Available online at www.sciencedirect.com
ScienceDirect
Organisms, Diversity & Evolution 8 (2008) 130–145
ORGANISMS DIVERSITY & EVOLUTION
www.elsevier.de/ode

Molecular phylogeography of the viperine snake *Natrix maura* (Serpentes: Colubridae): Evidence for strong intraspecific differentiation

Daniela Guicking[a,*], Ulrich Joger[b], Michael Wink[a]

[a]*Institut für Pharmazie und Molekulare Biotechnologie, Abt. Biologie, Universität Heidelberg, Im Neuenheimer Feld 364, 69120 Heidelberg, Germany*
[b]*Staatliches Naturhistorisches Museum, Pockelsstr. 10, 38106 Braunschweig, Germany*

Received 23 October 2006; accepted 31 May 2007

Abstract

The molecular phylogeography of the viperine snake, *Natrix maura* (Linnaeus, 1758), was investigated using complete sequences of the mitochondrial cytochrome *b* gene and genomic ISSR-PCR fingerprinting. In a total of 120 samples, 44 unique cytochrome *b* haplotypes were found which defined three major genetic lineages associated with samples from Morocco, Tunisia and Europe, respectively. The same lineages were supported by nuclear data. A possible fourth lineage exists in southern Spain. Genetic distances of cytochrome *b* sequences between the three main lineages were in the range of 3.9–5.6%, suggesting independent evolution since the early Pliocene. Distinction of the three major lineages at the subspecies or species level is discussed to account taxonomically for the high intraspecific variation in the viperine snake. A more detailed analysis of the European samples based on genetic diversity indices and a network reconstruction suggests a complex Pleistocene history for the viperine snake in Europe. Clear differentiation was found between populations south and north of the central Iberian mountain ranges, suggesting Pleistocene glacial refugia both in the southern and northern Iberian peninsula. In the south, genetic diversity was associated with the main river valleys, whereas northern haplotypes were more broadly distributed, indicating gene flow or postglacial range expansions. Unexpectedly high levels of genetic variation in southeastern France and northwestern Italy would be compatible with the hypothesis of a glacial refugium north of the Pyrenees or in Italy. However, due to the dependence of *N. maura* on warm climates, the assumption of a northern refugium seems unwarranted. We believe that further sampling in northern Spain is likely to reveal genetically diverse populations which could have served as sources for postglacial recolonization of France and Italy.
© 2008 Gesellschaft für Biologische Systematik. Published by Elsevier GmbH. All rights reserved.

Keywords: *Natrix maura*; Cytochrome *b*; ISSR-PCR; Phylogeny; Northwestern Africa; Iberian peninsula

Introduction

Growing evidence exists from numerous molecular systematic and phylogeographic studies that most European vertebrate species are much more ancient than has long been thought (Blondel and Aronson 1999). For the majority of the species studied, a Miocene

*Corresponding author. Current address: University of Kassel, Systematik und Morphologie der Pflanzen, Heinrich-Plett-Str. 40, 34132 Kassel, Germany. Fax: +49 561 8044200.
E-mail address: daniela.guicking@gmx.de (D. Guicking).

1439-6092/$ - see front matter © 2008 Gesellschaft für Biologische Systematik. Published by Elsevier GmbH. All rights reserved.
doi:10.1016/j.ode.2007.05.001

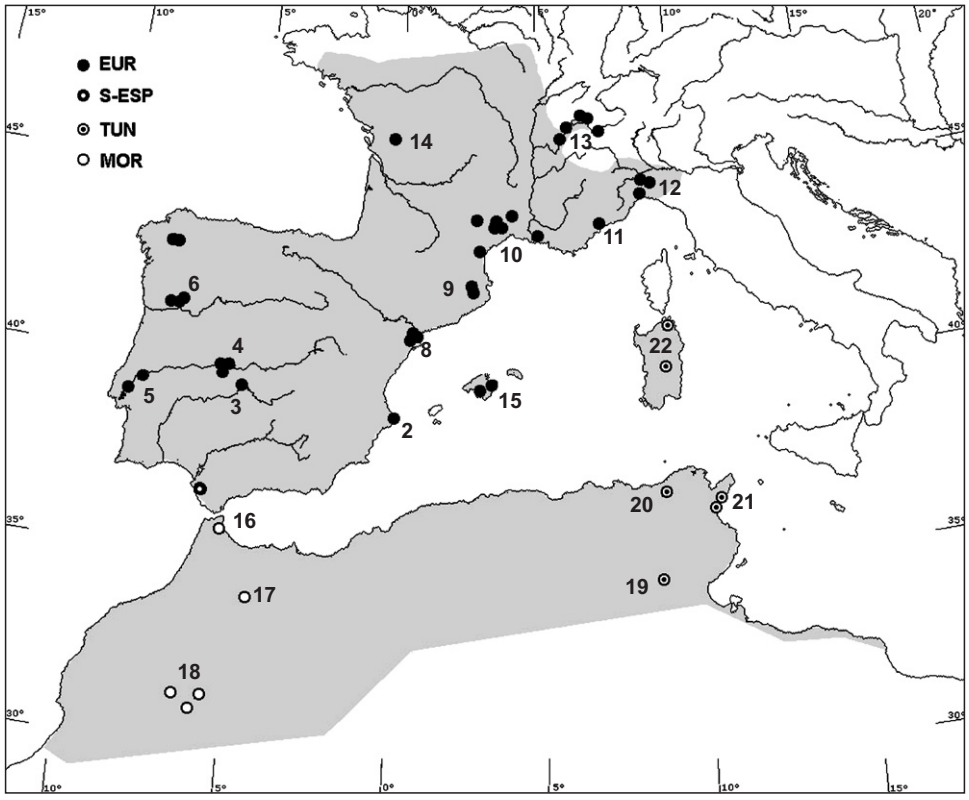

Fig. 1. Locations of sampling sites and populations defined for phylogeographic analysis. Numbers in parentheses indicate sample sizes. Gray area shows distribution range of *N. maura*. Different symbols refer to different evolutionary lineages as defined in Fig. 2. EUR = European clade, MOR = Moroccan clade, TUN = Tunisian clade, S-ESP = southern Spanish clade.

or Pliocene origin has been postulated, whereas Pleistocene differentiation has mostly been restricted to the intraspecific level. A similar scenario also seems to apply to the three species of western Palearctic water snakes within the genus *Natrix*: the viperine snake *N. maura* (Linnaeus, 1758), the dice snake *N. tessellata* (Laurenti, 1768), and the grass snake *N. natrix* (Linnaeus, 1758) (Guicking et al. 2006a). In a detailed analysis of the genus phylogeny, evidence was found that the last common ancestor of the three extant *Natrix* species probably lived during the late early to middle Miocene, much earlier than has generally been assumed (Guicking et al. 2006a). We hypothesized that after the collision of the African and Eurasian continents about 18–20 million years ago, the ancestral *Natrix* population expanded from southwestern Asia to Africa. Subsequent isolation of the African populations resulted in the independent evolution of an African lineage, which finally gave rise to the extant *N. maura*. The populations on the Eurasian continent evolved into *N. tessellata* and *N. natrix*. Intraspecific divergence in all three species most likely started at the Miocene–Pliocene boundary. If these age estimations were correct, and given a generally low mobility of reptiles, clear patterns of intraspecific differentiation are expected.

The viperine snake, *N. maura*, is the smallest and least broadly distributed of the three *Natrix* species. It is confined to aquatic or marshy habitats in climatically favored regions, where it feeds on amphibians and fishes as its most important prey organisms (Schätti 1999). The range of *N. maura* includes southern France, northwestern Italy, the Iberian Peninsula and northwestern Africa (Fig. 1). The only detailed and geographically comprehensive study on intraspecific variation in this species has been carried out by Schätti (1982). He analyzed a number of internal and external morphological and meristic characters as well as patterns of coloration in more than 500 specimens, covering the whole distribution range. His results showed that phenotypical variation is weak in the viperine snake,

and that most variation occurs only regionally. Slight differences in scale parameters were observed between European and African animals. Because phenotypical variation does not show clear geographic patterns, the viperine snake is considered a monotypic species at the moment (Schätti 1999).

In the present paper, we readdress the question of intraspecific differentiation in the viperine snake, using mitochondrial cytochrome *b* sequences and genomic inter-simple-sequence-repeats polymerase chain reaction (ISSR-PCR) fingerprinting as genetic markers. A general approach of phylogenetic inference is used to identify independent evolutionary lineages in the viperine snake and to detect an association of genetic and geographic variation. A closer look at the geographic distribution of genetic variability in Europe allows first hypotheses on the processes that have shaped the genetic structure of European *N. maura*. Our data provide clear evidence of strong intraspecific variation in the viperine snake. To account for this high variability taxonomic distinction of the major evolutionary lineages is suggested.

Material and methods

Sampling

A total of 120 samples from across most of the species' distribution range was analyzed in this study (see Fig. 1 and the Appendix for locality information). As a source of DNA, whole blood drawn from living snakes, tissue samples of dead specimens or shed skin was used. Samples were stored in EDTA buffer or 70% ethanol and kept at −16 °C. Isolation of total genomic DNA followed standard protocols (Sambrook and Russell 2001).

For tree rooting purposes, initially six species of new world natricine snakes and both other species of *Natrix* were used as outgroups. Because the choice of the outgroup did not influence tree topology of the ingroup, in the final analyses three sequences of *N. natrix* and five sequences of *N. tessellata* were chosen for outgroup rooting.

