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ScienceDirect
Organisms, Diversity & Evolution 9 (2009) 201–214
ORGANISMS DIVERSITY & EVOLUTION
www.elsevier.de/ode

Cryptic diversity in a Eurasian water snake (*Natrix tessellata*, Serpentes: Colubridae): Evidence from mitochondrial sequence data and nuclear ISSR-PCR fingerprinting

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Received 8 August 2008; accepted 6 March 2009

Abstract

The dice snake, *Natrix tessellata* (Laurenti, 1768), is a suitable study organism to address questions of Eurasian phylogeography due to its wide Palearctic distribution. We analysed complete mitochondrial cytochrome *b* sequences and nuclear ISSR-PCR fingerprints of more than 300 specimens representing nearly the entire geographic range. Nine major mitochondrial lineages were discovered based on mtDNA sequences. The three most basal lineages comprised populations from Iran, Jordan–Egypt, and Greece, respectively. Other lineages were associated with samples from the Turkish peninsula, the Caucasus, the Aral Sea, and eastern Kazakhstan. A sister-group relationship was found between two lineages from Crete and the European mainland. Assuming an evolutionary rate of 1.35% sequence divergence per million years, among-lineage p-distances of 1.7–8.4% suggest that intraspecific differentiation might date back as far as the Miocene/Pliocene transition 5–6 million years ago. The pattern of genetic differentiation in mitochondrial phylogeny with regard to Asia Minor and the region of the Aral Sea was not congruent with the results of the nuclear ISSR-PCR analyses, and suggests admixing within some mtDNA clades at contact zones. The taxonomic implications of the high intraspecific variation in the dice snake are discussed.

Keywords: Aral Sea; Cytochrome *b*; *Natrix tessellata*; Phylogeography; Pleistocene refugia; Taxonomy

Introduction

Studies of genetic differentiation and geographic association of genealogical lineages have contributed considerably to our understanding of how species evolve and diverge, and have revealed unexpected genetic structuring within many species complexes (Bickford et al. 2007).

The phylogeography of European biota has been studied extensively against the background of Pleistocene climatic oscillations. Large-scale extinctions in the north with survival of refugial populations in the south during cold periods, and post-glacial northward range expansions of southern populations may explain present patterns of genetic diversity in the European fauna and flora. The most important glacial refugia were located in the south of the continent, ie. in Iberia, Italy, the Balkans, or further to

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doi:10.1016/j.ode.2009.03.001

the east, around the Caucasus Mountains (reviewed in Hewitt 1996, 2000; Taberlet et al. 1998). Less attention has been paid to species with a broader, Eurasian distribution range. In many cold-tolerant species, Asian populations are derived from post-Pleistocene, eastward range expansions of north European lineages with relatively little differentiation. Some examples are the field vole, *Microtus agrestis* (see Jaarola and Searle 2002); the adder, *Vipera berus* (Kalyabina-Hauf et al. 2004; Ursenbacher et al. 2006); and the grass snake, *Natrix natrix* (Guicking et al. 2006). In contrast, Eurasian species with a western Asian origin show more complex phylogeographies in Asia, e.g. the badger, *Meles meles* (see Marmi et al. 2006); and stripe-necked terrapins, genus *Mauremys* (Fritz et al. 2008). This is because Pleistocene glacial periods were less severe in Middle Asia than in Europe, as is suggested by paleobotanical (Tarasov et al. 2000) and paleogeological evidence (Kashiwaya et al. 2001). Consequently, better long-term survival and stronger genetic differentiation may be expected in southern Asian than in European biota.

The dice snake, *Natrix tessellata* (Laurenti, 1768), ranges from Italy across the Balkans, Middle Asia, and the Near East into northwestern China (Bannikov et al. 1977; Gruschwitz et al. 1999; Fig. 1). It is confined to aquatic or marshy habitats and feeds mainly on fish and amphibians. The dice snake has been reported also from brackish water, e.g. from various river mouths along the Mediterranean coast, from the Black Sea, the Caspian, and the former Aral Sea (Gruschwitz et al. 1999). To date, only few studies have addressed questions of intraspecific variation in the dice snake. They have been locally restricted or have relied exclusively on phenotypic traits (morphology, scalation, and colour patterns) that show only weak and mainly clinal geographic variation (Laňka 1975; Mebert 1993). Consequently, little is known about geographical structuring in *N. tessellata*, and a satisfactory subspecies concept is lacking (Gruschwitz et al. 1999). The only subspecies that has been described, apart from the nominotypical subspecies, is *N. t. heinrothi* (see Hecht 1930) and refers to the dice snake population on the small Ukrainian island Serpilor in the Black Sea. However, the validity of this subspecies has been refuted, because re-evaluation of the features originally described for distinction of *N. t. heinrothi* did not yield diagnostic differences between the island and mainland populations. Therefore, a revision of the subspecies concept of *N. tessellata* is strongly recommended (Gruschwitz et al. 1999).

The wide Palearctic distribution and the fact that most squamate reptiles show strong geographic structuring (e.g. Burbrink et al. 2000; Paulo et al. 2002; Ursenbacher et al. 2006; Guicking et al. 2008) turn the dice snake into an interesting study organism to address questions of Eurasian phylogeography. Here, we present data obtained from mitochondrial cytochrome *b* sequences of more than 300 specimens representing almost the entire distribution range. These data allow us to characterise nine independent mitochondrial lineages. Furthermore, inter-simple-sequence-repeat polymerase chain reaction (ISSR-PCR) genomic fingerprinting (Gupta et al. 1994; Wu et al. 1994; Zietkiewicz et al. 1994) was used to compare the mitochondrial with nuclear data, and to locate regions of admixture among mtDNA lineages. The regional structuring of the populations offers insights into barriers to dispersal, refugia, colonisation routes, and secondary contact zones at a geographical level that is not much explored.

Finally, our data provide guidelines for a taxonomic revision of *N. tessellata*. Well-defined geographic distribution ranges and long-term independence of mitochondrial lineages suggest that the dice snake may consist of a number of distinct taxonomic entities.

