Biological Journal of the Linnean Society, 2006, **87**, 127–143. With 5 figures

Evolution and phylogeny of the genus *Natrix* (Serpentes: Colubridae)

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Received 14 May 2004; accepted for publication 1 February 2005

Some aspects of the natural history of snakes of the colubrid genus *Natrix* have been well studied. With their extensive European distribution and relative abundance, their ecology, reproduction and behaviour are well known. Yet other facets of their biology remain poorly understood. These include knowledge of *Natrix* phylogeny, hypotheses explaining the current distribution of the three extant members of the genus, and their evolution and relationships. In this study we used molecular data, the nucleotide sequences of four protein-coding mitochondrial genes (3806 bp total), to provide a well-supported phylogeny for the genus *Natrix*. With these molecular data, evidence from the fossil record, and knowledge of palaeogeological events, we used two approaches in designing a time scale which we used to date the major events in *Natrix* speciation and intraspecific variation. Our data strongly support a phylogeny for the genus in which *N. maura* is basal with *N. natrix* and *N. tessellata* being sister species. The calibrated molecular clock suggests that *N. maura* diverged from the common ancestor of the three species 18–27 mya and that *N. natrix* and *N. tessellata* diverged 13–22 mya. Although the ranges of these estimates are large they support an early Miocene to late Oligocene origin for the three species. Intraspecific divergence is estimated to have commenced 5.3, 6.0 and 6.7 mya with evolutionary rates of 1 : 1.25 : 1.35% per million years for *N. maura, N. natrix* and *N. tessellata*, respectively. *Biological Journal of the Linnean Society*, 2006, **87**, 127–143.

ADDITIONAL KEYWORDS: cytochrome *b* – molecular clock – mtDNA – ND1 – ND2 – ND4 – snakes – systematics.

INTRODUCTION

A BRIEF HISTORY OF *NATRIX* SYSTEMATICS

Following its establishment (Laurenti, 1768) the genus *Natrix* was, prior to revisions commencing in 1958, a large group which included rather divergent taxa. When Taylor & Elbel (1958) assigned several east Asian species previously included within *Natrix* to the genus *Rhabdophis* the genus became more cohesive. With reassignment of additional Asian species to three more genera, *Natrix* was further reduced to include some 21 species with either Old World or New World distributions (Malnate, 1960).

Using data from immunological studies, karyology, and aspects of hemipenial morphology, Rossman & Eberle (1977) demonstrated a clear distinction between Old and New World taxa. The African species, *N. anoscopus*, was shown to be distinct and was placed in a new genus *Afronatrix,* with the remaining strictly Asian species of *Natrix* grouped into a new genus, *Sinonatrix*. New World taxa were assigned to several new genera and these, together with the remaining New World genera of natricine snakes, were grouped to form a monophyletic tribe, the Thamnophiini.

Now reduced to three well-established species, *Natrix* is restricted to Eurasia, with the grass snake *N. natrix* (Linnaeus, 1758) being the type species, and

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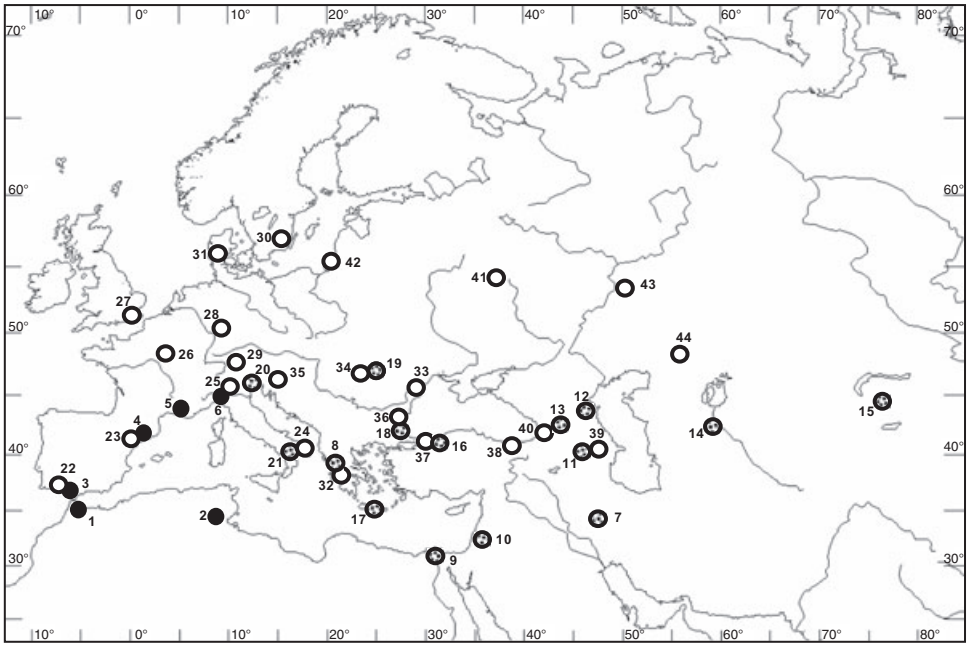

Figure 1. Localities for samples included in this study; black symbols represent localities of *Natrix maura*, dotted symbols *N. tessellata* and open circles *N. natrix*. Numbers refer to samples indicated in the Appendix.

the viperine snake *N. maura* (Linnaeus, 1758) and the dice snake *N. tessellata* (Laurenti, 1768) being its other representatives. A fourth species, *N. megalocephala* Orlow & Tunijew, 1987 has been described, but its validity remains open to question (Böhme, 1999).

N. natrix has both the greatest ecological tolerance (Mertens, 1947; Kabisch, 1999) and the most northerly distribution. It occurs in England and Wales, northern Scandinavia and Finland, and eastward through Siberia to Lake Baikal (Thorpe, 1984b; Kabisch, 1999), and is distributed throughout southern Europe and the Middle East, extending into Africa north of the Sahara (Kabisch, 1999). In most of the European countryside it can be the most commonly encountered snake species. Historically *N. natrix* has been divided into many subspecies (Hecht, 1930; Mertens, 1947; Nilson & Andren, 1981; see Kabisch, 1999 for a review). However, Thorpe (1975, 1979, 1984a, 1984b), after an extensive multivariate study of both internal and external morphological and meristic characters, argues for the recognition of but four subspecies. Two are island endemics, *N. n. corsi* and *N. n. cetti* of Corsica and Sardinia, respectively. The populations of the Eurasian mainland, North Africa and islands of the eastern Mediterranean Sea are divided into eastern, *N. n. natrix,* and western, *N. n. helvetica*, subspecies. These two subspecies, regarded by Thorpe (1984a) as semispecies or incipient species, have a rather narrow contact zone which extends from north-eastern Italy northward through western Germany to the North Sea west of the River Rhine (Thorpe, 1984b; Fig. 1).

N. tessellata and *N. maura* occupy similar ecological niches and are confined to aquatic or marshy habitats (Busack & Jaksic, 1982; Gruschwitz *et al*., 1999; Schätti, 1999). *N. maura* is monotypic and is restricted to the southern two thirds of France, the Liguria region of north-western Italy, the whole of the Iberian Peninsula and adjacent North Africa (Schätti, 1999). *N. tessellata*, with one weakly diverged subspecies, *N. t. heinrothi* from the Ukrainian Black Sea island of Ostrov Zmeinyi (Hecht, 1930), whose validity has been disputed (Gruschwitz *et al*., 1999), has a more extensive distribution. This includes southern Europe (except for Iberia and France), the countries of the Middle East and the Nile Delta and east across Kazakhstan into China (Bannikow *et al*., 1977; Gruschwitz *et al*., 1999).

N. megalocephala, if truly valid (see above) is monotypic and is confined to the mountains north-east and east of the Black Sea (Orlow & Tunijew, 1999).

N. natrix is sympatric with all of the other *Natrix* species over all or most of their distributions. *N. tessellata* and *N. maura* are almost parapatric with only a small range overlap in Liguria.

