The phylogenetic signal in cranial morphology of *Vipera aspis*: a contribution from geometric morphometrics

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Morphological variation in the frontal bone and cranial base of *Vipera aspis* was studied using geometric morphometrics. Significant differences in shape were found among samples from subspecies present in Italy (*V. a. aspis*, *V. a. francisciredi*, *V. a. hugyi*). Sexual dimorphism was negligible as well as allometry and size differences. The most divergent subspecies was *V. a. aspis*, possibly in relation to its recent history of geographic isolation in a glacial refugium. Shape clusters were in good agreement with clusters from studies of external morphology and completely congruent with results from molecular studies of mtDNA.

**Key words:** asp viper, cranial shape and size, Italy, phylogeny, systematics

**INTRODUCTION**

The asp viper, *Vipera aspis* (Linnaeus, 1758), is among the best studied Palaearctic vipers (Mallow et al., 2003). It is a small to medium-sized snake, averaging 60–70 cm total length (50–63 cm snout–vent length), up to 82 cm maximum length (Saint Girons, 1978). It is only found in western and southern Europe (Zuffi, 2002; Fig. 1) from sea level to 3000 m (Saint Girons, 1997). Geographic variability in morphological features has been described (Phisalix, 1968; Kramer, 1980; Zuffi & Bonnet, 1999; Zuffi, 2002). For instance, Zuffi (2002) studied characters of the external morphology and copulatory apparatus, and found differences in the number of dorsal bars and of ventral and subcaudal scales, together with a distinctive hemipenal form. Thus, he suggested that *Vipera aspis* may indeed represent a species complex (*sensu* Hermann et al., 1999) including *Vipera aspis*, *V. atra* Meisner 1820, *V. hugyi* Schinz 1833 and *V. zinnikeri* Kramer 1958. A taxonomic revision was also carried out using immunological data by Pozio (1980), who showed that electrophoretic patterns of venom compositions were very different between *V. aspis atra*, *V. aspis francisciredi* Laurenti, 1768, and *V. aspis hugyi*, whereas the latter had the same electrophoretic pattern as *V. aspis montecristi* Mertens, 1956. However, the reproductive isolation of populations within the *V. aspis* species group has been questioned by some authors focusing on neurotoxins. For instance, Guillemín et al. (2003) found that interbreeding may have occurred even between two clearly separated species, *V. aspis* from southeastern France and the sand viper, *Vipera ammodytes* (Linnaeus 1758). On the other hand, De Haro et al. (2002) showed that in France at least one population of *V. aspis* had a specific venom profile distinct from those of other French populations. Garrigues et al. (2005) also made a preliminary comparison using mitochondrial cytochrome b and ND2 DNA sequences. However, the limited number of samples analysed by Garrigues et al. (2005) did not allow any robust reconstruction of relationships within *V. aspis*. In contrast, a recent study on mtDNA variability investigating the phylogeography of the asp viper across its whole distribution range (Ursenbacher et al., 2006) indicated that *V. aspis* can actually be separated into four distinct groups. Moreover, they considered *V. atra*, characterized by a relatively high number of ventral scales and a marked black dorsal pattern, and distributed in northwestern Italy (Zuffi, 2002), as a synonym of *V. a. aspis*. Thus, the four well-supported molecular clades, corresponding to *V. a. aspis*, *V. a. francisciredi*, *V. a. hugyi* and *V. a. zinnikeri*, were considered as subspecies.

Whether populations that are recognized using soft-tissue anatomy and molecular markers can also be discriminated using bone morphology has never been accurately investigated. Moreover, incongruences between results from previous studies are present, requiring further analyses and the clarification of independent characters.