Sequencing of the cytochrome *b* gene

Amplification of the target sequence was performed in 50 µl PCR volume containing 0.75 units of *Taq* polymerase, 0.2 mM of each dNTP, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5% Triton X-100, and 10 mM Tris–HCl (pH 8.5). A 10 pmol of primers L14724NAT (5′-GAC CTG CGG TCC GAA AAA CCA-3′; Guicking et al. 2002) and H16064 (5′-CTT TGG TTT ACA AGA ACA ATG CTT TA-3′; Burbrink et al. 2000), and 50–100 ng of total genomic DNA were used. PCR reactions were performed in a Biometra thermoblock with an initial denaturation at 94 °C for 5 min, 36 cycles of denaturing at 94 °C for 50 s, primer annealing at 50 °C for 30 s, and elongation at 72 °C for 90 s. A final elongation step was included for 5 min at 72 °C. PCR products were sequenced directly on automated sequencers (ALF Express II, Amersham Biosciences, Freiburg, and ABI Prism 3100, Applied Biosystems, Amsterdam) according to the manufacturer's instructions and with primers given in Guicking et al. (2006a).

The obtained sequences were aligned manually. To check for sequence errors, all sequences from closely related populations were compared and variable sites were checked individually in the sequence printouts. All sequences obtained an open reading frame without unexpected stop codons and a strong bias against guanidine on the L-strand, as is typical for mitochondrial DNA. It therefore may be inferred that the sequences represent the functional genes rather than nuclear pseudogenes.

In total, 116 samples were sequenced. Four additional sequences were extracted from GenBank or kindly provided by R. Lawson (Osher Foundation Laboratory, San Francisco, USA). All unique haplotypes identified in this study are deposited in GenBank under accession numbers AY487681 to AY487722 (see Appendix).

ISSR-PCR genomic fingerprinting

ISSR-PCR involves amplification of DNA using primers that are designed from di-, tri- or tetranucleotide repeat motifs and thus are complementary to microsatellites. ISSR-PCR takes advantage of the abundance of microsatellites in the genome: when two microsatellites are present within an amplifiable distance and in an inverted orientation, a single complementary oligonucleotide will prime amplification of the intervening DNA segment. As usually more than one such region occurs in the genome, several fragments of different lengths are amplified. Gel-electrophoretic separation of PCR products thus generates characteristic multi-locus fingerprint patterns of the template DNA. The main advantages of ISSR-PCR refer to a relatively good reproducibility and to the ease and cost-effective procedure of the amplification reaction in which no specific primers are needed. A disadvantage is that the loci are interpreted as dominant markers and no genotypic allele information is acquired.

In the present study, ISSR-PCR genomic fingerprinting was performed radioactively on high-resolution polyacrylamide gels. Three primers were chosen which produced polymorphic and well-resolved fingerprint patterns in *N. maura*: $(GACA)_4$, $(GAA)_5$, and $(GGAT)_4$. For PCR reactions, 50 ng of total DNA

was used as template, plus 6 pmol $(GACA)_4$, 12 pmol $(GGAT)_4$ or 14 pmol $(GAA)_5$ primer, 1.5 mM $MgCl_2$, 0.1 mM of dGTP, dCTP, and dTTP, 0.045 mM dATP, 1 μCi ^{33}P-alpha-dATP, 2.5 μl of 10× amplification buffer and 1 unit *Taq* polymerase in a total volume of 25 μl. The reactions were performed with an initial denaturation at 94 °C for 5 min; this was followed by 27 cycles for $(GACA)_4$ and $(GGAT)_4$ or 31 cycles for $(GAA)_5$ of 94 °C for 50 s, annealing for 30 s, and elongation at 72 °C for 2 min. A final elongation step at 72 °C for 25 min was included. Primer-specific annealing temperatures were chosen in a gradient PCR: 55 °C $(GACA)_4$, 46 °C $(GGAT)_4$, and 42 °C $(GAA)_5$. PCR products were separated on a denaturing Sequagel matrix at 65 W for 2.5 or 4 h (length 40 cm). After drying, an X-ray film was exposed to the gel for 1–3 days and developed.

Seventy-seven samples could be analyzed by ISSR-PCR. In the remaining samples either DNA concentration was too low or the DNA was degraded, so that amplification of ISSR-PCR fragments was not possible. Repeated analyses showed that ISSR-PCR is highly reproducible. Analysis of ISSR-PCR autoradiograms was done visually. A 0/1 matrix was produced indicating absence or presence of each analyzed fragment in every sample. Only fragments that appeared in at least two individuals and that occurred as strong and distinct bands were included in ISSR-PCR data analysis.

Phylogenetic reconstruction

For phylogenetic reconstruction from the cytochrome *b* sequences, three methods were employed: maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference. MP and ML analyses were used as implemented in the program Paup 4.0b10 (Swofford 2001). Bayesian phylogenetic analysis was performed using the program MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). Because prior analyses have shown that saturation effects are negligible in our data set (Guicking et al. 2006a), all characters were weighted equally in the parsimony analysis. A ML tree was calculated using the TIM + I + G model as selected under the Akaike Information Criterion in Modeltest 3.06 (Posada and Crandall 1998), using heuristic searches with tree-bisection–reconnection branch swapping and 10 random addition sequences replicates. Bayesian analysis was performed in four chains for 1,500,000 generations with the same parameter settings as for the maximum likelihood calculation (using the prset command). Every 100th tree was sampled, and the final consensus was drawn from the last 13,501 trees.

From ISSR-PCR data, a neighbor joining tree with the mean number of pairwise character differences as distance measurement was calculated using Paup 4.0b10 (Swofford 2001).

Bootstrap values (Felsenstein 1985) with 1000 replicates were calculated under the maximum parsimony criterion for cytochrome *b* data, and with the neighbor joining algorithm for ISSR-PCR data.

Differentiation of the major clades

Genetic distances between mitochondrial cytochrome *b* haplotypes (uncorrected *p*-distances and corrected distances according to the TIM + I + G model and the parameter values as chosen by Modeltest) were used to compare levels of genetic diversity within and among major phylogenetic clades, using the programs Mega 2.1 (Kumar et al. 2001) and PAUP 4.0b10. An analysis of molecular variance (AMOVA; Excoffier et al. 1992) was performed with the program Arlequin 2.0 (Schneider et al. 2000) to compare the components of genetic diversity imputable to the variance among and within evolutionary lineages.

Genetic variation in European *Natrix maura*

To allow for a more detailed analysis of the phylogeography and evolutionary history of European *N. maura*, 14 locality groups (hereafter called populations) were defined on the European mainland, in which samples from close localities were pooled (Fig. 1). Samples from Mallorca were not considered further in these analyses because of the probably introduced status of the Mallorcan *N. maura* (see below; Guicking et al. 2006b).

To characterize and compare mitochondrial genetic diversity between different European populations, the number of haplotypes (h), number of polymorphic sites (p), nucleotide diversity (π), and mean number of pairwise differences (d) were calculated using the program Arlequin 2.0 (Schneider et al. 2000).

Intraspecific gene evolution was estimated by a network reconstruction with the program TCS 1.18 (Clement et al. 2000). MtDNA haplotypes differing by fewer than 15 mutations had a probability >95% of being connected in a most parsimonious manner. Accordingly, all mainland European samples could be included in the network, except for one sample from Cádiz region in southern Spain that constituted an independent evolutionary lineage (see below). To indicate the most likely root of the network, the closest connections to the southern Spanish sample are shown.

Arbitrary groups of haplotypes were defined to respectively comprise haplotypes separated by no more than one missing intermediate haplotype. The geographic distribution of these haplotype groups helps to

visualize patterns of phylogeographic substructure in European *N. maura*.

Results

A range-wide phylogeography of *Natrix maura*

In 120 samples of *N. maura* from across the entire distribution range, 44 unique cytochrome *b* haplotypes were identified. These differed at 114 variable sites and 95 parsimony informative sites. The number of substitutions between different haplotypes ranged from 1 to 53, corresponding to a net proportion of nucleotide substitutions of up to 4.75%.

To resolve intraspecific differentiation according to cytochrome *b* sequences, phylogenetic trees were calculated under the maximum parsimony and maximum likelihood criteria, and using Bayesian inference (Fig. 2). All reconstructions yielded nearly identical tree topologies. Four major clades could be distinguished, and confirmed by high support values (Figs. 1 and 2), that corresponded to samples from Morocco (MOR), Tunisia (TUN), southern Spain (S-ESP), and the remainder of Europe (EUR), respectively. All reconstructions clearly

