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Barred, blotched or intermediate? A photographic-based survey on the spatial distribution of *Vipera aspis* phenotypes from central-southern Italy

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Variation in animal colouration, which holds ecological and evolutionary significance, is evident in snakes, including the polytypic and highly polymorphic asp viper *Vipera aspis*. This species, currently acknowledged with four subspecies, features three of them (*V. a. aspis, V. a. francisciredi* and *V. a. hugyi*) within the Italian territory, each displaying distinct dorsal patterns. Analysing 143 georeferenced photos from personal surveys and citizen science, the present study explores transition areas between *V. a. francisciredi* and *V. a. hugyi*, revealing the coexistence of three dorsal pattern phenotypes (i.e. *francisciredi, hugyi,* and intermediate) across central-southern Italy. The outcome of the work reveals occurrences of *francisciredi* phenotypes considerably beyond the southern limit indicated in recent literature. Remarkably, intermediate phenotypes cover a significant portion of southern Italy, including Apulia (three records), Basilicata (six records) and Campania (three records) regions, and overlapping with both *hugyi* and *francisciredi* ranges. These findings suggest asymmetrical gene flow from northern to southern populations, resembling patterns observed in other reptiles. The intricate distribution of colour phenotypes underscores the necessity for molecular investigations to comprehend the genetic context and evolutionary history of *V. aspis* populations from central-southern Italy.

Key words: admixture; asp viper; *francisciredi*; hybridization; *hugyi*; transition.

Colour and pattern variation in animals holds diverse ecological and evolutionary significance (Stuart-Fox & Moussalli, 2009). This can be categorised into several key aspects, including communication (Galan, 2000; Ligon & McGraw, 2013), camouflage (Marshall *et al.*, 2016; Troscianko *et al.*, 2016), and thermoregulation (Vences *et al.*, 2002; Clusella-Trullas *et al.*, 2007). Similar to numerous other taxa,

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polychromatism is a recurring phenomenon among snakes (Bechtel, 1978, and references therein; Wolf & Werner, 1994; Regnet *et al.*, 2022) and is often considered an evolutionary advantage associated with a broad array of adaptive options (Pizzatto & Dubey, 2012; Forsman, 2016). Intraspecific colour and pattern variations may manifest within populations (Duarte *et al.*, 2015; Reynolds *et al.*, 2020; Di Nicola *et al.*, 2023) and between populations, potentially exhibiting a geographic pattern (Sweet, 1985; Zuffi, 2008; Lattanzio & Buontempo, 2021; Storniolo *et al.*, 2023). The intraspecific geographical variation in colour among snakes has traditionally contributed to taxonomic implications over time (Cope, 1900; Cattaneo, 1985; Geniez *et al.*, 2006). In several cases, these implications have been supported by molecular or phylogenetic approaches (e.g. Fritz & Ihlow, 2022; Storniolo *et al.*, 2023), while in other contexts no correspondences have emerged (e.g. Ursenbacher et al., 2006; Faraone *et al.* 2020).

The asp viper *Vipera aspis* (Linnaeus, 1758) is a polytypic and highly polymorphic species and four subspecies are currently recognised, both supported by morphological (Zuffi & Bonnet, 1999; Zuffi, 2014) and genetic inferences (Ursenbacher *et al.*, 2006; Barbanera *et al.*, 2009): *Vipera aspis aspis* (Linnaeus, 1758); *Vipera aspis francisciredi* Laurenti, 1768; *Vipera aspis hugyi* Schinz, 1833; and *Vipera aspis zinnikeri* Kramer, 1958. The three former subspecies are present in Italy (Zuffi *et al.*, 2011; Di Nicola *et al.*, 2021; Sindaco & Razzetti, 2021) and their distributional ranges and general dorsal colour variation patterns are detailed below.

Vipera aspis aspis (formerly known as ssp. *atra*, Meisner, 1820 in its Italian populations) is widespread in the Italian, French and Swiss western Alps, as well as in a large part of France (Zuffi, 2002; Ursenbacher *et al.*, 2006). *Vipera a. aspis* exhibits a highly variable dorsal pattern (Mebert *et al.*, 2011; Dubey *et al.*, 2015), which can include transverse bars, rounded blotches or none of these designs, particularly in patternless and melanic specimens (Mebert *et al.*, 2011; Tessa, 2016).

