Head shape variation in eastern and western Montpellier snakes

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Abstract. The Montpellier snake Malpolon monspessulanus is a wide-ranging species that inhabits Western and Eastern Europe, North Africa and Middle East. Four clades have been recognised as two species, M. insignitus and M. monspessulanus, each with two subspecies. Clades have been substantially identified on the basis of molecular data, pholidosis and colouration, while morphometric traits have been ignored. We compared head shape of 54 specimens belonging to three out of the four clades (M. insignitus insignitus, M. i. fuscus, and M. monspessulanus monspessulanus) by means of geometric morphometrics. We found a significant differentiation: the supraocular and frontal area showed the largest amount of variation, being respectively much thinner in M. i. insignitus, a bit less thin in M. i. fuscus and definitely wider in M. m. monspessulanus. Our findings are fully in agreement with the genetic studies and phylogeny explains more than 20% of the observed variation, supporting the taxonomic distinction inside the genus Malpolon. The functional and/or adaptive meaning of the observed differences is not clear, but it seems unlikely that it may be related to diet. Combining morphological data with phylogeography and environmental features, we formulated an explanatory hypothesis that allowed a precise and testable prediction.

Keywords. Taxonomy, phylogeography, Psammophiinae, Malpolon, Mediterranean, Geometric morphometrics, non-parametric MANOVA, variation partitioning.

INTRODUCTION

The genus Malpolon Fitzinger 1826 belongs to the subfamily Psammophiinae (family Lamprophiidae; Pyron et al., 2013), a taxonomic group of snakes which includes 49 species (Uetz and Hošek, 2013) with an Afro-asiatic distribution (Sindaco et al., 2013; Uetz and Hošek, 2013). The genus currently counts two Mediterranean species (Böhme and De Pury, 2011), which show allopatric geographic distributions (Fig. 1): Malpolon monspessulanus Hermann 1804 (western Montpellier snake), which ranges from Liguria (W Italy) through France, Iberian Peninsula, Morocco, and Western Sahara (Fahd and Pleguezuelos, 2001; Geniez et al., 2006; Sindaco et al., 2013); M. insignitus Geoffroy-Sant-Hilaire 1827 (eastern Montpellier snake), which spreads from eastern Morocco eastward around the Mediterranean Sea, reaching Anatolia and Balkans, Middle East, Iraq and Iran, and also Caucasus and Transcaucasia (Sindaco et al., 2013). Two subspecies are currently recognized within each species (Fig. 1, see also Sindaco et al., 2013; Uetz and Hošek, 2013): M. insignitus insignitus Geoffroy-Sant-Hilaire 1827, which occurs in the eastern coast of Mediterranean Africa (from eastern Morocco to Egypt) and Middle East (De Haan, 1999; Carranza et al., 2006); and M. i. fuscus Fleischmann 1831, which occupies the remnant part of the areal (Balkans, Anatolia, Caucasus and Transcaucasia, Iraq and Iran; Fig. 1; De Haan, 1999); M. monspessulanus monspessulanus Hermann 1804, which ranges from eastern Morocco to South-western Europe, and M. m. saharatlanticus Geniez,
Specific and subspecific distinctions are based on molecular data and phenotypic traits. The phylogenetic reconstruction using mitochondrial DNA (cyt b and 12S rRNA; Carranza et al., 2006) revealed that i) the genetic distance between the eastern (M. insignitus) and western (M. monspessulanus) clades justifies their raising to the status of distinct species (Carranza et al., 2006), ii) the occurrence in the eastern clade of a greater genetic diversity, which suggests maintaining the subspecies M. i. fuscus, and iii) the genetic variability of the western clade is far lower, even comparing Moroccan populations to European ones, supporting the hypothesis that current European populations are descendant of a recent colonization from Morocco through Gibraltar (80,000–170,000 years ago; Carranza et al., 2006). Since it was not possible to obtain a precise localization of each analysed specimen, two types of localities are shown: unmistakably identified localities (dots), and localities arbitrarily chosen inside the State where specimens were collected (stars). Right: Phylogenetic tree inferred from cytochrome b and 12S rRNA with recognizable clades (modified from Carranza et al., 2006). Names of the tips of the tree are the original ones by Carranza et al., 2006 and correspond to the accepted scientific names at the time of their publication. Clades are named with the current nomenclature.
axis is a surrogate of the specimen's size, thus clades differentiation along PC1 represents the linear size difference among subsamples (directly appreciable from table 2 in Geniez et al., 2006); iii) PC2 axis is weakly correlated to morphometric measures; iv) coherently with the points above, the authors do not use morphometrics to explain the observed differentiation (see Table 6 and discussion in Geniez et al., 2006). In other words, the analysis, by merging size with shape, does not allow detecting the actual occurrence of morphometric differentiation, therefore a study explicitly addressing this issue and comparing *Malpolon* clades from a morphometric perspective is still lacking.

