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Pro-opiomelanocortin gene and melanin-based colour polymorphism in a reptile

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Colour polymorphism is widespread among vertebrates and plays important roles in prey–predator interactions, thermoregulation, social competition, and sexual selection. However, the genetic mechanisms involved in colour variation have been studied mainly in domestic mammals and birds, whereas information on wild animals remains scarce. Interestingly, the pro-opiomelanocortin gene (*POMC*) gives rise to melanocortin hormones that trigger melanogenesis (by binding the melanocortin-1-receptor; Mc1r) and other physiological and behavioural functions (by binding the melanocortin receptors Mc1-5rs). Owing to its pleiotropic effect, the *POMC* gene could therefore account for the numerous covariations between pigmentation and other phenotypic traits. We screened the *POMC* and *Mc1r* genes in 107 wild asp vipers (*Vipera aspis*) that can exhibit four discrete colour morphs (two unpatterned morphs: concolor or melanistic; two patterned morphs: blotched or lined) in a single population. Our study revealed a correlation between a single nucleotide polymorphism situated within the 3′-untranslated region of the *POMC* gene and colour variation, whereas *Mc1r* was not found to be polymorphic. To the best of our knowledge, we disclose for the first time a relationship between a mutation at the *POMC* gene and coloration in a wild animal, as well as a correlation between a genetic marker and coloration in a snake species. Interestingly, similar mutations within the *POMC* 3′-untranslated region are linked to human obesity and alcohol and drug dependence. Combined with our results, this suggests that the 3′-untranslated region of the *POMC* gene may play a role in its regulation in distant vertebrates. *Biological Journal of the Linnean Society*, 2014, **111**, 160–168.

ADDITIONAL KEYWORDS: *Mc1r* – melanocortin-1-receptor gene – melanogenesis – *POMC* – snake – *Vipera aspis*.

INTRODUCTION

Intraspecific melanin-based colour polymorphism is widespread among vertebrates and plays crucial roles in social competition, sexual selection, prey–predator interactions, and thermoregulation (Hoffman & Blouin, 2000; Roulin, 2004; Clusella-Trullas, van Wyk & Spotila, 2007; Caro, 2009). However, the genetic mechanisms involved in colour variation have been studied mainly in humans and domestic animals (Lyon *et al*., 1992; Lu *et al*., 1994; Krude *et al*., 1998; Lin & Fisher, 2007; Trut, Oskina & Kharlamova, 2009; Guo *et al*., 2010) and the few studies carried out in wild organisms have usually focused on the melanocortin-1-receptor (*Mc1r*), a gene with major effects on coloration, but very few pleiotropic effects (Ducrest, Keller & Roulin, 2008; Hubbard *et al*., 2010).

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Therefore, information on wild animals remains scarce (Vage *et al*., 1997; Rosenblum, Hoekstra & Nachman, 2004; Gross, Borowsky & Tabin, 2009; Rosenblum *et al*., 2010), whereas it is fundamental for unravelling the mechanisms involved in the evolution of colour polymorphism (McKinnon & Pierotti, 2010). Indeed, genes of the melanogenesis pathway, particularly those of the melanocortin system, are known for their pleiotropic effects in various vertebrate taxa. Hence, such genes might be responsible for the observed association between melanin-based coloration, physiology, behaviour, and life-history traits (Ducrest *et al*., 2008). For example, in a number of vertebrates, coloration is known to be associated with multiple traits such as reproductive behaviour, fitness, body size, hormone levels, aggressiveness, anti-predator behaviour, and humoral immunocompetence (Endler, 1995; Roulin, 2004; McKinnon & Pierotti, 2010; Roulin & Ducrest, 2011; Kittilsen *et al*., 2012; for an example showing that colour polymorphism was not found to be associated with life-history traits, see Cooke, Rockwell & Lank, 1995).