Cranial form provides potentially interesting traits for investigating the systematic relationships of vipers (Guo & Zhao, 2006). In particular, the akinetic portion of the skull has been considered more suitable for taxonomic comparisons in snakes (Kramer, 1980; Gloyd & Conant, 1990). This is because the neurocranium, which forms the akinetic portion of the skull, is less strongly influenced by selective pressures related to diet (prey swallowing) and it is not directly associated with the mechanics of envenomation. Such studies, however, either did not focus on
European vipers or were limited by the paucity of available information in terms of study specimens and number of metric characters. Morphometric tools are presently available that allow accurate comparisons of the geometric shape of organisms or of their organs (Rohlf & Marcus, 1993; Adams et al., 2004). These methods, known as geometric morphometrics, have been successfully applied to detect subtle variation among closely related taxa (Duarte et al., 2000; Cardini, 2003; Frost et al., 2003; Klingenberg et al., 2003; Nicola et al., 2003; Macholán, 2006).

Thus, we applied geometric morphometrics to explore size and shape variation in the cranium of Italian populations of *V. aspis* that include three of the four subspecies supported by the molecular analysis of Ursenbacher et al. (2006). The analysis was performed by measuring crania using two-dimensional coordinates of anatomical landmarks in representatives of *V. a. aspis*, *V. a. francisciredi* and *V. a. hugyi*. The main aims of the study were to assess the magnitude of the variation and the significance of differences in cranial size and shape among these subspecies, and to reconstruct their similarity relationships.

**MATERIALS AND METHODS**

We considered only adult specimens (*n*=61) recognized and classified following Zuffi & Bonnet (1999). *Vipera a. aspis* (4 males, 3 females), *V. a. francisciredi* (10 males, 8 females) and *V. a. hugyi* (14 males, 9 females) (sensu Ursenbacher et al., 2006) were analysed. Individuals from contact areas between species, also showing intermediate numbers of dorsal bars and markings and numbers of ventral scales, were considered separately, and indicated as *V. a. aspis* *francisciredi* in the north, and *V. a. francisciredi* *hugyi* in the south (see Appendix 1 for references).
on the frontal bone and cranial base were used as morphometric descriptors (Fig. 2, Table 1). Information on only one side of the cranium was used in order to avoid redundancy in symmetric structures. Five almost coplanar and clearly visible landmarks were used for describing the shape of the frontal bone (Fig. 2A). Similarly, five approximately coplanar landmarks were used to describe the shape of the basiophenoid and basioccipital bones in the cranial base (Fig. 2B). Crania were not always well preserved and either ventral or dorsal bones were missing in a minority of specimens. For these, either only the frontal bone or the cranial base was therefore measured.

We applied geometric morphometrics techniques (Bookstein, 1991; Dryden and Mardia, 1998; Zelditch et al., 2004), implementing linear statistical models (Rohlf, 1998; Klingenberg & Monteiro, 2005). Differences in landmark coordinates due to position or size were removed with a generalized procrustes analysis (GPA – Rohlf & Slice, 1990), thus leaving only information on shape in the landmark configurations. Size was computed as centroid size (CS), which is a measure of the dispersion of landmark coordinates due to position or size were removed. Significance of regressions was tested with a permutation test for the generalized Goodall’s F (Goodall, 1991; Rohlf, 2005). Average shapes for the frontal and cranial base were computed for each species with pooled sexes. The two data sets were then combined (Adams, 1999) and phenetic relationships among viper taxa were summarized with multi-dimensional scaling (MDS) and cluster analysis (performed on the matrix of Euclidean distances between mean shapes). A minimum-length spanning tree (MST; Rohlf, 1970) was superimposed on the MDS scatterplot to help detect local distortions. The matrix correlation (a Pearson correlation coefficient) was computed on unfolded diagonal symmetric matrices between the original matrix of Euclidean distances (all shape variables) and the one based on the position of the species in the three-dimensional MDS scatterplot was 0.960. Among different clustering methods, the unweighted pair-group method using centroid (UPGMC) had the highest cophenetic correlation (Rohlf, 1970) to the original distance matrix ($r=0.856$); thus, UPGMC was used for the cluster analysis. All analyses were performed in SPSS and NTSYSpC 2.1.