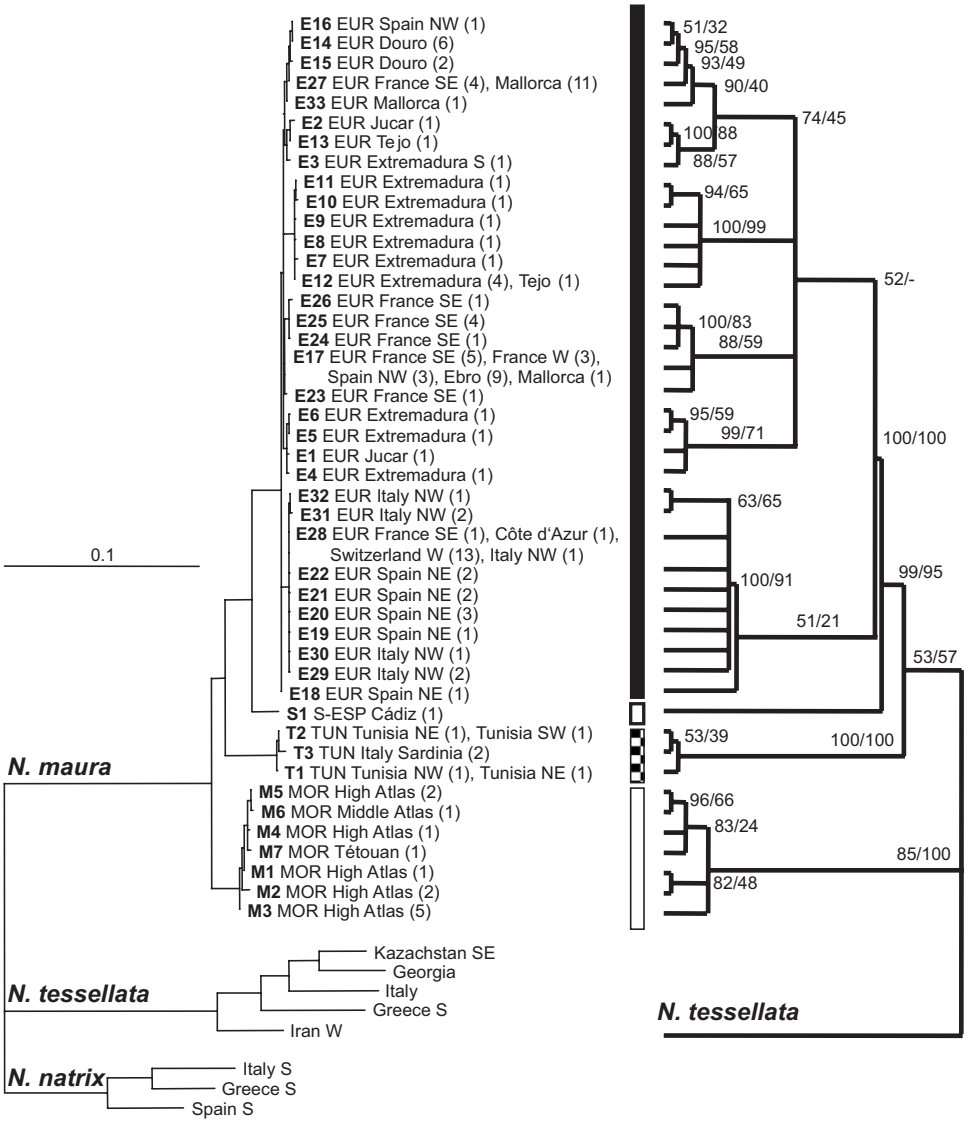

Fig. 2. Phylogenetic trees of *N. maura* reconstructed from cytochrome *b* haplotypes; left: maximum likelihood phylogram, right: Bayesian cladogram. Sequences from *N. natrix* and *N. tessellata* were used for outgroup rooting. Support values shown as Bayesian/MP bootstrap. Haplotype identifications as in Table 2 and Fig. 4. Numbers in parentheses are frequencies of haplotypes. Vertical bars indicate the four major lineages defined by the data — black bar = European clade; white with wide borders = southern Spanish clade (S-ESP); checkered = Tunisian clade; white with narrow borders = Moroccan clade. Note that the single sequence in clade S-ESP poses some uncertainty as to the distinction of an independent southern Spanish lineage (see text).

Table 1. Genetic distances among cytochrome *b* sequences of major phylogenetic clades of *N. maura*

	MOR	TUN	S-ESP	EUR
MOR	0.0090/0.0090	0.0441–0.0536	0.0429–0.0442	0.0438–0.0552
TUN	0.0394–0.0466	0.0045/0.0044	0.0465–0.0500	0.0453–0.0561
S-ESP	0.0385–0.0394	0.0412–0.0439	–	0.0257–0.0332
EUR	0.0385–0.0474	0.0394–0.0466	0.0242–0.0304	0.0134/0.0137

Below diagonal: ranges of between-clade distances (uncorrected *p*-distances). On diagonal: maximum within-clade distances (uncorrected *p*-distances/corrected ML distances). Above diagonal: ranges of between-clade distances (corrected ML distances). ML distances corrected according to the parameters chosen by Modeltest (see text). MOR = Moroccan clade, TUN = Tunisian clade, S-ESP = southern Spanish clade, EUR = European clade.

supported a sister relationship between the southern Spanish and the European clade. However, it should be noticed that only one sequence from southern Spain was available for this study. Therefore, interpretations regarding this lineage are only preliminary. Because distinction of this sample from other European samples was supported by three further mitochondrial gene sequences (NADH dehydrogenase subunits 1, 2, and 4; Guicking et al. 2006a), we presume that the observed differences were not caused by sequencing errors. Less clear was the relationship between the European and African lineages. Support was low for either a Moroccan–European or a Tunisian–European sister relationship, independent of the reconstruction method used (Fig. 2). Weak resolution of these relationships and very low support values demonstrated that the relative placement of the European to the African clades cannot readily be inferred from the cytochrome *b* data, although with longer sequence information the Moroccan–European relationship was clearly favored (Guicking et al. 2006a).

The island populations of Sardinia and Mallorca showed very close relationships to populations from Tunisia and the European mainland, respectively. Two samples from Sardinia differed at only four nucleotide positions from the most closely related haplotype in Tunisia. On Mallorca, three haplotypes were found in a total of 13 samples, and these differed from each other by a maximum of four nucleotides. Two of the haplotypes were also detected on the European mainland.

Pairwise genetic distances based on cytochrome *b* sequences are summarized as interclade and intraclade comparisons in Table 1. The MOR, TUN, and EUR clades were separated from each other by uncorrected *p*-distances of 3.9–4.7% and corrected TIM + I + G distances of 4.4–5.6%, respectively. Between the southern Spanish sample from Cádiz (clade S-ESP) and the remaining European samples (clade EUR) genetic distances were in the ranges of 2.4–3.0% and 2.6–3.3%. Intraclade distances were highest in the European clade, reaching a maximum of 1.3%. Genetic distances lower within the African clade than in the European one do not necessarily indicate less diversity, but might be due to smaller sample sizes and more local sampling. Considerably lower within-clade than among-clade distances (Table 1) and generally shallow branch topologies (Fig. 2) suggest that differentiation into extant haplotypes occurred in comparatively recent times in all major clades.

With each haplotype considered only once, AMOVA ascribed 85% of the observed variation to among-clade variation and only 15% to within-clade variation, indicating highly significant differentiation of the four major clades ($\Phi_{st} = 0.846$, $P < 0.001$).

Genetic evidence from nuclear ISSR-PCR markers supported the findings from mitochondrial sequence data. ISSR-PCR analyses from 77 samples yielded 48 polymorphic bands which could be reliably scored from the gels. Pairwise comparisons of ISSR-PCR fingerprints resulted in 0–30 differences, corresponding to a maximum proportion of different fragments of 63% between two samples.

Phylogenetic reconstruction inferred from ISSR-PCR fingerprints supported intraspecific subdivision into two African and one European clade with high bootstrap values (Moroccan clade: 92%, Tunisian clade: 70%, European clade: 90%; Fig. 3). Unfortunately, no sample material from southern Spain was available for genomic fingerprinting. In congruence to the mitochondrial data, ISSR-PCR results supported the close association of the populations from the Mediterranean islands of Mallorca and Sardinia to populations from the European mainland and Tunisia, respectively.

Population structure of European *Natrix maura*

A total of 33 distinct cytochrome *b* haplotypes were detected within the European clade. The geographic distribution of these haplotypes among 14 locality groups (populations) is shown in Table 2. With only two exceptions, all haplotypes occurred only locally. These exceptions were haplotype E17, found in northeastern Spain, southeastern and western France, and haplotype E28, which occurred throughout the northeastern distribution range of *N. maura*. Especially in the southern populations, many distinct haplotypes were

