Short Notes

Phylogeography of the European Whip Snake, *Hierophis viridiflavus* (Colubridae), using mtDNA and nuclear DNA sequences

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Abstract. The phylogeography of the colubrid snake *Hierophis viridiflavus* was estimated using ND4 mtDNA sequences and a fragment of the nuclear marker β-fibrinogen intron 7. This species has a wide distribution across the Mediterranean region, and is characterized by three distinct colour patterns ("viridiflavus", abundistic, and melanic-melanotic) with a quite well defined geographic distribution. The "viridiflavus" pattern occurs in the northwestern and central areas of the species' range, the abundistic pattern is typical of the individuals from Corsica and Sardinia, and the melanic-melanotic coloration is present in northeastern and southern Italy, and Sicily. A total of 42 individuals from most of the species' range (including Pyrenees, central France, Italian Peninsula, Corsica, Sardinia and Sicily) were analyzed. Results support the existence of two different mtDNA lineages, one from Spain, France, Corsica, Sardinia, and continental Italy mainly west of the Apennines, and another one occurring in the southern part of Italy and northeast of the Apennines, with a 4% genetic divergence between them. Since both mtDNA lineages are found in northwestern Italy, where the "viridiflavus" colour pattern is almost exclusively found, it is clear that colour pattern is not linked to the mtDNA clades. The same is evident for β-fibrinogen, which is not subdivided geographically in the way that the mtDNA lineages are. Regarding Corsica and Sardinia, the limited genetic differentiation of island populations from the western continental lineage, indicates that these populations are a result of a recent colonization in accordance with recent described phylogeographic patterns.

Keywords: β-fibrinogen intron 7, colour pattern, glacial refugia, *Hierophis viridiflavus*, ND4, phylogeography.

The European Whip Snake, *Hierophis viridiflavus* (Lacépède, 1789) is a Mediterranean colubrid snake, ranging from the Pyrenees to Istria including: central and southern France, southern Switzerland, Italy (Sardinia, Sicily and most of the Italian islands included), Malta, Pelagosa (Vanni and Nistri, 2006; Zuffi, 2007) and other Croatian islands, southwestern Slovenia, and one introduced population in Gyaros island, Cyclades, Greece (Utiger and Schätti, 2004). This snake is characterized by different colour patterns ranging from the so-called "viridiflavus" bottle green and yellow pattern, to a darker fully melanic one (Zuffi, 2008). These colour morphotypes are distributed in clearly defined areas, with the "viridiflavus" pattern mostly in the northwestern and central parts of its range, while the abundistic (dark) pattern is especially found in Corsica and Sardinia; and the black one in northeastern and southern Italy, and Sicily (Zuffi, 2008).

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Hierophis viridiflavus has undergone many taxonomical revisions in the past; Boulenger (1913) grouped the species with *Hierophis gemonensis* (Laurenti, 1768) and considered all the morphotypes as mere colour varieties while, later Mertens and Müller (1928, 1940) and Mertens and Wermuth (1960) separated *H. gemonensis* from *H. viridiflavus*, and recognised for the latter form two subspecies *viridiflavus* and *carbonarius.*

For the last 20 years *Hierophis viridiflavus* has been consistently considered monotypic in most of its distribution range (Schätti and Vanni, 1986), while recent preliminary data suggested that the Sardinian, southern Italian and Sicilian populations belong to different subspecies (Scali et al., 2003), reviving the scenario proposed by Mertens and Müller (1928, 1940).

According to a phylogeographic study performed by Nagy et al. (2003), two different genetic groups were identified within *H. viridiflavus*, a western one occurring in France, Switzerland and Italy west of the Apennines, and an eastern one found in Croatia, eastern and southern Italy. With the present study, we attempt to resolve the phylogeography of *H. viridiflavus* more precisely by sampling additional areas not available in previous studies, such as around Salerno (Campania, southern Italy), Corsica and Sardinia, and to determine if there is any correspondence between the phylogeographic history and the existing colour patterns, particularly the forms proposed by Mertens and Müller (1928, 1940), using mtDNA and nuclear markers.

Genomic DNA was extracted from 42 individuals of *H. viridiflavus* (geographic localities of the tissue samples are given in table 1 and fig. 1) following standard high-salt protocols (Sambrook et al., 1989). A fragment including the terminal portion of the ND4 gene and the tRNAs for Serine, Histamine and Leucine was amplified by PCR using the primers published by Arévalo et al. (1994). Amplification conditions were the same as described by Pinho et al. (2006). To amplify the seventh intron from the β-fibrinogen gene (7IβFIB), we used the primers and thermal cycling parameters published by Creer et al. (2006).