Vipera aspis francisciredi is widespread from central Italy to the central-eastern Italian Alps, with some populations in Switzerland and Slovenia (Kumar, 2009; Di Nicola *et al.* 2021). The predominant dorsal pattern of *V. a. francisciredi* consists of four transverse series of dark bars, offset from each other, with two on the back and two on the sides (Bruno, 1985; De Smedt, 2006). Melanic individuals of *V. a. francisciredi* have been documented in central Italy (Di Nicola *et al.*, 2024).

Vipera aspis hugyi is endemic to southern Italy and Sicily, being prevalent in Campania, Apulia, Basilicata, Calabria and Sicily as well as on Montecristo Island (Tuscan Archipelago), where it was introduced in historical times (Masseti & Zuffi, 2011; Di Nicola *et al.*, 2021). The dorsal pattern of this subspecies is highly distinctive, characterised by a vertebral series of rounded blotches and offset series of dark circles on the flanks (Zuffi *et al.*, 2011). Patternless and melanic individuals of *V. a. hugyi* have been documented (Di Nicola & Meier, 2013; Di Nicola & Faraone, 2020).

The examination of intergradation or contact zones between different taxa requires substantial sampling efforts and the management of extensive datasets. Despite the challenges involved, such endeavours frequently yield compelling results. This holds true also for snakes, including vipers, where investigations at both interspecific and intraspecific levels have revealed the presence of intermediate phenotypes and varying degrees of gene flow (e.g. MARTÍNEZ-FREIRÍA *et al.*, 2009; GUILLER *et al.*, 2017; SALVI *et al.*, 2017; SCHULTZE *et al.*, 2019; ASZTALOS *et al.*, 2020; ZWAHLEN *et al.* 2022; MEIER *et al.*, 2023). The delineation of *V. aspis* subspecies boundaries in Italy has been a focal point of study, approached with varying degrees of specificity. The transition area between *V. a. aspis* and *V. a. francisciredi* in northern Italy has been specifically described using morphological criteria (BRUNO, 1985; ZUFFI & BONNET, 1999; ZUFFI *et al.*, 2002), and subsequently confirmed by mitochondrial markers (URSENBACHER *et al.*, 2006; BARBANERA *et al.*, 2009). It roughly corresponds to the border between Lombardy and Piedmont. The transition area between *V. a. francisciredi* and *V. a. hugyi* is currently defined as a line that approximately extends from the Mount Vesuvius (central Campania, Tyrrhenian coast) to the northern slopes of the Gargano promontory (Apulia, Adriatic coast) (LANZA & VANNI, 1989; ZUFFI & BONNET, 1999; BARBANERA *et al.*, 2009). Although without providing specific data or mapping, BRUNO (1985) previously asserted that *V. a. francisciredi* extends even farther south, encompassing the Cilento mountains in southern Campania. BARBANERA *et al.* (2009) provided genetic support for the existence of *V. a. francisciredi* and *V. a. hugyi* morphotypes. BARBANERA *et al.* (2009) showed that nuclear markers (microsatellites) fully support *V. a. francisciredi* and *V. a. hugyi* morphotypes, while mitochondrial markers highlight the paraphyletic status of the latter morphotype, probably due to ancient, asymmetrical introgression processes between these subspecies. However, the sampling coverage in that study was insufficient to precisely define the transition area between these two subspecies.

Currently, citizen science represents a significant opportunity for collecting a large amount of high-quality data. As a result, it is widely employed in zoology and ecology (FARAONE *et al.*, 2017; HACKLAY *et al.*, 2021), including various studies on snakes (e.g. FRITZ & IHLOW, 2022; DI NICOLA *et al.*, 2022, 2023; STORNIOLO *et al.*, 2023). In this study, we adopted the citizen science approach to achieve two main objectives. Firstly, we employed a colour phenotype-based methodology to deduce the transition area between *V. a. francisciredi* and *V. a. hugyi* subspecies in southern Italy. This approach aimed to compare our findings with those traditionally reported in the literature, expecting a more comprehensive spatial coverage and aiming to establish a more accurate depiction of this contact area. Subsequently, our focus turned to identifying the distribution of intermediate phenotypes and examining the extent of their overlap with the geographic ranges of the two subspecies. The outcomes of our study are expected to contribute to a clearer understanding, encouraging further examination through detailed morphological analysis and biomolecular surveys specifically targeted at the intergradation zone between *V. a. francisciredi* and *V. a. hugyi*.

