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ORIGINAL ARTICLE

No association between candidate genes for color determination and color phenotype in *Hierophis viridiflavus*, and characterization of a contact zone

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Abstract

Genetic and phenotypic differentiation in allopatric conditions can be explained either by neutral phenomena or adaptative processes driven by selection. In reptiles, coloration can affect aspects directly related to their survival, representing a classical character under selection. In this context, secondary contact areas are natural laboratory to understand evolutionary processes underlying genetic permeability, especially when populations differ in phenotypic traits such as coloration. The western whip snake *Hierophis viridiflavus* presents two divergent mitochondrial clades, characterized by the presence of one of two main color phenotypes, namely one with black and yellow stripes and a fully melanic one. Here, we investigated whether melanogenesis-linked genes are determinant of the chromatic differences observed across the phenotypic variation of the species. In addition, we used a multilocus dataset, including 134 original *ND4* sequences, to better define the overall genetic structure and to provide a characterization of a contact zone identified in Central Italy by estimating the amount of nuclear gene exchange. While we found no evidence supporting a direct association between target genes and coloration, a non-synonymous substitution polymorphism, at high frequency, was detected in the β melanocyte-stimulating hormone whose possible function has been discussed. Concerning the genetic structure, both mtDNA and nuDNA were partly concordant indicating introgression events occurring at the contact zone. When we measured the nuclear gene flow, we found a significant amount of gene exchange, mainly guided from one clade to the other, that is, asymmetric. These results might suggest the presence of ecological and/or behavioral processes driving the observed directional gene flow.

KEYWORDS
color determination, contact zone, *Hierophis viridiflavus*, hybridization, western whip snake

1 | INTRODUCTION

Under allopatry, genetic differences between populations generally result from either neutral phenomena or by adaptative processes driven by different selective pressures (e.g., Nosil et al., 2009). In both cases, when diverging populations come in contact, the formation of secondary contact zones could allow hybridization between the differentiating biological entities (Hewitt, 1996). Depending on

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the strength of disruptive natural selection, however, diversification can still occur, even in presence of gene flow (Nosil, 2008; Pinho & Hey, 2010). Understanding processes prevailing on genes during population divergence, either neutral or selection-mediated, is a hot topic in evolutionary biology (Leinonen et al., 2008).

Coloration is a classic example of a character under strong selection in animals, playing an important role in a wide range of ecological processes, including mate choice, camouflage, and physiological functions (Clusella-Trullas et al., 2008; Gray & McKinnon, 2007; Luiselli, 1992; Martínez-Freiría et al., 2017). Furthermore, in ectotherm animals such as reptiles, coloration may play an additional role, with darker colorations potentially being associated with a benefit in terms of thermoregulation, as suggested by the so-called "thermal melanism hypothesis" (Bittner et al., 2002; Clusella-Trullas et al., 2008; Clusella-Trullas et al., 2007). Despite this variety of crucial roles, the genetic basis of pigmentation has so far been scarcely investigated in reptiles, but has been considerably studied in homeotherms (Olsson et al., 2013). In mammals and birds, the genetic basis of pigmentation has been demonstrated to be associated with several genes having an active role, either constitutive or regulatory (Campagna et al., 2017; Hubbard et al., 2010; Senczuk et al., 2020). Putative melanising mutations in the *melanocortin-1 receptor* (*MC1R*), in particular, have been characterized in many vertebrate groups (Anderson et al., 2009; Hoekstra, 2006; Hofreiter & Schöneberg, 2010; Hubbard et al., 2010, and references therein). With regard to reptiles, most case studies failed to determine any putative association between target genes and coloration (Corso et al., 2012; Cox et al., 2013; Micheletti et al., 2012; Raia et al., 2010), with only few exceptions (Nunes et al., 2011; Rosenblum et al., 2004, 2010). Nevertheless, a significant association between color polymorphism and a single nucleotide polymorphism (SNP) in the *pro-opiomelanocortin* gene (*POMC*), the precursor of the melanocyte-stimulating (MSHs) and adrenocorticotropic (ACTH) hormones (Millington, 2006), has been found in the asp viper *Vipera aspis* (Ducrest et al., 2014). In this species, the frequencies of alternative mutation have been found to differ between diverging, syntopic color morphs. Finally, another study found that genetic changes in the *sepiapterin reductase* gene and in the *beta-carotene oxygenase 2* locus determined throat color polymorphism in the common wall lizard *Podarcis muralis* (Andrade et al., 2019).

The western whip snake *Hierophis viridiflavus* is a medium-sized (120–150 cm in length, on average) colubrid snake ranging from the Pyrenees to Croatia, throughout France, Corsica, Switzerland, Slovenia, and Italy, where it is present across the whole mainland and the islands of Sardinia, Sicily, Elba, and the Pontine archipelago (Speybroeck et al., 2016). Two deeply divergent mitochondrial clades, largely characterized by different coloration, have been identified within this species (Nagy et al., 2002; Rato et al., 2009). The main lineage-splitting coincides with potential allopatric events from eastern Italy to western Italy, Iberian Peninsula, and continental France, probably driven by paleoclimatic factors (Mezzasalma et al., 2018). Because of evidences provided by molecular (mtDNA), morphological and karyological data, these two clades have recently been elevated to species rank under the names of *H. viridiflavus* and *H. carbonarius*, corresponding to western (W) and eastern (E) clade, respectively (Mezzasalma et al., 2015). A very recent update of the European herpetofauna, however, has questioned the validity of this taxonomic change, proposing to consider the two clades as subspecies, under the names of *H. viridiflavus viridiflavus* and *H. viridiflavus carbonarius* (Speybroeck et al., 2020). The two subspecies are mostly parapatric: The range of *H. v. viridiflavus* goes from the central Tyrrhenian side of the Italian Peninsula up to northern Italy, including the main island of Corsica and Sardinia, central and southern France and northern Spain; *H. v. carbonarius* occurs in southern Italy, Sicily included, and all the Adriatic side up to Istria and Dalmatia (Figure 1). In addition, while *H. v. viridiflavus* generally shows a brown/blackish, yellow-striped color pattern, with darker populations described for some Tyrrhenian islands (Avella et al., 2017; Rato et al., 2009; Zuffi, 2008), *H. v. carbonarius* is typically melanic, with few exceptions (Vanni & Zuffi, 2011; Figure 2). Despite the parapatric distribution, there is no perfect correspondence between color phenotype and mitochondrial clade in northern areas (Po Valley), where the two taxa come into contact (Rato et al., 2009). In light of this, the western whip snake represents a suitable model organism to investigate the role of neutral or, alternatively, adaptive processes acting on candidate melanogenesis-related genes in driving genetic divergence.