In order to start filling the gap, we decided to focus on the head for at least two reasons. Firstly, the snake head is thought to be subject to strong ecological and evolutionary constraints (Shine, 1991; Bonnet et al., 2001; Aubret et al., 2004; Manier, 2004; Vincent et al., 2006; Gentilli et al., 2009; Hampton, 2011; Llorente et al., 2012; Henderson et al., 2013), so the different evolutionary histories undergone by each clade may have left traces on the head shape (Shine, 1991; Grudzien et al., 1992; Forsman and Shine, 1997; Hibbits and Fitzgerald, 2005; Vincent et al., 2006a; Herrel et al., 2008). Secondly, the genus *Malpolon* is characterised by a peculiar head shape: the combination of large eyes and oblique supraocular scales gives this snake a typical “grim facial expression” (Kreiner, 2007). This trait is shared with other species of Psammophiinae (even if not so accentuated; Coborn, 1991), suggesting its possible genetic base and thus a potential systematic value. In this scenario, our study aims to answer three main questions: i) does head shape vary among the *Malpolon* clades? ii) Is the morphometric variation in agreement with the phylogenetic relationship among clades? iii) Is there any relationship between geographic pattern of head shape variation and environmental features?

<table>
<thead>
<tr>
<th>Species</th>
<th>Subspecies</th>
<th>Saddle-complex</th>
<th>No. of scales at mid-body</th>
<th>Basioccipital processes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. monspessulanus</em></td>
<td>monspessulanus</td>
<td>present, limited to the first third of the trunk</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
<td><em>M. monspessulanus</em></td>
<td>saharatlanticus</td>
<td>present, extended on the major part of the body</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
<td><em>M. insignitus</em></td>
<td>insignitus</td>
<td>Absent</td>
<td>19</td>
<td>2</td>
</tr>
<tr>
<td><em>M. insignitus</em></td>
<td>fuscus</td>
<td>Absent</td>
<td>17</td>
<td>2</td>
</tr>
</tbody>
</table>

**MATERIALS AND METHODS**

**Samples and geometric morphometrics**

We analysed 54 specimens (36 males and 18 females) obtained from the collections preserved in the Natural History Museums of Milan (MSNM), Florence (“La Specola”, MZUF) and Genoa (“Giacomo Doria”, MSNG; Table 2; Table S1). Only adult males and females were considered (age was assessed following Feriche et al., 2008) and specimens from Lampedusa and Cyprus were excluded in order to avoid a possible “island” effect on the size and shape of the head (Bobak, 2006). Unfortunately, all collections lacked specimens of the subspecies *M. m. saharatlanticus*, which, consequently, was not included in our comparison. Each specimen was assigned to one of the remnant three subspecies on a geographic base (Fig. 1; De Haan, 1999; Carranza et al., 2006; Geniez et al., 2006; Sindaco et al., 2013). The species identification was confirmed by pholidosis and colouration, whenever possible.

For each specimen we recorded sex and snout-to-vent length (SVL) and we took a picture of the dorsal view of the head, using a Nikon D50 Professional camera at 6.1 megapixel resolution equipped with a Nikkor 60 mm AF-S Micro lens, at a fixed distance of 18 cm. Photos were subsequently used to perform the analysis of the shape by means of geometric morphometrics. Geometric morphometrics is a well-established technique (Claude, 2008; Kaliotzopoulou, 2011; Adams et al., 2012) that effectively allows removing non-shape information from geometric structures (Bookstein, 1997; Dryden and Mardia, 1998) and has been proved to be more powerful than traditional morphometrics (Rohlf, 2000). Fourteen two-dimensional landmarks and eight semi-landmarks (Fig. 2; Table S2) were digitized for each specimen using TpsDig2 (Rohlf, 2010; available at: http://life.bio.sunysb.edu/morph/), which resulted in 22 pairs of coordinates that together described head shape. These configurations were superimposed using a general procrustes analysis (GPA; Rohlf and Slice, 1990) in order to remove information concerning size and orientation and to preserve only information related to shape (Dryden and Mardia, 1998; Claude, 2008). Since head is a bilaterally symmetric structure and we were not interested in the quantification of the deviation from this condition, we followed the procedure suggested by Klingenberg et al. (2002) to remove left-right asymmetry from
the global shape. The procedure involves three steps: 1) generating a reflected copy of each configuration; 2) superimposing the combined collection of original and mirrored configurations; 3) averaging the coordinates of corresponding landmarks in the original and mirrored configuration of each specimen in order to obtain a perfectly symmetric configuration. We specifically implemented the first and third step in R (version 3.0.1; R core Team, 2013), while general procrustes superimposition (second step) was performed by the “gpagen” function of the “geomorph” R-package (Adams and Otarola-Castillo, 2013).