To quantify the congruence between shape similarity relationships and phylogeny, we computed a matrix correlation between patristic distances from the UPGMC phenogram and those from the gene tree of Ursenbacher et al. (2006) as proxy for phylogeny based on independent data. The phylogenetic tree was built using different haplotypes for each species; for the comparison, we selected haplotypes by choosing individuals from localities closest to our study samples, i.e. haplotype H1 for V. a. aspis, H16 for V. a. francisciredi and H20 for V. a. hugyi (Ursenbacher et al., 2006).

<table>
<thead>
<tr>
<th>Table 1.</th>
<th>Anatomical description of landmarks of dorsal and ventral bones in viperid cranium.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsal</td>
<td>Description</td>
</tr>
<tr>
<td>1</td>
<td>Proximal margin of the suture between the frontal bone</td>
</tr>
<tr>
<td>2</td>
<td>Outermost point between prefrontals and frontals</td>
</tr>
<tr>
<td>3</td>
<td>Point between the frontal and postfrontal bones</td>
</tr>
<tr>
<td>4</td>
<td>Suture point between the frontal, postfrontal and parietal bones</td>
</tr>
<tr>
<td>5</td>
<td>Suture point between frontals and parietals</td>
</tr>
<tr>
<td>Ventral</td>
<td>Description</td>
</tr>
<tr>
<td>1-2-3-4</td>
<td>Higher points of the basisphenoid lateral crest</td>
</tr>
<tr>
<td>5</td>
<td>Teeth of the basioccipital</td>
</tr>
</tbody>
</table>

We applied geometric morphometrics techniques (Bookstein, 1991; Dryden and Mardia, 1998; Zelditch et al., 2004), implementing linear statistical models (Rohlf, 1998; Klingenberg & Monteiro, 2005). Differences in landmark coordinates due to position or size were removed with a generalized procrustes analysis (GPA – Rohlf & Slice, 1990), thus leaving only information on shape in the landmark configurations. Size was computed as centroid size (CS), which is a measure of the dispersion of landmarks around the barycentre of the configuration. Digitizing error and tangent space approximation to the space shape, estimated as in Cardini & Tongiorgi (2003), were found not to introduce any appreciable error.

Differences between groups were described with thin plate spline (TPS) deformation grids (Bookstein, 1991; Adams et al., 2004). After testing for differences in shape of frontal and ventral bones among groups (sex × species MANOVA for V. a. aspis, V. a. francisciredi, V. a. hugyi, and V. a. aspis*francisciredi), the two data sets were combined by appending the matrices of frontal and cranial base shape variables (Adams, 1999), and MANOVA (sex × species) and CVA (pooled sexes) were performed to test the significance of group differences using all available information. A scatterplot of the principal components of the shape was used to show graphically the relationships among individuals and groups. The relationship between size and shape in adults (static allometry) was assessed in the two largest samples (V. a. francisciredi and V. a. hugyi) by regressing shape variables onto CS. Significance of regressions was tested with a permutation test for the generalized Goodall’s F (Goodall, 1991; Rohlf, 2005). Average shapes for the frontal and cranial base were computed for each species with pooled sexes. The two data sets were then combined (Adams, 1999) and phenetic relationships among viper taxa were summarized with multi-dimensional scaling (MDS) and cluster analysis (performed on the matrix of Euclidean distances between mean shapes). A minimum-length spanning tree (MST; Rohlf, 1970) was superimposed on the MDS scatterplot to help detect local distortions. The matrix correlation (a Pearson correlation coefficient) was computed on unfolded diagonal symmetric matrices between the original matrix of Euclidean distances (all shape variables) and the one based on the position of the species in the three-dimensional MDS scatterplot was 0.960. Among different clustering methods, the unweighted pair-group method using centroid (UPGMC) had the highest cophenetic correlation (Rohlf, 1970) to the original distance matrix ($r=0.856$); thus, UPGMC was used for the cluster analysis. All analyses were performed in SPSS and NTSYSpC 2.1.