In vertebrates, the melanogenic pro-opiomelanocortin (*POMC*) gene encodes the melanocyte-stimulating hormones (α, β, γ-MSH), the adrenocorticotrophic hormones (ACTH) and endorphins (α, β-endorphin). In tetrapods, the *POMC* gene is highly conserved and its tissue distribution and functions are similar across species (Schiöth *et al*., 2005; Kobayashi *et al*., 2007). In ectothermic tetrapods, *POMC* was sequenced in brown tree snakes (*Boiga irregularis*), American alligators (*Alligator mississippiensis*), and turtles (Kobayashi *et al*., 2007; Becker, Valverde & Crother, 2011), and contains α-, β- and γ-MSH as well as β-endorphin (Kobayashi *et al*., 2007). The *POMC* gene is also found in ectothermic vertebrates such as fish and invertebrates consisting of coding regions for theee MSHs and one endorphin (Salzet *et al*., 1997; Stefano, Salzet-Raveillon & Salzet, 1999; Takahashi *et al*., 2006). The melanocortin peptides can bind five different melanocortin receptors (Mc1-5Rs) with α-MSH binding Mc1r, triggering the production of eumelanin. In mammals, McRs have preference for the MSHs, whereas, in birds and fishes, they have higher affinity for ACTH-derived peptides (Klovins *et al*., 2004; Ling *et al*., 2004). Because of the central role of *POMC* in melanogenesis and its multiple pleiotropic effects, as well as in the interaction between α-MSH and Mc1r, these two genes should be simultaneously considered in studies focusing on the genetic mechanisms of melanin-based coloration.

In the present study, we screened the *POMC* and *Mc1r* genes in wild individuals of a snake species, the polymorphic European asp viper (*Vipera aspis*). Its abundance and wide distribution in Europe have made this species one of the most intensively studied reptiles in the Northern Hemisphere (Monney, Luiselli & Capula, 1996; Bonnet *et al*., 1998; Lourdais *et al*., 2004; Ursenbacher *et al*., 2006; Zuffi *et al*., 2009). This species is considered to be one of the most chromatically variable snakes in the world (Phisalix, 1968; Brodmann, 1987). The viper occurs over a wide altitudinal gradient throughout its range, from sea level to more than 2000 m a.s.l. (Meyer, Zumbach & Schmidt, 2009) with dorsal coloration varying among as well as within populations. For example, in some high elevation populations or in humid forested habitats, up to 50% of individuals are melanistic, whereas blotched individuals (possibly more cryptic; Andrén & Nilson, 1981; fig. 1) are more frequent in other populations (Monney *et al*., 1996). Several studies have hypothesized that melanistic coloration in such taxa is not only an adaptation to enhance rates of heat transfer during basking, but also plays a role in predator–prey interactions (Andrén & Nilson, 1981; Monney *et al*., 1996; Clusella-Trullas *et al*., 2007). Aside from these two colour morphs, concolor (uniformly light coloured) and lined individuals are found in few montane populations, where they can be locally abundant with up to 50% of concolor and lined individuals (Mebert *et al*., 2011). For these reasons, this species appears to be an appropriate model system for studying the evolution of colour polymorphism in wild organisms.

MATERIAL AND METHODS

Sampling

From 2000 to 2012, we collected DNA samples from buccal swabs of 107 adult asp vipers (*Vipera aspis aspis*) over a geographical continuum (160 × 160 km^2) encompassing central and western Switzerland and the Mont Blanc Massif area in France (next to the Swiss border; from 350 to 2100 m a.s.l.). Four different colour morphs (Fig. 1) co-occur in this region: two patterned (blotched: $N = 42$; lined: $N = 13$) and two patternless (melanistic: $N = 26$; concolor: $N = 26$; for more details on the geographical location of samples, see Supporting information, Table S1). Concolor and lined individuals are uncommon and only found in populations of the Mont Blanc Massif. Several individuals scored the phenotype of snakes simultaneously and none of the 107 individuals used in the present study had an ambiguous phenotype.

