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Who are you? The genetic identity of some insular populations of *Hierophis viridiflavus* s.l. from the Tyrrhenian Sea

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Abstract. This work investigates the genetic identity of *Hierophis viridiflavus* s.l. specimens from insular populations, to determine which of the two previously identified species is present on each island. Here, the authors hypothesise about times and modes of colonization and discuss the faunistic value of the obtained results. This follows the recent proposal to consider the two clades as two different species. Specimens from the islands of Favignana, Lipari and Vulcano belong to *H. carbonarius* and probably all belong to putative Sicilian source populations. Conversely, all individuals from the Pontine Islands (Ponza, Palmarola, Ventotene) should be considered to belong to *H. viridiflavus*. Even if genetically identical to the specimens from the Tyrrhenian Italian coast, these individuals show a darker colouration, very similar to the one usually shown by *H. carbonarius* specimens. Considering that the Pontine *H. viridiflavus* populations probably have a very recent origin, the dark livery of these individuals could be the result of a rapid morphological adaptation to insular environments.

Keywords. Colour pattern, *Hierophis viridiflavus*, islands, nd4, phylogeography.

The western whip snake *Hierophis viridiflavus* s.l. (Lacépède, 1789) is a colubrid snake with a wide distribution range. It can be found in Central Europe from Eastern Spain to Central France, Luxemburg, Switzerland, Slovenia and Croatia. Its range also includes all of the Italian Peninsula, Sicily, Sardinia and most of the smaller Italian islands, Corsica and some Croatian islands (Vanni and Nistri, 2006; Zuffi, 2007). The species is found from sea level to 1500-1800 m a.s.l., although it is extremely rare above 1500 m in the Alps (Farinello and Bonato, 2000). Interestingly, individuals of this species show two main phenotypes, one named "*viridiflavus*" (usually brown/blackish with yellow stripes and spots) and the other named "*carbonarius*", typically melanic (completely black with blackish/grey ventral colouration) or melanotic (almost completely black livery, but paler/yellowish head scales and ventral surface). Individuals from some of the islands of the Tuscan Archipelago, Sardinia and Corsica show a third phenotype, a middle ground between "*carbonarius*" and "*viridiflavus*", called "*abundistic*" (Zuffi, 2008). In the past, four *H. viridiflavus* s.l. subspecies have been described for Italy, mainly due to the chromatic pattern of the analysed individuals: *H. v. viridiflavus* (Rimpp, 1979), *H. v. carbonarius* (Bonaparte, 1833), *H. v. kratzeri* (Kramer, 1971) and *H. v. antoniimanueli* (Capolongo, 1984). However, based on mitochondrial and nuclear DNA evidences, they have all been recently rejected (Vanni and Zuffi, 2011). Indeed, Nagy et al. (2002) and Rato et al. (2009) showed the presence of two mitochondrial distinct haplogroups: the first, roughly corresponding to the subspecies *H. v. viridiflavus* (clade W) includes individuals from Spain, France, Corsica, Sardinia and central to north-western Italy on the West side of the Apennines; the second, in part matching with the subspecies *H. v. carbonarius* (clade E), occurs on the other side of the Apennines, from north-eastern

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Italy to southern Italy (including Sicily). More recently, a study based on morphometric, genetic and karyological data, proposed the elevation of the two genetic groups to species status (Mezzasalma et al., 2015). In particular, the authors emphasized significant differences in sexual chromosomes, as while females from the Eastern group have a submetacentric W sex chromosome, females from the Western group have a telocentric W sex chromosome. Thus, individuals from the Eastern clade have been recognized as *Hierophis carbonarius*, while individuals from the Western clade have been recognized as *Hierophis viridiflavus*. Interestingly, the relationship between colour variation and genetic repartition does not match completely (Zuffi, 2008). The brown/blackish colouration with yellow stripes and spots pattern, generally almost exclusive of the *H. viridiflavus* range, can be found also in *H. carbonarius* specimens (Rato et al., 2009). Although the distribution of the two species in the Italian Peninsula and on the largest Mediterranean islands (Sardinia, Corsica and Sicily) has already been studied (Nagy et al., 2002; Rato et al., 2009; Mezzasalma et al., 2015), there is a lack of molecular data from smaller Italian archipelagos. The aim of the present work is to determine the genetic identity of individuals collected in some Tyrrhenian islands including: Ponza, Palmarola and Ventotene from the Pontine Archipelago; Vulcano and Lipari from the Aeolian Islands; and Favignana from the Aegadian Islands, in order to update the distribution of the two species of whip snakes.

