

Mitochondrial phylogeography, contact zones and taxonomy of grass snakes (*Natrix natrix*, *N. megalocephala*)

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Grass snakes (*Natrix natrix*) represent one of the most widely distributed snake species of the Palearctic region, ranging from the North African Maghreb region and the Iberian Peninsula through most of Europe and western Asia eastward to the region of Lake Baikal in Central Asia. Within *N. natrix*, up to 14 distinct subspecies are regarded as valid. In addition, some authors recognize big-headed grass snakes from western Transcaucasia as a distinct species, *N. megalocephala*. Based on phylogenetic analyses of a 1984-bp-long alignment of mtDNA sequences (ND4+tRNAs, cyt *b*) of 410 grass snakes, a nearly range-wide phylogeography is presented for both species. Within *N. natrix*, 16 terminal mitochondrial clades were identified, most of which conflict with morphologically defined subspecies. These 16 clades correspond to three more inclusive clades from (i) the Iberian Peninsula plus North Africa, (ii) East Europe and Asia and (iii) West Europe including Corso-Sardinia, the Apennine Peninsula and Sicily. Hypotheses regarding glacial refugia and postglacial range expansions are presented. Refugia were most likely located in each of the southern European peninsulas, Corso-Sardinia, North Africa, Anatolia and the neighbouring Near and Middle East, where the greatest extant genetic diversity occurs. Multiple distinct microrefugia are inferred for continental Italy plus Sicily, the Balkan Peninsula, Anatolia and the Near and Middle East. Holocene range expansions led to the colonization of more northerly regions and the formation of secondary contact zones. Western Europe was invaded from a refuge within southern France, while Central Europe was reached by two distinct range expansions from the Balkan Peninsula. In Central Europe, there are two contact zones of three distinct mitochondrial clades, and one of these contact zones was theretofore completely unknown. Another contact zone is hypothesized for Eastern Europe, which was colonized, like north-western Asia, from the Caucasus region. Further contact zones were identified for southern Italy, the Balkans and Transcaucasia. In agreement with previous studies using morphological characters and allozymes, there is no evidence for the distinctiveness of *N. megalocephala*. Therefore, *N. megalocephala* is synonymized with *N. natrix*.

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Introduction

While the knowledge of the phylogeography of many European taxa has made great progress over the past 20 years (Hewitt 1996, 2000, 2011; Taberlet *et al.* 1998; Joger *et al.* 2007; Schmitt 2007; Lymberakis & Poulakakis 2010; Schmitt & Varga 2012), there still remain some wide-ranging iconic taxa understudied. One of these species is the grass snake (*Natrix natrix*). It is one of the most widely distributed snake species of the Palaearctic region, ranging from the North African Maghreb region and the Iberian Peninsula through most of Europe and western Asia eastward to the region of Lake Baikal in Central Asia (Bannikov *et al.* 1977; Kabisch 1999). Grass snakes are more or less associated with aquatic habitats, but they are not true water snakes like the related species *N. maura* and *N. tessellata*. Grass snakes usually reach a total length of up to 120 cm, with maximum sizes of some 200 cm, and feed on amphibians, fish and small mammals or nestling birds (Bannikov *et al.* 1977; Engelmann *et al.* 1986; Gruber 1989; Kabisch 1999; Arnold & Ovenden 2002; Kreiner 2007).

Traditionally, many morphologically defined grass snake subspecies have been distinguished, which differ mainly in body proportions, colouration and size (Hecht 1930; Mertens 1947, 1957, 1966; Mertens & Wermuth 1960; Kramer 1970; Bannikov *et al.* 1977). However, in a sweeping revision using multivariate statistics, Thorpe (1979) reduced the number of subspecies to four (Table 1). Within this four subspecies model, *N. n. natrix* is distributed in the east of the species' range, and *N. n. helvetica* occupies most of the western range, except Corsica and Sardinia. Thorpe (1979) confirmed the earlier view (Mertens & Wermuth 1960) that the two subspecies *natrix* and *helvetica* meet and hybridize in the Rhine region. In addition to these two subspecies, Thorpe (1979) recognized only the morphologically highly distinctive grass snakes from Corsica and Sardinia as further subspecies (*N. n. corsa*, *N. n. cetti*). Nevertheless, other authors contin-

ued to treat up to 14 subspecies as valid (Fig. 1; Table 1; Nilson & Andrén 1981; Engelmann *et al.* 1986; Gruber 1989; Kabisch 1999; Arnold & Ovenden 2002; Kreiner 2007; Baier *et al.* 2009). Furthermore, two additional species, *N. cetti* (Corsica, Sardinia) and *N. megalcephala* (western Transcaucasia), are sometimes recognized (Orlov & Tuniyev 1987, 1999; Vanni & Cimmaruta 2010). However, the validity of *N. megalcephala* has been repeatedly doubted (Hille 1997; Böhme 1999; Jandzik 2005; Frotzler *et al.* 2011; Göçmen *et al.* 2011), and the status of *N. c. cetti* and *N. c. corsa* as subspecies of *N. natrix* was recently reinstated (Fritz *et al.* 2012).

Until today, there are only few studies tackling genetic differentiation of grass snakes. In a pioneering study, Hille (1997) examined some grass snake subspecies and *N. megalcephala* using allozyme data. Although he confirmed an east-west differentiation within *N. natrix* and found no evidence for the validity of *N. megalcephala*, his results were largely inconclusive. Using mitochondrial DNA sequences of some 25 specimens, Guicking *et al.* (2006, 2008a) provided a first preliminary phylogeography and compared their findings with morphologically defined subspecies. Fritz *et al.* (2012) added to the data set of Guicking *et al.* (2006, 2008a) sequences of Corsican, Sardinian and continental Italian grass snakes and concluded that Corso-Sardinian snakes do not represent a distinct species owing to their close phylogenetic relationship with *N. n. helvetica*. Furthermore, in the light of the observed mtDNA variation, Guicking *et al.* (2008a) and Fritz *et al.* (2012) agreed that Thorpe's (1979) four subspecies model evidently underestimates genetic and taxonomic variation within *N. natrix*. However, obvious mismatches between preliminary mtDNA data and any subspecies delineation also underscored the need for further investigations using an expanded sampling to achieve a better understanding of the phylogeography and taxonomy of grass snakes (Guicking *et al.* 2008a; Fritz *et al.* 2012).

