What can goby otolith morphology tell us?

by

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Abstract. – The taxonomic information inscribed in otoliths has been widely ignored in ichthyological research, especially in descriptions of new fish species. One reason for this is that otolith descriptions are *per se* qualitative, and only a few studies have presented quantitative data that can support assignments of otoliths to individual species or permit differentiation between higher taxonomic levels. On the other hand, in palaeontology, otoliths have been employed for the identification and taxonomic placement of fossil fish species for over 100 years. However, palaeontological otolith data is generally regarded with suspicion by ichthyologists. This is unfortunate because, in the Cenozoic, the fossil otolith record is much richer than that based on skeletons. Thus fossil otoliths are a unique source of information to advance our understanding of the origin, biogeographical history and diversification of the Teleostei. This case study deals with otoliths of the Oxudercidae, which, together with the Gobiidae, encompasses the 5-branchiostegal-rayed gobiiforms. The objective was to determine whether the five lineages of the Oxudercidae, and individual species of the European *Pomatoschistus* lineage, could be distinguished based on the quantification of otolith variations. The data set comprises otoliths from a total of 84 specimens belonging to 20 recent species, which represent all five lineages of the Oxudercidae (*Mugilogobius*, *Acanthogobius*, *Pomatoschistus*, *Stenogobius*, *Periophthalmus*), and five fossil otoliths of †*Pomatoschistus* sp. (sensu Brzobohatý, 1994). Ten measurements were taken on each otolith and 23 otolith variables were computed and used for univariate and multivariate analyses. The results indicate that otolith morphometry (i) is capable of identifying the *Pomatoschistus* and *Periophthalmus* lineages among the Oxudercidae, but is of limited use in the separation of the other three lineages; (ii) can reliably distinguish the sand gobies (a distinct clade within the *Pomatoschistus* lineage) from other members of the *Pomatoschistus* lineage; and (iii) supports a previous assignment of fossil otoliths to †*Pomatoschistus* sp. as an ancient genus of the sand gobies. With its middle Miocene age (15 m.y. ago), †*Pomatoschistus* sp. represents the oldest record of a sand goby species to date. We discuss possible relationships between distinct otolith morphologies, biogeographic distribution and lifestyles for *Pomatoschistus* and the *Periophthalmus* lineage, and also for the sand gobies. We conclude that otolith morphology, combined with morphometry, can be considered as an autapomorphy for an individual species from the studied groups. In addition, it also appears to contain a phylogenetic signal, but more work is needed to evaluate this fully.

Résumé. – Que révèle la morphologie des otolithes de gobies ?

L'information taxonomique inscrite dans les otolithes a longtemps été ignorée dans les recherches en ichtyologie, surtout dans la description de nouvelles espèces de poissons. Une des raisons est que la description des otolithes est souvent qualitative, et il n'existe que peu d'études qui ont présenté des données quantitatives permettant d'assigner un otolithe à une espèce, ou une discrimination à des niveaux taxonomiques plus élevés. À l'inverse, en paléontologie, les otolithes sont utilisés depuis plus de 100 ans pour l'identification et le replacement taxonomique de poissons fossiles. Cependant, les données d'otolithes paléontologiques sont généralement vues de manière suspicieuse par les ichtyologues. C'est bien dommage car, pour le Cénozoïque, il existe beaucoup plus de d'otolithes fossiles que de squelettes. Ainsi, les otolithes fossiles représentent une source unique d'information qui peut nous aider à comprendre l'origine, l'histoire biogéographique et la diversification des téléostéens. Cette étude porte sur les otolithes d'Oxudercidae, qui, avec les Gobiidae, englobent les gobiiformes à cinq rayons branchiostèges. L'objectif de ce travail est de déterminer si les cinq lignées d'Oxudercidae, et des espèces européennes de la lignée *Pomatoschistus*, peuvent être différenciées grâce à des variables quantitatives des otolithes. Le jeu de données comprend des otolithes de 84 spécimens appartenant à 20 espèces récentes représentant les cinq lignées d'Oxudercidae (*Mugilogobius*, *Acanthogobius*, *Pomatoschistus*, *Stenogobius*, *Periophthalmus*), et cinq otolithes fossiles de †*Pomatoschistus* sp. (sensu Brzobohaty, 1994). Dix mesures ont été prises sur chaque otolithe et 23 variables ont été mises au point pour des analyses statistiques uni- et multivariées. Les résultats montrent que l'analyse morphométrique des otolithes permet (i) d'identifier les lignées de *Pomatoschistus* et *Periophthalmus* parmi les Oxudercidae, mais qu'elle est d'intérêt limité pour la discrimination des trois autres lignées ; (ii) de distinguer de manière sûre les "sand gobies" (gobies vivant sur fonds sableux) des autres membres de la lignée *Pomatoschistus* ; (iii) de valider l'assignation des otolithes fossiles de †*Pomatoschistus* à un genre ancien de "sand gobies". Datant du Miocène moyen (–15 Ma), †*Pomatoschistus* sp.

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représente l'enregistrement le plus ancien de "sand gobies" connu à ce jour. Notre étude permet de discuter les relations possibles entre des morphologies distinctes d'otolithes, la distribution géographique des espèces ainsi que leur mode de vie pour les lignées de *Pomatoschistus* et *Periophthalmus*, ainsi qu'au sein des "sand gobies". Nous concluons que la morphologie des otolithes, combinée à leur analyse morphométrique, peut constituer une autapomorphie spécifique au sein des groupes étudiés. De plus, il semblerait qu'il puisse y avoir un signal phylogénétique, mais des études approfondies sont nécessaires afin de clarifier ce point.

INTRODUCTION

Fish otoliths are aragonitic structures that develop independently from the skeleton as integral components of the hearing and balance organs of bony fishes (see Nolf, 1985; Popper and Lu, 2000). In the inner ear of teleost fishes, they are arranged in three pairs referred to as the saccular (sagittae), utricular (lapilli) and lagenar otoliths (asterisci), respectively (see Schulz-Mirbach *et al*., 2011). Examples for the usage of otoliths in biology include otolith shape analysis for fish stock discrimination (*e.g*. Campana and Casselman, 1993; Lord *et al*., 2012), the study of otolith chemistry to gain information about life history and habitats (*e.g*. Tzeng and Tsai, 1994; Avigliano *et al*., 2017), and use of otolith growth rings for estimation of age and duration of larval phases (*e.g*. Campana, 2005; Lord *et al*., 2010).

The overall shape and detailed morphology of the sagitta ('otolith' in the following) provide taxonomically useful characters for species identification in most teleosts. However, although several atlases are available for the otoliths of recent species (*e.g*. Volpedo and Echeverría, 2000; Campana, 2004; Smale *et al*. 2005; Tuset *et al.,* 2008), the taxonomic information inscribed in otoliths has been widely ignored in ichthyological research, especially in descriptions of new fish species (for exceptions *e.g*., see Teimori *et al*., 2012, 2014; Esmaeili *et al*., 2014; Gholami *et al*., 2014). On the other hand, in palaeontology, otoliths have been employed precisely for this purpose for over 100 years because fossil otoliths are much more abundant than fossil fish skeletons (Nolf, 1985). Koken (1884) was one of the first to publish a comprehensive study of fossil otoliths, and some 1800 fossil fish species have now been documented solely on the basis of otoliths (Nolf, 2013). Their identification mostly relies on comparative studies of the inner face of the sagitta, since this is the biggest otolith and exhibits the largest number of distinguishable features (except in Cyprinidae and Siluridae, see Assis, 2003, 2005; Schulz-Mirbach and Reichenbacher, 2006). As yet, however, only a few studies have attempted to collect quantitative data that could provide further support for otolith-based taxonomic assignments (Volpedo and Echeverría, 2000; Reichenbacher *et al*., 2007; Schwarzhans, 2014).

