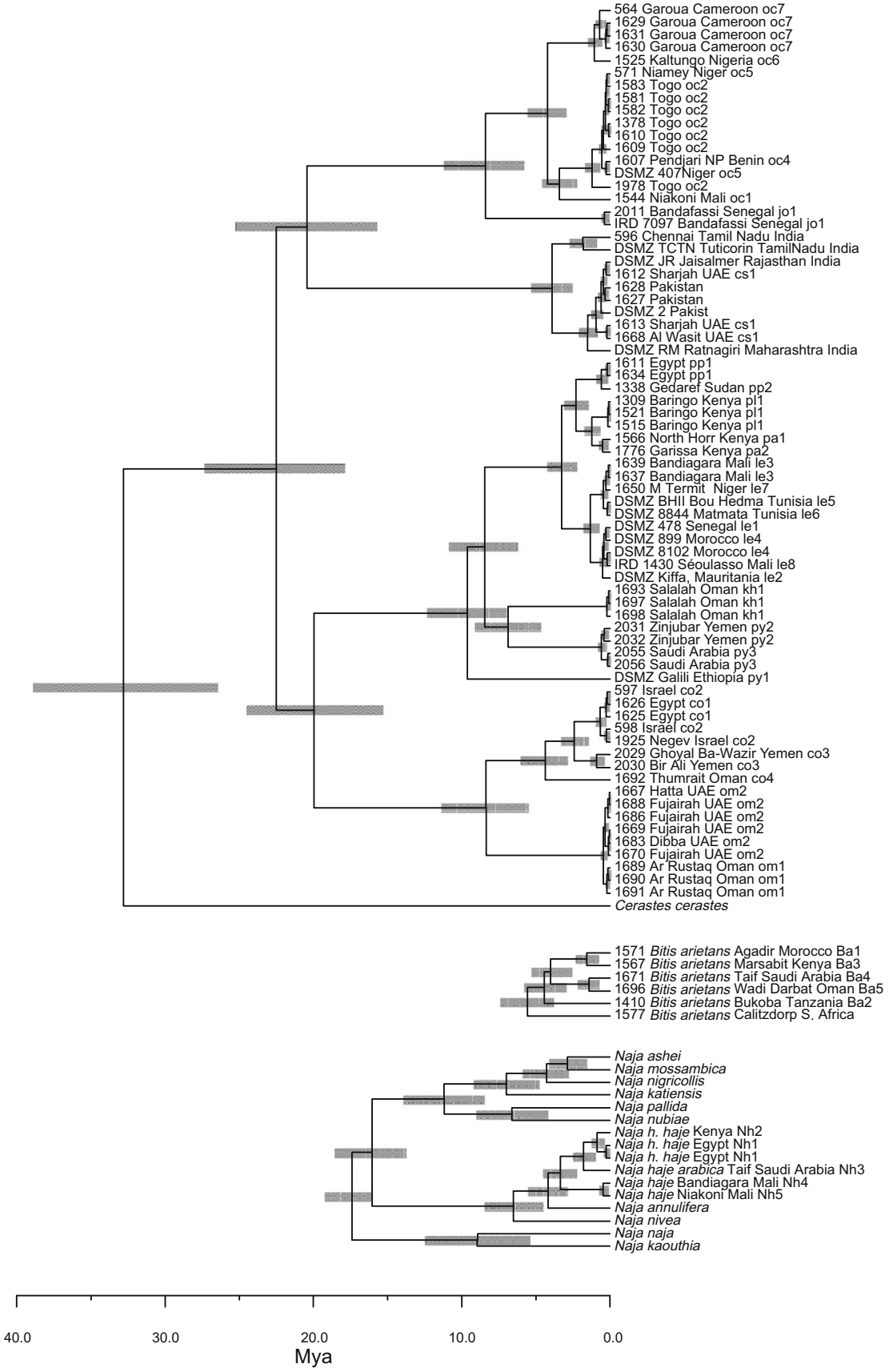

Fig. 4. BEAST maximum credibility ultrametric tree showing the timing of the evolution of *Echis*, the co-distributed viper *Bitis arietans*, and the genus *Naja*.

some *Atheris* species (Joger and Courage, 1999) must be regarded as a convergent structure to the analogous scales in *Echis* and *Cerastes*.

The final aim of any systematic revision of a genus must be the delimitation of species. The present study was based solely mitochondrial DNA sequences. There is growing consensus that species cannot be delimited based on mtDNA data alone (e.g., Moritz, 1994; Puorto et al., 2001; Sites and Marshall, 2004). Since all mitochondrial genes are inherited as a single linkage group, multiple genes cannot provide independent evidence of population history (Moore, 1995), and multiple mtDNA haplotype clades need not correspond to or indicate multiple organismal lineages when gene flow is predominantly mediated by males (Puorto et al., 2001; Irwin, 2002; Ogden and Thorpe, 2002). On the other hand, mtDNA has the important advantage in species delimitation that its smaller effective population size (*Ne*) will result in four times more rapid coalescence of the haplotypes of a given lineage, compared to nuclear markers (Moore, 1995; Wiens and Penkrot, 2002; Zink and Barrowclough, 2008). This suggests that, especially in the case of allopatric populations, where questions on the nature of contact zones between taxa are not applicable, mtDNA should be particularly valuable in revealing independently evolving historical lineages that might be much more difficult to detect with nuclear markers. Consequently, although mtDNA sequences alone cannot provide definitive evidence for or against the presence of multiple species, the presence of deep and reciprocally monophyletic haplotype clades does suggest that the presence of multiple taxa is at least a tenable working hypothesis that should then be tested with additional evidence (morphology, nuclear markers). In this study, we have used mtDNA sequences to flag likely distinct lineages, but refrain from making firm taxonomic recommendations unless there is additional published evidence of congruent morphological variation.

