

Phylogenetic relationships of the Italian populations of Horseshoe Whip Snake *Hemorrhois hippocrepis* (Serpentes, Colubridae)

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Abstract. *Hemorrhois hippocrepis* is a colubrid snake with a West Mediterranean distribution. It is widespread in the Iberian Peninsula and Northwest Africa. The only Italian populations are found on the islands of Sardinia and Pantelleria. The phylogenetic relationships of these insular populations have been analysed for the first time on the basis of the mitochondrial DNA cytochrome *b* gene. The sequences were compared with those available from the geographic range of this species. The analyses showed that the Italian samples are part of a lineage that groups Tunisian and East Algerian samples, with which they share the same haplotype. These results strongly support the hypothesis of a recent origin of the Italian populations of *Hemorrhois hippocrepis*, probably determined by human-mediated dispersal from North Africa.

Keywords. Horseshoe Whip Snake, phylogeny, colubridae, cytochrome *b*.

The Horseshoe Whip Snake, *Hemorrhois hippocrepis* Linnaeus, 1758, is a medium to large size colubrid with a Western Mediterranean distribution. It is widespread across the eastern, central and southern Iberian Peninsula, and in the northern mesic parts of Morocco, Algeria, and Tunisia. In Italy, its presence is limited to the islands of Sardinia and Pantelleria (Sindaco et al., 2013).

The first reports of *H. hippocrepis* in Sardinia are found in Gené (1834, 1839) and refer to the southwestern part of the island. Later, Mertens and Wermuth (1960) considered the species extinct, but subsequently, Bruno and Hotz (1976) rediscovered it in a territory between Cagliari and Oristano, and its presence there

was also confirmed by Puddu et al. (1988) and Bruno and Maugeri, (1990). *Hemorrhois hippocrepis* in the San Pietro satellite islet was reported by Stefani (1971) and Poggesi et al. (1995) and is still mentioned in recent references (Corti et al., 2006; Di Nicola and Mezzadri, 2018), although its presence is doubtful given the absence of corroborating observations (Corti et al., 2000). More recently, *H. hippocrepis* appears to be present with certainty only a few localities of the Cagliari Province (Corti et al., 2000; Zuffi, 2006; Di Nicola and Mezzadri, 2018; Di Nicola et al., 2019).

The Horseshoe Whip Snake has been known to inhabit the island of Pantelleria (Sicily) since the late

1800s (Doderlein, 1881; Camerano, 1891; Minà Palumbo, 1893) and it is widespread throughout the island (Cattaneo, 1985; authors' unpublished data).

The population of Pantelleria has been attributed on a morphological basis to the endemic subspecies *H. h. nigrescens* (Cattaneo, 1985), due to the melanotic color morph of adults, the large dimensions of both hatchlings and adults, and to certain distinctive pholidotic features. Later, Corti et al. (2000), in comparing various populations of *H. hippocrepis*, found no substantial morphological differences between the populations of Pantelleria, Sardinia and Tunisia. According to some authors, the Italian populations of Horseshoe Whip Snake may have been introduced by man in historical times (Bruno and Hotz, 1976; Zuffi, 2006; Cattaneo, 2015). Therefore, the ssp. *nigrescens* is currently considered doubtful, and *H. hippocrepis* is widely reported as a monotypic species (Venchi and Sindaco, 2006; Luiselli et al., 2011; Di Nicola, 2019; Di Nicola et al., 2019), as indicated by the genetic studies of Carranza et al. (2006), which however did not include samples from Italian populations.

In this work, we have analyzed for the first time the Italian populations of *H. hippocrepis* from a genetic point of view, with the aim of clarifying their phylogenetic relationships and testing if their origin can be related to human mediated dispersal as we expect.

In order to infer the phylogenetic relationships between the Italian samples and those of the other populations, we analysed the mitochondrial DNA cytochrome *b* (cyt *b*) sequence, a fragment often used for snakes in this type of analysis (Carranza et al., 2004, 2006; Faraone et al., 2020). Tissue samples ranging from 2 to 20 mg were collected from six specimens originating from Pantelleria and one from Sardinia, and stored in ethanol until processing. All snakes were found as roadkills between 2013 and 2019. DNA was extracted as described in Tagliavia et al. (2016). 3 ml of crude lysate were used as template for PCR amplification. Reactions were carried out in 30 µl in presence of 200 mM dNTPs and 0.15 mM primers CB1F and CB2R (respectively: 5'-CCATC-CAACATCTCAGCATGATGAAA-3' and 5'-CCCTCA-GAATGATATTTGTCTCA-3') (Korcher et al., 1989). The PCR conditions were 94 °C for 3 min, followed by 40 cycles at 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min and by 5 min of final extension at 72 °C. PCR products were analysed by electrophoresis onto 1% agarose gel containing 0.5 µg/ml ethidium bromide and sequenced with primer CB1F (BMR Genomics).