Table 1 Morphologically defined taxa (*Natrix natrix* subspecies, *N. megalcephala*) compared with mitochondrial clades

Thorpe (1979)	Kabisch (1999), Orlov & Tuniyev (1999)	Kreiner (2007)	Mitochondrial clades (this study)
<i>Natrix natrix natrix</i>	<i>Natrix natrix natrix</i>	<i>Natrix natrix natrix</i>	3, 4, 8
<i>Natrix natrix natrix</i>	<i>Natrix natrix persa</i>	<i>Natrix natrix cypriaca</i>	7
<i>Natrix natrix natrix</i>	<i>Natrix natrix fusca</i>	<i>Natrix natrix fusca</i>	?
<i>Natrix natrix natrix</i>	<i>Natrix natrix gotlandica</i>	<i>Natrix natrix gotlandica</i>	3, 4
<i>Natrix natrix natrix</i>	<i>Natrix natrix persa</i>	<i>Natrix natrix persa</i>	1, 2, 3, 4, 5, 7, 8
<i>Natrix natrix natrix</i>	<i>Natrix natrix schweizeri</i>	<i>Natrix natrix schweizeri</i>	3
<i>Natrix natrix natrix</i>	<i>Natrix natrix scutata</i>	<i>Natrix natrix scutata</i>	8
<i>Natrix natrix natrix</i>	<i>Natrix natrix syriaca</i>	<i>Natrix natrix syriaca</i>	6
<i>Natrix natrix helvetica</i>	<i>Natrix natrix astreptophora</i>	<i>Natrix natrix astreptophora</i>	Tu, Eu
<i>Natrix natrix helvetica</i>	<i>Natrix natrix helvetica</i>	<i>Natrix natrix helvetica</i>	4, C, E
<i>Natrix natrix helvetica</i>	<i>Natrix natrix lanzai</i>	<i>Natrix natrix lanzai</i>	D, F
<i>Natrix natrix helvetica</i>	<i>Natrix natrix sicula</i>	<i>Natrix natrix sicula</i>	A, F
<i>Natrix natrix cetti</i>	<i>Natrix natrix cetti</i>	<i>Natrix natrix cetti</i>	B
<i>Natrix natrix corsa</i>	<i>Natrix natrix corsa</i>	<i>Natrix natrix corsa</i>	B
—	<i>Natrix megalcephala</i>	—	8

Different names on the same line indicate synonymy of the respective taxa according to the different authors.

The present study aims to fulfil this task. Here we use a nearly range-wide sampling of 410 grass snakes to generate a comprehensive phylogeography based on two mitochondrial markers, the NADH dehydrogenase subunit 4 gene (ND4) and the cytochrome *b* gene (*cyt b*). Our samples represent all but one of the 14 nominal *N. natrix* subspecies recognized by some recent authors (Kabisch 1999; Arnold & Ovenden 2002; Kreiner 2007; Baier *et al.* 2009) and *N. megalcephala* (Table 1), and allow comparing mitochondrial differentiation and morphologically defined taxa. Only the subspecies *N. n. fusca*, endemic to the island of Kea, Cyclades, is missing in our sampling.

Materials and methods

Sampling, gene selection and laboratory procedures

Three hundred and eighty tissue, blood and saliva samples of grass snakes were studied. Besides fresh material, the samples included tissues from museum specimens that had been, as a rule, ethanol-preserved for no more than 30 years (Table S1). Two mitochondrial genes were sequenced that were previously successfully used for phylogeographic purposes in *Natrix natrix* and the allied species *N. maura* and *N. tessellata* (Guicking *et al.* 2006, 2008a, b, 2009; Fritz *et al.* 2012), viz. the partial ND4 gene and the *cyt b* gene. The DNA sequence containing the partial ND4 gene embraced also the flanking DNA coding for tRNA-His, tRNA-Ser and tRNA-Leu. The obtained sequences varied in length between 437 and 696 bp (ND4), between 0 and 117 bp (adjacent tRNAs), and between 311 and 1117 bp (*cyt b*). For some samples, sequences of only one of the two genes could be generated (Table S1), due to bad DNA quality or small quantity. Remaining samples and DNA are stored at -80°C in the tissue collection of the Museum of Zoology, Senckenberg Dresden.

Total genomic DNA was extracted using either the DTAB method (Gustincich *et al.* 1991), the innuPREP DNA Mini Kit or the innuPREP Blood DNA Mini Kit (both Analytik Jena AG, Jena, Germany). DNA fragments were amplified using the primers given in Table S2. When the primers of Guicking *et al.* (2006) did not yield PCR products, newly designed primers were applied to amplify up to three shorter overlapping PCR products for the DNA fragment embracing ND4+tRNAs and up to four PCR products for *cyt b*. For primer combinations and PCR conditions, see Table S3.

PCR was carried out in a total volume of 25 μL containing 1 unit *Taq* polymerase (Bioron, Ludwigshafen, Germany), 1x buffer as recommended by the supplier, 0.4 μM of each primer (Biomers, Ulm, Germany) and 0.2 mM of each dNTP (Thermo-Scientific, St. Leon-Rot, Germany). Challenging samples were additionally treated with 10 μg BSA (Thermo-Scientific). PCR products were purified using the ExoSAP-IT enzymatic clean-up (USB Europe GmbH, Staufien, Germany; modified protocol: 30 min at 37 $^{\circ}\text{C}$, 15 min at 80 $^{\circ}\text{C}$) and sequenced on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the PCR primers and the BigDye Terminator version 3.1 Cycle Sequencing Kit (Life Technologies, Darmstadt, Germany). Cycle sequencing reactions were purified by ethanol/sodium acetate precipitation or using Sephadex (GE Healthcare, München, Germany).

Alignment, partitioning and phylogenetic analyses

All sequences were aligned and inspected using BIOEDIT 7.0.9.0 (Hall 1999) and MEGA 5.1 (Tamura *et al.* 2011). Thirty homologous GenBank sequences of known-locality grass snakes corresponding to the data set of Fritz *et al.* (2012) were aligned with our newly generated sequences,

