Phylogeography of the European Whip Snake *Coluber (Hierophis) viridiflavus* as inferred from nucleotide sequences of the mitochondrial cytochrome *b* gene and ISSR genomic fingerprinting

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Abstract
The intraspecific phylogeography of European Whip Snake *Coluber (Hierophis) viridiflavus* was reconstructed using complete sequences (1117 bp) of the protein-coding mitochondrial cytochrome *b* gene. *C. (H.) gemonensis* (Laurenti) and *C. (H.) caspius* Gmelin were used as outgroups. Additionally, a microsatellite-based genomic fingerprint method, ISSR, was employed to check for gene flow between populations. Two clearly different genetic clades could be identified within European Whip Snake, a western one occurring in France, Switzerland and Italy west of the Apennines, and an eastern one found in Croatia, eastern and southern Italy. The latter one could be further subdivided into three subgroups, two of which occur in southernmost Italy only (southern Calabria and Sicily). These two distinct entities were probably formed during a long continuous settlement of the European Whip Snake in these climatically favourable areas, whereas the more northern populations experienced enormous shrinking of their ranges during cold periods in the Pleistocene. Potential glacial refuge areas are discussed.

Key words: European Whip Snake, Coluber (Hierophis) viridiflavus, phylogeography

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INTRODUCTION

The taxonomy and systematics of the European Whip Snake, *Coluber (Hierophis) viridiflavus* Lacépède, 1789, and of the other species of the genus *Coluber* (sensu lato), have been intensively debated in the last centuries. Although the European whip snake exists exclusively in Europe (Heimes 1993, Naulleau 1997), it shows remarkable morphological variation. Many of these characteristics can change from population to population (Schätti & Vanni 1986), and therefore a multitude of morphological forms (insular and colour forms first of all) at species and subspecies levels have been reported in the literature (Suckow 1798, Bonaparte 1833, De Betta 1874, Boulenger 1893, Mertens & Müller 1928, Mertens & Wermuth 1960, Kramer 1971, Capolongo 1984, etc.). While Bruno (1975 & 1980) considers that two separate taxa ("*viridiflavus*" and "*carbonarius*" - on the basis of melanism) at species level might be possible, Schätti & Vanni (1986) among others question the existence of subspecies - due to lack of evidence in pholidotic and hemipenis-morphological data. The occurence of the melanistic form ("*carbonarius*") presents an intricate taxonomic problem. Several authors have tried to explain the distribution areas of the (sub)species in accordance with melanism. However, it has became clear that a taxonomic subdivision in this case cannot be based on melanism (Schätti & Vanni 1986, Nagy & Korsós 2001).

Intraspecific division of *Coluber (Hierophis) viridiflavus* has been investigated until now strictly with respect to morphological or morphometric characters. Therefore, we wanted to analyze the intraspecific evolution and phylogeography using genetic data. Our analysis of 22 specimens of *Coluber (Hierophis) viridiflavus* relied on two methods: nucleotide sequencing of a mitochondrial marker gene, which shows adequate resolution at intraspecific level (cytochrome *b* = cytb), and ISSR-PCR fingerprinting of nuclear DNA, respectively, to evaluate gene flow and potential hybridizations (Wink et al. 2000).

MATERIAL AND METHODS

1. Samples

Tissue samples originate from four countries (Croatia, France, Italy and

Table 1. Tissue samples included in the present study.
* - no proper data available