Materials and Methods

Data collection and pattern evaluation

The central-southern Italy range where to investigate the contact and admixture among *Vipera aspis* phenotypes approximately coincides with the territories of central and southern Campania, Basilicata and northern Apulia. This area was identified based on our own experience and the boundaries between *V. a. francisciredi* and *V. a. hugyi* subspecies as indicated in the literature (Zuffi & Bonnet, 1999; Zuffi, 2002). Additionally, the genetic data provided by Barbanera *et al.* (2009), i.e. the contact zone between clades B and D shown in Fig. 2 of that study, were considered, despite their sample coverage was very limited in the territories potentially affected by the contact of the two subspecies.

The precise distribution limits of *V. a. francisciredi* towards the south and *V. a. hugyi* towards the north are not definitively established (Golay *et al.*, 2008). Consequently, two northern and southern "buffer" areas were incorporated into the investigated range, where only the presence of the expected dorsal pattern phenotype was ascertained. This approach allowed to avoid the exclusion of any eligible records outside the initially hypothesised range. The designated area was arbitrarily delimited to the north by a straight line between the coordinates 42.4301°N, 14.2779°E and 41.3821°N, 12.9193°E (in the southern sectors of Lazio and Abruzzo) and to the south by the coastal borders of mainland Apulia and Calabria.

Georeferenced photos of *Vipera aspis* within the established range (n = 143) were obtained from personal (8%; n = 12) and third-party (92%; n = 131) contributions. Third-party photos were obtained in November 2023 through direct request to collaborators and members of the Facebook group *Identificazione Anfibi e Rettili* (https://www.facebook.com/groups/283231695476830) (49%; n = 70). Additional pictures were retrieved from the citizen science social network iNaturalist.org (43%; n = 61). Personal or directly requested records were considered eligible if they included exact coordinates, photos of sufficient quality (i.e. in which the dorsal pattern of at least half of the animal's anterior half was visible), and information about the observation period. Records obtained from iNaturalist.org were considered eligible if they met the criteria for research-grade observations, which includes having photos, date, and coordinates (with an accuracy of 5 km), and receiving community consensus on identification.

An expert-based assessment of dorsal patterns through photographs was conducted to discriminate between the *francisciredi* (barred, Fig. 1A), *hugyi* (blotched, Fig. 1B), and intermediate (Fig. 1C) phenotypes. The phenotypes were assessed as either *francisciredi* or *hugyi* based on the literature (e.g. Bruno, 1985; De Smedt, 2006; Zuffi *et al.* 2011; Di Nicola, 2019; Di Nicola *et al.* 2021). The classification as intermediate was assigned when the dorsal pattern exhibited, for over half the length of the animal, a design considered intermediate between the first two phenotypes (i.e. quadrangular dorsal blotches showing a geometric shape with marked angles, which therefore are clearly distinguished from the rounded ones typical of

