NORTH-WESTERN JOURNAL OF ZOOLOGY 8 (2): 374-377
Article No.: 121209

http://biozoojournals.3x.ro/nwjz/index.html

Molecular data confirm recent fluctuations of northern border of dice snake (*Natrix tessellata)* range in Eastern Europe

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Received: 09. February 2012 / Accepted: 28. July 2012 / Available online: 10. August 2012 / Printed: December 2012

Abstract. Herein we present the cytochrome oxidase subunit I (COI) based phylogeography of the dice snake (*Natrix tessellata*) in Eastern Europe and The Balkans. Eight haplotypes were identified. Phylogenetic reconstruction has showed 3 major clades: 1) Greece, 2) Balkans, Central and Eastern Europe, 3) Eastern Europe (Ukraine and Russia only). Our data are in concordance with the results of other studies of the species' phylogeography. The northernmost isolated population of the dice snake in Lipetsk province, Russia have no differences from neighboring Ukrainian populations and seems to be the result of recent colonization during the Holocene climatic optimum.

Key words: COI, phylogeography, colonization, *Natrix tessellata.*

The dice snake, *Natrix tessellata* is distributed from Italy over the Balkans, Near East, to China (Gasc et al. 1997, Gruschwitz et al. 1999, Vlcek et al. 2010, Mebert 2011). It prefers aquatic or marshy habitats, including brackish water (Gruschwitz et al. 1999, Tuniyev et al. 2011). In Eastern Europe, the northern border of continuous range of the species is between 49° N and 50° N latitude. However, there are some isolated populations more to the north, with the most well known of them in Galichya Gora Nature Reserve, the upper Don current, Lipetsk province, Russia (Lada and Nedosekin 1997, Kotenko et al. 2011, Litvinov et al. 2011) and near Samara, Russia (Litvinov et al. 2011).

According to the available literature, the population genetic structure of dice snake was analyzed in details in isolated German (Guicking et al. 2004) and Swiss (Gautschi et al. 2000) populations, as well as in southern Austria (Guicking & Smole-Wiener 2003). Unusual degree of divergence was detected among some isolated populations in Germany, perhaps as result of microevolutionary processes (Guicking et al. 2004), whereas the isolated Swiss population suffered from loss of genetic diversity likely due to its pass through a bottleneck (Gautschi et al. 2002). However, clear evidences of recent postglacial expansion from the Balkans were found in mitochondrial DNA sequences by Guicking et al. (2006, 2009) and Guicking & Joger (2011). They presented a range wide phylogeography and phylogeny of the species, based on the mitochondrial cytochrome b gene and ISSR-PCR fingerprinting.

In our study we used the cytochrome oxidase subunit 1 barcoding region to elucidate the phylogenetic relationship between previously uncovered *Natrix tessellata* populations from Eastern Europe and the Balkans. We were able to compare the concordance between topology of the phylogenetic tree based on the COI sequences and the phylogenetic tree based on the cytochrome b from the study of Guicking et al. (2009).

Samples were taken from animals caught in the field, which were safely released after the procedure. DNA was isolated from tissue samples of up to 5 mm of distal part of the tail. We also sampled tissues from specimens of museum collections.

We used in total 15 specimens of *N. tessellata* (Fig. 1): n = 5 specimens from Romania (Ro1, Ro2, Ro3, Ro4 - 44°33′N, 28°44′E,- Histria and Ro5 – 46°57′ N, 22°44′E,- Negreni), n = 3 specimens from Serbia (Ser1 – 46°06′ N, 20°06′E, - Divcibare, Ser2 - 44°35′ N, 20°35′ E, - Belgrade, Ser3 - 43°05′ N, 22°30′ E, - Luznica valley), n = 1 specimen from Italy (Ita - 44°37′ N, 09°19′ E, Ottone - , from the Natural History Museum of Pavia), n = 2 specimens from Ukraine (The Museum of Nature, Kharkiv National

University – MNKhNU: Ukr1: G-1287 – 49°05′ N, 37°27′ E, - Kharkivska oblast, Ukr2: G-1230 - 48°34′ N, 39°45′ E, - Luhanska oblast), n=1 specimen from Russia (MNKhNU: Rus: G-1446 – 52°6′ N, 39°8′ E, - Galichya Gora) and n = 3 specimens from Greece (The Natural History Museum of Crete –NMHC: Gr1: 80.3.35.16 - 39°38′ N, 21°13′ E, - Trikala, Gr2: 80.3.35.1 - 37°51′ N, 22°27′ E, - Korinthias, Gr3: 80.3.35.14 - 35°16′ N, 25°21′ E, - Crete). In addition, we also used n = 1 specimen of *N. natrix* (46°57′ N, 22°44′ E, - Negreni) for outgroup.