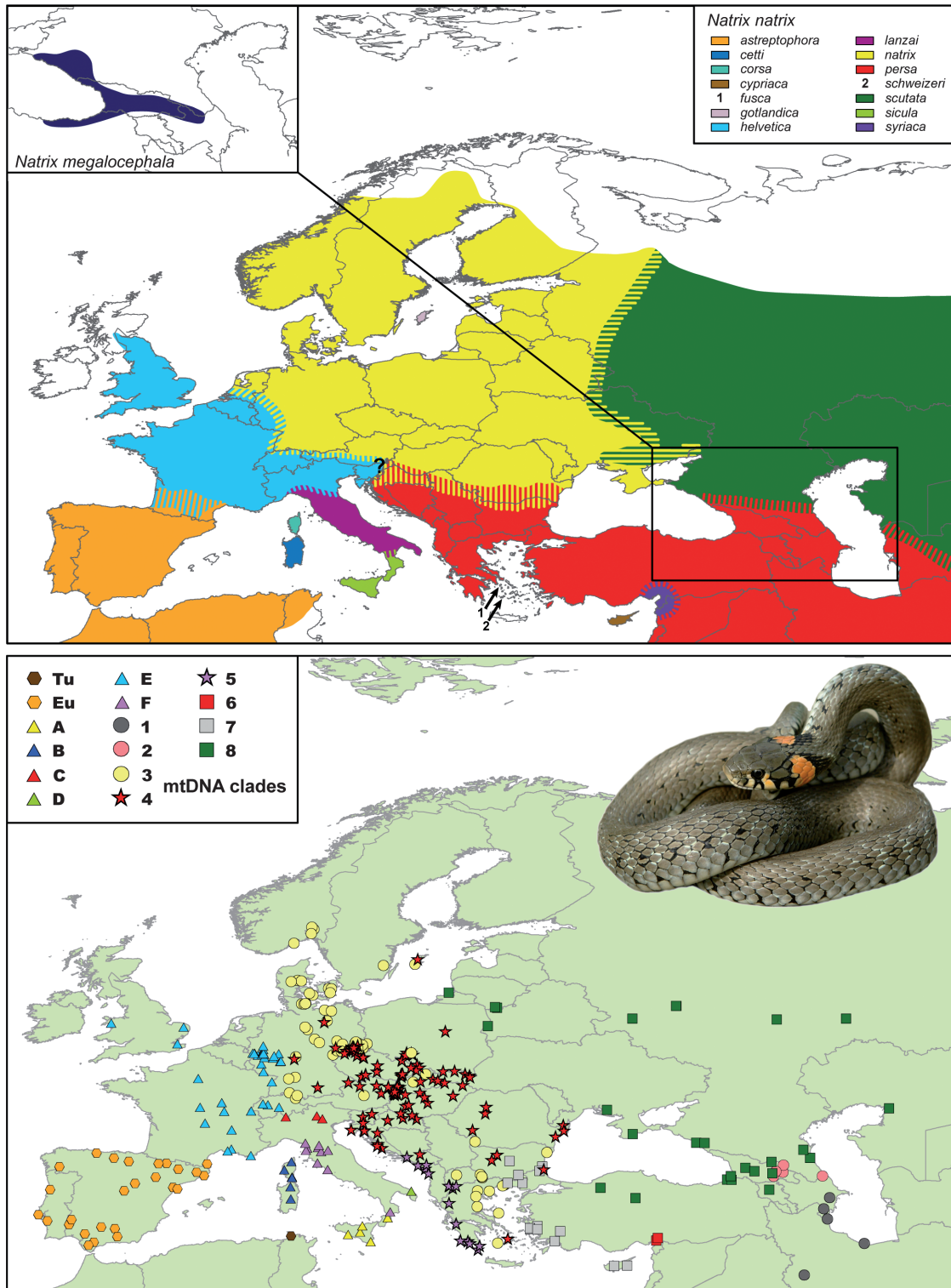


Fig. 1 Distribution of subspecies of *Natrix natrix* and *N. megaloccephala* (top) and mitochondrial clades (bottom). Hatching along range borders of *N. natrix* subspecies indicates putative contact or hybrid zones. Distribution ranges combined from Kabisch (1999), Orlov & Tuniyev (1999), Kreiner (2007), Baier *et al.* (2009) and Göçmen *et al.* (2011). Inset (bottom): *N. natrix* from Mtskheta, Georgia (photograph: M. Auer).

resulting in a total of 410 sequences. *Natrix maura*, *N. tessellata* and the more distantly related North American water snake *Nerodia sipedon* were added as outgroups; *N. sipedon* was used for tree rooting. GenBank accession numbers of all sequences are listed in Table S1.

Phylogenetic relationships were inferred for sequences of all 410 grass snakes (Table S1) using three data sets, that is, for each mtDNA fragment alone (ND4+tRNAs vs. *cyt b*) and for the two fragments concatenated. For the combined analyses and for the mtDNA fragment embracing the partial ND4 gene plus adjacent DNA coding for tRNAs, the data were partitioned by gene; the DNA coding for tRNAs was treated as a single partition. In the alignment of concatenated sequences (1984 bp total length), 696 bp corresponded to the partial ND4 gene, 171 bp to the DNA coding for tRNA-His, tRNA-Ser and tRNA-Leu, and 1117 bp to the *cyt b* gene. The 867-bp-long ND4+tRNA sequences contained 175 variable sites, and the 1117-bp-long *cyt b* sequences, 270 variable sites (ingroup sequences only). The best evolutionary model was determined for each partition using the Akaike information criterion of MRMODELTEST 2.3 (Nylander 2004), resulting in the GTR+I+G model for ND4 and *cyt b* and the HKY+I model for the combined tRNAs. Phylogenetic trees were calculated using MRBAYES 3.2.1 (Ronquist et al. 2012) and the implemented Metropolis-coupled Markov chain Monte Carlo algorithm. Two parallel runs, each with one cold and three heated chains, were conducted. The chains ran for 10^7 generations, with every 100th generation sampled. However, using the default settings of MRBAYES the two runs for *cyt b* and the concatenated data set did not converge on a stationary level. Therefore, analyses were rerun setting the heating parameter λ to 0.05. For generating the final 50% majority rule consensus, a burn-in of 25% was used to sample only the most likely trees. In addition, phylogenetic relationships were inferred using the Maximum Likelihood (ML) approach as implemented in RAxML 7.2.8 (Stamatakis 2006). Using the GTR+G model across all partitions, five independent ML searches were run with different starting conditions and the fast bootstrap algorithm. The robustness of the branching patterns was examined by comparing the best trees. Subsequently, 1000 nonparametric thorough bootstrap replicates were calculated and the values plotted against the tree with the highest likelihood value. In addition, uncorrected *p* distances were computed using MEGA and the 'pairwise deletion' option.

Results

For each data set (ND4+tRNAs and *cyt b* alone or concatenated), there are no significant differences in the topologies of the Maximum Likelihood and Bayesian trees, and the

trees for the combined data set have completely identical topologies. Differences between the trees based on each mtDNA fragment alone and the trees based on the concatenated sequence data occur only with respect to the weakly resolved deeper nodes, while the placement of individual sequences in terminal clades is consistent. In the trees based on the combined sequences, some of the more basal branching patterns are better supported than in the trees based on ND4+tRNAs and *cyt b* alone. However, some deeper nodes remain also then weakly supported (Fig. 2). In agreement with previous results (Guicking et al. 2006), *Natrix tessellata* and *N. maura* constitute the successive sister taxa of a well-supported clade corresponding to *N. natrix* (Fig. S1). Our three sequences of *N. megalocephalocephala* are consistently embedded within *N. natrix*, and there are 16 major subordinated clades within this paraphyletic group (clades Tu, Eu, 1–8 and A–F; Fig. 2). With respect to ND4+tRNAs, uncorrected average *p* distances among these 16 clades amount to 0.29–7.29%, while divergences of 0–0.63% are observed within the clades. For *cyt b* sequences, the respective values are 0.28–7.51% and 0–0.62% (Table S4).

The sequences of *N. megalocephalocephala* cluster in a well-supported subordinated clade containing also sequences of *N. n. natrix*, *N. n. persa* and *N. n. scutata* (clade 8 in Fig. 2). A closer inspection of clade 8 reveals that the average divergence between the three sequences of *N. megalocephalocephala* and the 35 sequences of *N. natrix* subspecies is only 0.26% (ND4+tRNAs and *cyt b* concatenated). The sequences of *N. megalocephalocephala* represent three haplotypes, and the same haplotypes also occur among the sequences of *N. natrix* within clade 8.

