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Evolutionary Relationships among the True Vipers (Reptilia: Viperidae) Inferred from Mitochondrial DNA Sequences

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Nucleotide sequences of mitochondrial cytochrome *b* and 16S rRNA genes, totaling 946 bp, were used to reconstruct a molecular phylogeny of 42 species of the subfamily Viperinae representing 12 of the 13 recognized genera. Maximum-parsimony and maximum-likelihood were used as methods for phylogeny reconstruction with and without *a posteriori* weighting. When representatives of the Causinae were taken as outgroup, five major monophyletic groups were consistently identified: *Bitis*, *Cerastes*, *Echis*, the Atherini (*Atheris* s.l.), and the Eurasian viperines. *Proatheris* was affiliated with *Atheris*, and *Adenorhinos* clustered within *Atheris*. The African *Bitis* consisted of at least three monophyletic groups: (i) the *B. gabonica* group, (ii) the *B. caudalis* group, and (iii) the *B. cornuta* group. *B. worthingtoni* and *B. arietans* are not included in any of these lineages. Eurasian viperines could be unambiguously devided into four monophyletic groups: (i) *Pseudocerastes* and *Eristicophis*, (ii) European vipers (*Vipera* s.str.), (iii) Middle East *Macrovipera* plus *Montivipera* (*Vipera xanthina* group), and (iv) North African *Macrovipera* plus *Vipera palaestinae* and *Daboia russelii*. These evolutionary lineages are consistent with historical biogeographical patterns. According to our analyses, the viperines originated in the Oligocene in Africa and successively underwent a first radiation leading to the five basal groups. The radiation might have been driven by the possession of an effective venom apparatus and a foraging startegy (sit–wait–strike) superior in most African biomes and might have been adaptive. The next diversifications led to the *Proatheris–Atheris* furcation, the basal *Bitis* splitting, and the emergence of the basal lineages within the Eurasian stock. Thereafter, lineages within *Echis*, *Atheris*, and *Cerastes* evolved. The emergence of three groups within *Vipera* s.l. might have been forced by the existence of three land masses during the early Miocene in the area of the Paratethys and the Mediterranean Seas. Taxonomic consequences of these findings are discussed. © 2001 Academic Press

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INTRODUCTION

The taxonomy and phylogeny of viperine snakes has been controversially discussed in recent years because morphology-based and molecular trees differ fundamentally in the branching orders proposed (Herrmann and Joger, 1997; Herrmann *et al.*, 1999). However, a consensus excluding the primitive genera *Azemiops* and *Causus* from the Viperinae was reached and separate subfamilies were set up for both of them (Cadle, 1988; Groombridge, 1986; Heise *et al.*, 1995; Liem *et al.*, 1971).

Within true vipers, Groombridge (1986) distinguished between an African group and a Palaearctic group and considered *Echis* and *Cerastes* separate entities that were tentatively attached to the African group. Ashe and Marx (1988) and Marx *et al.* (1988), however, emphasized characters that united *Cerastes* with the Palaearctic *Eristicophis* and *Pseudocerastes*, but placed *Echis* together with the oriental *Daboia russelii* and with the Afrotropical genus *Bitis*. An even stronger disagreement with biogeographical patterns was found in these authors' disruption of *Vipera* s.l. (*Vipera*, *Macrovipera*, and *Daboia*) into a polyphyletic assemblage of independent clades. Herrmann and Joger (1997) reanalyzed both data sets of Groombridge and of Ashe and Marx but found meaningful phylogenetic signals only in Groombridge's data. Later, Herrmann *et al.* (1999) enriched Groombridge's data set with cytochrome *b* amino acid sequence data, confirming the monophyly of the African and the Palaearctic group and the affiliation of *Echis* and *Cerastes* to the African group. Microdermatoglyphic patterns supported an association of *Echis* (and to a lesser degree of *Cerastes*) with *Atheris* (Joger and Courage, 1999).

However, immunological data contradict this view, as they position *Echis* and *Cerastes* closer to the Palae-

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arctic *Macrovipera* and the Oriental *Daboia* (Herrmann and Joger, 1995, 1997). A preliminary analysis of partial cytochrome *b* gene sequences was ambiguous, depending on the particular tree reconstruction algorithm used, and even positioned *Cerastes* as a basal taxon in the viperine tree (Herrmann *et al.*, 1999).

In this study we have analyzed nucleotide sequences of the mitochondrial cytochrome *b* and 16S rRNA genes and used them in a combined data set. The aim of this study was to further elucidate phylogenetic relationships in the viperine complex.

We use the term "Eurasian vipers" for palaearctic genera (*Eristicophis, Pseudocerastes, Vipera* and *Macrovipera*) plus *Daboia*. The term "*Vipera* s.l." is applied to *Vipera, Macrovipera,* and *Daboia*. The latter two were taxonomically separated from *Vipera* by Herrmann *et al.* (1992). The unit "Atherini" includes the genera *Atheris, Proatheris, Montatheris* (not studied here), and *Adenorhinos* (see Fig. 1).

MATERIAL AND METHODS

DNA was extracted from blood collected by caudal vein puncture as described in Joger and Lenk (1995) from living specimens or tissue samples removed from freshly dissected or ethanol-preserved animals. The dataset includes 42 species representing 12 of the 13 recognized viperine genera with the exception of *Montatheris,* a monotypic genus of East Africa that has been recently separated from *Atheris* (Broadley, 1996).

The selection of outgroup species was done on the strength of taxonomical considerations. We regarded the genus *Causus* as the most important candidate, as it is classified in a separate subfamily and lacks apomorphic states of viperines in several characters (venom apparatus, topology of arteries, shape of pupils, scalation, reproduction), but simultaneously shares with viperines some synapomorphic states in other characters (discussed in Underwood, 1999) which give reason to assume a sister group relationship.