Sample	Species	Country of origin	Place of origin	Latitude	Longitude	Date
P7 (1)	*C. (H.)viridiflavus*	Croatia	Krk	*	*	05/1994
J19 (5)	"	France	Quincy	*	*	05/1997
J36 (3)	"	Italy	Toscana	*	*	*
J39 (5)	"	France	Quincy	*	*	05/1997
J40 (5)	"	France	Quincy	*	*	05/1997
DG8 (7)	"	Italy	Grado, RN Canavata	45.42N	13.28E	04/2000
DG9 (7)	"	Italy	Grado, RN Canavata	45.42N	13.28E	04/2000
DG16 (9)	"	Italy	Lago di Lesina	41.51N	15.21E	04/2000
DG17 (9)	"	Italy	Lago di Lesina	41.51N	15.21E	04/2000
DG21 (11)	"	Italy	Monte St. Angelo	41.44N	15.58E	04/2000
DG22 (12)	"	Italy	Torre San Gennaro	40.32N	18.05E	04/2000
DG38 (14)	"	Italy	Sicily, betw. Cesaro & Troina	37.49N	14.38E	04/2000
DG45 (15)	"	Italy	Sicily, betw. Randazzo & Castiglione	37.53N	15.02E	04/2000
DG53 (16)	"	Italy	Calabria, Bagaladi	38.02N	15.49E	04/2000
DG55 (17)	"	Italy	Calabria, F. Crati	39.40N	16.30E	04/2000
DG65 (18)	"	Italy	T. Platano, Baragiano	40.39N	15.37E	04/2000
DG66 (18)	"	Italy	T. Platano, Baragiano	40.39N	15.37E	04/2000
DG71 (20)	"	Italy	Rapallo	44.22N	09.15E	05/2000
DG77 (21)	"	Italy	F. Staffora, betw. Varzi & Voghera	44.55N	09.06E	05/2000
DG86 (22)	"	Switzerland	R. L'Allondon, Maval	46.14N	06.08E	05/2000
A1 (23)	"	France	Rive de l'Artuby, Canguers	*	*	04/2000
J55 (24)	"	Italy	Canossa	*	*	05/1999
J3	*C. (H.) gemonensis*	Croatia	Istria	*	*	*
KCC1	*C. (H.) caspius*	Greece	Serifos	*	*	*

Figure 1. Map with localities of specimens used for the analysis.

Switzerland, see Table 1 and Figure 1 for details). In most cases a blood sample was taken from the tail of living specimens at the capture site (Joger & Lenk 1997); in addition, different tissue samples were taken (a small piece of liver, heart, or tail) from recently killed animals (snakes that were run over by cars). The distribution area of the European whip snake (Heimes 1993, Naulleau 1997) was more or less covered by sampling - with the exception of the Western islands of the Mediterranean Sea, such as Corsica, Sardinia etc. Samples from the two closely related species (Schätti 1988): *Coluber (Hierophis) gemonensis* (Laurenti, 1768) and *Coluber (Hierophis) caspius* (Gmelin, 1789), were analyzed as outgroups.

2. Isolation of DNA

Tissue samples were stored until processing either in 80% ethanol or in EDTA buffer (Arctander 1988). The isolation of total genomic DNA was carried out with proteinase K digestion, followed by cleaning steps with phenol-chloroform and guanidine-thiocyanate (Sambrook et al. 1989). The extracted DNA was dissolved and stored in Tris-EDTA (10 mM Tris and 1mM EDTA).

3. PCR and nucleotide sequencing

The whole cytb gene was amplified with polymerase chain reaction in an end volume of 50 µl. PCR primers are listed in Table 2. PCR was carried out under standard conditions: initial denaturating (4 min at 94°C), 31-33 cycles with denaturating (45 s, 94°C), elongation (2 min, 72°C) and annealing step (50 s, 41-45°C), followed by a final elongation step for 10 min at 72°C. PCR products were stored at 4°C, and were directly used as templates in a cycle sequencing reaction (Lenk et al. 1999). Typical conditions were: 3 min at 94°C, 26 cycles with denaturating (30 s, 94°C) and annealing and elongation step (45 s, 60°C). The sequencing products were analyzed in an automated sequencer ALFExpress II (Amersham Pharmacia Biotech). Sequences were checked for errors and

Table 2. Primers used for PCR amplification and sequencing (cy: primer used in cycle sequencing only).

