Identifying sagittae otoliths of Mediterranean Sea gobies: variability among phylogenetic lineages

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Gobiidae is the most species rich teleost family in the Mediterranean Sea, where this family is characterized by high taxonomic complexity. Gobies are also an important but often-underestimated part of coastal marine food webs. In this study, we describe and analyse the morphology of the sagittae, the largest otoliths, of 25 species inhabiting the Adriatic and northwestern Mediterranean seas. Our goal was to test the usefulness and efficiency of sagittae otoliths for species identification. Our analysis of otolith contours was based on mathematical descriptors called wavelets, which are related to multi-scale decompositions of contours. Two methods of classification were used: an iterative system based on 10 wavelets that searches the Anàlisi de Formes d'Otòlits (AFORO) database, and a discriminant method based only on the fifth wavelet. With the exception of paedomorphic species, the results showed that otolith anatomy and morphometry can be used as diagnostic characters distinguishing the three Mediterranean phylogenetic goby lineages (*Pomatoschistus*-lineage, or sand gobies, *Aphia*-lineage and *Gobius*-lineage). The main anatomical differences were related to overall shape (square to rhomboid), the development and shape of the posterodorsal and anteroventral lobes, and the degree of convexity of dorsal and ventral margins. Iterative classifications and discriminant analysis of otolith contour provided very similar results. In both cases, more than 70% of specimens were correctly classified to species and more than 80% to genus. Iterations in the larger AFORO database (including 216 families of teleostean fishes) attained a 100% correct classification at the family level.

Key words: otolith shape; morphology; contour; gobiids; phylogeny; Mediterranean Sea.

INTRODUCTION

The family Gobiidae, together with several other families, belongs to the suborder Gobiioidei (gobies in the broader sense) (Nelson, 2006). Phylogenetic affinities and sister groups of the Gobiioidei were proposed by Thacker (2009) based on molecular phylogenetic evidence; however, the consequent classification of gobies remains highly variable among authors. According to Nelson (2006), the suborder Gobiioidei should be placed in the order Perciformes. Thacker (2009) proposed that Gobiioidei be placed in the order Gobiiformes. Wiley & Johnson (2010) recognized the order Gobiiformes as *incertae sedis* in the Percomorphacea division, and Betancur *et al.* (2013) placed order Gobiiformes in the supraordinal group Gobiomorpharia. Phylogenetic relationships within Gobiioidei are also still not fully resolved, and results of some recent studies of the Gobiioidei differ from studies of just European lineages (Thacker, 2009, 2013; Thacker & Roje, 2011; Agorreta *et al.*, 2013; Tornabene *et al.*, 2013).

Classification schemes within Gobiioidei, which have been reviewed by Van Tassel *et al.* (2011) and Agorreta *et al.* (2013), also do not agree on the number of recognized families, with studies based on osteological vs. molecular data arriving at different conclusions. Gill & Mooi (2012) provided a provisional classification based on molecular data, and found that all native European gobies can be divided into three distinct lineages within the family Gobiidae: *Pomatoschistus*-like, *Aphia*-like and *Gobius*-like lineages (Gill & Mooi, 2012; Agorreta *et al.*, 2013).

The phylogeny of European gobies has been most effectively studied by molecular techniques (reviewed in Agorreta *et al.*, 2013), although various studies have combined molecular data with additional characters, such as osteology and meristics (Thacker, 2013) and lateral line variation (McKay & Miller, 1997). Contributions to the phylogeny of exclusively European gobies using non-molecular data are few. Simonović (1999) studied the relationship between Ponto-Caspian and Atlantic-Mediterranean gobies using external morphological, osteological and karyological data. Malavasi *et al.* (2012) used behavioural and life history data to build a phylogeny for the European gobiid lineages, and Kramer *et al.* (2012) studied the potential for teeth to inform phylogenetic relationships of European gobiids. Gobiidae is the most species rich fish family both in the Mediterranean Sea and among marine fishes more generally, with numbers of described gobiid species constantly increasing (Kovačić *et al.*, 2016, 2017). Gobies play an important ecological role in coastal ecosystems because of their diversity and abundance (Zander, 2011), but their significance is often underestimated because they are small, benthic, and cryptically coloured making them easy to overlook (Patzner *et al.*, 2011; Glavičić *et al.*, 2016).

The inner ear of bony fishes is involved in hearing, mechanoreception and equilibrium (Popper & Combs, 1982; Popper *et al.*, 2005). Fish hearing involves one to three, paired organs of the inner ear: the sacculus, utricle, and lagena. Each of these organs contains a densely mineralized aragonite mass called an otolith, and these are respectively known as the sagittae, lapilli and asterisci (Platt & Popper, 1981). Anatomical and geometric studies of sagittae shape variation have made important contributions to understanding the evolution and phylogeny of various marine teleost groups, such as gadids (Gaemers, 1984), merlucciids (Lombarte & Castellón, 1991), sciaenids (Monteiro *et al.*, 2005) and cyprinodontids

(Reichenbacher *et al.*, 2014). However, despite the need for additional phylogenetically informative characters to improve understanding of goby evolution, no study has examined gobiid otoliths in a taxonomic or phylogenetic context. Studies of gobiid otoliths and their use for species identification are also valuable given the significant role of gobies in coastal food webs (Bell & Harmelin-Vivien, 1983; Heymer & Zander, 1994; Kovačić & La Mesa, 2008). Otoliths could prove especially valuable when specimens have been poorly preserved or are missing other key taxonomic characters (Miller, 1986). Our goals in this study are threefold: (i) to describe and analyse the shapes of sagittae otoliths from gobies inhabiting the Mediterranean Sea, (ii) to test the efficacy of otolith contour as a taxonomic characteristic capable of differentiating between species and genera, and (iii) to evaluate whether sagittae otolith shape similarity is consistent with phylogenetic relatedness as determined by independent data sets (Agorreta *et al.*, 2013).