In the present study, we assess whether the two melanogenesis-linked genes *POMC* and *MC1R* are determinant of the chromatic differences observed across the phenotypic variation showed by *Hierophis viridiflavus*. Furthermore, in combination with mtDNA sequences, we better define the overall distribution of the western whip snake genetic lineages and we provide a characterization of a contact zone identified in Central Italy by estimating the extent and the amount of nuclear gene exchange.

2 | MATERIALS AND METHODS

2.1 | Sampling

We sampled 134 individuals of *H. viridiflavus* between 2013 and 2017, mainly during spring and late summer (see Table S1 for our sampling information). We surveyed the whole Italian Peninsula, sampling with particular effort in the areas of Central-Southern Italy where the two clades are supposed to be in contact (Mezzasalma et al., 2018). Snakes were captured by hand and released after pattern identification and tissue sample collection (i.e., scale clipping). Other tissue samples were obtained from roadkills and shed skins. All tissue samples were preserved in ethanol 80% and stored at 4°C, and no voucher specimens were kept. The color pattern of each individual was assessed based on the following four categories: "*carbonarius*" (completely black dorsally and generally paler ventral portions), "*abundistic*" (black/very dark pattern with some yellowish portions), "*viridiflavus*" (mainly yellowish pattern with few darker portions), and "brown" (completely brown pattern). Given the ontogenetic change in color pattern

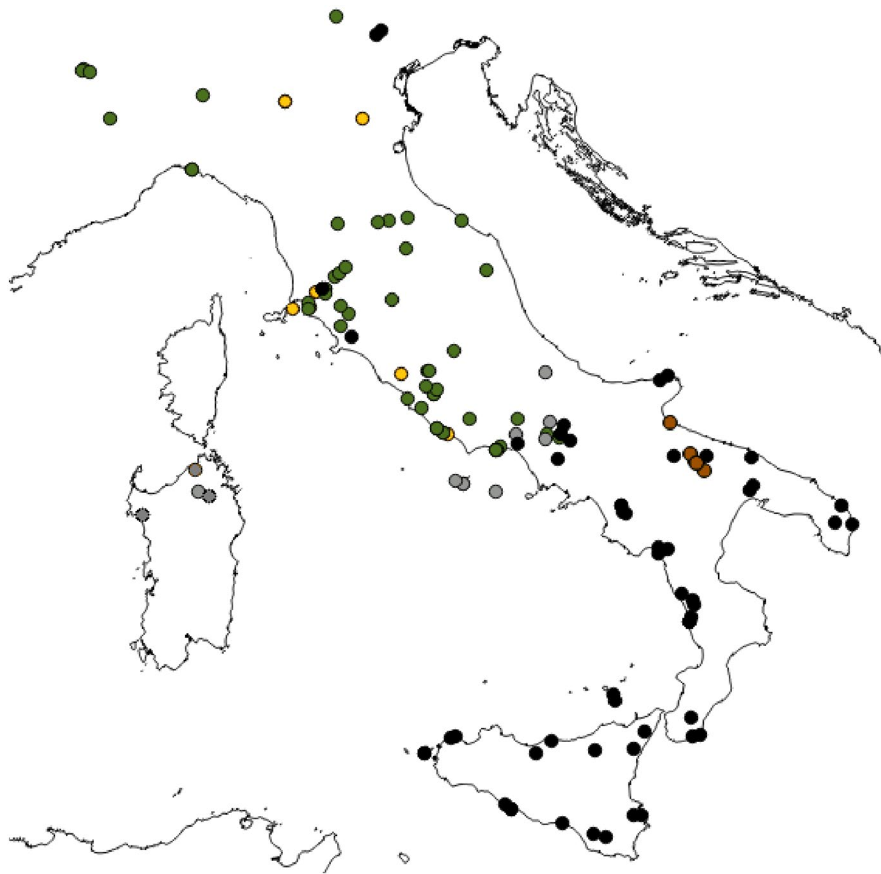

FIGURE 1 Original samples used in this study: green = *viridiflavus*; black = *carbonarius*; yellow = Juveniles (ND); brown = brown pattern; and gray = *"abundistic"*

and the impossibility to properly assess the color phenotype from shed skin (Schätti & Vanni, 1986), we did not take into account the coloration pattern from skin samples, juveniles, and subadult specimens, as reported in Table S1 (SVL > 75 cm, following Capizzi et al., 2008).

2.2 | Laboratory procedures and data analysis

Genomic DNA was extracted from all the collected tissue samples using the universal extraction protocol described by Aljanabi and Martinez (1997), consisting in incubation at 56°C with proteinase K and precipitation with isopropanol. The quality of the template has been checked through electrophoretic runs.