Additional analyses were performed with other outgroups (i.e., the viperid *Azemiops feae* and the colubrid *Dinodon semicarinatus* as more distantly related alternatives). Table 1 shows all specimens used.

Tissue samples were stored in 95% ethanol or EDTA–buffer (10% EDTA, 0.5% sodium fluoride, 0.5% thymol, 1% Tris, pH 7.0; Arctander, 1988) at −20°C. Total genomic DNA was prepared following standard proteinase K and phenol chloroform protocols (Sambrook *et al.*, 1989). Portions of the cytochrome *b* and 16S rRNA gene were amplified using the polymerase chain reaction. Primers listed in Table 2 were used in varying combinations to obtain the desired cytochrome *b* fragment of all species. The 16S rRNA fragment was amplified with the primer combination L 2510 and H 3062. Sequencing primers (labeled with a fluorescent dye cy5) used were L 14846, H 15556/7, H 15149, L 15162, L 2510, and H 3062.

For amplification, total DNA was used as a template plus 25 pmol of the above-mentioned primers, 1.5 mM $MgCl_2$, 0.1 mM each dNTP, 5 μl amplification buffer, and 1.5 U *Taq* DNA Polymerase (Amersham Pharmacia Biotech) in a total volume of 50 μl. After an initial denaturation step (4 min at 94°C) 30 cycles of 30 s at 95°C, 30 s at 45°C, and 90 s at 72°C were performed on a Biometra thermocycler. After 30 cycles the reaction temperature was maintained at 72°C for 4 min and than lowered to 4°C for further storage. The quality of the resulting PCR products was controlled by electrophoresis on a 1.5% agarose gel (Qualex Gold Agarose; FMC Bioproducts). Depending on the strength of the band, 1 to 6 μl of the PCR product was used to perform direct cycle sequencing of the PCR products employing the "ThermoSequenase flourescent labeled primer cycle sequencing kit with 7-deaza-dGTP" (Amersham Pharmacia Biotech) according to the protocol of the manufacturer. Cycle sequencing was performed at 94°C for 3 min, followed by 25 cycles of 94°C for 30 s and 50°C for 90 s. The sequencing products were loaded on longranger acrylamide gel (FMC Bioproducts) without further purification and run on an ALF EXPRESS II sequencer (Amersham Pharmacia Biotech) for automatic fluorescence detection of the nucleotide sequence. Both heavy and light strands were sequenced in such a way that large overlapping segments (80%) were obtained. All sequence outputs were compared with the electrophoretograms and aligned manually using the human sequence (Accession No. J01415) as reference (Anderson *et al.*, 1981). 16S rRNA sequences were aligned with the aid of the secondary structure model of the human sequence (Gutell and Fox, 1988) to optimize site homology in the alignment. Sequences were deposited at GenBank/EMBL (Accession Nos. AJ275679–AJ275784; see Table 1).

Because the resolution of the 16S rRNA data set alone was limited due maybe to the low number of informative characters, we combined the sequences from the cytochrome *b* and 16S rRNA genes into one data set. We used MEGA version 1.01 (Kumar *et al.*, 1993) to calculate base compositional frequencies for both genes. Sequence divergences were calculated with PAUP* 4b3a (Swofford, 1998). To test the amount of phylogenetic information in the data set, the g1 value (Hillis and Huelsenbeck, 1992) derived from 10,000 random trees was determined with PAUP* 4b3a.

Many philosophies and algorithms concerning techniques for extracting maximum information from DNA sequence data exist. Systematic analyses should principally avoid assumptions about evolutionary processes in their methods (Mindell and Thacker, 1996). However, multiple substitutions can lead to homoplasy, which may often impede analyses, especially if higher order relationships are analyzed or fast evolving genes are used (Brown

TABLE 1

Specimens Included in This Study, Their Taxonomic Identity, Origin, Accession No., Catalogue No. of Specimen, and Catalogue No. of DNA