Nucleotide sequences, each of about 320 nucleotides, were analysed and manually proofread with the software Chromas v. 2.6.6 (Technelysium Pty. Ltd. 1998, Queensland, Australia). Coding gene fragments of cyt *b*

were translated into amino acids to assess the lack of stop codons. Cyt *b* sequences of *H. hippocrepis* were searched and downloaded from GenBank (Nagy et al., 2004; Carranza et al., 2006; Beddek et al., 2018) from the native geographic range of the species (Figs 1 and 2). Additionally, cyt *b* sequences of *Lytorhynchus diadema*, *Hierophis viridiflavus*, *Hemorrhhois algirus*, *Coronella girondica*, *Psammophis shokari*, *Rhagerhis moilensis* and *Malpolon monspessulanus* were downloaded and used as outgroup. Nucleotide sequences were aligned using ClustalW with default parameters.

Phylogenetic analysis was performed with the Maximum Likelihood (ML) method and the Akaike Information Criterion, using "Smart Model Selection" (SMS) (Lefort et al., 2017), implemented in PhyML v. 3 (Guindon et al., 2010). Support for nodes was estimated using bootstrapping (Felsenstein, 1985) with 1000 replicates. The most appropriate evolutionary model was the GTR+G+I model (-Log likelihood value -1397.47), with a 0.30 estimate of invariable sites and a 0.819 discrete approximation of the gamma distribution.

Including the outgroups, 67 sequences of 294bp total length were analysed. All the Italian samples showed the same haplotype (GenBank accession numbers MT498647-MT498653), which is also shared with all the Tunisian and the easternmost Algerian samples.

The results obtained confirm the same overall *H. hippocrepis* phylogenetic structure detected by previous studies on mitochondrial sequences (Carranza et al., 2006; Beddek et al., 2018), i.e., the existence of a western clade (subclade A) that includes the Iberian Peninsula, Morocco, western Algeria and non-native populations of the Balearic islands (see Silva-Rocha et al., 2015), and an eastern clade (subclade B) that includes eastern Algeria and Tunisia. The western clade has greater haplotype diversity while the eastern clade is characterized by a single haplotype, shared by all the Italian samples. The phylogenetic structure obtained also reflects the morphological variation within this species. In fact, the eastern populations (Sardinia, Pantelleria and Tunisia) differ from the western ones (Spain, Portugal and Morocco) in that they have a greater number of ventral scales (Cattaneo, 1985; Corti et al., 2000).

The sharing of the same cyt *b* haplotype by the Tunisian and Italian populations of *H. hippocrepis* strongly supports a recent origin of the latter. This was probably determined by human-mediated fauna translocation, as previously speculated (Bruno and Hotz, 1976; Zuffi, 2006; Cattaneo, 2015) and suggested by the biogeographical and historical features of the two islands. Although Sardinian fossil ophidiofauna is well represented and several findings of 'colubrinae' are known, there are no