For all 134 specimens, a fragment of the mitochondrial gene encoding *NADH dehydrogenase subunit 4* (*ND4*) with flanking tRNAs coding for tRNA-His, tRNA-Ser, and tRNA-Leu (824 bp) was amplified with standard PCR procedures. We amplified five nuclear markers: fragments of the two genes candidate for color determination; the *pro-opiomelanocortin* gene (*POMC*; 870 bp) and the *melanocortin 1 receptor* gene (*MC1R*; 602 bp), and three nuclear markers commonly employed for phylogeographic studies: the *RAG1* gene encoding for *recombination activating gene 1* protein (975 bp), the *oocyte maturation factor* gene (*c-mos*; 528 bp) and the catenin (*cadherin-associated protein*) beta 1 gene (*CTNNB1*). For the latter, three couple of primers were used to amplify different segments: *CTNNB1_a* (944 bp), *CTNNB1_b* (646 bp), and *CTNNB1_c* (763 bp).

All primers and PCR conditions are reported in Table S2. With regard to the nuclear genes, preliminary amplifications and sequencing were obtained for a low number of specimens (5 to 10 specimens for each mtDNA clade), in order to explore nucleotide variability and fixed alternative SNPs. All the templates were purified with the Sure Clean (Bioline) purification kit and then sequenced by Macrogen© (www.macrogen.com). The obtained electropherograms were checked for ambiguous positions, and IUPAC codes were used for nuclear heterozygous calls. With regard to the mtDNA data, 91 additional *ND4* sequences from previous studies were retrieved from GenBank (Mezzasalma et al., 2015; Rato et al., 2009) and added to our dataset. To resolve the gametic phase of the nuclear markers we used the software PHASE 2.1 (Stephens & Donnelly, 2003) with the following parameters: 10^3 iterations discarded as burn-in, 1 as thinning interval, and 10^3 post-burn-in iterations. Consensus sequences and alignments for each data were performed using the software MEGA 6 (Tamura et al., 2013).

FIGURE 2 Phenotypic variation in *Hierophis viridiflavus* s.l. (a) Brown/blackish, yellow-striped color pattern of *H. viridiflavus*. (b) Melanic color pattern of *H. carbonarius*. (c) Dark color pattern of *H. viridiflavus* ("*abundistic*"). (d) Completely brown color pattern of *H. carbonarius*. Photographs taken by Gabriele Senczuk (a), Frank Deschandol (b, d), and Gregoire Meier (c)

For both the mitochondrial and nuclear datasets, we performed neutrality tests (Tajima's D, Fu and Li's D and Fu and Li's F) using DnaSP v5 (Librado & Rozas, 2009), to assess whether the chosen genes were under natural selection or their variability was driven by neutral evolution.

2.3 | Implication in coloration of candidate gene *POMC*

The fixed or nearly fixed SNPs of *POMC* between the two different chromatic forms cannot be considered as a proof of their involvement in color determination, since they could be a mere consequence of geographic isolation (see Ducrest et al., 2014). For this reason, we focused on individuals showing a discordance between mitochondrial and nuclear haplotypes. By doing so, it was possible to understand whether a given SNP on a candidate gene was related to a certain color rather than to the mitochondrial line of belonging.

The sequenced *POMC* fragment (785pb) includes sections coding for the following peptides: the α melanocyte-stimulating hormone (*α-MSH*), the corticotropin-like intermediate peptide (*CLIP*) region, the *β-lipotropic hormone* (*LPH*) segment containing *β-MSH* and *β-END* segments, and a segment of 278 bp downstream the untranslated 3'end (Kobayashi et al., 2007).

Some of the analysis focused on β-MSH, since only in this *POMC* product a non-synonymous substitution polymorphism at high frequency was found (see results). To identify variable versus conserved amino acids among snakes, an alignment was built with MEGA including β-MSH sequences from the two *Hierophis* subspecies and other available orthologous sequences available from various snake species: *Boiga irregularis* (AB280537), *Agkistrodon piscivorus* (GU586286), *Thamnophis elegans* (XM032216464), *Malayopython reticulatus* (AF369042), *Protobothrops mucrosquamatus* (XM015819997), *Python bivittatus* (XM025173659), *Notechis scutatus* (XM026671536), *Thamnophis sirtalis* (XM014057309), *V. aspis* (KC511125), and *Ophiophagus hannah* (AZIM01126306). The mean of synonymous (dS) and non-synonymous (dN) substitutions for the *β-MSH* was calculated using MEGA6. The Nei-Gojobori method was used to test the hypothesis of dN > dS (i.e., a deviation from strict neutrality), and variance was computed using 1,000 bootstrap replicates. Additionally, in order to find potential differences in the predicted secondary structure of different forms of the *β-MSH* peptides, we performed a prediction study of the secondary structure of the mutated *β-MSH* peptide with the GOR4 program (National Institutes of Health©).

2.4 | Gene flow at the contact zone

To explore gene genealogies, we built statistical networks for each informative gene using the statistical parsimony approach as implemented in PopART v1.7 (Leigh & Bryant, 2015).

Nucleotide (π) and haplotype diversity (*H*) for each gene, clade, and haplogroup as identified by the statistical networks were estimated using DnaSP.

To assess presence and extent of nuclear gene flow between the two lineages, we used a Bayesian coalescent-based approach as implemented in the software MIGRATE-n v3.6.11 (Beerli, 2009). In doing so, we performed two separate multilocus analysis, one including the whole dataset (170 *POMC* and 100 *RAG1* phased sequences) and the other using a subset of individuals from the contact zone in Central Italy (36 *POMC* and 28 *RAG1* phased sequences). We performed five independent runs using four parallel chains and a static heating scheme of 10,000.00 3.00 1.20 1.00. Finally, to determine the direction of the gene flow, we generated four migration models using the flags "x" or "0" to allow migration or not between populations: (a) gene flow from Clade W to Clade E; (b) gene flow from Clade E to Clade W; (c) "complete isolation" with no migration; and (d) "full migration". To evaluate the most supported scenario, we used the marginal likelihood to calculate each model probability following the software guidelines (Beerli & Palczewski, 2010).