Species	Geographic origin	cytb Accession No.	16S rRNA Accession No.	Voucher specimen	Voucher DNA
Adenorhinos barbouri	Masisiwe, Tanzania	AJ275686	AJ275739	ZMK R68297	5038
Atheris ceratophora	Usambara Mountains, Tanzania	AJ275682	AJ275735	Collection Germot Vogel, 322	5031
Atheris chlorechis	Togo	AJ275679	AJ275732	HLMD RA-2892	4991
Atheris desaixi	Mt. Kenya, Kenya	AJ275680	AJ275733	NHMN, no number	4987
Atheris hispida	Kakamega, Kenya	AJ275681	AJ275734	Collection Klaus Zahn, no number	4997
Atheris nitschei	Ruanda	AJ275683	AJ275736	HLMD RA-1675	5020
Atheris squamigera	Togo	AJ275684	AJ275737	HLMD RA-2908	5018
Azemiops feae	Vietnam	AJ275687	AJ275740	HLMD RA-2910	4990
Bitis arietans	Kigali, Ruanda	AJ275689	AJ275742	Collection Harald Hinkel, no number	5010
Bitis atropos	Swartburg, South Africa	AJ275691	AJ275744	PEM, no number	4985
Bitis caudalis	Swakopmund, South Africa	AJ275693	AJ275746	ZFMK 65212	5003
Bitis cornuta	Luderitz, Namibia	AJ275694	AJ275747	TM 71197	4996
Bitis gabonica	Irangi, Kivu, Democratic Republic Congo	AJ275695	AJ275748	ZFMK 64335	4986
Bitis nasicornis	Irangi, Kivu, Democratic Republic Congo	AJ275697	AJ275750	ZFMK 64888	5029
Bitis peringueyi	Swakopmund, South Africa	AJ275698	AJ275751	TM F1212	5019
Bitis rhinoceros	Togo	AJ275696	AJ275749	HLMD RA-2909	5021
Bitis worthingtoni	Kenya	AJ275692	AJ275745	NHMN, no number	5036
Causus resimus	Burundi	AJ275700	AJ275753	Collection Harald Hinkel, no number	5032
Causus rhombeatus	South Africa	AJ275699	AJ275752	HLMD RA-1583	4995
Cerastes cerastes	Erfoud, Morocco	AJ275703	AJ275755	HLMD RA-1453	5014
Cerastes gasperetti	Isreal,	AJ275704	AJ275756	HLMD RA-1593	5025
Cerastes vipera	Djebil, Tunisia	AJ275705	AJ275757	HLMD RA-1432	4984
Daboia russelii	Pakistan	AJ275723	AJ275776	HLMD RA-2899	5045
Dinodon semicarinatus	—	NC_001945	NC_001945	—	—
Echis carinatus	Pakistan	AJ275706	AJ275758	HLMD RA-2871	4983
Echis coloratus	Wadi Rishrash, Egypt	AJ275708	AJ275760	ZFMK 64324	5015
Echis multisquamatus	Turkmenistan	AJ275702	AJ275763	Collection Göran Nilson, no number	10882
Echis ocellatus	Mali	AJ275710	AJ275762	HLMD RA-1594	5028
Echis pyramidum	Egypt	AJ275709	AJ275761	HLMD RA-1591	5026
Echis sp.	Yemen	AJ275707	AJ275759	MHNG, no number	5024
Eristicophis macmahoni	Pakistan	AJ275711	AJ275764	HLMD RA-2890	4993
Macrovipera deserti	Bou Hedma, Tunisia	AJ275712	AJ275765	HLMD RA-2385	5011
Macrovipera lebetina	Kopet-Dagh, Turkmenistan	AJ275713	AJ275766	Collection Göran Nilson, no number	5006
Macrovipera mauritanica	Between Goulimine and Tan-Tan, Moroco	AJ275714	AJ275767	HLMD RA-1182	5009
Macrovipera schweizeri	Milos, Greece	AJ275715	AJ275768	Collection Göran Nilson, no number	5004
Proatheris superciliaris	Malawi	AJ275685	AJ275738	HLMD RA-2880	5012
Pseudocerastes fieldii	Israel	AJ275716	AJ275769	Collection Michael Lehmann, no number	5030
Pseudocerastes persicus	Pakistan	AJ275717	AJ275770	HLMD RA-1724	5002
Vipera ammodytes	Borcka, Turkey	AJ275718	AJ275771	Collection Göran Nilson, no number	5008
Vipera berus	Göteborg, Sweden	AJ275719	AJ275772	HLMD RA-1665	5001
Vipera dinniki	Georgia	AJ275720	AJ275773	HLMD RA-1610	4999
Vipera palaestinae	Israel	AJ275722	AJ275775	HLMD RA-1904	4988
Vipera raddei	Ararat, Turkey	AJ275730	AJ275784	Collection Mario Schweiger, no number	5034
Vipera seoanei	San Sebastian, Spain	AJ275729	AJ275782	HLMD RA-2875	5005
Vipera wagneri	Karakurt, Turkey	AJ275725	AJ275778	Collection Mario Schweiger, no number	4982
Vipera xanthina	Ciglikara, Turkey	AJ275724	AJ275777	Collection Göran Nilson, no number	5016

Note. Museum acronyms: HLMD, Hessisches Landesmuseum, Darmstadt; MHNG, Muséum d'Histoire Naturelle, Genève; NHMN, Natural History Museum Nairobi; TM, Transvaal Museum, Pretoria; PEM, Port Elizabeth Museum; ZFMK, Zoologisches Forschungsinstitut und Museum A. Koenig, Bonn; ZMK, Zoological Museum, Kopenhagen. The sequences from *Dinodon semicarinatus* were obtained from GenBank (Kumazawa *et al.*, 1996). Many samples are identical with those used in Herrmann and Joger (1995), (1997) and in Herrmann *et al.* (1999), (1992).

TABLE 2

Primers Used for PCR and DNA Sequencing (Marked with an Asterisk)

Primers for cytochrome *b*	
L 14846* (two variants)	5′-CAACATCTCA GCATGATGAA ACTTCG-3′ (Kocher *et al.*, 1989)
	5′-CTCCCAGCCC CATCCAACAT CTCAGCATGA TGAAACTTCG-3′
L 14841	5′-CATCCAACAT CTCAKCATKA TGAAA-3′
L 14845	5′-AAACATTTCA ACCTGRTGAA ATTTC-3′
L 15162*	5′-GCAAGTCTTC TACCATGAGG ACAAATATC-3′
H 15149*	5′-AAACTGCAGC CCCTCAGAAT GATATTTGT CCTCA-3′ (Kocher *et al.*, 1989)
H 15557*	5′-AATAGGAAGT ATCATTCGGG TTTGATG-3′
H 15556*	5′-AATAGGAAGT ATCATTCTGG TTTGAT-3′
H 15556*	5′-AAATAGGAAA TATCATTCTG GTTTAAT-3′ (Moritz *et al.*, 1992)
H 15553	5′-GCAAATAGGA AGTATCATTC TGGTTT-3′
Primers for 16S rRNA	
L 2510	5′-CGCCTGTTTA TCAAAAACAT-3′ (Simon *et al.*, 1994)
H 3062	5′-CCGGTTTGAA CTCAGATCA-3′

Note. Numbers refer to the corresponding position of the human mitochondrial genome of the 3′ nucleotide of the primer.

et al., 1979; Miyamoto and Boyle, 1989). Downweighting homoplasic positions and frequent substitution types is an appropriate and widely used tool to reduce effects of homoplasy in DNA sequence data (Fitch and Ye, 1991; Hillis *et al.*, 1994). Our strategy for finding a reliable viperine phylogeny was to search for consistent and statistically significant features under different algorithms and different evolutionary assumptions (i.e., exclusion or use of weighting schemes).