Material and methods

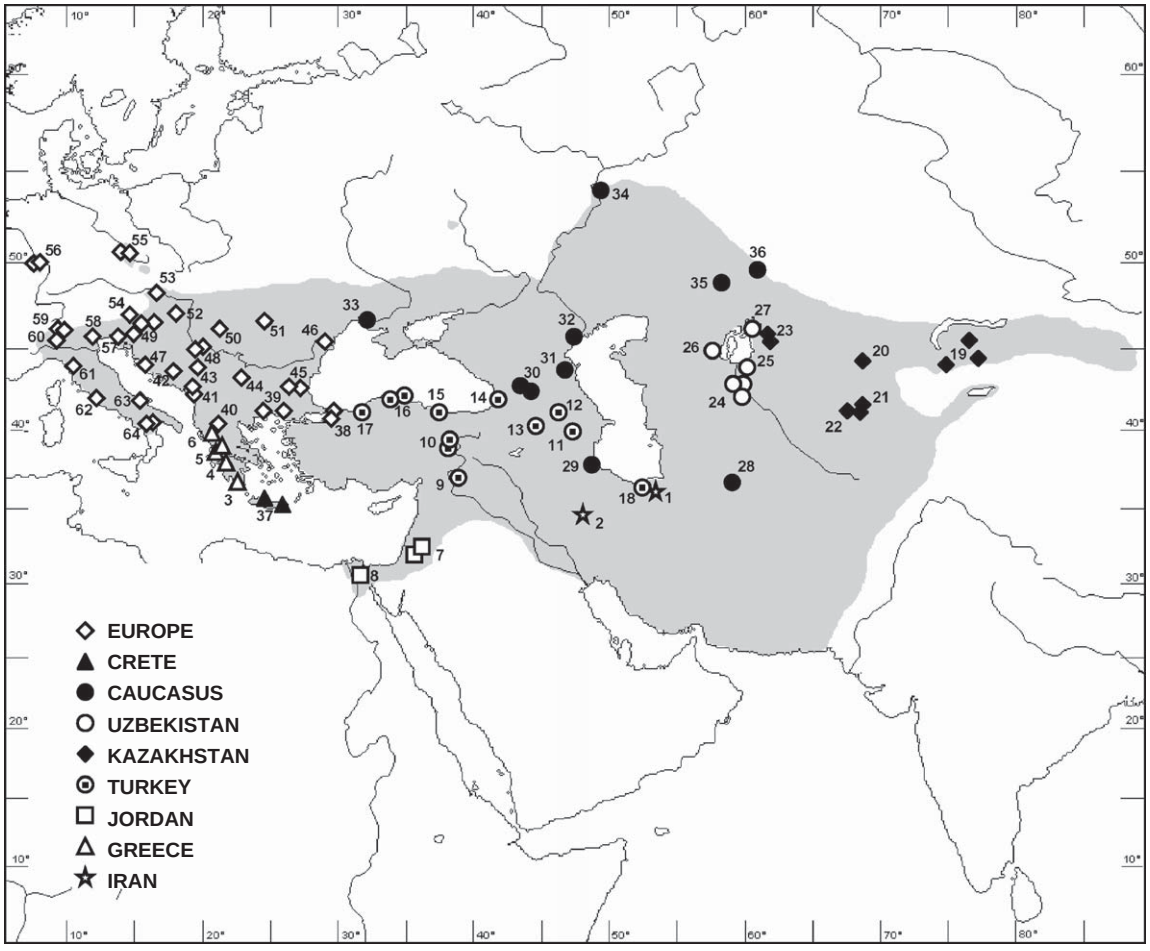
Sample material and DNA isolation

305 samples of *Natrix tessellata* from 26 countries were included, covering most of the species' distribution range (Fig. 1; Appendix 1, see Supplementary material in the online edition of this paper). Samples of blood were taken from the caudal vein of living snakes, or small pieces of tissue from roadkills and museum specimens were used, or shed skin. Samples were stored in 70% ethanol or EDTA buffer at −16 °C. Whole genomic DNA was extracted following standard protocols (Sambrook and Russell 2001). Small aliquots of sample material were digested in the presence of proteinase K and 1% SDS at 50 °C overnight. Cell fragments and proteins were removed from the extract by precipitation with NaCl and centrifugation, or by standard phenol/chloroform extraction. The DNA was precipitated with isopropanol, washed and resuspended in TE buffer.

Two additional *Natrix* species were included as outgroups in order to root the maximum-parsimony and maximum-likelihood cytochrome *b* trees: three sequences of *N. maura* (Linnaeus, 1758) from Tunisia (AY487681), Morocco (AF420077), and Spain (AY487704); and three sequences of *N. natrix* (Linnaeus, 1758) from Spain (AY866535), Italy (AY487733), and Greece (AY487725). For outgroup rooting of a MrBayes tree, a single sequence of *N. maura* from Tunisia was used.

MtDNA sequencing

The complete cytochrome *b* gene was amplified by polymerase chain reaction (PCR) using specific primers situated in the flanking regions of the gene: L14724NAT (5′-GAC CTG CGG TCC GAA AAA CCA-3′ (Guicking



Iran
1 IR Lar Valley (1)
2 IR Kermanshah (2)

Greece
3 GR Gialova (1)
4 GR Kalogria (8)
5 GR Golf of Arta (12)
GR Etoloakarnania (1)
6 GR Lake Ioannina (5)

Jordan
7 JOR River Zarqa (3)
8 Egypt (1)

Turkey
9 TR Birecik (1)
10 TR Malatya (1)
TR Kemah (1)
11 AZ Zangelan (1)
12 Armenia (2)
13 TR Ararat (2)
14 GE Batumi (1)
15 TR Sarkale (6)
16 TR Sinop (1)
TR Catalzeytin (1)
17 TR Eskicaga (1)
TR Lake Yenicaga (5)
18 IR Lar Valley (1)

Kazakhstan
19 KZ Almaty Region (5)
KZ Ili River (2)
KZ W Almaty (1)
20 KZ Karatau mountains (2)
21 UZ Gazalkent (6)
22 UZ Tashkent (1)
23 KZ Lake Kamyslebas (2)
KZ Raim (3)

Uzbekistan
24 UZ Urganc (2)
UZ Kungrad (4)
25 UZ Amu Darja Delta (5)
UZ Djanpyk (1)
UZ Lake Ashikol (1)
26 UZ Aral Sea (2)
27 KZ Baskara (1)
KZ Lake Tschebas (1)
KZ Syr Darja Delta (1)

Caucasus
28 IR Mashhad (2)
29 IR Guilan Prov. (1)
IR Lemir (4)
30 GE Agara (1)
GE Tblisi (3)
GE Rustavi (7)
31 Chechnia (1)
32 RUS Astrakhan (3)
33 UA Nikolaev (1)
34 RUS Samara (1)
35 KZ River Kaulschur (2)
36 KZ River Turgai (1)

Crete
37 GR Crete Á. Nicoláos (3)
GR Crete Finika (1)