Monney *et al*. (1996) found that individuals are not sexually dimorphic in terms of colour morph (in Swiss populations characterized by melanistic and blotched individuals). In addition, based on a total of 253 observations made around the Mont Blanc Massif, where the four colour morphs co-occur

Figure 1. Colour polymorphism in the asp viper (*Vipera aspis*; A, concolor; B, lined; C, blotched, D, melanistic).

(S. Ursenbacher, J. C. Monney, P. Golay and K. Mebert, unpubl. data; blotched: $N = 194$; lined: $N = 19$; concolor: $N = 45$; melanistic: $N = 26$), we found no significant sex bias in morph frequency ($\chi^2 = 12.32$; $P = 0.20$).

According to a previous phylogeographical study, all our sampling locations are situated within the distribution of a single mitochondrial lineage (1B), strictly represented by the nominal subspecies, and where only one haplotype has been detected (H1), as a result of a recent postglacial recolonization of the Alps (Ursenbacher *et al.*, 2006). Our population of vipers is therefore genetically homogeneous.

DNA extraction and amplification

We extracted total cellular DNA using the DNAeasy Tissue kit (Qiagen). Double-stranded DNA amplifications of *Mc1r* and *POMC* were performed with the primer pairs *Mc1r*f1 (5′-AGGTGRGTGTTTGGGGCA-3′)/*Mc1r*r1 (5′-CGCTGGATGGTCATGAT-3′) and *Mc1r*f4 (5′-ACCGCTACATCACCATCTT-3′)/*Mc1r*r5 (5′-GTCACAAAGASTCACCTGCT-3′) and *POMC*f1 (5′-ATTCAGAAGTACATCCACTGG-3′)/*POMC*r1 (5′-TAACTTTAATCCTTTCATTTACAG-3′), specifically designed for the present study. Polymerase chain reaction amplifications were performed in a 9800 Fast thermal cycler (Applied Biosystems) with 0.05 U of Taq DNA polymerase (Qiagen) and consisted of 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 55 °C (50 °C for *Mc1r*f1/*Mc1r*r1) and 105 s extension at 72 °C. PCR fragments were further purified with the Wizard SV Gel and PCR clean-up system (Promega) and sequenced at GATC sequencing service (Cologne, D; http://www.gatc-biotech.com).

Statistical analysis

We first tested for an association between the different colour morphs and each single nucleotide polymorphism (SNP) with chi-squared tests. Because the different colour morphs may not be homogeneously distributed in the study area, we added the three covariates latitude, longitude and elevation where each individual was collected in a logistic regression to examine whether the statistic association between colour and SNP is independent of geography. We also performed two additional analyses where we restricted our dataset to samples: (1) strictly collected in the Mont Blanc massif where the four morphs co-occur (blotched: $N = 28$; lined: $N = 13$; melanistic: $N = 9$; concolor: $N = 26$) or (2) excluding low elevation sites (< 1000 m a.s.l.) where only the blotched morph is present (blotched: $N = 35$; lined: $N = 13$; melanistic: $N = 26$; concolor: $N = 26$). All the statistical analyses were performed using JMP, version 8.0 and SAS, version 9.2 (SAS Institute). $P < 0.05$ (two-tailed) was considered statistically significant.