FOSSIL HISTORY OF THE GENUS *NATRIX*

The first records of the family Colubridae are from the late Eocene in Thailand (Rage & Auge, 1993; Ivanov,

2001). The oldest representatives of the family apparently invaded North America and Asia from south-east Asia. Central Europe was probably reached in the course of 'La Grande Coupure' across the Mazury–Mazowsze land bridge in present-day Poland, which separated the north European and south Ukrainian marine basins in the early Oligocene (Ivanov, 1997, 2001). At this time colubrid snakes, among them *N. mlynarski* from France [the earliest *Natrix* species known (Rage & Auge, 1993; Ivanov, 2001)], first appeared in the fossil record of Europe. During the early Miocene, the European snake fauna underwent massive adaptive radiation probably as a result of improving climatic conditions (Ivanov *et al*., 2000). During and following this period, several new species appeared in the fossil record, among them *N. sansaniensis*, *N. merkurensis* and *Palaeonatrix lehmani* (Rage & Auge, 1993; Ivanov, 2002a). The first rich European colubrid fauna appeared at the end of the early Miocene (Ivanov, 2002b), and it is presumed that these more advanced representatives of the Colubridae expanded outward from Asia and Africa into Europe (Ivanov *et al*., 2000).

A mid-Miocene record of a specimen closely resembling *N. longivertebrata* has been reported by Rage & Auge (1993). Considered a direct ancestor of the extant *N. natrix* by Szyndlar (1991a, 1991b), this specimen may represent an extinct subspecies of *N. natrix*, suggesting that *N. natrix* might have already existed in the middle Miocene (Ivanov, 1999, 2002a). During the Pliocene an impoverishment of the fauna occurred that can be correlated with ongoing climate deterioration (Rage & Auge, 1993; Ivanov *et al*., 2000). A number of representatives of extant colubrid species have been described from the mid-Pliocene and later (Szyndlar, 1991b), but, except for *N. longivertebrata* from the mid-Miocene, fossils of extant species of *Natrix* (*N. natrix*; Zerova & Chkjikvadze, 1984; Szyndlar, 1991a, and *N. tessellata* Zerova & Chkjikvadze, 1984; Markert, 1976) have not been recorded earlier than the upper Pliocene and Pleistocene.

Hypotheses for the evolutionary history of the extant *Natrix* species

Although the genus *Natrix* is undoubtedly monophyletic, its relationship to other natricine genera is not clear, nor are relationships among the three extant taxa. An east–west differentiation is observed in many European animal species, attributed to vicariant evolution in different Pleistocene refugia in southern Europe. These species and subspecies typically form hybrid zones where their ranges meet [e.g. *Bombina variegata*/*B. bombina*, *Lacerta viridis*/*L. bilineata*, *Corvus (corone) corone*/*C. (c.) cornix*]. Thorpe (1979, 1984a, 1984b) hypothesized Pleistocene vicariance as the cause for differentiation between the eastern and western mainland subspecies of *N. natrix*. A different scenario, however, has been proposed recently for the evolution of this species (Ivanov, 2002b). Referring to the supposed record of *N. natrix* from the middle Miocene, Ivanov has postulated a more ancient origin for the species, suggesting that divergence between the eastern and western subspecies might reflect independent invasions of Europe, one from the east and one from north-west Africa.

Considering the ecological adaptations, near parapatric distribution, and morphological similarities, *N. maura* and *N. tessellata* have often been described as sister species. Differentiation between the two seems to have been ascribed anecdotally to a similar scenario of Pleistocene vicariance as described above (K. Mebert, pers. comm.), even though there is apparently no reference for this hypothesis in the literature. In this context, the question of whether *N. maura* and *N. tessellata* hybridize under natural conditions, as is the case in other species with Pleistocene lineage divergence (e.g. Szymura, Uzzell & Spolsky, 2000), could be important. Hybridization has been documented between all three species combinations, but all records refer to observations in captivity, and there is no evidence for hybridization in nature (see Kabisch, 1999; Schätti, 1999). D. Guicking, U. Joger and M. Wink (unpubl. data) used genomic markers (ISSR-PCR) to investigate hybridization in more than 40 specimens of *N. tessellata* and *N. maura* from Lake Geneva, Switzerland, and north-west Italy, where both species are syntopic. None of the study specimens showed signs of hybridization, supporting earlier observations that hybridization in nature either is very rare or does not occur at all (Koller & Ursenbacher, 1996).

Early molecular studies of Natrix

Prior to the modern DNA work of Guicking, Joger & Wink (2002) on *N. tessellata* and *N. maura*, there had been few published attempts to apply molecular methods to *Natrix* phylogeny and phylogeography. Lawson (1986) analysed data from several sources in a study of the systematics of Old World natricine snakes. These analyses included allozyme data from all three species of *Natrix*. Trees based on genetic distances were ambiguous with regard to the placement of each species relative to the others. Furthermore, because of the very small sample sizes, these distance-based trees suffer from a serious methodological flaw. However, a cladistic analysis of the same data based on shared derived alleles as character states did place *N. maura* basal to a sister taxon pair composed of *N. natrix* and *N. tessellata*. This study was based on a total of 25 samples. In another allozyme study, Hille (1997) examined gene flow and genetic divergence in

N. natrix in a transect across the contact zone between *N. n. natrix* and *N. n. helvetica*. This study also included specimens of *N. megalocephala* and island populations from Sardinia, Greece and Cyprus. There was no genic differentiation between *N. megalocephala* and *N. n. natrix*, but a clear indication of a genetic break between *N. n. helvetica* and *N. n. natrix* populations, as well as the genetic distinctiveness of the three island endemics.

Aim of this study

The aim of this study was to investigate the evolutionary history of the genus *Natrix* and to test the hypotheses formulated above. Based on sequences of four mitochondrial genes, we reconstructed a molecular phylogeny for the genus. To establish dates for the most important divergence events, a molecular clock was calibrated using information from related species, palaeogeographical evidence, and the fossil record.

These data suggest a basal position for *N. maura* and a sister relationship between *N. tessellata* and *N. natrix*. The three species most likely diverged as early as the middle Miocene, intraspecific differentiation starting in the late Miocene to early Pliocene. We use this information to postulate a new scenario for the evolutionary history of the genus.

MATERIAL AND METHODS

Selection of specimens

To take into account the possible effects of intraspecific variation on phylogeny reconstruction, we sampled snakes from numerous geographical regions within their ranges. For each species, we attempted to obtain individuals that were representative of their entire distributional range; samples from the easternmost populations of *N. natrix* and from *N. megalocephala* were not available. Included were 23 *N. natrix*, six *N. maura* and 15 *N. tessellata* (see Fig. 1 for map and the Appendix for locality and voucher number information).

Initially, *Nerodia fasciata*, *Xenochrophis punctulatus*, *Amphiesma stolata* and *Rhabdophis tigrinus* were investigated and tested, both separately and together, with each of the four genes for suitability as outgroups for tree-rooting purposes. As none of these taxa changed the topology of the *Natrix* ingroup clade, and *Ne. fasciata* was consistently closer to the ingroup, a single specimen of this taxon was chosen as the outgroup for all analyses presented herein.

Laboratory procedures

Snake tissue (liver, blood, shed skin or tail tip) was collected and stored in 95% ethanol or storage buffer (Arctander, 1988) until needed for DNA extraction. DNA extraction, gene amplification and sequencing were carried out in laboratories in Germany and California; standard methods were used for these procedures (Sambrook & Russell, 2001) and only insignificant differences in technique occurred between the two laboratories. DNA was obtained by proteinase K digestion followed by two rounds of extraction with phenol/$CHCl_3$ and one of $CHCl_3$ alone (Sambrook & Russell, 2001). The DNA was precipitated by the addition of three volumes of cold 100% ethanol. Preparation of DNA template for the polymerase chain reaction (PCR), PCR product purification and cycle sequencing were carried out as detailed in Burbrink, Lawson & Slowinski (2000). Nucleotide sequences were determined using the ABI model 3100 Genetic Analyser; gene segments were edited and assembled using the computer program Sequencher version 4.0 (Gene Codes Corp., 1999, Ann Arbor, Michigan). Alignment of each of these four protein-coding genes was accomplished easily by eye using the computer program Xesee (Cabot & Beckenbach, 1989).

