External shape analyses in Atherina boyeri (Risso, 1810) from different environments

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External shape analyses in *Atherina boyeri* (Risso, 1810) from different environments

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Abstract

In this study, specimens of the large-scale sand smelts *Atherina boyeri*, a fish species occurring in different environments (freshwater lakes, coastal lagoons, sea), were examined for their body shape and the differences observed were analysed statistically. The variation of external morphology was investigated in 11 populations from different environments. The morphology was characterized by means of geometric morphometrics and compared; results showed that the phenotypic (morphometric) distances among samples differed significantly by Relative Warp Analysis, specimens from the coastal lagoon being clearly separated from those belonging to lake and marine populations on the first RW1 axis and the latter ones separated on the RW2 axis. The results obtained also show the absence of a gradient of shape differences according to lake–lagoon–sea origin or eidonamic convergence in fish from environments with similar (high) trophic productivity. An integration of geometric morphometric data in a multidisciplinary approach with genetic, biochemical, geographical, molecular and other morphological analyses is necessary to describe the phylogenetic relationships of Atherinidae, shedding light on some ambiguous taxonomic ranks.

Keywords: *Atherina boyeri*, ecomorphology, geometric morphometric, image analysis, fish ecology

Introduction

Sand smelt (*Atherina boyeri*) is characterized by a high polymorphism and this generates continuous debates about its systematics (Kiener & Spillmann 1969; Trabelsi et al. 2002a,b). The large-scale sand smelt *Atherina boyeri*, Risso 1810, is a coastal fish, widely distributed throughout the Mediterranean and Black Sea and in the eastern Atlantic Ocean. This species is gregarious and carnivorous, feeding on small crustaceans, worms, molluscs and fish larvae (Froese & Pauly 2007). It is a euryhaline species which spends a relevant part of its lifecycle in brackish coastal environments (Bamber & Henderson 1988); occasionally, it can be found in freshwater habitats. Reproduction can occur in all these environments. During the larval and juvenile stages the fishes reside in coastal lagoons; before the cold season, the adults preferably leave the inshore waters to move towards the sea (Mistri & Colombo 1988).

The taxonomy of genus *Atherina* is controversial due to its high morphological variability (Kiener & Spillmann 1969; Marfin 1982; Bamber & Henderson 1988; Creech 1991; Francisco et al. 2008), with a relevant phenotypic polymorphism in *A. boyeri* (Focant et al. 1999; Astolfi et al. 2005). Before 1969 nearly 20 distinct species were described in the literature, until Kiener and Spillman (1969) considered them synonymous of *A. boyeri*. Klossa-Kilia et al. (2007) pointed out that the Family Atherinidae is represented in Europe by a single genus consisting of three species, *Atherina boyeri* Risso 1810, *A. hepsetus* L. 1758 and *A. presbyter* Cuvier 1829. Other recent studies (Trabelsi et al. 2002a) and molecular investigations (Avise & Wollenberg 1997; Gilles et al. 2000) clearly identified three groups, which were proposed to be elevated to the rank of species, distinguished on the basis of morphology, morphometry and biochemistry. The first species-group consisted...
of non-punctuated marine specimens (*A. boyeri*), the second species of punctuated marine specimens (*A. punctata*), and the third, lagoon specimens (*A. lagunae*) (Trabelsi et al. 2002b). Klossa-Kilia et al. (2002) demonstrated, through mitochondrial DNA analyses, the existence of two separate populations: the lagoon and the marine populations. However, at present, the taxonomic identification of this polymorphic species is still based on the keys of Mercader et al. (2003), which are based on colour, the number of soft rays and spines of the dorsal and anal fins, the number of scales in the longitudinal series and the eye diameter referred to snout length as diagnostic characters. Recently, the high morphological variation of this taxonomic group has been associated with high genetic diversity among populations, even if occasional exchanges of individuals among populations cannot be ruled out, favoured by the migration of the adults to the coastal sea during the winter (Congiu et al. 2002). Francisco et al. (2008) demonstrated that *A. boyeri* is a complex clade composed of clear geographic subdivisions. Also, the presence of an isolation-by-distance pattern in the Italian populations of large-scale sand smelt (Congiu et al. 2002) seems to suggest that the homogenizing effect of gene flow counteracts the reproductive isolation as a consequence of adaptive divergence in this region.

Because of the heterogeneity of environments in which this species lives and the remarkable complexity of the taxonomic relationships within genus *Atherina*, an ecomorphological approach to this species could improve knowledge about *Atherina*.