Our results show that all the included populations of *Echis* fall into four main species groups, the *E. carinatus*, *E. ocellatus*, *E. pyramidum* and *E. coloratus* groups. The interrelationships among the four species groups are incompletely resolved: our analyses provide significant support for the sister-group relationship between the *E. coloratus* and *E. pyramidum* groups, but the relationships between this clade and the *E. carinatus* and *E. ocellatus* groups remain unresolved. Barlow et al. (2009), using a combination of nuclear and mitochondrial genes, but only one representative for each species group, recovered the *E. carinatus* group as the sister group of all other *Echis*, and the *E. ocellatus* group as the sister group to the *E. coloratus-pyramidum* clade.

The discovery of the four species groups allows us to evaluate Cherlin's (1990) division of *Echis* into three subgenera (Table 1). Our results show clearly that Cherlin's subgenus arrangement does not reflect the phylogeny of the genus: his *Turanechis* is non-monophyletic as *E. sochureki* and *E. multisquamatus* group with *E. carinatus*, not the *E. coloratus* group, and his *Toxicoa* is non-monophyletic as the *E. pyramidum* group appears to be the sister group of the *E. coloratus* group, not the *E. ocellatus* group. In view of the conserved morphology of the genus, we do not consider the recognition of subgenera in *Echis* useful at this point.

All four main species groups contain substantial additional phylogenetic structure within them. Within each, our data can at least partly address a number of unresolved taxonomic issues (see Table 1 for our proposed taxonomy). All genetic distances refer to CYTB and NADH4 only to facilitate comparison with other studies using these genes.

4.1.1. The Echis carinatus group

The *Echis carinatus* group is the clade containing the least divergence between constituent populations. Cherlin recognized three species within this group, as defined here (Table 1), *E. carinatus* (southern India, Sri Lanka), *E. sochureki* (northern India to Pakistan, and *E. multisquamatus* (Central Asia, Iran). Auffenberg and Rehman (1991) found clinal variation across the range of the group, and considered all populations as subspecies of *E. carinatus*, and Lenk et al. (2001) found very low levels of divergence between *E. carinatus sochureki* from Pakistan and *E. multisquamatus* from Turkmenistan.

Our results indicate that the *E. carinatus* group diverged relatively recently, and that the most divergent populations occupy the south of the range of the complex (*p*-distance from northern haplotypes: 0.049). Our *E. multisquamatus* haplotype is nested among other haplotypes from northern India, Pakistan and Arabia. Given our results and the reported clinal variation in morphology, we synonymize *E. multisquamatus* Cherlin, 1981 with *E. carinatus sochureki* Stemmler, 1969. Whether the latter should be recognized as distinct from southern Indian *E. c. carinatus* at species or subspecies level or at all will require denser sampling across India and a combination of multiple molecular markers and/or further detailed morphological analyses. We suggest retaining it as a subspecies, *E. c. sochureki*, until additional evidence emerges. We were unable to include samples of the Sri Lankan *E. carinatus sinhaleyus* or the island endemic *E. c. astolae* from Pakistan, but on biogeographical grounds (both islands were linked to the nearest mainland during Pleistocene times of low sea level), it seems relatively unlikely that either would change the general picture.

The *E. carinatus* clade is notable for consisting of the ovoviviparous populations of the genus, whereas all other *Echis* appear to be oviparous (Mallow et al., 2003). This suggests that ovoviviparity may have arisen individually in the *E. carinatus* clade, but the evolution of reproductive mode is difficult to reconstruct due to the poor phylogenetic resolution among the major clades of the Viperinae (Wüster et al., 2008).

4.1.2. The Echis coloratus group

In the *E. coloratus* group, the major divergence (*p*-distance 0.087) is between *E. omanensis* and the remainder of the group. *Echis omanensis* was described on the basis of morphological differences from *E. coloratus* (Babocsay, 2004), and our data confirm its status as a long-separated lineage. Within *E. coloratus* sensu stricto, Israeli haplotypes representing populations described as *E. c. terraesanctae* by Babocsay (2003) do not cluster separately from other Israeli and Egyptian haplotypes, suggesting that this taxon does not represent a separate historical lineage. The *E. coloratus* haplotype from southern Oman represents a divergent (*p*-distance 0.043) sister group to all other *E. coloratus*. Whether further distinctive lineages can be defined and recognized within *E. coloratus* will only be answerable through denser morphological and molecular analysis of the species' large Arabian range.

4.1.3. The Echis ocellatus group

The most divergent haplotypes in the *E. ocellatus* group originate from eastern Senegal. Trape and Mané (2006) considered these populations as representing the taxon *Echis jogeri* Cherlin, 1990, based on their very low ventral scale counts. They interpreted this as representing the extreme end of an apparent east-west cline in ventral scale counts within *E. ocellatus* (Hughes, 1976), and therefore synonymized *E. jogeri* with *E. ocellatus*. However, there has been no detailed investigation of finer patterns of morphological variation in *E. ocellatus*. The very deep divergence (*p*-distance 0.099) revealed here suggests that the western populations with low ventral scale counts from western Mali, Senegal and northern Guinea may represent a separate historical lineage within the *E. ocellatus* group. Pending more fine-grained analyses of the group, we consider recognition of the westernmost populations of the complex as a separate species, *E. jogeri*, the most plausible working hypothesis. We agree with Trape and Mané (2006) that

the stated type locality of *E. jogeri* (Timbuktu, Mali) is probably in error, since the distribution of the *E. ocellatus* complex is largely restricted to the Sudan and Guinea savanna of West Africa (Trape and Mané, 2006), thus excluding the much more xeric region of Timbuktu.