We sampled a total of seven individuals of *H. viridiflavus* s.l. from six different islands between March 2014 and July 2015 (geographic locations are reported in Table 1 and showed in Fig. 1). Snakes were caught and handled following standard protocols (Fowler, 1978), and some ventral scales were removed and preserved in pure ethanol. In one case (RS296 from Lipari), the tissue was obtained from a shedded skin.

Genomic DNA was extracted following the protocol described in Aljanabi and Martinez (1997). A fragment including the terminal portion of the NADH dehydrogenase subunit 4 (*nd4*) was amplified by standard PCR protocols using primers published by Arèvalo et al. (1994). Amplification conditions were the same as described by Pinho et al. (2006). The PCR products were purified with a Sure Clean (Bioline©) purification kit and the sequencing reactions were run under Big-Dye TM Terminator cycling conditions by a commercial company, Macrogen (www.macrogen.com). The electropherograms were checked using the software FinchTV (http://www.geospiza.com/finchtv/) to ensure the absence of double peaks and ambiguous positions. The obtained sequences were deposited to GenBank (accession numbers: KY923281-KY923287) and joined with additional 91 *nd4* sequences of *Hierophis viridiflavus* s.l. retrieved from GenBank (accession numbers: FJ430621-FJ430660, Rato et al., 2009; LN552045-LN552095, Mezzasalma et al., 2015). Nucleotide sequences were translated into amino acids with MEGA 6.0 (Tamura et al., 2013) using the vertebrate mitochondrial genetic code in order to assess the absence of pseudogenes.

One *nd4* sequence of *Hierophis gemonensis* (Laurenti, 1768) was downloaded from GenBank (accession number: AY487044, Nagy et al., 2004) and included in the analysis as outgroup, as it is considered the closest related species to *H. viridiflavus* (Schätti, 1988).

The software jModelTest (Posada, 2008) was used to determine the most appropriate model of sequence evolution for the *nd4* dataset. According to the Akaike information criterion (AIC), the most supported evolutionary model was the TrN + I, therefore applied in the subsequent analysis. To reconstruct phylogenetic relationships, we used a coalescent Bayesian approach as implemented in MrBayes 3.2.6 (Ronquist et al., 2012). We run 2 million generations, with 4 Markov chains sampling every 1000 steps. After a burn-in of 10%, the remaining trees were used to compute a 50% majority rule consensus tree.

In addition, a statistical parsimony network under 95% probability connection limits was constructed using TCS 1.21 (Clement et al., 2000). Number of haplotypes, nucleotide diversity (π) and haplotype diversity (H) were

Table 1. individuals analysed including sampling location, group of islands, colour pattern, haplotype number and haplogroup.

Sample code	Locality	Archipelago	Colour pattern	Haplotype	Species
RL22	Ponza	Pontine	*"abundistic"*	H1	*H. viridiflavus*
RL66	Ponza	Pontine	*"abundistic"*	H1	*H. viridiflavus*
RL79	Ventotene	Pontine	*"abundistic"*	H1	*H. viridiflavus*
RL80	Palmarola	Pontine	*"abundistic"*	H1	*H. viridiflavus*
RS85	Favignana	Aegadian	*"carbonarius"*	H10	*H. carbonarius*
RS276	Vulcano	Aeolian	*"carbonarius"*	H9	*H. carbonarius*
RS296	Lipari	Aeolian	*"carbonarius"*	H9	*H. carbonarius*