Ecomorphology is defined by Motta and Kotrschal (1992) as the study of the relationship between shape (expressed as functional design by Wainwright 1991) and habitat (Motta & Kotrschal 1992), the habitat being expressed as the set of abiotic and biotic parameters with respect to the organism itself (Motta et al. 1995; Norton et al. 1995; Antonucci et al. 2007). In particular, ecomorphology addresses the relationship between the morphology of individuals, populations or higher taxa on one hand and their ecology on the other (Leisler & Winkler 1985). One tenet central to studies in ecomorphology is that morphology limits the performance or capabilities of an individual and hence its patterns of resource use (Wainwright 1991; Long 1995; Chan 2001). Due to the habitat differences, trophic preferences and behaviour of fishes, ecomorphological studies of this group represent a stimulating topic in biology (Costa & Cataudella 2006; Antonucci et al. 2009).

In this scenario, this study is aimed at assessing morphological differences in body shape in 11 populations of large-scale sand smelts (*A. boyeri*) from freshwater, brackish and marine environments, in an ecomorphological framework.

### Materials and methods

**Samples**

The external shape of 523 specimens of large-scale sand smelt (*A. boyeri* determined following the key of Mercader et al. 2003) from 11 different sites in Italy were analysed (Figure 1). Table I reports the information on the number of specimens per site and their mean size (standard length: cm). The following diagnostic characters were considered: colours (also in terms of punctuation), number of soft rays and spines of the dorsal and anal fins, number of scales in the longitudinal series, eye diameter with respect to snout length. No specimen presented punctuations. All sampled individuals were adult and comparable, as were all at the saturating size (i.e. the size after which the shape remains constant: isometry), considering their growth trajectory *sensu* Alberch et al. (1979) and Loy et al. (2001). Individuals were sampled during the same, non-reproductive period (December–January) (Boscolo 1970; Fernandez-Delgado et al. 1988; Costa et al. 2005), in order to avoid shape variations due to gonad maturity (i.e. abdomen swelling).

The available information on some of the environmental characteristics of the sampling sites are reported in Table II. At all sites with the exclusion of Siniscola, adults are naturally (lakes) or artificially (lagoons) prevented from migrating towards the open sea in winter.

**Shape analysis**

Differences among specimens were analysed by means of geometric morphometry (Bookstein 1991; Loy et al. 2000; Costa et al. 2004, 2006; Zelditch et al. 2004), which allows the visualization and quantification of morphological differences by representing the deformations of a morphometric point (landmarks) configuration in a coordinate space. Landmarks are defined as homologous points bearing information on the geometry of biological forms (Bookstein 1991; Loy et al. 2001).

Coordinates of landmarks were aligned by Generalized Procrustes Analysis, a procedure consisting of three steps: the translation of point coordinates to a common centroid located at the origin (0, 0) of a reference system of coordinates; the scaling of each outline with the unitary centroid size; and finally the rotation of coordinates to minimize the sum of square distances between correspondent
landmarks (Bookstein 1991; Antonucci et al. 2011). On the photograph of each individual, 22 landmarks were digitized using the software TpsDig (Rohlf 2004a) (Figure 2). The consensus configurations (an average shape) of the individuals belonging to each site were visualized by ‘unwarping’ the images of each species, so that the landmarks coincide with their positions in a reference configuration, using the software TpsSuper (Rohlf 2004b; Costa & Cataudella 2006). To explore the differences among large-scale sand smelt samples, a Relative Warp Analysis (RWA) was performed using the software TpsRelw (Rohlf 2006). The relative warps are computed to summarize the variation among the specimens with respect to their partial warp scores. This method is analogous to a principal component analysis and is used to quantify changes in shape and pattern of morphometric variation within and among groups, when each individual is considered as fitting into a consensus configuration (Cadrin 2000).

A cladogram of Procrustes morphological distances was performed with the complete linkage algorithm to observe distances among the different samples (Bookstein 1991). The splines (deformation grids) of the extremes of each RWA axis and cladograms were extracted using TpsRelw (Rohlf 2006).

The significance of the correlation between each of the first two RW axes and each available environmental variable of the different sampling sites (temperature and salinity at sampling time, maximum water depth) was tested with Spearman’s cross-correlation (Costa & Cataudella 2006).
Table I. Sampling sites, labels, number of specimens and mean size (standard length (SL) in cm) ± SD.

