

Phylogeography of the Caspian whipsnake in Europe with emphasis on the westernmost populations

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Abstract. The Caspian whipsnake (Squamata: Serpentes: *Dolichophis caspius*) reaches its northwestern distribution limit in Hungary where it is the single representative of its genus. Recently, several new localities of the species were discovered in Hungary. Focusing on these edge populations and on other ones in the western parts of the distribution, a phylogeographic study based on cytochrome *b* sequences was carried out. Thereby, a clear mitochondrial divergence was observed in Europe, with eastern and western haplotype groups found by analysing a number of specimens and populations. Our haplotype network analysis suggests a rather rapid postglacial (re)colonization of Europe by this snake species. In Hungary, three different haplotypes were detected.

Keywords: *Dolichophis caspius*, haplotype network, Hungary, mtDNA, Serbia.

Introduction

The Caspian whipsnake *Dolichophis caspius* (Gmelin, 1789) is a large-sized colubrid species with a remarkably rapid mobility. Its adult colouration is usually uniformly brownish (yellowish or reddish brown) above, and pale yellow below, but juveniles show typical spotted patterns. The species inhabits parts of south-eastern Europe including the Balkan Peninsula, Turkey (Istanbul region, Black Sea region, Central and East Anatolia; Budak and Göçmen, 2005), further regions around the Black Sea and the Caspian Sea (Schcherbak and Böhme, 1993, see inset in fig. 1). Recently, several new localities were discovered in Romania (Strugariu and Gherghel, 2007; Covaciu-Marcov and David, 2010) as well as in Croatia (Kletečki et al., 2009).

The Caspian whipsnake inhabits forest-steppe and steppe habitats in lowland and also on some hilly and mountaneous areas of Serbia (Radovanović, 1951). There are viable populations in the provinces of Vojvodina, especially on the banks along the river Danube, and also in eastern Serbia and Kosovo, as well as Metohija provinces. It reaches its northwestern distribution border in Hungary where it is the single representative of its genus, and considered to be a typical Ponto-Caspian faunal element (Varga, 1995; Korsós, 2007). In Hungary, it was previously known from only two regions: the Szársomlyó Hill (part of the Villányi Hills, South Hungary), and the Budai Hills (Frigyás, 1865; Dely, 1997). A few other, remote locality records were considered to be dubious (Dely, 1997), but in the past few years several new and isolated populations were discovered in possible historical connection with the existing ones (Korsós, Mara and Traser, 2002; Tóth, 2002; Bellaagh, Korsós and Szelényi, 2008). They are all located along the western (right) bank of the river Danube, at its middle section in Hungary (Paks, Dunaújváros, Dunaszekcső and Dunaföldvár; fig. 1). In the nature conservation legislation, the Caspian whipsnake has a strictly protected status in Hungary, and it is listed in Appendix 2 of the Berne Convention

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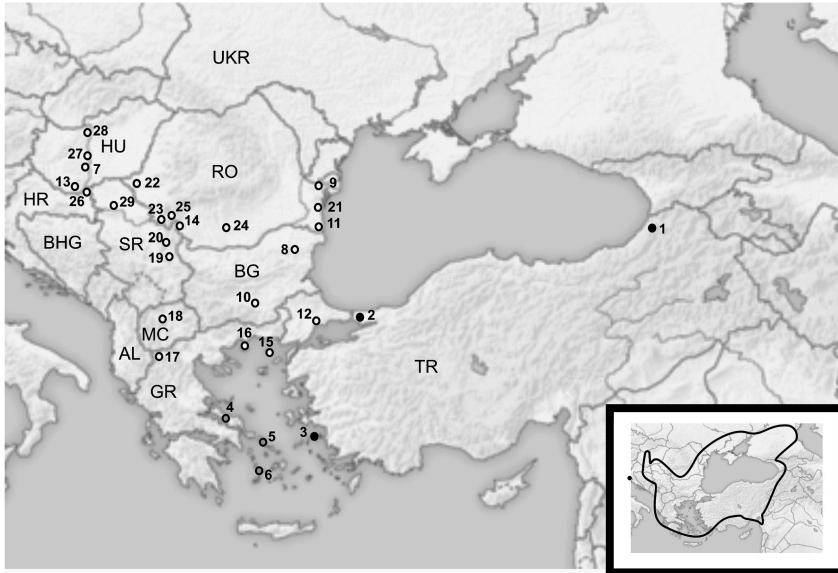


