A preliminary analysis of phylogenetic relationships and biogeography of the dangerously venomous Carpet Vipers, *Echis* (Squamata, Serpentes, Viperidae) based on mitochondrial DNA sequences

E. Nicholas Arnold1, Michael D. Robinson2, Salvador Carranza3,*

Abstract. Phylogenetic analysis of 1117 bp of mitochondrial DNA sequences (731 bp of cytochrome *b* and 386 bp of 16S rRNA) indicate that *Echis* consists of four main clades: *E. ocellatus*, and the *E. coloratus*, *E. pyramidum*, and *E. carinatus* groups. In the *E. coloratus* group, *E. coloratus* itself shows substantial genetic divergence from *E. omanensis*, corroborating their separate species status. In the *E. pyramidum* clade, *E. pyramidum* from Egypt and *E. leucogaster* from West Africa are genetically very similar, even though samples are separated by 4000 km. South Arabian populations of the *E. pyramidum* group are much better differentiated from these and two species may be present, animals from Dhofar, southern Oman probably being referable to *E. khosatzkii*. In the *E. carinatus* group, specimens of *E. carinatus sochureki* and *E. multisquamatus* are very similar in their DNA. The phylogeny indicates that the split between the main groups of *Echis* was followed by separation of African and Arabian members of the *E. pyramidum* group, and of *E. coloratus* and *E. omanensis*. The last disjunction probably took place at the lowlands that run southwest of the North Oman mountains, which are likely to have been intermittently covered by marine incursions; they also separate the *E. pyramidum* and *E. carinatus* groups and several sister taxa of other reptiles. The *E. carinatus* group may have spread quite recently from North Oman into its very extensive southwest Asian range, and there appears to have been similar expansion of *E. pyramidum* (including *E. leucogaster*) in North Africa. Both these events are likely to be associated with the marked climatic changes of the Pleistocene or late Pliocene. Similar dramatic expansions have also recently occurred in three snake species in Iberia.

Keywords: biogeography, *Echis*, evolution, mitochondrial DNA, snakes, taxonomy, venom.

Introduction

Carpet vipers (*Echis*) are found across the semi-arid regions of west, northern and east Africa, west, south and east Arabia, parts of Iran and Afghanistan north to Uzbekistan, and in Pakistan, India and Sri Lanka (fig. 1). They are often abundant and in many areas are a common cause of fatal snake bite in people (Gasperetti, 1988; Spawls and Branch, 1995). It has been shown that antivenoms raised against venom from a population of *Echis* in one area may be ineffective in treating bites elsewhere (Warrell and Arnett, 1976; Gillissen et al., 1994). Sometimes this is because different species of *Echis* are likely to have been involved (Gillissen et al., 1994), for venom chemistry sometimes varies greatly between them. Consequently, a good appreciation of the taxonomy of the genus is essential for efficient treatment.

For a long time, just two species of *Echis* were recognised: *E. coloratus* (Günther, 1878) found in Arabia, Jordan, Israel and eastern Egypt, and *E. carinatus* (Schneider, 1801), which was believed to occur over most of the range of the genus. In *E. coloratus*, populations from Israel and west Jordan have recently been described as a subspecies, *E. c. terraesanctae* (Babocsay, 2003) and populations from northern Oman given full species status as *E. omanensis* (Babocsay, 2004), both taxa being recognised on the basis of morphology. The systematics of *Echis carinatus* in its broad sense has...
Figure 1. Distribution of main taxa of Carpet vipers (*Echis*). Star indicates distinctive population in north Algeria (see below). Maps based on Gasperetti (1988); Spawls and Branch (1995); Babocsay (2004); Geniez et al. (2004); Mazuch (2005); and Trape and Mané (2006).