<table>
<thead>
<tr>
<th>Sampling sites</th>
<th>Labels</th>
<th>No. specimens</th>
<th>Mean size (SL) ± SD (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bracciano</td>
<td>BR</td>
<td>29</td>
<td>8.9 ± 2.4</td>
</tr>
<tr>
<td>Trasimeno</td>
<td>TR</td>
<td>59</td>
<td>9.6 ± 2.3</td>
</tr>
<tr>
<td>Lesina</td>
<td>LE</td>
<td>29</td>
<td>8.6 ± 2.5</td>
</tr>
<tr>
<td>Orbetello</td>
<td>OR</td>
<td>59</td>
<td>8.4 ± 2.7</td>
</tr>
<tr>
<td>Varano</td>
<td>VA</td>
<td>53</td>
<td>9.5 ± 2.1</td>
</tr>
<tr>
<td>Santa Gilla</td>
<td>SG</td>
<td>56</td>
<td>8.6 ± 2.5</td>
</tr>
<tr>
<td>Monaci</td>
<td>MO</td>
<td>25</td>
<td>8.1 ± 2.7</td>
</tr>
<tr>
<td>Valle Figheri</td>
<td>VF</td>
<td>45</td>
<td>8.0 ± 2.4</td>
</tr>
<tr>
<td>Caprolace</td>
<td>CP</td>
<td>74</td>
<td>7.4 ± 2.4</td>
</tr>
<tr>
<td>Siniscola</td>
<td>SI</td>
<td>57</td>
<td>9.4 ± 2.0</td>
</tr>
<tr>
<td>Fogliano</td>
<td>FO</td>
<td>37</td>
<td>7.7 ± 2.7</td>
</tr>
</tbody>
</table>

To test the sample’s homogeneity in terms of size, a multivariate test for a general linear model (GLM) predicting shape variation (as captured by the partial warps and the uniform shape component) as a function of an independent size variable (computed as centroid size; Rohlf & Slice 1990) was computed with the software TpsRegr (Rohlf 2005). The above-mentioned resulting probability of the multivariate test was compared with the results of a permutation test (1000 random permutations) for Wilks’ Lambda (Costa et al. 2004). The shape variation with size was decomposed into uniform (stretching and shearing of shape) and non-uniform (localized shape changes) shape components; the absolute contribute of the uniform component was calculated.

Results

The consensus configurations referring to the 11 different populations are shown in Figure 3. In the ‘average’ images, those areas that appear fuzzy and ‘out of focus’ correspond to the body regions that vary from specimen to specimen in a way not well correlated with the variation in the positions of the landmarks (Rohlf 2004b).

Table II. Environmental descriptors of environments where samples were harvested; salinity ranges during the year, temperature and salinity (ppt) at sampling time, maximum depth (m) and surface (km²).

<table>
<thead>
<tr>
<th>Labels</th>
<th>Typology</th>
<th>Salinity range (ppt)</th>
<th>Salinity at sampling (ppt)</th>
<th>Temperature at sampling (°C)</th>
<th>Max. depth (m)</th>
<th>Surface (km²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR</td>
<td>Lake</td>
<td>&lt;5</td>
<td>0</td>
<td>12</td>
<td>165</td>
<td>57</td>
</tr>
<tr>
<td>TR</td>
<td>Lake</td>
<td>&lt;5</td>
<td>0</td>
<td>12</td>
<td>5</td>
<td>121</td>
</tr>
<tr>
<td>LE</td>
<td>Coastal lagoon</td>
<td>8–37</td>
<td>12</td>
<td>14</td>
<td>1.15</td>
<td>51</td>
</tr>
<tr>
<td>OR</td>
<td>Coastal lagoon</td>
<td>23–40</td>
<td>25</td>
<td>14</td>
<td>1.5</td>
<td>27</td>
</tr>
<tr>
<td>VA</td>
<td>Coastal lagoon</td>
<td>23–35</td>
<td>26</td>
<td>14</td>
<td>5</td>
<td>65</td>
</tr>
<tr>
<td>SG</td>
<td>Coastal lagoon</td>
<td>30–42</td>
<td>35</td>
<td>14</td>
<td>2.5</td>
<td>15</td>
</tr>
<tr>
<td>MO</td>
<td>Coastal lagoon</td>
<td>29–40</td>
<td>32</td>
<td>14</td>
<td>1</td>
<td>0.9</td>
</tr>
<tr>
<td>VF</td>
<td>Coastal lagoon</td>
<td>12–36</td>
<td>20</td>
<td>15</td>
<td>2</td>
<td>35</td>
</tr>
<tr>
<td>CP</td>
<td>Coastal lagoon</td>
<td>35–40</td>
<td>26</td>
<td>14</td>
<td>2.3</td>
<td>3</td>
</tr>
<tr>
<td>SI</td>
<td>Marine site</td>
<td>38</td>
<td>38</td>
<td>13</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>FO</td>
<td>Coastal lagoon</td>
<td>38–40</td>
<td>38</td>
<td>14</td>
<td>3</td>
<td>39</td>
</tr>
</tbody>
</table>