Sequences from both strands were obtained on an automated sequencer (ABI 310) and were submitted to GenBank (accession numbers from FJ430603 to FJ430660). One ND4 sequence of *Hierophis gemonensis* and *Hierophis caspius* (Gmelin, 1789) were downloaded from GenBank (AY487044 and AY487039, respectively) and included in the analyses as outgroups, since they are considered as the closest related species to *H. viridiflavus* (Schätti, 1988).

For this fragment of β fibrinogen some individuals were heterozygous. Haplotype reconstruction was performed using PHASE version 2.1 (Stephens et al., 2001; Stephens and Donnelly, 2003). All sequences were exported to Bioedit v.5.0.9. (Hall, 1999) and aligned manually. For the β-fibrinogen dataset, since levels of variation were low a haplotype network was constructed with the software Network 4.5.0.1 using the median joining algorithm (Bandelt et al., 1999) with the parameter epsilon set to 0. Before importing the data into Network, this was transformed in a Roehl data file using the software DnaSP 4.50 (Rozas et al., 2003), without considering the gaps/missing data and removing the invariable sites.

The ND4 and adjacent tRNA's sequences were imported into PAUP* 4.0b10 (Swofford, 2003). Maximum Parsimony (MP) analysis was performed using heuristic searches involving tree bisection and reconnection (TBR) branch swapping. Robustness of these trees was assessed by bootstrap analysis (Felsenstein, 1985) involving 1000 pseudo-replications. The model of evolution that best fits the data was estimated with GARLI 0.95 (Zwickl, 2006) during Maximum Likelihood (ML) analysis. Multiple searches were conducted, each resulting in a single best tree. The resulting likelihood values were compared, selecting from among these the tree with the highest likelihood score. Bootstrap support was calculated from 1000 bootstrap replicates.

For Bayesian analysis, the model of sequence evolution was calculated using ModelTest v.3.7 (Posada and Crandall, 1998) under the Akaike Information Criterion (following Posada and Buckley, 2004), and the phylogenetic analysis was performed using MrBayes v.3.0 (Huelsenbeck and Ronquist, 2001). Parameters were estimated as part of the analysis with four Markov chains incrementally heated with the default heating values. All analyses started with randomly generated trees and ran for 2 million generations, saving one tree in each 100 generations. The log-likelihood values of the sample point were plotted against the generation time and all the trees prior to reaching stationarity were discarded, ensuring that burn-in samples were not retained. Combining the remaining trees, a 50% majority consensus tree was generated. The frequency of any particular clade of the consensus tree represents the posterior probability of that clade (Huelsenbeck and Ronquist, 2001). Two independent replicates were conducted and inspected for consistency to check for local optima (Huelsenbeck and Bollback, 2001).

All basic sequence statistics and uncorrected p-distances were calculated using the software Mega version 4 (Tamura et al., 2007).

Considering the ND4 gene fragments, a total of 42 individuals were analysed resulting in aligned sequences of 797 bp. Maximum parsimony analysis recovered 6 equally most parsimonious trees (48 informative sites, 145 steps),

Table 1. Code of the tissue samples used in the study, mtDNA and nDNA haplotypes, location and GenBank accession number for ND4 and β-fibrinogen gene fragments. Both mtDNA and nDNA haplotype columns indicate to which sample code (on the ML tree) and haplotype number (in the network) the tissue samples correspond to, in terms of haplotype match.