Europe
38 TR Sile (1)
TR Sapanca (2)
39 GR Seres (2)
Greece NE (1)
40 GR Kastoria (1)
41 MON Lake Scutari (3)
MON Spuz (1)
42 BIH Lake Jezero (2)
BIH Konjic (1)
43 SER Veliki Rzav (2)
SER Crna Kamenica (1)
44 SER Luznicka dolina (1)
45 Bulgaria (2)
BG Sozopol (1)
46 RO Danube Delta (8)
47 HR Zadar (1)
48 SER Tresnja (1)
SER Kostolac (1)
49 SLO Zalec (1)
SLO Celje (1)
SLO Vrbno (1)
SLO River Drava (4)
50 RO Banat (1)
51 RO Cluj (1)
52 H Lake Balaton (3)
53 SK Bratislava (5)
54 A Carinthia (17)
55 CZ River Eger (16)
CZ River Berounka (6)
56 D River Lahn (11)
D River Mosel (10)
D River Nahe (7)
57 SLO Lepena (1)
SLO River Sava (11)
SLO Ljubljana (1)
SLO River Soca (1)
SLO Polovnik (3)
SLO Istria (2)
SLO Mirna Pec (3)
SLO River Dragonija (1)
SLO Portoroz (1)
58 I Grado (5)
I Tezze (1)
59 I Lake Garda (5)
CH Lake Lugano (5)
60 I Castelleone (1)
I Ottono (1)
I Varzi (2)
61 I Vigevano (1)
I Somma Lombardo (1)
I Lari (2)
62 I Lazio (4)
63 I Lesina (4)
64 I Taranto (3)
I Baragiano (1)

Fig. 1. Sampling localities and affiliation to major clades in *Natrix tessellata* based on cytochrome *b* sequences; sample numbers per locality listed in parentheses. Area shaded in grey represents approximate distribution range of *N. tessellata* according to Bannikov et al. (1977) and Gruschwitz et al. (1999).

et al. 2002), and H16064 (5′-CTT TGG TTT ACA AGA ACA ATG CTT TA-3′ (Burbrink et al. 2000). Additional primers situated within the cytochrome *b* gene (L 15161: 5′-TAA TAC AAA AYA CCC AYG CAA TTG G-3′, L15444: 5′-CCA ACC TTA ACA CGA TTC TTT GCC-3′, H15307: 5′-GCC CAG AAM GAT ATT TGT CCT CA-3′, H15713: 5′-TAG GCA AAT AGR AAG TAT CAT TCT GG-3′) were used to amplify small overlapping fragments from highly degraded DNA samples. PCR was performed in 50 µl volumes each 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). Five pmol of each primer and 50–100 ng of total DNA were used. PCR reactions were performed in a Biometra thermoblock with an initial denaturation step for 5 min at 94 °C, followed by 36 cycles of 50 s at 94 °C, 30 s at 50 °C and 90 s at 72 °C, and a final elongation step for 5 min at 72 °C. PCR products were sequenced directly on automated sequencers following the manufacturer's instructions (ALF Express II, Amersham Biosciences; or ABI Prism 310 and ABI Prism 3100, Applied Biosystems) and with primers given in Guicking et al. (2006). Sequences were aligned manually. All sequences were checked for unexpected stop codons, and variable sites of sequences from closely related populations were checked individually in the sequence chromatographs, to make sure that the sequences were mitochondrial and not pseudogenes. All cytochrome *b* haplotypes included in this study can be found in GenBank, under accession numbers AY487574–AY487680 and EU119167–EU119171.

ISSR-PCR genomic fingerprinting

We conducted nuclear genomic fingerprinting using ISSR-PCR. The latter has been used primarily in plant systematic studies (e.g. Tsumura et al. 1996; Fang and Roose 1997; Wolfe et al. 1998; Schrader and Graves 2004), to a much lower degree in studies of natural populations of animals (e.g. Wink et al. 2001; Nagy et al. 2002; Fritz et al. 2005, 2008; Hundsdörfer et al. 2005, Guicking et al. 2008). ISSR-PCR employs a single primer designed from di-, tri- or tetra-nucleotide repeat motifs (microsatellites), which are abundant in the eukaryotic genome (Tautz and Renz 1984). Amplified regions correspond to the sequence between two microsatellites orientated on opposite DNA strands (Gupta et al. 1994; Wu et al. 1994; Zietkiewicz et al. 1994). Gel-electrophoretic separation of PCR products generates characteristic multi-locus fingerprint patterns of the template DNA due to the ubiquitous occurrence of microsatellites in the genome. The main advantages of ISSR-PCR lie in the easy 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.

ISSR-PCR was performed with radioactively labelled nucleotides on denaturing high-resolution polyacrylamide gels. Suitable primers and reaction conditions were determined in a number of optimising reactions. Good repeatability of ISSR-PCR fingerprints was ensured through using the same DNA template in several independent reactions. For final analysis, primers $(GACA)_4$, $(GAA)_5$ and $(GGAT)_4$ were chosen, because these produced polymorphic and well-resolved fingerprint patterns in *N. tessellata*. PCR reactions were performed in a total volume of 25 µl, containing 50–100 ng of total DNA, 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 10x amplification buffer, and 1 unit *Taq* polymerase. The reactions were carried out in a Biometra thermocycler, using the following procedure: initial denaturation for 5 min at 94 °C, followed by 27 cycles for $(GACA)_4$ and $(GGAT)_4$ or 31 cycles for $(GAA)_5$ of 50 s at 94 °C, 30 s at the primer-specific annealing temperature, and 120 s at 72 °C. Final elongation was included for 25 min at 72 °C. Primer-specific annealing temperatures were 55 °C for $(GACA)_4$, 46 °C for $(GGAT)_4$, and 42 °C for $(GAA)_5$. PCR products were separated on a denaturing Sequagel XR matrix (National Diagnostics) at 65 W for 2.5 h or 4 h (length 40 cm). After drying, the gel was exposed to an X-ray film for 1–3 days and developed. ISSR-PCR needs non-degraded DNA for successful amplification of fragments; therefore, only samples with good DNA quality could be included in this analysis (see Appendix 1). Autoradiograms were analyzed by eye and scored to produce a 0/1 matrix coding for absence/presence of individual fragments in each sample. Only strong and distinct polymorphic bands were included in the matrix.

Phylogenetic analysis of mtDNA sequences

Maximum-parsimony (MP) and maximum-likelihood (ML) trees were reconstructed using PAUP 4.0b10 (Swofford 2002), and by Bayesian inference using MrBayes 3.0b4 (Ronquist and Huelsenbeck 2003). The MP analysis was performed with all sites weighted equally, because saturation effects were negligible in our data set (see Guicking et al. 2006). The most appropriate model of sequence evolution for the ML and Bayesian analyses was chosen using Modeltest 3.06 (Posada and Crandall 1998). The $TrN+I+\Gamma$ model was found to best fit the data. For MP and ML analyses, heuristic searches were performed with the starting trees obtained by neighbour-joining, tree-bisection-reconnection branch swapping, and ten random-addition sequences replicates.

The robustness of the branches of the shortest MP trees was assessed by nonparametric bootstrapping (Felsenstein 1985) performed with 1000 replicates. Bayesian analysis was performed by Markov Chain Monte Carlo (MCMC) randomisation with four chains for 1,500,000 generations with a codon-site dependent rate variation. Every 100th tree was sampled, and the burn-in was set to 1500; thus, the final consensus was drawn from the last 13,501 trees.