Figure 1. Localities of the investigated samples of *Dolichophis caspius*. Inset picture shows the approximate distribution range of the species.

of the Council of Europe. In Serbia, the Caspian whipsnake is protected by the Order on Protection of Natural Rarities of Serbia.

With regards to the taxonomical status of whipsnakes, several revisions have been made in recent years. Based on detailed morphological (mainly hemipenial characteristics) and molecular genetic analyses, the former snake genus *Coluber* Linnaeus, 1758 was split into several different genera (Schätti and Utiger, 2001; Nagy et al., 2004). These and other analyses (Nagy et al., 2003a, 2004), however, also showed that the genus *Hierophis* sensu Schätti and Utiger (2001) remained paraphyletic, as dwarf snakes (genus *Eirenis* Jan, 1863) were phylogenetically nested in this group. Following the recommendations by Nagy et al. (2003a, 2004), the most appropriate taxonomic solution is the subdivision of those species into three monophyletic genera: *Hierophis* Fitzinger, 1834, *Dolichophis* Gistel, 1868 and *Eirenis*. *Dolichophis* in that sense includes the species *D. jugularis* (Linnaeus, 1758), *D. caspius* (Gmelin, 1789), *D. schmidti* (Nikolskij, 1908) and *D. cypriensis* (Schätti,

1985) (Nagy et al., 2003a, 2004; Utiger and Schätti, 2004).

In contrast to the species-level taxonomy, surprisingly little is known about the intraspecific variation of these snakes investigated by molecular genetic methods. In this context, studying edge populations may play a crucial role in conservation genetic issues. In the current study, we present the results of a phylogeographic analysis of *Dolichophis caspius*, focusing on the westernmost populations.

Materials and methods

For this study, several southeastern European localities of the Caspian whipsnake were investigated (see fig. 1 and Appendix 1 for details). Blood samples collected from living specimens, tail tips or muscle tissues of dead animals (e.g., road-kills) were used for DNA analyses. These types of tissue samples were preserved in 95%-99% ethanol. Additionally, shed skins found in relatively fresh and dry condition were also successfully used as sources of DNA for PCR and sequencing. This material was stored dry with silica gel as desiccating agent or frozen. No specimens were killed for this study, and therefore no voucher specimens are deposited in collections.

A standard phenol-chloroform method was used for total genomic DNA extraction (modified after Sambrook, Fritsch and Maniatis, 1989). Alternatively, different DNA extraction kits of Qiagen were used. The complete cytochrome

b gene was amplified with primers L14910, L14919 and H16064 (Burbrink, Lawson and Slowinski, 2000; modified by de Queiroz, Lawson, Lemos-Espinal, 2002). PCR products were sequenced with L14903 (a 5' end of L14910), L-410, H-391 (Nagy et al., 2003b) on various automated capillary sequencers following the manufacturers' instructions.

DNA sequences were checked for their quality manually, and for their mitochondrial origin by translating them into amino acids. A median-joining haplotype network (Bandelt, Forster and Röhl, 1999) based on the cytochrome *b* sequences was calculated by Network 4.5.1 (www.fluxus-engineering.com).