Primer	Source	Sequence (5'-3')
L14724	Meyer et al. (1990)	CGA AGC TTG ATA TGA AAA ACC ATC
smtA (L-14846)	after Kocher et al. (1989), Lenk &Wink (1997)	CAA CAT CTC AGC ATG ATG AAA CTT CG
mtE (H-15556)	Lenk et al. (2001)	AAT AGG AAG TAT CAT TCT GGT TTG AT
smtF (H-16060)	modified after Wink (1995)	TCA GTT TTTGGT TTA CAA GAC CAA TG
L-15570cy	This study	GAY AAA ATC CCA TTY CAC CC
H-15305cy	This study	AAT GAT ATT TGT CCT CAT GG

aligned manually.
The statistical analyses of the nucleotide sequences were carried out with the PAUP* 4.0b8 (Swofford 2001) programme package using maximum parsimony method with heuristic search and bootstrap analysis.

4. ISSR-PCR fingerprinting

ISSR (Inter simple sequence repeat) genomic fingerprinting was performed to verify results obtained from mitochondrial cytochrome *b* sequences and to evaluate potential hybridisation. ISSR-PCR uses single primers designed from short tandem repeats to amplify stretches of DNA between adjacent microsatellites. Given that microsatellites are scattered throughout the genome (Tautz & Renz 1984) and in such density that adjacent microsatellites lie within the limits of *Taq* polymerase processing, during ISSR-PCR a large number of fragments is generated. Once separated on polyacrylamide gel, ISSR-PCR products appear as polymorphic multiband fingerprints.
Five ISSR-primers that are known to generate polymorphic fingerprints in several vertebrate taxa were tested under varying PCR conditions on *Coluber (Hierophis)* samples. $(CA)_{10}$- and $(GACA)_4$-primers gave most useful results. The reactions were carried out according to the protocol of Wink et al. (2000). In a PCR volume of 25 µl, 50-100 ng of total DNA were used as template, plus 6 pmol $(GACA)_4$ or 20 pmol $(CA)_{10}$ primer, 1,5 mM $MgCl_2$, 0,1 mM of dCTP, dGTP, and dTTP, 0,045 mM dATP, 1 µCi ^{33}P-alpha-dATP, 2,5 µl 10x amplification buffer and 1 unit *Taq* polymerase (Amersham Pharmacia Biotech). PCR programmes were set for 5 min at 94°C, followed by 26 cycles of 30 s at 94°C, 20 s at annealing-temperature, and 50 s at 72°C. After completion, the temperature was set to 72°C for 5 min and then lowered to 4°C for further storage. Optimal annealing-temperatures, as found out by a 2°C temperature gradient PCR (40°C-60°C), were 40°C for $(CA)_{10}$-primer and 55°C for $(GACA)_4$-primer. PCR products were separated on a denaturing Sequagel matrix at 65 W for 4 h and visualised by autoradiography. Fingerprint patterns were evaluated visually.

RESULTS

1. Analysis of cytochrome *b* sequences

The complete mitochondrial gene cytb (1117 base pairs) was amplified from 24 samples, including the outgroups.
The phylogeographic analysis of *Coluber (Hierophis) viridiflavus* revealed four haplotype groups in two main clades, which are clearly separated genetically and by geographic areas (Figure 2). These clades are supported with significant bootstrap

values (96-100%).
Among our sequences 57 variable nucleotides (5.10% of the cytb gene, excluding outgroups) were found (Figure 3); the two main groups are separated by - at least - 2.95% (33 different nucleotides). They represent a Western and an Eastern clade. The Western group (clade W in Figure 2) includes animals from France, Switzerland and Italy west of the Apennines (i.e. Tuscany province and Riviera coast). This group possesses a quite uniform haplotype - only single nucleotide mutation sites were detected - which appears to be monomorphic without further subdivisions.
The Eastern group is divided into further subgroups: 1. Italy, east of the Apennines and the island Krk (clade E on Figure 2), 2. southern Calabria, and 3. Sicily (both of them represented in clade S). At the Eastern border of the distribution area live - sympatrically with *C. (H.) gemonensis* - the Slovenian and Croatian populations of *C. (H.) viridiflavus*. Our sample from the island Krk shows the closest relationship with the eastern Italian samples. In contrast, the Southern (South-Eastern) populations belong to a clearly separated group: the South Calabrian and Sicilian populations differ not only from the northeastern ones, but also from each other.