Genetic diversity of mtDNA lineages

Levels of genetic diversity among major mtDNA lineages were compared by calculating mean and minimum-to-maximum among-clade uncorrected p-distances and corrected ML distances, applying the $TrN+I+\Gamma$ model as chosen by Modeltest. A hierarchical analysis of variance (AMOVA; Excoffier et al. 1992) was performed with the program Arlequin 3.11 (Excoffier et al. 2005) to compare the component of genetic diversity imputable to the variance among lineages to that observed within lineages. Number of haplotypes, haplotype diversity (h), percentage of polymorphic sites, and nucleotide diversity (π) were calculated for six clades with sample size $N>15$ using the program DnaSP 4.10 (Rozas et al. 2003). To test for recent population expansion, we compared the observed distribution of pairwise nucleotide differences among haplotypes (mismatch distribution) to the distribution expected from a sudden-expansion model (Rogers and Harpending 1992; Rogers 1995), using the program Arlequin 3.11 (Excoffier et al. 2005). Whereas the mismatch distribution from populations at demographic equilibrium is typically multimodal, a unimodal distribution is indicative of a recent demographic expansion (Rogers and Harpending 1992). As a result of restricted sampling in the other lineages, the analysis of mismatch distribution was performed only in the European lineage of the dice snake.

Analysis of nuclear ISSR-PCR data

Genomic ISSR-PCR data were analysed using the program Structure 2.2 (Pritchard et al. 2000; Falush et al. 2003, 2007) to investigate possible admixture among mtDNA lineages. Structure 2.2 makes use of multilocus genotype data to infer population structure and assign individuals to populations based on Bayesian principles. First, information on mtDNA lineage affiliation was included as a prior to identify admixed individuals. Second, to infer population structure no prior information on mtDNA lineage affiliation was included, but a series of independent runs was conducted under an admixture model with different proposals for K (the number of clusters), testing all values from $K=1$ to $K=14$, with ten replicates for each value of K. The estimated number of clusters was taken to be the value of K with the highest estimated log probability. All runs used a burn-in period of 30,000 followed by 100,000 iterations.

Results

MtDNA phylogeny

Complete sequences of the mitochondrial cytochrome *b* gene (1117 base pairs, bp) were obtained from 305 specimens. 116 unique haplotypes were identified, with 273 (24.4%) variable sites, of which 238 (21.3%) were parsimony informative. Including the six outgroup sequences, there were 387 variable sites (34.6%), of which 322 (28.8%) were parsimony informative.

Phylogenetic reconstruction by ML (not shown) and by Bayesian inference (Fig. 2) yielded nearly identical topologies with very slight differences in branch lengths and resolution of terminal nodes. Nine well-supported major clades were revealed; these were labelled 'Iran' (I), 'Greece' (G), 'Jordan' (J), 'Turkey' (T), 'Kazakhstan' (K), 'Uzbekistan' (U), 'Caucasus' (A), 'Crete' (C), and 'Europe' (E), referring to the respective main distribution range (Fig. 1). Reconstruction based on MP revealed the same clades. All major clades were supported by Bayesian posterior probability values of 100% and by MP bootstrap values of 99–100% (Fig. 2). The only exception was the 'Turkey' clade with posterior-probability and bootstrap values of 97% and 87%, respectively.

In all trees, clade 'Iran' appeared at the most basal position. Strong evidence of sister relationships was found for clades 'Crete' and 'Europe' as well as for 'Caucasus' and 'Uzbekistan'. The latter two combined formed a well-supported sister group to 'Kazakhstan', and these three together were the sister group to 'Turkey' (Fig. 2).

MtDNA genetic diversity

Genetic diversity among mtDNA clades clearly exceeded within-clade diversities. The AMOVA ascribed 92.8% of the cytochrome *b* variation to among-clade variation, and 7.2% to within-clade variation, indicating highly significant genetic substructuring in the dice snake ($\Phi_{st}=0.928$, $p<0.001$). Average among-clade genetic distances (uncorrected p-distances/corrected ML distances) ranged from 1.70%/1.77% ('Uzbekistan' vs 'Caucasus') to 8.06%/11.2% ('Iran' vs 'Crete') (Table 1). Within-clade genetic diversity was low for clades 'Iran', 'Jordan', and 'Crete' (Table 1), but all three clades had very low sample sizes. Levels of genetic diversity (uncorrected p-distances) were in the range of 0.7–2.1% in clades 'Greece', 'Kazakhstan', 'Uzbekistan', 'Caucasus', and 'Europe'; the highest values