MATERIALS AND METHODS

SAMPLE COLLECTION

A total of 25 gobiid species from the Mediterranean Sea, belonging to 14 genera, were studied. From the northwestern Mediterranean (Iberian Peninsula and Balearic Islands), 69 specimens from 18 samples including 10 species were obtained from fisheries discards of seine or trawl boats from 2000 to 2011. From the eastern Adriatic Sea, 173 specimens from 22 samples including 18 species were collected in the period from 2006 to 2011 by combinations of: SCUBA diving using a hand net and anaesthetic, beach seining and by trawl fishing (Table I). Specimens from both areas were identified in the laboratory based on external morphological characters and were measured for total length (L_T in mm) using a stereomicroscope.

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127 Examined species belonged to three evolutionary lineages: a) the *Pomatochistus*-like
128 lineage, including species from the genera *Buenia*, *Cystallogobius*, *Deltentosteus*,
129 *Knipowitschia*, *Pomatoschistus* and *Pseudaphya*, b) the *Aphia*-like lineage, including *Aphia*
130 and *Lesueurigobius*, and c) the *Gobius*-like lineage, including the genera *Chromogobius*,
131 *Gobius*, *Odondebuenia*, *Thorogobius*, *Zebrus* and *Zosterisessor* (Table I).

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134 Sagittae otoliths were removed, washed, dried and stored in labelled plastic vials. Otoliths
135 from the left side of each fish were oriented on slides with the inner side (*sulcus acusticus*) up
136 in order to digitize their form using a microscope attached to an image analyser with
137 magnification depending on otolith size. Otolith length (L_O , in mm) was obtained directly
138 from a morphometric program available from the Anàlisi de Formes d'Otòlits (AFORO)
139 website <http://isis.cmima.csic.es/aforo/> (Lombarte *et al.*, 2006). Each image and all associated
140 data were stored in a database (Table I). Otolith shape terminology followed Tuset *et al.*
141 (2008), and specific gobioid terminology for the orientation and anatomical description of the
142 sagittae followed Schwarzhans (2014a), Bratishko *et al.* (2015) and Schwarzhans *et al.*
143 (2015). Nevertheless, we have defined some concepts for understanding otolith descriptions:
144 *sulcus acusticus* is a longitudinal depression in the otolith that commonly divides it into two
145 parts, one anterior (*ostium*) and other posterior (*cauda*); if the *sulcus* is clearly differentiated
146 in two parts it is considered heterosulcoid, and if it is positioned longitudinally it is described
147 as median (Tuset *et al.* 2008).

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150 OTOLITH CONTOUR ANALYSES

In order to standardize the contour measurements, the mathematical description of the outline started in the preventral projection as an initial point in all analysed otoliths. The outline obtained was analysed using the wavelet transform (*WT*) (see Parisi-Baradad *et al.*, 2005, 2010; Sadighzadeh *et al.*, 2014; Tuset *et al.*, 2015). This procedure is based on expanding the contour into a family of functions obtained as dilations and translations of a unique function known as the mother wavelet (Mallat, 1991). The advantage of this multi-scale analysis is the possibility of identifying areas or single morphological points (landmarks) located on the x-axis (between one and 512) along the contour where the rostrum is the origin of the contour (Parisi-Baradad *et al.*, 2005, 2010). Image processing was performed using the software Age and Shape (v. 1.0, Infaimon SL[®], Spain). For each contour, ten wavelet scales were obtained (Parisi-Baradad *et al.*, 2005; Sadighzadeh *et al.* 2014).

Two methods of otolith classification were used: a) an iterative system based on nine wavelets that searches the AFORO database (over 4,500 specimens of 216 families) and b) a canonical discriminant method based on *WT* signal number five, which is suitable for interspecific identifications (Sadighzadeh *et al.* 2014; Tuset *et al.* 2015). The first method is an Automated Taxon Identification (ATI) system (see Parisi-Baradad *et al.*, 2010, Tuset *et al.*, 2013), which searches the AFORO database iteratively, from the coarsest to the finest wavelet scale, to find otoliths that most resemble the tested otolith. For each iteration, the approximation signal of the tested otolith is compared with each otolith in the database, using a Euclidean distance (ED) to order the database otoliths from most to least similar to the specimen in order of increasing ED.

For the second method, a principal component analysis (PCA) based on a variance-covariance matrix was performed to reduce wavelet functions without losing information (Tuset *et al.*, 2015). Following Gauldie & Crampton (2002), significant eigenvectors were identified by plotting the percentage of total variation explained by the eigenvectors *versus* the expected proportion of variance explained under a broken-stick model. Similar interspecific differences might be attributed to allometry, so Pearson's correlations between otolith lengths and the PC variables were tested (Stransky & MacLellan, 2005; Burke *et al.*, 2008; Maderbacher *et al.*, 2008). The effect of otolith length was removed using the residuals of the common within-group slopes of the linear regressions of each component on otolith length, building a new PCA matrix.