The remainder of the *E. ocellatus* complex shows considerable additional phylogeographic structure, with three phylogroups (*p*-distances 0.054–0.065) being apparent, consisting, from west to east, of a single haplotype from southern Mali, a number of haplotypes from eastern Burkina Faso, Togo, Benin and western Niger, and a haplotype clade from eastern Nigeria and adjoining northern Cameroon. Given the variation in the morphology of *E. ocellatus* across its range (Hughes, 1976), the possibility that these may represent additional organismal lineages within the group cannot be excluded. Resolution of this problem will require fine-grained additional sampling and concurrent usage of multiple independent morphological and molecular markers, but is of considerable relevance due to the medical importance of the *E. ocellatus* complex as the major cause of snakebite mortality and morbidity in West Africa (Warrell and Arnett, 1976; Warrell, 1995).

4.1.4. The Echis pyramidum group

The *E. pyramidum* group is the most complex of the four main species groups, both in terms of phylogeographic pattern as well as in terms of taxonomic history. We were unable to include specimens unquestionably attributable to Cherlin's (1990) species *E. hughesi* (northeastern Somalia) and *E. megalocephalus* (Eritrea). These were considered to be potentially conspecific with *E. pyramidum* by Nilson (in McDiarmid et al., 1999), and, based on morphology (Pook et al., unpublished data), we regard them as part of this complex. In addition to these two species, Cherlin (1990) recognized four further species with six subspecies in this group: *E. pyramidum* from Egypt, Libya, Tunisia and northwestern Kenya only; *E. arenicola* (including *E. leucogaster* as a ssp.) from the western part of Saharan and Sahelian Africa; *E. khosatzkii* from eastern Yemen and southern Oman; and *E. varius* (emended from *E. varia*: Schätti and Gasperetti, 1994) from Ethiopia, the Sudanese Red Sea coast and southwestern Arabia as well as northeastern Kenya.

Schätti and Gasperetti (1994) criticized Cherlin's work, noting that *E. arenicola* is a synonym of *E. pyramidum*, and did not consider *E. varius* a separate species from *E. pyramidum*. Similarly, Mazuch (2005) questioned the distinctness of *E. pyramidum leakeyi* and *E. p. aliaborri*, assigned by Cherlin (1990) to *E. pyramidum* and *E. varius*, respectively.

Our results indicate extensive genetic variation in the *E. pyramidum* group. The Arabian haplotypes form a strongly supported monophyletic group that differs profoundly from its African sister clade (*p*-distance 0.102). The Arabian clade contains two highly divergent (*p*-distance 0.094) haplotype groups: one from southern Oman, the other southwestern Yemen and southwestern Saudi Arabia. Arnold (1980), Cherlin (1990) and Schätti and Gasperetti (1994) noted extensive morphological variation in the Arabian populations of the *E. pyramidum* group, especially between the easternmost populations (Hadramaut and southern Oman) and those from western Yemen and southwestern Saudi Arabia. The considerable sequence divergence between these two forms and between them and their African sister group suggest that these represent two independently evolving, endemic Arabian species: *Echis khosatzkii* (Cherlin, 1990) from southern Oman and eastern Yemen, and *Echis borkini* (Cherlin, 1990) from western Yemen and southwestern Saudi Arabia. The precise distribution limits of the two forms, particularly in southern Yemen, require further study.

Within mainland Africa, our results revealed a single, highly divergent haplotype from Ethiopia, which appears to form the sister group to the rest of the *E. pyramidum* group (*p*-distance 0.103). The name *Echis varius* Reuss, 1834 may be applicable to this form, but further evidence from morphology and inspection of type material will be required to confirm this. Within the rest of the *E. pyramidum* complex, haplotypes assignable to *E. leucogaster* and *E. pyramidum* form sister clades. However, divergence levels between the two are relatively low (*p*-distance 0.045). This is consistent with the hypothesis that *E. leucogaster* represents an independently evolving lineage, but additional evidence from denser sampling, especially parts of Central Africa such as Chad and Sudan, will be needed to fully resolve the status of this taxon.

Within *E. pyramidum* sensu stricto, specimens from Kenya form the sister clade to those from Egypt and eastern Sudan. Our results do not support the existence of two sympatric species (*E. pyramidum* and *E. varius*) in much of eastern Africa, as proposed by Cherlin: our samples from Gedaref, Sudan, and northeastern Kenya (Garissa and possibly North Horr) would be attributable to *E. v. varius* and *E .v. aliaborri*, respectively, under Cherlin's classification, whereas material from Egypt and Baringo would be attributable to *E. p. pyramidum* and *E. p. leakeyi*. Our results show all haplotypes involved to form a tightly knit clade with low divergences (*p*-distance 0.033 between Kenyan and Egyptian samples), giving little indication of multiple species being present. We therefore consider them as part of the single species *E. pyramidum*.

The presence of a highly divergent Ethiopian haplotype of unclear affinities in a single specimen indicates that our study may not have revealed all lineages within the *E. pyramidum* complex. The clustering of multiple highly divergent mtDNA lineages around the southern Red Sea and Gulf of Aden and the recognition of the apparently morphologically distinct species *E. hughesi* and *E. megalocephalus* from the same region (Cherlin, 1990) suggest that considerable further diversity in the *E. pyramidum* complex may yet be identified in the Gulf of Aden-Red Sea area, and emphasizes the key importance of this region in the evolution and diversification of the *E. pyramidum* complex.

4.2. Phylogeny of Echis and venom variation

Our results provide an evolutionary and phylogenetic background to the extensive variability of venom composition and lack of antivenom cross-neutralization in this genus. Even though *Echis* is so morphologically conserved that most populations were considered conspecific until the 1970s, it is in fact an old genus, dating back to an initial divergence approximately 22 Mya. Consequently, the four major lineages have had ample time to accumulate differences in venom composition. In addition, the evolution of different diet composition in the major lineages provides a basis for selection for adaptive differences in venom composition (Barlow et al., 2009).