Phylogenetic analyses were performed using the maximum-parsimony (MP) and maximum-likelihood (ML) approaches of PAUP* 4b3a (Swofford, 1998). These widely applied phylogeny reconstruction methods follow different philosophies and warrant sufficiently different perspectives in data analyses. Thus, congruent features found in both methods can be considered meaningful. Whereas the uncorrected approaches were run with the default settings, we had to select appropriate correction types for the weighted approaches. In studies dealing with comparable questions on snake phylogeny (Kraus *et al.*, 1996; Vidal *et al.*, 1997; Keogh *et al.*, 1998; Parkinson, 1999; Malhotra and Thorpe, 2000), workers used varied strategies. In our opinion the strict application of *a posteriori* weightings, i.e., corrections based on preliminary data analyses, is an important step toward objectivity in data analyses. Therefore, all corrections made in this study were based on parameters derived solely from the data set.

In all corrected analyses, data matrix transformations that incorporate both character site- and substitution-type weighting were applied. To find an appropriate correction modus we employed MODELTEST (Posada and Crandall, 1998). This program uses an alignment as input and performs comparisons on different evolutionary models based on the likelihood ratio test statistic. The best-fitting model is sorted out and suggestions for character site weightings are made. The results were used directly to determine the settings of the maximum-likelihood calculation. To transfer this scheme to maximum-parsimony, a step matrix based on the inverse of the instantaneous substitution rate matrix (Q matrix) of the suggested model was constructed. The matrix was made symmetric using the median of each value pair, rounded to the nearest integer value and adjusted to satisfy the triangle inequality after the suggestion of Sankoff (1975). Character site weighting in the parsimony analysis was conducted with the "successive approximation" of Farris (1969) using the rescaled consistency index as a measure of homoplasy. We omitted codon position weighting in the cytochrome *b* gene due to its problematic nature in transmembrane protein-coding genes (Naylor *et al.*, 1995). We also did not differentiate between paired and unpaired structural motifs in the 16S rRNA sequences as the effect on a potential bias of compensatory change in paired rRNA regions has been shown to be relatively small (Macey *et al.*, 1997; Mindell *et al.*, 1991).

Heuristic searches were done under each optimality criterion (Swofford, 1998). Because these searches were not exact, they could not guarantee finding the optimal tree (Swofford *et al.*, 1996). To increase the chance to find the global optimum rather than local optima, each MP and unweighted ML search was replicated by 100 different randomly chosen trees as start positions for branch swapping using the "tree bisection and reconnection" (TBR) algorithm.

To test the statistical reliability of the maximum-parsimony trees, we used the bootstrap test (Felsenstein, 1985; Nei *et al.*, 1998) with 1000 pseudoreplicates. For maximum-likelihood, computational constraints did not allow a bootstrap analysis.

RESULTS AND DISCUSSION

Sequence Statistics

From the whole data set a 597-nt portion of the cytochrome *b* gene and 401 nt of the 16S rRNA gene could be used for phylogeny reconstructions. Within

the cytochrome *b* gene of the presented sequences, no insertions and deletions and no premature stop codons or ambiguous nucleotides were encountered. A few cytochrome *b* gene sequences actually contained short repeated insertions. They were indicated to be possibly paralogous and not processed any further. As expected, the alignment of the 16S rRNA gene revealed several insertions and deletions. Although alignments of these regions could be made for subsets of our data matrix, an unequivocal alignment for the whole data set was impossible. For the sake of objectivity, these sites (within and adjacent to loop10 after the scheme of Horovitz and Meyer, (1995)) were omitted from data analyses. The final data set of the combined genes comprised 946 characters that met the requirements of the phylogenetic analyses.

Light-strand base compositions among species ranged 24.3–29.8% (A), 24.5–33.7% (T), 25.3–36.2% (C), and 11.2–14.4% (G) for cytochrome *b* and 33.9–36.5% (A), 22.1–26.4% (T), 20.6–24.4% (C), and 17.5–19.8% (G) for 16S rDNA. The bias against guanine is characteristic of the light strand of mitochondrial DNA and supports the authenticity of mitochondrial sequences. A total of 417 characters were variable and 334 were parsimony informative. A maximum uncorrected sequence divergence of 31% was found between *Bitis worthingtoni* and *Causus resimus* in the cytochrome *b* gene and 14% between *Echis multisquamatus* and *Bitis caudalis* in the 16S rRNA gene. Overall pairwise sequence divergences of the combined data set reached 22% between *B. worthingtoni* and *C. resimus*. Average genetic divergences between all taxa amounted to 15% nucleotide sites. In the combined data set the distribution of 10,000 random trees displayed a left skewness, indicating a significant phylogenetic signal in the data set. The g1 value of the combined data set amounted to -0.60 ($P = 0.01$) (Hillis and Huelsenbeck, 1992). The best-fitting evolutionary model for our data set found by MODELTEST was the general time reversible (GTR) model (Yang, 1994). It allows for six substitution types in the substitution rate matrix. The program suggested further character site rates to be assumed to follow a gamma distribution with the shape parameter and the proportion of invariable sites to be estimated (GTR + G + I).

Phylogenetic Analysis

Phylogeny within the Basal Viperine Lineages

Atherini. In our analysis the Atherini are represented by three genera (*Adenorhinos, Atheris,* and *Proatheris*). *Proatheris* clustered as the sister of *Atheris* (including *Adenorhinos*). The distinct status of *Proatheris* has already been recognized by Groombridge (1986) based on morphology and high immunological distances of plasma albumins (Herrmann and Joger, 1997) and has led to its formal recognition as a separate genus (Broadley, 1997).