RESULTS

We sequenced a total of 910 bp of the *POMC* gene, including 556 bp of the exon 3 coding for the different

active peptides and 354 bp downstream of the gene. Our first screening of *POMC* in 15 individuals of different colour morphs revealed three SNPs: site 608 (*aa*, *ag* or *gg*), site 620 (*aa*, *ag* or *gg*), and site 817 (*tt*, *ct* or *cc*) situated within the 3′-untranslated region (GenBank accession number: KC511125) 52 to 259 bp downstream of the stop codon, respectively. Based on these findings, we genotyped all 107 individuals of the four different colour morphs for these three SNPs. The frequency of the different alleles at the three sites was not significantly different between the two sexes (site 608: $\chi^2 = 2.36$, $P = 0.31$; site 620: $\chi^2 = 0.24$, $P = 0.89$; site 817: $\chi^2 = 0.01$; $P = 0.99$). In addition, the frequency of homozygotes was identical between sexes for sites 608 and 817 and marginally significant for site 620 (site 608: $\chi = 0.02$, $P = 0.88$; site 620: $\chi^2 = 4.05$, $P = 0.05$; site 817: $\chi^2 = 0.002$; $P = 0.97$), indicating that this gene is located on an autosome. Thus, we excluded sex from subsequent analyses. As expected, these three mutations were in linkage disequilibrium, but it was only marginally significant for sites 608–817 (sites 608–620: $\chi^2 = 11.05$, $P = 0.026$; sites 620–817: $\chi^2 = 124.06$, $P < 0.0001$; sites 608–817: $\chi^2 = 9.040$, $P = 0.06$).

Only mutations at site 608 were significantly associated with the four colour morphs ($\chi^2 = 14.76$, $P = 0.022$), which was not the case with mutations at sites 620 and 817 ($P > 0.47$). Figure 2 shows that at site 608 the frequency of homozygotes *aa* and *gg* and of heterozygotes *ag* is significantly different between patterned individuals (i.e. lined and blotched morphs) compared to unpatterned individuals (i.e. concolor and melanistic morphs; $\chi^2 = 9.97$; $P = 0.0068$); the difference mainly results from the high frequency of *gg* individuals compared to the pooled frequency of *aa* and *ag* in patterned versus unpatterned snakes, respectively ($\chi^2 = 8.84$; $P = 0.0029$). Because we collected individuals in different locations, we also performed additional analyses where the geographical coordinates (latitude and longitude) and elevation of the collected specimens were included in order to insure that our significant result was not confounded by a nonrandom spatial and elevational distribution of the different colour morphs (for spatial distribution of homozygotes and heterozygotes, see Fig. 3). This analysis was qualitatively similar, in that the frequency of the different mutations at site 608 (*gg* versus *aa* and *ag*) was still significantly associated with the presence/absence of patterns (logistic regression: $\chi^2 = 11.65$; $P = 0.0006$). However, in this model, we also found a significant interaction between elevation and site 608 (elevation × site 608: $\chi^2 = 7.83$, $P = 0.0051$). In addition, latitude and longitude were not significant alone with site 608 (all $P > 0.78$), although the interactions of the elevation with longitude and latitude were significant ($\chi^2 = 9.03$, $P = 0.0027$; $\chi^2 = 14.3$, $P = 0.0002$). These significant interactions can be explained by the restriction of unpatterned snakes to high elevation sites (from 1000 to 1961 m a.s.l.) compared to patterned snakes, which are distributed at low and high elevations (from 355 to 2100 m a.s.l.), as well as to a nonrandom distribution of mountains through our sampling site. For this reason, we performed an additional analysis where we excluded low elevation sites where only one morph is present (blotched; < 1000 m a.s.l.). This analysis yielded qualitatively similar results (site 608: *gg* versus *aa* and *ag*: $N = 100$, $\chi^2 = 8.81$, $P = 0.003$). Also because all four morphs are found only in the Mont Blanc massif, we performed a last analysis restricted to animals collected there. Again, we found qualitatively similar results ($N = 76$, $\chi^2 = 6.95$, $P = 0.0084$) demonstrating that the association between mutations at the *POMC* gene and colour morph is robust.

Concerning *Mc1r*, our first screening of the entire coding region (942 bp) of the gene of 15 differently coloured individuals (six blotched, five melanistics, two lined, and two concolors) revealed no polymorphism (GenBank accession number: KC511124). Consequently, no further analyses were performed.