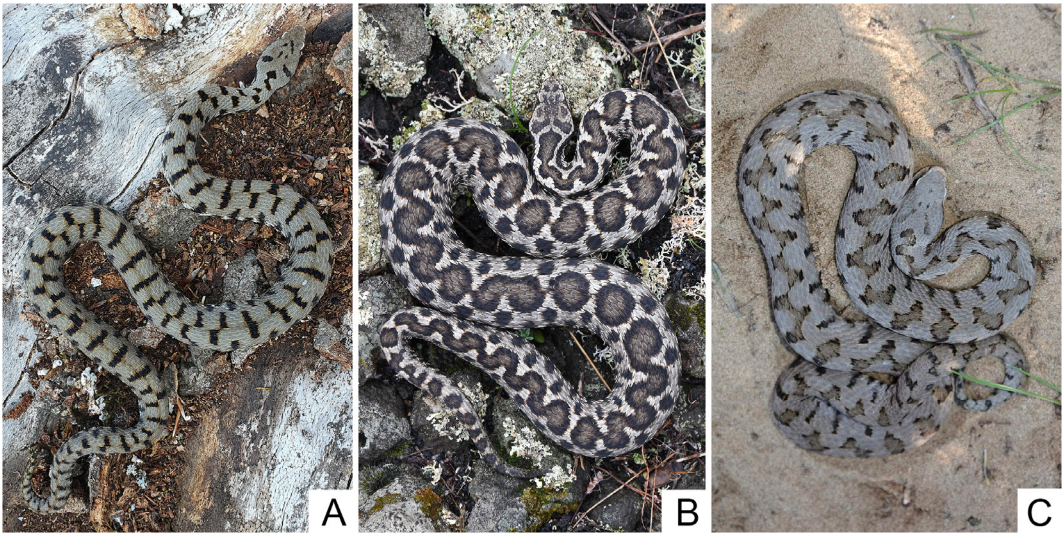

Figure 1: Examples of dorsal patterns in (A) *Vipera aspis francisciredi*, (B) *V. a. hugyi*, and (C) in an individual with an intermediate phenotype. Photo credits: Matteo Riccardo Di Nicola (A, B); Laura Ricci (C).

hugyi phenotypes and from the transversal bars —albeit of varying thickness and, in some cases, more or less fused together— of *francisciredi*; Fig. 1). The dorsal ground colour was not considered, as it is not adequately indicative of a given phenotype. Additionally, it may vary based on the moulting state and is not reliably measurable without standardised photographs.

Each of the four authors independently assessed the 143 pictures according to dorsal pattern phenotypes, and not knowing any information about the geographical location of each record; by doing so we limited any *a priori* bias in image classification associated with the source of records (i.e. putative association between phenotype and mitochondrial lineage). A collective evaluation was then carried out to address any discordant data until consensus was reached for all records.

Mapping and spatial analysis

After classification, all photos were matched with their coordinates *a posteriori* to avoid any bias in phenotypic assignment induced by geographic location of each record. Subsequently, for each phenotype, we computed the convex hull to contain all points and estimated the covered area via the *chull* function of *grDevices* base R package. These hulls were then used to build georeferenced spatial polygons via the *spPolygons* function of the *raster* package (HIJMANS, 2023a). Upon visual inspections, polygons overlapped with marine areas, so we restricted the polygons to mainland via the *intersect* function of the *raster* package.

We intersected polygons and calculated the percentage and area of overlap between distributional ranges of phenotypes.