The Gobiiformes, as defined in the latest edition of "Fishes of the World" by Nelson *et al*. (2016), constitute one of the largest orders of teleost fishes, with approximately 2,200 species and a worldwide distribution. In this case study, we deal with the Oxudercidae, which together with the Gobiidae forms the group of the more derived 5-branchiostegal-rayed gobiiforms (see Gierl and Reichenbacher, 2017: fig. 1). Molecular work has recognised five lineages among the Oxudercidae, which are named for the genera *Acanthogobius*, *Mugilogobius*, *Periophthalmus*, *Pomatoschistus* and *Stenogobius*, respectively (Agorreta *et al*., 2013; Thacker, 2015). The *Acanthogobius* lineage is a non-tropical taxon, distributed in the temperate Northern Pacific, the *Mugilogobius* lineage is distributed in the Indo-Pacific realm, and the *Periophthalmus* and *Stenogobius* lineages share a global tropical distribution (Thacker, 2015); none of these lineages is present in Europe (Agorreta *et al*., 2013). In contrast, the *Pomatoschistus* lineage is represented in Europe by several species and additionally present in Asia (Agorreta *et al*., 2013; Thacker, 2015).

Within the *Pomatoschistus* lineage the 'sand gobies' (for which no formal name exists) form a distinct clade, based on both morphological characters (McKay and Miller, 1997) and molecular data (Agorreta *et al*., 2013). The sand gobies include the species of *Pomatoschistus*, *Gobiusculus*, *Knipowitschia*, *Economidichthys* and *Hyrcanogobius*; with the exception of some females of *Gobiusculus flavescens*, they all share the apomorphy of a premaxilla without a postmaxillary process (McKay and Miller, 1997).

Whether or not fossil otoliths of the Oxudercidae can be assigned to one of the five newly recognized lineages or used to reliably distinguish sand gobies from non-sand gobies in the *Pomatoschistus* lineage has not yet been rigorously examined. The ability to do so would significantly advance our understanding of the origin, biogeographic history and diversification of the Oxudercidae clade (see Thacker, 2015). The objectives of this study are to test whether otolith morphometry might be suitable (i) for identifying lineages among the Oxudercidae, (ii) distinguishing the sand gobies from other members of the *Pomatoschistus* lineage, and (iii) for supporting previous assignments of fossil otoliths as †*Pomatoschistus* sp.

Institutional Abbreviations: NHM, Natural History Museum, Vienna, Austria; NMP, National Museum Prague, Czech Republic; ZM-CBSU, Zoological Museum of Shiraz University, Collection of Biology Department, Iran; ZSM, Zoological State Collection, Munich, Germany.

MATERIALS AND METHODS

Material

The material discussed here comprises saccular otoliths isolated from a total of 84 specimens belonging to 20 recent species representing all five lineages of the Oxudercidae, and five fossil otoliths of †*Pomatoschistus* sp. (sensu Brzobohatý, 1994). The *Stenogobius* lineage is represented by three species, the *Acanthogobius* and *Mugilogobius* lineages by four species each, the *Pomatoschistus* lineage by six and the *Periophthalmus* lineage by three species (see Tab. I). Only the saccular otoliths (referred to as otoliths in the following) of the left head side were used for this study (totally 89 left otoliths).

Methods

Otolith dissection

For otolith dissection, we followed standard protocols (*e.g.* Gholami *et al.*, 2014), with some slight modifications concerning cleaning and washing of the otoliths. The skull was opened dorsally with a scalpel and the otoliths were carefully removed using tweezers. Then the otoliths were treated with 5% KOH solution for at least 30 minutes to clean them from organic residuals. Subsequently, the otoliths were rinsed about five times with distilled water in a sample chamber under the microscope, and then air-dried.

SEM imaging

All otoliths from the recent species were coated with gold, and SEM images were taken using a LEO 1430 VP (15 kV) at the Zoologische Staatssammlung München or a HITACHI SU 5000 Schottky FE-SEM at the Department of Earth and Environmental Sciences (LMU Munich). The fossil otoliths of †*Pomatoschistus* sensu Brzobohatý (1994) were not gold-coated (because they represent the type material of Brzobohatý, 1994) and images were taken using the low vacuum SEM facility (JSM-6490LV, 20 kV) at the Senckenberg Museum and Research Institute, Frankfurt am Main. Based on the SEM images of the studied otoliths we prepared morphological descriptions and qualitative comparisons.

Table I. – Material used to analyse otolith characters in different lineages; n, number of fish individuals per species and number of otoliths in the case of †*Pomatoschistus*.

Lineage	Species	n	Designation
Stenogobius	*Awaous flavus* (Valenciennes, 1837)	2	ZSM-PIS-43853 (P-GO-1050 L; 1051 L)
	Gobioides broussonnetii Lacepède, 1800	2	ZSM-PIS-43852 (P-GO-1048 L; 1049 L)
	Stiphodon atropurpureus (Herre, 1927)	2	ZSM_PIS-43862 (P-GO-1070 L, 1071 L)
Acanthogobius	*Rhinogobius candidianus* (Regan, 1908)	4	ZSM-PIS-43858 (P-GO-1060 L; 1060,61-1 L; 1060,61-2 L; 1061 L)
	Rhinogobius formosanus Oshima, 1919	4	ZSM-PIS-43864 (P-GO-1072 L; 1072,73-1 L; 1072,73-3 L; 1073 L)
	Rhinogobius rubromaculatus Lee & Chang, 1996	4	ZSM-PIS-43860 (P-GO-1064 L; 1064,65-2 L; 1064,65-3 L; 1065 L)
	Rhinogobius zhoui Li & Zhong, 2009	4	ZSM-PIS-43859 (P-GO-1062 L; 1062,63-2 L; 1062,63-6 L; 1063 L)
Mugilogobius	*Brachygobius xanthozonus* (Bleeker, 1849)	2	ZSM-PIS-43865 (P-GO-1074 L; 1075 L)
	Chlamydogobius eremius (Zietz, 1896)	2	ZSM-PIS-43854 (P-GO-1052 L; 1053 L)
	Schismatogobius roxasi Herre, 1936	2	ZSM-PIS-43866 (P-GO-1076 L; 1077 L)
	Stigmatogobius sadanundio (Hamilton, 1822)	2	ZSM-PIS-43856 (P-GO-1056 L; 1057 L)
Pomatoschistus	*Buenia affinis* Iljin, 1930	4	NMP P6d 30/2017-3, -6, -7, -9
	Deltentosteus quadrimaculatus (Valenciennes, 1837)	9	NMP P6d 34/2017-1-3, -5-10
	Gobiusculus flavescens (Fabricius, 1779)	4	NMP P6V 142775-142778
	Knipowitschia croatica Mrakovčić *et al.*, 1996	6	NMP P6d 31/2017-1-4, -6, -7
	Pomatoschistus marmoratus (Risso, 1810)	7	NMP P6d 32/2017-1-7
	Pomatoschistus quagga (Heckel, 1839)	7	NMP P6d 33/2017-4-10
Periophthalmus	*Bolephthalmus dussumieri* Valenciennes, 1837	4	ZM-CBSU Khamir 38, 40, 41, 54
	Periophthalmus waltoni Koumans, 1941	8	ZM-CBSU Gowater 1734, 1736, 1739, 1741, 1743, 1745, 1748, 1751
	Scartelaos tenuis (Day, 1876)	5	ZM-CBSU Helleh 82, 84-86, 89
Fossil	†*Pomatoschistus* sp. (Brzobohatý, 1994)	5	NHM 1993/140/4-8