Results

We obtained a data matrix of 44 specimens and DNA sequences of 1117 bp. We did not find any signal of contamination or sequences of nuclear genomic origin. In total 14 haplotypes were detected (fig. 2, Appendix 2). Clear mitochondrial divergence was found in Europe based on cytochrome *b* sequences (fig. 2), with an eastern (E1-E4) and a western (W1-W10) haplotype group, and by analysing a number of specimens and populations. The two main groups are separated by at least 14 substitution steps in their cytochrome *b* sequence (*p* distances between both groups range between 1.3%-1.9%), and physically by the Aegean Sea as well as probably by the Bosphorus. Localities along the Turkish

Black Sea coast, and those on East Aegean islands (Samos) are inhabited by specimens possessing the eastern haplotypes (and marked with black circles in figs 1-2). The western haplotype group includes distinctly different haplotypes from the Cyclades (Andros, Serifos; haplotype W2), from Euboea (haplotype W1; both localities are situated in the western Aegean Sea, and are marked by grey circles in figs 1-2), and from several, mostly mainland localities in southeastern Europe (from Greece to Hungary, to Turkey, and to Ukraine, marked by empty circles in figs 1-2), respectively. In the latter group, the majority of the screened samples are characterized by sharing the same single haplotype (W4 in fig. 2), and localities cover a wide geographic range from mainland Greece, Serbia, Croatia, Romania, Macedonia, Ukraine and Hungary (in the latter case, only some populations along the Danube river). Furthermore, we found some slightly divergent haplotypes (W3, W5-W10 in fig. 2) which are closely related to the previous one, but show a few (1-3) substitution steps. Focusing on Hungary, three different haplotypes were detected in close geographic proximity, but they were clearly linked to discrete localities; Paks (W3), Szársomlyó (W8; both populations possessing unique haplotypes which were

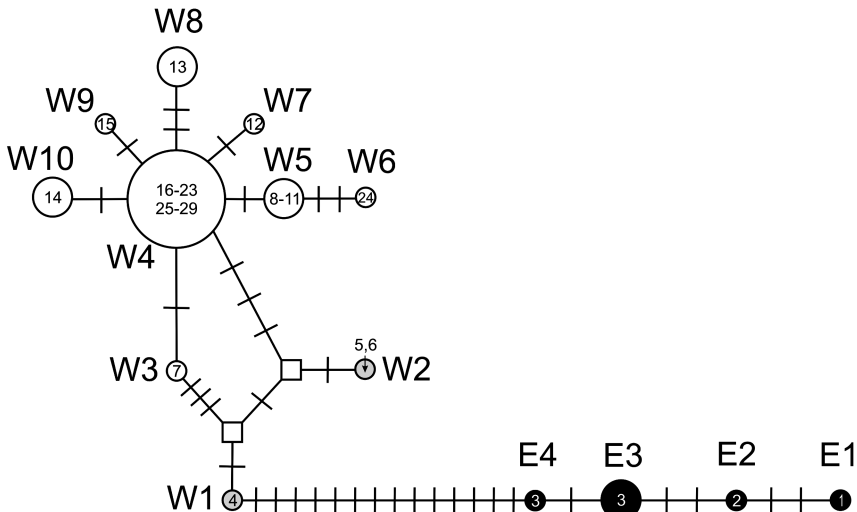


Figure 2. Haplotype network of *Dolichophis caspius* based on cytochrome *b* sequences. Eastern and western haplotypes are marked with E1-E4 and W1-W10, respectively. Numbers within circles represent localities as in fig. 1.

not found anywhere else) and the populations along the Danube river (W4) were separated by a few (1-3) mutational steps in their cytochrome *b* sequence.