In order to obtain partial COI gene sequence, we used the degenerate primer pair V1F and V1R published by Smith et al (2008). After PCR optimization the amplification was carried out in 50µl solution, using the GoTaq® Flexi DNA Polymerase Kit (Promega, USA) at 1.5 mM $MgCl_2$ concentration with an initial denaturation at 94°C for 5 mins; followed by 35 cycles each of a denaturation at 94ºC for 40 sec, primer annealing at 59º C for 40 sec and extension at 72ºC for 60 sec; and finished with a single extension for 5 mins at 72ºC. The PCR products were extracted from gel using the Nucleospin® Extract II Kit and subjected to direct sequencing using the V1F primer at the Macrogen Inc. (Seoul, Korea).

We used Kimura 2-parameter model to calculate sequence distance matrix and to construct phylogenetic tree with the Maximum likelihood method. All sequence analyses were performed with MEGA 5 (Tamura et al. 2011).

Out of the 15 specimens we have found 8 haplotypes provided with the following accession numbers: A (JN871603): Ro1 – Ro5, Ser1, Ser2; B (JN871604): Ser3; C (JN871605): Ita; D (JN871606): Gr3, E (JN871607): Ukr1, Rus; F (JN871608): Ukr2; G (JN871609): Gr1; H (JN871610): Gr2 (Fig. 1).

Alignment of the sequences yielded 48 variable nucleotide positions out of 621 nucleotides.

The highest value of 0.056 for the pairwise divergence was found between sequences of Gr1 and Ukr1, Gr1 and Rus, and Gr1 and Ser3 (Table 1). In general the highest values (those > 0.05)

Figure 1. Identified haplotypes (after GoogleEarth).

Table1: Estimates of pairwise divergences between sequences based on Kimura 2-parameter model.

	Gr1	Gr2	Gr3	Ita	Ro1	Ro2	Ro3	Ro4	Ro5	Rus	Ser1	Ser2	Ser3	Ukr1	Ukr2
Gr1															
Gr2	0.003														
Gr3	0.052	0.050													
Ita	0.052	0.050	0.013												
Ro1	0.054	0.052	0.011	0.002											
Ro2	0.054	0.052	0.011	0.002	0.000										
Ro3	0.054	0.052	0.011	0.002	0.000	0.000									
Ro4	0.054	0.052	0.011	0.002	0.000	0.000	0.000								
Ro5	0.054	0.052	0.011	0.002	0.000	0.000	0.000	0.000							
Rus	0.056	0.054	0.040	0.043	0.045	0.045	0.045	0.045	0.045						
Ser1	0.054	0.052	0.011	0.002	0.000	0.000	0.000	0.000	0.000	0.045					
Ser2	0.054	0.052	0.011	0.002	0.000	0.000	0.000	0.000	0.000	0.045	0.000				
Ser3	0.056	0.054	0.013	0.003	0.002	0.002	0.002	0.002	0.002	0.047	0.002	0.002			
Ukr1	0.056	0.054	0.040	0.043	0.045	0.045	0.045	0.045	0.045	0.000	0.045	0.045	0.047		
Ukr2	0.054	0.052	0.038	0.042	0.043	0.043	0.043	0.043	0.043	0.002	0.043	0.043	0.045	0.002	