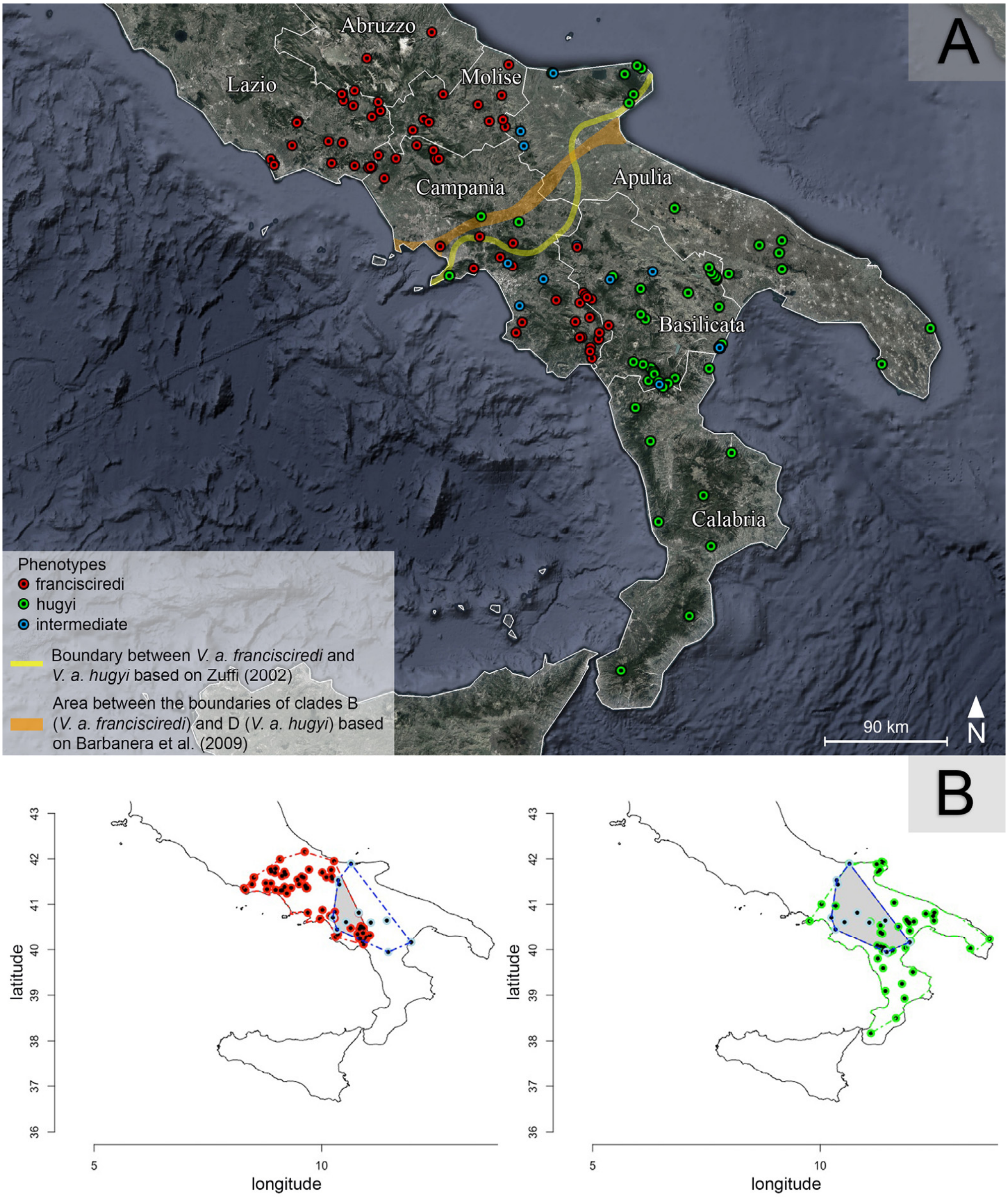

Figure 2: Maps of central and southern mainland Italy with the distribution of the 143 *Vipera aspis* individuals included in the study, differentiated by phenotype. (A) Spatial distribution of records of each *V. aspis* phenotype in southern Italy, including boundaries between the subspecies *V. a. francisciredi* and *V. a. hugyi* reported by previous studies. (B) Convex hulls computed to encompass all records from each phenotype (see legend in part A for colour coding), including intersections between hulls shaded in grey (*intermediate* and *francisciredi* phenotypes in the left panel; *hugyi* and intermediate phenotypes in the right panel). Map credit: Google Earth [Data SIO, NOAA, U.S. Navy, NGA, GEBCO Image Landsat / Copernicus], modified.

Table 1: *Vipera aspis* phenotypes divided by region. Percentages in the column of total count of records are relative to the distribution of records by regions, whereas percentages in the phenotype columns are relative to the distribution by phenotypes within each region.

Region	N of records	*franciscired*i	*hugyi*	intermediate
Abruzzo	3 (2.1%)	3 (100%)	0	0
Apulia	18 (12.6%)	1 (5.6%)	14 (77.8%)	3 (16.7%)
Basilicata	42 (29.4%)	6 (14.3%)	30 (71.4%)	6 (14.3%)
Calabria	9 (6.3%)	0	9 (100%)	0
Campania	35 (24.4%)	29 (82.8%)	3 (8.6%)	3 (8.6%)
Lazio	27 (18.9%)	27 (100%)	0	0
Molise	9 (6.3%)	9 (100%)	0	0

It must be noticed, however, that because the study was restricted to central-southern Italy, the range sampled for *franciscired*i and *hugyi* did not correspond to their whole distribution, which spreads beyond the area considered in the present study. Areas of convex hulls and polygons were calculated using the *area* function of the *terra* package (HIJMANS, 2023b). All spatial analyses were performed in the R version 4.2.2 (R CORE TEAM, 2022).

RESULTS

From the dorsal pattern evaluation of the 143 photos, 75 franciscired phenotypes (52.4%), 56 hugyi phenotypes (39.2%), and 12 intermediate phenotypes (8.4%) were identified. The distribution of records, separated by phenotype, is shown in Fig. 2A and regionally summarised in Table 1. Details for each record are shown in Table S1.