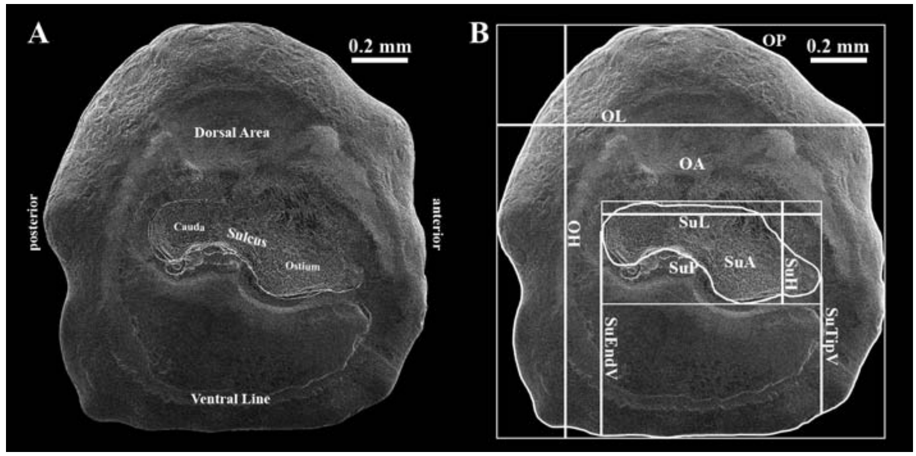

Figure 1. – Left otolith of *Rhinogobius candidianus* (ZSM-PIS-43858 (P-GO-1060 L)): nomenclature (**A**) and measurements (**B**). Abbreviations: OA, otolith area; OH, otolith height; OL, otolith length; OP, otolith perimeter; SuA, sulcus area; SuH, sulcus height; SuL, sulcus length; SuP, sulcus perimeter; SuEndV, distance from sulcus end to the ventral margin; SuTipV, distance from sulcus tip to the ventral margin.

Otolith measurements

The SEM images of the otoliths were oriented so that the ventral margin was essentially horizontal (Fig. 1A). Only in the case of *Gobiusculus flavescens* was the posterior margin used as the base. Ten measurements were taken on each otolith (Fig. 1B) using imageJ 1.51r (Rasband, 1997-2017). To measure the otolith length (OL), otolith height (OH), sulcus length (SuL) and sulcus height (SuH), a rectangle was drawn that enclosed the most dorsal, most ventral, most anterior and most posterior points of the otolith and the sulcus, respectively (yellow lines in Fig. 1B). The lengths of this rectangle's edges represent the maximum length and height of the otolith and sulcus, respectively. Measurements were also made of the otolith perimeter (OP), otolith area (OA), sulcus perimeter (SuP), sulcus area (SuA), distance from sulcus tip to the ventral margin (SuTipV) and distance from sulcus end to the ventral margin (SuEndV). The outline of the sulcus is usually recognisable because it is encompassed by a faintly etched line (Fig. 1A). Measurements of the SuP were taken along this line, or, if multiple lines were present, at the outermost margin (see Fig. 1B). In cases where parts of the sulcus were not clearly defined by a thin line, the sulcus perimeter was estimated by comparison with other otoliths of the same species. All measurements are presented in the Supplementary Information.

Morphometrics

For the statistical analysis, 23 otolith variables in all were computed, based on the ratios between the individual otolith measurements (Tab. II).

Table II. – Definitions of the 23 otolith variables used in this study. For measurements, see figure 1B.

Otolith variable	Abbreviation
Ratio of otolith length and otolith height	OL/OH
Ratio of otolith perimeter and otolith length	OP/OL
Ratio of otolith perimeter and otolith height	OP/OH
Ratio of sulcus area and otolith area	SuA/OA
Ratio of sulcus perimeter and otolith perimeter	SuP/OP
Ratio of sulcus perimeter and distance from sulcus tip to the ventral margin	SuP/SuTipV
Ratio of sulcus perimeter and distance from sulcus end to the ventral margin	SuP/SuEndV
Ratio of sulcus length and otolith length	SuL/OL
Ratio of sulcus length and otolith height	SuL/OH
Ratio of sulcus length and sulcus height	SuL/SuH
Ratio of sulcus length and distance from sulcus tip to the ventral margin	SuL/SuTipV
Ratio of sulcus length and distance from sulcus end to the ventral margin	SuL/SuEndV
Ratio of sulcus length and otolith perimeter	SuL/OP
Ratio of sulcus length and sulcus perimeter	SuL/SuP
Ratio of sulcus height and otolith length	SuH/OL
Ratio of sulcus height and otolith height	SuH/OH
Ratio of sulcus height and distance from sulcus tip to the ventral margin	SuH/SuTipV
Ratio of sulcus height and distance from sulcus end to the ventral margin	SuH/SuEndV
Ratio of sulcus height and otolith perimeter	SuH/OP
Ratio of sulcus height and sulcus perimeter	SuH/SuP
Ratio of distance from sulcus tip to the ventral margin and otolith perimeter	SuTipV/OP
Ratio of distance from sulcus tip to the ventral margin and distance from sulcus end to the ventral margin	SuTipV/SuEndV
Ratio of distance from sulcus end to the ventral margin and otolith perimeter	SuEndV/OP

Statistics

All otolith variables were analysed using SPSS 24.0 (IBM Corp., 2016) and PAST 3.17 (Hammer *et al.*, 2001). The Shapiro-Wilk test ($p < 0.05$) indicated that several otolith variables are not normally distributed across the lineages and species, respectively. We suspect that the non-normally distributed variables represent artefacts resulting from the relatively small sample sizes; consequently these data were not normalized. We used Mann-Whitney ($p < 0.05$) to examine the variation of non-normally distributed otolith variables and ANOVA with Dunnett T3 post-hoc test ($p < 0.05$) in case of normally distributed otolith variables. In addition, multivariate analysis based on Principal Components Analysis was done to find out whether the identification of the fossil otoliths as †*Pomatoschistus* sp. (sensu Brzobohatý, 1994) is supported by our morphometric approach.

RESULTS

Otolith morphology among Oxudercidae lineages

Apart from those of the *Periophthalmus* lineage, the otoliths of the species studied here share a rectangular to slightly rounded overall shape and have mostly smooth rims. Accordingly, they show no posterodorsal or preventral projection. In contrast, species of the *Periophthalmus* lineage have otoliths with crenulated rims and a posterodorsal projection is present (Fig. 2).

