Statolith identification of Mediterranean Octopodidae, Sepiidae, Loliginidae, Ommastrephidae and Enoploteuthidae based on warp analyses

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The statoliths of 14 species (193 right statoliths from subadult to adult individuals), belonging to five Cephalopoda families (Sepiidae, Loliginidae, Enoploteuthidae, Ommastrephidae and Octopodidae) were analysed using morphometric methods based on landmarks (geometric morphometry). The aim of the current study is to determine the discriminating power of statolith shape analysis in species identification of Mediterranean cephalopods. Discriminant analyses of the partial warps were able to fully identify (100% discrimination) the species of all families, except Octopodidae which showed some misclassification (correctly classified about 68–90%). These results were also shown by relative warp analysis. Octopodidae statoliths were studied for the first time using geometric landmark-based methods. Greatest differences in statolith shape between Octopodidae species, were in the area that unites the statolith dome with the flat wing. Landmark analysis applied to statoliths can be a useful taxonomic tool in the identification of closely related species.

INTRODUCTION

Cephalopods statoliths are paired calcareous structures composed of aragonite and calcite, associated with sensory epithelia and located inside two adjacent cavities, the statocysts (Clarke, 1978; Arkhipkin & Bizikov, 2000). The statocysts are organs associated with the detection of gravity and angular acceleration. Some authors believe that these organs are also sensitive to vibrational stimulation (Hanlon & Budelmann, 1987).

Similar to fish otoliths, statoliths have been used in age identification (Lipinski, 1986; Villanueva, 1992; Sánchez, 1995 among others). Statoliths are more precise for species identification of both recent and fossil cephalopods, compared to other hard structures, such as beaks and gladii (Clarke, 1978; Clarke & Fitch, 1979). Thus, morphological analyses of statoliths are useful in studies of cephalopod biology. In the first studies, the shape description of the statoliths was qualitative, without any quantification of the degree of similarity (Clarke, 1978). In these works, statolith nomenclature was established, i.e. in orders Sepiida and Teuthida the statolith structure is formed by the lateral dome, dorsal dome, rostrum, spur, ventral rostrum and wing, whereas in order Octopoda, the statolith shows a limpet or cone shape on one side and a flat attachment area on the other side.

With the advances of image processing systems, quantitative analytical methods for studying the shape of statoliths have been developed. Lombarte et al. (1997) studied the statolith intraspecific variability of three cephalopod species through contour analysis (outlines) of *Eledone cirrhosa*, *Sepia officinalis* and *Todarodes sagittatus*. However, with this method, that is based on Fourier's harmonics, it is not possible to determine the morphological features responsible for shape changes (Adams et al., 2003).

Dommergues et al. (2000) studied statoliths from the families Sepiidae, Sepiolidae, Loliginidae, Enoploteuthidae and Ommastrephidae based on the comparison of homologous points (landmarks). Landmark based methods provide variables with a morphological interpretation (Adams et al., 2003). A similar methodology was employed in the morphometric study of the shells of three Mediterranean cuttlefish species: *Sepia officinalis*, *Sepia elegans* and *Sepia orbignyana* (Neige & Boletzky, 1997).

Considering the specific characteristics of the statolith's morphology and the efficiency of shape analysis based on landmarks the objectives of the present work are: (1) to describe the main specificities of Octopodidae statoliths and the most characteristic landmarks of this group; and (2) to determine the adequacy of landmark analysis for discriminating species according to the statolith shape in Mediterranean cephalopod families.

MATERIALS AND METHODS

Subadult and adult individuals of 14 species of the western Mediterranean Sea, belonging to the families Sepiidae: *Sepia orbignyana* (7 right statoliths), *Sepia officinalis* (19) and *Sepia elegans* (10); Loliginidae: *Loligo (Alloteuthis) media* (16), *Loligo (Alloteuthis) subulata* (9) and *Loligo (Loligo) vulgaris* (14); Enoploteuthidae: *Abralia veranyi* (6); Ommastrephidae: *Illex coindetii* (8), *Todarodes sagittatus* (35) and *Todaropsis eblanae* (3); Octopodidae: *Eledone cirrhosa* (19), *Eledone moschata* (14), *Octopus salutii* (18) and *Octopus vulgaris* (15) were analysed. A total of 193 right statoliths was dissected and stored in a dry state. In Sepiida and Teuthida statoliths an orthogonal projection image of the anterior view (Clarke & Fitch, 1979) was digitized, and in Octopoda, a lateral view projection (Clarke, 1978) was used. Both groups were digitized

Figure 1. Location of the landmarks and pseudo-landmarks on the statoliths. (A) Lateral view of an Octopoda statolith; (B) anterior view of a Teuthiida statolith.

using a Sony High-res CCD video camera coupled to a Leica Wild microscope. Processing of digital images, calibration and analysis was carried out using Optimas 6.0 software.

