

# Phylogenetic relationships among European ratsnakes of the genus *Elaphe* Fitzinger based on mitochondrial DNA sequence comparisons

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**Abstract.** In order to elucidate the phylogenetic relationships in European ratsnakes of the genus *Elaphe*, we analyzed a 597 bp part of the mitochondrial cytochrome *b* gene of eight West Eurasian and one East Asian species. *Lampropeltis* served as outgroup. Maximum parsimony and maximum likelihood suggest the existence of four lineages: 1) *E. scalaris*; 2) the *E. longissima* species group comprising *E. longissima*, *E. lineata*, *E. situla*, *E. hohenackeri*, and *E. persica*; 3) *E. quatuorlineata* and 4) *E. dione* as a sister group to 3). *Elaphe scalaris* is basal and shows no closer affiliation with any other analyzed species. The Middle Eastern *E. persica* and *E. hohenackeri* appear basal within the *E. longissima* group. *Elaphe lineata* differs by 8% nucleotide substitutions from *E. longissima*, supporting the hypothesis that both taxa represent distinct species. *Elaphe situla* is associated with *Elaphe longissima* and *E. lineata*. Three analyzed subspecies of *E. quatuorlineata* are represented by distinct haplotypes. The extent of divergence gives reason to assign species status to the taxon *sauromates*. Besides, we found two very distinct haplotypes within the range of *E. (q.) sauromates*, indicating the existence of a third, so far unidentified, species within the *E. quatuorlineata* complex. The East Asian *E. porphyracea* clusters with the *E. longissima* group. This, as well as comparisons with supplementary sequences of Asian *Elaphe* species, document the multiple origins of European *Elaphe*.

**Zusammenfassung.** Um die phylogenetischen Beziehungen europäischer Kletternattern der Gattung *Elaphe* zu erhellen, sequenzierten und analysierten wir ein 597 Nukleotide messendes Stück des mitochondrialen Cytochrom *b* Gens von acht west-eurasischen und einer ostasiatischen Art. *Lampropeltis* diente als Außengruppe. Maximum parsimony und maximum likelihood Berechnungen zeigen die Existenz von vier genetischen Linien auf: a) *E. scalaris*; b) die *E. longissima* Artengruppe mit *E. longissima*, *E. lineata*, *E. situla*, *E. hohenackeri*, und *E. persica*; c) *E. quatuorlineata* und d) *E. dione* als Schwestergruppe zu c). *Elaphe scalaris* nimmt eine basale Position ein und scheint mit keiner untersuchten Art näher verwandt zu sein. Die westasiatischen Arten *E. persica* und *E. hohenackeri* sind die Schwestergruppe zu *E. longissima*, *E. lineata* und *E. situla*. *Elaphe lineata* unterscheidet sich durch 8% Nukleotidaustausche von *E. longissima*, wodurch der Artstatus von *E. lineata* unterstützt wird. *Elaphe longissima*, *E. lineata* und *E. situla* bilden eine Abstammungsgemeinschaft. Auch die Haplotypen der drei untersuchten *E. quatuorlineata*-Unterarten unterscheiden sich so stark, daß Artstatus für das Taxon *sauromates* zu fordern ist. Darüberhinaus fanden wir im Gebiet von *E. (q.) sauromates* zwei stark unterschiedliche Haplotypen, die die Existenz einer dritten bislang unidentifizierten Art nahelegen. Die ostasiatische *E. porphyracea* scheint der *E. longissima*-Gruppe nahe zu stehen. Dies, sowie der Vergleich mit ergänzenden Sequenzdaten asiatischer *Elaphe*-Arten, belegt den mehrfachen Ursprung europäischer *Elaphe*.