2. ISSR-PCR patterns

ISSR-primers $(GACA)_4$ and $(CA)_{10}$ produced informative fingerprints with several polymorphic bands. ISSR profiles were more diverse with the $(GACA)_4$-primer (Figure 4) than with the $(CA)_{10}$-primer (not shown). Both primers generate a series of species-specific bands as

Figure 2. Phylogeny of the cytochrome b gene of Coluber (Hierophis) viridiflavus. Strict consensus tree resulted from maximum parsimony analysis, tree length=251. Bootstrap proportions >50 (100 replications) are presented.

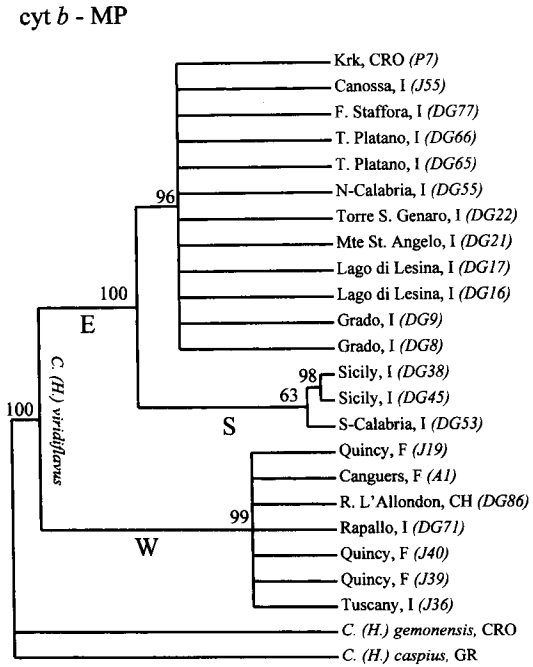

Figure 3. Variable nucleotides of C. (H.) viridiflavus samples (cytochrome b gene with position numbering). E: eastern group; W: western group; Sic: Sicily; Cal: South-Calabria; var: variable sequences with single nucleotide mutations.

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                                                    1111
          111 1111222233 3344444445 5555666666 7778888999 9990000
         2244458122 4569056811 3834566671 3457146889 0261447011 5790467
         4527814709 7658487828 0127302577 1929829341 8934366658 5492721

#C_virid_E       GCACACGCGA TTTGGCTCGG TGTTATAGTC GGACCAATTC ACGAGGCAAT ATGAGTC
#C_virid_E_var   .......... .......... ......R..Y ..W....... ..R....... .Y.....
#C_virid_E_Sic   ...G.TRT.. ....AT.T.. .AC....... ....T.G... ...GA..... ..AG...
#C_virid_E_Cal   ...G...TA. .C..A.C... .AC....... .....G.C.. .......... G.A..C.

#C_virid_W       ATGGG.A..G ..CAA.C.AA CA.CGA.AC. AA.T....CT GT..AA.G.C ....A.T
#C_virid_W_var     ATGGG.A..G Y.CAA.C.AA CA.CGA.AC. AA.T....CT GT..AAYGMC ....A.T
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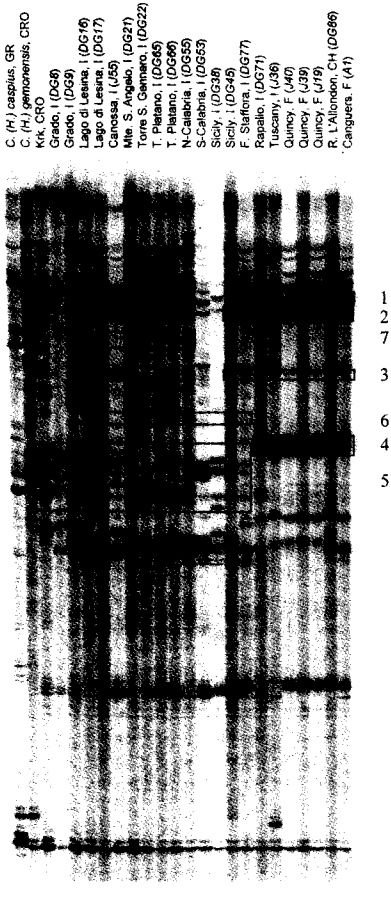
Figure 4. ISSR PCR fingerprint made with (GACA)4 primer. See text for details.