Figure 2. Description of the landmarks used to analyse the shape of large-scale sand smelt: (1) snout tip; (2) anterior insertion of the first dorsal fin; (3) posterior insertion of the second dorsal fin; (4) and (6) points of maximum curvature of the caudal peduncle; (5) posterior-most trunk extremity; (7, 8) posterior and anterior insertion of the anal fin, respectively; (9) insertion of the pelvic fin; (10) ventro-lateral insertion of the opercular plate; (11) posterior-most extremity of premaxillar; (12) centre of the eye; (13) upper limit of the opercular plate; (14) lateral maximum extension of the opercular plate; (15, 16) upper (dorsal) and lower (ventral) insertion of the pectoral fin, respectively; (17, 18) upper (dorsal) and lower (ventral) margin of the eye, respectively; (19) uppermost limit of pre-operculum; (20) posterior (caudal) insertion of the first dorsal fin; (21) anterior (cephalic) insertion of the second dorsal fin; (22) anus.
Figure 3. Averaging the unwarped images pixel by pixel (consensus configurations) of the 11 different populations obtained with the software TpsSuper.

Figure 4. Relative Warp scores centroids of the specimens of *A. boyeri* belonging to 11 different sites. Vertical and horizontal lines show standard errors (SE) for each group. On the left and central right of the graph, the extreme of the RW1 splines are reported. On the upper and lower part of the right side of the graph, the extreme of the RW2 splines are reported.

The multivariate test for a GLM predicting shape variation as a function of an independent size variables (computed as centroid size) revealed a low percentage explained by this model (13.06% including the uniform component). The result of the permutation test showed a Wilks’ Lambda value of 0.29 (0.10%; smaller percentages imply significance). The absolute contribution of the uniform component was 3.81.

The plot of the first two axes of the RWA computed on individuals sampled in the 11 different sites is shown in Figure 4. Coastal lagoon specimens (CP, MO, OR, SG, LE, VA and VF) are mainly aggregated near the axes origin, whilst lake and marine populations plotted in the positive side of RW1. These latter are separated on the RW2, where marine (SI) and Lake Trasimeno (TR) lots are positioned on the negative side, in quadrant IV, while Bracciano lake (BR) is on the positive semi-axis.

The spline relative to the BR lake population (on the top right side of the Figure 4) is characterized by a large eye, a small mouth gap upper-positioned, a short caudal peduncle and a pelvic fin caudal located below the centre of the mass (see also Figure 3).

The spline of the marine population SI (on the bottom right side of the Figure 4) has a taller body profile, a smaller eye, a larger and more ventral mouth gap, a longer caudal peduncle and the insertion of the pelvic fin more cephalic located with respect to the BR lot (see also Figure 3). The spline of the other lake population (TR; on the centre right of the Figure 4) has, with respect to the marine population SI, a higher body profile, a shorter caudal peduncle and the insertion of the pelvic fin backward-positioned.

The spline of the coastal lagoon populations (in the top left side of Figure 4) has a larger head region, a larger mouth gap less upper-positioned, a larger eye and a larger pectoral fin when compared with the others.

The correlation between the mean value for each population on each of the first two RW axes and each environmental characteristic of the different sampling sites, tested with Spearman’s cross-correlation (Table III), shows a low significant correlation only for RW1 and maximum depth (Spearman $r$ value = 0.535; $p = 0.041$).

The cladogram extracted from the Procrustes distances of the eight coastal lagoon populations of *A.
A. boyeri is reported in Figure 5. It is possible to observe that (i) the population from Lesina is the most distant from all the others; (ii) the populations from central Tyrrhenian coastal lagoons (CP, FO, MO and OR) are less distant among them; (iii) the Orbetello population remained more distant from the Pontini Lake lots. The shared spline relative to the Tyrrhenian populations (on the bottom right side of Figure 5) is characterized by a higher body profile, a larger eye and mouth gap with respect to the shape of the other populations (Adriatic ones and SG).