In most otoliths of all lineages, the sulcus has the typical gobioid shape ('shoe sole-like'). Only two of the species examined, *Rhinogobius rubromaculatus* (Fig. 2I) and *Buenia affinis* (Fig. 2N), show a short thickening of the crista inferior along the cauda ('subcaudal iugum' sensu Schwarzhans, 2014). Furthermore, some species display modifications of the general sulcus shape. Within the *Mugilogobius* lineage, this holds for *Chlamydogobius eremius* with its triangular ostium and thin, ventrally bent, appendix-like prolongation at the end of the cauda (Fig. 2A), as well as for *Stigmatogobius sadanundio* which has a relatively long cauda that is slender in the middle and widened at the end (Fig. 2B). Examples from the *Stenogobius* lineage are *Awaous flavus* (Fig. 2F, G) and *Stiphodon atropurpureus* (Fig. 2H), both of which display a very shallow sulcus; indeed, the sulcus is barely recognisable in the latter. Within the *Pomatoschistus* lineage, the cauda in both *Pomatoschistus marmoratus* (Fig. 2M) and *Gobiusculus flavescens* (Fig. 2O) is reduced in size, whereas in *Deltentosteus quadrimaculatus* there is a notable triangular extension of the upper ostial rim (Fig. 2P). Furthermore, one species of the *Mugilogobius* lineage, *i.e.* *Schismatogobius roxasi* (Fig. 2D) and one of the *Acanthogobius* lineage, *i.e.* *Rhinogobius zhoui* (Fig. 2J) lack a clear subdivision of the sulcus into ostium and cauda. Moreover, the four species of *Rhinogobius* differ among themselves in their sulcus morphology. Most distinct is the sagitta of *R. zhoui*, which has a slender and slightly pointed but relatively small sulcus lacking the constriction between ostium and cauda (Fig. 2J). In the remaining three species of *Rhinogobius*, the sulcus is clearly divided, but differences are recognisable in the contour of the ostium. In *R. formosanus* (Fig. 2L), the lower rim of the ostium has a distinctive, U-shaped indentation, in *R. candidianus* (Fig. 2K) a similar but less pronounced indentation is present, whereas no such structure is developed in *R. rubromaculatus* (Fig. 2I).

Otolith morphology of recent sand-gobies (*Pomatoschistus* lineage) and of †*Pomatoschistus* sp. (sensu Brzobohatý, 1994)

The otoliths of four recent species of sand gobies were used for this study, *i.e.* *Gobiusculus flavescens* (Fig. 2O, 3J-L), *Knipowitschia croatica* (Fig. 3M-O), *Pomatoschistus marmoratus* (Fig. 2M, 3D-F), and *P. quagga* (Fig. 3G-I). In all species, the sulcus has the typical 'shoe sole' shape, but it is not centred (as is usual in the lineages of the Oxudercidae) and lies slightly closer to the anterior than to the posterior otolith margin. A further difference relative to the general 'shoe sole' sulcus condition concerns the cauda, which is distinctively smaller in width and length than the ostium.

The otoliths of *G. flavescens* are rounded-rectangular, with the height exceeding the length (Fig. 2O, 3J-L). The dorsal margin is rounded and smooth, the anterior margin straight but with a distinct convexity in the middle. The ventral margin is smooth or presents a few slight incisions; its deepest part is at the posterior end (and not in the middle), which gives the otolith a slightly irregular shape. The posterior margin is straight or has a small concavity approximately in the middle. The sulcus is as described above, but the cauda is straight or slightly inclined towards the ventral rim (instead of slightly inclined to the dorsal rim as in the other sand goby species).

The otoliths of *K. croatica* are almost rectangular in shape, but have a slightly rounded dorsal margin, and are higher than long (Fig. 3M-O). The dorsal margin sometimes displays a slight posterodorsal projection. The anterior margin is straight or shows a slightly undulating contour. The smooth ventral margin is straight in the middle and anteriorly and posteriorly curved. The posterior margin presents a slight posteroventral kink in some otoliths; otherwise it is straight or slightly concave.

The otoliths of *P. marmoratus* and *P. quagga* are similar to each other (Fig. 3D-F vs. 3G-I). The main difference is that *P. quagga* is higher than long, while *P. marmoratus* is approximately square-shaped. Both species display a dorsal margin that can be either rounded or presents a small ridge in the middle or slightly behind the middle; a short, broad posterodorsal projection is present in most specimens. Again in both species the anterior margin is slightly undulating, while