The remaining taxa of the Atherini appeared to be monophyletic as supported by high bootstrap values (Fig. 1) above the significance level. Overall genetic distances (GTR) within this group (excluding *Proatheris*) ranged from 9.0 to 13.6% nucleotide substitutions. *Adenorhinos barbouri* is consistently affiliated with *Atheris ceratophora* in all reconstructions. This association is surprising as *Adenorhinos* is morphologically divergent (Broadley, 1996; Groombridge, 1986) from the other members of the genus *Atheris* (Rasmussen and Howell, 1998). However, both taxa share a common distribution in east Tanzania out of the range of most other *Atheris* species and they prefer terrestrial habitats rather than arboreal (Broadley, 1997). According to our data, *A. barbouri* (which had not been analyzed in previous molecular studies) should be included in *Atheris*; otherwise, *Atheris* would become paraphyletic.

A third recurrent feature was found in the consistent sister relationship between *Atheris squamigera* and *A. hispida*. This confirms the common ancestry of the rough-scaled *Atheris* as found in previous morphological and immunological studies (Groombridge, 1986; Herrmann and Joger, 1997) and in a combination of morphology and amino acid sequence data (Herrmann *et al.*, 1999). Hence, the present results are congruent with the existing data.

Bitis. The genus *Bitis* appeared to be comparatively diverse. Intrageneric genetic distances were the highest among all genera studied and ranged from 7.9 to 17.5% (GTR). Also, bootstrap support for the monophyly of the entire *Bitis* complex was weak (74% at maximum; Fig. 1). The *Bitis* species clustered in distinct groups. The three West African taxa of the *gabonica* clade (*gabonica, rhinoceros, nasicornis*) formed a monophyletic group in all reconstructions, although the relationships within this group could not be resolved unambiguously. Four representatives of the group of small *Bitis* species (*B. atropos, B. caudalis, B. cornuta, B. peringueyi*) emerged consistently as a monophyletic clade, but significant associations were found in the Karoo–Kalaharian sand-dwelling species (*B. caudalis, B. peringueyi*) or the rock-dwelling species (*B. atropos, B. cornuta*) only. The fifth small species, *B. worthingtoni,* emerged as a rather distinct taxon with no established relationships to the remaining species of *Bitis*; it took different positions within the *Bitis* complex. This feature reflects its isolated distribution in Kenya, distant from other small *Bitis* species, and is in congruence with morphological findings (Groombridge, 1986). Also, the position of *B. arietans* was inconsistent under different tree reconstructions. An affinity to the other large-bodied species of the *gabonica* group was gained in the weighted ML, but the alliances between