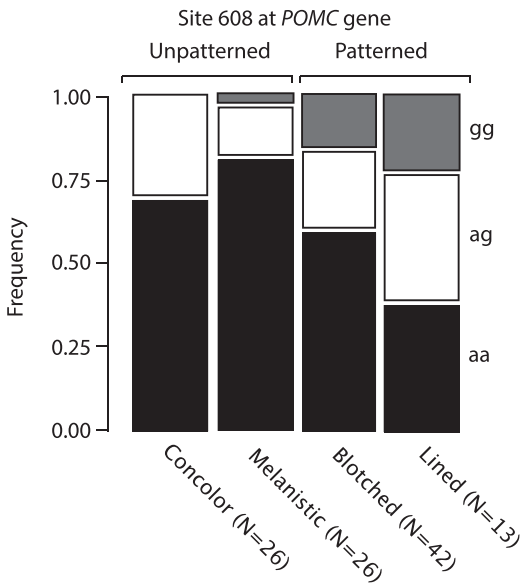

Figure 2. Frequency of homozygotes (*aa*, black; *gg*, grey) and heterozygotes (*ag*, white) at site 608 of the pro-opiomelanocortin gene (*POMC*) in the four asp viper colour morphs (concolor, melanistic, blotched, and lined).

DISCUSSION

The present study is the first to reveal a link between a mutation in the *POMC* gene and colour morphs in wild organisms. To date, such relationships were known only in humans and mice, causing red hair

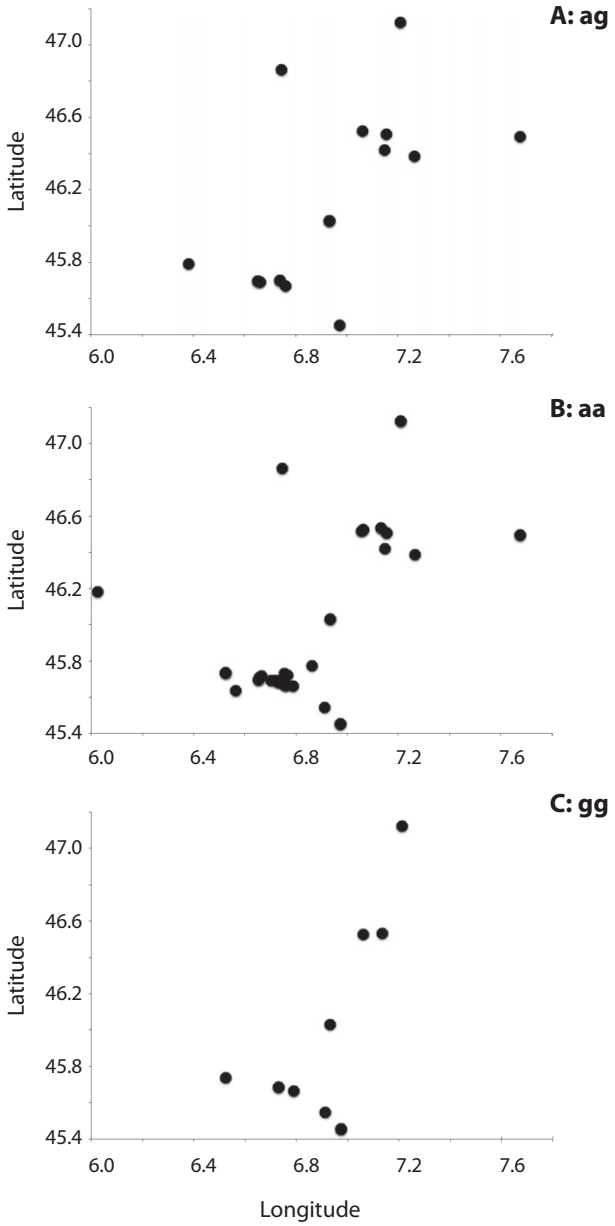

Figure 3. Spatial distribution of asp viper heterozygotes (A, *ag*) and homozygotes (B, *aa*; C, *gg*) and at site 608 of the pro-opiomelanocortin gene.