Multivariate analyses were performed in order to detect morphometric differences in the otolith shapes of gobiids. Canonical variate analysis (CVA) was computed on the reduced PCA matrix to summarise the variation among species by maximising their distances (Linde *et al.*, 2004). In addition, otoliths were classified using a jackknife (leave-one-out) approach. The classification accuracy was determined by comparing the jackknife predicted group membership to the actual group membership and calculating the percentage of individuals that were correctly classified (Tuset *et al.*, 2015). Finally, to evaluate the correspondence between otolith shape similarity and phylogenetic relatedness, a UPGMA cluster analysis was performed on Euclidean distances drawn from the fifth mean wavelet of the otolith outline. This multivariate algorithm clustered species based on overall otolith shape similarity, and enabled us to compare species similarity based on otolith shape with their phylogenetic relatedness (Clabaut *et al.*, 2007). All statistical analyses were performed in XLStat 2012, a statistical plug-in for MS Excel 2011 and PAST (PAleontological STatistics, version v1.81;

Hammer *et al.*, 2001). Significance level for all statistical tests was set at 0.05.

RESULTS

ANATOMICAL DESCRIPTION

Morphological features of the sagittae otoliths allowed us to identify two, clearly distinct otolith patterns among Mediterranean gobies. The first group was composed of *Pomatochistus*-like (Fig. 1) and *Aphia*-like (Fig. 2) gobies, whose otoliths were discoidal to square or, in the case of *Knipowitschia panizzae* (Verga, 1841), pentagonal (Tuset *et al.*, 2008). The sagittae of all species except *Aphia minuta* (Risso, 1810), *Crystallogobius linearis* (Düben, 1845) and *Pseudaphya ferreri* (de Buen & Fage, 1908) were longer than higher. In relation with the outline, the ventral margin was usually flat or slightly convex, whereas the dorsal margin was round, except in *Lesueurigobius* spp. and *Deltentosteus*, which showed a lobate pattern.

In all species, the *sulcus acusticus* had the characteristic sole-shape observed in Gobiidae (Schwarzahns, 2014a) a heterosulcoid, mesial, median (supramedian in *Lesueurigobius suerii*) and slightly inclined structure, with a differentiated *ostium* and *cauda*. The *ostium* was as long as the *cauda*, both with a round or oval shape, with an ostial lobe in the dorsal margin, except in *Crystallogobius linearis* and *Aphia minuta*. Particularly valuable characters are concentrated on the anterior and dorsal margins. *A. minuta* showed a flattened posterior margin, with this margin being more convex in *Cr. linearis* and slightly concave in *Pseudaphya ferreri*. Both *Pomatochistus quagga* (Heckel, 1837), and *Knipowitschia panizzae* had a flattened posterior margin, but it was divided by a shallow notch. *Pomatoschistus*

226 *marmoratus* (Risso, 1810), *Buenia affinis* (Iljin, 1930), *Lesueurigobius friesii* (Malm, 1810)
 227 and *L. suerii* (Risso, 1810) all show a rounded posterior margin with a postdorsal projection
 228 separated from the postventral angle by a notch. With respect to the anterior margin:
 229 *Pomatoschistus quagga* had a flattened margin; in *A. minuta* and *Ps. ferreri* it was slightly
 230 convex; it had a small pointed *preventral projection* in *Cr. linearis*; it was slightly concave in
 231 *K. panizae* and *B. affinis*; and it had two projections separated by a notch in *P. marmoratus*,
 232 *L. friesii* and *L. suerii*. *Deltentosteus quadrimaculatus* (Valenciennes, 1837), belonging to the
 233 *Pomatoschistus*-like group, shared an intermediate shape with the *Gobius*-like group (Fig. 1
 234 and 3), that is, squared to rectangular (longer than tall) with lobed margins especially
 235 developed in the posterodorsal area.

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238 The third group (Figs. 3 and 4), which belongs to the *Gobius*-like lineage, had sagittae
 239 otoliths with an approximately rhomboidal shape (in *Gobius auratus* Risso, 1810, *G. couchi*
 240 Miller & El-Tawil, 1974, *G. cruentatus* Gmelin, 1837, *G. geniporus* Valenciennes, 1837, *G.*
 241 *niger* Linnaeus, 1758, *G. vittatus* Vinciguerra, 1883, *Odondebuenia balearica* (Pellegrin &
 242 Fage, 1907) and *Zebrus zebrus* (Risso, 1827)), or a more rectangular rhomboidal shape
 243 (*Chromogobius zebratus* (Kolombatovic, 1891), *Gobius buccichi* Steindachner, 1870, *G.*
 244 *cobitis* Pallas, 1814, *G. paganellus* Linnaeus, 1758, *G. roulei* de Buen, 1928, *Thorogobius*
 245 *macrolepis* (Kolombatovic, 1891) and *Zosterisessor ophiocephalus* (Pallas, 1814)), longer
 246 than high with a well-defined, postdorsal projection. The ventral margin or rim could be flat
 247 to slightly convex, although in *O. balearica* it was remarkably convex. Dorsal margins had
 248 more specific differences: they were flat with a small convexity in *Ch. zebratus*, *G. buccichi*,
 249 *G. cobitis*, *G. vittatus*, *O. balearica*, *T. macrolepis*, *Ze. zebrus* and *Zo. ophiocephalus*; they
 250 are round in *G. auratus*, *G. couchi* and *G. geniporus*; and they had lobes in *G. auratus*, *G.*

