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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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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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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