Figure 1A shows the location of the seven landmarks and pseudolandmarks selected in Octopoda statoliths. The pseudolandmarks in Octopoda statoliths (2, 6 and 7) were based on the points of maximum inflexion of the outline (Adams et al., 2003). Figure 1B shows the 18 landmarks and pseudo-landmarks in Teuthida and Sepiida statoliths considered by Dommergues et al. (2000). Six landmarks were positioned in the lateral dome, eight in the wing, three in the ventral rostrum and one in the dorsal dome.

Details on the procedures for geometrical morphometric analysis can be found in Adams et al. (2003), nevertheless a brief description is given here. After digitizing, landmark maps were rotated, scaled (to unit centroid size) and translated through a generalized least squares superimposition (GLS) procedure (generalized procrustes) to eliminate scale and orientation distortions (using tpsRel 1.24 Rohlf, 2001). A thin-splate spline procedure was used to fit an interpolated function to an average map (consensus configuration) of the carapace shape and derive the uniform and non-uniform (partial warps) components of shape variation. The two uniform components describe differences that affect all parts of the carapace equally (global differences). The magnitude of the first of these indicates the degree of stretching along the X-axis relative to the average carapace map. Whereas the magnitude of the second indicates compressions or dilatations along the Y-axis. The non-uniform shape components (partial warps) describe localized departures from the average carapace map. Both partial warps and uniform components were used as shape variables in a discriminant analysis and a multivariate analysis of variance (MANOVA), within each group. Shape changes were visualized through relative warp analysis (similar to principal components). The consensus configuration statolith shape of each species was estimated.

RESULTS

The greatest differences in shape, among species, were found in the concavities of the statolith outlines (points of maximum inflection) (Table 1A,B, Figure 1). The geometric morphometric analyses carried out on the statoliths within each taxonomic group: Sepiidae, Loliginidae, Enoploteuthidae, Ommastrephidae (both families from the suborder Oegopsina were analysed together) and Octopodidae, are described below.

Table 1. Relative contribution of each landmark in the relative warp analyses. (A) Octopodidae, represented by 7 landmarks. (B) Sepiidae, Loliginidae, Ommastrephidae and Enoploteuthidae (last two families below to suborder Oegopsina) represented by 18 landmarks. The landmarks with maximum variability are indicated in bold print. Landmark locations are shown in Figure 1.

A.		B.			
landmak	Octopodidae	landmark	Sepiidae	Loliginidae	Oegopsina
1	0.004	1	0.214	0.080	0.067
2	0.015	2	0.256	0.212	0.172
3	0.123	3	0.029	0.033	0.018
4	0.563	4	0.019	0.031	0.015
5	0.194	5	0.048	0.091	0.096
6	0.070	6	0.042	0.051	0.063
7	0.032	7	0.037	0.062	0.063
		8	0.052	0.104	0.150
		9	0.028	0.071	0.093
		10	0.004	0.025	0.024
		11	0.008	0.026	0.022
		12	0.062	0.039	0.046
		13	0.031	0.023	0.018
		14	0.014	0.019	0.012
		15	0.012	0.007	0.006
		16	0.079	0.074	0.098
		17	0.051	0.043	0.030
		18	0.015	0.006	0.007

Figure 2. Landmark analyses of Sepiidae statoliths: (A) plots of the first two relative warps; and (B) consensus landmark figure of each analysed species (*Sepia officinalis*, *Sepia orbignyana* and *Sepia elegans*).