cruentatus, *G. niger* and *G. roulei*. The anterior margin was flattened in *Ch. zebratus*, *G. cobitis*, *G. paganellus*, *T. macrolepis* and *Zo. ophiocephalus*, but the anterior margin more commonly shows an oblique margin with a concavity or a shallow notch between two projections as in *G. couchi*, *G. cruentatus*, *G. geniporus*, *G. vittatus* and *O. balearica*. In *G. auratus*, *G. buccichi*, *G. niger*, *G. roulei* and *Ze. zebrus* the preventral projection ends in a sharp point. Posterior margins in this group were oblique (flattened in *G. cruentatus* and *G. paganellus*) with a shallow notch at the middle separating a round postventral angle and a pointed postdorsal projection. In *G. niger* and *T. macrolepis* there was a round postdorsal projection on the posterior margin; in *G. auratus* and *G. geniporus* the postdorsal projection was blunt, and in *G. cobitis* and *G. cruentatus* it was squared. In all *Gobius* species the *sulcus acusticus* was sole-shaped, supramedian and very moderately inclined, whereas it was median in all other genera. In *G. auratus*, *G. buccichi*, *G. niger*, *G. paganellus* and *G. roulei*, the *sulcus* was clearly inclined. In all species, the *sulcus acusticus* is characterized by being sole-shaped with an *ostium* equal to or longer than the *cauda* and wider, the *cauda* had a round to oval shape and the *ostium* had an angular dorsal lobe.

AFORO CLASSIFICATION SYSTEM (10 WAVELETS)

The otolith contour analysis classified 72.1% of the gobiid specimens correctly to species, 84.7% to genus, 93.7% to lineage and 100% to the Gobiidae (Table II) in making comparisons to over 4,500 otolith specimens from the 1,460 species and 216 families included in the AFORO database. At lineage-level, all specimens of the *Gobius*-like lineage were correctly identified; the other two lineages also showed a high level of correct classification (83.3% and 88.4%). Even at the genus level the automatic classification routine got a high percentage of identifications correct for 10 genera (77.8–100%). Exceptions

included species of *Knipowitschia* (50·0%), closely related species of *Pomatoschistus*. (63·3%) and the neotenic genus *Aphia* (66·7%). Finally, at the species level the method provided an acceptable automatic classification (70·6–100%) to species for the genera *Buenia*, *Crystallogobius* and *Deltentosteus* within the *Pomatoschistus*-like lineage; of *Lesueurigobius* within the *Aphia*-like lineage; of *Thorogobius*, *Zebrus*, *Zosterisessor* and two *Gobius* species (*G. geniporus*, *G. niger*) within the *Gobius*-like lineage. Other *Gobius* species did not present clear species-level differentiation in otolith shape (40–60% of correct classifications). These methods especially highlighted the genera *Chromogobius* and *Odondebuenia* as having very distinctive sagittae shapes: sagittae of *Chromogobius* were rectangular, and sagittae of *Odondebuenia* were rhomboidal. The sagittae of both species had a markedly convex ventral margin. Species of *Chromogobius* and *Odondebuenia* were always correctly identified by the automated, 10-wavelet classification routine using the AFORO database.

CLASSIFYING SPECIES WITH THE FIFTH WAVELET

A total of twelve principal component (PC) variables were selected from the analysis of wavelet scores. Of these, PC1–PC4, PC7 and PC12 showed significant correlations to otolith length. A new PCA matrix was therefore built using residuals of the common within-group slopes of the linear regressions for each component. Significant differences in the otolith shape were found between species (MANOVA, Wilks's $k = 2·944 \cdot 10^{-4}$, $F_{252,2041} = 9·517$, $P < 0·0001$). Interspecific classification analysis (jackknife validated from CVA) of otolith contours classified 63·0% of all cases correctly (Table III). Classification efficiency reached the highest values when the sagittae were from *Odondebuenia balearica* (90·0%), *Zebrus zebrus* (90·0%), *Thorogobius macrolepis* (88·9%) and *Chromogobius zebratus* (87·5%).

Identification of *Gobius* spp. was much less accurate for *G. auratus* and *G. niger* ($\cong 20.0\%$), and other *Gobius* varied between 50.0% for *G. cruentatus* and 77.8% for *G. roulei*. In this group, the case of *G. niger* was especially noteworthy, as it was classified as *G. roulei* in 38.8% of cases.

For *Aphia*-like gobies, the fifth wavelet routine had uneven success: *Lesueurigobius suerii* and *L. friesii* were acceptably classified (100% and 73.3%, respectively), whereas *Aphius minuta* only received 44.5% correct classifications and was classified as *Crystallogobius linearis* in 33.3% of cases. Finally, among *Pomatoschistus*-like gobies, *Pomatoschistus quagga* and *Knipowitschia panizae*, showed the lowest percentages of classification (33.3% and 37.5%, respectively), whereas *Cr. linearis* had the highest success rate (90.0%). In most cases, the highest percentages of assignment error were between species of the same phyletic group (Table III).