While mismatches between taxonomy and phylogenetic placement within *N. natrix* are the rule and not the exception (Table 1), there are a few monophyletic taxa. The well-supported most basal clade (Fig. 2) corresponds to sequences of *N. n. astreptophora* from North Africa and the Iberian Peninsula plus adjacent France. Within this clade, a sequence of a Tunisian grass snake (Tu) is deeply divergent from all European *N. n. astreptophora* (Eu). Also most sequences of *N. n. sicula* (with one exception) represent a deeply divergent clade (clade A), and the sequences of Corso-Sardinian grass snakes constitute another well-supported clade (clade B). However, the four sequences from Corsica (subspecies *corsa*) and the three sequences from Sardinia (subspecies *cetti*) are not reciprocally monophyletic (Fig. S1). Furthermore, our only two sequences of *N. n. syriaca* represent a deeply divergent clade (clade 6).

The sister group of *N. n. astreptophora* is a major clade whose monophyly is well supported only in Bayesian analyses (Fig. 2). This clade comprises grass snake sequences from most of the species' range (except North Africa,

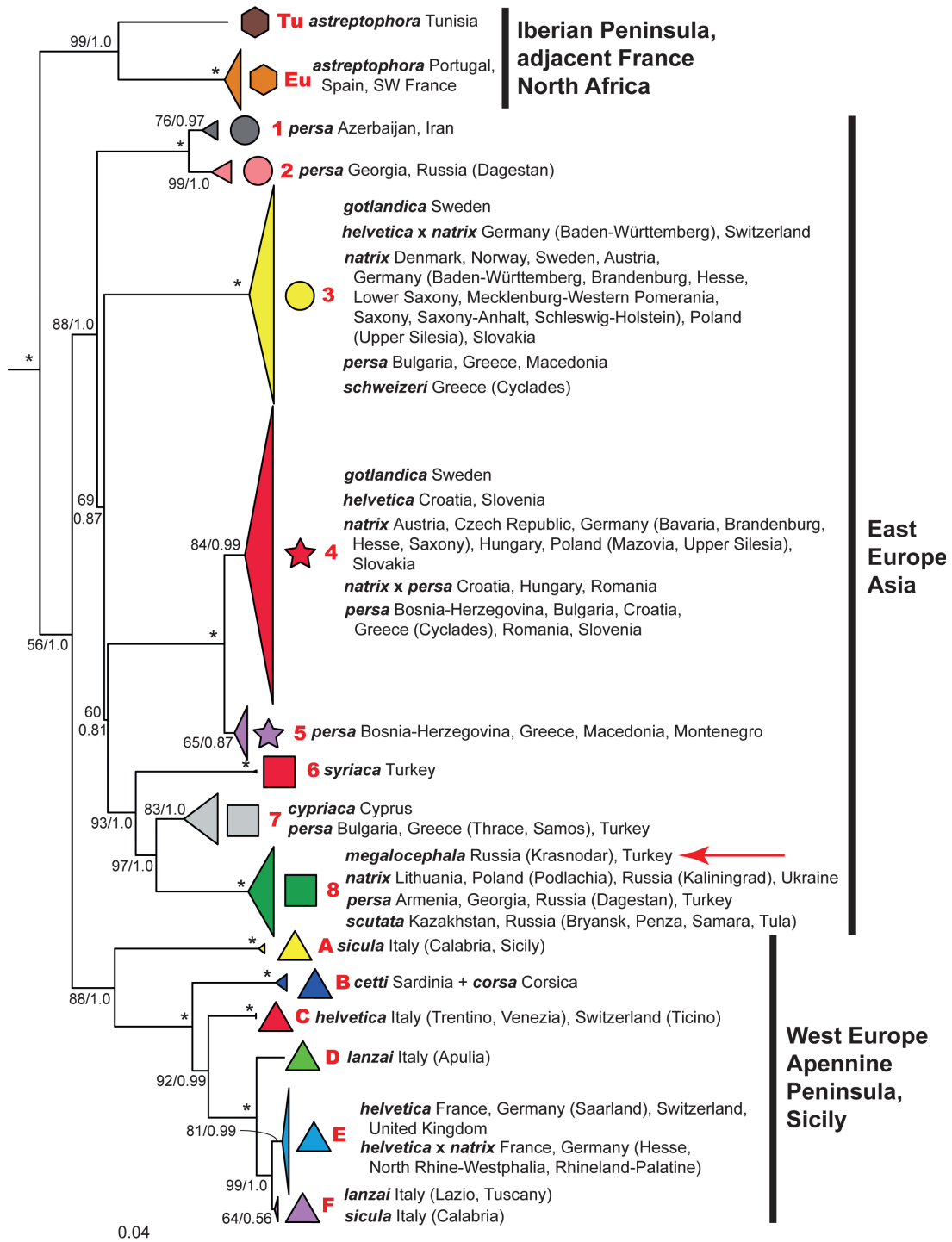


Fig. 2 Mitochondrial phylogeny of grass snakes inferred from Maximum Likelihood analyses using 1984-bp mtDNA (ND4+tRNAs, *cyt b*) of 407 samples of *Natrix natrix* and three samples of *N. megalocephala*. Terminal clades collapsed to cartoons. Outgroups (*N. maura*, *N. tessellata*, *Nerodia sipedon*) removed for clarity; for a complete tree, see Fig. S1. Numbers along nodes indicate branch support under Maximum Likelihood (1000 bootstrap replicates) and Bayesian analyses (posterior probabilities). Asterisks indicate maximum support under both tree-building methods. Clade symbols correspond to Fig. 1; red letters and numbers preceding taxon names refer to the text and Table 1. Red arrow highlights placement of *Natrix megalocephala* among *N. natrix*.

Iberian Peninsula and adjacent France = *N. n. astreptophora*). Within this clade, there are two other subordinated major clades, one well-supported clade from the eastern part and another well-supported clade from the western part of the range (including Apennine Peninsula and Sicily). The clade from the east contains the eight subordinated clades 1–8, and the clade from the west, the six subordinated clades A–F. Most of these 14 clades conflict with subspecies delineations (Table 1).

Some deeper nodes of the eastern clade are weakly resolved and have very short branch lengths. By contrast, seven of the eight terminal clades (1–4, 6–8) are well supported (Fig. 2). However, clade 5, corresponding to sequences of *N. n. persa* from Bosnia and Herzegovina, Greece, Macedonia and Montenegro, receives only weak support, and mean uncorrected *p* distances between clade 5 and its sister group (clade 4) amount only to 0.55% (ND4+tRNAs) and 1.38% (cyt *b*; Table S4). Within the eastern clade, a similarly weak divergence occurs only between the well-supported sister clades 1 and 2 (ND4+tRNAs: 0.72%, cyt *b*: 1.43%); among the other terminal clades, much higher values of 2.37–5.56% (ND4+tRNAs) and 3.23–5.74% (cyt *b*) are observed (Table S4).