Tissue samples code	mtDNA haplotype	nDNA haplotype	Location	GenBank accession nos. ND4/β-fibrinogen
V1	V3	H1	Cascina Settimo, Lombardy, NW Italy	FJ430644/FJ430611
V2	V11	H1/H5	Voltaggio, Piedmont, NW Italy	FJ430648/FJ430610
V3	V3	H6/H8	Ardenno, Lombardy, NW Italy	FJ430622/FJ430617
V4	V11	–	Isola di Montecristo, Central Italy	FJ430631
V6	V11	–	Isola di Montecristo, Central Italy	FJ430630
V7	V11	–	Isola di Montecristo, Central Italy	FJ430627
V8	V11	–	Isola di Montecristo, Central Italy	FJ430632
V9	V11	–	Chizé, Villiers en Bois, Western France	FJ430628
V10	V11	–	Chizé, Villiers en Bois, Western France	FJ430629
V11	V11	–	Chizé, Villiers en Bois, Western France	FJ430621
V12	V11	–	Isola dell'Asinara, Sardinia, Italy	FJ430642
V13	V3	H1/V7	Lentiai, Veneto, NE Italy	FJ430641/FJ430609
V14	V3	H1/V7	Lentiai, Veneto, NE Italy	FJ430643/FJ430612
V15	V3	H1/H5	Lentiai, Veneto, NE Italy	FJ430645/FJ430608
V16	V3	H5/H6	Lentiai, Veneto, NE Italy	FJ430646/FJ430607
V17	V17	–	Salerno, Campania, SW Italy	FJ430647
V18	V3	H1/H3	Capiago Intimiano, Lombardy, NW Italy	FJ430651/FJ430605
V19	V19	–	Doberdò del Lago, Friuli-Venezia Giulia, NE Italy	FJ430655
V20	V3	–	Travacò Siccomario, Lombardy, NW Italy	FJ430633
V21	V21	H1/H7	Briga Novarese, Piemonte, NW Italy	FJ430634/FJ430614
V22	V11	–	Isola dell'Asinara, Sardinia, Italy	FJ430640
V23	V3	–	Comacchio, Emilia Romagna, NE Italy	FJ430635
V24	V3	H1/H2	Avio, Trentino Alto-Adige, N-NE Italy	FJ430636/FJ430604
V25	V3	H1/H4	Mombello heatland, Limbiate, Lombardy, NW Italy	FJ430637/FJ430606
V26	V3	–	Mombello heatland, Limbiate, Lombardy, NW Italy	FJ430638
V27	V3	H5	Travo, Piacenza, Emilia Romagna, NW Italy	FJ430639/FJ430616
V28	V11	–	Corsica, France	FJ430654
V29	V11	–	Molinos, Vall Fosca, Spain	FJ430623
V30	V11	H5/H7	Molinos, Vall Fosca, Spain	FJ430624/FJ430613
V31	V11	–	Molinos, Vall Fosca, Spain	FJ430625
V32	V11	H1/H7	Pobellà, Catalonia, Spain	FJ430626/FJ430615
V33	V33	–	Pisa, Tuscany, NW Italy	FJ430652
V35	V17	–	Avella, Avellino, Campania, SW Italy	FJ430649
V36	V17	–	Amalfi, Napoli, Campania, SW Italy	FJ430650
V37	V37	–	Lago Spartà, Sicily, SW Italy	FJ430653
V38	V38	H1/H7	Roccamassima, Latium, C Italy	FJ430658/FJ430619
V39	V39	–	Lago Laceno, Campania, SW Italy	FJ430656
V40	V3	H9/H10	Presso Lago di Lesina, Apulia, SE Italy	FJ430657/FJ430618
V41	V41	H1	Teulada, Sardinia, Italy	FJ430659/FJ430603
V42	V42	H1	Villalvernia, Piedmont, NW Italy	FJ430660/FJ430620

and the ML heuristic search found a single tree of $-\ln 1790.4818$ (fig. 2). Bayesian analysis produced an identical estimate of relationships. For the β-fibrinogen fragment a total of 32 individuals were analysed with an aligned length of 474 bp. 14 individuals were heterozygous for this locus, affecting 10 nucleotide positions.

From the analysis of the tree based on mtDNA, the existence of two well defined groups is quite evident, one regrouping individuals from northwestern and southern Italy, northeast of the Apennines, and Sicily ("eastern" group); and another clade or namely the "western" group, in which are included the individuals from Spain, France, Corsica, Sardinia, northwestern Italy and west of the Apennines. The mean genetic distance between these two clades is 4%, which is less than reported be-