3 | RESULTS

The obtained final alignments included: 134 *ND4* original sequences (568 bp) and 91 sequences obtained from the NCBI portal (Mezzasalma et al., 2015; Rato et al., 2009); 222 *POMC* sequences (785 bp), 114 *RAG1* sequences (899 bp), and 50 *MC1R* sequences (588 bp) (Alignment S1–S4). We also obtained 15 *c-mos*, 5 *CTNNB1_b*, and 6 *CTNNB1_c* sequences, which resulted to be invariant and were therefore excluded from further analyses. Concerning *CTNNB1_a* (5 sequences), we found differences in sequence length between the two sexes due to an INDEL polymorphism (males ~ 900 bp and females ~ 520 bp), because linked to sex chromosomes as previously reported (Matsubara et al., 2016). All the sequences are accessible on GenBank (Accession numbers: MW297553–MW297679 for *ND4;* MW314439–MW314549 for *POMC*; MW314550–MW314575 for *MC1R*, and MW314382 - MW314438 for *RAG1*).

The neutrality tests did not result to be significant (*p*-value always higher than 0.05, see Table 1); therefore, absence of natural selection on all the studied genes can be assumed.

3.1 | Candidate gene for dark coloration

In our sampling sessions, we found 54 adult specimens with "*viridiflavus*" phenotype and 53 adults with "*carbonarius*" phenotype. Tissues were also collected from 11 juveniles (ND), 12 adult specimens with "*abundistic*" phenotype, and 4 adult "brown" individuals (Figure 1 and Table S1). In five specimens from Central Italy (RC56, RC46, RC47, RC50, and RC45) and in one specimen from North East Italy (RTR01), we found discordance between color phenotype and mtDNA clade. In fact, while these specimens presented a typical "*viridiflavus*" coloration, they belonged to the clade E. An opposite discordance ("*carbonarius*" coloration in snakes belonging to the clade W) was found in two samples from Tuscany (RT16 and RT23). Although the intermediate phenotype called "*abundistic*" had previously been described in some Tyrrhenian islands (Avella et al., 2017; Rato et al., 2009; Zuffi, 2008), we found individuals showing this color pattern also in central Italy (RL235, RMO01, and RMO03).

TABLE 1 Results of the neutrality tests (Tajima's D, Fu and Li's D and Fu and Li's F) performed for each gene (*ND4*, *POMC*, *RAG1*, and *MC1R*)

	ND4 (*n* = 225)	*POMC* (*n* = 222)	*RAG1* (*n* = 114)	*MC1R* (*n* = 50)
Tajima's D	2.078	−0.0377	0.666	0.87079
	$0.10 > p > 0.05$	$p > 0.10$	$p > 0.10$	$p > 0.10$
Fu and Li's D	−1.493	0.227	−0.29	0.7473
	$p > 0.10$	$p > 0.10$	$p > 0.10$	$p > 0.10$
Fu and Li's F	0.077	0.152	0.02	0.31303
	$p > 0.10$	$p > 0.10$	$p > 0.10$	$p > 0.10$

Note: All the *p*-values are higher than 0.05; therefore, none of the results is significant. The number of sequences used (*n*) is indicated for each gene.

Out of the 25 *MC1R* sequenced samples, 7 showed the "*viridiflavus*" phenotype, 16 the "*carbonarius*" and 2 the "*abundistic*." Only three haplotypes have been found for this gene, with one of them occurring at high frequency (98%) and shared between specimens with different coloration (Figure S1). However, it should be mentioned that only half of the *MC1R* was sequenced (588 bp instead of total 942 bp of coding sequence), and these results should be taken as preliminary.

Among the 111 *POMC* samples, 48 showed the "*viridiflavus*" phenotype, 47 the "*carbonarius*," 9 the "*abundistic*," and 7 were juveniles. This gene showed a total of 14 SNPs. Nine SNPs are due to low-frequency substitution, below 4% (with two singletons), while five SNPs occurred at high frequency: SNP123, SNP371, SNP562, SNP640, and SNP732. SNP123 and SNP 732 were eliminated in subsequent analysis since they cannot be involved in color determination. In fact, SNP123 (C/T) is a synonymous substitution of the *pro-γ-MSH* peptide. SNP732 (G/A), occurring in the 3' untranslated region, is only found in Sicily, where only melanic individuals are found, but is very rare in melanic populations of the Italian Peninsula, with the exception of two individuals from Central Italy. Three additional SNPs (371, 562, and 640) showed different frequencies between the two mtDNA clades (Table 2) and were, therefore, considered as candidate for color determination. SNP371 (A/G) determines an amino acid change (*H Histidin -R Arginin*) in the *β-MSH* peptide. Almost the totality of the "*carbonarius*" specimens (41 out of 47, 87.23%) carried the H (Histidin) aminoacid in homozygous condition, while 34 out of 48 "*viridiflavus*" specimens (70.83%) showed an R (Arginin) allele in homozygous or heterozygous condition. These proportions mirror those observed between the two mtDNA lineages and, as mentioned above, may be due to divergence by geographic isolation (Table 2). In the light of this result, we focused our attention on specimens showing a

TABLE 2 Numbers of specimens with different genotypes at three SNPs (SNP371, SNP562, and SNP640), in the *POMC* gene fragment, for the two studied mtDNA clades and for the two phenotypes "*viridiflavus*" and "*carbonarius*"

	SNP371			SNP562			SNP640		
	AA	AG	GG	CC	AC	AA	CC	CT	TT
mtDNA Clade W	12	16	31	23	8	28	7	8	44
mtDNA Clade E	48	3	1	51	1	0	47	5	0
"*viridiflavus*" phenotype	14	15	19	21	8	19	12	5	31
"*carbonarius*" phenotype	41	3	3	44	1	2	40	5	2