been confused and unstable in recent decades. Within this assemblage, a number of taxa have been described on the basis of external features, including *E. carinatus leakeyi* (Stemmler and Sochurek, 1969) and *E. c. aliaborri* (Drewes and Sacherer, 1974) from Kenya. *Echis c. sochureki* (Stemmler, 1969) was described from Pakistan, Iran and North Oman and *E. c. astolae* (Mertens, 1971) from Astola Island near Pakistan. *Echis ocellatus* (Stemmler, 1970) of the savannahs of West Africa was recognised as occurring from Senegal eastwards to at least western Chad (Wüster et al., 1997), while populations assigned to *E. pyramidum* (Geoffroy Saint-Hilaire and Geoffroy Saint-Hilaire, 1827), which is based on Egyptian material. Morphology indicated that nearly all *Echis* populations from Egypt to Kenya belonging to the western group could be regarded as a single rather variable form, and that *E. leucogaster* was very similar to these (Arnold, 1980a). On the other hand, *E. ocellatus* appeared to be a separate species distinguished by scale counts, pattern and hemipenial structure. It was also noted that an animal from Biskra, northern Algeria (Natural History Museum, London: BMNH 1907.4.6.55) has a distinctive hemipenis and may represent yet another taxon (fig. 1B).

A broad study of *Echis* anatomy, incorporating features of the hemipenis and dorsal scale shape (Arnold, 1980a), made it clear that *E. carinatus* in its broad sense is morphologically disjunct. The eastern forms (at that time assigned to *E. c. carinatus* in India and Sri Lanka, *E. c. sochureki* and *E. c. astolae*) are very different in these aspects from the more western ones, the two assemblages approaching each other in Oman. There are also differences in reproduction, eastern animals apparently being viviparous while western ones are egg-laying (Smith, 1943; Minton, 1966; Stemmler, 1971; Spawls and Branch, 1995). Among the western populations, the oldest available name is *E. pyramidum* (Geoffroy Saint-Hilaire and Geoffroy Saint-Hilaire, 1827), which is based on Egyptian material. Morphology indicated that nearly all *Echis* populations from Egypt to Kenya belonging to the western group could be regarded as a single rather variable form, and that *E. leucogaster* was very similar to these (Arnold, 1980a). On the other hand, *E. ocellatus* appeared to be a separate species distinguished by scale counts, pattern and hemipenial structure. It was also noted that an animal from Biskra, northern Algeria (Natural History Museum, London: BMNH 1907.4.6.55) has a distinctive hemipenis and may represent yet another taxon (fig. 1B).

Subsequent to this survey, *Echis multisquamatus* Cherlin, 1981, was described from Turkmenistan and *Echis* was later the subject of an extensive revision (Cherlin, 1990; Cherlin and Borkin, 1990). In this, a total of twelve species
and a further eight subspecies were recognised, seven of these taxa being new. For example, animals from southwest Arabia previously placed in *E. pyramidum* were assigned to *E. khosatzkii* Cherlin, 1990 and *E. varia borkini* Cherlin, 1990. Of Cherlin’s new forms, *E. multisquamatus* was shown to be close to *E. carinatus* sochureki in its external morphology (Auffenberg and Rehman, 1991). But many of Cherlin’s other radical taxonomic changes have never been formally reassessed, and the systematics of the *Echis carinatus* complex has remained unstable. Species-level distinction between *E. carinatus* and the more western forms has generally been accepted (see for instance Gasperetti, 1988; Schätti and Gasperetti, 1994), but many authors use conflicting taxonomic arrangements elsewhere in the genus, especially in Africa. Thus, Largen and Rasmussen (1993), Spawls and Branch (1995), and Mazuch (2005) all vary in the names they use for some populations, and the last two publications do not incorporate some of Cherlin’s changes in the regions they cover. Regrettably, confusion in the systematics of *Echis* has probably been greatest in the medical and toxinological literature (Wüster et al., 1997). For example, less than 50% of experimental venoms and animals involved in bites that were mentioned could be confidently assigned to the currently more widely accepted species (Wüster and McCarthy, 1996).

Because of these problems, the relationships of some of the more critical populations of *Echis* are surveyed here using mitochondrial DNA sequences. So far, their use in the genus has been limited, although individuals assigned to six taxa have been investigated using fragments of cytochrome *b* (*cytb*) and 16S rRNA (Lenk et al., 2001). In the course of a continuing broader molecular study on the Arabian reptile fauna, tissue samples were collected by the present authors from three taxa of *Echis* in Oman during 2005. The DNA sequences produced from these were combined with other new ones from Mauritania, Niger and with those of Lenk et al. (2001) and Malhotra and Thorpe (2000) in Genbank, and with another sequence of *E. ocellatus* deposited there by W. Wüster. The results of these preliminary analyses were used to assess aspects of the systematics and biogeography of *Echis*.