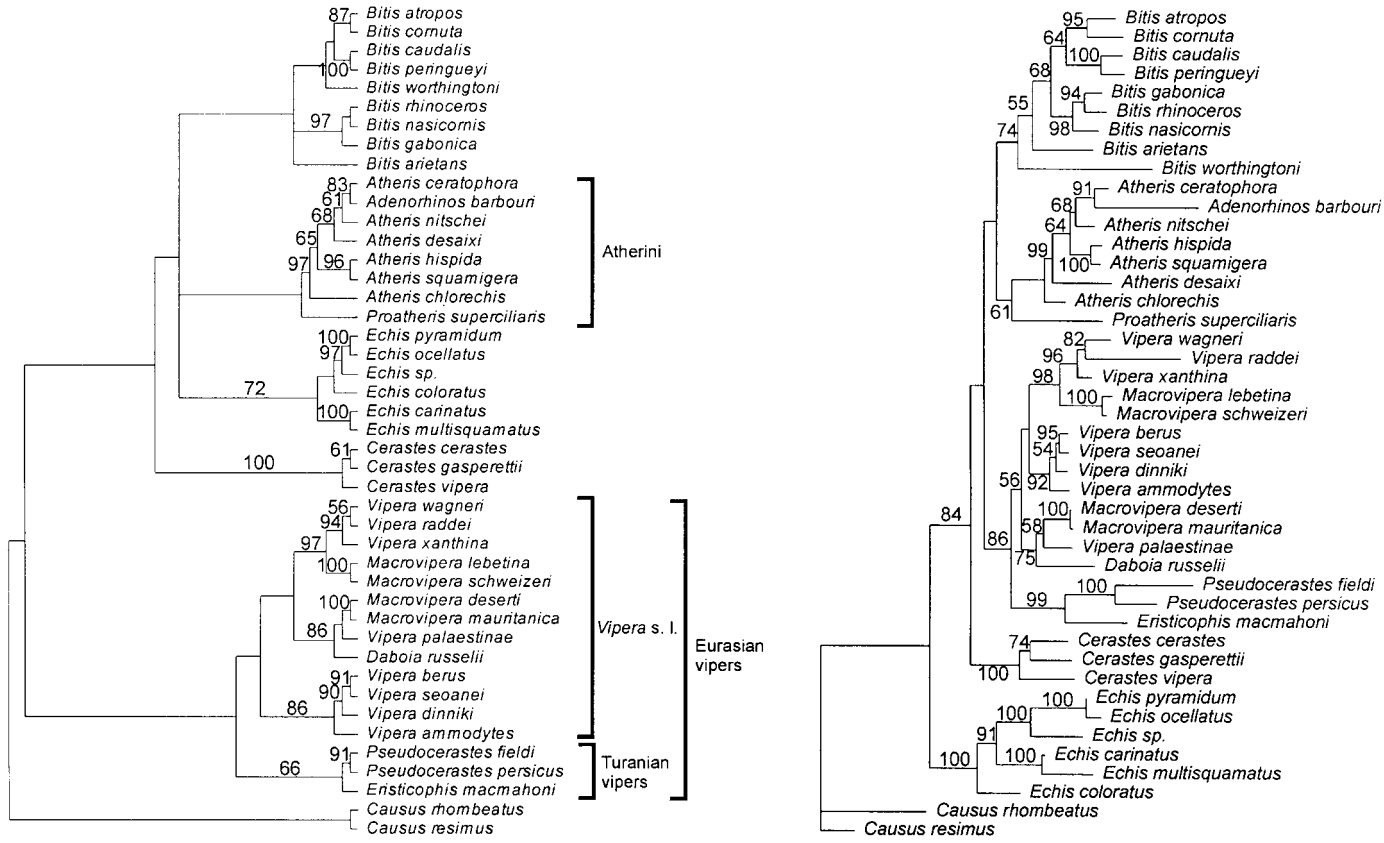

FIG. 1. Maximum-parsimony trees with *Causus* serving as outgroup. (Left) The 75% majority-rule cladogram of the unweighted maximum-parsimony analysis. Five equally parsimonious trees each of 2346 steps in length were obtained. Numbers indicate bootstrap values based on 1000 pseudoreplicates. (CI = 0.27, HI = 0.73, RI = 0.47, RC = 0.13.) Terms used in the text are indicated on the right. (Right) Weighted maximum-parsimony phylogram based on a GTR-adjusted step matrix and the "successive approximation" approach (Farris, 1969). Numbers indicate bootstrap values based on 1000 pseudoreplicates. The analysis yielded a single shortest tree. (CI = 0.59, HI = 0.41, RI = 0.70, RC = 0.41.)

arietans and any other *Bitis* are not supported by significant bootstrap values. The monophyletic groups within *Bitis* found by all tree construction methods correspond to subgenera defined in a previous study (Lenk *et al.*, 1999). They are identical to species groups that had already been distinguished morphologically (Groombridge, 1986) and immunologically (Herrmann and Joger, 1997).

Cerastes. The specialized genus is a species-poor but distinct monophyletic viperine assemblage (supported by 95–100% bootstrap). The internal phylogeny of *Cerastes* was consistent in all analyses. *C. vipera* was the sister group of *C. gasperetti* and *C. cerastes* (Figs. 1 and 2). Genetic distances among *Cerastes* species varied between 9.4 and 11.2% (GTR).

Echis. The taxonomy of this medically important genus is unclear. Originally, the species *E. coloratus* and *E. carinatus* were the only species recognized. Today, some workers follow the system of Cherlin (1990) who split *E. carinatus* into a number of species, whereas others take intermediate positions. In our analysis, we consistently found three groups. These were (1) *Echis coloratus,* (2) a clade comprising the Asian *E. carinatus* and *E. multisquamatus,* and (3) another clade consisting of *Echis* sp., *E. pyramidum,* and *E. ocellatus.* These groups were well supported by significant bootstrap values. Intrageneric genetic distances ranged from 0.8 to 15.8%. The groups within *Echis* make sense with respect to biogeography, as Asian species are clearly distinct from Afro–Arabian species and confirm those groups defined by Cherlin (1990). An undefined *Echis* sample from the Arabian peninsula (*Echis* sp.—possibly *E. khosatzkii*) revealed a striking distinctness relative to its near relatives, the African *E. pyramidum* and *E. ocellatus.*

Eurasian viperines. All our analyses produced almost identical phylogenetic patterns for the Eurasian viperines; only two exchangeable tree positions were observed (*V. ammodytes* versus *V. dinniki*) (Figs. 1 and 2). The extent of the genetic distance within this group was up to 16.5%.