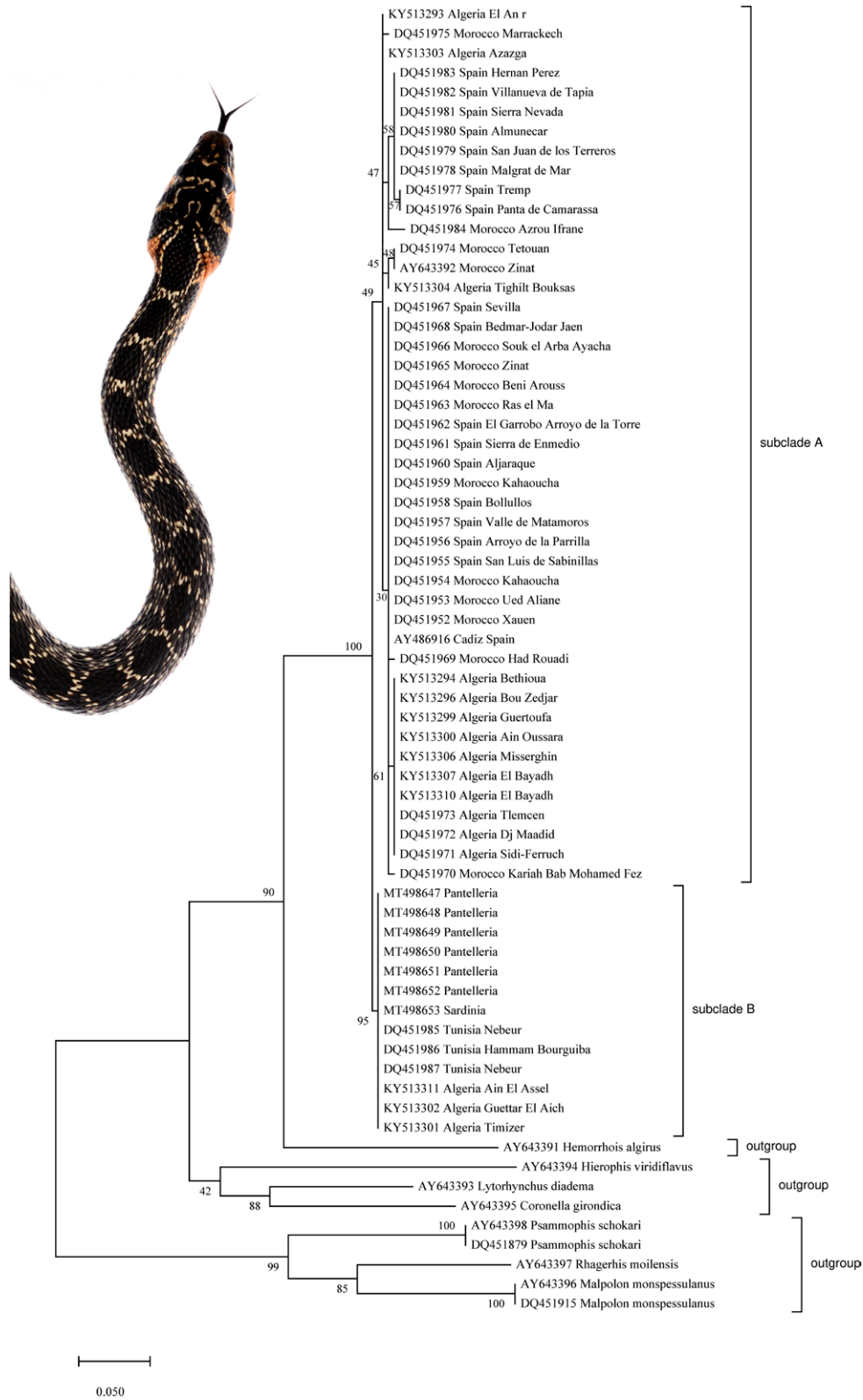


Fig. 1. Maximum Likelihood (ML) tree of *Hemorrois hippocrepis* inferred from the mitochondrial cytochrome *b* gene. The numbers at nodes are ML bootstrap values. Except for the samples from Pantelleria and Sardinia, the remaining *cyt b* GenBank accession numbers are from Nagy et al. (2004), Carranza et al. (2004, 2006) and Beddek et al. (2018).