**Material and methods**

A total of 17 specimens of the genus *Echis* were included in this study, among them representatives of at least seven nominal species covering much of the geographical distribution of the genus. One *Cerastes cerastes* (Linnaeus, 1758) and one *C. vipera* (Linnaeus, 1758) were used as outgroups. Specimen data are given in table 1.

**Extraction of DNA and PCR amplification**

The DNeasy tissue kit, Qiagen, was used to extract genomic DNA from the samples, following the manufacturers’ instructions. Primers used in both amplification and sequencing were L14846F1 (5′-CAA CAT CTC AGC ATG ATG TGA TCG TAG GAT GGC GTA-3′; Lenk et al., 2001) and S2 (5′-TGG GAT TGA TCG TAG GAT GCC GTA-3′) for the cytochrome *b* (*cytb*) gene, and L2510 (5′-CGC CTG TTT ATC AAA AAC AT-3′) and H3062 (5′-CGC GTT TGA ACT CAG ATC A-3′) for 16S rRNA (Lenk et al., 2001). The two gene fragments were amplified using PCR procedures described by Carranza et al. (1999, 2001) and processed with an ABI 377 automated sequencer following the manufacturer’s protocols.

**Phylogenetic analyses**

DNA sequences were aligned using ClustalX (Thompson et al., 1997) with default parameters (gap opening = 10; gap extension = 0.2). The *cytb* alignment included no gaps and no stop codons were observed when the sequences were translated into amino acids using the vertebrate mitochondrial code, suggesting that all the *cytb* sequences analyzed were functional. Only five gaps had to be postulated to unambiguously align all the 16S rRNA sequences; therefore all the positions were included in the phylogenetic analyses.

Topological incongruence among partitions was tested using the incongruence length difference (ILD) test (Michkevich and Farris, 1981; Farris et al., 1994). In this, 10,000 heuristic searches were carried out after removing all invariable characters from the dataset (Cunningham, 1997). To test for incongruence among data sets we also used a reciprocal 70% bootstrap proportion (Mason-Gamer and Kellogg, 1996) or a 95% Bayesian posterior probability threshold. Topological conflicts were considered significant if two different relationships for the same set of taxa were both supported with bootstrap values > 70%, or posterior probability values > 95%.
Table 1. Details of material and sequences used in the present study. The question mark indicates that the cyt\(b\) and 16S rRNA sequences of the specimen of \textit{E. ocellatus} by Lenk et al. (2001) are probably the result of a misidentification of an \textit{E. leucogaster} specimen as an \textit{E. ocellatus} (see fig. 2).

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Locality</th>
<th>Accession numbers cyt(b)/16S rRNA</th>
<th>References</th>
</tr>
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<td>AJ275708/AJ275760</td>
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Three methods were employed for phylogenetic analyses of the two separate gene fragments and for those in which they were combined. These were: maximum-likelihood (ML), maximum-parsimony (MP) and Bayesian analysis. Modeltest (Posada and Crandall, 1998) was used to select the most appropriate model of sequence evolution for the ML and Bayesian analyses under the Akaike Information Criterion. This was the GTR model taking into account gamma distribution and the number of invariable sites for each independent partition (cyt\(b\) and 16S) and for the combined data set (cyt\(b\) + 16S).

Both ML and MP analyses were performed in PAUP*4.0b10 (Swofford, 1998) and included searches involving tree bisection and reconnection (TBR) branch swapping with 10 and 100 step-wise additions of taxa, respectively. In the MP analyses, gaps were included as a fifth state and transitions and transversions were given the same weight. Bayesian analyses were performed on MRBAYES v.3.1.2 (Huelsenbeck and Ronquist, 2001) and each partition had its own model of sequence evolution and model parameters (see above). Four incrementally heated Markov chains with the default heating values were used. All analyses started with randomly generated trees and ran for 2\(\times\)10\(^6\) generations in two independent runs with samplings at intervals of 100 generations that produced 20 000 trees. After verifying that stationarity had been reached, both in terms of likelihood scores and parameter estimation, the first 5000 trees were discarded in both independent runs of the cyt\(b\) and 16S rRNA and the combined analyses and a majority rule consensus tree was generated from the remaining 15 000 post-burnin trees. The frequency of any particular clade among the individual trees contributing to the consensus tree represents the posterior probability of that clade (Huelsenbeck and Ronquist, 2001); only values equal or above 95% were considered to indicate sufficient support (Wilcox et al., 2002).