A plot of sagittal otolith morphospace (Fig. 5) based on the first two CVA axes (which together explained 66.2% of the variance) revealed that levels of shape variation differed among species and phylogenetic lineages (Fig. 5). This was highlighted in *Chromogobius zebratus*, which had otoliths clearly more rhomboidal in shape than other species, and *Lesueurigobius suerii*, which had more of a square otolith. Although the convex hull delimiting *Gobius*-lineage otolith morphospace demonstrated that this group was the most variable in its shape, a separation between *Gobius* spp. and remaining species was nevertheless noted. By contrast, the *Pomatoschistus*-like group was clustered closely in morphospace. However, the discoidal-to-squared otolith shapes in these latter species drove

high variability due to changes in otolith height. This is why these shapes were less successfully classified. Finally, *Crystallogobius linearis* and *Aphius minuta* were distributed away from their groups and close to each other in otolith morphospace (Fig. 5). The cluster analysis demonstrated that otolith shape was heavily influenced by phylogenetic relatedness (Fig. 6). Members of the *Gobius*-like lineage were clearly separated from members of the *Pomatoschistus*-like and *Aphia*-like lineages. In this second group, *Cr. linearis* and *A. minuta* were also clearly separated from all others.

DISCUSSION

Differences in otolith shape have been used to identify teleost species for over 130 years, since the studies of Koken (1884), and our analyses extend the usefulness of otolith shape data to differentiation of a diverse assemblage of Mediterranean gobies. Specifically, our results demonstrate that analysis of otolith contours using wavelet functions is an efficient tool for automated discrimination and identification of Mediterranean goby genera and species. An iterative classification method and canonical variate analysis showed very similar results. Although the average percentage of correct identification may seem low (61.8%), otolith shape can still be considered useful due to the large number of species analysed (n= 25). In fact, there is an inverse association between identification success and number of possible species in any shape-based identification analysis. Tuset *et al.* (2016) used otolith shape to correctly identify 58.1% of 42 rockfish species (Sebastidae), and Sadighzadeh *et al.* (2012b) used otolith shape to correctly identify 65.5% of 12 snapper species (Lutjanidae). By contrast, when analyses are limited to only two to five species, the percentage of correct classification noticeably increases to ~70% (Tuset *et al.*, 2006, 2013). Among previous studies of gobiids, Lord *et al.* (2012) consistently classified all of three congeneric species

(*Sicyopterus lagocephalus*, *S. aiensis* and *S. sarasini*) from the South Pacific Ocean; Bani *et al.* (2013) separated three species (*Neogobius caspius*, *Ponticola bathybius* and *Ponticola gorlap*) from the Caspian Sea with 94.7% classification success; and Xin *et al.* (2014) classified five species from northern Chinese coastal waters with 98.6% accuracy (*Ctenotrypauchen chinensis*, *Odontamblyopus lacepedii*, *Amblychaeturichthys hexanema*, *Chaeturichthys stigmatias*, and *Acanthogobius hasta*).

As in previous descriptions of gobiid otoliths from southern African waters (Smale *et al.*, 1995) and the Western Pacific (Lin & Chang, 2012), otolith anatomical characters can be used to discriminate species. Recently such characters have even been used to support the morphological redescription of *Ponticola iljini* (Vasilyeva *et al.*, 2016). In Mediterranean goby lineages, the main differences among otoliths are related to general shape, i.e., square to rhomboidal; the development and shape of posterodorsal and anteroventral lobes; and the degrees of convexity of dorsal and ventral margins. By contrast, in other lineages, such as the genera *Gobiodon*, *Myersina*, *Oxyurichthys*, *Trypauchen* and *Valenciennea*, different general shapes characterised by high dorsal expansions can be found (Smale *et al.*, 1995, Lombarte *et al.*, 2006, Lin & Chang, 2012).

The round otoliths that occur in fish larval stages (Modin *et al.*, 1996), and that are retained to adulthood in *A. minuta*, *Cr. linearis* and *Ps. ferrerii* (Giovanotti *et al.*, 2007), are a result of the neotenic evolution of these pelagic species. A convergent evolutionary process has been observed in other paedomorphic teleosts, such as the notothenid *Pleuragramma antarcticum* (Lombarte *et al.*, 2010). These morphologies are clearly separated in our

morphospace, which may relate to the relative sizes of the skull and the shapes of the otoliths (Bani *et al.*, 2013). Recently, Schwarzhans (2014b) observed, within the genus *Hymenocephalus*, trends of parallel polarity in certain head and otolith features and discussed the connection with both structures. In any case, a relationship makes sense, because the head shape is, directly or indirectly, related to both feeding habits (Hobson, 2006) and otolith shape (Tuset *et al.*, 2016).

Especially common otolith characters shared in the gobiid family are the heterosulcoid and mesial *sulcus acusticus* and the development of posterodorsal and anteroventral lobes on the margins. These uniquely confirm Gobiidae as a differentiated basal group of modern actinopterygian fishes (Miya *et al.*, 2003; Betancur *et al.*, 2013). Only the phylogenetically close family Eleotrididae and the unrelated Hoplichthyidae (Nelson 2006; Betancur *et al.*, 2013) show a similar *sulcus acusticus* anatomy (Smale *et al.*, 1995; Nolf, 2013), although eleotrids differ from gobiids by having marked indentations in the otolith margins. These highly differentiated otolith shapes compared to other Perciformes families support the most recent molecular data. From those it has been proposed that gobiids and related groups constitute a new taxonomic order, Gobiiformes (Thacker, 2009; Wiley & Johnson 2010, Betancur *et al.*, 2013), distinct from Perciformes.