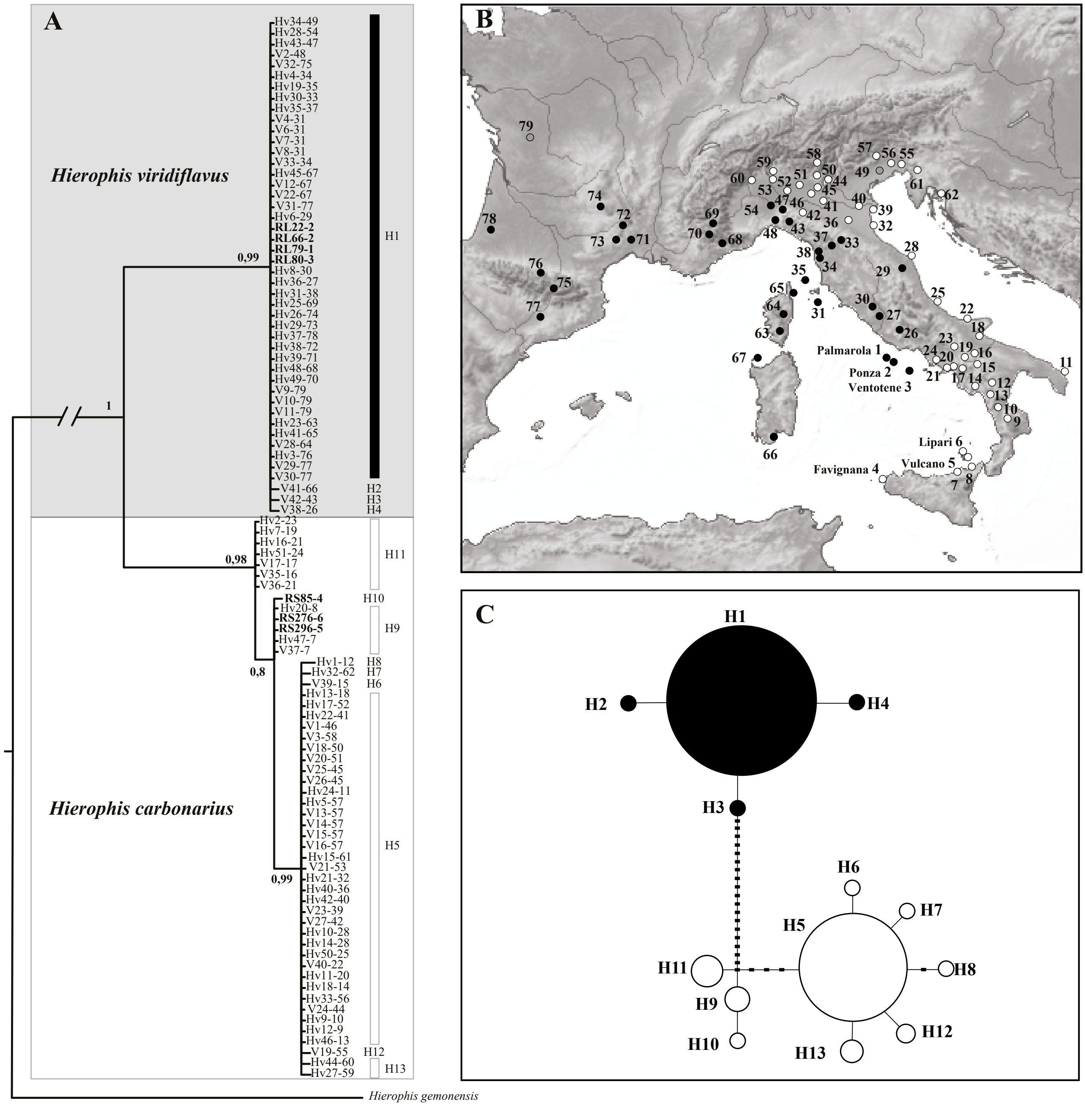

Fig. 1. Bayesian phylogenetic tree (A) based on *nd4* sequences for 98 ingroup specimens of *H. viridiflavus* s.l. and one outgroup (*H. gemonensis*). The posterior probabilities are indicated at each node. Each label indicates the specimen code, the locality number and the relative haplotype. Insular individuals are shown in bold. Geographic distribution (B) of the two mitochondrial lineages corresponding to *H. viridiflavus* (blue) and *H. carbonarius* (red). Statistical parsimony network (C) connecting haplotypes. The circle size is proportional to the sequence frequencies and each filled rectangle represent one substitution.

also calculated for each group using DnaSP 5.1 (Librado and Rozas, 2009).

The final *nd4* alignment (568 bp) of 99 sequences returned 67 polymorphic sites and 13 haplotypes. The phylogenetic analysis confirmed the presence of two well defined mitochondrial clades (Fig. 1A), as already stated in previous works and corresponding to the two species *H. viridiflavus* and *H. carbonarius* (Rato et al., 2009;

Mezzasalma et al., 2015). Nei's standard genetic distance between the two species was 4.2%.