![Fig. 2. Landmark configurations for frontal bone (A) and cranial base (B) of the vipers.](image-url)
RESULTS

Separate analyses of frontal bone and cranial base

Size differences (results not shown) between groups were generally negligible in both datasets (frontal bone and cranial base). Only V. a. francisciredi and V. a. hugyi showed appreciable though very small differences in frontal bone size (the former being the largest species in the group). Size was also generally homogeneous in each group as suggested by coefficients of variation (ratio between SD and mean) ranging between 5.2% and 15.8%. Group differences were significant for shape of both frontal bone and cranial base of V. a. aspis, V. a. francisciredi, V. a. hugyi and V. a. aspis*francisciredi, while sexual dimorphism and the interaction term were not (Table 2). The same outcome was found in the combined data set (Table 2). Sexual dimorphism was negligible compared to group differences. Thus, groups were compared with pooled sexes in further analyses.

Static allometry was investigated in V. a. francisciredi and V. a. hugyi. The proportion of shape variation correlated to size was small (7.2% and 6.1% respectively) and not significant. Similar results were found when sexes were analysed separately.

Combined dataset

Differences among V. a. francisciredi, V. a. aspis, V. a. hugyi and V. a. aspis*francisciredi cranial shapes were significant (CVA, $\lambda_{wss} = 0.0430, F_{36,53}=2.848, P<0.001$, total hit ratio 90.9%). Tests of pairwise differences based on Mahalanobis distances were significant, except for the comparison between V. a. francisciredi and V. a. aspis*francisciredi (Table 3). Also, V. a. francisciredi and V. a. hugyi are relatively similar ($P>0.05$). Group separation in the scatterplot of the specimens along the first two PCA axes (Fig. 3) suggests that Vipera a. aspis is most distinctive.

The MDS scatterplot and UPGMC phenogram of mean shapes suggest phenetic relationships congruent with those presented above (Figs. 4–5). Vipera a. francisciredi* hugyi is close to V. a. hugyi; both are characterized by medially elongated frontal bones with a relatively narrow posterior region. This region is comparatively larger in V. a. francisciredi and V. a. aspis*francisciredi, whose strong similarity are suggested by both the ordination and the phenogram. As in the PCA of individual specimens (Fig. 3), V. a. aspis is distinctive, especially in the cranial base, and intermediate between the outgroups and all other subspecies. Finally, as expected, outgroup species (V. ammodytes and V. berus), with their small anterior basiophenoid (landmarks 1–4) and elongated posterior cranial base (Fig. 5), are distinct from V. aspis.

Patristic distances of V. a. francisciredi, V. a. aspis and V. a. hugyi from the UPGMC phenogram are strongly in agreement with those from the phylogenetic tree of Ursenbacher et al. (2006) ($r=0.992$).

DISCUSSION

Tests of sexual dimorphism were performed to decide whether pooling samples regardless of sex was warranted. Indeed, sexual dimorphism was not evident in our analysis and this is consistent with results from ecological studies that found no dietary differences between female and male vipers (Luiselli & Agrimi, 1991). Besides, selective pressures that might increase sexual dimorphism may be moderate in this species as bites are not

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Table 2. MANOVA sex × species of shape variables for frontal and cranial base of V. a. francisciredi, V. a. aspis, V. a. hugyi and V. a. aspis* francisciredi and for the combined data set. Significant values: *$P<0.05$; **$P<0.01$.