well as several group-specific bands (Figure 4, indicated with numbers) that support the differentiation into a western (Figure 4, bands No. 1, 2, 3, 4) and an eastern clade (bands No. 5: several bands in a specific size range, to a certain extent also No. 6, 7). Western and eastern clades, as suggested by ISSR-analysis, conform to those suggested by cytochrome *b* sequences.

Furthermore, ISSR patterns suggest a hybrid status for some samples. This is evident in samples J55 and DG77, both from Northwestern Italy. According to cytochrome *b* sequences, both samples belong to the eastern clade, whereas ISSR-profiles show both eastern clade specific bands (Figure 4, no. 5) as well as western clade specific bands (in sample DG77 No. 2 and 3, in sample J55 No. 1, 2, and 3). These results suggest recent gene flow between the eastern and western clade in Northwestern Italy.

The separation of the Sicilian and southern Calabrian samples (DG38, DG45 and DG53, respectively) from the rest of the eastern clade is less evident according to ISSR profiles than it is according to cytochrome *b* sequences. The band pattern of the Sicilian sample DG45 may show genetic introgression from the west, too (Figure 4, bands No. 2, 3).

DISCUSSION

The sequence analysis (confirmed by

ISSR-PCR fingerprinting) unambiguously shows that there are discrete phylogeographic units within the distribution range of the European Whip Snake. Spatial and historic factors might be responsible for their isolation.

Two bigger groups are apparently separated on the genetic level: The Western and Eastern populations are divided by the mountain ranges of the Alps and the Apennines acting as strict geographic barriers (Figure 1). Apparently this species does not cross mountain ranges easily (though it has been reported from elevations above 1,800 m [Heimes 1993]). Results based on cytb sequences support the conclusion that the Western group might be monotypic; however, no samples from the Western Mediterranean islands (see above), or from the Northern border of the distribution zone (e.g. Luxembourg) were available for our research. An important aspect is that the distribution of this colubrid species is continuous between France and the Western foot of the Apennines; it includes a recent connection along the narrow coastal strip of Côte d'Azur and Riviera di Ponente.

The mitochondrial sequences confirm a close relationship between *Coluber (Hierophis) gemonensis* and *Coluber (Hierophis) viridiflavus*. This result suggests also a Western-Eastern divergence: *Coluber (Hierophis) viridiflavus* is representative of the central Mediterranean, while *Coluber (Hierophis) gemonensis* of the eastern Mediterranean, respectively. In addition, it is also possible that both of them share a common ancestor with *C. (H.) caspius* and *C. (H.) jugularis*, as Schätti (1988) believes.

As the sister species are distributed in the Balkans, it is justified to assume the geographic origin of *C. (H.) viridiflavus* at the eastern edge of its distribution range. Many eastern European species that reach Italy do not cross the Apennines; a vertebrate example is the Hooded Crow *Corvus corone cornix*. In the turtle *Emys orbicularis*, genetically different subspecies inhabit eastern and western Italy (Lenk et al. 1999).