The distribution of phenotypes was checked against the distributional range of the respective subspecies, namely *V. a. franciscired*i and *V. a. hugyi*. While the distribution of *hugyi* phenotypes generally aligned with its distributional range, with the exception of six records in the northern portion of Gargano peninsula (representing 10.7% of the records from this phenotype; Fig. 2A), a significant portion of the observed records of *franciscired*i phenotypes (26 out of 71, 34.6%; Fig. 2A) extended beyond the putative contact zone between subspecies in central and southern Campania, where all phenotypes occurred simultaneously.

After constructing the convex hulls for each phenotype, the areas were calculated. Firstly, the area of intersection between *franciscired*i and *hugyi* within the region considered in the present study (i.e. central -southern Italy) covered 8348.04 km^2. Secondly, we focused on the distribution range of intermediate phenotypes, which encompassed an area of 15 777.8 km^2 in southern Italy and occurred within the regions of Apulia, Basilicata, Campania and Calabria (Fig. 2B). This range partially overlapped with that of *franciscired*i phenotypes in an area of 5456.03 km^2, corresponding to 34.6% of the total distribution range of intermediate phenotypes, and largely overlapped with that of *hugyi* phenotypes in an area of 15 749.31 km^2, which corresponded to 99.8% of the total intermediate phenotype range. Lastly, we calculated the area where only the two non-intermediate phenotypes overlapped by subtracting the area of overlap among the three phenotypes from the area of overlap of *franciscíredi* and *hugyi*, obtaining a surface area of 2892 km^2.

DISCUSSION

The analyses conducted in this study revealed the coexistence of three distinct *V. aspis* dorsal pattern phenotypes in central-southern Italy (see Fig. 2A). In detail, the *francisciredi* phenotype occurred mainly in the northernmost part of the survey area in the regions of Lazio, Molise, Marche and Campania; nevertheless, a cluster of *francisciredi* phenotypes was detected in southern Campania (central-west of the study area), where all phenotypes occurred. Meanwhile, *hugyi* phenotypes were limited to Apulia, Basilicata and Calabria, as expected. Interestingly, 8.4% of total records were intermediate phenotypes; their range covered a remarkable portion of southern Italy (15 777.8 km^2) and largely overlapped with that of *hugyi* phenotypes. A small portion of Campania coast and inland areas showed a mixture of phenotypes that included only non-intermediate ones.

From the phylogenetic perspective, the investigation by BARBANERA *et al.* (2009) showed a discordance between mitochondrial and nuclear DNA in the delimitation of morphological subspecies. As regards to southern Italy, only the nuclear markers aligned with the morphological identity of the subspecies, while the mitochondrial ones indicated a large, ancient introgression.

Dorsal colourations in the asp viper are markedly variable among clades as well as among populations, especially in the *aspis* and *francisciredi* subspecies (BRUNO, 1985; ZUFFI & BONNET, 1999; ZUFFI *et al.*, 2011; DI NICOLA *et al.*, 2021). The ecological significance of dorsal colouration in reptiles has been extensively investigated, particularly in vipers. The evolution of colourations and patterns in these snakes is frequently attributed to selective forces such as predator avoidance (NISKANEN & MAPPES, 2005) or thermoregulation (ANDRÉN & NILSON, 1981). For instance, dorsal patterns often consist of disruptive ornamentations that allow snakes to be concealed by background matching (SANTOS *et al.*, 2014) and thus boost fitness in terms of long-time survivorship. On the other hand, survivorship in squamates is also favoured by thermal efficiency, which is hypothesised to depend on increased melanisation (Thermal Melanism Hypothesis as in CLUSELLA-TRULLAS *et al.*, 2007). MARTÍNEZ-FREIRÍA *et al.* (2020) showed the ecological benefits associated with the darkening of dorsal patterns in vipers. The authors found that darkening was evolutionarily related to colder climatic conditions and thus were favoured to compensate sub-optimal conditions. Additionally, thermal efficiency significantly affects performance (ZAMORA-CAMACHO *et al.*, 2015) and immunocompetence (HUYGHE *et al.*, 2010), hence an increase in dorsal darkening is expected to be positively selected over time. The *hugyi* phenotype is characterised by broad dark dorsal blotches that might therefore boost thermal efficiency and thus be under positive selection. However, the potentially favoured phenotype occurred only in southernmost Italy, where climatic conditions are expected to be less stringent towards ectotherms, with higher average temperatures and less seasonality compared to other parts of the country (Fratianni & Acquaotta, 2017; Pinna, 2017). Furthermore, under this hypothesis, melanistic phenotypes should have been more frequent in the northernmost portion of the species range, where instead melanic vipers occur sporadically, mainly clustered in some mountain areas of central and northern Italy, and never predominant compared to standard phenotypes (Castella *et al.*, 2013; Di Nicola *et al.*, 2024). Hence, the thermal melanism hypothesis in this geographic context seems unsuitable to justify the spatial distribution of phenotypes.