TABLE 3 Numbers of specimens with different genotypes at three SNPs (SNP371, SNP562, and SNP640), in the *POMC* gene fragment, for the specimens showing mtDNA/*POMC* discordance (i.e., specimens belonging to one mtDNA Clade but carrying a genotype at *POMC* fragment typical of the other mtDNA clade)

	SNP371			SNP562			SNP640		
	AA	AG	GG	CC	AC	AA	CC	CT	TT
mtDNA Clade W "*carbonarius*" phenotype	0	0	2	0	0	2	0	0	2
mtDNA Clade E "*viridiflavus*" phenotype	5	0	0	5	0	0	5	0	0

"mtDNA/SNP371 discordance" (Table 3). These specimens show the SNP371 congruent with the mtDNA clade rather than with the color morph. In particular, five "*viridiflavus*" specimens clustering with the Clade E (RTR01, RC56, RC47, RC50, and RC45) are homozygous for Histidin, and two "*carbonarius*" specimens clustering with Clade W (RT16 and RT23) are homozygous for Arginin.

SNP562 (C/A) is at the same nucleotide position of the SNP that Ducrest et al. (2014) found to be associated with different coloration patterns in the asp viper *V. aspis*. Almost the totality of the "*carbonarius*" specimens (44 out of 48, 91.66%) carried the CC genotype, while the AA and AC genotypes are found in 57% of the "*viridiflavus*" specimens (Table 2). Also in this case, when considering the specimens with mtDNA/SNP562 discordance, the substitution followed the mtDNA clade and not the color phenotype (Table 3).

Finally, SNP640 (C/T), found in the 3' untranslated region, mirrors the situation of the previous two SNPs. The CC genotype has been found in 40 out 47 "*carbonarius*" specimens and the TT genotypes in 31 out 48 "*viridiflavus*" specimens. In the specimens with mtDNA/SNP640 discordance, the substitution followed the mtDNA clade and not the color phenotype (Table 3).

The alignment of the β-*MSH* products among various snake species showed that in all of them the peptide is composed by 18 amino acids. Six variable positions have been observed. The changes SNP371 (A/G) in *H. viridiflavus* determine an amino acid change (*H Histidin - R Arginin*) adjacent 5' to the conserved motif H-F-R-W. This position is moderately conserved in snakes being an H in all the to date studied species with exception of *P. bivittatus*.

The dN/dS ratio of the β-*MSH* peptide was 3.256, and the Z test for selection returned no significant values (p = .61).

The prediction study of the secondary structure of the β-*MSH* peptide indicated a slight difference in structure, with an increase in the extended strand structure (55.5% vs. 38.9%) and a decrease in the random coil structure (44.4% vs. 61.1%) for the H- versus the R-carrying peptides, respectively.

3.2 | Mitochondrial and nuclear characterization at the contact zone

Among the 225 mtDNA sequences obtained, 109 were identified as belonging to *H. v. viridiflavus* (clade W) and 116 to *H. v. carbonarius* (clade E). We found a total of 28 haplotypes (of which 12 new), 8 belonging to the clade W and 20 to the clade E. Mitochondrial genetic diversity resulted to be higher in the clade E rather than in the clade W (Table 4). The distribution of the main clades confirms the one reported in previous works (Figure 3). However, the higher number of samples at our disposal allowed us to identify additional haplogroups. Indeed, within the clade E, three slightly differentiated haplogroups (E1, E2, and E3), restricted to three distinct geographic areas can be detected (Figure 3). Moreover, the more detailed sampling allowed the identification of a contact zone between the two main clades in central Italy (Figure 4).

Genetic diversity indices for the nuclear genes are reported in Table 4. *POMC* and *MC1R* showed higher values of nucleotide diversity in clade W while for RAG1 the diversity is higher in the E clade. The *MC1R* gene showed the presence of three haplotypes, with one (M2) representing 96% of the sample, while *RAG1* showed six haplotypes with shallow diversification. However, the *RAG1* gene exhibits a certain pattern in its distribution, with three haplotypes (R1, R4, and R5) more common in the E clade and the other three haplotypes (R2, R3, and R6) more frequently found

TABLE 4 Number of sequences obtained (*n*), number of haplotypes identified (*h*), nucleotide diversity with standard deviation ($\pi \pm SD$) and haplotype diversity with standard deviation ($H \pm SD$) for each gene, clade (W and E), and haplogroup (E1, E2, and E3)

Gene/Haplogroup	*n*	*h*	$\pi \pm SD$	$H \pm SD$
ND4/W	109	8	0.00026 ± 0.00010	0.125 ± 0.044
ND4/E	116	20	0.00493 ± 0.00034	0.744 ± 0.038
ND4/E1	16	2	0.00022 ± 0.00019	0.125 ± 0.106
ND4/E2	24	8	0.00191 ± 0.00040	0.721 ± 0.084
ND4/E3	76	10	0.00104 ± 0.00019	0.464 ± 0.068
POMC	222	21	0.00294 ± 0.00007	0.819 ± 0.015
POMC/W	118	12	0.00204 ± 0.00016	0.697 ± 0.038
POMC/E	104	16	0.00143 ± 0.00016	0.699 ± 0.042
RAG1	114	6	0.00113 ± 0.00006	0.733 ± 0.019
RAG1/W	70	5	0.00079 ± 0.00007	0.610 ± 0.031
RAG1/E	44	5	0.00137 ± 0.00009	0.755 ± 0.028
MC1R	50	3	0.00039 ± 0.00014	0.222 ± 0.075
MC1R/W	12	2	0.00082 ± 0.00018	0.485 ± 0.106
MC1R/E	38	2	0.00017 ± 0.00011	0.102 ± 0.065