Results

The two gene partitions used (cyt\(b\) and 16S) were shown to be congruent using the ILD-test \( (P = 0.90)\), and independent analyses of the two gene partitions confirmed there were no topological conflicts (Mason-Gamer and Kellogg, 1996). Therefore, both mitochondrial fragments were combined for further analyses. Of the 1117 bp of the combined data set (731 of cyt\(b\) and 386 of 16S), 327 were variable and 276 parsimony-informative.

The results of the phylogenetic analyses are shown in figure 2. ML, MP and Bayesian phylogenetic analyses all produced trees with identical topologies in which four well-supported main units are discernable. They are the \textit{E. ocellatus}, \textit{E. pyramidum}, \textit{E. coloratus}, and \textit{E. carinatus} groups which have uncorrected genetic divergences of about 12-18\% from each
Figure 2. Relationships of Carpet vipers (*Echis*) based on 731 base pairs of cytochrome *b* and 386 of 16S rRNA mitochondrial genes. Two other viperines, *Cerastes cerastes* and *C. v soulara* are used as outgroups. Maximum likelihood (ML), maximum parsimony (MP) and Bayesian analyses all gave identical topologies. Figures close to nodes are: ML bootstrap value/MP bootstrap value/Bayesian posterior probability value (only values equal or higher than 0.95 are indicated with an asterisk “*”). The question mark indicates that the cytb and 16S rRNA sequences of the specimen of *E. ocellatus* by Lenk et al. 2001 (see table 1) are probably the result of misidentification of an *E. leucogaster*.

Phylogeny of *Echis* 277

other in the cytb fragment investigated (see table 2). In the *E. pyramidum* group, there is a quite deep divergence between African and Arabian representatives (11-13%). In North Africa, a specimen of *E. pyramidum* from Egypt is very similar to two assigned to *E. leucogaster* from Mauritania (5%). A further snake from Mali, identified in GenBank as *E. ocellatus* (Lenk et al., 2001), is also close to *E. pyramidum* (1%) and *E. leucogaster* (5%) and very divergent from the other *E. ocellatus* included in this study (14-16%); it may consequently actually also be an *E. leucogaster*. Overall divergence in these four widely separated North African snakes is only about 2-5%.

The two south Arabian members of the *E. pyramidum* group, from Yemen and Dhofar in south Oman, are sisters but show a divergence
Table 2. Uncorrected genetic distances for the cytochrome \( b \) gene fragment used in this study.

<table>
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</table>

of about 10%. In the \( E. \) coloratus clade, there is a deep dichotomy between the western \( E. \) coloratus itself and \( E. \) omanensis of North Oman, with a divergence of about 9-10%. In the \( E. \) carinatus group, animals assigned to \( E. \) multisquamatus from Turkmenistan and \( E. \) c. sochureki from North Oman are very similar (about 2%) and a further specimen, apparently from Pakistan shows a divergence of 3% from these.

Discussion

Systematics

The great divergence between samples of \( E. \) ocellatus and \( E. \) pyramidum considered to be valid here indicates that they are not closely related as has been previously suggested (Lenk et al., 2001). As noted, the specimen assigned to \( E. \) ocellatus on which this relationship was based is more likely to be assignable to the \( E. \) multisquamatus from Turkmenistan and \( E. \) c. sochureki from North Oman are very similar (about 2%) and a further specimen, apparently from Pakistan shows a divergence of 3% from these.