Sepiidae

The relative warps of the three species of *Sepia* in the western Mediterranean (Figure 2A) showed that the first axis (20.96% of the variability) separated the *Sepia officinalis* (with negative values) from *Sepia orbignyana* and *Sepia elegans* (with positive values). The three species were differentiated mainly by the relative position of the landmark 1, the wing fissure (landmark 2) and the relative position of the lateral and dorsal dome (landmarks 12, 13, 14, 15, 16, 17 and 18). *Sepia officinalis* showed a smaller lateral dome and more developed dorsal dome than *S. elegans*, whereas *S. orbignyana* displayed intermediate features. *Sepia officinalis* could also be distinguished from the other species by the reduced wing fissure (Figure 2B).

A MANOVA of the partial warps showed significant differences between the statolith morphology of each species (Wilks's $\Lambda=0.202$; approximate $F_{[44,32]}=11.003$; $P<0.001$). The discriminating function was able to correctly assign all specimens to the respective species based on the statolith shape, thus the probability for a correct prediction is 100% in each species.

Loliginidae

In the relative warps analysis of the three Loliginidae species (*Loligo (Loligo) vulgaris*, *Loligo (Alloteuthis) media* and

Figure 3. Landmark analyses of Loliginidae statoliths: (A) plots of the first two relative warps; and (B) consensus landmark figure for each analysed species (*Loligo (Alloteuthis) media*, *Loligo (Alloteuthis) subulata* and *Loligo (Loligo) vulgaris*).

Loligo (Alloteuthis) subulata) the two subgenera (*Alloteuthis* and *Loligo*) were separated by the first relative warp, while the two species of the subgenus *Alloteuthis* (*L. media* and *L. subulata*) were separated by the second relative warp (Figure 3A). The shape differences observed were mainly in the statolith width and in the extension of the wing fissure (landmark 2). *Loligo vulgaris* had wider statoliths, deeper spur and wing fissures than the subgenus *Alloteuthis* ones (Figure 4B). The main shape difference between *L. subulata* and *L. media* is in the relative position of the dorsal and lateral dome, hence, statoliths from *L. subulata* are characterized by a more protruding dorsal dome than *L. media* (Figure 3B).

A MANOVA of the partial warps showed significant differences between the statolith morphology of each species (Wilks's $\Lambda=0.001$; approximate $F_{[64,10]}=6.360$; $P<0.001$). As in Sepiidae, the discriminating function was able to correctly classify all individuals into the respective species based on the statolith shape, thus the probability for a correct prediction is 100% in each species.

Ommastrephidae and Enoploteuthidae (Oegopsina)

The relative warp analysis of the four species of suborder Oegopsina from the western Mediterranean (*Abralia veranyi*, *Illex coindetii*, *Todaropsis eblanae*, *Todarodes sagittatus*) revealed clear differences between species

Figure 4. Landmark analyses for Ommastrephidae and Enoploteuthidae (suborder Oegopsina) statoliths: (A) plots of the first two relative warps; and (B) consensus landmark figure for each analysed species (*Illex coindetii*, *Todaropsis eblanae*, *Tadarodes sagittatus* and *Abralia veranyi*).

(Figure 4A). The first relative warp (23.49% of the total variability) separated the three species of the family Ommastrephidae according to the wing position. *Illex coindetii* showed a statolith wing close to the ventral rostrum and far from the dorsal dome. *Tadarodes sagittatus* statoliths had a wing that was closer to dorsal dome than in *I. coindetii* and *Todaropsis eblanae*. The shape of the dorsal dome in *T. eblanae* was intermediate between *I. coindetii* and *Tadarodes sagittatus* (Figure 4B). The second axis of the morphospace (11.59% of the total variability) placed the statoliths from the family Enoploteuthidae (*A. veranyi*) at the opposite extreme to the Ommastrephidae statoliths (*I. coindetii*, *Todaropsis eblanae*, *Tadarodes sagittatus*). *Abralia veranyi* statoliths were elongated with a small wing fissure.

A MANOVA of the partial warps showed significant differences between the statolith morphology of each species (Wilks's $\Lambda=0.0001$; approximate $F_{[96,51]}=11.0029$; $P<0.001$). The linear discriminant function assigned a 100% probability of correctly classifying each species.

Octopodidae

Relative warp analysis of the four species of family Octopodidae (*Eledone cirrhosa*, *Eledone moschata*, *Octopus vulgaris* and *Octopus salutii*) showed that, combining the two axes together the genus *Octopus* is located on both negative sides (of warp 1 and 2), whereas the genus *Eledone* in both positive sides, except for two individuals of

Figure 5. Landmark analyses of Octopodidae statoliths: (A) plots of the first two relative warps; and (B) consensus landmark configuration (average configuration) of each analysed species (*Octopus vulgaris*, *Octopus salutii*, *Eledone moschata* and *Eledone cirrhosa*).