The population living on Krk island - at the eastern border of the distribution zone - shows a petty divergence compared to Italian populations which belong to the eastern group. A continuous presence of the species during the Pleistocene is, however, unlikely in this northern part of its range. Therefore it is probably derived from a southern Italian stock (The Balkans' own glacial refugia were probably occupied by *C. (H.) gemonensis)*. North-South movements of such reptiles along the coasts of Italy are unhindered. The existence of South(east)ern subgroup(s) is, however, again paralleled by *Emys orbicularis* (Lenk et al. 1999) as well as by the snakes of the *Elaphe longissima* complex (Lenk & Wüster 1999). Such common zoogeographical patterns can be traced back to the glacial zoogeographic history in all likelihood (Lenk et al. 1999, Schätti 1988).

The southern Italian and Sicilian regions could have functioned as refugia during cold periods of the Pleistocene. A refuge for the eastern group could have been at the Gulf of Taranto and in Calabria. Our two Calabrian samples belong to slightly different haplotypes: DG53 ("South"), and DG55 ("North"), respectively. Both are different from the Sicilian sample. This variation could be explained by a longer continuous evolutionary history in these possible glacial refuges. A possible refuge for the western group could have been in the region of Naples-Salerno. However, we could not check samples from that area.

The evolution of the southern populations could also continue unhindered during glacial periods. This may have resulted in higher genetic differences, in other words, in a mosaic of genetically different forms. A few of these forms (in fact only

one on each side of the Apennines) would have used the climatically favorable interglacials to spread into more northern areas. Pleistocene fossils attributed to *C. (H.) viridiflavus* have been found in southern Germany and Austria; in the Pliocene, this species reached the territories of today's Czech Republic and Poland (Ivanov 1997, Schätti 1988). Climatic conditions during the glacial periods forced it to retreat into the above named refuges in southern Italy. After the last glacial period, re-immigration to the northern Adriatic region (eastern group) as well as to France and Belgium (western group) would have happened in a comparatively short time, which may not have allowed a significant genetic differentiation of northern populations to occur.

ISSR-PCR has useful applications for examining group specificity and hybridization events. Because of its detection of nuclear genetic rearrangements, it provides a valuable complement to data obtained from the maternally inherited mitochondrial genome. It is especially powerful in detecting hybridization events (Wink et al. 2000).

In our study, it allowed us to re-examine the separation of *Coluber (Hierophis) viridiflavus* samples into a western and an eastern clade, which is strongly supported by ISSR-analysis, as well as suggesting a gene flow between both groups in Northwestern Italy. However, ISSR-fingerprints provided less evidence for the separation of Sicilian and South Calabrian samples from other eastern clade samples than is suggested by cytochrome *b* sequence data.

Because of the evident, but not complete, genetic isolation, we recommend once more the recognition of two subspecies of the European whip snake sensu Mertens & Wermuth (1960). If further studies in the contact zones of both subspecies prove that an intrinsic genetic barrier exists, the two main clades could even be raised to species level (see Joger et al. 1998). The applicable name for the western populations is *Coluber (Hierophis) viridiflavus viridiflavus Lacépède*, 1789 (terra typica: southern France). The Eastern stocks were traditionally assigned to the subspecies *Coluber (Hierophis) viridiflavus carbonarius* Bonaparte 1833 (nomen conservandum, terra typica restricta Mertens & Müller 1928: Monti Euganei, Padua, Italy); however, the subspecific name has no more (or has far less) ethymological importance (an allusion to the melanism). The border between both subspecies is the Apennines. The South Italian populations are less differentiated within the Eastern group (1.16% [13 different nucleotides] and 1.34% [15] by Calabrian and Sicilian samples, respectively, at cytb level), so in our view taxonomical consequences are not justified. This, however, would mean that older names apply to the eastern subspecies: *Coluber (Hierophis) profulax Costa*, 1828 (terra typica Aspromonte, southern Calabria) or *C. (H.) xanthurus* Rafinesque-Schmaltz, 1810 (terra typica Sicily). An even older name is *C. (H.) sardus* Suckow 1798 (Terra typica Sardinia). As we have not studied Sardinian material, we cannot decide to which form it applies. In all cases, the morphology of the European whip snake has to be studied in more detail in order to find reliable characters to define subspecies.

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