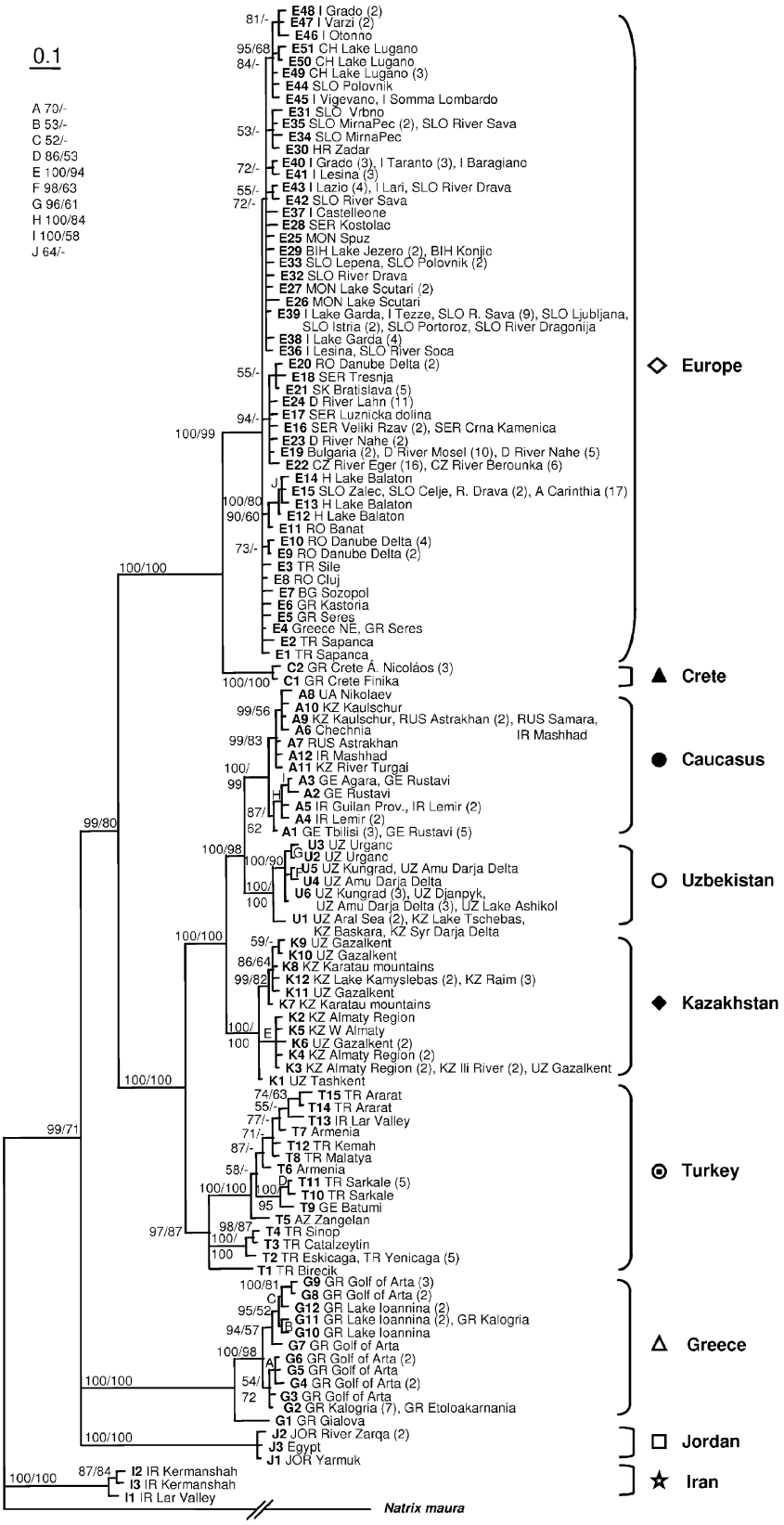


Fig. 2. Bayesian phylogram of 116 cytochrome *b* haplotypes detected in *Natrix tessellata*. Nine major lineages indicated by same symbols as in Fig. 1. Numbers at nodes: Bayesian clade credibility values/maximum parsimony bootstrap values. Numbers in parentheses following locality names are numbers of identical haplotypes from the corresponding locality.

Table 1. Genetic distances among nine major phylogenetic clades of *Natrix tessellata*.

Lineage	I	G	J	T	K	U	A	C	E
Iran (I)	4 (1) 0.0054/0.0053	15 (0)	n.a.	16 (1)	18 (2)	20 (2)	21 (2)	13 (0)	24 (1)
Greece (G)	0.0752–0.0797 0.0995–0.1076	11 (6) 0.0206/0.0216	n.a.	18 (6)	23 (4)	23 (6)	24 (6)	16 (4)	24 (8)
Jordan (J)	0.0734–0.0770 0.0991–0.1050	0.0752–0.0824 0.0984–0.1110	n.a. 0.0027/0.0027	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Turkey (T)	0.0680–0.0788 0.0884–0.1074	0.0707–0.0833 0.0907–0.1135	0.0702–0.0798 0.0944–0.1059	13 (6) 0.0323/0.0340	23 (6)	23 (8)	19 (13)	19 (3)	22 (12)
Kazakhstan (K)	0.0734–0.0788 0.0995–0.1089	0.0725–0.0824 0.0952–0.1118	0.0725–0.0761 0.0976–0.1047	0.0331–0.0501 0.0364–0.0586	16 (11) 0.0107/0.0108	20 (14)	28 (7)	24 (1)	30 (7)
Uzbekistan (U)	0.0761–0.0815 0.1040–0.1132	0.0725–0.0797 0.0946–0.1090	0.0716–0.0770 0.0962–0.1049	0.0367–0.0457 0.0399–0.0524	0.0233–0.0313 0.0274–0.0343	18 (10) 0.0072/0.0071	28 (9)	24 (3)	30 (9)
Caucasus (A)	0.0743–0.0806 0.1004–0.1119	0.0770–0.0842 0.1022–0.1166	0.0725–0.0788 0.0975–0.1088	0.0323–0.0474 0.0379–0.0550	0.0197–0.0286 0.0207–0.0314	0.0170–0.0215 0.0177–0.0228	19 (17) 0.0081/0.0081	25 (3)	27 (13)
Crete (C)	0.0806–0.0824 0.1120–0.1157	0.0770–0.0824 0.1026–0.1122	0.0716–0.0735 0.0941–0.0973	0.0539–0.0697 0.0676–0.0873	0.0654–0.0708 0.0833–0.0922	0.0680–0.0708 0.0872–0.0916	0.0662–0.0699 0.0847–0.0914	9 (0) 0.0009/0.0009	25 (5)
Europe (E)	0.0771–0.0824 0.1045–0.1161	0.0717–0.0833 0.0945–0.1148	0.0671–0.0725 0.0863–0.0944	0.0540–0.0707 0.0733–0.0912	0.0636–0.0726 0.0800–0.0956	0.0645–0.0725 0.0813–0.0942	0.0636–0.0708 0.0802–0.0936	0.0233–0.0296 0.0245–0.0320	21 (20) 0.0090/0.0090

Within-lineage values on the table diagonal from top left to bottom right: Respective upper value = total number of ISSR-PCR fragments within the clade, and number of polymorphic bands in parenthesis; lower value = maximum within-clade p-/ML-distances based on mitochondrial cytochrome *b* sequences.

Between-lineage values below and to the left of the table diagonal: Genetic-distance ranges based on cytochrome *b*; respective upper value = uncorrected p-distance, lower value = corrected ML-distance.

Between-lineage values above and to the right of the table diagonal: Total number of fragments present in the respective clade pair, and number of shared fragments in parenthesis.

n.a. = not applicable.

Table 2. Genetic diversity in cytochrome *b* sequences from six clades of *Natrix tessellata* analysed with sample sizes N > 15.

Lineage	N	Hap	*h*	%ps	π
Greece (G)	27	12	0.889 (0.047)	2.957	0.0049 (0.0011)
Turkey (T)	24	15	0.881 (0.053)	5.627	0.0172 (0.0012)
Kazakhstan (K)	22	12	0.905 (0.042)	1.434	0.0050 (0.0003)
Uzbekistan (U)	18	6	0.745 (0.079)	0.895	0.0029 (0.0005)
Caucasus (A)	27	12	0.880 (0.047)	1.436	0.0034 (0.0003)
Europe (E)	176	51	0.946 (0.007)	4.488	0.0037 (0.0001)

Hap = number of haplotypes; *h* = haplotype diversity; %ps = percentage of polymorphic sites; π = nucleotide diversity. Standard deviations given in parentheses.

were found in the 'Turkey' clade (Table 1). Haplotype diversities (*h*) and nucleotide diversities (π) are presented in Table 2 for six clades with N > 15. Values of π ranged from 0.3% ('Uzbekistan') to 1.7% ('Turkey'). Values of *h* were comparatively high for all clades ($h > 0.7$). Following Grant and Bowen (1998), high *h* and π values may be interpreted as suggestive of demographically stable populations with long evolutionary histories or geographic divergence. These conditions were found in the 'Turkey' clade, and to a certain degree in the 'Kazakhstan' and 'Greece' clades. In contrast, high *h* values and lower π values may be suggestive of a rapid expansion after a period of low effective population size (Grant and Bowen 1998). These conditions may apply to clades 'Uzbekistan', 'Caucasus', and 'Europe'. The mismatch distribution of the European lineage was clearly unimodal (data not shown) and fitted well the predicted distribution under a model of sudden expansion (SSD = 0.0025, P(SSDobs) = 0.193; Raggedness index = 0.0182, P(Ragobs) = 0.463). The estimate of Tajima's (1989) D statistic was significantly negative ($D = -1.96$, $p < 0.05$), thus providing further support for the hypothesis of rapid demographic expansion.