and yellow pigmentations, respectively, as a result of a structural loss of functions and hence a lack of POMC-derived peptides (Krude *et al.*, 1998; Yaswen *et al.*, 1999). In the asp viper, we found an association between colour variation and a SNP situated within the 3′-untranslated region of the *POMC* gene. Unpatterned individuals, either melanistic or concolor (Figs 1, 2), were mostly homozygotes '*aa*' or heterozygotes '*ag*' (proportion of '*gg*': 1.9%), whereas patterned individuals, either blotched or lined, were most often homozygotes '*gg*' (18%). Hence, the presence of the allele '*a*' was usually associated with an absence of pattern in snakes. However, because this SNP is not fully associated with the colour variation observed in the asp viper, additional genes than the *POMC* gene or a closely linked gene may be involved in the observed polymorphism. In addition, even if individuals can be easily categorized in four discrete morphs, some intramorph variations do exist. Because these kinds of variations were not quantified in the present study, we cannot exclude that it could, to some extent, impact upon our results and explain the lack of a full association between this SNP and colour variation.

Genes of the melanogenesis pathway are known for their pleiotropic effects and examples of associations between melanin-based coloration and other traits have been described in various vertebrates, potentially suggesting a role of the melanocortin system (Roulin & Ducrest, 2011). Given that the *POMC* gene is highly conserved in vertebrates, it would be interesting to sequence this gene in other animals to look for polymorphism. A key aspect is whether the polymorphism associated with coloration in *V. aspis* occurs in other species. Another key aspect is to identify the potential physiological function of the SNP we found in *V. aspis*. Accordingly, in humans, mutations within the same region (*POMC* 3′-untranslated region; 3′ UTR) are linked with obesity and alcohol and drug dependence (Sutton *et al.*, 2005; Zhang *et al.*, 2009). Because, in vertebrates, 3′ UTRs are often involved in post-transcriptional regulation (Adeli, 2011), we speculate that the observed mutation in the *V. aspis POMC* may act in a similar way. Precisely, the *POMC* gene is expressed predominantly in the anterior and intermediate pituitary gland, and subsidiarily in the central nervous system in mammals. Post-transcriptional regulations that involve 3′ UTRs can control neurone signalling (Goldie & Cairns, 2012) or repress translation initiation during mouse oocyte maturation via a loop structure containing proteins and 3′ UTR interacting with the 5′ UTR (Kang & Han, 2011). In addition, microRNAs (miRNAs) may bind the 3′ UTR and induce mRNA degradation, repression of translation initiation, and deadenylation (Fabian, Sonenberg & Filipowicz, 2010; http://www.microrna.org/microrna/getGeneForm.do), and variation in 3′ UTR length may influence binding miRNAs and proteins and thus RNA stability (Di Giammartino, Nishida & Manley, 2011). Hence, the 3′ UTR of the *POMC* gene appears to play a major role in its regulation in distant vertebrate species and, as the gene and its functions are well conserved from fish to mammals (Schiöth *et al.*, 2005), it may be also the case in snakes.

The *POMC* gene is known to be involved in thermoregulation mechanisms in mice and rats (Sinha, Schioth & Tatro, 2004; Creemers *et al*., 2006; Skibicka & Grill, 2008), further emphasizing the potential primary role of this gene in ectothermic snakes. It would therefore be interesting to investigate the potential role of the mutations that we found in the asp viper in energetic processes. Indeed, in ectotherms such as reptiles, melanism and colour polymorphism play an important role in thermoregulation processes and in their capacity to face environmental modifications (Clusella-Trullas *et al*., 2007). A possible research design would be to analyze thermoregulation in snakes with various *POMC* mutations under different thermal regimes.