Irrespective of climate and geography, *V. aspis* phenotypes are always cryptic and disruptive as they are normally characterised by geometric shapes such as blotches or bars (as it commonly occurs in *V. berus* for instance; Andrén & Nilson, 1981) that increase background matching against predators alongside secretive behaviours, suggesting that the major driving force of dorsal ornamentations in this species is predator avoidance by camouflage (e.g. Andrén & Nilson, 1981; Santos *et al.*, 2014; Pizzigalli *et al.*, 2020). Consequently, the occurrence of *francisciredi* phenotypes beyond the contact zone between the two subspecies hypothesised in the works of Zuffi and colleagues (e.g. Zuffi, 2002; Barbanera *et al.*, 2009), as well as the occurrence of intermediate ones, should reasonably be addressed to an alternative phenomenon to either camouflage or thermal ecology. For instance, the evolutionary history of a species, namely the divergence between different lineages over time, is often tightly related to phenotypic differentiation (Pfennig *et al.*, 2010; Derryberry *et al.*, 2011; Lee *et al.*, 2016; Zamudio *et al.*, 2016). In our case, the secondary contact between the two lineages might be causing multiple gene flow events that results in admixed populations showing phenotypes differing from those typically observed for *hugyi*, according to what was already stated by Barbanera *et al.* (2009). Interestingly, no *hugyi* and intermediate phenotypes were detected north to the contact zone, suggesting that gene flow, if any, might be asymmetrical from northern to southern populations of *V. aspis* as showed by Barbanera *et al.* (2009). Similar patterns of mito-nuclear discordance are known in other species (e.g. Yang *et al.*, 2020; Meier *et al.*, 2023), and have taken place also in the area considered here, sometimes with asymmetric introgression in opposite direction (Bisconti *et al.*, 2018; Schultze *et al.*, 2020).

In conclusion, the evidence we collected concerning the spatial distribution of dorsal pattern phenotypes in the asp viper from southern Italy described a complex geographical pattern compared to that of mtDNA lineages, so that multiple phenotypes were detected across the contact zone between *V. a. francisciredi* and *V. a. hugyi* subspecies. In detail, *hugyi* phenotypes almost exclusively occurred within the traditional range of their subspecies, while *francisciredi* ones were also found beyond the contact zone, expanding up to southern Campania. Another relevant finding revealed the occurrence of intermediate phenotypes that overlapped with both *francisciredi* and *hugyi* ones, and almost entirely southward of the contact zone. The area in Campania where both non-intermediate phenotypes occurred without the detected presence of intermediate phenotypes, which encompassed an extension of 2892 km^2, must be considered a focal area for future investigations to confirm the apparent absence of intermediate phenotypes, or alternatively to detect currently undetected individuals showing this phenotype. In this context, acknowledging that the phenotypic variability in dorsal patterns of *V. aspis* is marked, and that an approach based on citizen science data provides only preliminary information on such variability, a field investigation implementing a photographic standardised data collection, analysed under a quantitative approach, appears necessary to broaden the scope of our work. Additionally, in depth molecular investigations on individuals exhibiting each dorsal pattern phenotype seem crucial to assess whether the spatial pattern we observed concerning phenotypes reflects the genetic history of *V. aspis* populations at both the nuclear and mitochondrial level. This approach should aim to reconstruct the evolutionary history that has shaped the current distribution of phenotypes among these populations.

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