Sequences of *N. n. persa* and of grass snakes from the putative hybrid zone between *N. n. natrix* and *N. n. persa* occur in not less than seven distinct, in part deeply divergent, clades (clades 1–5 and 7–8). Sequences of the nominotypical subspecies *N. n. natrix* occur together with sequences of *N. n. persa* and other taxa in the well-supported clades 3, 4 and 8 (Fig. 2).

The deeper branching patterns within the western clade are distinctly better resolved than in the eastern clade, and branch lengths of the respective deeper nodes are longer than in most of the deeper nodes of the eastern clade. Within the western clade, sequences of Calabrian and Sicilian grass snakes of the subspecies *N. n. sicula* (clade A) constitute the sister group of the remaining sequences. The successive clades are B (Corso-Sardinian grass snakes, *N. n. corsa* and *N. n. cetti*), C (*N. n. helvetica* from Trentino and Venezia in north-eastern Italy and Ticino, Switzerland) and D (corresponding to a single sequence of a grass snake from Apulia, Italy). The terminal crown group is formed by clade E (*N. n. helvetica* and individuals from the hybrid zone of *N. n. helvetica* and *N. n. natrix*) and clade F (sequences of *N. n. lanzai* from Lazio and Tuscany, Italy, and a sequence of *N. n. sicula* from Calabria). However, compared with the basal clades A, B and C, the divergences among clades D, E and F are distinctly less pronounced and also weaker than between most other clades (Table S4). This is mirrored by shorter branch lengths (Fig. 2) and uncorrected *p* distances of only 0.29–0.80%

(ND4+tRNAs) and 0.28–1.16% (cyt *b*) for clades D, E and F, opposed to values of 2.12–6.12% (ND4+tRNAs) and 2.06–5.36% (cyt *b*) when clades A, B and C are compared with one another and with clades D, E and F (Table S4).

Discussion

Phylogeography

Our results, based on a nearly range-wide sampling, demonstrate considerable phylogeographic differentiation of grass snakes. Uncorrected *p* distances for the two studied mitochondrial markers (Table S4) resemble or clearly exceed divergences observed within the other two western Palaearctic *Natrix* species, both having a pronounced phylogeographic structure (Guicking *et al.* 2008b, 2009). With respect to the cyt *b* gene, mean divergences of 0.28–7.51% were observed among grass snake clades (Table S4). The maximum uncorrected *p* distance described for cyt *b* sequences of *Natrix maura* is 4.74% (European vs. Moroccan snakes; Guicking *et al.* 2008b), and a maximum value of 8.42% was observed when *N. tessellata* from the Caucasus and Greece were compared (Guicking *et al.* 2009). However, these values are maxima, and not mean values, as reported in the present study.

Based on mtDNA sequences of 410 grass snakes, our phylogenetic analyses revealed 16 distinct terminal clades (Figs 1 and 2). One of these clades includes the three sequences of *N. megalcephala* together with *N. natrix* sequences. These 16 clades correspond to three more inclusive clades from (i) the Iberian Peninsula plus North Africa, (ii) East Europe and Asia and (iii) West Europe including Corso-Sardinia, the Apennine Peninsula and Sicily (Fig. 2). These three major clades had already been identified in previous studies (Guicking *et al.* 2006, 2008a; Fritz *et al.* 2012).

Guicking *et al.* (2006, 2008a) and Fritz *et al.* (2012) had distinguished within clades (i), (ii) and (iii) a maximum of eight terminal clades. However, these studies had used a quite restricted sampling of 23–30 individuals and therefore had lumped for phylogeographic purposes some distinct lineages together, which were represented by single sequences only. If the terminal clades and lineages of Guicking *et al.* (2006, 2008a) and Fritz *et al.* (2012) are compared with our 16 clades, it is clear that just the following four clades are missing: clade Tu (*N. n. astreptophora* from Tunisia), clade 2 (*N. n. persa* from Georgia and Dagestan), clade 6 (*N. n. syriaca*) and clade A (*N. n. sicula* from Sicily and Calabria).

Based on molecular clock calculations, Fritz *et al.* (2012) had inferred that the basal clades (i), (ii) and (iii) diverged some 7–11 million years ago, while the then known 12 terminal lineages were dated to the Late Miocene to the Early Pleistocene, a time frame matching also well with the

branch lengths of the four newly discovered terminal clades (compare Fig. 2 of this study with Figs 1 and 3 in Fritz *et al.* 2012). It is obvious that the branch lengths of some deeper nodes of the East European–Asian clade (ii) are distinctly shorter than in the West European clade (iii), suggestive of more rapid radiation. In the West European clade, however, the terminal subordinated clades D, E and F also have rather short branches, indicating a later divergence compared with the remaining clades A, B and C.

The deep divergence between the Tunisian clade Tu and European samples of *N. n. astreptophora* (clade Eu; Fig. 2; Table S4) suggests an old separation of these two lineages. However, without studying grass snakes from Morocco and Algeria, any interpretation of this pattern has to remain highly speculative. It is well known that Algerian and Tunisian populations are often highly distinct from Moroccan ones (e.g. *Pleurodeles* spp., Carranza & Arnold 2004; Veith *et al.* 2004; *Hyla meridionalis*, Recuero *et al.* 2007; *Mauremys leprosa*, Fritz *et al.* 2006; *Testudo graeca*, Fritz *et al.* 2009a; *Chamaeleo chamaeleon*, Dimaki *et al.* 2008; *Timon* spp., Paulo *et al.* 2008; *Coronella girondica*, Santos *et al.* 2012; *Macroprotodon* spp., Carranza *et al.* 2004; *Malpolon* spp., Carranza *et al.* 2006; *Natrix maura*, Guicking *et al.* 2008b; *Vipera latastei* complex, Velo-Antón *et al.* 2012), while the differentiation between Moroccan and Iberian populations can be sometimes quite shallow (e.g. *Pleurodeles waltl*, Carranza & Arnold 2004; Veith *et al.* 2004; *Emys orbicularis*, Fritz *et al.* 2007a; *Mauremys leprosa*, Fritz *et al.* 2006; *Macroprotodon brevis*, Carranza *et al.* 2004; *Malpolon monspessulanus*, Carranza *et al.* 2006).

Due to a patchy sampling in south-western France, we were unable to confirm the putative contact zone between clade Eu (*N. n. astreptophora*) and the neighbouring clade E (*N. n. helvetica*). However, thanks to our much denser sampling in other regions, not only some new clades were discovered. Also the distribution ranges of previously identified clades became much clearer (Fig. 1), allowing to refine and to expand the preliminary phylogeographic considerations of Fritz *et al.* (2012). These authors concluded that, in agreement with general phylogeographic patterns of the western Palaearctic (Hewitt 1996, 2000, 2011; Taberlet *et al.* 1998; Joger *et al.* 2007; Schmitt 2007; Schmitt & Varga 2012), at least one glacial refuge was located in each of the southern European peninsulas and in Anatolia. Fritz *et al.* (2012) suggested that multiple refugia existed south of the Alps and in the Balkan Peninsula. This is confirmed by our present study. However, the situation is even more complex than thought before.