Most documented cases of lack of antivenom cross-neutralization concern the use of *E. carinatus* antivenom to treat bites by *E. ocellatus* in W. Africa (Warrell and Arnett, 1976; Visser et al., 2008; Warrell, 2008), but envenoming by a Tunisian *E. leucogaster* failed to respond to a number of antivenoms, including some apparently raised against the same or closely related species (Gillissen et al., 1994). Nevertheless, we suggest that our results could potentially serve as a "phylogenetic road map" to guide the search for candidate antivenoms for different species of *Echis*, or for the initial selection of antivenoms for the treatment of patients bitten by species of *Echis* for which specific antivenom is unavailable.

4.3. Molecular dating and the historical biogeography of Echis

Our data provide a new perspective on the historical biogeography of the saw-scaled vipers. *Echis* is of particular biogeographical interest due to its distribution across a tectonically active zone that contains both the collision zone between Afro-Arabia and Eurasia,

and also the active rifting zone causing the splitting of the Arabian Peninsula from Africa through the opening of the Red Sea. The early cladogenic events within *Echis* that gave rise to the four species groups took place at the beginning of the Miocene, around 20 Mya, and coincide with major tectonic activity at the time. Africa and the Arabian Peninsula, which had been isolated from Eurasia by the Tethys Sea since the Mesozoic, are thought to have collided with the Anatolian plate approximately 19 Mya (Rögl, 1999; Amer and Kumazawa, 2005; Harzhauser et al., 2007), although some authors have argued for a much earlier start to the collision (Allen and Armstrong, 2008). The resulting *Gomphotherium* landbridge facilitated range expansion and divergence of numerous taxa across Afro-Asia. At the same time, the progressive anticlockwise rotation of the Arabian Peninsula and associated formation of the Red Sea and Gulf of Aden, and the formation of the East African Rift system, also affected organismal distributions (Chorowicz, 2005). Reptile genera with broad Afro-Asian distributions are thus fitting subjects for historical biogeographic studies of the region.

Our reconstruction of the historical biogeography of the genus *Echis* suffers from the problem that the geographical origin of the genus is insufficiently understood, and that the data presented here provide inadequate resolution of the basal splits in the genus. Wüster et al. (2008) inferred an African origin for the Viperinae, and also for the genus *Echis*. However, the weak resolution of the basal relationships among the Viperinae makes those inferences tentative. The increased support for a sister-group relationship between *Echis* and *Cerastes* found here strengthens the hypothesis of an African origin, as the latter genus has a principally Afro-Arabian distribution, and the Arabian species *C. gasperettii* is nested among the African species *C. cerastes* and *C. vipera* (Lenk et al., 2001). Within *Echis*, the addition of a nuclear locus provided evidence that *E. carinatus* represents the sister group of all other *Echis* (Barlow et al., 2009).

The close succession of the basal splits in *Echis*, coupled with the present-day distribution of the four species groups, suggests that a scenario of rapid range expansion following the Afro-Arabian – Eurasian collision, followed by later vicariance may be most likely. Under this scenario, the *E. carinatus* group became isolated in Asia approximately 20 Mya. Considering the confidence intervals around our dating estimates, this is suggestive of an origin during the time of the *Gomphotherium* landbridge (Harzhauser et al., 2007), but also consistent with suggested faunal exchanges around the Oligocene–Miocene boundary (Kappelman et al., 2003; Harzhauser et al., 2007). Subsequent mid-Miocene marine connections between the Mediterranean Sea, Indian Ocean and the Paratethys (Rögl, 1999), separating the Indian subcontinent from Afro-Arabia, may have helped maintain the long-standing isolation of the *E. carinatus* group in southern Asia.

The distributions of the *E. coloratus* and *E. pyramidum* complexes extend primarily along the opposing sides of the Red Sea and the Gulf of Aden. The age of the split between these two sister groups is estimated at approximately 19.4 Mya (Fig. 4) in our analysis. This corresponds to or immediately follows the beginning of rifting in the Gulf of Aden and the southernmost Red Sea in the Oligocene, and the northward propagation of the rifting to the northern end of the Red Sea by the early Miocene (Hughes et al., 1991; Bosworth et al., 2005; Chorowicz, 2005). It thus seems likely that this rifting acted as a vicariance event separating these two clades on different sides of the opening Red Sea.

The early history of the genus *Echis* in relation to the collision between Afro-Arabia and Eurasia has echoes in other groups of reptiles. Amer and Kumazawa (2005) calculated a divergence time of approximately 25–29 Mya between Asian and African *Uromastyx*, which would fit in better with an early landbridge around the Oligocene–Miocene boundary. In the Elapid genus *Naja*, fossil evidence indicates that the Asian clade had split from the African clade by approximately 16 Mya (Szyndlar and Rage, 1990), consistent with dispersal from Africa after the uplift of the *Gomphotherium* landbridge (Wüster et al., 2007). The molecular dating presented here estimates the split between Asian *Naja* and their sister group at approximately 17.4 Mya. This is younger than estimated by Wüster et al. (2007), but the wide confidence intervals in that study are not inconsistent with differentiation following dispersal over the *Gomphotherium* landbridge. The divergence between African and Arabian clades of *Uromastyx* was dated at approximately 15–12 Mya by Amer and Kumazawa (2005), rather later than our estimate of the *E. coloratus–pyramidum* split, but Joger (1986) dated the *Uromastyx* split at about 18 Mya, based on an immunological 'albumin clock'.