## Introduction

Ratsnakes of the genus *Elaphe* (Fitzinger, 1853) have three distribution centres: North America, Southeast Asia and Europe with parts of West Asia. The latter assemblage, here called “European ratsnakes”, comprise eight European and West Asian species. Mertens and Müller (1928) were the first who established a solid nomenclature of this group, and stopped the confusing accumulation of synonyms of the 19th and early 20th century. The taxonomy of Mertens and Müller (1928) persists up to date, except that the former subspecies *E. longissima persica* (Werner, 1913) and *E. longissima romana* (Suckow, 1798) are now considered distinct species: *E. persica* and *E. lineata* (Camerano, 1891) respectively (Nilson and Andren, 1984; Lenk and Wüster, 1999).

Available data on intrageneric relationships of ratsnakes are rare and scattered in the literature. Before Mertens and Müller, three groups, sometimes treated as genera (De Betta, 1874; Strauch, 1874; Bedriaga, 1879; Mojsisovics, 1888; Camerano, 1891), had been recognized within the European *Elaphe*: 1) *Elaphe scalaris* (Schinz, 1822) as sole representative of the genus *Rhinechis*; 2) *Elaphe longissima* (Laurenti, 1768) / *E. situla* (Linnaeus, 1758) / *E. hohenackeri* (Strauch, 1873) often attributed to *Coluber* (or *Callopetlis*); and 3) *E. dione* (Pallas, 1773) / *E. quatuorlineata* (Lacépède, 1789) in the combination with the genus name *Elaphis*. This classification was based on head scalation patterns (Schreiber, 1912) and is supported by more recent workers (Nilson and Andren, 1984; Schulz, 1995). These pholidosis types, characteristic in western Palaearctic *Elaphe* species (Schulz, 1995), are not unique within the genus as a whole and their taxonomic value needs to be confirmed on a broader basis.

The position of *E. scalaris* within the genus remains controversial. Lawson and Dessauer (1981) suggested a basal position of *E. scalaris* within the entire genus because of a striking distinctiveness of plasma proteins between *E. scalaris* and the Asian *E. radiata*. Dowling et al. (1983) noted that *E. radiata* itself is an atypical representative of *Elaphe* distinct from other forms. A closer relationship of *E. scalaris* to European lineages was stated by Schulz (1995) who indicated several morphological similarities between the latter species and *E. hohenackeri*, *E. persica* and *E. situla*.

Further questions emerge considering the phylogeny at the species and subspecies level. In an earlier study we showed that all western Eurasian species can be readily distinguished by specific electrophoretic patterns of plasma proteins (Lenk and Joger, 1994). But subspecies of *E. quatuorlineata* and *E. longissima* exhibited such distinct patterns as well. In the case of *E. longissima*, electrophoretic results were concordant with differences in morphology and resulted in separating *E. lineata* as a distinct species (Lenk and Wüster, 1999). The taxonomic ranks of *E. quatuorlineata* subspecies, however, are still under discussion (Werner, 1932; Böhme and Ščerbak, 1993; Clark, 1994; Hingley, 1995).

In order to reveal the phylogenetic affinities among these *Elaphe* taxa, we analyzed nucleotide sequences of cytochrome *b*, a protein-coding mitochondrial DNA gene, which has become a powerful tool in animal systematics (Brown, 1983; Avise et al., 1987;

Mindell and Thacker 1996). Cytochrome *b* plays an important role in energy control of the cell. This functional constraint has a conserving effect on the evolution of the protein's structure or its gene respectively. Nucleotide substitutions which occur at sites of lesser functional constraints are, however, frequently observed. These sites evolve faster (Kimura, 1983) and hence provide important sources of phylogenetic information.

## Material and methods

**Sampling.** Samples were derived from 15 specimens of the eight western palaeartic *Elaphe* species, including most of the recognized subspecies (table 1). An East Asian species (*Elaphe porphyracea*) was included to test whether the European taxa are monophyletic. *Lampropeltis triangulum* served as outgroup as this genus is considered to be related to *Elaphe* (Dowling et al., 1983; Lopez and Maxson, 1996). Additional sequences of some Asian species were obtained from Genbank entries AF036010-21.