Our additional samples indicate the existence of not less than four distinct refugia in the Apennine Peninsula and Sicily: (i) in western Italy (corresponding to clade F; Figs 1 and 2), (ii) in north-eastern Italy plus adjacent Switzerland

(clade C), (iii) in south-eastern Italy (clade D), and (iv) a newly identified refuge in Sicily and Calabria (clade A).

This fine-scale pattern of ‘refugia-within-refugia’ (Gómez & Lunt 2007) or ‘microrefugia’ (Joger *et al.* 2007) in Italy and adjacent regions is at first glance unexpected because Schmitt (2007) argued that the phylogeographic patterns within the Apennine Peninsula are simpler than in the other, larger, southern European peninsulas. Pedall *et al.* (2011) contradicted this view and listed many plants and animals for which more than one refuge has to be postulated in the Italian peninsula. If the putatively four continental Italian and Sicilian refugia of *N. natrix* are compared with other taxa, it is obvious that this is by far not an idiosyncratic pattern. A phylogeographic differentiation in lineages east and west of the Apennine chain, paralleling clades C and F, occurs in some other reptiles, for instance, in European pond turtles (*E. orbicularis*, Pedall *et al.* 2011), wall lizards (*Podarcis siculus*, Podnar *et al.* 2005) and whip snakes (*Hierophis viridiflavus*, Rato *et al.* 2009). A distinct ‘Padano-Venetian refuge’, suggested by the distribution of our clade C, is further supported by the occurrence of the endemic frog species *Rana latastei* (Grossenbacher 2004) and endemic mitochondrial clades of the Italian crested newt *Triturus carnifex* (Canestrelli *et al.* 2012a), the pool frog *Pelophylax lessonae* (Canestrelli & Nascetti 2008), the spadefoot toad *Pelobates fuscus* (Crottini *et al.* 2007), the tree frog *Hyla intermedia* (Canestrelli *et al.* 2007) and the Italian wall lizard *Podarcis siculus* (Podnar *et al.* 2005). Finally, the distinct refuge in Sicily and adjacent southernmost continental Italy (Calabria), harbouring the most divergent clade A within the western group of *N. natrix* (Figs 1 and 2), is corroborated by quite a number of other taxa. Besides the endemic Sicilian species *Emys trinacris* (Fritz *et al.* 2005; Pedall *et al.* 2011), *Podarcis waglerianus* (Böhme 1986) and *Bufo siculus* (Stöck *et al.* 2008), there are deeply divergent mitochondrial clades in Sicily and Calabria in species such as hedgehogs (*Erinaceus europaeus*, Seddon *et al.* 2001), Italian hares (*Lepus corsicanus*, Pierpaoli *et al.* 1999), bank voles (*Myodes glareolus*, Colangelo *et al.* 2012), red squirrels (*Sciurus vulgaris*, Grill *et al.* 2009), green and wall lizards (*Lacerta bilineata*, Godinho *et al.* 2005; Böhme *et al.* 2007 and *P. siculus*, Podnar *et al.* 2005), newts (*Lissotriton italicus* and *T. carnifex*, Canestrelli *et al.* 2012a,b) and frogs (*H. intermedia*, Canestrelli *et al.* 2007; *Pelophylax lessonae*, Canestrelli & Nascetti 2008; *Rana italica*, Canestrelli *et al.* 2008).

Fritz *et al.* (2012) concluded that western Central Europe was colonized in the postglacial by grass snakes originating from the western Apennine refuge. However, our expanded sampling rather suggests that the endemic clade F, distributed in the north-western Italian peninsula (Fig. 1), did not expand into western Central Europe. The closely related

clade E from West Europe (including southern France) is clearly distinct (Fig. 2), and it seems more likely that its extant range results from a range expansion from within the south of its present distribution range (southern France).

Based on morphological evidence, it is generally accepted that the western subspecies *N. n. helvetica* meets and intergrades with the eastern subspecies *N. n. natrix* in the Rhine region (e.g. Thorpe 1979; Kabisch 1999; Kreiner 2007). Our mitochondrial data generally confirm this pattern in that the geographical distribution of the western clade E abuts the range of the eastern clade 3 there. However, what was completely unexpected is the finding that another eastern clade (clade 4) encroaches deeply into Central Europe (Fig. 1). Haplotypes of clades 3 and 4 occur in broad sympatry throughout southern and eastern Germany, on Gotland (Sweden), in southern Poland, Austria and Slovakia, indicating another Central European contact zone. Yet, to the south, the range of clade 4 seems to interrupt the distribution of clade 3. Records of clade 3 are lacking for the western central Balkan Peninsula, where clade 4 is widely distributed. In the south-eastern Balkans, there are again many records for clade 3, which occurs there in part in close proximity to haplotypes of clade 4 and another clade (clade 7). Moreover, in the south-western Balkans, clade 4 is replaced by the allied clade 5. We cannot exclude that the disjunct range of clade 3 is an artefact and that we simply missed to sample clade 3 in some regions. Moreover, the new records for clade 3 in the south-eastern Balkan Peninsula contradict the hypothesis (Fritz *et al.* 2012) that grass snakes harbouring haplotypes of this clade survived the last glaciation north of the Alps and suggest rather a northward range expansion from a Balkanic refuge.

In summary, this complicated phylogeographic pattern indicates that four, and not only two (Fritz *et al.* 2012), distinct glacial refugia were located in the Balkan Peninsula, which harboured grass snakes of clades 3, 4, 5 and 7. The exact location of these refugia is difficult to determine because the extant distribution ranges seem to be blurred by extensive Holocene range expansions and, perhaps, range shifts. The relatively restricted distribution range of clade 5 suggests that its refuge was in the south-west of the Balkan Peninsula. The refugia of the other three clades (3, 4, 7) were most probably further east, somewhere south of the boundary of permafrost or deep seasonal freezing (Frenzel *et al.* 1992), that is, south of the Danube Basin. Therefore, it is possible that the refugial range of clade 7 differed not too much from its extant range, embracing the northern Aegean region, western and southern Anatolia and Cyprus. The relatively pronounced differentiation within clade 7 (Fig. S1; Table S4) could indicate several

microrefugia in its extant range, being richly structured by sea straits and mountain chains. The disjunct range of clade 3 suggests that it originated in the south-eastern Balkan Peninsula. It resembles the distribution range of a certain mitochondrial lineage of the European pond turtle (*Emys orbicularis* lineage II), which is thought to have spread northward via the Axios/Vardar and Danube Rivers and the Moravian Gate (Fritz *et al.* 2007a; Sommer *et al.* 2009). It is plausible that *N. natrix*, living in similar habitats as *E. orbicularis*, used the same pathway.