The Turanian *Eristicophis* and *Pseudocerastes*

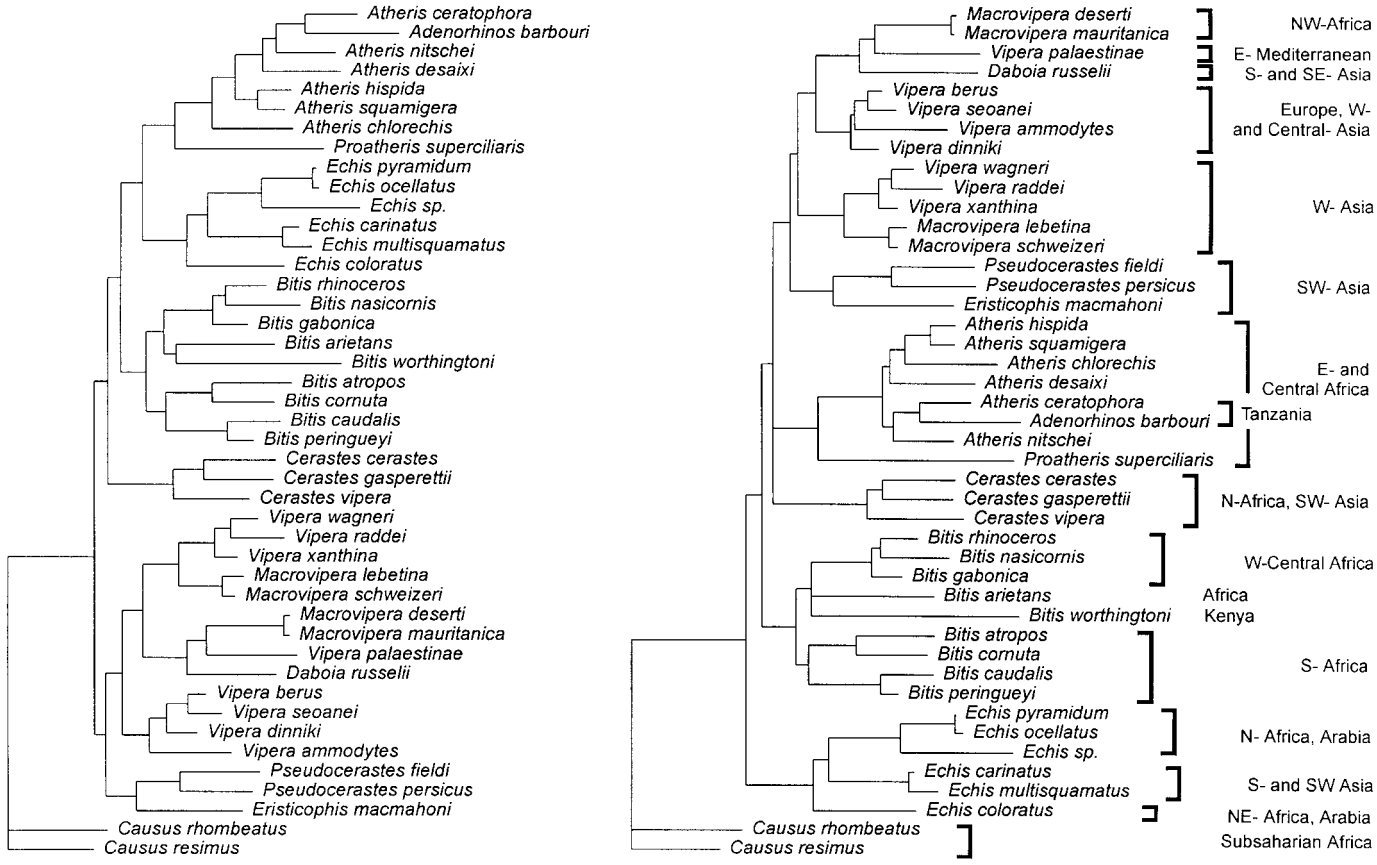

FIG. 2. Maximum-likelihood trees with *Causus* serving as outgroup. (Left) Unweighted maximum-likelihood phylogram (one single tree was obtained) using the default settings of the program. (Right) Weighted maximum-likelihood phylogram. The analysis, which considers the six substitution types of GTR (Yang, 1994), the gamma-distributed character site rates (shape parameter = 0.63), and the proportion of invariable sites = 0.49, yielded a single most likely tree. Bootstrap analyses were not performed because of computational constraints. The geographical main distributions of clusters are indicated on the right.

formed a monophyletic clade (maximum of 99% bootstrap support) and clustered as the sister of *Vipera* s.l. Within *Vipera* s.l., three clades seemed to be well supported in all analyses: (1) the small Eurasian *Vipera* species (including both the *aspis* complex represented by *V. ammodytes* and the *Pelias* complex represented by *V. berus* and *V. seoanei*), (2) the *V. xanthina* and *Macrovipera lebetina* complex of the Middle East, and (3) the big African–Oriental species.

Within the small viper clade (1), *V. dinniki* was almost consistently the sister of *V. berus* and *V. seoanei*, and *V. ammodytes* was the basal offbranch. In the second cluster (2), *M. lebetina* and *M. schweizeri* formed a sister clade to the mountain adders, with *V. xanthina* as sister to the *V. raddei* and *V. wagneri* pair. Within the third group (3) only two African species, *Macrovipera mauritanica* and *M. deserti* appeared to be closely related. Genetic distances between either of them and *D. russelii* or *V. palaestinae* were large (Figs. 1 and 2).

According to morphological analyses, the Eurasian viperines appeared either as a polyphyletic (Ashe and Marx, 1988) or as a monophyletic (Groombridge, 1986; Herrmann *et al.*, 1999) group. Our data clearly support monophyly, but the particular topology in which *Vipera* s.l. came up as sister group to the Turanian *Eristicophis–Pseudocerastes* clade is a new feature.

The affiliation among *Daboia russelii, Vipera palaestinae,* and the North African *Macrovipera* on one hand and the relationships of the *M. lebetina* complex with the subgenus *Montivipera* (Nilson *et al.*, 1999) on the other hand need discussion. *D. russelii* and *V. palaestinae* had already been placed together in a common genus by Groombridge (1986) because they share apomorphic similarities in snout shape and scalation of the nasal region, head color pattern, and reduction of the peritoneal pigmentation. North African *Macrovipera* resemble *Daboia* in head pattern and in high numbers of midbody scale rows (27 as opposed to 23–25 in Middle East *Macrovipera*). The relationships between *Montivipera* and the *M. lebetina* complex are in congruence with osteological findings (Szyndlar and Rage, 1999) and are biogeographically well founded. Apparently, current *Vipera* and *Macrovipera* represent

polyphyletic assemblages. We propose to restrict "*Macrovipera* (Reuss, 1927)" to *lebetina* and *schweizeri*, whereas the genus *Daboia* (Gray, 1842) should be assigned to *russelii, palaestinae, mauritanica,* and *deserti.*

After the separation of the above-mentioned species/genera, the European *Vipera* would remain as a monophyletic genus, *Vipera* s.str. As this is in good agreement with immunological data (Herrmann *et al.*, 1992), the morphologically based alternative, polyphyly or paraphyly of *Vipera* s.str. (Ashe and Marx, 1988), can be rejected. The morphological assignment was mainly due to interpretation of small head scales as a shared derived character of non-European *Vipera.* We should rather accept the more plausible hypothesis of independent parallel subdivisions of large head scales in different viperine groups.

Macrophylogeny of Viperines

Although all analyses virtually recovered a framework of five main clades (Atherini, *Bitis, Cerastes, Echis,* and Eurasian vipers), they did not resolve a consistent evolutionary pattern of the deep viperine evolution. Also, the use of more distant outgroups (*Dinodon* or *Azemiops*) did not improve the resolution of the phylogenetic reconstructions (not shown). Character weightings, indicated by bootstrap values and improved consistency indices (Fig. 1), showed a slightly increased resolution compared to the uncorrected approaches, but basal tree topologies were largely incongruent under the weighted MP and ML.

For example, both MP and ML changed the most basal OTU from Eurasian vipers (unweighted) to *Echis* (weighted). The basal position of *Echis* is now supported by 84% bootstrap in MP, whereas the bootstrap value of the basal position of Eurasian vipers was less than 50% in the uncorrected reconstructions (Fig. 1). A similar pattern arose for the monophyly of the Eurasian vipers, which were better resolved in the weighted MP approach (86% bootstrap support). This might indicate an improved resolution of the deep cladogenesis under the weighted aproaches, but the effect seems to be too weak to yield a clear picture of that part of viperine evolution, as indicated by bootstrap values still below the significance level (Fig. 1) and incongruences in other parts of both trees.