Blood samples were obtained by caudal vein puncture as described in Joger and Lenk (1995) or from dissected ethanol preserved animals. Samples were stored in 95% ethanol or EDTA buffer (10% EDTA, 0.5% sodiumfluoride, 0.5% thymol, 1% tris, pH 7.0) (Arctander, 1988). Total genomic DNA was extracted from 50 to 100  $\mu$ l aliquots following standard proteinase K and phenol chloroform protocols (Sambrook et al., 1989).

**PCR and sequencing.** Polymerase chain reaction (PCR) was used to amplify a roughly 700 bp portion of the mitochondrial cytochrome *b* gene. Amplifications were performed with 25 pmol of primers L-14846 (5'-CAACATCTCAGCATGATGAACTTCG-3') and H-15555 (5'-AATAGGAAGTATCATTCGGGTTGTG-3'). Typically a reaction mixture of 50  $\mu$ l volume containing 25 pmol of each primer, 2 units of DNA polymerase (Amersham Pharmacia Biotech), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris-HCl (pH 9), 0.02% BSA, and approximately 2  $\mu$ g template snake DNA was prepared and subjected to the following cycling program: initial denaturing step for 5 min at 95°C, 32 cycles with annealing 45 s at 47°C, primer extension 120 s at 72°C, and denaturing 45 s at 95°C. PCR products were sequenced using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech). For cycle sequencing a two stage program containing an initial denaturing step at 95°C for 2 min and ten cycles at 45°C (45 s), 72°C (120 s) and 95°C (45 s) followed by 15 cycles of 60°C (120 s) and 95°C (45 s) denaturing was used. The sequencing primers employed are CY5-L-14846, CY5-H-15555, and CY5-H-15149 (5'-CCTCAGAATG ATATTTGTCC TCA-3'). PCR-products were processed in an automated sequencer (Amersham Pharmacia Biotech, ALF-Express II). Sequences of 597 nucleotides in length were obtained directly from ALF-Express and aligned manually. Deletions, insertions or inversions were not encountered. The nucleotide sequence data reported in this paper are deposited in the DDBJ/EMBL/GenBank Nucleotide Sequence Database under accession numbers AJ277670-AJ277686.

**Phylogenetic analysis.** All phylogenetic calculations were done using the program package PAUP\*4.0b3a (Swofford, 1998). DNA sequences compared among organisms may contain phylogenetic signal, or they may be randomized with respect to phylogenetic history. We tested the phylogenetic signal with the g1 statistic of Hillis and Huelsenbeck (1992) for skewness of tree length distributions, estimated from 10,000 random trees. A significant result indicates that the tree length distributions of the dataset are skewed to the shorter lengths, whereas those composed of random noise are closer to symmetry. Critical values are provided in Hillis and Huelsenbeck (1992). Maximum parsimony and maximum likelihood were employed as optimality criteria to infer the phylogenetic relationships with heuristic searches. The selection of two different methods (and philosophies) warrants sufficiently different perspectives for phylogenetic inferences and congruent results can be considered meaningful.

In parsimony analysis the influence of random noise was minimized with the successive approximation approach after Farris (1969). The principle of this method is to give weights to characters according to their consistency within the current calculation. Thus the rescaled consistency index which represents a linear measure of consistency was determined for each character with the 'reweight' option of PAUP\*4b3a and attributed as correction factor for a successive phylogeny calculation. This calculation was iteratively continued until the rescaled consistency index could not be improved any further. Additional indices were used as descriptive

**Table 1.** Localities, vouchers, and accession numbers of specimens used in this study. NMW = Naturhistorisches Museum Wien. DNA voucher samples are available after request at the Institut für Pharmazeutische Biologie, Universität Heidelberg. Because of nature conservation restrictions, most samples were collected on free ranging or captive specimens (photographic documentation).