The strong divergence of the two Arabian populations of the \( E. \) pyramidum group studied here, from African ones and from each other, indicates they represent at least one separate species. The name \( E. \) khosatzkii (Cherlin, 1990) may be available for the population in Dhofar, southern Oman (fig. 1B). The locality of the holotype of \( E. \) khosatzkii, is ‘Hadhramaut’ (in fact, somewhere on an itinerary from Mukallah on the coast to the Hadhramaut valley and back to the sea at Ash Shihir, *fide* Anderson, 1896). The paratype comes from Ghayl Ba Wazir in the same general area of eastern Yemen, and a similar specimen has been reported 170 km to the east at Sayhut (Schätti and Desvoignes, 1999). These specimens come from 300-500 km west of the Dhofar ones. They share with these a distinctive range of dorsal patterns and, compared with most other Arabian animals of the \( E. \) pyramidum group from further west, relatively high ventral and subcaudal scale counts (East Yemen and Dhofar: 165-179 ventral scales and 39-46 subcaudal scales in males \((n = 6)\), and respectively 183-189, and 36-39 in females \((n = 4)\).
Western Yemen and adjoining southwest Saudi Arabia: usually 145-169 ventral scales and 29-37 subcaudal scales in males \((n = 5)\) and respectively 158-170 and 29-38 in females \((n = 6)\) – E.N. Arnold, unpublished data; Gasperetti, 1988; Schätti and Gasperetti, 1994). However, adults from eastern Yemen have narrow heads, whereas they are broad in the ones from Dhofar (Arnold, 1980a). The other genetically distinct southern Arabian member of the \(E. \) \textit{pyramidum} group investigated here does not have a precise locality within Yemen (Lenk et al., 2001), but its genetic divergence makes it unlikely to be conspecific with the Dhofar population. It could conceivably be a representative of the populations in western Yemen for which the name \(E. \) \textit{borkini} (Cherlin, 1990) (type locality Lahej near Aden) may be available (\(E. \) sp. in fig. 1B). More DNA sampling of the \(E. \) \textit{pyramidum} group in Yemen and Oman is required before these problems of nomenclature can be resolved. However, it is quite possible that at least two species of \(Echis\) could occur in southwest Arabia, for some other reptile taxa have two or more closely related allopatric or parapatric species there distributed from west to east. These include spiny-tailed agamids (\textit{Uromastyx yemenensis} and \textit{U. benti} – Wilms and Schmitz, 2007) and semaphore geckos (\textit{Pristurus carteri} group – Arnold, 1986a). In other cases more distantly related congeneric occur allopatrically in this region, for example in \textit{Hemidactylus} and \textit{ Ptyodactylus} geckos (Carranza and Arnold, unpublished data).

The strong genetic divergence between \(Echis \) \textit{coloratus} and the recently distinguished \(E. \) \textit{omanensis} corroborates the species status of these forms. In contrast, similarity in DNA sequence confirms an assessment from morphology (Auf- fenberg and Rehman, 1991) that \(Echis \) \textit{multisquamatus} is conspecific with \(E. \) \textit{carinatus sochureki}. The genetic similarity of \(E. \) \textit{multisquamatus} and \(E. \) \textit{c. sochureki} has already been noted by Lenk et al. (2001).

**Historical biogeography**

\(Echis\) belongs to the Viperinae, a group for which an African origin has been postulated (Lenk et al., 2001). \textit{Causus}, the sister group of the Viperinae is found in Africa and, of the five units that comprise the subfamily, \textit{Atheris}, \textit{Bitis} and \textit{ Cerastes} also occur there with the last two extending into Arabia, an area that is geologically part of that continent. The only unit of the Viperinae to occur predominantly outside Africa is made up of \textit{Vipera}, \textit{Pseudocerastes} and \textit{Eristicophis} and mainly occurs in Europe and Asia. \(Echis\) itself has three of its main units entirely in the African-Arabian region and the fourth, the mainly Asian \(E. \) \textit{carinatus} group, is also represented there in North Oman (fig. 1D). Given the overall distribution pattern of taxa within the Viperinae, and the topology of the phylogenetic tree from figure 2 in which the \(E. \) \textit{coloratus} group is basal to all the other three groups, it is most parsimonious to assume an African-Arabian origin for \(Echis\). This contrasts with an Asian genesis in the Irano-Turanian region suggested by Cherlin (1990). But that hypothesis did not take the distribution of the relations of \(Echis\) into account and the putative phylogeny of the genus on which it was based (Cherlin, 1990, fig. 6) is different from the one presented here.