Head shape variation among clades

Differences in head shapes were analysed by means of a non-parametric distance-based MANOVA (hereafter NP-MANOVA, Anderson, 2001; McArdle and Anderson, 2001). This method substitutes the usual raw data matrix of the dependent variables of MANOVA with a distance matrix computed between each pair of observations; hence it decomposes the sum of the square pairwise distances in an ANOVA-like way (see Anderson, 2001 for details). The technique allows covariates and interactions and the P-values of the model terms are assessed via permutation of the observations (Anderson, 2001). We applied the NP-MANOVA to the matrix of the pairwise Procrustes distances (Claude, 2008) computed on the superimposed configurations, using “clade” (three levels factor: *M. m. monspessulanus*, *M. i. insignitus*, *M. i. fuscus*), “SVL” (covariate) and their interaction as independent variable. SVL was included to take into account a possible allometric effect and it was log-transformed according to the non-linearity of the general allometric function (Gayon, 2000; Claude, 2008). The variable “sex” was excluded from the model because of the low and unbalanced number of females in our sample (we had only two adult females for *M. i. fuscus*; Table 2). Since “sex” could affect head shape, we tried to ensure the reliability of the results by assessing the coherence of the outcomes of a model that considers only males (MALE) with those of a model that considers both sexes (BOTH). Both models were based on the same set of independent variables, i.e., clade, SVL and their interaction. MALE tests if head shape of males varies among the clades, taking into account allometry; BOTH should allow a generalization of MALE results under the *a priori* assumption that no sexual dimorphism occurs in head shape. To give some support to this assumption, we tested the occurrence of intersexual difference in head shape in the *M. m. monspessulanus* clade, where males and females were balanced and numerous enough (Table 2). NP-MANOVA was performed with the function “adonis” of the “vegan” R package (Oksanen et al., 2013), and we tested the assumption of homogeneity of dispersions among clades.

**Fig. 2.** Configuration of the landmarks (black dots) and semi-landmarks (white dots) taken on each image. For a detailed definition of each landmark see Table S2.
RESULTS

Head shape variation among clades

The difference in head shape among the three *Malpolon* clades was highly significant for both MALE and BOTH models (Table 3). No sexual dimorphism in head shape was detected in *M. m. monspessulanus* (*sex*: $F_{1,20} = 0.90, P > 0.05$, no. of permutations = 9999).

The visual comparison of the predicted forms for each group allowed recognizing two main patterns of variation, loaded by different regions of the head (Fig. 3A; Fig. S1). The most evident one regarded the supraocular area and the external margin of the parietal scales, which showed a clear differentiation between the two species (*M. i. insignitus* and *M. i. fuscus* on one side and *M. m. monspessulanus* on the other side; Fig. 3B, 3C). In particular the two subspecies of *M. insignitus* had: i) a less flared frontal scale; ii) thinner and longer supraocular scales, which determine a more acute silhouette of the anterior part of the head; iii) a more arched margin between the frontal and supraocular scales; iv) a less curved posterolateral margin of the parietal scales. The difference between the subspecies of *M. insignitus* were less evident and were loaded by the prefrontal and pari-
etal regions: the intersection point between prefrontal and frontal scales as well as the intersection between the posterior margin of the parietals were shifted to the front in *M. i. fuscus* with respect to *M. i. insignitus* (Fig. 3D).