The split between Arabian and African sister clades of the *E. pyramidum* complex appears to date back to a single event approximately 8 Mya, followed by further diversification within Arabia approximately 6.6 Mya (Fig. 4). However, the paucity of sampling along the Red Sea coast of Sudan, Eritrea and Somalia leaves open the possibility that additional haplotype lineages may be found that alter this emerging picture of the origins of the Arabian species of the *E. pyramidum*. The present date estimate corresponds to the inferred age of the trans-Red Sea sister-group relationship between *Uromastyx ornata* and *U. ocellata* (Amer and Kumazawa, 2005). This time period in the late Miocene corresponds to the deposition of evaporites in the northern Red Sea (Orszag-Sperber et al., 1994, 2001; Bosworth et al., 2005), which indicate the isolation of the northern Red Sea from the Indian Ocean, and thus a land connection between Arabia and Africa. The separation between the Arabian *E. khosatzkii–E. borkini* clade and its African sister clade may thus be the result of vicariance due to the severing of that landbridge. The stronger affinity of the *E. coloratus* group for rocky habitats may have prevented a reciprocal crossing from Arabia to Africa; it most likely reached Egypt by a northern route through Sinai.

Interestingly, the ages of African–Arabian splits in other co-distributed snake species complexes included in this study differ sharply (Fig. 4). Whereas African and Arabian *E. pyramidum*-group populations diverged approximately 8 Mya, our dating estimates suggest that the corresponding date in *Bitis arietans* is approximately 4.0 Mya, and that between African and Arabian *N. haje* only 1.75 Mya. The confidence intervals for the three groups do not overlap, suggesting that these events are indeed the result of dispersal at different times.

Before molecular clock data were available, the presence of afrotropical species on both sides of the southern Red Sea (but not at its northern end) had been interpreted as a result of a direct land connection across the Bab El Mandeb during periods of Pleistocene sea level lowering (Joger, 1987; Winney et al., 2004). However, the available sedimentary record suggests an absence of post-Miocene land connections across the southern Red Sea (Fernandes et al., 2006), so that any later dispersal would have had to involve either overwater dispersal or dispersal around the northern end of the Red Sea. However, the Strait of Bab El Mandeb is believed to have been greatly narrowed during times of lowered eustatic sea levels corresponding to high latitude glaciations at several points during the last 500,000 years (Rohling et al., 1998; Siddall et al., 2003; Fernandes et al., 2006), which may have facilitated dispersal. The same phenomenon has also been recorded from pre-Pleistocene periods, particularly the Piacenzian (Pliocene; 3.6–2.6 Mya; Haq et al., 1987).

Our divergence date estimates thus suggest that the Afro-Arabian distribution of the *E. pyramidum* complex is most likely the result of vicariance due to the last late Miocene sea level rises. In contrast, *B. arietans* appears to have achieved its distribution

on both sides of the Red Sea after the end of the last land connection across the southern Red Sea, but this may have been assisted by a narrowing of the connecting passage in the Pliocene. Finally, *N. haje* may have dispersed from Egypt, where the species currently occurs along the Mediterranean coast and the Nile Valley, along the Red Sea coast to southern Arabia, followed by later extinction northwestern Arabia. Our results thus demonstrate considerable variation in the ages and likely routes of dispersal in the face of similar geological changes, a pattern that has been found in other regions and snake species complexes as well (e.g., Wüster et al., 2005a; Williams et al., 2008). The presence of *E. megalocephalus* on the Dahlak archipelago can be explained by the shallow sea depth between Eritrea and the archipelago (about 100 m). It was probably connected to the mainland during Pleistocene low sea level stages which reached 120 m below present (Fairbanks, 1989).

At approximately the same time when the *E. carinatus*, *E. pyramidum* and *E. coloratus* groups diverged in connection with tectonic events associated with the East African Rift and the Afro-Arabia–Eurasia collision, the *E. ocellatus* group diverged from other *Echis* in what are now the savanna regions of West Africa. The reasons for this divergence remain poorly understood. A number of other African reptiles occupy similar ranges restricted to the savannas of West Africa, yet there are few obvious distribution barriers that would impede continuity with the open formations of eastern Africa. It seems likely that the ancestors of the *E .ocellatus* and *E. pyramidum* groups (now both present in West Africa) differentiated in separate regions, West and East Africa, respectively. This is supported by the restricted distribution of the *E. ocellatus* group, which remains confined to the savanna regions of West Africa, and the East African/Arabian origin of all basal haplotypes in the *E. pyramidum* group. A possible barrier between them could have been Lake Chad with its associated wetlands, the extension of which had been much greater before the aridification of the Sahara (Griffin, 2006). After the Pliocene shrinking of the Lake, *E. leucogaster* may have been able to colonize the northern part of West Africa, while *E. ocellatus* remained in the southern part. The core area of the *E. ocellatus* group is a rocky area of relatively high endemism in western Mali, eastern Senegal and northern Guinea. Other endemic reptiles of this area are *Agama weidholzi* and *A. sankaranica* (Agamidae), *Hemitheconyx caudicinctus* (Eublepharidae), *Tarentola parvicarinata* (Gekkonidae), *Chalcides thierryi* (Scincidae), *Bamanophis dorri* (Colubridae) and *Naja katiensis* (Elapidae) (see Joger and Lambert, 1997).

However, our molecular dating of co-distributed taxa does not indicate a common pattern in the timing of the divergence of endemic West African savanna species: whereas *Echis ocellatus* diverged from its sister clade approximately 20 Mya, the co-distributed *Naja katiensis* diverged approximately 7 Mya (at the lower end of the confidence interval estimated for this event by Wüster et al., 2007), and in *Naja haje*, a divergent West African haplotype clade diverged from its sister group approximately 3.5 Mya (Fig. 4). This clearly indicates that the divergence of these Western African savanna endemics happened at different times, and that a single common cause cannot be invoked for these shared distribution patterns. Moreover, the deep divergences observed within the *E. ocellatus* group suggest that there may have been one or several subdivisions of the West African savanna biome over the last 8 million years.