Taxon	Origin	Voucher specimen	Voucher DNA	EMBL/GenBank/DBJ accession number
<i>Elaphe dione</i> 1	Asia-central	specimen alive, collection P. Lenk	5073	AJ277683
<i>Elaphe dione</i> 2	Kazakhstan, SW of Alma Ata	–	5075	AJ277684
<i>Elaphe hohenackeri</i>	Turkey, Aydin, near Karak/Kagizman, Aras valley	NMW 33583	5076	AJ277670
<i>Elaphe lineata</i>	Italy/Vesuvius	specimen alive, collection P. Lenk	5085	AJ277674
<i>Elaphe longissima</i> 1	France/Villiers en Bois	–	5138	AJ277671
<i>Elaphe longissima</i> 2	Turkey/Ararat (Aralik, Igridir)	NMW 33394	5131	AJ277672
<i>Elaphe persica</i>	Azerbaijan/Lenkoran	specimen alive, collection K.-D. Schulz	5147	AJ277673
<i>Elaphe porphyracea</i>	China/Zhejiang province	Gernot Vogel	5148	AJ277685
<i>Elaphe quatuorlineata quatuorlineata</i>	Italy/Sassod'Ombrone	–	5159	AJ277681
<i>Elaphe quatuorlineata muenteri</i> 1	Greece, Amorgos “striped specimen”	specimen alive, collection K. Hingley	5144	AJ277680
<i>Elaphe quatuorlineata muenteri</i> 2	Greece, Amorgos “unstriped specimen”	specimen alive, collection K. Hingley	5151	AJ277679
<i>Elaphe quatuorlineata sauromates</i> 1	Turkey, Aydin, near Karak/Kagizman	specimen alive, collection M. Schweiger	5150	AJ277677
<i>Elaphe quatuorlineata sauromates</i> 2	Kazakhstan	specimen alive, collection K. Hingley	5166	AJ277678
<i>Elaphe scalaris</i> 1	Portugal-central	–	5168	AJ277675
<i>Elaphe scalaris</i> 2	Spain	specimen alive, collection R. Butler	5167	AJ277676
<i>Elaphe situla</i>	Black Sea coast	specimen alive, collection E. Thiel	5170	AJ277682
<i>Lampropeltis triangulum</i>	SW-Sonora, Sinaloa, SW-Chihuahua (Mexico)	–	5177	AJ277686

parameters of the goodness of parsimony trees (Kluge and Farris, 1969; Farris 1989): consistency index (CI), homoplasy index (HI), retention index (RI). To examine the statistical robustness of tree branches bootstrap tests using 1000 pseudoreplicates were performed.

Maximum likelihood assumptions were derived from an evolutionary model selected with the PAUP-associated program MODELTEST (Posada and Crandall, 1998). The program performs comparisons of different evolutionary models on a given DNA data set based on the likelihood ratio test statistic. The best fitting model is selected and suggestions for character site weightings are made.

For additional information we included some sequences of Asian species obtained from Genbank (see above). The combination of the two datasets produced an overlap of 263 nucleotide sites which were subjected to parsimony analysis, only.

## Results

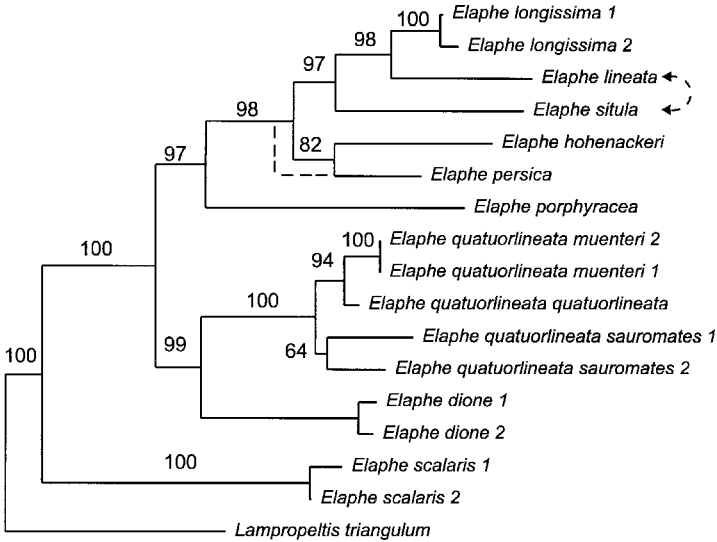
The dataset consisted of 597 characters of which 374 were constant, 55 variable but not informative, and 168 parsimony informative. The skewness of 1000 randomly chosen trees of our dataset was  $g_1 = -0.94$  ( $P = 0.01$ ) suggesting a significant phylogenetic signal within the cytb sequences (Hillis and Huelsenbeck, 1992).