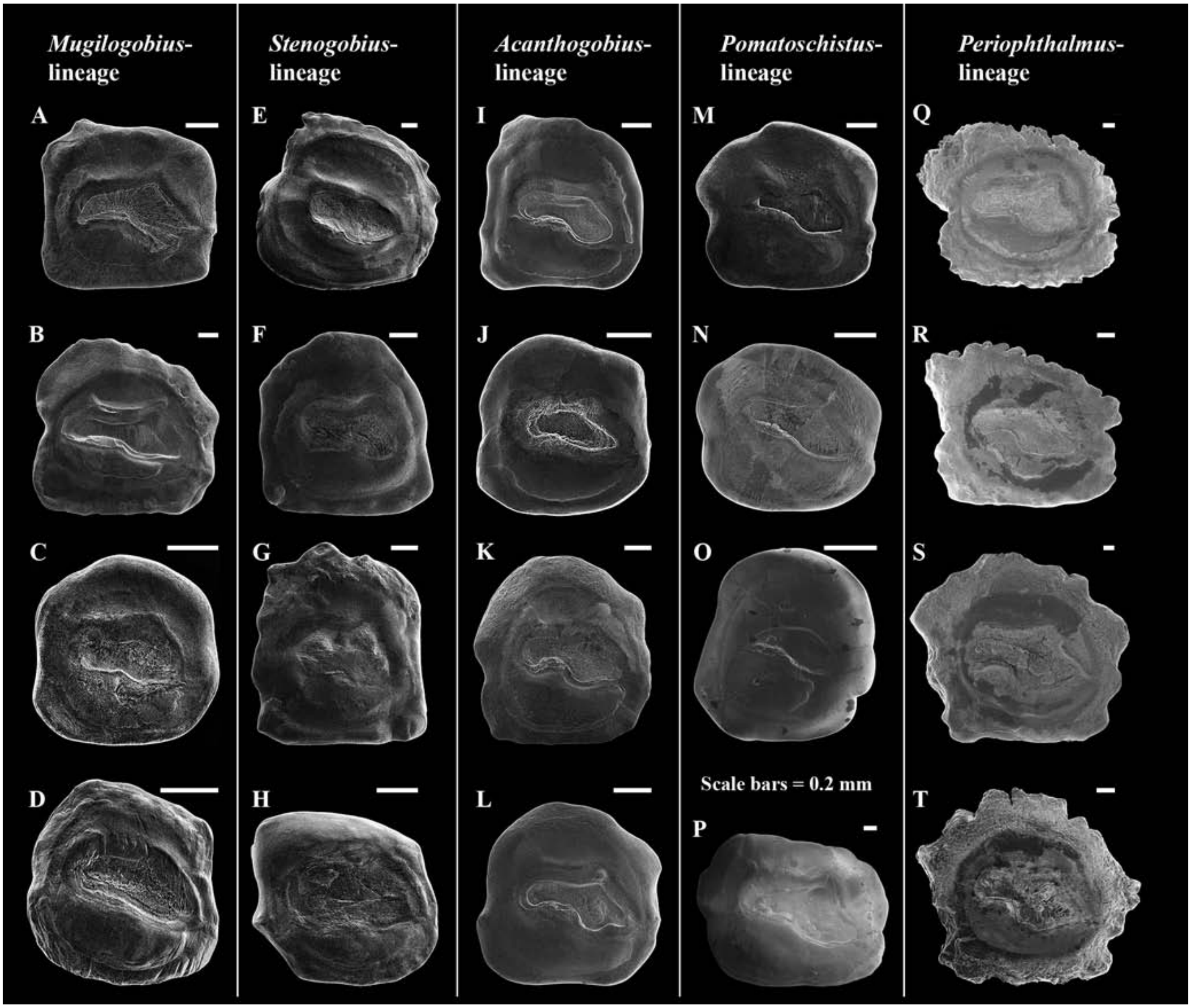

Figure 2. – Otolith morphology of the studied oxudercid species (left sagittae, inner face). *Mugilogobius* lineage: **A**: *Chlamydogobius eremius* ZSM-PIS-43854 (P-GO-1052). **B**: *Stigmatogobius sadanundio* ZSM-PIS-43856 (P-GO-1056). **C**: *Brachygobius xanthozonus* ZSM-PIS-43865 (P-GO-1075). **D**: *Schismatogobius roxasi* ZSM-PIS-43866 (P-GO-1077). *Stenogobius* lineage: **E**: *Gobioides broussonnetii* ZSM-PIS-43852 (P-GO-1048). **F, G**: *Awaous flavus* ZSM-PIS-43853 (P-GO-1051, -1050). **H**: *Stiphodon atropurpureus* ZSM-PIS-43862 (P-GO-1070). *Acanthogobius* lineage: **I**: *Rhinogobius rubromaculatus* ZSM-PIS-43860 (P-GO-1064). **J**: *R. zhoui* ZSM-PIS-43859 (P-GO-1062/63-2). **K**: *R. candidianus* ZSM-PIS-43858 (P-GO-1060). **L**: *R. formosanus* ZSM-PIS-43864 (P-GO-1073). *Pomatoschistus* lineage: **M**: *Pomatoschistus marmoratus* NMP P6d 32/2017-7. **N**: *Buenia affinis* NMP P6d 30/2017-6. **O**: *Gobiusculus flavescens* NMP P6V 142775. **P**: *Deltentosteus quadrimaculatus* NMP P6d 34/2017-9. *Periophthalmus* lineage: **Q**: *Boleophthalmus dussumieri* ZM-CBSU Khamirr 38. **R**: *Scartelaos tenuis* ZM-CBSU Helleh 86. **S, T**: *Periophthalmus waltoni* ZM-CBSU Gowater 1745 (male), ZM-CBSU Gowater 1736 (female).

the ventral margin is straight or slightly indented in the middle, posteriorly curved, but anteriorly angled or rounded. *Pomatoschistus marmoratus* has a cauda whose contour is mostly straight or slightly inclined towards the ventral rim (as seen in *G. flavescens*), while most otoliths of *P. quagga* have a cauda that is slightly inclined towards the dorsal rim.

The otoliths of †*Pomatoschistus* sp. are pictured in Brzobohatý (1994: pl. 6, figs. 9-119), but not been described. The sulcus has the same shape and position as described above for the recent sand goby species, but the cauda is straight or slightly inclined towards the ventral rim (Fig. 3A-C), as seen in *G. flavescens* and *P. marmoratus*. The otoliths are approximately square in overall shape. The rounded dorsal margin is smooth or mildly crenulated; a slight predorsal kink and a short posterodorsal projection are present in some otoliths. The anterior margin is straight or slightly inclined towards the ventral margin. The smooth ventral margin is straight or slightly notched in the middle

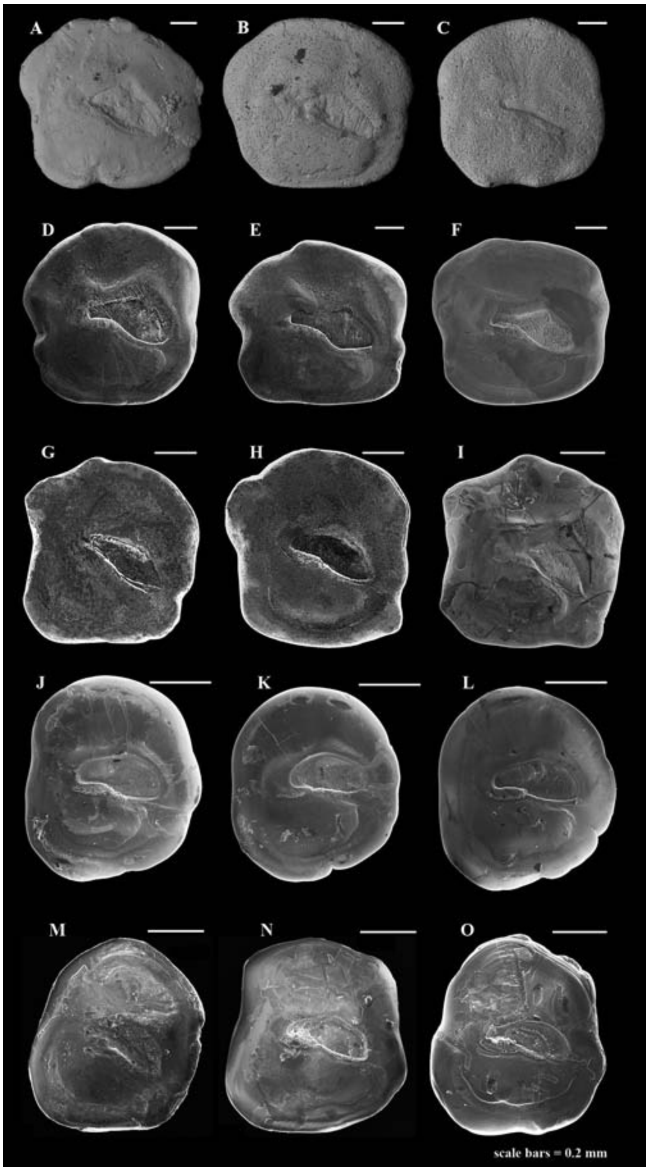

Figure 3. – Otolith morphology of †*Pomatoschistus* sp. (**A-C**: NHM 1993/140/4, NHM 1993/140/6, NHM 1993/140/8, all three mirrored) and the studied recent species of the sand-gobies, *i.e*. *Pomatoschistus marmoratus* (**D-F**: NMP P6d 32/2017-4, NMP P6d 32/2017-7, NMP P6d 32/2017-1), *P. quagga* (**G-I**: NMP P6d 33/2017-4, NMP P6d 33/2017-5, NMP P6d 33/2017-7), *Gobiusculus flavescens* (**J-L**: NMP P6V 142777, NMP P6V 142778, NMP P6V 142776) and *Knipowitschia croatica* (**M-O**: NMP P6d 31/2017-7, NMP P6d 31/2017-2, NMP P6d 31/2017-3). All images show the left sagitta from the inner face.

and anteriorly and posteriorly curved. The posterior margin is smooth and can show a slight concavity.

Variation of otolith variables between the oxudercid lineages and †*Pomatoschistus* sp. (sensu Brzobohatý, 1994)

We used non-parametric tests (Mann-Whitney, $p < 0.05$) to examine the significance of differences in otolith variables between the five recent lineages and †*Pomatoschistus* sp., because some variables were not normally distributed (Shapiro-Wilk, $p < 0.05$). The results indicate that eight out of the 23 otolith variables revealed differences between at least two of the studied groups (Fig. 4, Tab. III). Three of these eight variables incorporate the sulcus length (SuL/OL, SuL/SuEndV, SuL/OP), two the sulcus perimeter (SuP/OP, SuP/SuEndV), and the remainder are SuA/OA, SuTipV/SuEndV, and SuEndV/OP.