<table>
<thead>
<tr>
<th>View</th>
<th>Effect</th>
<th>$\lambda_{wss}$</th>
<th>$F$</th>
<th>d.f.</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal</td>
<td>Sex</td>
<td>0.792</td>
<td>1.663</td>
<td>6, 38</td>
<td>0.157</td>
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<tr>
<td></td>
<td>Group</td>
<td>0.386</td>
<td>2.403</td>
<td>18, 108</td>
<td>0.003**</td>
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<tr>
<td></td>
<td>Sex × group</td>
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<td>1.274</td>
<td>18, 108</td>
<td>0.219</td>
</tr>
<tr>
<td>Cranial base</td>
<td>Sex</td>
<td>0.770</td>
<td>1.097</td>
<td>6, 22</td>
<td>0.395</td>
</tr>
<tr>
<td></td>
<td>Group</td>
<td>0.246</td>
<td>2.234</td>
<td>18, 62.7</td>
<td>0.010*</td>
</tr>
<tr>
<td></td>
<td>Sex × group</td>
<td>0.654</td>
<td>0.565</td>
<td>18, 62.7</td>
<td>0.911</td>
</tr>
<tr>
<td>Combined</td>
<td>Sex</td>
<td>0.028</td>
<td>1.401</td>
<td>12, 14</td>
<td>0.271</td>
</tr>
<tr>
<td></td>
<td>Group</td>
<td>0.454</td>
<td>2.733</td>
<td>36, 42</td>
<td>0.001**</td>
</tr>
<tr>
<td></td>
<td>Sex × group</td>
<td>0.232</td>
<td>0.748</td>
<td>36, 42</td>
<td>0.811</td>
</tr>
</tbody>
</table>

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Fig. 3. Species discrimination in the V. aspis group using the combined data set (frontal bone + cranial base). First two axes of a PCA of all shape variables. PCA1 explains 44.2%; PCA2 explains 21.1%.
used during courtship and combat by males (Andrén, 1986). The main focus of the study was to assess whether subspecies distinguished on the basis of external morphology (Zuffi & Bonnet, 1999; Zuffi, 2002; Zuffi et al., 2003), and supported by a recent molecular analysis (Ursenbacher et al., 2006), were also supported by variation in cranial size and shape. Indeed, shape was significantly different and relationships among Italian subspecies of \textit{V. aspis} were completely congruent with molecular clades (Ursenbacher et al., 2006). \textit{V. a. francisciredi} and \textit{V. a. hugyi} were more similar to each other than any were to \textit{V. a. aspis}, as in the mtDNA phylogenetic tree by Ursenbacher et al. (2006). Moreover, even among subspecies the relationships identified by the analysis of cranial shape were congruent with results from mtDNA phylogeny. For example, the population from Montecristo Island, traditionally believed a valid subspecific taxon (\textit{V. a. montecristi}), had the same cranial shape as the specimens of \textit{V. a. hugyi} we analysed from southern Italy. We thus confirmed that the population from Montecristo Island may be considered a recent introduction from populations of \textit{V. a. hugyi} (Zuffi, 2002; see also Barbanera et al., 2009), as also previously suggested by its venom composition (Pozio, 1980). The monotypy of \textit{V. a. aspis} was also confirmed: populations from the western Alps had the same cranial shape as those from northern France, as expected from results on mtDNA phylogeny, and from external morphological analyses of scale and dorsal pattern variation (Golay et al., 2008).

Size, in contrast, did not show appreciable group differences. Cranial size might be evolutionarily more labile than shape and thus often unable to preserve a strong phylogenetic signal. This might be explained by the higher complexity of shape. Oxnard (2000) observed that morphometric comparisons of individual skeletal units (such as arms, limbs or teeth) tend to produce clusters that indicate functional convergences of anatomical parts in primates. In contrast, when variables from different anatomical regions are combined in a single analysis, separations of species mostly reflect evolutionary relatedness. He suggested that phylogenetic information within a structure is relatively small, whereas when several structures are analysed together, phylogenetic groups emerge (Oxnard, 2000). Similarly, the informativeness of a multivariate morphological descriptor like shape might be much larger than that of univariate size and thus more likely to pick up differences of phylogenetic interest. That shape might provide insight into the evolutionary history of this group was also suggested by the observation that \textit{V. a. aspis} had the