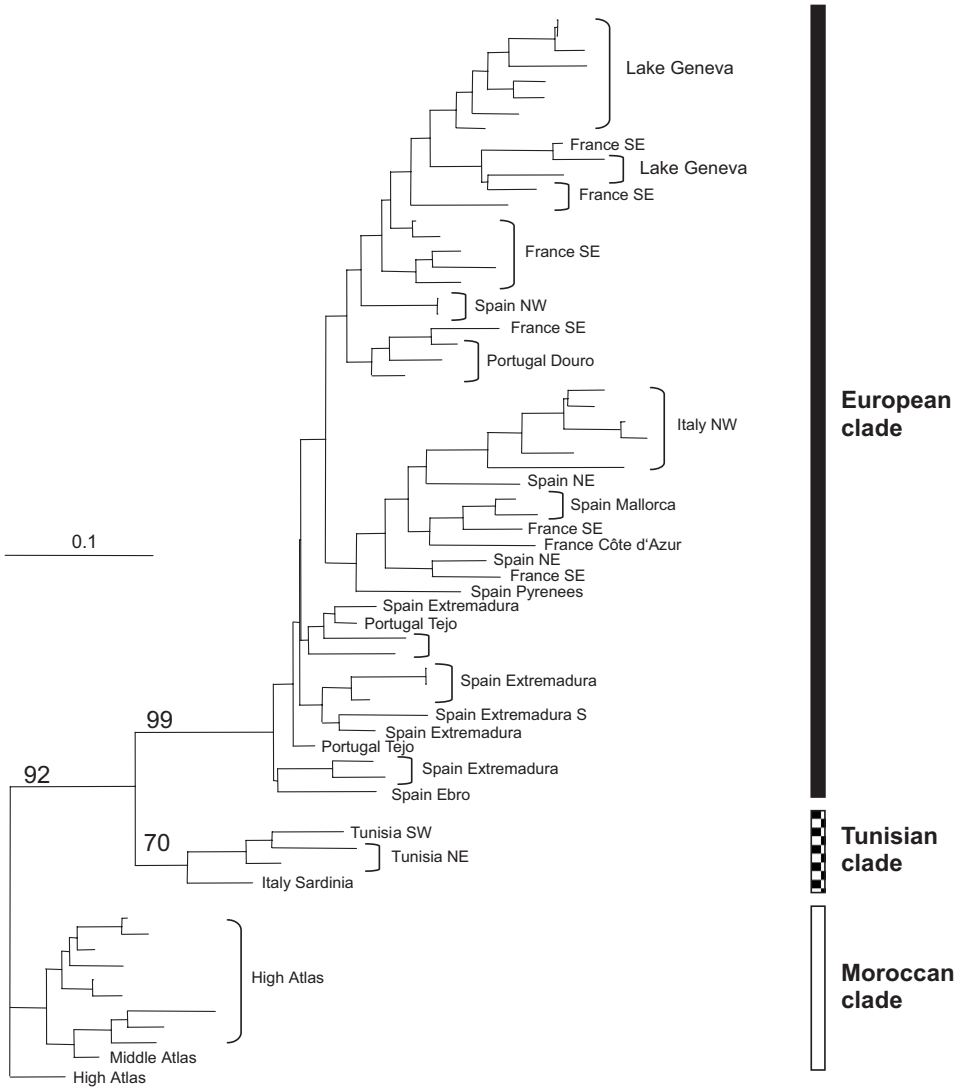

Fig. 3. Unrooted neighbor joining tree of *N. maura* ISSR-PCR data, reconstructed from 48 polymorphic bands detected with primers $(GACA)_4$, $(GAA)_5$, and $(GGAT)_4$. Bootstrap values based on 1000 replications are indicated for the major lineages.

found (e.g. in the population 'Extremadura' 9 different haplotypes were found among 12 individuals).

Genetic diversity indices for the 14 populations defined on the European mainland are given in Table 3. Based on the available samples, the highest diversities were found in the central Iberian populations (EUR Jucar, EUR Extremadura, and EUR Tejo), in northwestern Spain (EUR Spain NW), and in southeastern France (EUR SE France). Medium diversities were reported in northeastern Spain (EUR Spain NE) and Italy (EUR Italy NW). Very low or no diversity was found in the populations in northern Portugal (EUR Douro), in the Ebro delta (EUR Ebro), Switzerland (EUR Lake Geneva), and western France (EUR France W).

A statistical parsimony network illustrates the relationships of haplotypes in the European lineage (Fig. 4). Two equally close connections (27 mutational steps) exist between the haplotypes in the network and the southern Spanish sample, rooting the network at either the E18 or the E27 haplotype. When grouping all haplotypes separated by no more than one missing intermediate haplotype, seven groups resulted that were distributed either north or south of the central Iberian mountain ranges, but no haplotype group was found that occurred across the entire Iberian peninsula (Fig. 5). Furthermore, in the southern Iberian peninsula each individual haplotype group was associated with one of the major west–east orientated river systems, whereas north of the central Iberian mountains haplotype groups were more broadly distributed.

It seems noteworthy, though not supported by bootstrapping, that according to ISSR-PCR data the populations north of the central Iberian mountain ranges also formed a distinct group which was separated from all southern populations (Fig. 3). The only northern sample

Table 2. Geographic distribution of cytochrome *b* haplotypes in European *N. maura*, numbers of samples (*N*) per population and haplotype, and for each population (see Fig. 1) the respective numbers (*h*) of different haplotypes and unique haplotypes found

Populations	Haplotypes																																	*N* per population	Different *h*	Unique *h*
	S1	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E13	E14	E15	E16	E17	E18	E19	E20	E21	E22	E23	E24	E25	E26	E27	E28	E29	E30	E31	E32			
1 S-ESP Cádiz	1																																	1	1	1
2 EUR Jucar		1	1																															2	2	2
3 EUR Extremadura S				1																														1	1	1
4 EUR Extremadura					1	1	1	1	1	1	1	1	4																					12	9	8
5 EUR Tejo													1	1																				2	2	1
6 EUR Douro															6	2																		8	2	2
7 EUR Spain NW																	1	2																3	2	1
8 EUR Ebro																		9	1															10	2	1
9 EUR Spain NE																				1	3	2	2											8	4	4
10 EUR France SE																		5						1	1	4	1	4						16	6	5
11 EUR Côte d'Azur																													1					1	1	0
12 EUR Italy NW																													1	2	1	2	1	7	5	4
13 EUR Switzerland W																													13					13	1	0
14 EUR France W																		3																3	1	0
N per haplotype	1	1	1	1	1	1	1	1	1	1	1	1	5	1	6	2	1	19	1	1	3	2	2	1	1	4	1	4	15	2	1	2	1	87		

Table 3. Indices of genetic diversity among mitochondrial cytochrome *b* sequences from 13 European populations of *N. maura*

Population	*N*	*H*	*p*	$\pi \pm$S.D.	*d*
EUR Jucar	2	2	9	0.0081 ± 0.0085	9.00
EUR Extremadura S	1	1	–	–	–
EUR Extremadura	12	9	16	0.0044 ± 0.0026	4.91
EUR Tejo	2	2	10	0.0090 ± 0.0094	10.00
EUR Douro	8	2	1	0.0004 ± 0.0004	0.43
EUR Spain NW	3	2	6	0.0036 ± 0.0030	4.00
EUR Ebro	10	2	2	0.0004 ± 0.0004	0.40
EUR Spain NE	8	4	4	0.0015 ± 0.0011	1.64
EUR SE France	15	7	15	0.0032 ± 0.0019	3.60
EUR Côte d'Azur	1	1	–	–	–
EUR Italy NW	7	5	4	0.0016 ± 0.0012	1.81
EUR Switzerland W	13	1	0	0	0
EUR France W	3	1	0	0	0

N = number of individuals, *h* = number of haplotypes, *p* = number of polymorphic sites, $\pi \pm$S.D. = nucleotide diversity ± standard deviation, *d* = mean number of pairwise differences.

that clustered with the southern populations in the ISSR-PCR tree was one sample from the Ebro delta with haplotype E18, one of the two potentially ancient European haplotypes.

Discussion

Origin and relationships of major evolutionary lineages

Based on molecular markers, at least three (probably four) major clades can be distinguished in the viperine snake. These clades relate to geographic regions of Tunisia, Morocco, and Europe. Although phylogenetic relationships among the three major lineages were only weakly resolved by cytochrome *b* sequences, all inferences assigned basal status to the African clades, supporting our former hypothesis that the viperine snake originated in Africa (Guicking et al. 2006a). A fourth lineage that is located in southern Spain may be distinguished within the European clade. This lineage represents the phylogenetic sister group to all other European samples. So far, however, evidence for a southern Spanish lineage is based on a single cytochrome *b* sequence. Further sample material from the region would be desirable to draw conclusions on the distribution and distinctness of that lineage.

Three features of the phylogeographic pattern seen in *N. maura* merit further discussion: (1) the question of post-Messinian gene flow across the Strait of Gibraltar, (2) the role of the Maghreb region in northwestern

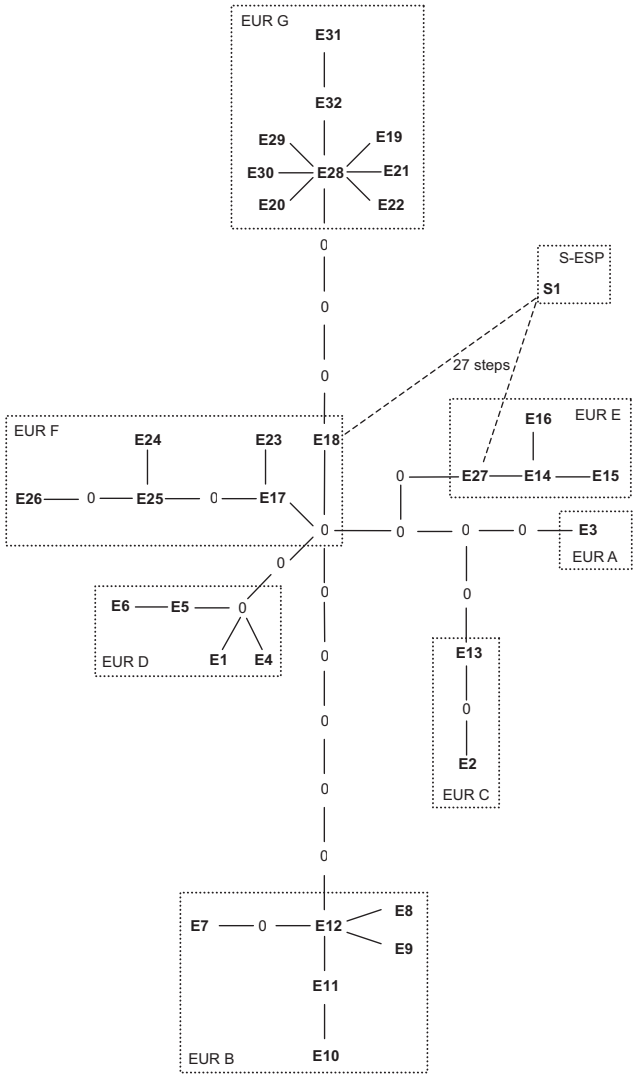

Fig. 4. Statistical parsimony network of all 33 cytochrome *b* haplotypes detected within the European lineage. Each line represents one mutational change. Zeros represent missing intermediate haplotypes. By grouping all haplotypes separated by no more than one missing intermediate, seven haplotype groups were defined (EUR A to EUR G). To show the most probable root to the network, the two shortest connections to the sample from Cadiz, southern Spain (haplotype S1), are indicated by broken lines.