The *Pomatoschistus* lineage and †*Pomatoschistus* sp. were clearly separated from the *Mugilogobius*, *Acanthogobius* and *Periophthalmus* lineages, but not from the *Stenogobius* lineage (Table III). The *Acanthogobius* and *Periophthalmus* lineages could be separated from each other, and from the *Pomatoschistus* lineage and †*Pomatoschistus* sp., but not from the *Mugilogobius* and *Stenogobius* lineages. While the *Mugilogobius* lineage could be separated at least from the *Pomatoschistus* lineage and †*Pomatoschistus* sp., no separation was possible between the *Stenogobius* lineage and the others, and only a single otolith variable separated this lineage from †*Pomatoschistus* sp. (see Tab. III).

Table III lists the otolith variables that were most useful in separating groups from one another. The most powerful parameter in this respect is SuL/OL, as it contributes to the separation of all groups that could actually be distinguished, and is the only variable that could discriminate †*Pomatoschistus* sp. from the *Stenogobius* lineage. The variables SuL/SuEndV and SuTipV/SuEndV could separate six groups, SuA/OA and SuEndV/OP were useful in the separation of five groups, and SuL/OP could still separate four groups. The two variables related to the sulcus perimeter (SuP/OP, SuP/SuEndV) showed only little discriminatory power by separating only one and two groups, respectively, from the rest (Tab. III).

Considering which groups cluster together, one finds that the SuEndV/OP discriminates between three groups, one comprising the *Stenogobius* and *Mugilogobius* lineages, the second one the *Acanthogobius* and *Pomatoschistus* lineages, and the third one the *Periophthalmus* lineage alone (Fig. 4H). Furthermore, the variables SuP/OP, SuL/OL and SuL/OP can distinguish *Stenogobius*+*Acanthogobius*+*Mugilogobius* from *Pomatoschistus*+*Periophthalmus* (Figs. 4B, D, F). With respect to all of these variables, fossil otoliths of †*Pomatoschistus* fall either within the limits of the *Pomatoschistus* lineage or nearest to it.

A multivariate analyses based on a PCA using all 23 otolith variables shows that all lineages overlap, but that the fossil otoliths of †*Pomatoschistus* sp. only overlap with the *Pomatoschistus* lineage (Fig. 5). Very similar results were obtained when only the eight otolith variables shown to be informative in the univariate analyses were employed in the PCA (plot not shown).

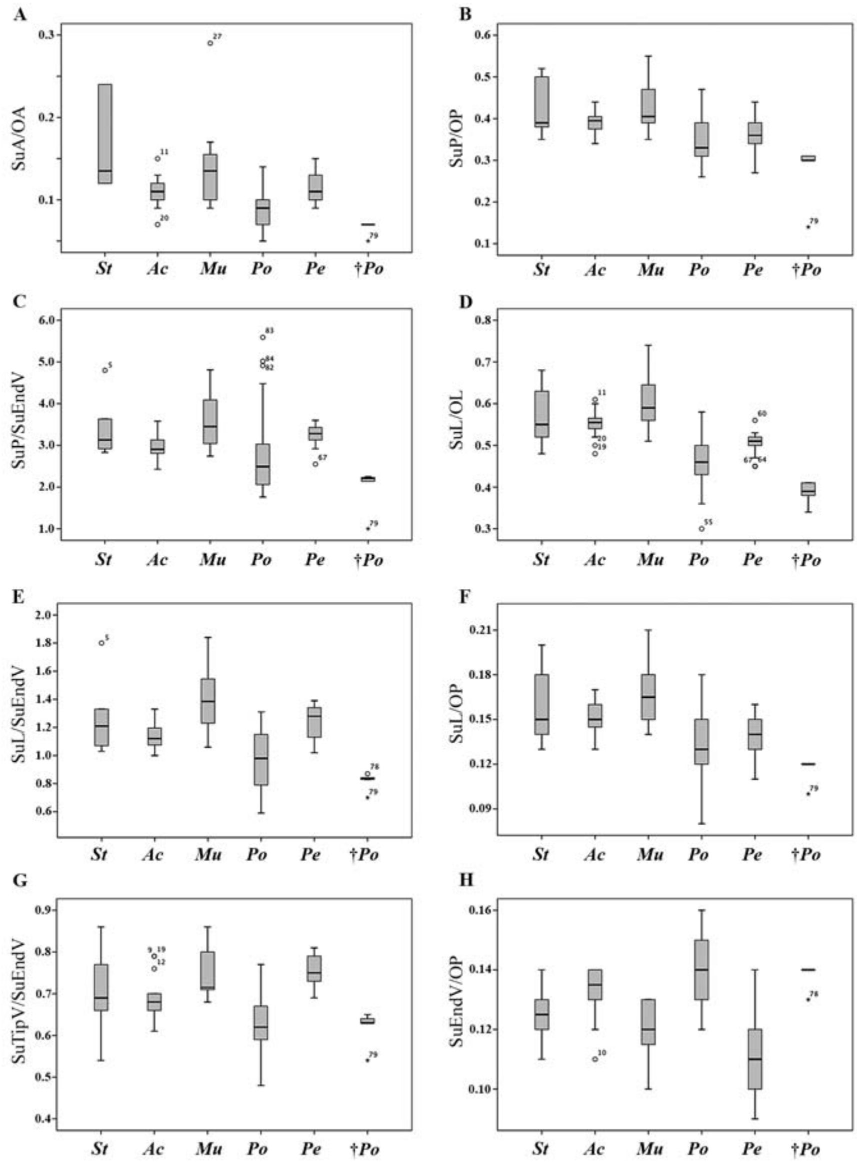

Figure 4. – Box plots of eight otolith variables that were useful in the separation of the five oxudercid lineages. **A**: Ratio of sulcus area and otolith area. **B**: Ratio of sulcus perimeter and otolith perimeter. **C**: Ratio of sulcus perimeter and distance from sulcus end to the ventral margin. **D**: Ratio of sulcus length and otolith length. **E**: Ratio of sulcus length and distance from sulcus end to the ventral margin. **F**: Ratio of sulcus length to otolith perimeter. **G**: Ratio of distance from sulcus tip to the ventral margin and distance from sulcus end to the ventral margin. **H**: Ratio of distance from sulcus end to the ventral margin and otolith perimeter. Abbreviations: *Ac*, *Acanthogobius* lineage; *Mu*, *Mugilogobius* lineage; *Pe*, *Periophthalmus* lineage; *Po*, *Pomatoschistus* lineage; *St*, *Stenogobius* lineage; †*Po*, †*Pomatoschistus* sp. from Brzobohatý (1994).

Variation of otolith variables between species of the *Pomatoschistus* lineage and †*Pomatoschistus* sp. (sensu Brzobohatý, 1994)

Most of the otolith variables obtained for the recent species of the *Pomatoschistus* lineage were normally distributed, but in case of †*Pomatoschistus* sp. only OL/OH and SuL/OH revealed normal distribution (Shapiro-Wilk, $p < 0.05$). Accordingly, we used either parametric tests (ANOVA with Dunnett T3 post-hoc test, $p < 0.05$) or Mann-Whitney ($p < 0.05$) to examine the significance of differences in the individual otolith variables between the six recent species and †*Pomatoschistus* sp. We selected the eight otolith variables that revealed clear differences between at least two of the studied groups (Fig. 6, Tab. IV). As in the analysis of the lineage groups, three of these parameters incorporate the sulcus length (SuL/OH, SuL/OP, SuL/SuEndV), two the sulcus perimeter (SuP/SuTipV, SuP/SuEndV) and the remainder are SuA/OA, OL/OH and SuH/SuP. Four otolith variables (OL/OH, SuL/OH, SuH/SuP, SuP/SuTipV) showed discriminatory power in this analysis, although they had not done so with respect to the lineage groups (see above).