We amplified and sequenced target DNA using previously published primers and those that we had developed specifically for work with snakes (Table 1). Both strands of the entire mitochondrial cytochrome *b* (cyt *b*; 1117 nucleotides), NADH dehydrogenase subunit 1 (ND1; 964 nucleotides), NADH dehydrogenase subunit 2 (ND2; 1029 nucleotides) genes and a partial sequence for the NADH dehydrogenase subunit 4 (ND4; 696 nucleotides) gene were amplified and sequenced for all specimens (a total of 3806 nucleotides).

Sequences generated for this study have been deposited in GenBank (accession numbers AY487681, AY487574, AY487585, AY487590, AY487590–591, AY487757–760, 487764–780, 487784–796, AY866529–544, AY870612–642, AY873705–772).

Data analysis

The ILD test, also known as the partition homogeneity test (Farris *et al*., 1994; Swofford, 2001), was applied to the dataset after all invariant characters were removed (Cunningham, 1997), to test for the advisability of combining data from all four genes. Because this test has received much criticism recently (Dolphin *et al*., 2000; Barker & Lutzoni, 2002), we examined congruence of tree topologies derived from the complete dataset as well as those derived from pairwise combinations of each of the four genes (Nagy *et al*., 2004).

Substitution saturation influences reliability of results obtained from DNA sequence analyses and we tested for saturation both graphically and statistically. Sequence statistics differed only slightly between the

Table 1. Primers used for amplification and sequencing of the mitochondrial cyt *b*, ND1, ND2 and ND4 genes

Primer	Sequence	Use	Position*	Reference
L14724NAT	5′-GACCTGCGGTCCGAAAAACCA-3′	amp./seq. cyt *b* (L)	14903 (tRNA-glu)	Guicking *et al*. (2002)
Dinoglu	5′-GACCTGTGATMTGAAAAACCAYCGTTGT-3′	amp. cyt *b* (L)	14910 (tRNA-glu)	de Queiroz *et al*. (2002)
Glu-f	5′-AACCACCGTTGTTATTCAACT-3′	seq. cyt *b* (L)	14919 (tRNA-glu)	Burbrink *et al*. (2000)
NATf516	5′-CCAACCTTAACACGATTCTTTGCC-3′	amp./seq. cyt *b* (L)	15444 (cyt *b*)	This study
Thrsnr2	5′-CTTTGGTTTACAAGAACAATGCTTTA-3′	amp./seq. cyt *b* (H)	16064 (tRNA-thr)	de Queiroz *et al*. (2002)
CB2	5′-CCCTCAGAATGATATTTGTCCTCA-3′	seq. cyt *b* (H)	15149 (cyt *b*)	Kocher *et al*. (1989)
619	5′-TCCCATTYCACCCATACCA-3′	seq. cyt *b* (L)	15584 (cyt *b*)	Burbrink *et al*. (2000)
815	5′-TCTGGTTTAATGTGTTG-3′	seq. cyt *b* (H)	15716 (cyt *b*)	Slowinski & Lawson (2005)
16Sb	5′-ACGTGATCTGAGTTCAGACCGG-3′	amp./seq. ND1 (L)	2376 (16S rRNA)	Palumbi (1996)
tRNA-ile	5′-CCGTGTCTACTCTATCAAGGTAGTC-3′	amp./seq. ND1 (H)	3518 (tRNA-ile)	de Queiroz *et al*. (2002)
505	5′-ATATCTGGAATATTCACGTA-3′	seq. ND1 (L)	2894 (ND1)	de Queiroz *et al*. (2002)
ND1-3	5′-AGTCADADGTGTTCTTGT-3′	seq. ND1 (H)	3056 (ND1)	de Queiroz *et al*. (2002) corrected.
L4437b	5′-CAGCTAAAAAAGCTATCGGGCCCATACC-3′	amp./seq. ND2 (L)	4801 (tRNA-met)	Kumazawa *et al*. (1996)
tRNA-trp	5′-GGCTTTCAAGGCTMCTAGTTT-3′	amp./seq. ND2 (H)	5877 (tRNA-trp)	de Queiroz *et al*. (2002)
419	5′-ACMTGACAAAAAATYGC-3′	seq. ND2 (L)	5238 (ND2)	de Queiroz *et al*. (2002)
569	5′-GTGTGGGCRATTGATGA-3′	seq. ND2 (H)	5382 (ND2)	de Queiroz *et al*. (2002)
ND4ab	5′-CACCTATGACTACCAAAAGCTCATGTAGAAGC-3′	amp./seq. ND4 (L)	11702 (ND4)	Arévalo, Davis & Sites (1994)
tRNA-leu	5′-CATTACTTTTACTTGGATTTGCACCA-3′	amp./seq. ND4 (H)	12569 (tRNA-leu)	Arévalo *et al*. (1994)
tRNA-his	5′-CACAGCTTGAYATTTTWTTTAAATTAC-3′	seq. ND4 (H)	12406 (tRNA-his)	This study

*Position of the 3′ nucleotide of the primer in the mitochondrial genome of the colubrid snake *Dinodon semicarinatus* (Kumazawa *et al*., 1998).
16S rRNA, ribosomal RNA-16S subunit; amp., amplification; Cyt *b*, cytochrome *b*; glu, glutamic acid; H, heavy strand; his, histidine; ile, isoleucine; L, light strand; leu, leucine; met, methionine; ND1, ND2 and ND4, NADH dehydrogenase subunits 1, 2 and 4; seq., sequencing; thr, threonine; tRNA, transfer RNA; trp, tryptophan.

four genes (see below) and we used the combined dataset in the statistical test. For graphical display, pairwise uncorrected p-distances were plotted against Kimura-2-parameter distances (K2P). Because K2P distances correct for different substitution rates of transitions and transversions, deviation of K2P distances from uncorrected genetic distance can be used to estimate saturation effects (Feldman & Parham, 2002; Nagy *et al*., 2003). Statistical analyses of saturation were performed on the combined dataset for all codon positions taken together and for each codon position considered separately. Testing for saturation was accomplished with the test of Xia *et al*. (2003) provided in the program package DAMBE v. 4.2.7 (Xia & Xie, 2001).

Phylogenetic reconstruction

We used MEGA v. 2.1 (Kumar *et al*., 2001) to obtain basic sequence statistics. Bayesian inference (Yang & Rannala, 1997) and the criterion-based methods of maximum parsimony (MP) and maximum likelihood (ML) were employed to infer phylogenies. We used MODELTEST v. 3.06 (Posada & Crandall, 1998) to select an appropriate evolutionary model for ML and Bayesian analyses; the most appropriate model (transferred into input files for Bayesian and ML analyses) for our data was the TrN + I + G model with base frequencies of A = 0.3537, C = 0.3249, G = 0.0794, T = 0.2420, six substitution types: A–C = 1.0000, A–*G* = 18.8678, A–T = 1.0000, C–*G* = 1.0000, C–T = 8.6769, G–T = 1.0000, and proportion of invariable sites I = 0.5086. Among-site rate variation followed a gamma distribution with shape parameter α= 1. 1856.

For Bayesian inference, MrBAYES v. 3.0b4 (Huelsenbeck & Ronquist, 2001) was used to analyse the data in four chains for 1 500 000 generations with every hundredth tree being sampled. 'Burnin' was set to 1500 so that a consensus was drawn from the last 13 501 trees sampled.

MP and ML analyses were conducted with PAUP 4.0b10 (Swofford, 2001), using heuristic searches, the tree-bisection-reconnection (TBR) algorithm for branch swapping and a neighbour joining tree as the starting tree. Because saturation was generally low (see below) we used equal weighting of codon positions for the MP analyses. Bootstrap values (Felsenstein, 1985) were calculated under the MP criterion for 1000 replicates both for the combined dataset and separately for each of the four genes.

Estimation of divergence times

Two independent approaches were used to estimate divergence times between the three *Natrix* species. For the first approach, the estimate was obtained from a molecular clock model constructed from amino acid distances with the first occurrence of Aniliidae 69–75 Mya (Rage, 1987) and the following divergence data providing calibration: snakes from saurians 130–150 Mya (Carroll, 1988), natricine from colubrine snakes 35–45 Mya (adapted from Szyndlar, 1991b and Ivanov, 2001), within *Hierophis* subgroups (including *Eirenis*) 18 Mya, (palaeoherpetological data of Ivanov, 2002a; after Nagy *et al*., 2004), and within *Hemorrhois* subgroups 16 Mya (Nagy *et al*., 2004). For these taxon pairs, amino acid distances were estimated from cyt *b* sequences taken from GenBank or kindly provided by Z. Nagy. Estimates for divergence times in *Natrix* were then inferred from the graph (see Fig. 2).