Our results also show that otolith morphometry can provide diagnostic characters distinguishing the three Mediterranean phylogenetic lineages (*Pomatoschistus*-lineage or sand gobies, *Aphia*-lineage and *Gobius*-lineage) that have been defined by molecular studies (Agorreta *et al.*, 2013). Similarly, the strong differences found by Bani *et al.* (2013) and Xin

et al. (2014) reflect the taxonomic distinctiveness of species in available gobiid molecular phylogenetic studies (Neilson & Stepien, 2009, Agorreta *et al.*, 2013).

The unique shapes of gobiid otoliths among those of all bony fishes are demonstrated by our morphometric study. Iterative comparisons to the AFORO database (including 216 families of teleostean fishes) gave a 100% correct classification at the family level. Moreover, gobiid otoliths were relatively large compared with other teleostean fishes (Lombarte & Cruz, 2007). Otolith size is associated with sound production (Arellano *et al.*, 1995; Lugli *et al.*, 1997) and to a capacity for acoustic communication that is recognized in both freshwater and marine species of the family, including *Padogobius bonelli*, *Padogobius martensii*, *Pomatoschistus minutus*, *G. nigricans*, *G. cruentatus*, *G. paganellus* and *Neogobius melanostomus* (Lugli *et al.*, 2003; Parmentier *et al.*, 2013; Pedroso *et al.*, 2013).

In conclusion, we have demonstrated that shape contours of gobiid otoliths can be used to automatically identify specimens to genus and even species. Moreover, we have detected an apparent phylogenetic clustering in the distinctive otolith patterns of the different lineages, as occurs in other fishes (Sadighzadeh *et al.*, 2014), such shape patterns can help with understanding the evolution of the group (Tuset *et al.*, 2016).

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TABLE I. Location of gobiidae species, phylogentic lineage, sampling characteristics. N, number of specimens; L_T , total length range expressed in mm

Species	Location	Phylogenetic lineage
<i>Buenia affinis</i>	Oštro, the Kvarner area, Northern Adriatic Sea	<i>Pomatoschistus</i> -like
<i>Crystallogobius linearis</i>	Blanes, Catalan Coast, MW Mediterranean	<i>Pomatoschistus</i> -like
<i>Deltentosteus quadrimaculatus</i>	River Llobregat Delta, Catalan Coast, NW Mediterranean	<i>Pomatoschistus</i> -like
<i>Knipowitschia panizzeae</i>	Mouth of the River Karišnica, the Zadar area, Adriatic Sea	<i>Pomatoschistus</i> -like
<i>Pomatoschistus marmoratus</i>	Klimno, the Kvarner area, Northern Adriatic Sea	<i>Pomatoschistus</i> -like
<i>Pomatoschistus quagga</i>	Oštro, the Kvarner area, Northern Adriatic Sea	<i>Pomatoschistus</i> -like
<i>Pseudaphya ferreri</i> *	Eivissa, Balearic Islands, MW Mediterranean	<i>Pomatoschistus</i> -like
<i>Aphia minuta</i>	Blanes, Catalan Coast, MW Mediterranean	<i>Aphia</i> -like
<i>Lesueurigobius friesii</i>	Vilanova i La Geltrú, Catalan Coast, NW Mediterranean	<i>Aphia</i> -like
<i>Lesueurigobius friesii</i>	Rijeka bay, the Kvarner area, Northern Adriatic Sea	<i>Aphia</i> -like
<i>Lesueurigobius suerii</i>	River Llobregat Delta, Catalan Coast, NW Mediterranean	<i>Aphia</i> -like
<i>Chromogobius zebratus</i>	Oštro, the Kvarner area, Northern Adriatic Sea	<i>Gobius</i> -like
<i>Gobius auratus</i>	Oštro, the Kvarner area, Northern Adriatic Sea	<i>Gobius</i> -like
<i>Gobius bucchichi</i>	Oštro, the Kvarner area, Northern Adriatic Sea	<i>Gobius</i> -like
<i>Gobius cobitis</i> *	Lloret, Catalan Coast, NW Mediterranean	<i>Gobius</i> -like
<i>Gobius couchi</i>	Podurinj, the Kvarner area, Northern Adriatic Sea	<i>Gobius</i> -like
<i>Gobius cruentatus</i>	Palma de Mallorca Bay, Balearic Islands, NW Mediterranean	<i>Gobius</i> -like
<i>Gobius cruentatus</i>	Oštro, the Kvarner area, Northern Adriatic Sea	<i>Gobius</i> -like
<i>Gobius geniporus</i>	Oštro, the Kvarner area, Northern Adriatic Sea	<i>Gobius</i> -like
<i>Gobius niger</i>	Vilanova i La Geltrú, Catalan Coast, NW Mediterranean	<i>Gobius</i> -like
<i>Gobius niger</i>	Mouth of the River Rječina, the Kvarner area, Northern Adriatic Sea	<i>Gobius</i> -like
<i>Gobius paganellus</i> *	Palma de Mallorca Bay, Balearic Islands, NW Mediterranean	<i>Gobius</i> -like
<i>Gobius roulei</i>	Oštro, the Kvarner area, Northern Adriatic Sea	<i>Gobius</i> -like
<i>Gobius vittatus</i>	Oštro, the Kvarner area, Northern Adriatic Sea	<i>Gobius</i> -like
<i>Odondebuenia balearica</i>	Oštro, the Kvarner area, Northern Adriatic Sea	<i>Gobius</i> -like
<i>Thorogobius macrolepis</i>	Oštro, the Kvarner area, Northern Adriatic Sea	<i>Gobius</i> -like
<i>Zebrus zebrus</i>	Oštro, the Kvarner area, Northern Adriatic Sea	<i>Gobius</i> -like
<i>Zosterisessor ophiocephalus</i>	Plemići cove, the Zadar area, Adriatic Sea	<i>Gobius</i> -like