These results showed that the sand gobies plus †*Pomatoschistus* sp. can be clearly discriminated from the non-sand gobies (*B. affinis*, *D. quadrimaculatus*), but that the separation of *P. marmoratus* between either *B. affinis* or *D. quadrimaculatus* is not as clearcut as that obtained for the other sand gobies (Fig. 6, Tab. IV). On the other hand, only three otolith variables, two of them related to the sulcus perimeter, separated *B. affinis* from *D. quadrimaculatus* (Tab. IV). In the case of the recent sand gobies, *P. marmoratus* could be

Table III. – Otolith variables that differed significantly between the studied groups (Mann-Whitney test, $p < 0.05$). To facilitate comparison, the otolith variables are arranged in each cell in the same order. The symbol -- indicates that the respective variable was not significantly different; the symbol --- indicates that none of the otolith variables was different. For abbreviations of otolith variables, see table II. Since the content of the Table shows a symmetric matrix, we shaded the upper part.

	Mugilogobius lineage	*Stenogobius* lineage	*Acanthogobius* lineage	*Pomatoschistus* lineage	*Periophthalmus* lineage	†*Pomatoschistus* sp.
Mugilogobius lineage	X	---	---	-- -- -- SuL/OL SuL/SuEndV -- SuTipV/SuEndV SuEndV/OP	---	-- -- SuP/SuEndV SuL/OL SuL/SuEndV SuL/OP SuTipV/SuEndV SuEndV/OP
Stenogobius lineage	---	X	---	---	---	-- -- -- SuL/OL -- -- -- --
Acanthogobius lineage	---	---	X	SuA/OA SuP/OP - SuL/OL SuL/SuEndV SuL/OP SuTipV/SuEndV -	-- -- -- SuL/OL -- -- SuTipV/SuEndV SuEndV/OP	SuA/OA -- -- SuL/OL SuL/SuEndV SuL/OP -- --
Pomatoschistus lineage	-- -- -- SuL/OL SuL/SuEndV -- SuTipV/SuEndV SuEndV/OP	---	SuA/OA SuP/OP -- SuL/OL SuL/SuEndV SuL/OP SuTipV/SuEndV --	X	SuA/OA -- -- SuL/OL SuL/SuEndV -- SuTipV/SuEndV SuEndV/OP	SuA/OA -- -- SuL/OL -- -- -- --
Periophthalmus lineage	---	---	-- -- -- SuL/OL -- -- SuTipV/SuEndV SuEndV/OP	SuA/OA -- -- SuL/OL SuL/SuEndV -- SuTipV/SuEndV SuEndV/OP	X	SuA/OA -- SuP/SuEndV SuL/OL SuL/SuEndV SuL/OP SuTipV/SuEndV SuEndV/OP
†*Pomatoschistus* sp.	-- -- SuP/SuEndV SuL/OL SuL/SuEndV SuL/OP SuTipV/SuEndV SuEndV/OP	-- -- -- SuL/OL -- -- -- --	SuA/OA -- -- SuL/OL SuL/SuEndV SuL/OP -- --	SuA/OA -- -- SuL/OL -- -- -- --	SuA/OA -- SuP/SuEndV SuL/OL SuL/SuEndV SuL/OP SuTipV/SuEndV SuEndV/OP	X

reliably separated from all studied sand gobies, and even from *P. quagga,* based on 5-7 otolith variables (see Tab. IV). In contrast, only two otolith variables discriminate between *G. flavescens* and *P. quagga*, and solely one otolith variable differs between *K. croatica* and *P. quagga* (SuH/SuP), and also between *G. flavescens* and *K. croatica* (SuA/OA) (Tab. IV). The fossil †*Pomatoschistus* sp. could be separated from *P. quagga* based on two variables (OL/OH, SuH/SuP),

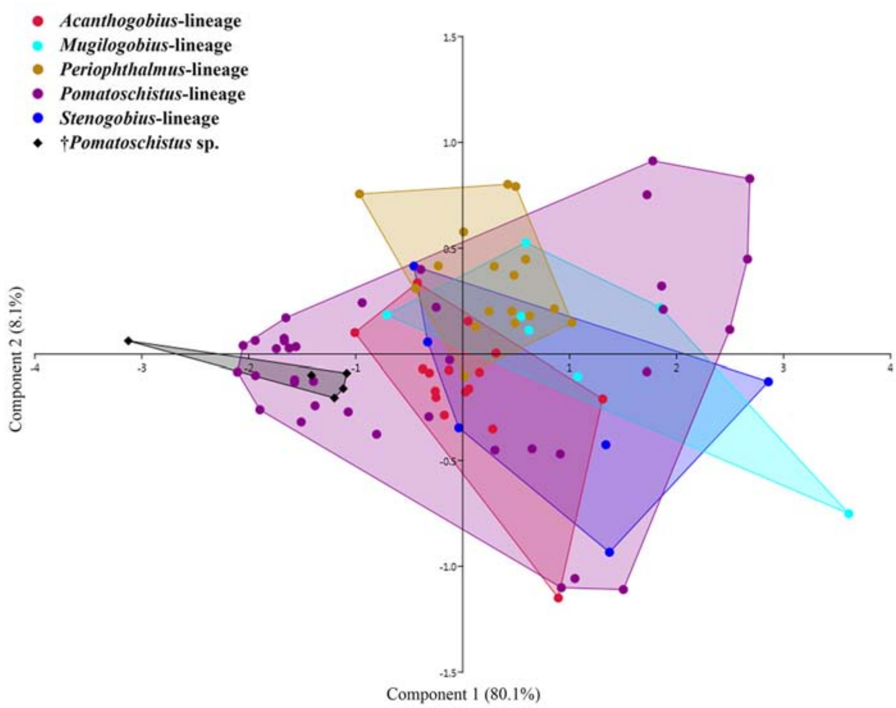

Figure 5. – PCA of the oxudercid lineages and †*Pomatoschistus* sp. based on all 23 otolith variables.

and only a single otolith variable separates it from *G. flavescens* (SuA/OA) and *K. croatica* (OL/OH).

Table IV lists the otolith variables that were most useful in the separation of the studied species of the *Pomatoschistus* lineage. It appears that the most powerful parameters are OL/OH, SuL/OP, SuL/OH, SuL/SuEndV and SuP/SuEndV, as they contribute to almost all the discriminations achieved for the recent species. SuH/SuP is involved in fewer cases of successful separation between groups, but is nevertheless meaningful because it is the sole variable that could separate *K. croatica* from *P. quagga* and, moreover, one of only two variables that discriminate between *B. affinis* and *P. marmoratus* and between *G. flavescens* and *P. quagga*. Of the remaining two variables, SuP/SuTipV appears to be less informative, whereas SuA/OA, although involved only in the separation between few groups, is the single variable that could discriminate between *G. flavescens* and *K. croatica* as well as between *G. flavescens* and †*Pomatoschistus* sp., and one of only two variables that separate *G. flavescens* from *P. quagga*.

The PCA based on all 23 otolith variables for the recent species of the *Pomatoschistus* lineage and †*Pomatoschistus* sp. resolves three groups (Fig. 7). One group consists only of *D. quadrimaculatus*. Within the second group, *B. affinis* overlaps to some extent with *P. marmoratus*. The third group comprises the sand gobies *G. flavescens*, *K. croatica* and *P. quagga* as well as †*Pomatoschistus* sp.