For the second approach, a linearized tree was calculated with the sequences of all four genes, using the molecular clock enforced option in the program PAUP vs. 4b10 (Swofford, 2001). Calibration of the clock for this tree assumed the split of African and European *N. maura* to be 5.3 Mya, corresponding to the reopening of the Strait of Gibraltar at the end of the Messinian salinity crisis (Krijgsman *et al*., 1999) and the split between *N. maura* and the other two species not to be earlier than 20–18 Mya, corresponding to the first collision of the African and Arabian plates with Eurasia (Rögl & Steininger, 1984). Because this approach assumes taxon rate homogeneity, the amount of rate variation inherent in the data was estimated by performing relative rate tests, both on pairwise sequence comparisons (Tajima, 1993), implemented in MEGA 2.1, and on groupwise sequence comparisons based on the two-cluster test of Takezaki, Rzhetsky & Nei (1995), in PHYLTEST v. 2.0 (Kumar, 1996). We used

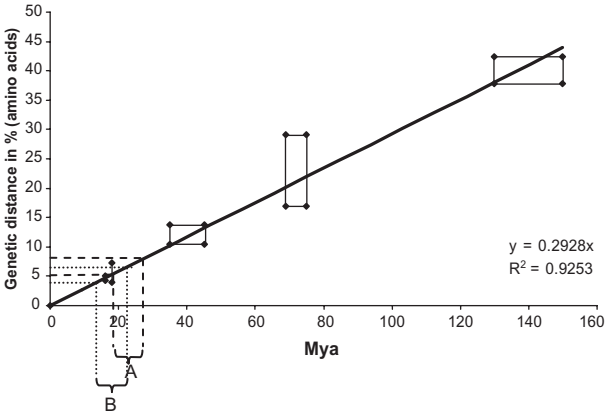

Figure 2. Divergence times estimates for *Natrix maura* from *N. natrix* and *N. tessellata* (A) and *N. natrix* from *N. tessellata* (B), based on amino acid distances. Correlation was based on assumption of linear relationship between amino acid distances and divergence times and utilizes five calibration points representing major divergences in snake evolution (see text).

likelihood ratio tests to compare the log-likelihoods between the clock enforced tree and the corresponding tree without assumption of a molecular clock (Huelsenbeck & Rannala, 1997).

Because relative rate tests indicated rate heterogeneity among species, it seemed desirable to estimate its extent. To achieve this, 'ratios of evolutionary rates' between species were estimated from the results of the individual-based pairwise relative rate tests (Tajima, 1993). In this test, m_ijj and m_iji represent the numbers of sequence-specific substitutions since the last common ancestor of the sequence pair. Pairwise ratios of evolutionary rates were estimated as m_ijj/m_iji for pairs of individuals, and the averages of these were calculated for each species pair based on 6×6 comparisons. Sequences used for pairwise comparisons were chosen to include both the fastest and the slowest evolving subsets of each species, as inferred from intraspecific relative rates, assuming these subsets to be representative for the whole dataset.

Despite some rate heterogeneity among species, the results obtained by the different approaches were sufficiently congruent to give satisfactory estimates of divergence times. As these estimates showed reasonable congruence to data obtained from related species as well as palaeogeographical and palaeontological evidence, it seemed unlikely that further analyses would provide much better estimates. Thus, we did not employ analyses based on non-synonymous sites or on non-parametric approaches as introduced by Sanderson (1997).

RESULTS

Sequence statistics

Forty-four unique haplotypes were identified among the 45 samples analysed for the entire dataset of 3806 nulceotides. Only the specimens of *N. natrix* from south-eastern England and north-eastern France were identical.

Empirical percentages of the four nucleotides differed only slightly among taxa and a strong bias against guanidine was recorded on the L-strand, typical for mitochondrial DNA (e.g. Desjardins & Morais, 1990; de Queiroz, Lawson & Lemos-Espinal, 2002; Doadrio, Carmona & Machordom, 2002). All sequences contained an open reading frame, and no non-terminal stop codons occurred. From this information, it may be inferred that the sequences represent the functional genes rather than nuclear pseudogenes (Zhang & Hewitt, 1996; Bensasson *et al*., 2001).

Of the 3806 characters obtained from the combined dataset, 1300 (34.2%) were variable and of these, 1090 (28.7%) were parsimony-informative. Estimated transition/transversion ratios ranged from 1.8 in ND2 to 2.9 in cyt *b*.

There was a single indel event detected in the entire dataset. All *N. tessellata* showed deletion of the 7th codon of the ND1 gene. Consequently, the length of the ND1 sequence in *N. tessellata* was 961 nucleotides compared with 964 nucleotides in the other species.

The signal for termination of translation in cyt *b* and ND1 was a terminal T which is post-transcriptionally adenylated to form a functional stop codon (TAA) in all taxa, a common mechanism in snakes (Burbrink *et al*., 2000; Nagy *et al*., 2004). The genes for ND2 and ND4 subunits included functional stop codons of types TAG and TAA, respectively. *Ne. fasciata* also had the stop codon TAG in the ND4 gene.

Saturation tests

Results of graphical saturation tests (not shown; available on request), indicated no saturation effect for all changes taken together as well as for first and second codon positions separately. The slight deviation of the graph from a straight line at the third codon position suggested some saturation effect. This was most pronounced for K2P > 0.5, reflecting comparisons of ingroup taxa with the outgroup taxon. Statistical tests based on the measurement introduced by Xia *et al*. (2003) resulted in significantly smaller I_{ss} than $I_{ss.c}$ values for all three codon positions in each of the four genes, as well as when taken together, indicating no significant saturation effect.

Justification for combining the datasets

ILD tests indicated no significant incongruence between any pair of genes or for the combined dataset of four genes (cyt *b* vs. ND1: $P = 0.908$; cyt *b* vs. ND2: $P = 0.984$, cyt *b* vs. ND4: $P = 805$; ND1 vs. ND2: $P = 0.442$; ND1 vs. ND4: $P = 0.467$; ND2 vs. ND4: $P = 0.676$; all four datasets analysed together: $P = 0.779$). Separate MP analyses of the four genes produced slightly different strict consensus trees (not shown). Bootstrap analyses, however, indicated no moderately or strongly supported conflict between any two genes (no conflicting clades that were supported in alternative trees by bootstrap values greater than 70%). The most notable difference was the basal placement of *N. tessellata* and derived sister group relationship of *N. maura* and *N. natrix* by ND4, which contrasted with the basal placement of *N. maura* and a derived sister group relationship of *N. natrix* and *N. tessellata* in the three remaining genes. Bootstrap support for these relationships within the ND4 tree was very low, however, at 53% for a *N. maura*–*N. natrix* sister group relationship compared with 29% for a sister relationship of *N. natrix* and *N. tessellata*. ND4 data alone clearly did not support any relationship between the three species. Further comparisons