Another parallel to *E. orbicularis* is the distribution of clade 8, which is largely congruent with the distribution range of another mitochondrial lineage of the pond turtle (lineage I; Fritz *et al.* 2007a, 2009b). The glacial refuge of both was most probably located somewhere close to the Caucasus Mountains. Also the localized range of clade 6 agrees perfectly with an endemic lineage of *E. orbicularis* (lineage X; Fritz *et al.* 2009b). However, unlike *E. orbicularis*, there are two distinct mitochondrial clades of *N. natrix* present in eastern Transcaucasia and northern Iran (clades 1 and 2), whereas only one lineage is present in the turtle (lineage VII; Fritz *et al.* 2007a, 2009b).

Taxonomy

It has been repeatedly argued that mtDNA sequences alone are by far no perfect proxy for phylogeography and taxonomic differentiation, among others due to their strictly maternal inheritance, the lack of recombination, their reduced effective population size and resulting bias caused by genetic drift, sex-specific dispersal and sometimes massive mitochondrial introgression, even across species borders (e.g. Ballard & Whitlock 2004; Edwards *et al.* 2005; Mallet 2005; Bazin *et al.* 2006; Currat *et al.* 2008). However, with regard to western Palaearctic amphibians and reptiles, there is a good agreement between taxonomic units (species, subspecies) and mitochondrial differentiation (Joger *et al.* 2007), with mismatches typically indicating bad taxonomy (e.g. Podnar *et al.* 2005; Ursenbacher *et al.* 2006, 2008; Fritz *et al.* 2007b, 2009b; Gvoždík *et al.* 2010a,b; Pedall *et al.* 2011). Other causes for mismatches are either hybridization and intraspecific gene flow (e.g. in the pond turtle *Emys orbicularis*, Pedall *et al.* 2011), interspecific mitochondrial introgression (e.g. in crested newts of the *Triturus cristatus* complex, Wielstra *et al.* 2013) or, rarely, complete mitochondrial replacement, like in the Carpathian newt *Lissotriton montandoni* (Zieliński *et al.* 2013), so that the application of mitochondrial markers, at least as a first step, is still well justified. This is in line with a careful review of recent literature on birds (Zink & Barrowclough 2008), showing that mtDNA delivers robust results for phylogeography and species limits in the vast majority of cases.

In grass snakes, most previous assessments of geographical variation were based on morphological investigations that used, with the notable exception of Thorpe (1979), small or heavily biased samples with respect to geographical coverage and age or sex classes (e.g. Hecht 1930; Mertens 1947, 1957, 1966; Kramer 1970; Nilson & Andrén 1981). Putatively diagnostic characters for subspecies of *Natrix natrix*, and for *N. megalcephala*, concern differences in total size, body proportions, colouration and pattern, while meristic characters typically broadly overlap (Kabisch 1999; Orlov & Tuniyev 1999). Apart from the publications by Hille (1997), Guicking *et al.* (2006, 2008a) and Fritz *et al.* (2012), no attempt has been undertaken yet to analyse geographically correlated genetic variation in *N. natrix*. Hille (1997) presented preliminary allozyme data for *N. megalcephala* and some subspecies of *N. natrix*, largely without novel insights. Based on a quite limited sampling of 23–30 snakes, Guicking *et al.* (2006, 2008a) and Fritz *et al.* (2012) presented phylogenetic analyses and molecular clock calculations using mtDNA sequences and found deeply divergent clades within *N. natrix* that often conflicted with traditionally recognized subspecies. However, our study provides for the first time a comprehensive and nearly range-wide examination of mitochondrial variation of grass snakes, based on sequences of 410 snakes. We included in our investigations samples of *N. megalcephala* and of 13 of the 14 subspecies of *N. natrix* recognized by some authors (Table 1).

If each taxon represents an evolutionarily distinct lineage, it should be expected that there is a general agreement between the geographical distribution of the mitochondrial clades and the 15 taxa, with the possible exception of the range borders of the subspecies of *N. natrix*, where the exchange of mitochondria could cause mismatches. In other words, it should be expected that the mitochondrial haplotypes in the core regions of the distribution ranges of each taxon are reciprocally monophyletic and constitute clearly distinct mitochondrial clades.

However, we found 16, and not 15, mitochondrial clades, and only few of them correspond well to any taxon (Figs 1 and 2; Table 1). Most notably, *N. megalcephala*, a taxon described as a distinct species (Orlov & Tuniyev 1987), does not represent a distinct clade. Rather, the three sequences of *N. megalcephala* are nested in the paraphyletic clade 8 containing also sequences of three subspecies of *N. natrix* (*N. n. natrix*, *N. n. persa*, *N. n. scutata*). Moreover, none of the three haplotypes of the *N. megalcephala* is unique; each haplotype is also represented by *N. natrix* sequences of clade 8. Using 32 allozyme loci, Hille (1997) found *N. megalcephala* undifferentiated from samples of *N. n. natrix* from Central Europe and *N. n. persa* from the Peloponnese, Greece, and the morphological distinctive-

ness of *N. megalcephala* was repeatedly questioned (Jandžík 2005; Frotzler *et al.* 2011; Göçmen *et al.* 2011). According to the original description (Orlov & Tuniyev 1987), *N. megalcephala* differs from *N. natrix* by its wider head, less distinct frontal and parietal scutes, and the more massive body (see also Orlov & Tuniyev 1999). However, there are intermediate specimens known (Jandžík 2005), and big-headed grass snakes also occur in other parts of the range of *N. natrix* (Göçmen *et al.* 2011), implying that the putatively diagnostic characters of *N. megalcephala* represent merely ontogenetic variation.

The only taxa being represented by reciprocally monophyletic mitochondrial clades are *N. n. astreptophora* and *N. n. syriaca*. However, the clade of *N. n. astreptophora* contains two deeply divergent lineages, one corresponding to the only studied North African grass snake and the other to all European samples of the subspecies *astreptophora*. This suggests that North African representatives of *N. n. astreptophora* could be taxonomically distinct and a morphological reassessment is warranted, as is the inclusion of Moroccan and Algerian samples in genetic investigations (see ‘Phylogeography’).

Also the putative range of *N. n. sicula* (Sicily, Calabria) agrees quite well with the distribution of clade A. The occurrence of clade A and clade C haplotypes in close proximity in Calabria indicates a contact zone in this region, which is paralleled by some other taxa (*Podarcis siculus*, Podnar *et al.* 2005; *Lisotriton italicus* and *Triturus carnifex*, Canestrelli *et al.* 2012a,b; *Hyla intermedia*, Canestrelli *et al.* 2007; *Pelophylax lessonae*, Canestrelli & Nascetti 2008; *Rana italica*, Canestrelli *et al.* 2008; perhaps *Emys orbicularis* and *E. trinacris*, Fritz *et al.* 2005).