Discussion

Phylogeography and colonization scenarios

The vast majority of the Central European amphibian and reptile species have probably gone locally extinct during the repetitive glacial cycles during the Pleistocene (Jánosy, 1986). During the warmer interglacial periods, however, several recolonization events have occurred from different refugia situated in southern Europe (Hewitt, 1999). On the other hand, different regions could have acted as refugia in subsequent glacial cycles. Based on our genetic results, the separation between these two snake lineages (east and west to the Aegean region) is probably much older than the western and northern dispersal of the western haplotype group. The Aegean area has undergone a complex geological history (e.g., Dermitzakis and Papanikolaou, 1981), since a formerly closed Aegean landmass begun to break up around 10-12 million years ago. Later on, in the Messinian, the Mediterranean Sea dried out (Hsü, Ryan and Cita, 1973), and migration of terrestrial animals became possible across the Aegean basin in the time frame of around 5.6-5.3 million years ago. At the end of this period, a potentially catastrophic flood refilled the Mediterranean basin (Garcia-Castellanos et al., 2009). In the Pleistocene, all Aegean islands reached their current positions, although significant sea level changes in glacial versus interglacials occurred. Anyway, the deep sea obviously acts as a barrier for these terrestrial snakes (at least our results based on the used mitochondrial marker argues for this). The separation of the main haplotype groups (E and W in fig. 2) is, however, probably younger than the period of the Messinian crisis, and may date back to Pleistocene only, as the estimated genetic diver-

gence between the groups is limited to about 1.3%-1.9% *p* distance (see, e.g., Carranza et al., 2000 for evolutionary rates, and Kasapidis et al., 2005; Poulakakis et al., 2005 for other reptile examples in this particular region). In comparison, a higher intraspecific divergence (around 3%) using the same gene was found in *Hierophis viridiflavus* (Nagy et al., 2002), another Mediterranean whipsnake species. These results for *H. viridiflavus* were confirmed by Rato et al. (2009) using another genetic marker(s). In order to draw any taxonomic conclusions (e.g., evaluation of intraspecific taxonomy which is questionable at present), additional genetic and morphological investigation of further specimens is necessary. Using nuclear genetic markers would assist to evaluate whether the isolation of the two haplotype groups is complete.

A rather low genetic divergence was observed within the western haplotype group with the notable exception of some Greek populations (Cyclades and Euboea). Caspian whipsnakes could have been survived in these regions during the glacials and persisted there since then. After the glacial period, the Caspian whipsnake colonized Central and southeastern Europe very likely out of the Balkan Peninsula (see also Hewitt, 1999), especially from the western Balkans. This species, similarly to many other snakes, has probably better dispersal capacities than small-sized reptiles, and therefore a relatively rapid colonization event seems to be likely which is reflected in the lower divergence values found. Towards the North, the species has reached Ukraine and in the Northwest Hungary representing the most extreme localities of this species in Europe (Schcherbak and Böhme, 1993).

It is surprising that Hungary – at the edge of the species' distribution range – is inhabited by populations with three different haplotypes. Moreover, all currently known localities in Hungary are situated on the right bank of the Danube River. The fact that no specimens or fossils of the species were found on the left bank so far may indicate that the species prob-

ably colonized the Carpathian Basin after the streambed of Danube has reached its current position, i.e., around 10 000 years ago (Karátson et al., 2006). It does not exclude the possibility, however, that “insular” populations could have been persisted under much favourable microclimatic conditions during glacials, and therefore they may be older than populations established by later (re)colonization events. In that context, the population of Paks (haplotype W3 in fig. 2) may represent a genetic link between the basal Greek populations (e.g., Euboea, W1) and the most common European haplotype (W4). On the other hand, the isolated South Hungarian calcareous hill Szársomlyó is characterized by Mediterranean-like climate conditions unlike other parts of Hungary, and might have been acted as ideal spot for retraction in moderately cold periods. However, to investigate this scenario in more detail, further genetic markers and a more dense sampling (whenever possible) may be necessary.

Alternatively, a paleoclimatic modelling of past distribution range would be useful.

Conservation implications in Hungary and Serbia

Hungarian localities of the Caspian whipsnake represent the northwestern boundary of the species' distribution. One of the main reasons of the fragmented distribution in this country may be the special habitat (and the corresponding ecological niche) preference of the species (Bellaagh, Báldi and Korsós, 2007) which is only present in a few geographic spots: that is a combination of warm microclimate, vertical orography, stony, rocky substrate or steepy hills covered by (semi-)open vegetation.