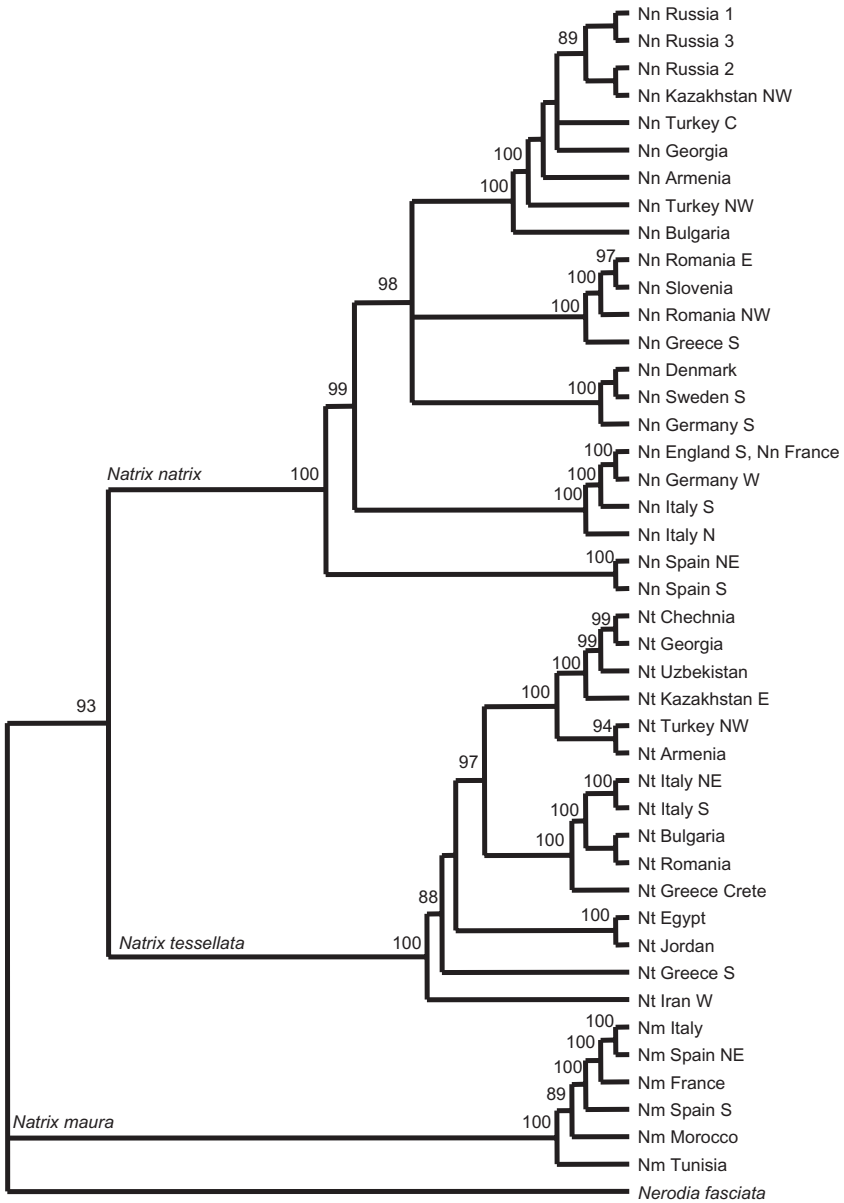

Figure 3. Maximum parsimony strict consensus cladogram of three most parsimonious trees. Bootstrap values (1000 replicates) are provided for the combined dataset. Only bootstrap values greater than 50% are given. Tree length: 3377 steps, CI = 0.5561, HI = 0.4439, RI = 0.8641.

between the four gene-specific topologies yielded no differences in the level of support for major groupings within the three species, and only minor differences in intragroup relationship among topologies. All differences were reflected in generally low bootstrap values for the respective clades, with little consequence for the interpretation of the overall generic phylogeny. Because these analyses gave no reason to keep the datasets separate, we focussed on results from analyses of all four genes combined.

PHYLOGENETIC INFERENCE

Figures 3 and 4 illustrate the strict consensus cladograms of the three most parsimonious trees of the MP analysis and the 50%-majority rule consensus tree from the last 13 501 Bayesian trees, respectively. Both reconstructions produced nearly identical topologies, and the tree obtained by ML analysis (not shown) was topologically identical to the Bayesian tree. All three species were clearly identified as monophyletic

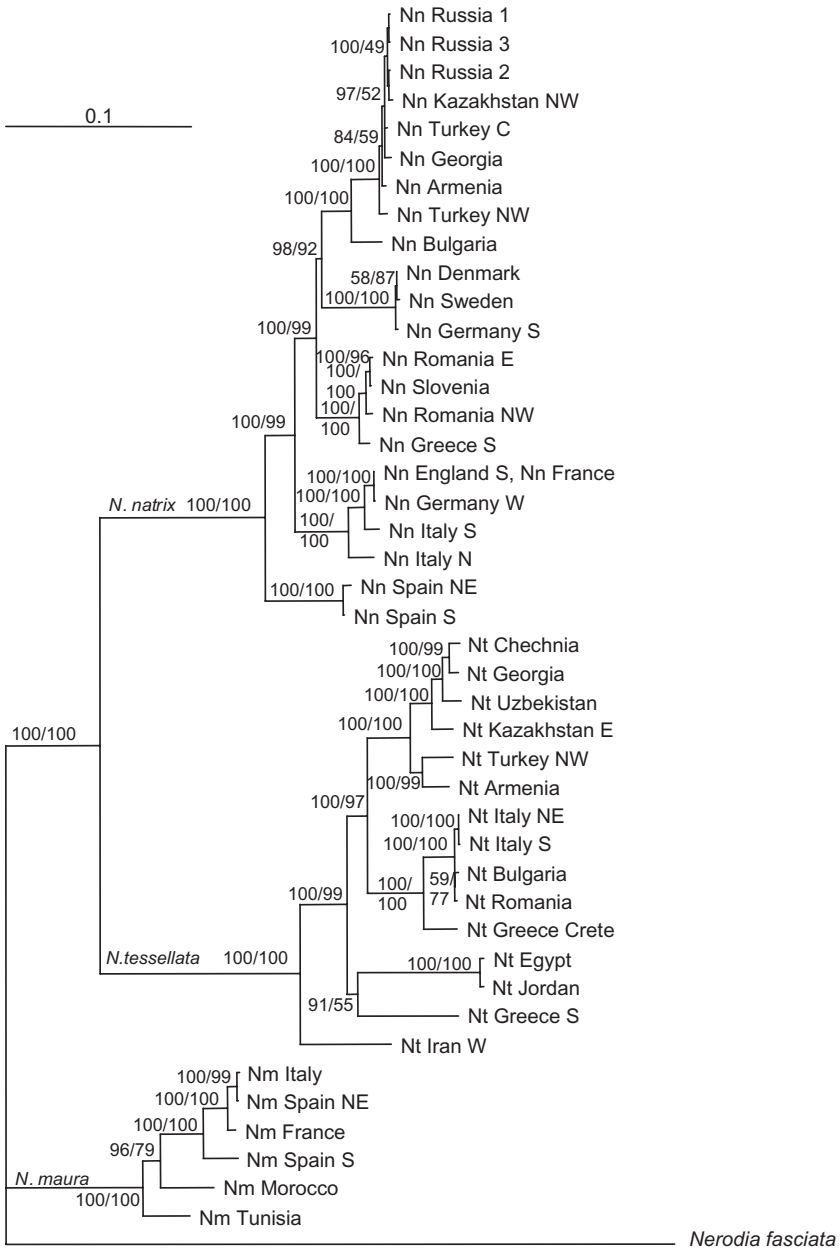

Figure 4. Bayesian inference phylogram (TrN + I + G model) based on the combined 3806 base pairs from cyt *b*, ND1, ND2 and ND4 with *Nerodia fasciata* as outgroup. Numbers are clade credibility values from the Bayesian analysis followed by maximum likelihood bootstrap values (500 replicates). Phylogram topology was identical to that obtained by ML analysis.

groups, each supported by high bootstrap values (Figs 3, 4). High support values were also obtained for a sister relationship of *N. tessellata* and *N. natrix*, and this clade was placed as the sister group to *N. maura*.

Intraspecific differentiation in all three species was strong, and support for major lineages was generally high. In *N. maura*, there was clear separation of the African and European populations, with further strong divergence of the two African samples and between the southern Spanish population and samples collected in more northern parts of the distribution range.