<table>
<thead>
<tr>
<th></th>
<th>\textit{V. a. francisciredi}</th>
<th>\textit{V. a. aspis}</th>
<th>\textit{V. a. hugyi}</th>
<th>\textit{V. a. aspis} \textit{francisciredi}</th>
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</thead>
<tbody>
<tr>
<td>\textit{V. a. francisciredi}</td>
<td>37.409</td>
<td>9.306</td>
<td>6.588</td>
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<tr>
<td>\textit{V. a. aspis}</td>
<td>0.003**</td>
<td>31.156</td>
<td>36.560</td>
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<tr>
<td>\textit{V. a. hugyi}</td>
<td>0.047*</td>
<td>0.005**</td>
<td>18.333</td>
<td></td>
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<tr>
<td>\textit{V. a. aspis} \textit{francisciredi}</td>
<td>0.451</td>
<td>0.008**</td>
<td>0.009**</td>
<td></td>
</tr>
</tbody>
</table>

(Fig. 4. Phenetic relationships among \textit{Vipera} species (including the outgroups and the two transition forms). MDS scatterplot of the mean shapes.)
most distinctive cranial shape. The geographic range of this subspecies in southeastern France and northwestern Italy overlaps with an area considered an important glacial refugium of the asp viper (Ursenbacher et al., 2006). Here, geographic isolation in a period of environmental change might have promoted morphological divergence.

In summary, the samples of *V. aspis* studied were significantly different, but clustered together to the exclusion of outgroup species. This was again congruent with the conclusion of Ursenbacher et al. (2006) that taxa within this group show differences smaller than those between well recognized separate species of *Vipera*.

Our samples also included two taxa that could not be unequivocally ascribed to defined subspecies, but had taxonomic features intermediate between two subspecies. One of them, *V. a. francisciredi* *francisciredi*, was very close to *V. a. hugyi*, while the other, *V. a. aspis* *francisciredi*, was very similar to *V. a. francisciredi*. We cannot support or refute the hypothesis of a supposed “hybrid” origin of these populations due to poor sampling; nevertheless, notwithstanding intermediate taxonomic features, cranial shape was very similar to only one of the supposed parental taxa. Larger samples will be needed to accurately quantify and compare cranial variation in *V. aspis* and carefully compare shape and size of individuals from contact areas. Indeed, the present study is aimed at providing a preliminary investigation of differences in *V. aspis* using hard-tissue morphology. The paucity of specimens from this group in museum collections strongly limited the size of our samples. This is a common problem in taxonomic analyses (Marcus, 1990), where preliminary investigations often help to stimulate extensive follow-up studies.

**ACKNOWLEDGEMENTS**

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REFERENCES


(L.) mediante analisi elettroforetica delle proteine contenute nel veleno. *Natura Milano* 71, 28–34.


**APPENDIX**

**Museum catalogue numbers and locality of collection for the specimens**

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Sex</th>
<th>Locality</th>
<th>Collection</th>
<th>Box</th>
<th>Catalogue number</th>
<th>Structure</th>
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</thead>
<tbody>
<tr>
<td>francisciredi</td>
<td>m</td>
<td>Valley of Sestaione (Lucca)</td>
<td>Calabresi 1</td>
<td>c96</td>
<td>frontal</td>
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<td>francisciredi</td>
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<td>Calabresi 4</td>
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Accepted: 7 May 2009
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1Sex: m, male; f, female.
2Locality (province).
3Collection: Calabresi – historical collection prepared by Enrica Calabresi in 1924 and presently housed at the ‘La Specola’ Zoological Museum of the University of Florence; MSNT – Museo di Storia Naturale e del Territorio of the University of Pisa.
4Box: reference number of specimens examined by Calabresi (1924).