Moreover, populations of the Caspian whipsnake in Hungary are seriously threatened mainly by habitat loss as a result of various human activities (e.g., limestone quarries, construction works, agriculture, tourism), but also through predation by feral pets, such as dogs and cats (e.g., Tóth, 2002). Currently, only the population from Szársomlyó seems to be vi-

able, from all other localities only sporadic data and reports of whipsnakes exist (see Introduction). Unfortunately, only two of the five proved Hungarian localities are protected legally, and in general, neither regular controls nor monitoring activities are being carried out.

Populations of the Caspian whipsnake in Serbia are also seriously threatened mainly by habitat loss as a result of various human activities (for similar causes as in Hungary). According to our observations, Caspian whipsnake specimens usually share their hibernacula and other group sites, and these are sometimes in human inhabited areas: wells, mines etc. Unfortunately, because of human fear and prejudice, many specimens are killed in these places (see, e.g., news at www.ekoforum.org/index/vest.asp?vID=146), despite the fact that the species is protected by law in Serbia.

Therefore we highlight the need for appropriate and effective conservation measure in both countries to protect this peculiar snake species.

Acknowledgements. We appreciate the kind assistance of several colleagues who provided tissue samples for this project: S.-D. Covaciu-Marcov, M. Dimaki, G. Dzukić, B. Halpern, Y. Ioannidis, U. Joger, L. Krecsák, W. Meyer, G. Popgeorgiev, I. Sas, B. Trócsányi and J. Vörös. MB's visit to Heidelberg, and field trips to collect tissue samples were supported by the Hungarian National R&D Programme, title: The origin and genesis of the fauna of the Carpathian Basin: diversity, biogeographical hotspots and nature conservation significance; contract no: 3B023-04. ZK's work in in Brussels (in the Joint Experimental Molecular Unit at the Royal Belgian Institute of Natural Sciences) was supported by the European Commission's (FP 6) Integrated Infrastructure Initiative programme SYNTHESYS (BE-TAF 3927). We also thank both referees for their valuable comments.

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Received: January 11, 2010. Accepted: April 14, 2010.

Appendix 1: List of localities

Abbreviations: BG: Bulgaria; GR: Greece; HR: Croatia; HU: Hungary; MC: Macedonia; RO: Romania; SR: Serbia; TR: Turkey.

1: Borcka, TR; 2: Sile, TR; 3: Samos, GR; 4: Euboea, GR; 5: Andros, GR; 6: Serifos, GR; 7: Paks, HU; 8: Shumen, BG; 9: Tulcea, RO; 10: Kolec, Chaskowo, BG; 11:

Hagiieni, Jud. Constanta, RO; 12: Tekirdag, TR; 13: Szársomlyó, HU; 14: Vajuga Milutinovac, SR and Portile de Fier II, Mehedinți, RO; 15: Samothraki, GR; 16: Thassos, GR; 17: Prespes, GR; 18: Veles, MC; 19: Brest. Banja, SR; 20: Zlot, SR; 21: Histria, RO; 22: Buzias, RO; 23: Eselnita, RO; 24: Draganesti, Jud. Olt, RO; 25: Toplet, RO; 26: Batina, HR; 27: Dunaújváros and Dunaföldvár, HU; 28: Budapest, Sas-hegy, HU; 29: Podcenta, SR.

For one sample collected in Turkey (showing haplotype E2) and two samples from Ukraine (both haplotype W4) no closer locality data are known.

Appendix 2: GenBank accession numbers of the observed haplotypes

E1: HM210776; E2: HM210777; E3: HM210778; E4: HM210779; W1: HM210780; W2: AY376739; W3: HM210781; W4: HM210782; W5: HM210783; W6: HM210784; W7: HM210785; W8: HM210786; W9: HM210787; W10: HM210788.