Africa for intraspecific differentiation, and (3) the origin of the island populations on Mallorca and Sardinia.

The phylogeographic importance of the Strait of Gibraltar has been a focus of many studies in the western Mediterranean (e.g. Busack 1986; Busack et al. 2005; Veith et al. 2004; Cosson et al. 2005; Fritz et al. 2006). During the Messinian period about 5–6 million years ago, the closure of the Strait of Gibraltar led to an almost complete desiccation of the Mediterranean, and to salt formation in all deep basins, the so-called Messinian salinity crisis (Hsü et al. 1977; Krijgsman et al. 1999). The resulting land corridor permitted faunal

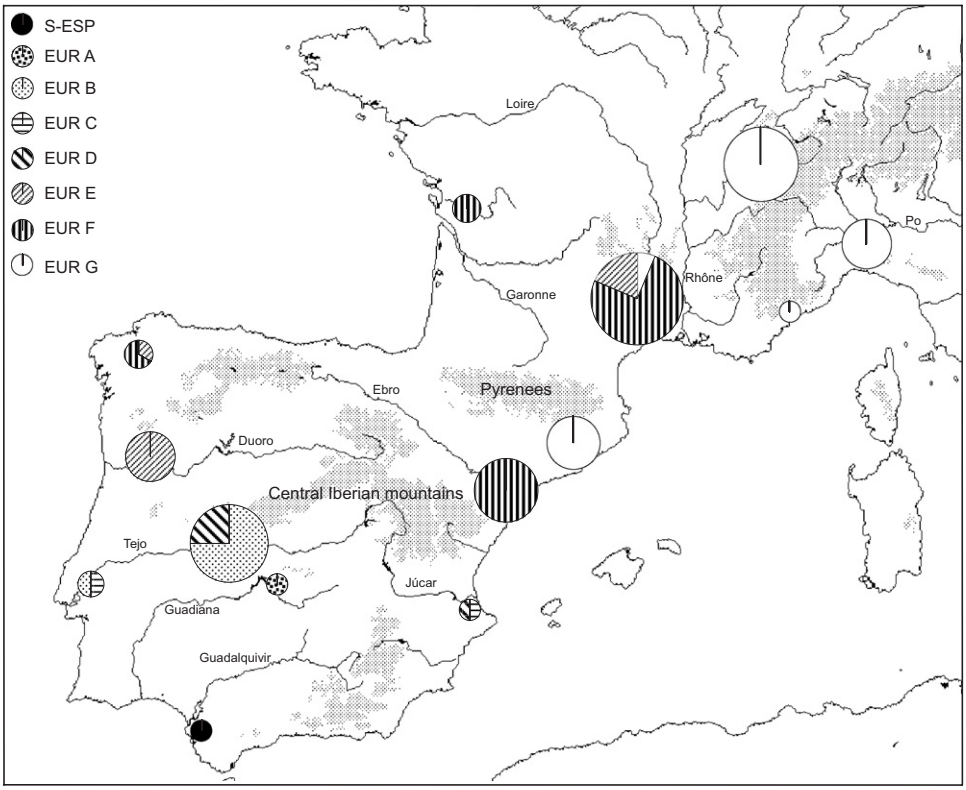

Fig. 5. Genetic variation of 14 population groups of *N. maura* in Europe, illustrated by occurrence and frequency of cytochrome *b* haplotypes belonging to the seven haplotype groups defined in Fig. 4. Sizes of circles correlate to sample sizes in the populations.

exchanges between southern Europe and the Maghreb region in northern Africa (Blondel and Aronson 1999; Dobson and Wright 2000). This biotic interchange came to an end when the strait reopened about 5 million years ago (Krijgsman et al. 1999), initiating vicariant evolution of faunal lineages on both sides of the strait (Blondel and Aronson 1999; Dobson and Wright 2000). We discussed a similar scenario for *N. maura*, hypothesizing that *N. maura* reached Europe during the Messinian salinity crisis 5–6 million years ago and has evolved independently on either side of the strait after the reflooding of the Mediterranean (Guicking et al. 2006a). However, in a number of terrestrial taxa evidence has been found that post-Messinian gene flow took place across the Strait of Gibraltar (e.g. Busack 1986; Harris et al. 2002; Cosson et al. 2005). In most cases, this gene flow has been interpreted as a result of transmarine dispersal facilitated by rafting. Evidence for transmarine dispersal in reptiles has been discussed by Houle (1998) and Lenk et al. (1999) and is evident in many island species (e.g. Sites et al. 1996; Carranza et al. 2000; Nagy et al. 2003; Glor et al. 2005).

The viperine snake is a semiaquatic snake that is usually found in freshwater habitats, but might also occur in coastal brackish waters (Schätti 1999; Lenk 2002). It therefore has been regarded as a species quite unlikely to be isolated completely from conspecifics by the strait (Busack 1986). However, neither mitochondrial nor nuclear data indicate post-Messinian gene flow (this study). Although this result may turn out to be an artefact simply because of too limited sampling on either side of the strait, the currently available data indicate that even if post-Messinian gene flow across the Strait of Gibraltar took place in the viperine snake, this would have little effect on the populations further inland, both in Africa and Europe.

The Maghreb region in northwestern Africa is formed by the three countries of Morocco, Algeria, and Tunisia and belongs to the Palaearctic biogeographical realm. Several studies of North African reptiles and amphibians show considerable genetic diversity between Moroccan, Algerian, and Tunisian forms, and it has been suggested that an important geographical barrier might exist around the Moulouya river basin in extreme eastern Morocco (Álvarez et al. 2000; Harris et al. 2002). Fritz et al. (2006) found evidence for distinct lineages in the stripe-necked terrapin, *Mauremys leprosa*, north and south of the Atlas mountains in Morocco.

The data currently available for *N. maura* do not allow any statement on where the biogeographic border between the Moroccan and Tunisian clades is most likely located. However, clear differentiation of the Tunisian and Moroccan samples is concordant with de Jong (1998), who identified the region of Morocco and

the Algerian/Tunisian region as two distinct 'areas of endemism' in northwestern Africa, based on a more general approach using area-cladograms. Provided our time estimations are correct (Guicking et al. 2006a), similar maximum genetic distances between the two African clades on the one hand and between the African and the European clade on the other hand suggest that the two African clades started to diverge at the end of the Messinian about 5 million years ago. This gives further support to the hypothesis of a strong biogeographic barrier between the two regions in northwestern Africa, because otherwise it seems unlikely that the two lineages could have evolved independently for several million years.

For the two Mediterranean island populations on Mallorca and Sardinia, a very recent origin has been suggested due to morphological similarities between the island and mainland specimens (Schätti 1982). This hypothesis is clearly supported by genetic data. Both islands had their last contact to the mainland during the Messinian salinity crisis, when Mallorca was connected to southeastern Spain, and Sardinia together with the island of Corsica was connected to Italy and Tunisia (Hsü et al. 1977; Krijgsman et al. 1999). Native origin of the island populations on Mallorca and Sardinia through terrestrial dispersal therefore would imply independent evolution for about 5 million years, and consequently strong genetic differentiation. The close genetic similarities of the Mallorcan and Sardinian *N. maura* with populations from the European mainland and Tunisia, as found in the present study, hence suggest a much more recent origin of the two island populations. Genetic data are most compatible with an anthropogenic origin for the Mallorcan population (Guicking et al. 2006b), and this hypothesis is further supported by historical data indicating that the viperine snake may have been introduced to Mallorca by the Romans (Gosàlbez 1987). Less clear information is available for the Sardinian population of *N. maura*. In this case, it is not yet possible to decide whether the viperine snake more likely reached the island by natural transmarine dispersal or with the help of humans (see also Schätti 1999).

Evolutionary history of *Natrix maura* in Europe

The phylogeographic history of European biota has been most extensively studied against the background of Pleistocene climatic oscillations. These studies highlight the consequences that large-scaled extinctions in the north and survival of refugial populations in the south during cold periods as well as recolonization of northern territories during interglacials had on the European fauna and flora (e.g. Hewitt 1996, 2000; Taberlet et al. 1998). Survival in southern refugia generally allowed long-term persistence of populations and thus resulted in high genetic diversity (Hewitt 1996; Taberlet et al. 1998). In contrast, northern parts of the distribution ranges that were uninhabitable during cold periods were colonized only after the last glacial retreat about 14,000–9000 years before present (Crowley and North 1991), and consequently exhibit a much reduced genetic diversity. Depending on the mode of expansion, population bottlenecks during range expansions might have led to further loss of genetic diversity in northward expanding populations (Hewitt 1996; Ibrahim et al. 1996).

A comparatively shallow branch topology for mtDNA in European *N. maura* suggests that differentiation into extant haplotypes in Europe started long after the split of the European from the African lineages. Assuming an evolutionary rate of 1% divergence per 1 million years (Guicking et al. 2006a), the separation of the southern Spanish sample from the remaining European ones can be dated roughly to 2.5 million years ago. The maximum intraclade genetic distance of 1.37% in the European clade suggests that diversification of this lineage took place in the middle and late Pleistocene, possibly in response to changing climatic conditions (Crowley and North 1991; Webb and Bartlein 1992; Jansson and Dynesius 2002).