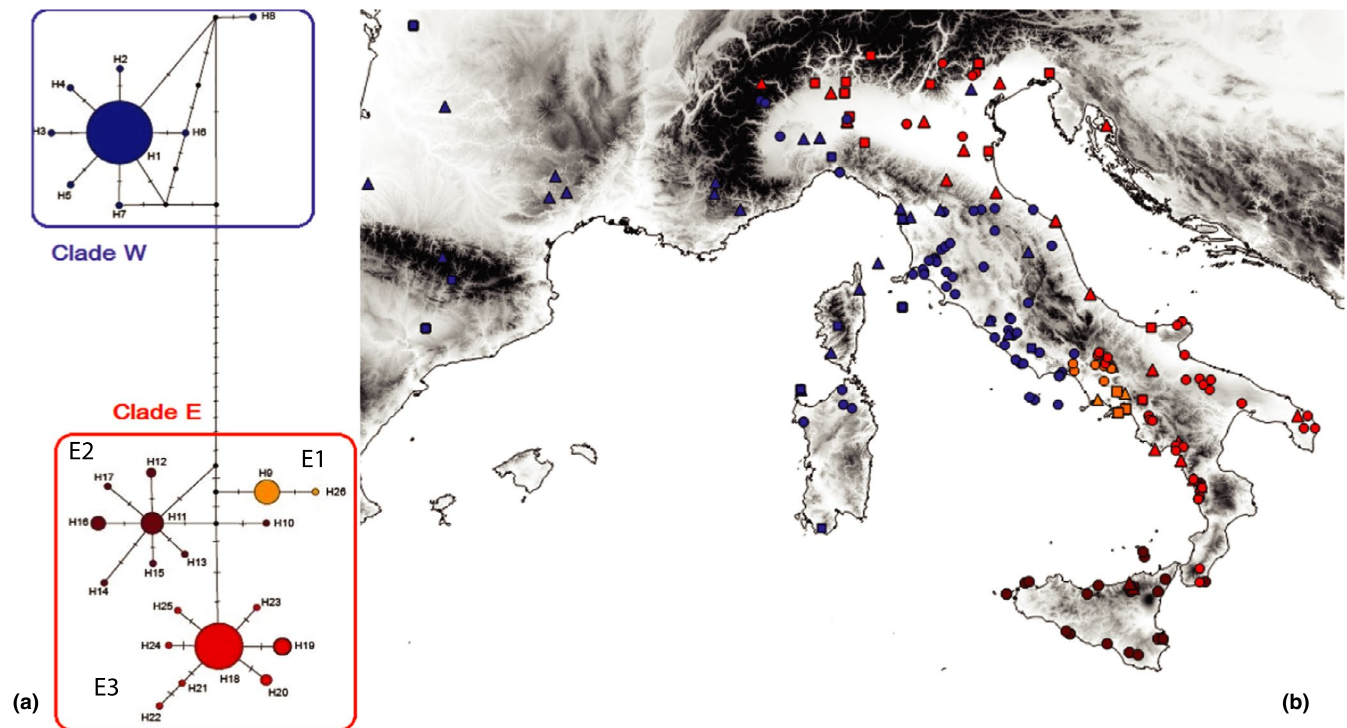

FIGURE 3 (a) *ND4* gene network with clades and haplogroups: Blue = clade W; Yellow = clade E (haplogroup E1); Brown = clade E (haplogroup E2); and Red = clade E (haplogroup E3). (b) Geographic distribution of the *ND4* haplogroups. Each symbol represents an individual, and the shape indicates the provenience of each sample: circles = original samples; squares = samples from Rato et al., 2009; triangles = samples from Mezzasalma et al., 2015

within the W clade (Figure 4b). On the other hand, the *POMC* gene showed a much higher variability, with 21 haplotypes recognized. For this gene, as for *RAG1*, differences in the distribution pattern can be observed, with some alleles being more frequent in one clade than in the other (Figure 4a). In particular, we found several heterozygous specimens in central Italy, localized in the contact area, showing one nuclear allele typical of clade E, and the other, typical of clade W.

The nuclear gene flow analyses returned comparable estimates from each independent run, showing the presence of gene exchange between the populations considered, either when considering the whole dataset or the contact zone dataset (Table 5). Although the