The DNA phylogeny (fig. 2) suggests that \(Echis\) spread and diversified relatively rapidly to produce its main groups, while disjunction of the \(E. \) \textit{pyramidum} group around the Red Sea, and the separation of \(E. \) \textit{coloratus} and \(E. \) \textit{omanensis} within Arabia, occurred rather later. The Red Sea is a site of disjunction for many other reptile clades, including ground geckos (\textit{Stenodactylus}), sand skinks (\textit{Scincus}), lacertids of the \textit{Acanthodactylus scutellatus} and \textit{Mesalina brevirostris-M. rubropunctata} groups, sand vipers (\textit{Cerastes}) and awl-headed snakes (\textit{Lytorhynchus}) (see Arnold, 1980b, 1986b, 1987). Similarly, the disjunction between \(E. \) \textit{coloratus} and \(E. \) \textit{omanensis} in eastern Arabia, is matched by many other groups including \textit{Bunopus}, \textit{Hemidactylus} and \textit{Pristurus} geckos.
(Arnold, 1980a; Arnold and Carranza, unpublished DNA data), sand skinks (Scincus m. mitranus and S. mitranus muscatensis – Carranza et al., 2008), and lacertids (Acanthodactylus schmidtii and A. blandfordii – Harris et al., 1998). Different degrees of genetic divergence between western and eastern representatives of these units suggest that there was not a single vicariance event but a series of temporary interruptions that affected different taxonomic groups at different times. This possibility gains some support from topography. The highland regions of Dhofar and North Oman are separated by a lowland area that stretches from the Arabian Gulf southeast to the Arabian Sea. A fault-line running through this area and extensive inland sabkhas (salt flats) suggest there may have been an intermittent shallow sea separating the more elevated regions on either side of it (Gasperetti, 1988). As already noted, a number of taxa, perhaps including Echis, have different species distributed along the southern coastal areas of southwest Arabia, indicating another region where some vicariance took place. If this really applies to Echis, genetic distances (table 2) suggest separation of the two forms concerned might have been around the time when the ancestor of E. coloratus and E. omanensis speciated.

Parsimony suggests E. carinatus may have originated by vicariance in North Oman and subsequently expanded eastwards, although the topology of the phylogeny (fig. 2) indicates this may have involved more than one invasion, or alternatively an invasion and a later back-colonisation. Such movement between North Oman and Iran and neighbouring areas may have taken place in other reptile groups. Hemi-dactylus persicus and Acanthodactylus blandfordii appear to have moved from Oman into Iran (Arnold and Carranza, unpublished data) and this may also have happened in Asaccus geckos (Arnold and Gardener, 1994). Movements in the opposite direction are likely to have occurred in the ancestor of Bunopus hajarensis and B. spatulurus, and in Ablepharus cf. panonicus and Pseudocerastes (Arnold and Gallagher, 1977).

Echis carinatus may have extended its range comparatively recently and rapidly in southwest Asia, as the North Oman and Turkmenistan animals investigated here are genetically similar, even though they are separated by at least 1200 km. It remains to see if spread into India was also comparatively recent. Such rapid expansion by snake species, once the edge of a new inhabitable region is colonised, has also occurred elsewhere. For example, DNA phylogenies show that the Montpellier snake (Malpolon monspessulanus), Horseshoe whip snake (Hemorrhois hippocrepis) and Western false smooth snake (Macroprotodon brevis) have only reached the Iberian peninsula from north Africa recently, but have spread extensively there (Carranza et al., 2004; Carranza and Arnold, 2006).

Limited genetic divergence (table 2) suggests that quite rapid range extension has also occurred in Echis pyramidum (including E. leucomaster) in North Africa. This may have happened after restriction by climatic change although, if so, the core area where E. pyramidum survived is not identifiable. There also appears to have been some restriction after expansion, as the range of E. pyramidum is very fragmented. These events are likely to have occurred during the well-substantiated climatic fluctuations that characterised the Pleistocene and late Pliocene (Sarnthein, 1978; Schuster et al., 2006). Movement of E. carinatus in southwest Asia may also have been around the same general period.

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