A detailed analysis of genetic variation in European *N. maura* has revealed a number of distinct haplotypes and locally high haplotype diversity (this study). This shows that the current sampling is insufficient to comprehensively illustrate the genetic diversity of this lineage. Inferences about the evolutionary history of European *N. maura* therefore must be regarded as preliminary. Nevertheless, some patterns observed in the geographic distribution of European haplotypes are noteworthy and may guide future studies on the evolution of *N. maura*.

A comparatively clear picture of north–south distinction and west–east relation was found in the Iberian *N. maura*. Such a pattern of predominantly west–east orientated gene flow and north–south differentiation is not surprising as it is in concordance with phylogeographic patterns in many aquatic or semiaquatic animals in the Iberian peninsula and is clearly attributable to the physiography of the Iberian peninsula, which is dominated by west–east orientated mountain ranges and river valleys (e.g. Machordom and Doadrio 2001, Gómez and Hunt 2007). Hence, in *N. maura* as in other Iberian species mountain ranges played a role as geographic barriers to dispersal, leading to fragmentation and subsequent vicariant evolution, whereas river basins facilitated migration and genetic exchange. The occurrence of distinct haplotypes or haplotype lineages in different river valleys, e.g. in the Guadalquivir, Guadiana and Tejo/Jucar river basins in the southern Iberian peninsula, suggests that *N. maura* survived Pleistocene

cold periods in different microrefugia associated with these river basins, resulting in allopatric differentiation.

Whereas in the southern Iberian peninsula haplotype groups were clearly associated with distinct river valleys, more widespread distribution of lineages was observed in areas north of the central Iberian mountain ranges. These broader geographic distribution ranges might reflect enhanced gene flow or postglacial range expansions of refugial populations. Postglacial migrations most likely followed major river systems, e.g. along the river Rhône, resulting in genetically impoverished northern populations as were found in eastern France and Switzerland. Closely related haplotypes south and north of the Pyrenees provide evidence that the Pyrenees have not prevented migration between northern Spain and France but were most likely circumvented along either the Atlantic or the Mediterranean coast or both.

Probably the most surprising result of our study were the comparatively high genetic diversities found in populations of southeastern France and Italy. Northern genetic richness in some cases has been interpreted as a consequence of long-term persistence of the northern populations (Stewart and Lister 2001). The observed pattern of genetic diversity in northern *N. maura* therefore could be interpreted as a result of Pleistocene glacial refugia north of the Pyrenees. A further argument in favor of a northern refugium is provided by the exclusively northern distribution of a presumably ancient haplotype. It is generally assumed that haplotypes at interior positions of a network are more ancient than tip haplotypes (Templeton et al. 1995), as applies to haplotype E28 in *N. maura*. E28, however, was found only north of the Pyrenees, whereas the population in northeastern Spain comprised only tip haplotypes that are presumably younger and were derived from E28 (E19, E20, E21, E22). According to general phylogeographic theory, this pattern of haplotype distribution is most compatible with a southward trans-Pyrenean range expansion. However, north of the Pyrenees mainly tundra and boreal forest is assumed to have occurred during the last glaciation (Crowley and North 1991; Hewitt 1996). Thus, it seems unlikely that a thermophilic species like *N. maura*, that is highly dependent on warm climates (Schätti 1999), was able to survive Pleistocene cold periods in southern France.

Alternatively, northern genetic richness in the viperine snake could be explained by a Pleistocene refugium in Italy from where postglacial colonization of northern Italy and southeastern France took place. The currently restricted distribution of *N. maura* in Italy seems to argue against an Italian refugium, but could be interpreted as a result of interspecific competition with *N. tessellata*, which is similar to *N. maura* ecologically but apparently more competitive when both occur in sympatry (Schätti 1999). To us, the most likely explanation for the observed genetic richness of northern populations in *N. maura* is insufficient sampling of northern Iberian populations. Further sampling in this area is likely to reveal the occurrence of genetically diverse populations that might have served as source populations for postglacial recolonization of areas north of the Pyrenees. Therefore, more data should be collected before hypotheses on the location of northern Pleistocene refugia are drawn.

Taxonomic conclusions

We may conclude that both our mitochondrial and nuclear data are consistent with the interpretation of three or four distinct phylogenetic taxa within what is currently recognized as *N. maura*. Genetic distances of up to more than 5% are in the range of distances reported among subspecies and even species in other natricine snakes (de Queiroz 1994; Alfaro and Arnold 2001). Taxonomic subdivision of *N. maura* into three or four phylogenetic taxa therefore seems justifiable. However, it still needs to be determined whether such distinction would be more appropriate at the species or subspecific level. We suggest to wait for additional information from further samples and phenotypic data before making final decisions on how many taxa at which hierarchical level should be distinguished within the viperine snake complex. To decide taxonomic issues, further samples for genetic analyses would be particularly desirable from southern Spain and the Maghreb region, in order to determine the geographic ranges of the currently under-represented major lineages and to ascertain whether gene flow takes place among adjacent lineages. Finally, tests should be implemented linking genotypic with phenotypic information to identify morphological differences among major lineages and to allow phenotypic delimitation of taxonomic entities.

Acknowledgements

We are very grateful to T. Amann, X. Bonnet, H.-P. Eckstein, W. Fiedler, Ph. Golay, R.A. Griffiths, A. Hille, A. Hundsdörfer, P. Lenk, A. Loureiro (BTVS-ICN, Portugal), A. Pieh, E. Razzetti, X. Santos (permission from the "Direcció General del Medi Natural, Generalitat de Catalunya" no. 6539, 26.10.1990), A. Sproll, S. Ursenbacher, and M. Vences for their contribution of sample material. We would further like to thank Robin Lawson for providing unpublished sequences and for much helpful advice. Our special thanks to Femmie Kraaijeveld for proof-reading an earlier version of this manuscript, and to two anonymous reviewers for their comments. Financial support for this study was provided by the Deutsche Forschungs-Gemeinschaft (Jo 134-7, Wi-719/18).

Appendix

List of samples, indicating sample and/or voucher number, species name, geographic origin, cytochrome *b* gene haplotype as defined in this study, GenBank accession number, and inclusion in ISSR-PCR analysis

IPMB no./Voucher	*Natrix* species	Origin	Latitude	Longitude	Haplo-type	AccCytb	ISSR-PCR
T158*	*N. tessellata*	Iran, Kermanshah Prov.	34.20N	47.00E		AY487574	
T262	*N. tessellata*	Greece, Golf of Arta	39.10N	20.45E		AY487588	
T159	*N. tessellata*	Georgia, Batumi	41.35N	41.38E		AY487599	
T004	*N. tessellata*	Kazakhstan, Almaty Region				AY487604	
T005	*N. tessellata*	Italy, Lazio	42.08N	11.54E		AY487671	
MNCN 13796*	*N. natrix*	Spain, Cádiz Prov.	36.08N	05.42W		AY866535	
N003*	*N. natrix*	Greece, Ioánnina	39.40N	20.50E		AY487725	
N015*	*N. natrix*	Italy, near Torre San Gennaro	40.32N	18.05E		AY487733	
M069	*N. maura*	Tunisia, near Sousse	36.00N	10.30E	T1	AY487682	+
M133/HLMD RA 1188	*N. maura*	Tunisia, Bulla Regia	36.34N	08.46E	T1		
M011*	*N. maura*	Tunisia, Tameghza	34.23N	07.54E	T2	AY487681	+
M068	*N. maura*	Tunisia, near Nabeul	36.30N	10.30E	T2		+
M073	*N. maura*	Italy, Sardinia			T3	AY487683	+
M075/HLMD 00580	*N. maura*	Italy, Sardinia, Arzachena	41.05N	09.23E	T3		
M003	*N. maura*	Morocco, High Atlas			M1	AY487684	+
M081	*N. maura*	Morocco, High Atlas, Tizi-n Tichka	30.58N	06.50W	M2	AY487688	
M093	*N. maura*	Morocco, High Atlas, Tizi-n Tichka	30.58N	06.50W	M2		
M004	*N. maura*	Morocco, Tabounaht	30.57N	06.50W	M3	AY487685	+
M006	*N. maura*	Morocco, Tabounaht	30.57N	06.50W	M3		+
M009	*N. maura*	Morocco, Tabounaht	30.57N	06.50W	M3		+
M010	*N. maura*	Morocco, Tabounaht	30.57N	06.50W	M3		+
M089	*N. maura*	Morocco, High Atlas, Tizi-n Tichka	30.58N	06.50W	M3		+
M007	*N. maura*	Morocco, Tabounaht	30.57N	06.50W	M4	AY487687	+
M005	*N. maura*	Morocco, Tabounaht	30.57N	06.50W	M5	AY487686	+
M008	*N. maura*	Morocco, Tabounaht	30.57N	06.50W	M5		+
M088	*N. maura*	Morocco, Middle Atlas	33.00N	04.43W	M6	AY487689	+
MVZ 178093*	*N. maura*	Morocco Tétuan Prov.	53.50N	05.34W	M7	AF420077	
MNCN 12016*	*N. maura*	Spain, Cádiz Prov.	36.08N	05.42W	S1	AY866530	
M076/HLMD 00101	*N. maura*	Spain, Benissa	38.34N	00.03E	E1	AY487699	
M078/HLMD 00102	*N. maura*	Spain, Benissa	38.34N	00.03E	E2	AY487701	
M050	*N. maura*	Spain, Extremadura	39.16N	05.02W	E3	AY487696	+
M101	*N. maura*	Spain, Extremadura	40.16N	05.51W	E4	AY487710	
M100	*N. maura*	Spain, Extremadura	39.50N	06.05W	E5	AY487709	+
M099	*N. maura*	Spain, Extremadura	39.50N	06.05W	E6	AY487708	+
M102	*N. maura*	Spain, Extremadura	39.50N	06.05W	E7	AY487711	+
M103	*N. maura*	Spain, Extremadura	39.50N	06.05W	E8	AY487712	+
M109	*N. maura*	Spain, Extremadura	39.50N	06.05W	E9	AY487715	
M107	*N. maura*	Spain, Extremadura	39.25N	06.05W	E10	AY487714	+
M106	*N. maura*	Spain, Extremadura	39.30N	06.40W	E11	AY487713	+
M077/HLMD 00694	*N. maura*	Spain, S			E12	AY487700	
M104	*N. maura*	Spain, Extremadura	39.37N	06.46W	E12		+
M105	*N. maura*	Spain, Extremadura	39.35N	06.20W	E12		+
M108	*N. maura*	Spain, Extremadura	39.25N	06.05W	E12		+
M135	*N. maura*	Portugal, Ulme	39.21N	08.29W	E12		+
M134	*N. maura*	Portugal, River Tejo	38.55N	09.00W	E13	AY487720	+
M136/BTVS-ICN 00-0024	*N. maura*	Portugal, Peneda Geres NP	41.48N	08.08W	E14	AY487721	
M137/BTVS-ICN 00-0031	*N. maura*	Portugal, Peneda Geres NP	41.44N	08.10W	E14		
M138/BTVS-ICN 00-0050	*N. maura*	Portugal, Peneda Geres NP	41.46N	08.09W	E14		
M140/BTVS-ICN 02-0042	*N. maura*	Portugal, Peneda Geres NP	41.45N	08.09W	E14		
M142/BTVS-ICN 02-0070	*N. maura*	Portugal, Peneda Geres NP	41.45N	08.09W	E14		+
M141/BTVS-ICN 02-0053	*N. maura*	Portugal, NW			E14		+
M139/BTVS-ICN 00-0060	*N. maura*	Portugal, Peneda Geres NP	41.46N	08.09W	E15	AY487722	+