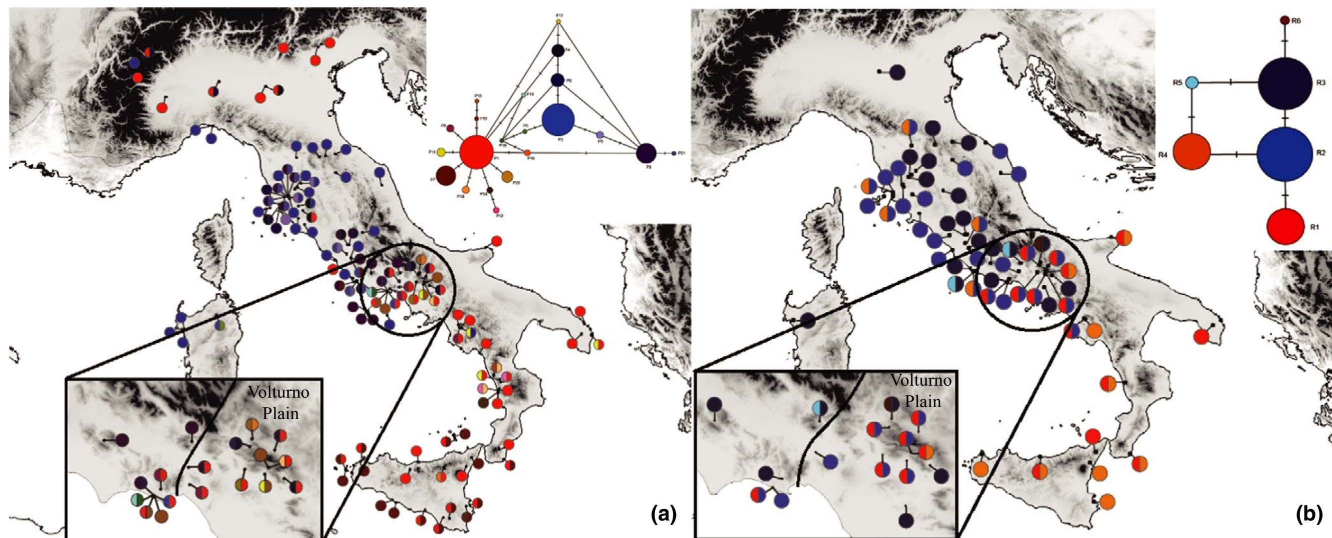

FIGURE 4 *POMC* (a) and *RAG1* (b) statistical parsimony networks and haplotype's geographic distribution with close up on the contact area. The size of the circles in the networks is proportional to allele frequencies. In the haplotype's geographic distribution map, each circle chart represents an individual with alleles colored according to the relative network

TABLE 5 Marginal likelihood, standard deviation and relative model probability for each of the tested gene flow scenario. Results are reported considering the whole dataset and the contact zone only

	W to E	E to W	No gene flow	Full migration model
Whole dataset				
Marginal likelihood ± *SD*	−2801.95 ± 0,511	−2814.79 ± 2.064	−2856.94 ± 4.270	−2801.21 ± 1.339
Model Probability	0.322	8.5308E−07	4.24021E−18	0.677
Contact zone dataset				
Marginal likelihood ± *SD*	−2524.07 ± 0.095	−2524.73 ± 0.043	−2567.91 ± 0.338	−2527.18 ± 1.887
Model Probability	0.640	3.310E−01	5.874E−14	0.028

"full migration" model showed higher model probabilities for the whole dataset, gene exchange from W clade to E clade showed higher probability when considering the two directional models only. Concerning the contact zone dataset, calculated model probabilities indicate migration from W clade to E clade as the most supported model of gene exchange (Table 5).

4 | DISCUSSION

Disentangling the nature of the processes driving allopatric differentiation and speciation is a crucial task in evolutionary biology. Among the main drivers of speciation are either historical or natural selection processes (Slavenko et al., 2016; Sobel et al., 2010; Wiens & Scholl, 2019). Under the first condition, a gradual separation driven by gene flow reduction might lead to speciation; under natural selection, on the other hand, only few genetic changes are usually required to ensure a complete reproductive isolation (Gavrilets, 2014). In this context, secondary contact zones represent natural laboratories to understand whether selection or, alternatively, neutrality is acting on genes thought to be involved in important phenotypic traits such as coloration. Our results indicate that the investigated target nuclear genes (*POMC* and *MC1R*) are not directly involved in color determination in *Hierophis viridiflavus*. This outcome is in contrast with what reported in previous works for other reptile species (Ducrest et al., 2014; Nunes et al., 2011; Rosenblum et al., 2004, 2010). Moreover, our comprehensive dataset allowed us to better define the overall distribution of the western whip snake clades/subspecies/taxa, and to provide a characterization of the contact zone by estimating the extent and the amount of nuclear gene exchange.

4.1 | Implication of candidate genes for coloration

Results from the analysis of the *MC1R* gene indicated no segregating substitutions between the two subspecies (Figure S1). This is in line with most studies carried out on this gene, since this marker has been shown to have a role in reptile color determination only in few reports (Nunes et al., 2011; Rosenblum et al., 2004, 2010). However, only half of this gene has been sequenced in this work and a role for coloration it cannot be completely ruled out. Concerning *POMC*, none of the three

identified candidate SNPs seems to be related to color polymorphism. This is highlighted by seven samples for which the mitochondrial clade does not coincide with the color phenotype. No association between target genes and color pattern is confirmed also for the "brown" phenotype found in Puglia, since we did not find any specific substitution in the two candidate nuclear genes. Therefore, the observed differences in *POMC* alleles may have followed the vicariance event determining the separation between *H. v. carbonarius* and *H. v. viridiflavus*. However, two interesting considerations need to be made about the identified *POMC* SNPs. Firstly, SNP562 is in the exact same position as the SNP found by Ducrest et al. (2014) in *V. aspis*, possibly indicating a highly polymorphic site, consequence of relaxed selection rather than a casual instance. Secondly, SNP371 determines an amino acid substitution (H/R) in the *β-MSH*. This hormone, as all the MSHs, is characterized by a common "core" structure, constituted by four amino acids (H-F-R-W), which facilitates binding to the melanocortin receptors (Kobayashi et al., 2007; Millington, 2006). SNP371 is adjacent to this conserved motif and its position is rather conserved in snakes, being the H present in almost all the studied species, with exception of *P. bivittatus*. Given the predicted secondary structure of the mutated 18-amino acid peptide, the H → R exchange could modify the secondary structure of the peptide, and being adjacent to the core melanocortin sequence H-F-R-W, we cannot exclude that this can alter the ligand's affinity for *MC4R*. It has long been known that genes responsible of differences in pigmentation have pleiotropic effects and are linked to physiological changes, such as an increase in aggression and sexual activity (Anderson et al., 2009; Ducrest et al., 2008; Raia et al., 2010; Roulin et al., 2010). In humans, *β-MSH* has been demonstrated to be the key ligand at the feeding-regulating MC4-R and that the inhibition of tonic release of β-MSH contributes to the insurgence of hunger in underfeeding conditions (Lee et al., 2006). For this reason, we can speculate that the H → R substitution observed at different frequencies in the two *Hierophis* subspecies may have a "physiological" effect, modifying the hormone's function. Field observations highlighted the overall better body conditions of melanistic western whip snakes, in terms of both size and weight (Luiselli, 1995). However, it is still unclear whether the larger size of melanistic individuals depends on faster growth rates or differential mortality rates between the two phenotypes. Therefore, although this genetic marker would seem not to be directly related to color determination processes, its role in other regulatory mechanisms cannot be ruled out (Gasparini et al., 2009; Jackson, 1999; Jacquin et al., 2011). Specific comparative functional studies of the two *β-MSH* peptides are required to verify potential physiological differences between individuals showing different phenotypes.