While most haplotypes were unique to single populations, a few were shared between two or more geographically close populations. In only three cases, identical haplotypes were found at very distant localities. These were haplotype 'K3' in Gazalkent (Uzbekistan) and at the Ili river in Kazakhstan (geographic distance 680 km); haplotype 'A9' in Kazakhstan at the Kaulschur river, in the Samara Region (Russia), the Wolga delta at the Caspian Sea, and in the Mashhad region of Iran (geographic distances 800 km to 2000 km); and haplotype 'E19' in Bulgaria and western Germany (about 1500 km apart).

ISSR-PCR analysis

163 samples were analysed by ISSR-PCR, including samples of all major cytochrome *b* clades except 'Jordan'. 47 polymorphic fragments were scored for data analysis; they yielded 124 unique genotypes. The highest percentages of shared ISSR-PCR fragments were found among lineages 'Uzbekistan' and 'Kazakhstan' as well as 'Caucasus', 'Turkey', and 'Europe' (Table 1).

An assignment test using the program Structure 2.2 (Pritchard et al. 2000), with prior information on mtDNA lineage affiliation, revealed seven individuals exhibiting clearly admixed genotypes (Fig. 3). Two individuals from the Syr Darja river delta northeast of the Aral Sea comprised alleles from the two mtDNA lineages 'Kazakhstan' and 'Uzbekistan'; two individuals originating from the Caucasus exhibited alleles from lineages 'Turkey' and 'Caucasus'; and three individuals from northwest Turkey comprised alleles either from lineages 'Europe' and 'Greece' or from 'Europe' and 'Turkey' (Fig. 3). Admixed individuals originated from the contact zones of the respective mtDNA lineages, with the exception of two individuals from northwest Turkey with admixed affiliation to mtDNA lineages 'Europe' and 'Greece', in which case it is unknown whether the range of the mtDNA lineage 'Greece' extends into western Turkey (Fig. 1).

When inferring population structure with the program Structure 2.2, the highest estimated log probability was found for K = 6 (Fig. 3). The two lineages 'Iran' and 'Crete' were not resolved. This is most likely due to low numbers of loci and/or sample sizes, since several ISSR-PCR fragments do support the genetic discreteness of these lineages when considered independently (Table 1). A genetically homogeneous cluster included all individuals from the 'Greece' mtDNA lineage. The 'Europe' lineage was also clearly separated from other lineages, although most individuals of this lineage displayed membership coefficients of two distinct clusters that showed no clear geographical relationship. This might reflect a low underlying genetic structure of European dice snakes. The mtDNA lineage pairs 'Kazakhstan' and 'Uzbekistan' on the one hand, and 'Turkey' and 'Caucasus' on the other, were not differentiated by nuclear data (Fig. 3). However, samples from north of the Caucasus mountain range (locality Nos. 32–36 in Fig. 1) formed a genetically distinct cluster within the 'Caucasus' lineage. This corroborates results from the mtDNA phylogeny (Fig. 2), in which these samples also formed a distinct subclade (that,

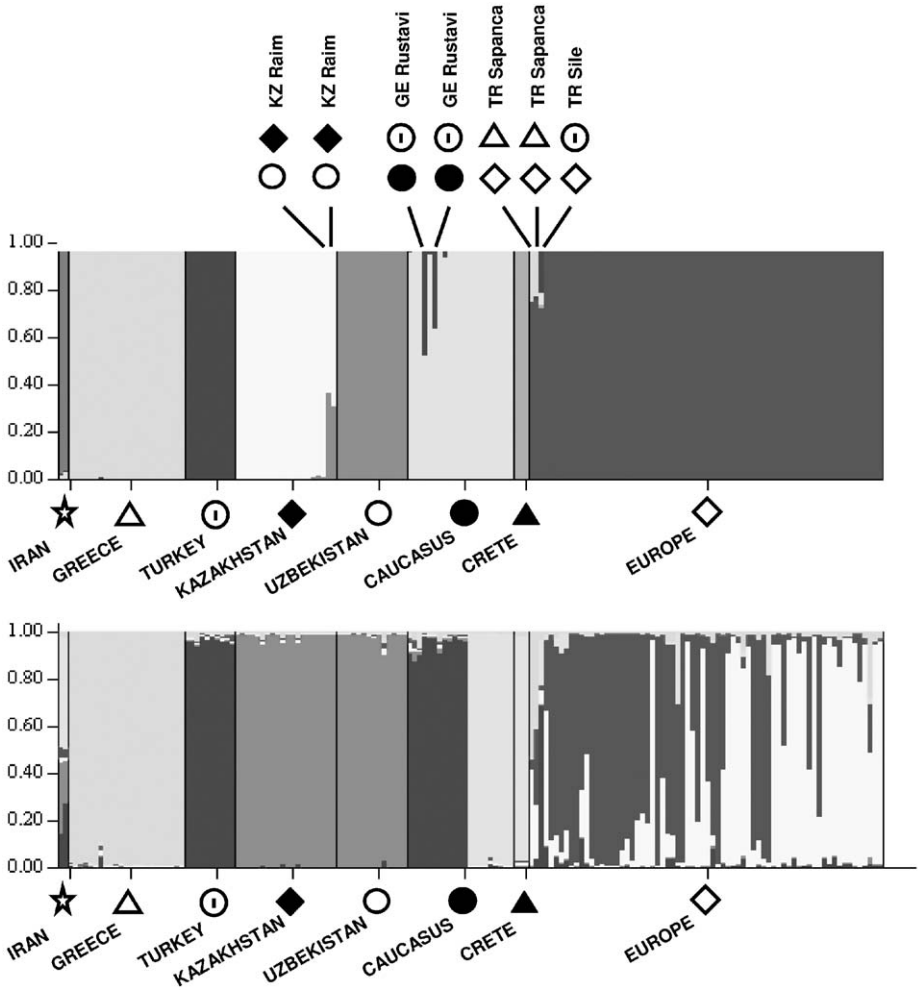


Fig. 3. Results of ISSR-PCR analysis using Structure 2.2 (Pritchard et al. 2000). Each individual is represented by a thin horizontal line divided into K segments that represent the individual's estimated membership fractions in K clusters. Each of K genetically discrete clusters is represented by a distinct shade of grey. Individuals are arranged by their mtDNA affiliation and geographic origin. Black lines separate mtDNA lineages as labelled below each subfigure. Top: Assignment test using mtDNA lineage affiliation as a prior; seven individuals were assigned admixed origin of each of two mtDNA lineages as indicated above. Bottom: Estimated population structure assuming an admixture model with six clusters (K = 6).

interestingly, also included two samples from locality No. 28 in eastern Iran).