Discussion

None of the A. boyeri individuals analysed here presented punctuation, seemingly contradicting the hypothesis of the three Atherina species formulated by Trabelsi et al. (2002b). Conversely, we found statistically quantified differences in body shape among the 11 groups of the large-scale sand smelts from different habitats (inland lakes, coastal lagoons and sea sites), for the first time. The observations based on RWA (Figure 4) demonstrated evident phenotypic (morphometric) differences among samples, and the analysis of the individuals plotted on the first axis of RWA allows exclusion of a clear modelling effect of the habitat on morphotypes. In fact, differences in morphotype do not follow the lake–lagoon–sea gradient: on the first axis (RW1), coastal lagoon specimens (CP, MO, FO, OR, SG, LE, VA, and VF) appear more similar (aggregated) than between lake and marine specimens; possibly this can be related to the fact that lake and marine populations are limited to more isolated regions. Again, we found that Bracciano large-scale sand smelt morphotype is not associated to the Trasimeno one in the same cluster, probably because of the different depth and width of these lakes. Conversely, we find quite unexpected similarities among the Trasimeno (lake) and Siniscola (sea) morphotypes: this could be explained by hypothesizing that the thinner shape of their caudal peduncle could help avoidance of predators in extended water bodies, such as the sea or Trasimeno lake. This is supported by the correlation between RW1 and maximum depth of the sampling site.

Samples from some coastal lagoons showed a large overlap. The shared coastal lagoon morphotype (top left side of Figure 4) was characterized by a taller body profile, a larger head region, larger and less upper-positioned mouth gap, larger pectoral fin and larger eye diameter, with respect to the other ones. However, by analysing the cladogram extracted from the Procrustes distances of the coastal lagoon specimens (Figure 5), it is also possible to infer some differences within this group. First, the Lesina population is different from those from other coastal lagoons, which could be split into two main groups: one comprising the central Tyrrhenian coastal lagoons (Pontini Lakes and Orbetello) lots and the other, the other coastal lagoons populations, grouped without any geographic rationale. Why the Lesina large-scale sand smelt differs in body shape from those of the other coastal lagoons could be ascribed to the peculiar physiographic, ecological and management characteristics of Lesina lake (SOGESID 2002). In fact, as many as 24 different waterways and 6 underground springs flow into this 46,000 ha lake, and some of these springs are further characterized by constant high water temperature (20–27°C) all through the year. All of these characteristics make Lesina a large eutrophic reservoir (productivity of about 40 kg/ha; Cataudella et al. 2001), with a permanent salinity gradient from east (8 ppt) to west (37 ppt), a concomitant intense and rich water circulation and very low anthropic pressure. However, if the high productivity of Lesina lagoon was the reason for hosting peculiarly shaped sand smelt, we should have to expect a similar shape in fish from Santa Gilla, one of the most productive Italian lagoon systems (319–620 kg/ha), but this is not the case. Lesina probably differs throughout the year in salinity gradient, water level and temperature stability.

Morphological and morphometric differences among populations from different lagoons may reflect not only phenotypic plasticity, but also the effect of isolation, selection and genetic drift (Maltagliati 1999). Such isolation between ecotypes can enable natural selection to enhance existing differences between alternative morphotypes (and also between populations that differ in the expression of such morphotypes) (Pfennig et al. 2010).

In summary, the differences in body shape of A. boyeri from different environments were demonstrated but the key modulating factors responsible for such differences were not ascertained, there being

Table III. Spearman’s cross-correlation between each of the first two RW axes and each environmental characteristic (as reported in Table II) of the different sampling sites (temperature and salinity at sampling time, maximum depth).

<table>
<thead>
<tr>
<th></th>
<th>RW1</th>
<th>RW2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
</tr>
<tr>
<td>Salinity at sampling</td>
<td>0.512</td>
<td>0.083</td>
</tr>
<tr>
<td>Temperature at sampling (°C)</td>
<td>0.295</td>
<td>0.078</td>
</tr>
<tr>
<td>Maximum depth (m)</td>
<td>0.535</td>
<td>0.041</td>
</tr>
</tbody>
</table>

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so many physical and chemical variables characteristic of lagoon, lake and sea environments. In this framework, geometric morphometric data could be integrated in a multidisciplinary approach, adding genetic, biochemical, geographical, molecular and other morphological approaches in order to describe the phylogenetic relationships of Atherinidae, and shed light on the ambiguous taxonomic attributions.

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