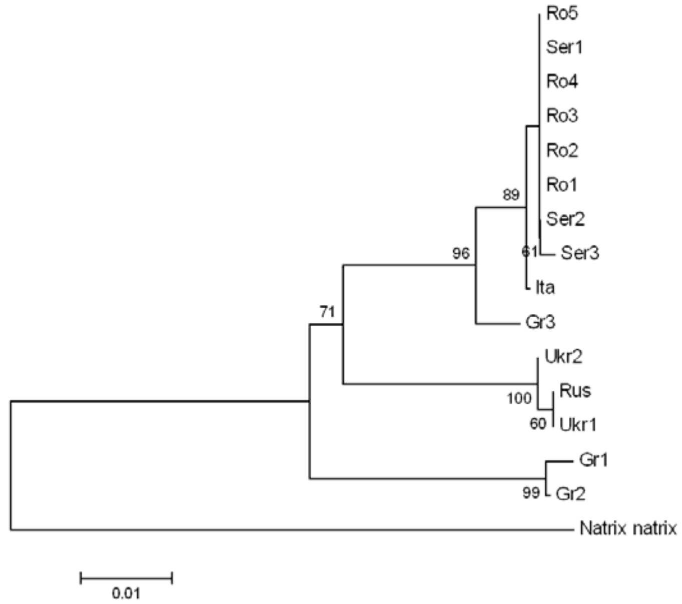

Figure 2. Phylogenetic analysis of the 15 *N. tessellata* COI sequences, based on Kimura 2-parameter model and Maximum likelihood method. The bootstrap consensus tree was inferred from 1000 replicates. *Natrix natrix* ((JN871611) sequence was used for outgroup.

were between either the Gr1 or Gr2 sequences paired with the rest of the sequences suggesting the basal position of the haplotypes from Greece (Table 1).

The phylogenetic analyses of the *N. tessellata* sequences indicate 3 major clades (Fig. 2). One group is formed by the Ukrainian and Russian sequences and another separate group by the Gr1 and Gr2 sequences, which are from the mainland of Greece. It was surprising that the third Greek sample (Gr3), from the island of Crete, which is spatially closer to the Greek mainland samples (Gr1 and Gr2) cluster together with distant samples from Romania, Italy and Serbia, The mainland Greek samples are geographically between the Crete Island to the south and the other Balkan-Italian samples to the north. These results confirm the study on cyt b by Guicking et al. (2009), in which Crete appears as a separate lineage, but as sister, and thus more closely related, to the European lineage. The concordance between our results and those of Guicking et al. (2009) emphasizes their conclusion that the colonization of Greece and Europe occurred in at least two waves. Consequently, the Gr1 and Gr2 COI haplotypes from our study probably represent the remnants of the first colonization and then survived isolated since the early Pliocene. The rest of Western and Central Europe was colonized in the Pleistocene, where possible routes connecting Crete, Eastern and Central Balkans and westwards to Europe could have led along the western coast of Turkey, as was already proposed in Guicking et al. (2009).

The Ukrainian and Russian *N. tessellata* haplotypes form a separate clade from the European dice snake haplotypes and this result is also in concordance with the findings of Guicking et al. (2009): the north of the Black Sea and the north east European habitats of the dice snake were colonized from the Caucasian and not the Balkan refugium. Our northernmost and well isolated population of *N. tessellata* from Galichya Gora (Rus) seems to be the result from a very recent and fast colonization event, as the time of isolation was not long enough to gain any genetic divergence from the nearest Ukrainian sample Ukr1. Similar to the isolated populations in Germany (Guicking et al. 2004), we hypothesize that the population from Galichya Gora had appeared due to expansion of the Dice snake range in the Holocene climatic optimum at 9-5 kya. As in the case of the German dice snakes, the colonization routes could have followed large rivers, generally oriented in a south-north direction (the Danube and Elbe in Germany and the Don and Volga in Russia). Relict populations' habitat is, in fact, part of a specific, more thermophilous landscape, rich in south-oriented slopes and stony outcrops etc. (Kotenko et al. 2011). Fossils of *N. tessellata* also support the scenario of several northern expansions of its range in Pleistocene (Ratnikov, 2009; Ratnikov and Mebert, 2011) and together with our data show almost instant (in geological scale) response on climatic changes and northern dispersion in the past.

Unfortunately, regarding the population from Galichya Gora, we have no information to compare its' genetic structure with data on German isolates, and, at this moment, we cannot look for similarities in microevolutionary processes in these spatially isolated northern populations. However, in spite the lack of any new variation in the mitochondrial genome of this Eastern European isolate in addition to the rest of the species' range, we emphasize the particular value and need of further studies of this population and similarly northern isolates in order to understand mechanisms and consequences of range fluctuations during climate changes and microevolutionary processes in isolated populations.

Acknowledgements. We are very grateful for Endre Jakab and Sergiu Chira for assistance with molecular protocols and Aleksandr Prisny, Eduard Snegin and Edoardo Razzetti for providing samples. JCI was supported by Grant nr 173025 of Ministry of education and science of Republic of Serbia. Finally, but not least, we want to thank Konrad Mebert and an anonymous referee for reviewing and correcting the manuscript.

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