The uncorrected maximum parsimony approach produced six equal shortest trees each of 503 steps in length (not shown, consistency index (CI) = 0.56, homoplasy index (HI) = 0.44, retention index (RI) = 0.63, rescaled consistency index (RC) = 0.35). The resolution of this method was remarkably improved under the successive approximation approach (fig. 1). This was indicated by bootstrap test as well as consistency indices which exhibited better results when homoplastic characters were downweighted. In bootstrap analyses all clades but three were supported at significance levels > 95%.

As most appropriate evolutionary model the Tamura Nei model (Tamura and Nei, 1993) was suggested by MODELTEST. Character site rates were assumed to follow a gamma distribution with the shape parameter = 0.2133. The maximum likelihood analysis was adjusted with these settings and yielded a tree that showed an almost identical topology with the maximum parsimony tree, with the exception that *E. hohenackeri* and *E. persica* did not cluster as sister species and *E. situla* was closer to *E. longissima* than *E. lineata* (fig. 1).

The two different approaches consistently identified three species groups arranged as follows. *Elaphe scalaris* was always the most basal taxon and was remarkably differentiated from the remaining species (to a similar extent as the outgroup from the ingroup). A second group consisted of *E. dione* and *E. quatuorlineata*. *Elaphe longissima*, *lineata*, *situla*, *persica*, and *hohenackeri* are recognized as the third group. The association of *E. porphyracea* to the *E. longissima* group indicated the deep phylogenetic divisions of European *Elaphe* species. A sister relationship between the West Asian *E. hohenackeri* and *E. persica*, as suggested by the parsimony tree, was not supported by maximum likelihood; however both taxa unequivocally shared common ancestry with the *E. longissima* / *lineata* / *situla* group.

When some more Asian species were added to the analysis, *E. dione* clustered with *E. bimaculata* as evidenced by 100% bootstrap value (fig. 2) and the monophyly *dione* /



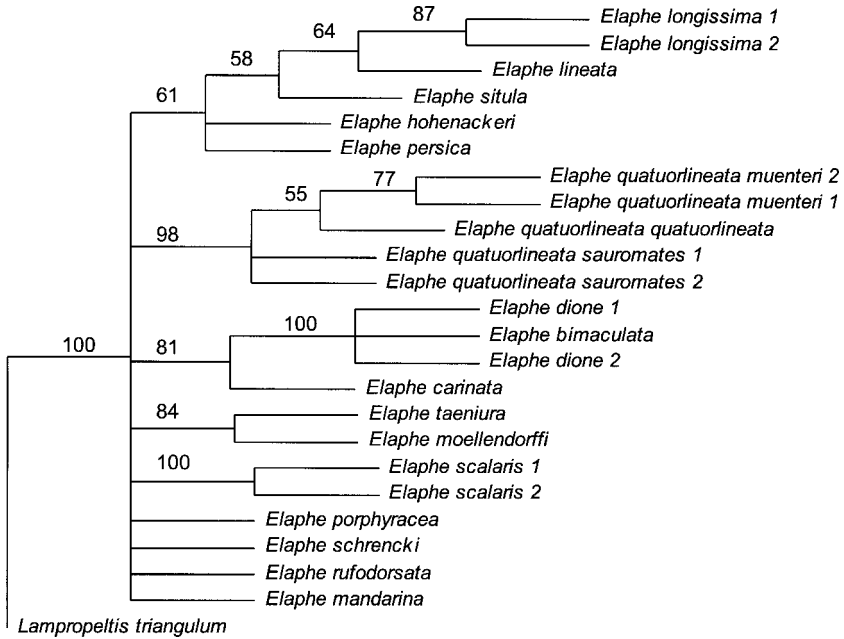
**Figure 1.** Maximum parsimony phylogram of European *Elaphe* species. A heuristic search (after the successive approximation approach of Farris (1969) with *Lampropeltis* serving as outgroup) resulted in a single tree. Bootstrap confidence limits after 1000 pseudoreplicates are added (tree length score: 200, CI = 0.82, HI = 0.18, RI = 0.83, RC = 0.68). Changes in tree topology under maximum likelihood are indicated by dashed lines.