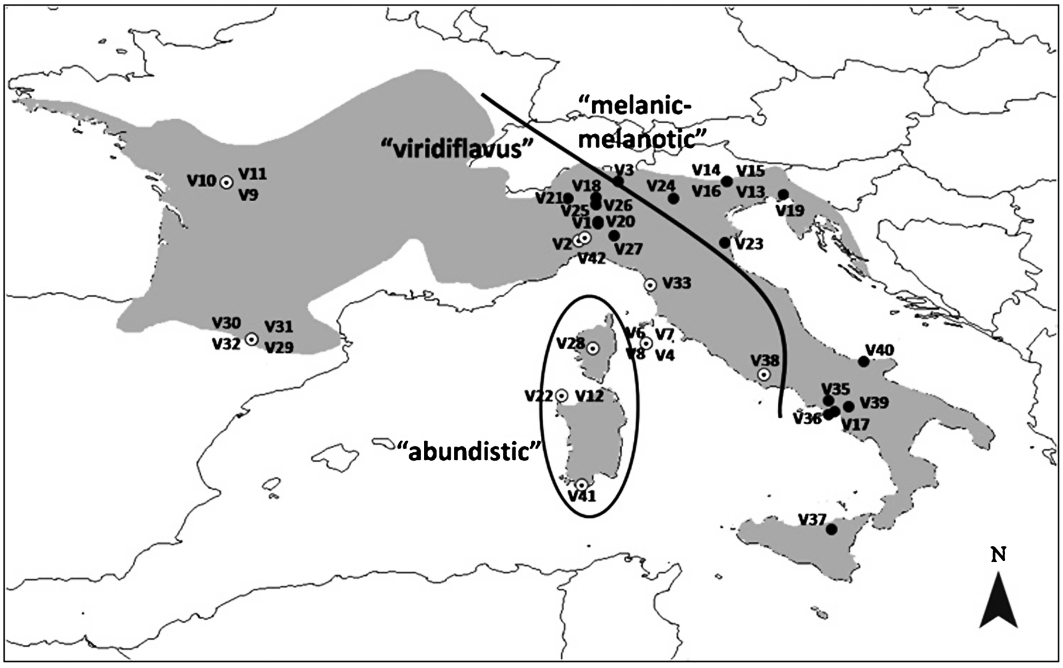

Figure 1. Map with geographic location of all the tissue samples used in this study. The symbols ⊙ and ● represent the individuals from the "Western" group, and the "Eastern" group, respectively. In light grey is represented the distribution area of *Hierophis viridiflavus*. The black line marks the division between the "viridiflavus" and "melanic-melanotic" patterns, and the ellipse around Corsica and Sardinia corresponds to the "abundistic" colour pattern present in these islands.

tween typical distinct species of colubrid snakes for the same gene (e.g., Guicking et al., 2006). In northwestern Italy, both mtDNA lineages are found in the same region, although they have not been recorded syntopically (closest distance 55 km, between Travo -V27- and Villalvernia -V42-). In this area, snakes with "viridiflavus" colour pattern are almost exclusively found, indicating that colour pattern is not linked to the mtDNA clades. The same is evident when analysing the network of β-fibrinogen intron 7 sequences (fig. 2) haplotypes 1, 5 and 7 are shared between clades, suggesting incomplete lineage sorting. With these evidences, both from mtDNA and nDNA, the data does not support the elevation of these two clades at the specific level, a fact already suggested by Schätti and Vanni (1986). Surprisingly, individuals from Corsica and Sardinia are included in the "western" clade, without evidence of subdivision; indeed, individuals V12 and V22 from Sardinia and V28 from Corsica have the same ND4 haplotype as other individuals from the mainland. Although there are no fossil records in Sardinia for this species (Delfino and Rook, 2008), we prefer to assume that the obtained phylogeographic pattern in these islands is the result of a recent colonization, possibly facilitated by the marine regressions during the Pleistocene's glacial maxima, a pattern already described for other colubrid snakes (e.g., Carranza et al., 2004, 2006). The overall genetic substructuring within *H. viridiflavus* could also have been the result of the Pleistocene's climate oscillations, leading to the establishment of several refugia where populations evolved and differentiated independently. This hypothesis has also been suggested by Nagy et al. (2003), and the same authors suggest southern Italy and Sicily as possible refugia. Although Nagy et al. (2003) suggested the region of Salerno (Campania, southern Italy), as possible glacial refugia for the "western" lineage, our data indicate that this is unlikely as snakes from this region