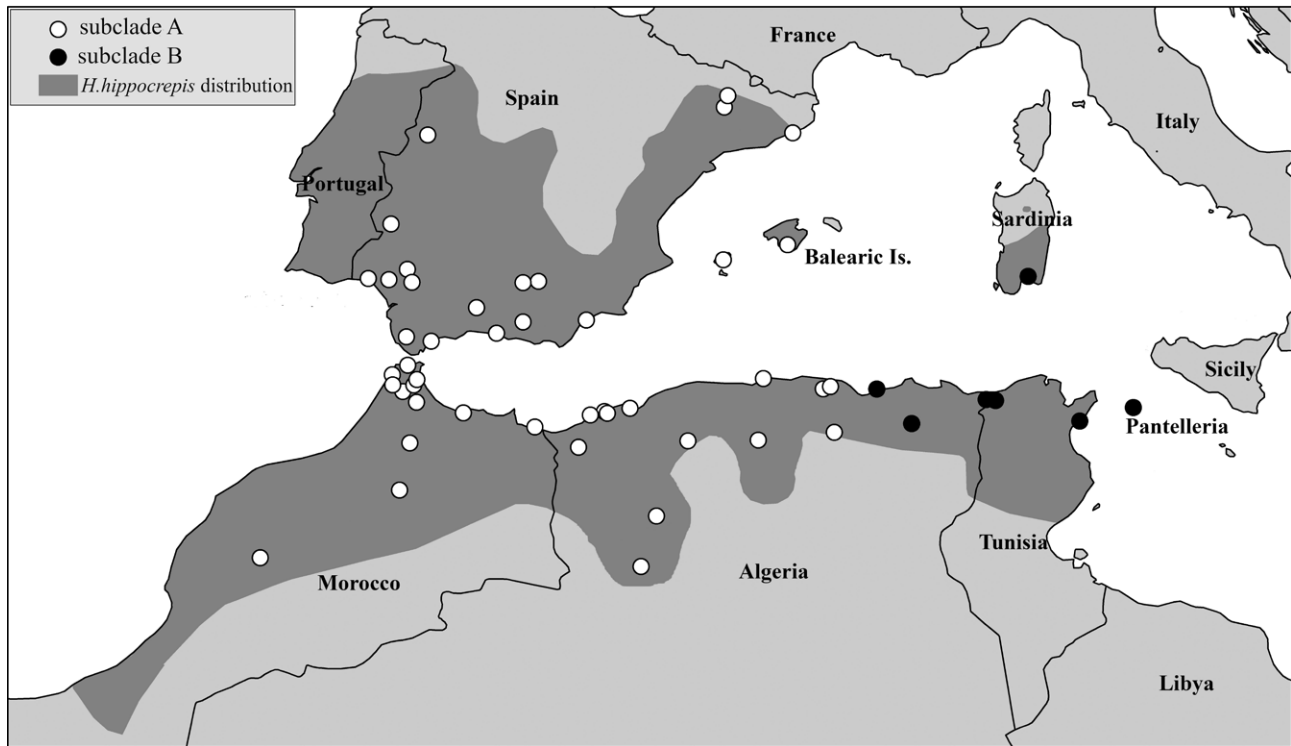


Fig. 2. Geographic range of *Hemorrhhois hippocrepsis* and distribution of all the samples included in the analysis, distinguished on the basis of the western and eastern lineages, respectively white and black dots.

remains clearly attributable to *H. hippocrepsis* (Kotsakis, 1980; Abbazzi et al., 2004; Georgalis et al., 2019). While the only remains of terrestrial vertebrates found in Pantelleria, belong to undetermined birds and Artiodactyla mammals dating back to the Bronze Age and linked to human farming and hunting activities (Wilkins, 1987). Furthermore, *H. hippocrepsis* is not listed in the overall fossil register of the Italian reptiles (Delfino, 2006, 2011). Several current reptile species found in Sardinia share close genetic affinities with Tunisian populations, and this suggests they are of recent origin. The origin of the Sardinian populations of *Natrix maura* are not completely clear, due to its slight variation compared to the Tunisian one (Guicking et al., 2008). For other species like *Tes-tudo graeca*, *Chalcides ocellatus*, and *Chalcides chalcides*, human-mediated dispersal appears to be the most probable process involved (Carranza et al., 2008; Fritz et al., 2009; Kornilios et al., 2010). As for Pantelleria, although some studies support ancient Pleistocene contacts with the Tunisian mainland (Pasa, 1953; Bordoni, 1973), and an active colonization of some species has been hypothesized (Lanza, 1973; Stöck et al., 2016), the onset of catastrophic eruptive events dating back to about 45,000 years ago probably caused the extinction of most of the native fauna (Agnesi and Federico, 1995; Massa, 1995;

Muscarella and Baragona, 2017). This suggests a subsequent origin of the reptile community through recent passive dispersion mechanisms (Cattaneo, 2015).

Wide cultural and commercial connections between Tunisia, Pantelleria and Sardinia throughout history, especially around the 6th-3rd century B.C.E. under the influence of the Carthaginians (Bechtold, 2013), may have played a role in the human-mediated exchanges of reptile fauna across the Mediterranean basin (Bruno, 1985; Masseti and Zuffi, 2011). It may be in this context that the Italian populations of *H. hippocrepsis* originated.

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