The second *Echis* clade occurring in western Africa is the *E. pyramidum* group. All basal haplotype lineages in this clade occur around the Red Sea and the Gulf of Aden, whereas only a single, more recently diverged haplotype clade, represented by the species *E. leucogaster*, occurs in the western three quarters of the group's range. This species diverged from *E. pyramidum* in the Pliocene, approximately 3.2 Mya. This suggests that the *E. pyramidum* clade originated in eastern Africa, and that the occupancy of open and xeric parts of western Africa is the result of a relatively recent westward range expansion, coinciding most likely with aridification and the spread of open vegetational formations across Africa as a result of the onset of climatic fluctuations in the Pliocene (Shackleton et al., 1984), as well as the shrinking of the Neogene Lake Chad (Griffin, 2006).

In the *Echis carinatus* group, our results show that southern haplotypes form the sister group to successively more exclusive northern groupings. Although additional sampling is required, this pattern is most suggestive of a present-day distribution resulting from a northward range expansion from Peninsular India. There is little obvious phylogeographic structure and only low divergence (maximum *p*-distance in CYTB and NADH4 is 0.025) between northwestern India, Pakistan, northeastern Arabia and Central Asia. Our analyses suggest that these haplotypes first diverged approximately 0.9 Mya. The lack of divergence between Arabian and Asian populations of *E. c. sochureki* is unsurprising, as the shallow Persian Gulf was entirely exposed during times of lowered sea level during Quaternary glacial maxima (Lambeck, 1996).

Within the *Echis coloratus* group, the main lineage splitting event was the divergence between *E. coloratus* and *E. omanensis* approximately 8.1 Mya. The distributions of these two primarily rock-dwelling species are separated by a flat, low-lying expanse of primarily sandy soils stretching from the coast of eastern Oman into the Rub Al Khali. The herpetofauna of southern Oman is generally more similar to the Yemeni fauna than to the fauna of northern Oman. Only 35 of 86 reptile species present in southwestern Arabia (including southern Oman) are also found in northern and eastern Oman (Joger, 1987). Southern Oman is inhabited by a number of afrotropical elements (including *E. pyramidum* group members, *N. haje* and *B. arietans*), whereas eastern Oman has palearctic and oriental relicts (such as *E. carinatus* and *Pseudocerastes persicus*). Thus a fundamental biogeographical and ecological barrier must have separated these two regions for a long time, and our time estimate for the two members of the *E. coloratus* group gives a first indication of the possible duration of this barrier.

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Appendix A

See Appendix.

Appendix I

Sample information for the *Echis* and other novel sequences used in the phylogenetic analysis in this paper. Data for additional taxa not listed here are given in Wüster et al. (2008). UAE, United Arab Emirates; Collections: DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; HLMD, Hessisches Landesmuseum Darmstadt; IRD, Institut de Recherche pour le Développement, Dakar; SNHM, Staatliches Naturhistorisches Museum, Braunschweig; WW, W. Wüster, personal collection.