The NP-MANOVA also detected a significant allometric effect (Table 3), which was shared by all clades (the interaction term was indeed not significant). Allometry seemed to act mainly on the prefrontal scales, leading to a relative elongation of the scales with increasing size (Fig. 4). Correspondingly, the anterior margin of the frontal scale and the posterior margin between the parietals became more acute.

**Relationship between head shape and phylogeny**

The partition of the determination coefficient assigned a remarkable part of variation to the factor “clade” (more than 20% in both models; Fig. 5A), while the effect of size appeared negligible (no more than 1%). The overlap between the two variables was null in both models (Fig. 5A), indicating that the pure effect of the phylogenetic factor weighted considerably more than allometry. The morphometric tree obtained from the mean predicted shapes showed that the two subspecies of *M. insignitus* were more similar to each other than to *M. m. monspessulanus* (Fig. 5B), i.e., the morphometric relationship among clades predicted by the factor “clade” matched the phylogenetic one.

**Relationship between head shape and environment**

The environmental features of the occurrence cells are significantly different among clades (*F*$_{3,349}$ = 60.28, *P* ≤ 0.0001, no. of permutations = 9999; Fig. 6A). In par-
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In particular, *M. m. monspessulanus* and *M. i. fuscus* inhabit relatively colder and rainier areas than the couple *M. m. saharatlanticus* – *M. i. insignitus*, which, conversely, share a drier and warmer environment (Fig. 6A). The analysis of the climatic distance confirmed this crossed resemblance between the least related clades (Fig. 6B).

**DISCUSSION**

The species and subspecies of the genus *Malpolon* have been substantially identified on the basis of molecular data and, to a less extent, on pholidosis and coloration pattern (De Haan, 1999; Carranza et al., 2006; Geniez et al., 2006). On the other hand, morphometric traits have been practically ignored, even though such information can be very helpful, not only for taxonomic purposes, but also in order to shed light on the evolutionary mechanisms that had driven the differentiation of *Malpolon* lineages. With this in mind, we looked for the occurrence of a morphometric diversification in three out of the four recognised clades of the Montpellier snakes, by focusing on head shape because of its potential genetic, evolutionary and adaptive meaning (Shine, 1991; Forsman and Shine, 1997; Bonnet et al., 2001; Manier, 2004; Vincent et al., 2006a; Herrel et al., 2008; Gentilli et al., 2009; Hampton, 2011; Llorente et al., 2012; Henderson et al., 2013). We found three important results, i.e., i) head shape varies significantly among the three clades; ii) the differences are fully in agreement with the previous phylogenetic analyses and phylogeny explains a relevant part of the observed shape variation; iii) the tree obtained from climatic data does not match that from phylogeny.

The largest difference in head shape occurs between the western and eastern clades and is loaded by the frontal and supraocular regions: the frontal scale is more flared in *M. insignitus* than in *M. monspessulanus* and, contemporarily, the supraocular scales are larger and with a continuous joint with the parietals. The two subspecies of the eastern species are less differentiated between each other, with *M. i. fuscus* showing a little enlargement of the pre-parietal zone and an anterior shift along the mid-line of the intersection point between the frontal and prefrontal scales as well as the posterior intersection between the parietal scales. All these differences cannot be ascribed neither to a combination of allometric effect nor to an inhomogeneous distribution of size classes in our sample (Table 2) because SVL is not statistically different among clades ($F_{3,33} = 1.42; P > 0.05$), allometry has an overall low effect (Fig. 5A), and it does not involve the same regions of clade (allometry involved mainly the prefrontal region, whereas clades were loaded by the frontal, supraocular and partly parietal one; compare Fig. 3 and Fig. 4). Even when some overlapping effect of clade and size occurs (eastern clades, where specimens have smaller SVL; Table 2), shape changes due to clade are opposite to what could be expected on the basis of size differences between samples (Fig. 3D and Fig. 4).

Our results suggest that head shape patterns of Montpellier snakes match the phylogenetic reconstruction based on genetic data. A similar result has been already obtained for other snakes (e.g., *Vipera aspis*, Gentilli et al., 2009; *Coronella austriaca*, Llorente et al., 2012), but we found that this effect seems more pronounced in the genus *Malpolon*. Indeed, the differences in head shapes showed in
Fig. 3A were not amplified, contrary to what was done by both Gentilli et al. (2009) and Llorente et al. (2012), supporting the intensity of the “clade” effect. Therefore, we can reliably state that our results corroborate the distinction of the two species (*M. monspessulanus* and *M. insignitus*) and also the recognition of two subspecies inside the *insignitus* clade, as suggested by both molecular analysis (Carranza et al., 2006) and pholidosis (De Haan, 1999).