*species not used in identification analysis

TABLE III. Results of canonical variate analysis (CVA) using wavelet 5th obtained from otolith contours of Mediterranean gobiid species. The membership within each group is in bold

Actual group	Predicted group membership (in percentage)																
	<i>Baff</i>	<i>Clin</i>	<i>Dqua</i>	<i>Kpan</i>	<i>Pmar</i>	<i>Pqua</i>	<i>Amin</i>	<i>Lfri</i>	<i>Lsue</i>	<i>Czeb</i>	<i>Obal</i>	<i>Tmac</i>	<i>Zzreb</i>	<i>Zoph</i>	<i>Gaur</i>	<i>Gbuc</i>	<i>Gcou</i>
<i>Buenia affinis</i> (<i>Baff</i>)	60·0	10·0	10·0	10·0	10·0												
<i>Crystallogobius linearis</i> (<i>Clin</i>)		90·0					10·0										
<i>Deltentosteus quadrimaculatus</i> (<i>Dqua</i>)	25·0		50·0		25·0												
<i>Knipowitschia panizzeae</i> (<i>Kpan</i>)				37·5	25·0	12·5											
<i>Pomatoschistus marmoratus</i> (<i>Pmar</i>)	10·0			10·0	80·0												
<i>Pomatoschistus quagga</i> (<i>Pqua</i>)				33·3	22·3	33·3											
<i>Aphia minuta</i> (<i>Amin</i>)		33·3					44·5										
<i>Lesueurigobius friesii</i> (<i>Lfri</i>)		6·7	13·3					73·3									
<i>Lesueurigobius suerii</i> (<i>Lsue</i>)									100								
<i>Chromogobius zebratus</i> (<i>Czeb</i>)										87·5			12·5				
<i>Odondebuenia balearica</i> (<i>Obal</i>)											90·0					10·0	
<i>Thorogobius macrolepis</i> (<i>Tmac</i>)												88·9					
<i>Zebrus zebrus</i> (<i>Zzeb</i>)											10·0		90·0				
<i>Zosterisessor ophiocephalus</i> (<i>Zoph</i>)											11·1			55·6			
<i>Gobius auratus</i> (<i>Gaur</i>)											10·0		10·0	20·0	20·0		10·0
<i>Gobius bucchichi</i> (<i>Gbuc</i>)													11·1		11·1	55·6	11·1
<i>Gobius couchi</i> (<i>Gcou</i>)						10·0										20·0	50·0
<i>Gobius cruentatus</i> (<i>Gcru</i>)						6·2										12·5	12·5
<i>Gobius geniporus</i> (<i>Ggen</i>)						10·0	10·0										
<i>Gobius niger</i> (<i>Gnig</i>)				11·1		5·6	5·6	11·1									
<i>Gobius roulei</i> (<i>Grou</i>)																11·1	
<i>Gobius vittatus</i> (<i>Gvit</i>)															10·0	10·0	20·0

TABLE II. Percentage of specimens correctly classified using AFORO system based on iterative analysys of wavelets obtained from otolith contours of Mediterranean gobiid species.

ED are mean Euclidian distance						
Lineage group/Species	Species	ED	Genus	Lineage	Family	Closest species ED
<i>Pomatoschistus</i>-like						
<i>Buenaia affinis</i>	80.0	0.64	80.0	90.0	100.0	<i>P. marmoratus</i> 0.95
<i>Crystallogobius linearis</i>	90.0	0.71	90.0	90.0	100.0	<i>A. minuta</i> 1.05
<i>Deltentosteus quadrimaculatu</i>	87.5	0.88	87.5	87.5	100.0	<i>L. friesii</i> 1.24
<i>Knipowischia panizzeae</i>	50.0	1.19	50.0	100.0	100.0	<i>P. marmoratus</i> 1.20
<i>Pomatoschistus marmoratus</i>	60.0	0.91	60.0	100.0	100.0	<i>K. panizzeae</i> 1.13
<i>Pomatoschistus quagga</i>	66.7	0.94	66.7	66.7	100.0	<i>L. friesii</i> 1.19
Genus <i>Pomatoschistus</i> mean	63.3	0.93	63.3	83.3	100.0	1.16
<i>Aphia</i>-like						
<i>Aphia minuta</i>	66.7	0.93	66.7	66.7	100.0	<i>C. linearis</i> 1.11
<i>Lesueurigobius friesii</i>	70.6	0.87	76.5	76.5	100.0	<i>P. quagga</i> 1.11
<i>Lesueurigobius suerii</i>	75.0	0.99	100.0	100.0	100.0	<i>L. friesii</i> 1.17
Genus <i>Lesueurigobius</i> mean	72.8	0.93	88.2	88.2	100.0	1.14
<i>Gobius</i>-like						
<i>Chromogobius zebratus</i>	100.0	0.79	100.0	100.0	100.0	<i>O. balearica</i> 1.33
<i>Odondebuena balearica</i>	100.0	0.82	100.0	100.0	100.0	<i>Z. ophiocephalu</i> 1.18
<i>Thorogobius macrolepis</i>	77.8	0.62	77.8	100.0	100.0	<i>G. geniporus</i> 1.17
<i>Zebrus zebrus</i>	80.0	0.80	80.0	100.0	100.0	<i>G. buechichi</i> 0.93
<i>Zosterisessor ophiocephalus</i>	88.9	1.03	88.9	100.0	100.0	<i>G. vittatus</i> 1.04
<i>Gobius auratus</i>	60.0	0.91	90.0	100.0	100.0	<i>G. couchi</i> 0.94
<i>Gobius buechichi</i>	55.6	0.96	88.9	100.0	100.0	<i>G. auratus</i> 0.92
<i>Gobius couchi</i>	40.0	0.98	100.0	100.0	100.0	<i>G. vittatus</i> 0.90
<i>Gobius cruentatus</i>	52.9	0.92	100.0	100.0	100.0	<i>G. buechichi</i> 1.06
<i>Gobius geniporus</i>	81.8	0.95	90.9	100.0	100.0	<i>G. roulei</i> 1.01
<i>Gobius niger</i>	72.2	0.89	94.4	100.0	100.0	<i>G. roulei</i> 0.92
<i>Gobius roulei</i>	44.5	0.90	100.0	100.0	100.0	<i>G. niger</i> 0.86
<i>Gobius vittatus</i>	80.0	0.85	100.0	100.0	100.0	<i>G. couchi</i> 1.09
Genus <i>Gobius</i> mean	60.9	0.92	95.5	100.0	100.0	1.08
Total mean	72.1	0.90	84.7	93.7	100.0	1.08
Total standard deviation	15.7	0.12	14.7	11.8	0.0	0.12