Sample/voucher	Species	Locality (code as in Fig. 1, 2 and 4)	GenBank Accession No. CYTB/NADH4/12S/16S
DSMZ 407	*Echis ocellatus*	Between Niamey and Tapoa, Niger (oc5)	GQ359431/GQ359520/GQ359599/GQ359681
DSMZ 419	*Echis ocellatus*	Fada-N'Gourma, Burkina Faso (oc3)	GQ359432/–/GQ359600/–
DSMZ JR	*Echis carinatus sochureki*	Jaisalmer, Rajasthan, India	GQ359434/GQ359522/GQ359602/GQ359683
DSMZ TCTN	*Echis carinatus carinatus*	Tuticorin, Tamil Nadu, India	GQ359435/GQ359523/GQ359603/GQ359684
DSMZ RM	*Echis carinatus carinatus*	Ratnagiri, Maharashtra, India	GQ359439/GQ359527/GQ359607/GQ359688
*Coll. Göran Nilson., no number	*Echis carinatus multisquamatus*	Turkmenistan (cm1)	AJ275702/–/–/AJ275763
SNHM N 40742	*Echis* cf. *pyramidum*	Galili, Afar triangle, Ethiopia (py1)	GQ359448/GQ359533/GQ359614/GQ359697
DSMZ 478	*Echis leucogaster*	Between Kidira and St Louis, Senegal (le1)	GQ359455/GQ359540/GQ359621/GQ359704
SNHM 40745	*Echis leucogaster*	Bou Hedma, Tunisia (le5)	GQ359456/GQ359541/GQ359622/GQ359705
DSMZ 8102	*Echis leucogaster*	Allougoum, Morocco (le4)	GQ359458/GQ359543/GQ359624/GQ359707
DSMZ 8844	*Echis leucogaster*	Matmata, Tunisia (le6)	GQ359459/GQ359544/GQ359625/GQ359708
DSMZ 899	*Echis leucogaster*	Morocco (le4)	GQ359457/GQ359542/GQ359623/GQ359706
SNHM 40743	*Echis leucogaster*	Kiffa, Mauritania (le2)	GQ359460/GQ359545/GQ359626/GQ359709
*DSMZ 2	*Echis carinatus sochureki*	Pakistan	GQ359441/GQ359528/GQ359608/GQ359690
WW 564	*Echis ocellatus*	Garoua, Cameroon (oc7)	GQ359418/GQ359508/GQ359587/GQ359668
WW 571	*Echis ocellatus*	Niamey, Niger (oc5)	GQ359419/GQ359509/GQ359588/GQ359669
WW 596	*Echis carinatus carinatus*	Chennai, Tamil Nadu, India	GQ359433/GQ359521/GQ359601/GQ359682
WW 597	*Echis coloratus*	Israel	GQ359461/EU624224/EU624256/GQ359710
WW 598	*Echis coloratus*	Israel	GQ359462/GQ359546/GQ359627/GQ359711
WW 1309	*Echis pyramidum leakeyi*	Baringo, Kenya (pl1)	GQ359445/GQ359530/GQ359611/GQ359694
WW 1338	*Echis pyramidum pyramidum*	Gedaref, Sudan (pp2)	GQ359444/–/GQ359610/GQ359693
WW 1378	*Echis ocellatus*	Togo (oc2)	GQ359421/EU624225/EU624257/GQ359671
WW 1515	*Echis pyramidum leakeyi*	Baringo, Kenya (pl1)	GQ359446/GQ359531/GQ359612/GQ359695
WW 1521	*Echis pyramidum leakeyi*	Baringo, Kenya (pl1)	EU852296/EU852302/EU852314/EU852320
WW 1525	*Echis ocellatus*	Kaltungo, Nigeria (oc6)	GQ359420/GQ359510/GQ359589/GQ359670
WW 1544	*Echis ocellatus*	Niakoni, Mali (oc1)	GQ359427/GQ359516/GQ359595/GQ359677
WW 1566	*Echis pyramidum aliaborri*	North Horr, Kenya (pa1)	GQ359447/GQ359532/GQ359613/GQ359696
WW 1581	*Echis ocellatus*	Togo (oc2)	GQ359422/GQ359511/GQ359590/GQ359672
WW 1582	*Echis ocellatus*	Togo (oc2)	GQ359423/GQ359512/GQ359591/GQ359673
WW 1583	*Echis ocellatus*	Togo (oc2)	GQ359424/GQ359513/GQ359592/GQ359674
WW 1607	*Echis ocellatus*	Pendjari National Park, Benin (oc4)	GQ359430/GQ359519/GQ359598/GQ359680
WW 1609	*Echis ocellatus*	Togo (oc2)	GQ359425/GQ359514/GQ359593/GQ359675
WW 1610	*Echis ocellatus*	Togo (oc2)	GQ359426/GQ359515/GQ359594/GQ359676
WW 1611	*Echis pyramidum pyramidum*	Egypt (pp1)	GQ359442/EU624226/EU624258/GQ359691
WW 1612	*Echis carinatus sochureki*	Sharjah, UAE (cs1)	GQ359436/GQ359524/GQ359604/GQ359685
WW 1613	*Echis carinatus sochureki*	Sharjah, UAE (cs1)	GQ359437/GQ359525/GQ359605/GQ359686
WW 1625	*Echis coloratus*	Sinai, Egypt (co1)	GQ359463/GQ359547/GQ359628/GQ359712
WW 1626	*Echis coloratus*	Sinai, Egypt (co1)	GQ359464/GQ359548/GQ359629/GQ359713
WW 1627	*Echis carinatus sochureki*	Pakistan	GQ359440/EU624223/EU624255/GQ359689
WW 1628	*Echis carinatus sochureki*	Pakistan	GQ359438/GQ359526/GQ359606/GQ359687
WW 1629	*Echis ocellatus*	Garoua, Cameroon (oc7)	EU852294/EU852300/EU852312/EU852318
WW 1630	*Echis ocellatus*	Garoua, Cameroon (oc7)	GQ359428/GQ359517/GQ359596/GQ359678
WW 1631	*Echis ocellatus*	Garoua, Cameroon (oc7)	GQ359429/GQ359518/GQ359597/GQ359679
WW 1634	*Echis pyramidum pyramidum*	Egypt (pp1)	GQ359443/GQ359529/GQ359609/GQ359692
WW 1637	*Echis leucogaster*	Bandiagara, Mali (le3)	GQ359454/GQ359539/GQ359620/GQ359703
WW 1639	*Echis leucogaster*	Bandiagara, Mali (le3)	GQ359452/GQ359537/GQ359618/GQ359701
WW 1650	*Echis leucogaster*	Massif de Termit, Niger (le7)	GQ359453/GQ359538/GQ359619/GQ359702
WW 1667	*Echis omanensis*	Hatta, UAE (om2)	GQ359466/GQ359550/GQ359631/GQ359715
WW 1668	*Echis carinatus sochureki*	Al Wasit, Sharjah, UAE (cs1)	EU852295/EU852301/EU852313/EU852319
WW 1669	*Echis omanensis*	Fujairah, UAE (om2)	GQ359467/GQ359551/GQ359632/GQ359716
WW 1670	*Echis omanensis*	Fujairah, UAE (om2)	GQ359468/GQ359552/GQ359633/GQ359717
WW 1683	*Echis omanensis*	Dibba, UAE (om2)	GQ359469/GQ359553/GQ359634/GQ359718
WW 1686	*Echis omanensis*	Fujairah, UAE (om2)	GQ359470/GQ359554/GQ359635/GQ359719
WW 1688	*Echis omanensis*	Fujairah, UAE (om2)	GQ359471/GQ359555/GQ359636/GQ359720
WW 1689	*Echis omanensis*	Ar Rustaq, Oman (om1)	GQ359472/GQ359556/GQ359637/GQ359721
WW 1690	*Echis omanensis*	Ar Rustaq, Oman (om1)	GQ359473/GQ359557/GQ359638/GQ359722
WW 1691	*Echis omanensis*	Ar Rustaq, Oman (om1)	GQ359474/GQ359558/GQ359639/GQ359723
WW 1692	*Echis coloratus*	Thumrait, Oman	GQ359465/GQ359549/GQ359630/GQ359714
WW 1693	*Echis khosatzkii*	Near Salalah, Oman (kh1)	GQ359449/GQ359534/GQ359615/GQ359698
WW 1697	*Echis khosatzkii*	Near Salalah, Oman (kh1)	GQ359450/GQ359535/GQ359616/GQ359699
WW 1698	*Echis khosatzkii*	Near Salalah, Oman (kh1)	GQ359451/GQ359536/GQ359617/GQ359700
WW 1776	*Echis pyramidum aliaborri*	Garissa, Kenya (pa2)	GQ359475/GQ359559/GQ359640/GQ359724
WW 1925	*Echis coloratus*	Negev, Nahal Paran, Israel (co2)	EU852297/EU852303/EU852315/EU852321
WW 1998	*Echis ocellatus*	Togo (oc2)	GQ359482/GQ359566/GQ359647/GQ359731
IRD TR 7097	*Echis jogeri*	Bandafassi, Senegal (jo1)	GQ359483/GQ359567/GQ359648/GQ359732
WW 2011	*Echis jogeri*	Bandafassi, Senegal (jo1)	GQ359476/GQ359560/GQ359641/GQ359725
IRD TR 1430	*Echis leucogaster*	Séoulasso, Mali (le8)	GQ359484/GQ359568/GQ359649/GQ359733