N. tessellata from Iran, southern Greece and Jordan/Egypt clearly formed independent lineages, with the Iranian sample representing the most basal haplotype. The remaining mainland European samples of

Table 2. Maximum genetic distances (Tamura-Nei's model with gamma = 1.1856) for intraspecific comparisons and mean distances ± SE (500 bootstrap replicates with the program MEGA 2.1) for interspecific comparisons for individual genes and all genes combined

	cyt *b*	ND1	ND2	ND4	All
N. maura	0.050	0.082	0.071	0.062	0.058
N. tessellata	0.097	0.113	0.117	0.109	0.099
N. natrix	0.085	0.089	0.094	0.088	0.085
N. maura–N. tessellata	0.184 ± 0.0161	0.188 ± 0.0144	0.181 ± 0.0143	0.204 ± 0.0205	0.187 ± 0.0080
N. maura–N. natrix	0.160 ± 0.0137	0.167 ± 0.0141	0.184 ± 0.0148	0.177 ± 0.0198	0.170 ± 0.0072
N. tessellata–N. natrix	0.181 ± 0.0159	0.157 ± 0.0132	0.188 ± 0.0144	0.196 ± 0.0189	0.178 ± 0.0075

N. tessellata were most closely related to the sample from Crete, and a final highly divergent clade comprised all samples from Turkey, extending to the easternmost parts of its range.

Five major lineages were distinguished in *N. natrix,* each corresponding to geographical regions, with (2) Iberian Peninsula, Italy and western Europe, (3) eastern Europe, (4) central Europe with Scandinavia, and (5) an Asian and eastern-most european clade defining distributions. One sample from Bulgaria clustered within the last group, but could represent a sixth independent lineage characterized by a fairly high genetic distance between it and the other individuals within the clade. In *N. natrix,* clades corresponding to the western parts of the distribution range were placed as the most basal groups.

Minor differences regarding the relative placement of major lineages within species were obtained with different reconstruction methods. Within *N. tessellata* the placement of sample 'Nt Greece S' as sister taxon to 'Nt Egypt' and 'Nt Jordan' in Bayesian inference contrasted with its placement basal to a different group in the MP analysis. Additionally, the placement of *N. natrix* from southern Germany and Scandinavia as sister group to snakes from the eastern part of their distribution range as found with Bayesian inference conflicted with the lack of resolution of these relationships in the MP analysis.

Genetic distances within and among species

Pairwise genetic distances within and among species are summarized in Table 2. Intraspecific distances were slightly higher in ND1 and ND2 genes than they were in cyt *b* and ND4 genes. Intraspecific divergence was highest in *N. tessellata*, with maximum values of more than 0.10 in the three ND genes and 0.09 in the cyt *b* gene. Differentiation was less distinct in *N. natrix*; this might have been due to the lack of sampling in the eastern parts of its Asiatic distributional range, the Near East and north-western Africa. The lowest intraspecific genetic distances, 0.05–0.08, were recorded in *N. maura*.

Among-species distances were similar for all three species pairs, ranging from 0.170 (*N. maura* vs. *N. natrix*) to 0.187 (*N. maura* vs. *N. tessellata*) for the combined dataset, and there were only slight differences in these values within each gene (Table 2). Genetic distances between ingroup taxa and the outgroup taxon, *Ne. fasciata,* ranged from 0.21 to 0.25.

Estimation of divergence times

Using our first technique (see above), divergence times between *Natrix* species were estimated based on five calibration points obtained from fossil evidence and by assuming a linear correlation of amino acid distances and divergence times. Plotting genetic distance against presumed divergence times for the calibration points showed that this assumption applied relatively well for evolution in snakes (Fig. 2). It therefore seemed justifiable to infer a first estimate of divergence times between the three *Natrix* species from our resulting graph. This approach yielded an estimate of 18–27 Myr for divergence of *N. maura* from the other two species and 13–22 Myr for that of *N. natrix* and *N. tessellata* (Fig. 2). Although intervals of these estimates are large, they support clearly a Miocene origin for the three species.

Calculation of a molecular clock enforced tree based on the sequence data of all four genes gave three equally good trees differing only slightly in a few terminal branch lengths; one of the trees is shown in Figure 5. Calibration of this tree assumed the split of African and European *N. maura* to have occurred about 5.3 Mya and the split between *N. maura* and the other two species to have been not earlier than 18–20 Mya when the African and Asian continents collided for the first time. Both dates were slightly discordant when dating the chronogram, this discordance most likely being attributable to a slower evolutionary rate in *N. maura* than in *N. natrix* and *N. tessellata* (see

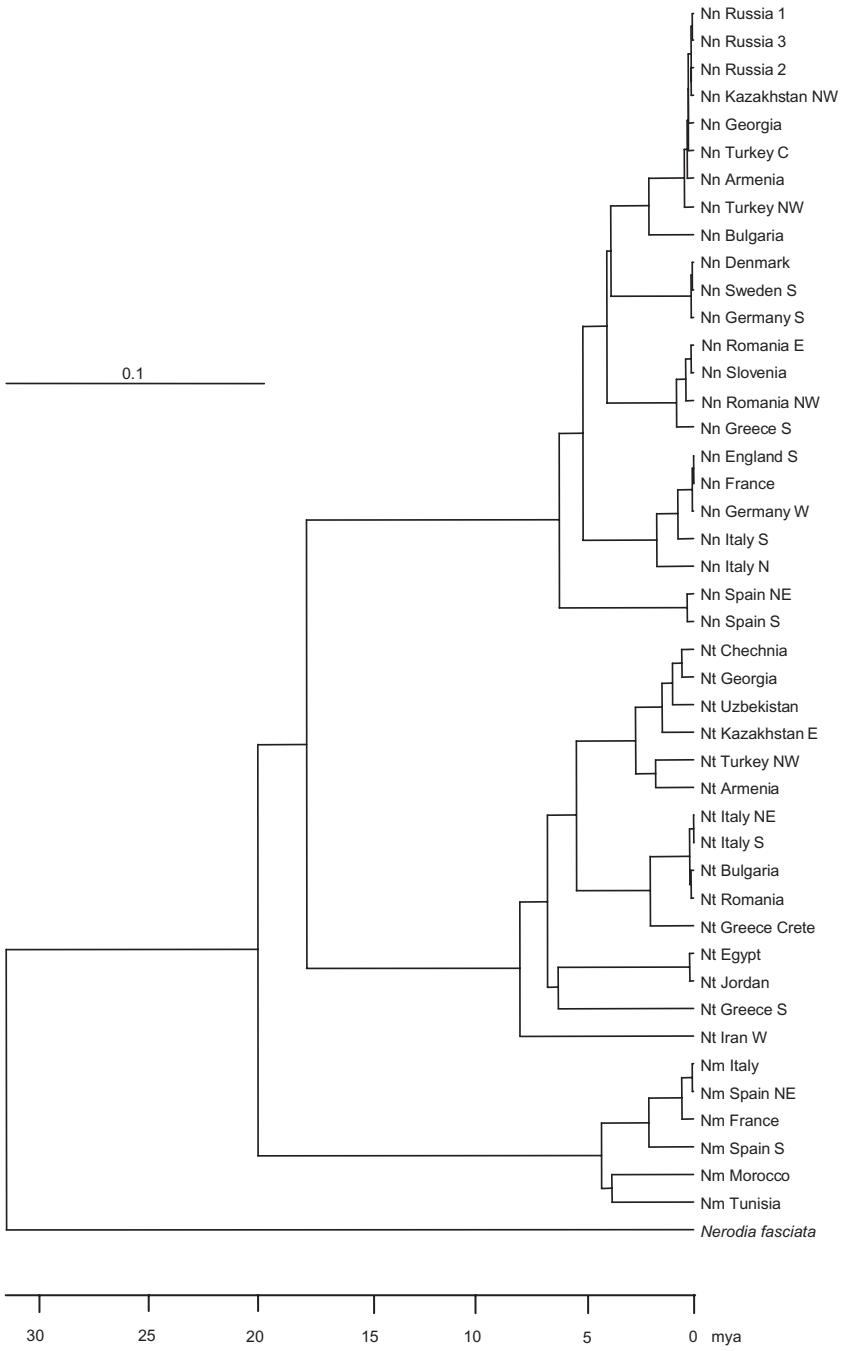

Figure 5. Maximum likelihood chronogram for evolution of the genus *Natrix*. Calibration of time scale based on palaeogeographical evidence (see text).

below). Nevertheless, the results were concordant with those obtained by the first approach to divergence time estimation, and placed the split between *N. tessellata* and *N. natrix* in the early Miocene (Burdigalian stage), and the major intraspecific divergences in all three species in the late Miocene and early Pliocene.