The functional and/or adaptive meaning of the observed shape differences is not clear. Herpetologists have usually related head shape modification in snakes to diet and prey size (Shine, 1991; Grudzien et al., 1992; Bon-
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net et al., 2001; King, 2002; Aubret et al., 2004; Boback, 2006; Vincent et al., 2006a, b; Hampton, 2011), but this idea does not seem to fit the Malpolon case because of two main reasons: firstly, the head features associated with prey size are mainly related to head width and snout length/width (Shine, 1991; Bonnet et al., 2001; Hampton, 2011); these traits are expected to mould the shape of the parietal scales, while our results focused mainly on the pre-parietal area. Secondly, the (scanty) data about Malpolon diet do not highlight any sort of difference between eastern and western populations and describe Montpellier snake as an opportunistic predator of items of various sizes (De Haan, 1999; Ottonello et al., 2006; Plezueguelos et al., 2009). The occurrence of a systematic difference in the size of available prey is a prerequisite for a diet-shape relationship (Aubret et al., 2004). Since it does not seem so, we do not think feeding is the major key to understand the observed pattern of head shape variation in Montpellier snakes, even though further studies are needed to exclude the diet divergence hypothesis.

An alternative hypothesis to explain the observed pattern of head shape variation may come from the combination of the morphological data with the phylogeography of the genus and with the environmental features of the geographical areas occupied by each clade of Malpolon. Each clade inhabits climatically and topographically different areas, suggesting that these environmental factors could be among the causal factors for the observed morphological variation. More interestingly, the comparison identifies two main climatic groups: M. i. fuscus and M. m. monspessulanus on the one hand, M. i. insignitus and M. m. saharatlanticus on the other hand. If we compare the mean head shape of the three analysed clades (Fig. 3), we notice that the enlargement of frontal and supraocular scales of M. i. fuscus with respect to M. i. insignitus seems to shift the insignitus head to the monspessulanus form (Fig. 5B). In the light of the phylogeographic reconstruction by Carranza et al. (2006), the two above considerations allow formulating a testable hypothesis: after the speciation event that led to the distinction of the eastern and western Montpellier snake, M. insignitus dispersed eastward along the Mediterranean coast of North Africa, penetrated into Middle-East and, through Turkey into Balkans and Caucasus. Here it encountered different environmental conditions, which have induced the modification of the head shape (this is the hypothetical assumption: the environment-shape causal relationship). Since the conditions of this region partially match those occurring in the western part of the Malpolon range, M. m. monspessulanus and M. i. fuscus may have shown a certain degree of evolutionary convergence in head shape. Even if this interpretation is quite speculative, it has the advantage of giving a symmetric prediction for the other two clades: under the same assumption head shape of M. m. saharatlanticus should slightly differ from that of the nominal subspecies in the direction that tends towards M. i. insignitus.

A further and complementary consideration is that the region of the head which shows the greatest variability involves the typical trait of the genus, i.e., the “grim facial expression” (Kreiner, 2007): this suggests that the key to the interpretation of the observed head shape variation may lie in the functional meaning of this trait. Even if no information is available about it (as far as we know), some indications may come from the hunting style of the Montpellier snakes: preys are spotted by sight and the first third of the trunk is often uplifted in order to have a better view of the surroundings (Arnold and Burton, 1985; De Haan, 1999; Kreiner, 2007). Such a tactic justifies the great development of the eyes and, consequently, the expansion of the supraoculars and the parallel reduction of the frontal. It is not improbable that also the degree of binocular vision could have taken advantage from this particular configuration, improving hunting performance (Hibbitts and Fitzgerald, 2005). So, different environmental characteristics may have required slightly different eyes-supraoculars-frontal packages and the observed among-clades differences may be the result of this adaptation.

In conclusion our study gives strong support to the taxonomic and phylogenetic relationships among three out of the four currently recognized clades inside the genus Malpolon; it highlights the potential importance of head shape in the reconstruction of the evolutionary history of the Montpellier snakes; it shows how geometric morphometrics can be a powerful instrument to validate molecular phylogeny and to investigate the underlying causal mechanisms (Rohlf, 2002; Kaliontzopoulou, 2011).

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