O. salutii (Figure 5A). Table 1A shows the relative importance of each landmark in the shape differences. Thus, the differences in the concavity (located between the flat wing area and the rostrum, landmarks 3, 4, 5, Figure 1B), permit separation of the genera. In *Octopus* the statolith's rostrum (landmark 5) is more distant from the flat wing area (landmark 3) than in *Eledone*. In *Eledone*, landmarks 3 and 5 are located in approximately the same plane whereas in *Octopus* the rostrum stands out sharply. Furthermore, the lateral view of *Eledone* statoliths is more elliptical than *Octopus* statoliths, which are sub-triangular (Figure 5B). The interspecific statolith shape differences within each genus were not clear.

A MANOVA of the partial warps showed significant differences between the statolith morphology of each species (Wilks's $\Lambda=0.202$; approximate $F_{[30,162]}=8.077$; $P<0.001$). The linear discriminant function correctly classified about 80% of the specimens. The *Eledone* statolith shape showed a greater discriminating power than the *Octopus* statolith (*Eledone* 85–90%, whereas in *Octopus* was 68–73%).

DISCUSSION

Different factors influence the statolith morphology. Their shape is an important phylogenetic value (Clarke, 1978;

Dommergues et al., 2000) and is considered to be associated with adaptive abilities related with mechanoreception (Budelmann et al., 1987; Arkhipkin & Bizikov, 2000).

Discriminant analyses of the partial warp weight matrix successfully identified and separated the statoliths according to the species and genus. In orders Sepiida and Teuthida, 100% of the statoliths were identified correctly, while in the order Octopoda nearly 80% of the statoliths were appropriately classified. These results show that landmark analysis of statoliths discriminates similar species, as Clarke (1978) discussed. This is particularly important in the separation of species that are difficult to classify, like the subgenus *Alloteuthis* (Laptikhovsky et al., 2003). Within Mediterranean Sepiidae, our results are in accordance with those obtained by Neige & Boletzky (1997) for the landmark analyses using the cuttlebones. In both studies *Sepia officinalis* showed morphological characteristics further apart from those of *Sepia elegans* and *Sepia orbygniana*, and thus these two species are more similar. The shape and position of the wing in relation to the dorsal dome and rostrum are the characteristics that better differentiate the species.

Unlike the statoliths of Sepiida and Teuthida, the shape of the Octopoda statoliths was not previously studied using the warp analysis. The form and ultrastructure of Octopoda statoliths are simpler than other groups studied. The Octopoda statolith ultrastructure did not show growth rings, unlike other cephalopods groups (Lipinski, 1986). As for their shape, the available descriptions are limited to a few genera such as *Eledone*, *Eledonella*, *Benthoctopus*, *Enteroctopus* and *Octopus* (Clarke, 1978; Clarke & Maddock, 1988; Villanueva et al., 1991; Sakaguchi et al., 2000). Previous studies found that Octopoda statoliths have similar morphology between species with a high individual variability within the species, which makes the classification difficult. This morphological variability is accentuated in the area of the excisura rostral that unites the superior part of the dome with the flat wing zone. Lombarte et al. (1997) associate the individual variability of the statolith rostral area with growth. The statoliths of the larger individuals were characterized by having greater development in the rostral flat wing (landmark 3 in Figure 1B) than in the rostral area of the dome (landmark 5 in Figure 1B). However, in the species of the genus *Octopus* this was not observed (Sakaguchi et al., 2000). Statoliths from *Eledone* and *Octopus* showed the main morphological differences in the general shape of the statolith lateral view. Statoliths of *Eledone* are more elliptical than those of *Octopus*, which are subtriangular.

Thus, landmark analysis applied to statoliths can be a useful tool in the identification of closely related species, in both modern and fossil specimens (Clarke, 1978; Clarke & Fitch, 1979). Also its potentiality can be extended to age identification, as during development the statolith shape changes from the paralarvae to the juvenile and subadult phases (Lombarte et al., 1997; Dommergues et al., 2000).

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