Comparison of the chronogram and phylogram by a likelihood ratio test did not yield significant differences in the likelihoods of these trees, suggesting that the error introduced by rate heterogeneity (see below) was not very great ($-\ln_{Chronogram} = 23411.28$, $-\ln_{Phylogram} = 23390.42$, LR = 41.72, d.f. = 43, $P > 0.05$).

Because evolutionary rates were similar across all four genes, relative rate tests were performed on the combined dataset (Table 2). Intraspecific pairwise relative rate tests (Tajima, 1993) yielded only slight dif-

Table 3. Pairwise comparisons of *Natrix maura*, *N. tessellata* and *N. natrix* 'relative evolutionary rates'

	cyt *b*	ND1	ND2	ND4	All
N. maura : *N. tessellata*	0.729 ± 0.076	0.762 ± 0.119	0.625 ± 0.095	0.895 ± 0.129	0.737 ± 0.042
N. maura : *N. natrix*	0.775 ± 0.084	0.797 ± 0.085	0.748 ± 0.113	0.954 ± 0.111	0.801 ± 0.053
N. tessellata : *N. natrix*	1.061 ± 0.116	1.043 ± 0.053	1.189 ± 0.138	1.128 ± 0.156	1.096 ± 0.100

Values represent mean ± SD for pairwise comparisons between six representative specimens for each species.

ferences, which were considered negligible for our purpose. Interspecific comparisons, however, yielded significant differences between evolutionary rates of the three species. The most pronounced differences occurred between *N. maura* and the other two species, with significantly slower evolutionary rates found in *N. maura*. Rate heterogeneity between *N. tessellata* and *N. natrix* was only slight but, in groupwise as well as in most pairwise comparisons it was still significant, with *N. tessellata* having faster rates. To compensate for this rate heterogeneity in estimating divergence times, 'relative ratios of evolutionary rates' between the three species were calculated (see Material and methods and Table 3). Using these values, ratios of evolutionary rates were estimated as *N. maura* : *N. natrix* : *N. tessellata* equal to 1 : 1.25 : 1.35.

With an assigned divergence time of 5.3 Myr for European and African *N. maura*, an evolutionary rate of 1% per million years is implied for *N. maura* (maximum uncorrected p-distances within *N. maura* were 5.3% sequence divergence). Assuming no change in species-specific evolutionary rate over the time of intraspecific differentiation, divergence times needed correcting for the observed faster evolutionary rates in *N. tessellata* and *N. natrix*. These corrections yielded molecular clock rates of about 1.35% per million years and 1.25% per million years for *N. tessellata* and *N. natrix*, respectively. Correspondingly, intraspecific differentiation was estimated to have been initiated 6.7 Mya and 6.0 Mya in the two species, respectively. These values were in good accord with those obtained from the molecular clock enforced tree (Fig. 5).

DISCUSSION

Phylogenetic relationships within *Natrix*

Mitochondrial DNA sequence differences of up to 9% and 15%, within and among species of *Natrix* are not surprising, considering results from phylogenetic studies in other snakes of the Colubridae family. Most studies report intrageneric mtDNA sequence divergences of between 3% and 16% (see Johns & Avise, 1998) and intraspecific divergence of between 3% and 7% (Alfaro & Arnold, 2001; Nagy *et al*., 2002), with Burbrink *et al*. (2000) reporting cyt *b* sequence divergence as high as 16% among lineages of *Elaphe obsoleta*.

The evidence presented here refutes the generally assumed sister relationship between *N. maura* and *N. tessellata*. Three of the four mitochondrial genes we investigated supported a sister relationship between *N. natrix* and *N. tessellata* and a basal position for *N. maura*; the fourth gene did not define clearly any phylogenetic resolution. Phenotypic similarity between *N. maura* and *N. tessellata* appears to reflect ecological niche adaptation (see Gruschwitz *et al*., 1999; Schätti, 1999) rather than phylogenetic inheritance. It appears that pheromonal or behavioural differences do not facilitate hybridization in the wild, in spite of some morphological traits being shared between the species.

The length of time since *N. natrix, N. maura* and *N. tessellata* shared a common ancestor suggests that hybridization in the wild is not achievable (Koller & Ursenbacher, 1996; Schätti, 1999; unpubl. data).

Estimation of a molecular clock for the genus *Natrix*

There are many ambiguities in the application of any molecular clock (for reviews see Avise *et al*., 1992; Easteal, Collet & Betty, 1995; Arbogast *et al*., 2002), and most phylogeographical scenarios can provide only approximations of divergence times. Our use of two independent approaches to estimate evolutionary rates and divergence times in *Natrix* clearly supports a Miocene origin and evolutionary rate estimates of 1–1.35% per one million years.

In addition to congruence between the two approaches, several other points seem to support the proposed time scale. The estimated evolutionary rates are within the range reported for vertebrates in general (Brown, George & Wilson, 1979; Martin & Palumbi, 1993) and are very similar to those suggested for other squamates (e.g. Oliverio, Bologna & Mariottini, 2000; Nagy *et al*., 2004). These estimated divergence times suggest a general scenario for the evolutionary history of the genus that corresponds well with the palaeogeographical history of the western Palearctic and with a Miocene origin for extant

Natrix species, as suggested by fossil evidence for *N. natrix* (Ivanov, 2002a). General support notwithstanding, there is some uncertainty inherent in our estimated divergence times; rate heterogeneity among the three *Natrix* species may have introduced errors in divergence time estimates. Evaluation of the extent of rate heterogeneity yielded differences of no greater than 30% (Table 3), however, and it seems unlikely that our estimate of a Miocene origin for the species and a late Miocene/early Pliocene intraspecific differentiation would have been greatly affected by errors of this nature.

Palaeontological or palaeogeographical evidence may provide only minimum or maximum estimates for times of divergence. Because separation of *N. maura* from the ancestors of *N. natrix* and *N. tessellata* could have occurred earlier or later than 18–20 Mya, when the African and Eurasian continents first collided (Rögl & Steininger, 1984) (Fig. 5), using 18–20 Mya as an estimate may be imprecise. Faunal exchange between north-western Africa and Iberia, initiated during the time of land connection and persisting for about a million years (Krijgsman *et al*., 1999), at the Messinian salinity crisis (5.3 Mya), probably provides a more precise calibration point. Using data from allozyme electrophoresis, Busack (1986; table 5) found that migration rates broadly overlapped mutation rates in *N. maura*. Harris *et al*. (2002) suggest that for many small animals migration across the Strait of Gibraltar by swimming or natural rafting cannot be ruled out, however. If migration actually did occur after continental separation was completed, African and European lineages could have diverged either later or earlier than our assumed 5.3 Myr, contributing additional uncertainty to this calibration point.

A HYPOTHESIS FOR THE EVOLUTIONARY HISTORY OF THE GENUS *NATRIX*

If estimations for the molecular clock rate are incorrect by a factor of two or three, an unlikely magnitude, the data presented here clearly favour a Miocene origin of the three *Natrix* species and pre-Pleistocene (± 700 000 years ago) climatic cycle induced intraspecific differentiation (Webb & Bartlein, 1992). Palaeontological evidence (Rage & Auge, 1993; Ivanov, 2001) and current distributions suggest southern Asia as the origin of the genus (see also Hecht, 1930). Intraspecific phylogenetic relationships indicate that ancestral populations of *N. maura* lived in Africa and later colonized Europe, suggesting an invasion of north-western Africa from the east (Dobson & Wright, 2000). Genetic data further suggest that ancestral populations of *N. tessellata* lived in south-western Asia, and that extant lineages of *N. natrix* likely originated due to fragmentation of an ancestral European population.

These observations, coupled with our estimated evolutionary time frame, suggest separation of ancestral *N. maura* from ancestral *N. tessellata* and *N. natrix* during the late-early or early-middle Miocene. This would have been as a result of range expansion of the ancestral *Natrix* into Africa across the landbridge that formed between Africa and Eurasia by collision of the Afro-Arabian and Eurasian plates 20–18 Mya (Rögl & Steininger, 1984; Steininger, Rabeder & Rögl, 1985). Subsequently, African lineages became isolated as the seaway between the Mediterranean and the Indo-Pacific reopened during the mid-Miocene (Rögl & Steininger, 1984). This isolation provided for independent evolution of the two populations, giving rise to *N. maura* in Africa and to *N. natrix* and *N. tessellata* in Eurasia.