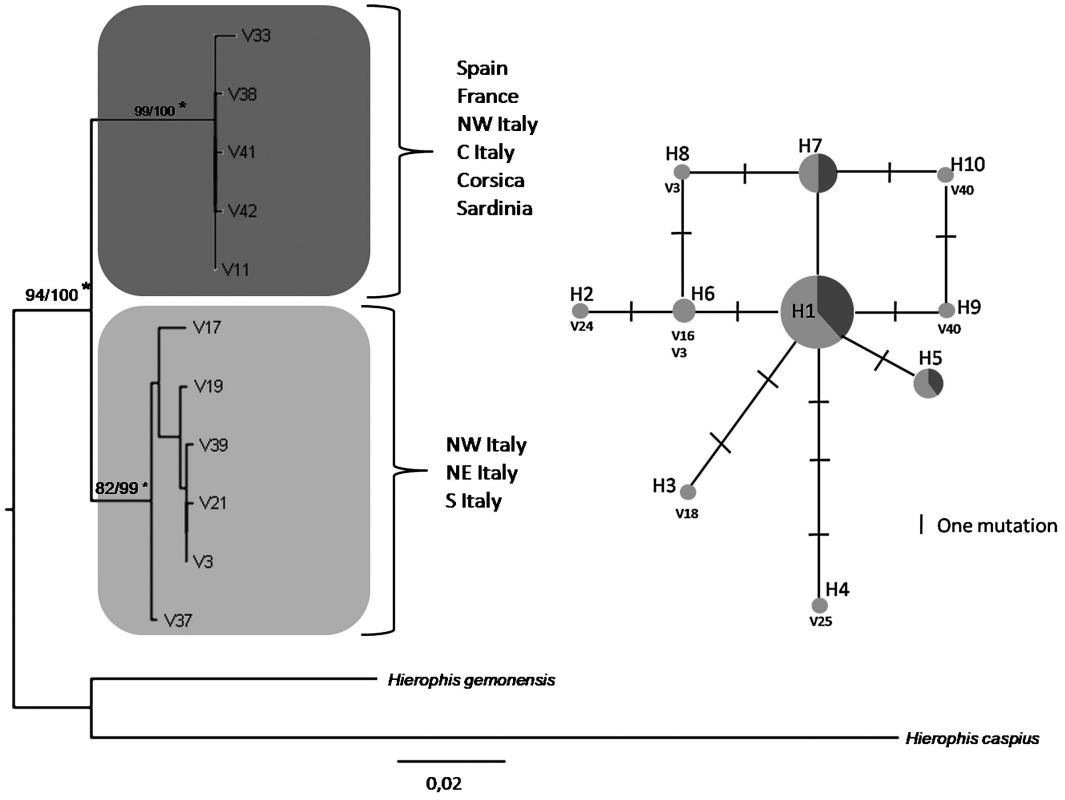

Figure 2. Tree derived from a ML analysis for the ND4 gene fragment using the model described in the text. Bootstrap values (>50%) for MP and ML are given above the branches (MP/ML), and Bayesian posterior probabilities with values > 95% are indicated with *. The tree was rooted using *Hierophis gemonensis* and *Hierophis caspius*. Below the tree is indicated the number of substitutions/site. On the right is represented the haplotype network for β-fibrinogen intron 7 variation. Individuals included in haplotypes 1, 5 and 7 are identified in the table 1. Each "l" corresponds to one mutated position. For both figures light grey colour corresponds to individuals from the Eastern group, and dark grey to the Western group.

belong to the "eastern" clade. The refugium for the "western" lineage was likely further north than this.

It does seem likely that southern Italy harboured both glacial refugia for this species. The existence of multiple refugia, or "refugia within refugia" (Gómez and Lunt, 2007) is well documented in the Iberian Peninsula, but less commonly reported in the Italian Peninsula (Podnar et al., 2005; Fritz et al., 2007). These results indicate that this region too may have more complex substructuring within the glacial refugia than was previously expected.

Our results also highlight the advantages of combining nuclear and mtDNA data in phylogeographic studies (Fritz et al., 2008a, b; Godinho et al., 2008). The lack of structure in the nuclear gene could be the result of differential migration, but incomplete lineage sorting seems more plausible without further evidence of this. Also, both molecular markers indicate that the "western" group has a lower genetic diversity, since it only displays three of the commonest 10 haplotypes of β-fibrinogen, and has a mtDNA nucleotide diversity ("Pi" using the Jukes and Cantor (1969) correction) of 0.00089 much lower than 0.01170 present in the "eastern" group.

Known colour pattern variation does not seem to be related to the major mtDNA lineages. A similar pattern was also highlighted for *Psammophis schokari* (Forskål, 1775), another colubrid snake with distinct colour patterns, and whose phylogeography is the result

of severe climate changes experienced by the Sahara desert between the Miocene and Pleistocene (Rato et al., 2007). As in *H. viridiflavus*, these distinct colour patterns could be interpreted as a by-product of selective pressures of local environmental conditions (Zuffi, 2007).

To conclude, our extended geographic sampling supports the previous finding of two mtDNA lineages within *H. viridiflavus* that possibly differentiated during the Pliocene. In this scenario populations from Corsica and Sardinia belong to the "western" clade, and may likely result from a recent colonization. Both mtDNA clades occur in proximity in northwestern Italy, and the nuclear marker β-fibrinogen showed limited nuclear differentiation, possibly due to incomplete lineage sorting. Colour variation is not associated with genetic divergence, but is more likely due to local adaptation.

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