Despite a significant association between the mutation at site 608 in the *POMC* gene and colour polymorphism in the asp viper, we cannot rule out the hypothesis that the observed pattern is confounded by other parameters, such as genes linked to *POMC* or geographical structure (e.g. with the presence of a particular mutation associated to a colour morph, distributed in sampling sites that are geographically and genetically isolated). However, this last hypothesis is unlikely, given that our analyses including the geographical coordinates and elevation of individuals or a dataset restricted to the Mont Blanc massif or only with the sites > 1000 m a.s.l. still showed a significant association between the *POMC* gene and colour morphs. In addition, our sampling can be considered as genetically homogeneous, because it is strictly represented by individual the nominal subspecies and only one mitochondrial haplotype has been detected (H1) in this area (Ursenbacher *et al*., 2006). Even if the *POMC* gene has not a direct effect on coloration, which could be the result of a linked gene, the present study has a number of evolutionary implications given the pleiotropic effects of the *POMC* gene (Ducrest *et al*., 2008). Indeed, it implies that differently coloured vipers may show different physiological and behavioural properties.

Turning specifically to ectothermic tetrapods, two previous studies highlighted mutations in *Mc1r* as responsible of the colour variations observed in lizard species (*Aspidoscelis inornata*, *Holbrookia maculata*, and *Sceloporus undulatus*), all of them from the White Sands area in New Mexico (USA), where individuals exhibit bleached colorations compared to specimens from adjacent populations (see Rosenblum *et al*., 2004, 2010). By contrast, Rosenblum *et al*. (2004), Corso, Gonçalves & de Freitas (2012) and Cox, Rabosky & Chippindale (2013) did not find any association between *Mc1r* mutations and colour polymorphism in Californian and South American lizard species (*Anniela pulchra*, *Liolaemus occipitalis*, *Liolaemus arambarensis*, *Phrynosoma platyrhinos*, and *Uta stansburiana*), as well as in two North American snake species (*Sonora semiannulata* and *Thamnophis sirtalis*), suggesting that this gene is not always involved in colour polymorphism, as confirmed in the present study. Consequently, colour variation in reptiles does not always involve *Mc1r* but may include *POMC* among other genes that remain to be discovered.

In a more general context, recent studies have shown that colour polymorphism plays a central role in the evolution of reptile species. For example, colour polymorphic species have larger geographical distributions, utilize more diverse habitat types, and are less threatened than monomorphic species (Forsman & Aberg, 2008; Forsman *et al*., 2008). In addition, colour polymorphic species are older (in terms of intraspecific diversification; Pizzatto & Dubey, 2012), meaning that such species are genetically more diverse than monomorphic species. Consequently, colour polymorphism in reptiles is involved in important populations processes. A recent study focusing on two colour polymorphic montane populations of asp vipers (characterized by melanistic and blotched individuals; Castella *et al*., 2013) has shown significant relationships between the colour morph, body condition, and sex of individuals, as well as differences in the frequency of gravid females between morphs. Moreover, colour morphs were not randomly distributed within populations but followed an elevational gradient. Such results indicate that colour polymorphism plays an important role in the ecology of this species and is not selectively neutral.

In conclusion, as a result of the key role of *POMC* in melanogenesis, and its multiple pleiotropic effects, this gene should be considered in studies focusing on the genetic mechanisms of melanin-based colorations in wild species, with the aim of understanding the mechanisms involved in the evolution of colour polymorphism. In addition, future studies should: (1) screen the *POMC* gene through the whole distribution of *V. aspis* to determine whether its association with coloration is still valid in other populations and if mutations are fixed in particular areas; (2) aim to understand the role of mutations at the *POMC* (e.g. effect on expression level); and (3) test for potential relationships between this mutation and other phenotypic characteristics.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Morph, latitude and longitude (decimal degree), elevation (m), and sex of samples used in the present study.