Considering the current state of the debate on viperine evolution (summarized by Herrmann *et al.*, 1999), none of our alternative trees is fully consistent with any previously published phylogenetic hypothesis. However, our data support the monophyly of Eurasian viperines as found by morphological (Groombridge, 1986; Herrmann and Joger, 1997) and immunological (Herrmann and Joger 1995) studies. A stronger partition into a Eurasian and an African clade (Detrait and Saint-Girons, 1979; Groombridge, 1986; Herrmann and Joger, 1997) was realized in our unweighted approaches only. The affiliation of *Echis* and *Cerastes* (Joger and Courage, 1999) based on the common occurence of serrated "rattling" scales could not be confirmed here. Thus, our data stabilize certain proposed phylogenetic groupings and provide additional input into the ongoing debate on viperine phylogeny.

To rule out possible disturbances by "problematic taxa" on phylogeny reconstructions, we constrained further searches to a tree backbone, which allowed rearrangements only among entire clades of *Atheris, Adenorhinos, Bitis, Cerastes, Echis,* Eurasian vipers, and *Proatheris.* However, neither consistency nor statistical stability were significantly enhanced (results not shown).

We conclude, based on mtDNA sequences, that the early cladogenesis cannot be resolved by our data set. This could be due to methodical terms (e.g., a reduced information content of our data set caused by saturated sites) or alternatively to a rapid radiation that took place within a short time period. In the latter case, the observed polytomy would be a plausible consequence. It is well known that evolutionary innovations that open up new niches can cause sudden radiations. Envenoming prey by a single strike is undoubtedly an example of a very successful invention in viperid snakes. Perhaps the hypothesis of an explosive diversification provides the key to the controversal debate on viperid phylogeny as produced by studies of internal and external morphology (Ashe and Marx, 1988; Groombridge, 1986; Marx and Rabb, 1965), immunoelectrophoretic investigations on venom proteins (Detrait and Saint-Girons, 1979), and immunological studies on serum albumins (Herrmann and Joger, 1997; Herrmann *et al.*, 1992).

However, we would like to emphasize patterns of genetic divergence and statistical cohesion of clades to reveal some cornerstones in this part of viperine evolution. Several clades, such as *Atheris, Cerastes,* and *Echis,* were statistically better supported than others, such as Atherini, *Bitis,* and Eurasian viperines. This pattern is paralleled by low average genetic distances within *Atheris, Cerastes,* and *Echis* and high distances within *Bitis,* Atherini, and the Eurasian viperines. This may indicate that the radiations of *Echis, Cerastes,* and *Atheris* are historically younger than those of Atherini, *Bitis,* and the European viperines. This is obviously accompanied by ecological features, as the diverse clades virtually all show specializations for various biomes and the less diverse clades are typically adapted to a single biome.

The Origin of Viperines

From a biogeographical point of view, the true vipers should have evolved mainly in Africa. In addition to the fact that the related Causinae are endemic to Africa, all the main lineages identified here either live entirely

in Africa or are represented on that continent by at least some species.

However, this feature is not supported by the fossil evidence. The oldest records stem from the early Miocene of Europe. In Africa, fossil viperines did not emerge until the middle Miocene, after Africa was already connected to Eurasia. Did the viperines therefore originate in Eurasia rather than in Africa? As shown by Szyndlar and Rage (1999), the oldest records of the early European Miocene already represented recent genera and were exclusively assigned to *Vipera*. Hence, it is obvious that the basal evolution of viperines already had taken place when the contemporaries of the oldest known fossils lived. Nevertheless, these fossils are good indicators for dating the emergence of the viperines to pre-Miocene times, probably to the Oligocene period (Szyndlar and Rage, 1999). During that time period Eurasia and Africa were still separated by a shallow sea, the narrowing Tethys. However, exceptional dispersal events via the sweepstake route across the Tethys, as reported for mammals during the Eocene and Oligocene (Bown and Simons, 1984; Coryndon and Savage, 1973), could also be assumed for viperines. This perspective would be in agreement with both hypotheses that the ancestors of all Viperidae (including Crotalinae and Azemiopinae) emerged in Asia (or India) and subsequently invaded Africa or vice versa. This obviously happened before Africa came into contact with Eurasia in the Miocene (Rögl and Steininger, 1983).

In our view, it is most likely that the Causinae and Viperinae diverged from each other in Africa during the Oligocene (see also Herrmann and Joger, 1997). The lack of fossils from that time period in Africa has been noted also for other terrestrial vertebrates (e.g., birds and mammals; see Peters and Storch, 1993). The possession of an effective venom apparatus allowed an adaptive radiation and a rapid dispersal across the biomes of Africa where no competitors (Crotalinae) were present. This first diversification might have led to the five main clades. It was followed by the development of the basal branches within the Atherini, *Bitis,* and the Eurasian vipers and, later on, the diversification within the genera *Atheris, Cerastes, Echis,* and *Vipera* s.l.

Eurasia was invaded only by members of *Cerastes, Echis,* and the Eurasian vipers. The biogeographically well-founded trifurcation within *Vipera* s.l. could have been favored due to a geographical separation of the three landmasses Europe, Middle East, and North Africa by the Mediterranean and Paratethys during the early Miocene (Rögl and Steininger, 1983). Accordingly, *Vipera* s.str. was restricted to the north, the related *Montivipera* and *lebetina* complexes occurred in the Middle East, and the ancestors of *Daboia, V. palaestinae,* and the African *Macrovipera* inhabited the areas south to the Mediterranean. Evidence for a former continuous distribution of *Daboia* across North Africa to Spain is provided by the discovery of the Pliocene *Vipera maxima,* a species very similar to extant *D. russelii* (Szyndlar, 1988) in Layna (Spain).

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