Discussion

Phylogeographic groups

Phylogenetic reconstructions of cytochrome *b* sequences revealed at least nine genetically distinct and geographically well-defined phylogeographic groups in *Natrix tessellata*. The basal position of Iranian samples in phylogenetic trees suggests that ancestral populations of the dice snake lived in the Near East (Guicking et al. 2006). To estimate divergence times in the genus *Natrix*, Guicking et al. (2006) applied a molecular-clock approach and estimated an evolutionary rate of 1.35% sequence divergence per million years for *N. tessellata* (details on the procedure of rate estimation are given in Guicking et al. 2006). Assuming that these rate estimations are reasonably correct, among-lineage distances of 1.9–8.1% (Table 1) suggest that the time frame involved in the separation of the major intraspecific clades identified in the present study spans the period between 1.4 and 6 million years ago. Although time estimates under the molecular-clock hypothesis must be taken with caution, we may conclude that major phylogeographic subdivisions in the dice snake occurred long before the onset of Pleistocene climatic oscillations and may date back as far as the Miocene/Pliocene transition. According to these considerations, only the most closely related sister-group relatives, 'Uzbekistan' and 'Caucasus', and 'Europe' and 'Crete', separated during the Pleistocene.

Origin and relationships of mitochondrial lineages in Europe

Assuming an origin of the dice snake in the Near East, genetic data clearly support two independent expansions into Europe that gave rise to the lineages 'Greece' and 'Crete plus Europe', respectively. An estimated divergence time of 5.5 million years between the lineage from southern Greece and other lineages suggests that a westward range expansion of the Asian population might have occurred during the Messinian salinity crisis between 5 and 6 million years ago (Hsü et al. 1977; Krijgsman et al. 1999). Subsequent flooding of the Mediterranean could have isolated the populations in southern Greece. The geographic restriction, strong genetic differentiation, and comparatively high genetic diversity of the 'Greece' clade, as supported by mitochondrial and nuclear markers, suggest independent evolution of this lineage since the early Pliocene.

The 2.6% genetic distance between clades 'Europe' and 'Crete' suggest that these two lineages separated in the late Pliocene or early Pleistocene, supporting the view that the Cretan dice snake population is autochthonous. There is good evidence from paleogeological and biogeographical data that the island of Crete has not had contact with the mainland since the end of the Messinian (Beerli et al. 1996). Colonisation to or from Crete, therefore, must have occurred across the water. Transmarine dispersal has been discussed for many island reptiles (e.g. by Carranza et al. 2000; Nagy et al. 2003a) and does not seem unlikely for the dice snake, since this species is believed to be reasonably tolerant of saline water (Gruschwitz et al. 1999).

The lack of phylogenetic and geographic resolution within the clade 'Europe', in both cytochrome *b* and ISSR-PCR data, contradicts expectations that European dice snakes may have persisted Pleistocene cold periods in two (or more) southern refugia on the Italian and Balkan peninsulas, as observed in other thermophilic European species (Hewitt 1996; Taberlet et al. 1998). Low intra-lineage divergence could either be the result of strong gene flow between populations or reflect a population expansion that occurred too recently for neutral DNA to diverge (Slatkin and Hudson 1991; Rogers and Harpending 1992). We regard the second alternative more likely, firstly because strong gene flow is generally not observed in reptiles (e.g. Lenk et al. 1999; Paulo et al. 2002; Guicking et al. 2008), and secondly since both the results of a mismatch analysis and high *h* and comparatively low π values are most consistent with a rapid expansion after a period of low effective population size (Bowen and Grant 1998). It seems conceivable that the European dice snakes have undergone large-scale extinctions during the late Pleistocene and survived in only one or few remnant populations in southern Europe or on the Turkish peninsula, where warmer climates persisted (Hewitt 1999). Postglacial range expansions into central Europe probably followed the river system of the Danube, as is supported by the finding of identical haplotypes in Bulgaria and western Germany, as well as by Holocene fossil records of *N. tessellata* from the upper Danube valley (Markert 1976).

Origin and relationships of Asian dice snakes

There is little doubt that the Asian dice snakes comprise some of the most ancestral lineages of the species. However, high levels of genetic variability and limited sample material especially from the Near East impede determination of the exact number and geographic boundaries of mitochondrial lineages in the region.

A distinct subdivision of populations in southwest Asia is consistent with observations in other species, e.g. in the fire salamander, *Salamandra salamandra* (see Steinfartz et al. 2000); dwarf snakes, genus *Eirenis* (Nagy et al. 2003b); and stripe-necked terrapins, genus *Mauremys* (Fritz et al. 2008). The highly structured topography probably has promoted fragmentation of populations in different mountain valleys. Furthermore, there is good evidence for better long-term survival of populations in valleys with steep slopes, because of local moderation of climate fluctuations and the possibility to track suitable habitats during climatic changes by comparatively short vertical movements (Hewitt 1996, 1999; Jansson and Dynesius 2002). This seems to be particularly true for the 'Turkey' clade, which exhibits high values of haplotype and nucleotide diversity indicative of a long evolutionary history in a large and stable population.

In contrast, the 'Uzbekistan' and 'Caucasus' clades, which both have a more northern distribution, showed high *h* but lower π values suggestive of rapid expansions after a period of low effective population size (Grant and Bowen 1998). These lineages probably were affected by Pleistocene climatic changes, resulting in northern extinctions during cold periods and range expansions during warm periods. In particular, good evidence was found for a glacial refugium of the dice snake in the Caucasus region, consistent with observations in other species (e.g. by Hewitt 1999; Orth et al. 2002; Fritz 2003). The current populations in the steppe habitats north of the Black and Caspian Seas are distributed in areas that were covered by permafrost and hence were uninhabitable during the latest glaciation (Hewitt 1999; Tarasov et al. 2000). These regions must have been colonised during post-glacial times. Most likely, refugial populations survived along the coasts of the Caspian and Black Seas, where the climate was ameliorated by the influence of the water bodies (Tarasov et al. 2000). Genetic discreteness and homogeneity of the respective animals might reflect the effects of a founder event.