*quatuorlineata* was no longer supported. Beside this no more inferences could be drawn from that dataset because of its limited information content (263 characters only).

## Discussion

### *Genetic relationships between Eurasian Ratsnakes*

The European ratsnakes are a polyphyletic group which is deeply split into different clades. The *E. longissima* group is divided into two basal species restricted to the Middle East, *E. hohenackeri* and *E. persica*, and the three almost entirely European species *E. longissima*, *E. lineata* and *E. situla*. The two Middle Eastern species are not closely related as indicated by long internodes (fig. 1). Surprisingly, *E. persica*, which once was treated as conspecific with *E. longissima*, is not even a close relative of *E. longissima* (as mentioned in Nilson and Andren, 1984). Instead *E. situla* appears to be next associated with the *E. longissima* / *E. lineata* clade. *Elaphe lineata* of Southern Italy, which was formerly treated as a subspecies of *E. longissima*, differs remarkably from this species. Genetic distances between *E. lineata* and *E. longissima* are eight times higher (8.0-8.5%) than the distances found between the two *E. longissima* specimens from the western and eastern limits of the range (Western France and Western Caucasus, 1.02% nucleotide difference).



**Figure 2.** 50% Majority rule cladogram of 1000 bootstrap (maximum parsimony) pseudoreplicates containing additional *Elaphe* sequences from Genbank entries AF036010-21 (Wang et al., 1997) with *Lampropeltis* as outgroup. This analysis was based on 263 characters only.

Thus, mitochondrial sequence data support the view that *E. lineata* represents a distinct species.

*Elaphe dione* is the next western Palaearctic relative of *E. quatuorlineata*. This confirms the validity of the preocular pholidosis as taxonomic marker, though the affiliation of the two taxa is not very close and other Asian species could be closer related with *E. dione* (at least *E. bimaculata*). Its occurrence in eastern Europe might be due to a recent expansion from the East.

*Elaphe quatuorlineata* is another species with weak affinities to other European *Elaphe*. In this taxon, five subspecies have been recognized (see Böhme and Ščerbak, 1993; Cattaneo, 1998; Cattaneo, 1999). At least two of these subspecies were found to be relatively distinct as indicated by blood protein electrophoresis (Lenk and Joger, 1994). Our DNA analysis revealed the existence of three main clades within *E. quatuorlineata*. The first lineage consists of the nominate subspecies *E. q. quatuorlineata* plus *E. q. muenteri* from the Cyclade islands. The nucleotide sequence of a striped specimen from the island Amorgos was almost identical (one nucleotide difference) to that of a uniformly coloured morph from the same locality which had become known as '*Elaphe rechingeri*' (Werner, 1932; see also Clark, 1994; Hingley, 1995; Cattaneo, 1999). The second lineage consists of *E. q. sauromates* from Asia Minor and the third is represented by an *E. q. sauromates*

specimen from Kazakhstan. We like to emphasize that the deep divergence among these two *E. q. sauromates* haplotypes is of similar magnitude as between either of them and *E. q. quatuorlineata*. Thus, our data indicate the existence of an additional and hitherto unidentified taxon within the *E. quatuorlineata* complex.