Sequences of the Corsican and Sardinian subspecies *N. n. corsa* and *N. n. cetti* together constitute the well-supported clade B (Fig. 2); however, sequences of the two subspecies are not reciprocally monophyletic (Fig. S1). These two subspecies differ significantly in colouration and pattern (Engelmann *et al.* 1986; Gruber 1989; Kabisch 1999; Arnold & Ovenden 2002; Kreiner 2007; Vanni & Cimmaruta 2010), suggesting that such characters may reflect rather population-specific characters.

All other subspecies of *N. natrix* conflict with mitochondrial clades, supporting the view (Guicking *et al.* 2008a; Fritz *et al.* 2012) that a taxonomic revision of grass snakes is required. Mitochondrial DNA sequences of Cypriot grass snakes (*N. n. cypriaca*), characterized by distinctive colouration and pattern (Baier *et al.* 2009), are not reciprocally monophyletic with respect to sequences of grass snakes from neighbouring eastern Mediterranean regions (clade 7; Figs 1 and S1). Also samples of the island-endemic subspecies *N. n. gotlandica* (Gotland, Sweden) and *N. n. schweizeri* (Cyclades, Greece) yielded no unique

haplotypes (Fig. 2; Table 1). The haplotypes detected in both subspecies belong to clades 3 and 4, which are also present in neighbouring mainland populations. This does not corroborate the validity of *N. n. gotlandica* and *N. n. schweizeri* and argues again rather in favour of population specificity of their distinctive colouration and pattern characters (Engelmann *et al.* 1986; Gruber 1989; Kabisch 1999; Arnold & Ovenden 2002; Kreiner 2007). The island of Gotland was completely inundated by the Baltic Ice Lake 11 000 years ago. It did not emerge before 10 300 years ago and was later not connected by land bridges to the surrounding Baltic coasts (Björck 1995). This implies that the Gotland population of grass snakes was founded only in the Holocene by oversea dispersal from what is now Poland, northern Germany, Denmark or Sweden, regions where grass snakes of clades 3 and 4 are currently widely distributed (Fig. 1), and that the distinctive colouration and pattern characters of the Gotland snakes evolved quite rapidly.

We cannot exclude that some mismatches between morphological taxon delimitation and clade assignment (Figs 1 and 2; Table 1) result from mitochondrial introgression. This could be, for instance, the case with respect to the conflicting sequences assigned to *N. n. helvetica* from westernmost Croatia and Slovenia, a region where several subspecies are thought to meet (*N. n. helvetica*, *N. n. natrix*, *N. n. persa*; Fig. 1). However, introgression is unlikely when large parts of a subspecies' range are occupied by endemic mitochondrial clades, as in the northern Italian part of the range of *N. n. helvetica* (Fig. 1: clade C). This suggests rather the occurrence of cryptic or overlooked distinct taxa there (see 'Phylogeography' for other endemic genetic lineages in the Padano-Venetian region). A similar, but more complex, situation refers to the striped subspecies *N. n. persa*, in whose range several endemic and locally restricted clades are distributed. Four of these clades are confined to the putative range of *N. n. persa* (clades 1, 2, 5, 7), while three further clades (clades 3, 4, 8) deeply encroach into the ranges of the two unstriped subspecies *N. n. natrix* and *N. n. scutata* (Fig. 1), rendering the situation even more complicated.

Striped grass snakes identified with *N. n. persa* are distributed over a vast range, from the Balkan Peninsula through Turkey and Transcaucasia to the south-eastern range border in Iran (Fig. 1). The considerable mitochondrial diversity within this vast territory implies that back stripes occur in several distinct taxa, which were historically lumped together due to similar morphology.

In the north of the distribution range of grass snakes (corresponding to the distribution ranges of the subspecies *N. n. helvetica*, *N. n. natrix* and *N. n. scutata*), back stripes are rare, but there are some records of striped snakes from

Central Europe (Austria: Grillitsch & Cabela 2001; Czech Republic: Werner 1929; Germany: Günther & Völkl 1996; Slovakia: Lác 1968; Rehák 1992). This situation suggests that clinal variation or selection could play a role.

For the time being, it is impossible to disentangle the conflicting patterns of morphological and mitochondrial variation. Further research employing nuclear markers, like nuclear genes and rapidly evolving microsatellite loci, may help to elucidate this confusing situation, also with respect to the newly discovered Central and South-east European contact zone between the mitochondrial clades 3 and 4 (Fig. 1). Also a morphological re-examination of striped grass snakes seems promising to unravel possibly overlooked morphological differences between representatives of the distinct mitochondrial clades.

Conclusions

Several distinct lines of evidence (morphology, allozymes, mtDNA) indicate that *Natrix megalcephala* Orlov & Tuniyev, 1987 is invalid. Therefore, this species is synonymized here with *N. natrix* (Linnaeus, 1758). From within the range of *N. natrix*, 16 terminal mitochondrial clades were identified, most of which conflict with morphologically defined subspecies. These 16 clades correspond to three more inclusive clades from (i) the Iberian Peninsula plus North Africa, (ii) East Europe and Asia and (iii) West Europe including Corso-Sardinia, the Apennine Peninsula and Sicily. The highest mitochondrial diversity is found in the south of the range, where the putative glacial refugia were located. Endemic mitochondrial clades occur in each of the southern peninsulas of Europe, on Corso-Sardinia, in northern Africa, Anatolia and the neighbouring Near and Middle East, suggesting that the respective glacial refugia were located there. While there is evidence for only one refuge in the Iberian Peninsula, the Apennine Peninsula and Sicily harboured most likely four distinct microrefugia. Also for the Balkan Peninsula, there is evidence for four microrefugia. Further multiple microrefugia were located in Anatolia and the neighbouring Near and Middle East. According to the extant distributions of mitochondrial clades, the north-west of the species' range was colonized from southern France, while Central Europe was reached by two distinct range expansions from within the southern Balkan Peninsula, resulting in two distinct secondary contact zones across Central Europe. One of these contact zones was theretofore unknown. Eastern Europe and north-western Asia were invaded from another refuge close to the Caucasus Mountains, and the existence of a third northern secondary contact zone in eastern Poland and Ukraine seems likely. Further, in part newly identified, secondary contact zones are in southern Italy, the Balkans and Transcaucasia. To disentangle the manifold conflicts

between morphologically defined subspecies and mitochondrial differentiation, further research is needed, including the application of nuclear genomic markers and a morphological re-examination of morphologically similar populations representing deeply divergent mitochondrial clades.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Maximum Likelihood tree, depicting relationships of all 410 samples of *Natrix natrix* and *N. megaloccephala*. Sample codes are GenBank accession numbers (Table S1). Colours correspond to Fig. 2 except for clade 3, which is shown in black. For further explanations, see Fig. 2.

Table S1. Grass snake samples studied and GenBank accession numbers of their DNA sequences.

Table S2. Primers used for PCR and sequencing. For primer combinations and PCR conditions, see Table S3.

Table S3. PCR protocols.

Table S4. Mean uncorrected *p* distances (percentages) between and within mtDNA clades of grass snakes (top: ND4+tRNAs, bottom: cyt *b*). Below the diagonal, divergences between groups; on the diagonal, within group divergences in boldface.