Differentiation of *N. natrix* and *N. tessellata* can be explained by fragmentation of the ancestral populations, one population to the north and one to the south of the Paratethys seaway that extended from central Europe to Middle Asia throughout most of the Miocene (Rögl & Steininger, 1984). As northern populations expanded into Europe and evolved into extant *N. natrix* lineages, the southern population remained in south-west Asia and evolved into *N. tessellata*. Intraspecific differentiation 5–6 Mya may have occurred as a result of the varying and fluctuating environmental conditions associated with deterioration of the climate at the end of the Miocene (Crowley & North, 1991). Climate cooling probably precipitated large-scale extinction events with fragmented populations surviving in climatically favoured regions. This scenario for evolution of *N. maura* and *N. tessellata* in southern regions and *N. natrix* in more northern regions explains the less-pronounced phenotypic divergence between *N. maura* and *N. tessellata* and stronger differentiation of *N. natrix* as an early adaptation to differing ecological niches and varying climatic conditions.

Range expansions across the desiccated Mediterranean during the Messinian salinity crisis (Hsü *et al*., 1977; Steininger *et al*., 1985) and subsequent isolation of populations on both sides of re-flooded areas are likely to have further promoted divergence. This was probably the case for African and European *N. maura* and might be true for *N. natrix* populations on the islands of Corsica and Sardinia. It also seems possible that colonization of southern Greece by *N. tessellata* took place during the Messinian.

Considering the uncertainty of molecular clock estimates in general and for *Natrix* in particular, final validation or refutation of the postulated history must await further insights from fossil records or availability of data from populations for which divergence times can be more precisely dated. Better evidence for times of divergence might be provided by investigating

mitochondrial DNA sequences of *N. natrix* from Corsica and Sardinia. As suggested by morphological studies, these islands support populations representing valid subspecies (Thorpe, 1979) and are most likely inhabited by ancient lineages of the grass snake. If these island populations were isolated more than 5 Mya after the Messinian salinity crisis, sequence divergence between *N. natrix* from Corsica, Sardinia and the European mainland should be in the magnitude of 5–6%. In addition, examination of African *N. natrix* could distinguish between our hypothesis that ancestral *N. natrix* colonized Africa from Europe and the hypothesis suggested by Ivanov (2002b) that the direction of migration was from Africa to Europe.

ACKNOWLEDGEMENTS

We are grateful to the following colleagues who generously donated specimens without which this study would have been far less complete. T. Amann, A. G. Bakiev, V. A. Beshkov, S. J. Busack, H. P. Eckstein, W. Fielder, P. G. Frank, J. Ghira, P. Gravlund, A. Hille, J. R. Macey, D. Modry, R. W. Murphy, Z. T. Nagy, G. Nilson, N. Orlov, T. G. Owen, T. J. Papenfuss, N. Rastegar-Pouyani, R. D. Sage, A. Sproll, and N. Vidal. We are grateful for laboratory assistance by J. Chen, C. Elliger and E. Visser. An earlier version of this paper was read by S. J. Busack who offered many helpful suggestions for its improvement. We thank F. T. Burbrink who kindly performed certain computations. Financial support was provided by grants from the Deutsche Forschungsgemeinschaft (Jo 134–7, Wi-719/18) and the California Academy of Sciences Research Fund.

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APPENDIX

Samples used in this study; voucher specimens from California Academy of Sciences (CAS), Museum of Vertebrate Zoology, University of California, Berkeley (MVZ), Museo Nacional de Ciencias Naturales, Madrid, Spain (MNCN), Royal Ontario Museum, Toronto (ROM), and Louisiana State University Museum of Natural Science, Baton Rouge (LSUMZ)

Map No.	ID for Fig.	Species	Collection locality	Voucher no.
	Nerodia fasciata	*Nerodia fasciata*	USA, Florida, Citrus Co.	CAS 211010
1	Nm Morocco	*Natrix maura*	Morocco, Tétuan Province	MVZ 178093
2	Nm Tunisia	*Natrix maura*	Tunisia, Tameghza	No voucher
3	Nm Spain S	*Natrix maura*	Spain, Cadiz Province	MNCN 12016
4	Nm Spain NE	*Natrix maura*	Spain, Katalonia, Osona District	MVZ 200533
5	Nm France	*Natrix maura*	France, Department Herault	No voucher
6	Nm Italy	*Natrix maura*	Italy, Voghera	No voucher
7	Nt Iran W	*Natrix tessellata*	Iran, Kermanshah	No voucher
8	Nt Greece S	*Natrix tessellata*	Greece, Ioánnina	No voucher
9	Nt Egypt	*Natrix tessellata*	Egypt	No voucher
10	Nt Jordan	*Natrix tessellata*	Jordan, Jarash	No voucher
11	Nt Armenia	*Natrix tessellata*	Armenia, Geolazar, Azat river	ROM 23418
12	Nt Chechnia	*Natrix tessellata*	Chechnia, Schelkovskaya District	CAS 182901
13	Nt Georgia	*Natrix tessellata*	Georgia, Agara	No voucher
14	Nt Uzbekistan	*Natrix tessellata*	Uzbekistan, Kungrad	No voucher
15	Nt Kazakhstan E	*Natrix tessellata*	Kazakhstan, Ili river	No voucher
16	Nt Turkey NW	*Natrix tessellata*	Turkey, Yenicaga	No voucher
17	Nt Greece Crete	*Natrix tessellata*	Greece, Crete	No voucher
18	Nt Bulgaria	*Natrix tessellata*	Bulgaria, Sozopol	CAS 219929
19	Nt Romania	*Natrix tessellata*	Romania, Cluj-Napoca	No voucher
20	Nt Italy NE	*Natrix tessellata*	Italy, Grado	No voucher
21	Nt Italy S	*Natrix tessellata*	Italy, Torre de Lato	No voucher
22	Nn Spain S	*Natrix natrix*	Spain, Cadiz Province	MNCN 13796
23	Nn Spain NE	*Natrix natrix*	Spain, Katalonia, Osona District	MVZ 200534
24	Nn Italy S	*Natrix natrix*	Italy, Torre San Gennaro	No voucher
25	Nn Italy N	*Natrix natrix*	Italy, Ticino	No voucher
26	Nn France	*Natrix natrix*	France, near Paris	No voucher
27	Nn England S	*Natrix natrix*	England, Kent, Sheppey Island	LSUMZ 41506
28	Nn Germany W	*Natrix natrix*	Germany, Kelkheim/Taunus	No voucher
29	Nn Germany S	*Natrix natrix*	Germany, Radolfzell	No voucher
30	Nn Sweden S	*Natrix natrix*	Sweden, Smaland	No voucher
31	Nn Denmark	*Natrix natrix*	Denmark	No voucher
32	Nn Greece S	*Natrix natrix*	Greece, Ioánnina	No voucher
33	Nn Romania E	*Natrix natrix*	Romania, Tulcea Region	No voucher
34	Nn Romania NW	*Natrix natrix*	Romania, Cluj-Napoca	No voucher
35	Nn Slovenia	*Natrix natrix*	Slovenia, Zalec	No voucher
36	Nn Bulgaria	*Natrix natrix*	Bulgaria, Malko Turnovo District	CAS 219930
37	Nn Turkey NW	*Natrix natrix*	Turkey, Yenicaga	No voucher
38	Nn Turkey C	*Natrix natrix*	Turkey, Sarkale	No voucher
39	Nn Armenia	*Natrix natrix*	Armenia, Ankavan	ROM 26842
40	Nn Georgia	*Natrix natrix*	Georgia, Batumi	No voucher
41	Nn Russia 1	*Natrix natrix*	Russia, Tula Region	CAS 175878
42	Nn Russia 2	*Natrix natrix*	Russia, Rybachy	No voucher
43	Nn Russia 3	*Natrix natrix*	Russia, Samara Region	No voucher
44	Nn Kazakhstan NW	*Natrix natrix*	Kazakhstan, Emba river	No voucher