The substantial genetic distances (5.0 to 6.5%) between *E. q. quatuorlineata* and *E. q. sauromates* revive the question whether *E. q. quatuorlineata* and *E. q. sauromates* actually present subspecies or full species as mentioned by Böhme and Ščerbak (1993). In comparison with other colubroid snakes, genetic distances would support species status for these taxa (Joger et al., 1997). The parapatric distribution pattern with a narrow contact zone in northern Greece (Böhme and Ščerbak, 1993) may also indicate reduced genetic compatibility between the two taxa (Barton and Hewitt, 1989). Subspecies are typically separated by extrinsic (geographical) barriers rather than by intrinsic (genetic) barriers which separate species (Avise, 1994; Joger et al., 1998). This situation in combination with our data suggests that the taxa *quatuorlineata* and *sauromates* should be treated as species. As a consequence the undescribed taxon would also take the rank of a species.

*Elaphe scalaris* always branched off at the base of the *Elaphe* tree and apparently represents a solitary phylogenetic lineage within the European stock. The observed genetic distances between *E. scalaris* and the remaining species imply that *E. scalaris* is a highly divergent representative of the European ratsnakes. This is congruent with the unique pholidosis of the snout region of this species, and supports the hypothesis of Minton (1976) that *E. scalaris* might represent a basal lineage within *Elaphe*.

#### *Stages of Elaphe evolution and an attempt at a molecular clock*

Fossil remains of *Elaphe* are comparatively frequent and have been reported from numerous sites in Europe (Szyndlar, 1991). They provide an opportunity to calibrate the molecular clock for *Elaphe*. Records belonging to extant species can be shown down to the mid-Pliocene 4.5 million years ago, when the osteological characters of ancient *E. longissima* already resembled those of the present form. Two putative ancestors of recent taxa are known from the uppermost Miocene 6.5-5.5 million years ago: *E. algorensis* and *E. praelongissima*. *Elaphe algorensis* has been considered an ancestor of *E. scalaris* (Ivanov, 1997) and *E. praelongissima* as an ancestor of *E. longissima*, *E. lineata* and *E. situla*.

Suggesting that *E. praelongissima* is the real ancestor of *E. situla* and *E. longissima/lineata*, these findings imply that *E. longissima* and *E. situla* have diverged in the early Pliocene, probably earlier. Dating the split to 5 million years ago, an average evolutionary rate of approximately 2.4% nucleotide substitutions per 1 million years can be obtained for the mitochondrial cytochrome *b* gene of these snakes (12.1% divergence). The separation of Amorgos / Cyclades from the Greece mainland which probably happened in the early Pleistocene (Schröder, 1986) could have been linked with the splitting of *E. q. quatuorlineata* and *E. q. muenterii* (3.4% nucleotide divergence). The dating of further cladogenetic events appears unreliable at present. Given this calibration, the basal

divergence of the *Elaphe* phylogeny would have happened in the early Miocene, the period of mass radiation of the Colubridae as evidenced by the fossil record (Rage, 1987).

### *Phylogeography of ratsnakes*

Taking into account the phylogeography of the European ratsnakes, it is evident that they do not form a monophyletic group (fig. 1). The European *Elaphe* fauna may stem from at least two, probably three or four, different invasions. This also holds when our data are compared with additional DNA sequences of Asian *Elaphe* species (Wang et al., 1997). Although the joined alignment was shorter than the initial one and, as a consequence, proved to be less informative, the combined analysis confirmed the monophyly of the *E. longissima* species group as well as the distinctness of *E. scalaris*, but uncovered a close relationship between (or even identity of) *E. dione* and *E. bimaculata* (fig. 2).

Thus among the western Palaearctic *Elaphe*, four phylogenetic lineages become apparent: 1) *E. scalaris*; 2) the *E. longissima* group with *E. longissima*, *E. lineata*, *E. situla*, *E. hohenackeri*, and *E. persica*; 3) *E. quatuorlineata* and 4) *E. dione* as sister group (if other Asian species were not considered) of 3). We conclude that the *E. longissima* species-group represents the sole indigenous western Palaearctic radiation. Affinities between this lineage and other European species like *E. quatuorlineata* or *E. dione* may exist, but the internal position of the East Asian taxon *E. porphyracea* supports the hypothesis of multiple (Asian) origins of European ratsnakes.

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