Furthermore, the presence of identical haplotypes in localities up to 2000 km apart is suggestive of a recent range expansion.

Sister relationships and relatively small among-lineage distances between the clades 'Turkey', 'Caucasus', 'Kazakhstan', and 'Uzbekistan' suggest two independent colonisation events from Asia Minor to the east during the late Pliocene and early Pleistocene or, alternatively, a range expansion of an ancestral eastern lineage to the west. The variable paleogeological history of the river systems between the Caspian Sea and the Aral Sea depression during the Pliocene (reviewed in Létolle and Mainguet 1996) might have facilitated both expansions and fragmentations in the region.

Comparison of mitochondrial and nuclear data

Analysis of nuclear ISSR-PCR data confirmed the presence of genetically discrete lineages within *N. tessellata*, indicating that the dice snake consists of a series of highly divergent clades. However, some discordance between mitochondrial and nuclear data was found, suggesting secondary contact of mtDNA lineages especially in Asia Minor and in the Aral Sea region.

Clades 'Greece' and 'Europe' (as well as 'Iran' and 'Crete' to a certain extent) were well resolved by phylogenetic and Bayesian approaches, which suggests their evolutionary independence. Inconsistencies between mitochondrial and nuclear data, as were found in Asia Minor (admixture of lineages 'Turkey' and 'Caucasus', and individuals of admixed genetic origin from lineages 'Turkey' and 'Europe' as well as 'Turkey' and 'Caucasus') and in the Aral Sea region (admixture of lineages 'Kazakhstan' and 'Uzbekistan'), may be explained either by incomplete lineage sorting of the nuclear genome or by gene flow among lineages. Due to a smaller effective population size of the mitochondrial genome (Moore 1995; Moritz and Hillis 1996), mtDNA usually shows stronger genetic differentiation than nuclear DNA. However, given the probably very high ages of the different lineages and the geographical restriction of admixed clades, we regard present or recent gene flow to be the more likely explanation.

A close relationship among samples from clades 'Turkey', 'Caucasus', 'Europe' and possibly 'Greece' in northern Turkey and the adjacent Caucasus region according to the ISSR data suggests gene flow among these clades, presumably following the southern coast of the Black Sea, a suitable habitat for the dice snake (Gruschwitz et al. 1999). Furthermore, gene flow across the Bosporus, as is indicated by three individuals from northwestern Turkey with admixed affiliation to mtDNA lineages 'Europe' and 'Turkey' or 'Greece' (Fig. 3), is not surprising because of the narrowness and probably young age of this strait (Kerey et al. 2004).

A second case of possible hybridisation among ancestral lineages may be postulated in the Middle Asian region of the Aral Sea, between clades 'Kazakhstan' and 'Uzbekistan'. Two animals collected in Raim in the Syr Darja river delta northeast of the Aral Sea represented mtDNA lineages from both 'Kazakhstan' and 'Uzbekistan' in the Structure 2.2 analysis of ISSR-PCR data (Fig. 3). Introgressive intergradation may have taken place between ancestral populations of the two lineages in the northeastern region of the Aral Sea, resulting in incongruent mitochondrial and nuclear affinities. This observation is of particular interest regarding conservation efforts around the Aral Sea. Haplotype identity (haplotype U1) between the southwestern and northeastern shores of the Aral Sea indicates that genetic exchange across the Aral Sea took place in former times. This is further corroborated by the former occurrence of *N. tessellata* on the island of Vozrozhdeniya in the central Aral Sea (Bannikov et al. 1977). Due to the desiccation and consequent salinisation of the Aral Sea over the past 40–50 years (reviewed in Létolle and Mainguet 1996), the populations are now geographically isolated so that no further genetic exchange can take place.

Conclusions

Our data are consistent with the notion of several distinct phylogenetic lineages within what is currently recognised as *N. tessellata*. Therefore, a taxonomic subdivision of *N. tessellata* seems desirable. However, additional information from further genetic and phenotypic analyses is required before final conclusions can be drawn as to how many taxa should be distinguished and at which hierarchical level (subspecies or species).

Similar questions, whether subspecies should be defined or independent lineages elevated to species level, remain open in the cases of *Natrix natrix* (see Böhme 1999) and *N. maura* (see Guicking et al. 2008). Most species concepts (e.g. the biological, phylogenetic, evolutionary, and cohesion concepts) basically agree on what species are: For sexual organisms, a species is a lineage which is unified primarily by sexual reproduction or gene flow among its constituent parts (Queiroz 1998; Coyne and Orr 2004). Therefore, a most important prerequisite for adequate species delimitation is to quantify the extent of gene flow among lineages. In our results, most lineages are well differentiated by both mitochondrial and nuclear data. However, we have found some evidence for gene flow among lineages in Asia Minor and Middle Asia. In order to decide whether any taxonomic entities to be based on those results deserve full species status, we consider it most important to perform comprehensive gene-flow analyses among animals from contact zones

of mitochondrial lineages. Additional genetic analyses including sample material from currently underrepresented areas (e.g. southwest Asia, Turkey) would be desirable, in order to determine the geographic ranges of different taxonomic entities. Finally, phenotypic studies including animals from the entire distribution range are essential, in order to determine whether the recognised genetic diversity is also reflected in morphology. With such data at hand, a comprehensive taxonomic revision of the dice snake would be possible.

Acknowledgments

Numerous people have contributed sample material or helped in one way or another to make sample material available to us. We are extremely grateful to all of them: T. Amann, A.G. Bakiev, B. Blosat, Y. Chikin, J. Crnobrnja Isailovic, G. Dücker, T. Dujsebaeva, H.-P. Eckstein, H. Enting, W. Fiedler, B. Gautschi, N. Godetsch, M. Gruschwitz, A. Herzberg, A. Hille, S. Kalyabina-Hauf, A. Kapla, L. Krecsag, P. Lenk, S. Lenz, D. Malakhov, G. Mantziou, W. Mayer, P. Mikulicek, D. Modry, G. Nilson, N. Orlov, E. Rastegar, N. Rastegar-Pouyani, E. Razetti, A. Schmidt, K. Smole-Wiener, A. Sproll, S. Tome, S. Ursenbacher, U. Utiger, A. Westerström, M. Vogrin, M.A.L. Zuffi. We would like to thank Robin Lawson for much helpful advice, and Eskandar Rastegar for sequencing some Iranian samples. D.G. thanks K. Mebert, H. Schäfer, and two anonymous reviewers for constructive comments on the manuscript. Cathy Wüster kindly helped with language editing. Financial support for this study was provided by the Deutsche Forschungsgemeinschaft (Jo 134-7, Jo 134-9, Wi 719-18), and by INTAS (436 RUS 113/665/0-1).

Appendix 1. Supplementary material

Supplementary data (Appendix 1) associated with this study can be found in the online edition at doi:10.1016/j.ode.2009.03.001

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