Specimens from Favignana, Vulcano and Lipari, belong to the species *H. carbonarius* (Fig. 1A). This clade showed the presence of nine haplotypes (Fig. 1C, see Table A1 in supplementary materials for all the haplotype references) with H = 0.623 ± 0.071, and $\pi = 0.00389 \pm 0.00057$ (mean ± SD). The specimens from Vulcano (RS276) and Lipari (RS296) shared the same haplotype (H9) with individuals from localities seven and eight (Fig. 1B), corresponding to Iria and Lago Spartà (Sicily). These results may suggest a recent colonization, either human-mediated or by oversea dispersal, from Sicily to the Aeolian islands. On the other hand, the specimen from the island of Favignana (RS85) showed a new private haplotype (H10), separated by one mutational step from haplotype H9. In this case, the single fixed substitution may have occurred on the island through a vicariant mechanism. Indeed, during the last glacial phase this island was connected to Sicily and become separated following the Last Glacial Maximum because of the sea level drop. A similar scenario has also been suggested to explain the genetic differentiation observed in other reptiles from Favignana (Mizan, 2015; Senczuk et al., 2017). However, due to the small sample size from Sicily, we cannot completely rule out that insular distinctiveness may have derived from a recent dispersal process of a haplotype not yet sampled in Sicily.

All the individuals from the Pontine Islands (RL22, RL66, RL79, RL80) shared one single haplotype (H1) and should therefore be recognized as belonging to *H. viridiflavus* (Fig. 1A). This clade is genetically less differentiated than *H. carbonarius* clade (H = 0.128 ± 0.067; $\pi = 0.00023 \pm 0.00012$; mean ± SD) and is composed by four haplotypes: a single highly represented haplotype (H1; with an allele frequency of 94%) and three derived and extremely localized haplotypes (H2, H3 and H4). This result may suggest an anthropic introduction in modern times or a recent colonization of the Pontine Islands from the Tyrrhenian coast of the Italian Peninsula.

Interestingly, the four specimens from the Pontine Islands showed a colour pattern which resemble the "*abundistic*" morph, which is in the middle between the "*carbonarius*" (melanic/melanotic) and the "*viridiflavus*" (black and yellowish) colour patterns. The "*abundistic*" phenotype was previously reported only in Sardinia, Corsica and the Tuscan Archipelago. However, Schätti and Vanni (1986) reported similarities between specimens from the Pontine Islands and the dark coloured ones from Emilia Romagna now considered belonging to *H. carbonarius*. In particular, the individual sampled from Ventotene (RL79, Fig. 2) had a very dark dorsal with a yellowish ventral colouration, showing a phenotype which could easily be mistaken with the one observed in many populations of *H. carbonarius*. This observation confirms that colour pattern alone cannot help identifying the species to which a specimen belongs.

Fig. 2. The specimen from Ventotene island (RL79). The individual was found stuck in a mist net trap and later released.

The four *H. viridiflavus* specimens from the Pontine Islands are genetically indistinguishable from the usually brown-yellowish western whip snakes located on the Tyrrhenian coast of Italy, but show a darker phenotype. It has been reported in previous works that colour variation in reptiles can be associated to adaptive processes (Norris and Lowe, 1964; Rosenblum et al., 2004). For example, darker or melanotic colouration may give a benefit in terms of thermoregulation (Trullas et al., 2007; Broennimann et al., 2014) and reproduction (Capula and Luiselli, 1994), and similar conclusion had been already drawn by Rato et al. (2009) and Zuffi (2007), as they consider the colour types in *Hierophis viridiflavus* s.l. a by-product of different environmental conditions. Therefore, the dark colouration of the snakes from the Pontine Islands could be the result of adaptive morphological evolution which occurred in a very short time, a phenomenon already observed in other insular reptile populations (Losos et al., 1997; Herrel et al., 2008). Finally, despite changes in colour polymorphism might also be the outcome of non-adaptive processes (King, 1988; Lorioux et al., 2008), the independent recurrence of the "abundistic" chromatism in all the northern Tyrrhenian Islands suggests a prominent role of adaptive forces acting in similar insular environmental conditions, which would deserve further studies.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found at <http://www.unipv.it/webshi/appendix> Manuscript number 20283.

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