4.2 | Mitochondrial and nuclear characterization at the contact zone

Previous and present data indicate that the evolutionary history of the western whip snake is dominated by an allopatric scenario of divergence driven by both paleoclimatic conditions and different refugial areas along the Italian Peninsula (Mezzasalma et al., 2018; Nagy et al., 2002; Rato et al., 2009). The comprehensive dataset we produced allowed us to better define the genetic distribution of the two subspecies of *H. viridiflavus* currently recognized. Concerning mtDNA, we highlight that haplogroup E3, which includes only haplotypes from Sicily, shows a star-like shape, with no trace of geographic structuring. The shallow divergence of this haplogroup, together with the observed homogeneous pattern in Sicily, may reflect a recent (i.e., late Pleistocene to Holocene) founder effect due to a single dispersal event because of land bridge connections. Regarding the remaining two haplogroups, E1 shows a certain degree of local diversification, restricted to a small geographic portion of the Central West coast, while E2 shows a much wider distribution, spanning from Southern to North-Eastern Italy.

The mtDNA signature of *H. viridiflavus* showed a marked genetic discontinuity in Central Italy were W clade and E clade are parapatrically distributed. This area correspond to the Volturno plain (Figure 4), which represents an important region of genetic discontinuity at both intraspecific and interspecific level, as a consequence of the repeated marine transgressions occurred during Mid-Pleistocene (Barra et al., 1996; Canestrelli et al., 2008; Mattoccia et al., 2011; Romano et al., 1994; Senczuk et al., 2017, 2018; Solano et al., 2016, 2018). Convergent evidences from both mtDNA and nuDNA indicate a certain level of genetic permeability occurring in this contact zone. Indeed, we found five specimens (RC56, RC46, RC47, RC50, and RC45) which showed a typical "*viridiflavus*" coloration, they belonged to the mtDNA clade E. Moreover, while nuDNA showed alternative nearly fixed haplotypes in most of the distribution area an high number of heterozygous individuals were identified in the contact zone (Figure 4). This genetic pattern could be generated by several factors including sex-biased dispersal capabilities or differences in mate choice. For example, if ongoing sex-biased dispersal occurs, the outcome will be in a mito-nuclear discordance in the direction of the dispersing sex-linked genome (Toews & Brelsford, 2012). Thus, the pattern we observe could be achieved either by preferential hybridization of females of the E clade into males of the W clade, or alternatively by nuclear introgression of males of the W clade into females of the E clade. Although generally, male snakes disperse further than females (Keogh et al., 2007), we should assume different dispersal capabilities between males of the two clades, to explain the observed pattern. Such a scenario appears unrealistic to speculate and we have no evidence to support it. On the other hand, when we measured the nuclear gene flow between the two mtDNA clades, we found a significant amount of gene exchange when considering either the whole dataset or the dataset restricted to the contact zone only (Table 5). Moreover, when we tested for directional models of gene exchange, migration from W to E clades was the most supported scenario for the contact zone dataset. The same results are also supported for the whole dataset when considering the two directional models only. In recent studies focusing on the common wall lizard *Podarcis muralis*, asymmetric gene flow between different lineages in a secondary contact area has been associated with different levels of competitive ability of males displaying divergent color phenotypes (While et al., 2015;

Yang et al., 2018). Therefore, the presence of a comparable mechanism driving directional gene flow between the two main color patterns in *H. viridiflavus* cannot be ruled out a priori.

Finally, in support to the reported genetic permeability, it should be noted that intermediate phenotypes recalling the "*abundistic*," as described for some large (Sardinia and Corsica) and small (Pontine islands and Tuscan archipelagos) Mediterranean islands (Avella et al., 2017; Zuffi, 2008) are also present at the contact zone.

Future research efforts should focus on contact zones between *H. v. viridiflavus* and *H. v. carbonarius*, to assess whether ecological and behavioral aspects might be involved in driving asymmetric gene flow. Finally, genome-wide association studies would be desirable to better understand genetic mechanisms involved in the still widely unresolved topic of color determination in reptiles.

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AUTHOR CONTRIBUTIONS

G.S. and R.C. conceived and designed the study. R.C. provided funding. G.S., L.G., I.A., E.M., M.M., G.A., and R.C. contributed to the collection of samples. G.S., L.G., and I.A. generated the data and performed the analyses. G.S. drafted the manuscript. All authors revised and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GenBank at https://www.ncbi.nlm.nih.gov/genbank, reference numbers: MW297553–MW297679 (*ND4*), MW314382–MW314438 (*RAG1*), MW314439–MW314549 (*POMC*), and MW314550–MW314575 (*MC1R*).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Alignment S1. *ND4* alignment (568 bp) including 134 *ND4* original sequences and 91 sequences obtained from the NCBI portal (Mezzasalma et al., 2015; Rato et. al., 2009)
Alignment S2. *POMC* alignment (785 bp) including 222 phased sequences.
Alignment S3. *RAG1* alignment (899 bp) including 114 phased sequences.
Alignment S4. *MC1R* alignment (588 bp) including 50 phased sequences.
Table S1. List of the analysed samples with sampling localities, colour phenotype and haplotypes corresponding to those reported in the statistical parsimony networks for each analysed gene.
Table S2. Primers information with relative references for all the amplified fragments of the genes.
Figure S1. *MC1R* statistical parsimony network and relative haplotype's geographic distribution. The size of the circles in the network is proportional to allele frequencies.

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