M143/BTVS-ICN 02-0072	*N. maura*	Portugal, Peneda Geres NP	41.42N	08.09W	E15		
M094	*N. maura*	Spain, Galicia, Vigo	42.14N	08.43W	E16	AY487707	
M002/HLMD RA 2704	*N. maura*	France, Camargue			E17	AY487691	
M065	*N. maura*	France, Lake Salagou	43.40N	03.21E	E17		+
M066	*N. maura*	France, Lake Salagou	43.40N	03.21E	E17		+
M124	*N. maura*	France, Lake Salagou	43.39N	03.20E	E17		+
M067	*N. maura*	France, River Aveyran	44.20N	02.30E	E17		+
M096	*N. maura*	France, near Niort	46.19N	00.27W	E17		
M097	*N. maura*	France, near Niort	46.19N	00.27W	E17		
M098	*N. maura*	France, near Niort	46.19N	00.27W	E17		
M053	*N. maura*	Spain, Ebro Delta	40.43N	00.54E	E17		
M055	*N. maura*	Spain, Ebro Delta	40.43N	00.54E	E17		
M056	*N. maura*	Spain, Ebro Delta	40.43N	00.54E	E17		
M057	*N. maura*	Spain, Ebro Delta	40.43N	00.54E	E17		
M058	*N. maura*	Spain, Ebro Delta	40.43N	00.54E	E17		
M059	*N. maura*	Spain, Ebro Delta	40.43N	00.54E	E17		
M060	*N. maura*	Spain, Ebro Delta	40.43N	00.54E	E17		
M061	*N. maura*	Spain, Ebro Delta	40.43N	00.54E	E17		
M062	*N. maura*	Spain, Ebro Delta	40.43N	00.54E	E17		
M095	*N. maura*	Spain, Pyrenees			E17		+
M082	*N. maura*	Spain, Galicia, Ponte Vedra	42.26N	08.38W	E17		+
M083	*N. maura*	Spain, Galicia, Ponte Vedra	42.26N	08.38W	E17		+
M110	*N. maura*	Spain, Mallorca, Alcudia	39.52N	03.07E	E17		+
M051	*N. maura*	Spain, River Ebro, Amposta	40.41N	00.44E	E18	AY487697	+
M080/ZFMK 58028	*N. maura*	Spain, Olot	42.11N	02.29E	E19	AY487703	
M085	*N. maura*	Spain, Olot	42.11N	02.29E	E20	AY487704	
M091	*N. maura*	Spain, Olot	42.11N	02.29E	E20		
MVZ 200533*	*N. maura*	Spain, Balena	41.51N	02.12E	E20		
M086	*N. maura*	Spain, Olot	42.11N	02.29E	E21	AY487705	+
M090	*N. maura*	Spain, Olot	42.11N	02.29E	E21		
M087	*N. maura*	Spain, Olot	42.11N	02.29E	E22	AY487706	+
M092	*N. maura*	Spain, Olot	42.11N	02.29E	E22		
M079	*N. maura*	France, Dept. Herault	43.15N	02.40E	E23	AY487702	
M126	*N. maura*	France, Lake Salagou	43.39N	03.20E	E24	AY487718	+
M127	*N. maura*	France, Lake Salagou	43.39N	03.20E	E25	AY487719	+
M147	*N. maura*	France, Lake Salagou	43.39N	03.20E	E25		+
M148	*N. maura*	France, Lake Salagou	43.39N	03.20E	E25		+
M149	*N. maura*	France, near Perpignan	42.51N	03.00E	E25		
M123	*N. maura*	France, Lake Salagou	43.39N	03.20E	E26	AY487717	+
M063	*N. maura*	France, Lake Salagou	43.40N	03.21E	E27	AY487698	+
M064	*N. maura*	France, Lake Salagou	43.40N	03.21E	E27		
M125	*N. maura*	France, Lake Salagou	43.39N	03.20E	E27		+
M150*	*N. maura*	France, Roche St. Secret Béconne	44.29N	05.01E	E27		
M118	*N. maura*	Spain, Mallorca, Pollenca	39.52N	03.01E	E27		+
M111	*N. maura*	Spain, Mallorca	39.30N	03.00E	E27		+
M112	*N. maura*	Spain, Mallorca	39.30N	03.00E	E27		+
M113	*N. maura*	Spain, Mallorca	39.30N	03.00E	E27		+
M114	*N. maura*	Spain, Mallorca	39.46N	02.47E	E27		+
M115	*N. maura*	Spain, Mallorca	39.46N	02.47E	E27		+
M116	*N. maura*	Spain, Mallorca	39.46N	02.47E	E27		+
M117	*N. maura*	Spain, Mallorca	39.30N	03.00E	E27		+
M119	*N. maura*	Spain, Mallorca	39.30N	03.00E	E27		+
M120	*N. maura*	Spain, Mallorca	39.47N	02.45E	E27		+
M122	*N. maura*	Spain, Mallorca	39.30N	03.00E	E27		+
M001	*N. maura*	Switzerland, Lake Geneva	46.28N	06.46E	E28	AY487690	
M020	*N. maura*	Switzerland, Lake Geneva	46.28N	06.45E	E28		+
M021	*N. maura*	Switzerland, Lake Geneva	46.28N	06.45E	E28		+
M022	*N. maura*	Switzerland, Lake Geneva	46.28N	06.45E	E28		+
M023	*N. maura*	Switzerland, Lake Geneva	46.28N	06.45E	E28		+
M043	*N. maura*	Switzerland, Lake Geneva	46.28N	06.47E	E28		+
M052	*N. maura*	Switzerland, River L'Allondon	46.15N	06.02E	E28		+
M128	*N. maura*	Switzerland, Martigny	46.06N	07.04E	E28		+
M129	*N. maura*	Switzerland, Martigny	46.06N	07.04E	E28		

M130	*N. maura*	Switzerland, Martigny	46.06N	07.04E	E28		+
M131	*N. maura*	Switzerland, Martigny	46.06N	07.04E	E28		+
M132	*N. maura*	Switzerland, Martigny	46.06N	07.04E	E28		
M019	*N. maura*	France, Lake Bourget	45.44N	05.52E	E28		+
M070	*N. maura*	France, Côte d'Azur			E28		+
M016	*N. maura*	Italy, Varzi	44.51N	09.05E	E28		+
M012	*N. maura*	Italy, Sta. Margherita	44.20N	09.12E	E29	AY487692	+
M013	*N. maura*	Italy, Sta. Margherita	44.20N	09.12E	E29		+
M014	*N. maura*	Italy, Sta. Margherita	44.20N	09.12E	E30	AY487693	+
M017*	*N. maura*	Italy, Voghera	44.59N	09.01E	E31	AY487695	+
M018	*N. maura*	Italy, Voghera	44.59N	09.01E	E31		
M015	*N. maura*	Italy, Ponte Organasco	44.41N	09.19E	E32	AY487694	+
M121	*N. maura*	Spain, Mallorca	39.30N	03.00E	E33	AY487716	+

Collections: BTVS-ICN = Banco de Tecidos de Vertebrados Selvagens, Instituto da Conservaçao, Portugal; HLMD = Hessisches Landesmuseum Darmstadt, Germany; IPMB = Institut für Pharmazie und Molekulare Biotechnologie, Heidelberg, Germany; MNCN = Museo Nacional de Ciencias Naturales, Madrid, Spain; MVZ = Museum of Vertebrate Zoology, Berkeley, U.S.A.; ZFMK = Zoologisches Forschungsinstitut und Museum Alexander König, Bonn, Germany.