Appendix I (*continued*)

Sample/voucher	Species	Locality (code as in Fig. 1, 2 and 4)	GenBank Accession No. CYTB/NADH4/12S/16S
WW 2029	*Echis coloratus*	Ghoyal Ba-Wazir, Yemen (co3)	GQ359477/GQ359561/GQ359642/GQ359726
WW 2030	*Echis coloratus*	Bir Ali, Yemen (co3)	GQ359478/GQ359562/GQ359643/GQ359727
WW 2031	*Echis* cf. *pyramidum*	Zinjubar, Yemen (py2)	GQ359479/GQ359563/GQ359644/GQ359728
WW 2032	*Echis* cf. *pyramidum*	Zinjubar, Yemen (py2)	GQ359480/GQ359564/GQ359645/GQ359729
WW 2033	*Echis* cf. *pyramidum*	Zinjubar, Yemen (py2)	GQ359481/GQ359565/GQ359646/GQ359730
WW 2055	*Echis* cf. *pyramidum*	Saudi Arabia (py3)	GQ359485/GQ359569/GQ359650/GQ359734
WW 2056	*Echis* cf. *pyramidum*	Saudi Arabia (py3)	GQ359486/GQ359570/GQ359651/GQ359735
WW 1567	*Bitis arietans somalica*	Marsabit, Kenya (Ba3)	GQ359490/EU624213/EU624245/GQ359739
WW 1571	*Bitis arietans arietans*	Agadir, Morocco (Ba1)	EU852298/EU852304/EU852316/EU852322
WW 1577	*Bitis arietans arietans*	Calitzdorp, Western Cape, South Africa	GQ359487/GQ359571/GQ359652/GQ359736
WW 1671	*Bitis arietans arietans*	Taif, Saudi Arabia (Ba4)	GQ359488/GQ359572/GQ359653/GQ359737
WW 1696	*Bitis arietans arietans*	Wadi Darbat, Dhofar, Oman (Ba5)	GQ359489/GQ359573/GQ359654/GQ359738
WW 1410	*Bitis arietans arietans*	Bukoba, Tanzania (Ba2)	GQ359491/GQ359574/GQ359655/GQ359740
WW 1430	*Naja ashei*	Watamu, Kenya	GQ359493/GQ359575/GQ359656/GQ359742
WW 1540	*Naja katiensis*	Doussoudiana, Mali	GQ359494/GQ359576/GQ359657/GQ359743
WW 191	*Naja mossambica*	Marromeu, Moçambique	GQ359495/GQ359577/GQ359658/GQ359744
WW 1080	*Naja pallida*	Tanzania	GQ359496/GQ359578/GQ359659/GQ359745
WW 837	*Naja nubiae*	unknown	GQ359497/GQ359579/GQ359660/GQ359746
WW 1263	*Naja haje haje*	Naivasha, Kenya (Nh2)	GQ359498/GQ359580/GQ359661/GQ359747
WW 1078	*Naja haje haje*	Egypt (Nh1)	GQ359499/GQ359581/GQ359662/GQ359748
WW 1681	*Naja haje arabica*	Taif, Saudi Arabia (Nh3)	GQ359500/GQ359582/GQ359663/GQ359749
WW 893	*Naja haje haje*	Egypt (Nh1)	GQ359501/GQ359583/GQ359664/GQ359750
WW 1079	*Naja haje haje*	Bandiagara, Mali (Nh4)	GQ359502/GQ359584/GQ359665/GQ359751
WW 1542	*Naja haje haje*	Niakoni, Mali (Nh5)	GQ359503/GQ359585/GQ359666/GQ359752
WW 881	*Naja annulifera*	Bulawayo, Zimbabwe	GQ359504/GQ359586/GQ359667/GQ359753
WW 1074	*Naja nigricollis*	Lara, Kaélé, Cameroon	GQ359505/AY713377/EU624237/GQ359754
WW 595	*Naja naja*	Nepal	GQ359506/AY713378/EU624236/GQ359756
WW 585	*Naja kaouthia*	Chumphon Province, Thailand	GQ359507/EU624209/EU624235/GQ359757
Unknown (NADH, CYTB)/ South Africa (16S, 12S)	*Naja nivea*		AF217827/AY058983/EU624238/GQ359755
WW 1640	*Cerastes cerastes*	Egypt	EU852299/EU852305/EU852317/EU852323
WW1313	*Vipera ammodytes*		DQ186520/EU624232/EU624266/EU624297
Unknown (CYTB)/HLMD RA-1665 (16S)/WW199 NADH4, 12S)	*Vipera berus*	France (CYTB)/Sweden (16s)/Anglesey, UK (NADH4, 12s)	AY321091/EU624233/EU624267/AJ275772
1318	*Bothrops asper*	Siquirres, Limón, Costa Rica	GQ359492/EU624210/EU624239/GQ372868
WW 1097 (NADH4, cyt*b*)/ WW1312 (16S, 12S)	*Crotalus simus*	Guanacaste Province, Costa Rica	AY704835/AY704885/EU624240/GQ372869
WW 787	*Porthidium lansbergii rozei*	San Antonio, Falcón, Venezuela	AY713375/AF393623/EU624242/GQ372870
WW 750	*Porthdium arcosae*	Salango, Manabí, Ecuador	AF292575/AF292613/EU624241/GQ372871

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