Identifying sagittae [1] otoliths of Mediterranean Sea gobies: variability among phylogenetic lineages

A. LOMBARTE*†, M. MILETIĆ‡, M. KOVAČIĆ§, J. L. OTERO-FERRER¶ AND V. M. TUSET*

Figure Legends

FIG. 1. Left otolith sagittae of the Pomatoschistus-like lineage. (a) *Crystalllogobius linearis* (29 mm LT); (b) *Pseudaphya ferrer* (32 mm LT); (c) *Pomatoschistus marmoratus* (34 mm LT); (d) *P. quagga* (37 mm LT); (e) *Knipowischia panizae* (41 mm LT); (f) *Buenia affinis* (26 mm LT); (g) *Deltentosteus quadrimaculatus* (84 mm LT). Scale bar = 1 mm.

FIG. 2. Left otolith sagittae of the *Aphia*-like lineage. (a) *Aphia minuta* (47 mm LT); (b) *Lesueurigobius friesii* (58 mm LT); (c) *L. suerii* (50 mm LT). Scale bar = 1 mm.

FIG. 3. Left otolith sagittae of the *Gobius*-like lineage. Species from the genus *Gobius*. (a) *Gobius auratus* (70 mm LT); (b) *G. buccichichi* (62 mm LT); (c) *G. couchi* (49 mm LT); (d) *G. cobitis* (97 mm LT); (e) *G. paganellus* (125 mm LT); (f) *G. roulei* (61 mm LT); (g) *G. cruentatus* (110 mm LT); (h) *G. geniporus* (115 mm LT); (i) *G. niger* (104 mm LT); (j) *G. vittatus* (43 mm LT). Scale bar = 1 mm.

FIG. 4. Left otolith sagittae of the *Gobius*-like lineage; the no-*Gobius* species. (a) *Thorogobius macrolepis* (56 mm LT); (b) *Zosterisessor ophiocephalus* (80 mm LT); (c) *Chromogobius zebratus* (40 mm LT); (d) *Odondebuenia balearica* (31 mm LT); (e) *Zebrus zebrus* (25 mm LT). Scale bar = 1 mm.

FIG. 5. Morphospace for the first two CVA axes based on the decomposition of the otolith contour using a 5th wavelet analysis. Colours show the phylogenetic lineages. (1) *Pomatoschistus*-like lineage: (Baff) *Buenia affinis*; (Clin) *Crystalllogobius linearis*; (Dqua) *Deltentosteus quadrimaculatus*; (Kpan) *Knipowischia panizae*; (Pmar) *Pomatoschistus marmoratus*; and (Pqua) *P. quagga*. (2) *Aphia*-like lineage: (Amin) *Aphia minuta*; (Lfrie) *Lesueurigobius friesii*; and (Lsue) *L. suerii*. (3) *Gobius*-like lineage: (Czeb) *Chromogobius zebratus*; (Gaur) *Gobius auratus*; (Gbuc) *G. buccichichi*; (Gcou) *G. couchi*; (Grou) *G. roulei*; (Gcru) *G. cruentatus*; (Ggen) *G. geniporus*; (Gnig) *G. niger*; (Gvit) *G. vittatus*; (Obal) *Odondebuenia balearica*; (Tmac) [2] *Thorogobius macrolepis*; (Zzeb) *Zebrus zebrus*; and (Zoph) *Zosterisessor ophiocephalus*.

FIG. 6. Dendrogram based on Euclidean distance and UPGMA linkage of the decomposition of the otolith contour using a 5th wavelet analysis. Colours show the phylogenetic lineages. Species abbreviations are the same than Fig.5.

Identifying sagittae otoliths of Mediterranean Sea gobies: variability among phylogenetic lineages

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Figure Legends

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Commented [A1]: Italicize species names throughout.

Commented [A2]: Correct total length abbreviations throughout.

Commented [A3]: Cite the phylogenetic study from which lineage information was obtained in the caption.