*Sample used in Guicking et al. (2006a).

References

Alfaro, M.E., Arnold, S.J., 2001. Molecular systematics and evolution of *Regina* and the thamnophiine snakes. Mol. Phyl. Evol. 21, 408–423.

Álvarez, Y., Mateo, J.A., Andreu, A.C., Díaz-Paniagua, C., Diez, A., Bautista, J.M., 2000. Mitochondrial DNA haplotyping of *Testudo graeca* on both continental sides of the Straits of Gibraltar. J. Hered. 91, 39–41.

Blondel, J., Aronson, J., 1999. Biology and Wildlife of the Mediterranean Region. Oxford University Press, New York.

Burbrink, F.T., Lawson, R., Slowinski, J.B., 2000. Mitochondrial DNA phylogeography of the polytypic North American Rat Snake (*Elaphe obsoleta*): a critique of the subspecies concept. Evolution 54, 2107–2118.

Busack, S.D., 1986. Biogeographic analysis of the herpetofauna separated by the formation of the Strait of Gibraltar. Natl. Geogr. Res. 2, 17–36.

Busack, S.D., Lawson, R., Arjo, W.M., 2005. Mitochondrial DNA, allozymes, morphology and historical biogeography in the *Podarcis vaucheri* (Lacertidae) species complex. Amphib.-Reptil. 26, 239–256.

Carranza, S., Arnold, E.N., Mateo, J.A., Lopez-Jurado, L.F., 2000. Long-distance colonization and radiation in gekkonid lizards, *Tarentola* (Reptilia: Gekkonidae), revealed by mitochondrial DNA sequences. Proc. Biol. Sci. 267, 637–649.

Clement, M., Posada, D., Crandall, K.A., 2000. TCS: a computer program to estimate gene genealogies. Mol. Ecol. 9, 1657–1659.

Cosson, J.-F., Hutterer, R., Libois, R., Sará, M., Taberlet, P., Vogel, P., 2005. Phylogeographical footprints of the Strait of Gibraltar and Quaternary climatic fluctuations in the western Mediterranean: a case study with the Greater White-toothed Shrew, *Crocidura russula* (Mammalia: Soricidae). Mol. Ecol. 14, 1151–1162.

Crowley, T.J., North, G.R., 1991. Paleoclimatology. Oxford University Press, New York.

de Jong, H., 1998. In search of historical biogeographic patterns in the western Mediterranean terrestrial fauna. Biol. J. Linn. Soc. 65, 99–164.

de Queiroz, A., 1994. Phylogenetic relationships of the garter snakes based on DNA sequence and allozyme variation. Biol. J. Linn. Soc. 53, 209–229.

Dobson, M., Wright, A., 2000. Faunal relationships and zoogeographical affinities of mammals in north-west Africa. J. Biogeogr. 27, 417–424.

Excoffier, L., Smouse, P.E., Quattro, J.M., 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131, 479–491.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Fritz, U., Barata, M., Busack, S.D., Fritzsch, G., Castilho, R., 2006. Impact of mountain chains, sea straits and peripheral populations on genetic and taxonomic structure of a freshwater turtle, *Mauremys leprosa* (Reptilia, Testudines, Geoemydidae). Zool. Scr. 35, 97–108.

Glor, R.E., Losos, J.B., Larson, A., 2005. Out of Cuba: overwater dispersal and speciation among lizards in the *Anolis carolinensis* subgroup. Mol. Ecol. 14, 2419–2432.

Gómez, A., Hunt, D.H., 2007. Refugia within refugia: patterns of phylogeographic concordance in the Iberian peninsula. In: Weiss, S., Ferrand, N. (Eds.), Phylogeography of Southern European Refugia. Springer, Dordrecht.

Gosàlbez, J., 1987. Amfibis, Rèptils i Mamífers. Història Natural dels Països Catalans, vol. 13. Enciclopèdia Catalana, Barcelona.

Guicking, D., Joger, U., Wink, M., 2002. Molecular phylogeography of the Viperine snake (*Natrix maura*) and the Dice snake (*Natrix tessellata*): first results. Biota 3, 49–59.

Guicking, D., Lawson, R., Joger, U., Wink, M., 2006a. Evolution and phylogeny of the genus *Natrix* (Serpentes: Colubridae). Biol. J. Linn. Soc. 87, 127–143.

Guicking, D., Griffiths, R.A., Moore, R.D., Joger, U., Wink, M., 2006b. Introduced alien or persecuted native?

Resolving the origin of the viperine snake (*Natrix maura*) on Mallorca. Biodivers. Conserv. 15, 3045–3054.

Harris, D.J., Carranza, S., Arnold, E.N., Pinho, C., Ferrand, N., 2002. Complex biogeographical distribution of genetic variation within *Podarcis* wall lizards across the Strait of Gibraltar. J. Biogeogr. 29, 1257–1262.

Hewitt, G.M., 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. Biol. J. Linn. Soc. 58, 247–276.

Hewitt, G.M., 2000. The genetic legacy of the Quaternary ice ages. Nature 405, 907–913.

Houle, A., 1998. Floating islands: a mode of long-distance dispersal for small and medium-sized terrestrial vertebrates. Divers. Distrib. 4, 201–216.

Hsü, K.J., Montadert, L., Bernoulli, D., Cita, M.B., Erickson, A., Garrison, R.E., Kidd, R.B., Mèlierés, F., Müller, C., Wright, R., 1977. History of the Mediterranean salinity crisis. Nature 267, 399–403.

Huelsenbeck, J.P., Ronquist, F., 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinf. Appl. Notes 17, 754–755.

Ibrahim, K.M., Nichols, R.A., Hewitt, G.M., 1996. Spatial patterns of genetic variation generated by different forms of dispersal during range expansion. Heredity 77, 282–291.

Jansson, R., Dynesius, M., 2002. The fate of clades in a world of recurrent climatic change: Milankovich oscillations and evolution. Annu. Rev. Ecol. Syst. 33, 741–777.

Krijgsman, W., Hilgen, F.J., Raffi, I., Sierro, F.J., Wilson, D.S., 1999. Chronology, causes and progression of the Messinian salinity crisis. Nature 400, 652–655.

Kumar, S., Tamura, K., Jakobsen, I.B., Nei, M., 2001. MEGA2: Molecular Evolutionary Genetics. Analysis Software. Arizona State University, Tempe.

Lenk, P., 2002. Verdriftung maurischer Bachschildkröten (*Mauremys leprosa*) und Vipernattern (*Natrix maura*) an der südportugiesischen Küste. Z. Feldherpetol. 9, 221–223.

Lenk, P., Fritz, U., Joger, U., Wink, M., 1999. Mitochondrial phylogeography of the European pond turtle, *Emys orbicularis* (Linnaeus, 1758). Mol. Ecol. 8, 1911–1922.

Machordom, A., Doadrio, I., 2001. Evidence of a Cenozoic Betic–Kabilian connection based on freshwater fish phylogeography (*Luciobarbus*, Cyprinidae). Mol. Phyl. Evol. 18, 252–263.

Nagy, Z.T., Joger, U., Wink, M., Glaw, F., Vences, M., 2003. Multiple colonizations of Madagascar and Socotra by colubrid snakes: evidence from nuclear and mitochondrial gene phylogenies. Proc. Biol. Sci. 270, 2613–2621.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinf. Appl. Notes 14, 817–818.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning. A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, New York.

Schätti, B., 1982. Bemerkungen zur Ökologie, Verbreitung und intraspezifischen Variation der Vipernatter, *Natrix maura* (Linné, 1758) (Reptilia, Serpentes). Rev. Suisse Zool. 89, 521–542.

Schätti, B., 1999. *Natrix maura* (Linnaeus 1758) – Vipernatter. In: Böhme, W. (Ed.), Handbuch der Reptilien und Amphibien Europas. Band 3/IIA: Schlangen II. Aula, Wiebelsheim, pp. 483–503.

Schneider, S., Roessli, D., Excoffier, L., 2000. Arlequin ver. 2.000: A Software for Population Genetic Analysis. Genetics and Biometry Laboratoy, University of Geneva, Switzerland.

Sites Jr., J.W., Davis, S.K., Guerra, T., Iverson, J.B., Snell, H.L., 1996. Character congruence and phylogenetic signal in molecular and morphological data sets: a case study in the living iguanas (Squamata, Iguanidae). Mol. Biol. Evol. 13, 1087–1105.

Stewart, J.R., Lister, A.M., 2001. Cryptic northern refugia and the origins of the modern biota. Trends Ecol. Evol. 16, 608–613.

Swofford, D.L., 2001. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4.0b10. Sinauer Associates, Sunderland.

Taberlet, P., Fumagalli, L., Wust-Saucy, A.-G., Cosson, J.-F., 1998. Comparative phylogeography and postglacial colonization routes in Europe. Mol. Ecol. 7, 453–464.

Templeton, A.R., Routman, E., Phillips, C.A., 1995. Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the Tiger Salamander, *Ambystoma tigrinum*. Genetics 140, 767–782.

Veith, M., Mayer, C., Samraoui, B., Donaire Barroso, D., Bogaerts, S., 2004. From Europe to Africa and vice versa: evidence for multiple intercontinental dispersal in ribbed salamanders (Genus *Pleurodeles*). J. Biogeogr. 31, 159–171.

Webb, T.I., Bartlein, P.J., 1992. Global changes during the last 3 million years: climatic controls and biotic responses. Annu. Rev. Ecol. Syst. 23, 141–173.