DISCUSSION

The intention of this study was to test whether the five lineages of the Oxudercidae, and individual species of the European *Pomatoschistus* lineage, could be distinguished based on the quantification of otolith variations. The approach was applied to otoliths from a total of 20 extant oxudercid species representing the five extant lineages, and fossil otoliths of †*Pomatoschistus* sp. (sensu Brzobohatý, 1994). Both otolith morphology (qualitative approach) and otolith morphometry (quantitative approach) were considered and a total of 23 otolith variables was assessed. The results showed that the otolith morphology of most Oxudercidae examined is clearly different from that found in their sister group, the Gobiidae (*e.g.* Agorreta *et al*., 2013), because no obvious posterodorsal projection is present (see Gierl *et al*., 2013: fig. 6D). However, the otolith samples obtained from the *Periophthalmus* lineage do have a prominent posterodorsal projection (see Fig. 2) and thus are similar to the otoliths of Gobiidae.

Separation at the lineage level using otoliths

Visual inspection of otolith morphology alone was insufficient to enable specimens to be assigned to an individual lineage, but the comparative analysis of otolith variables was partially successful. The overall rate of success is low (40%, see Tab. III), but appears to reflect to some extent the biogeographic distribution and, in the case of the *Periophthalmus* lineage, also a specific lifestyle (see below).

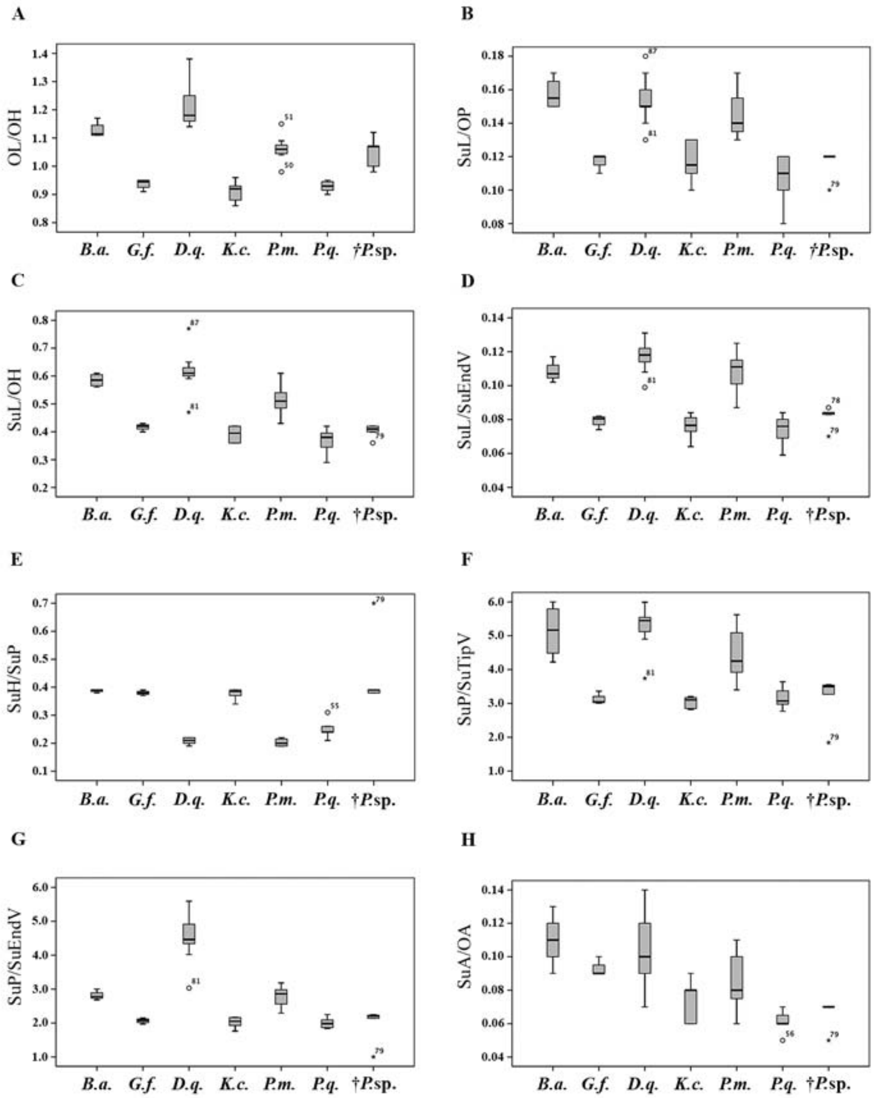

Figure 6. – Box plots of eight otolith variables that were useful in the separation of the recent species of the *Pomatoschistus* lineage and †*Pomatoschistus* sp. **A**: Ratio of otolith length and otolith height; **B**: ratio of sulcus length and otolith perimeter; **C**: Ratio of sulcus length and otolith height; **D**: Ratio of sulcus length and distance from sulcus end to the ventral margin; **E**: Ratio of sulcus height and sulcus perimeter; **F**: Ratio of sulcus perimeter and distance from sulcus tip to the ventral margin; **G**: Ratio of sulcus perimeter and distance from sulcus end to the ventral margin; **H**: Ratio of sulcus area and otolith area. Abbreviations: *B.a*., *Buenia affinis*; *G.f*., *Gobiusculus flavescens*; *D.q*., *Deltentosteus quadrimaculatus*; *K.c*., *Knipowitschia croatica*; *P.m*., *Pomatoschistus marmoratus*; *P.q*., *Pomatoschistus quagga*; †*P*. sp., †*Pomatoschistus* sp.

The *Pomatoschistus* lineage is the only lineage of the Oxudercidae that occurs in European waters (*Agorreta* et al., 2013). This biogeographic uniqueness may be reflected in its otoliths, as otolith variables separated the *Pomatoschistus* lineage from all others except the *Stenogobius* group (see Table III). Of the two tropical lineages, only the *Periophthalmus* lineage could be separated from the temperate ones (see Tab. III). This lineage includes the mudskippers, which are characterized by an amphibious lifestyle (Murdy, 1989). Among the ten mudskipper genera that are recognised, the members of *Boleophthalmus* Valenciennes, 1937, *Scartelaos* Swainson, 1839, *Periophthalmodon* Bleeker, 1874 and *Periophthalmus* Bloch & Schneider, 1801 (Murdy, 1989; Ishimatsu and Gonzales, 2011) are especially derived, because they can move on muddy substrates, climb rocks or mangrove roots, and display, forage and defend territories during low tides (Murdy, 2011; Jaafar and Parenti, 2017). The otoliths of the *Periophthalmus* lineage used in this study were obtained from three of these species (Tab. I). It can therefore be assumed that the highly distinctive nature of their otoliths, relative to those of the other oxudercid lineages, reflects a functional change in the sensory system of the inner ear that is related to their unique lifestyle.

Separation of species and †*Pomatoschistus* sp. (sensu Brzobohatý, 1994) within the *Pomatoschistus* lineage

The otoliths of the four recent species of the sand gobies used in our study (*Gobiusculus flavescens*, *Knipowitschia croatica*, *Pomatoschistus marmoratus*, *P. quagga*) all show a marked reduction in the size of the cauda (Figs 2, 3). Based on this character, they could be readily separated from the non-sand gobies studied here, i.e. *Deltentosteus quadrimaculatus* (Fig. 2P) and *Buenia affinis* (Fig. 2N). The name 'sand gobies' refers to their preference for sandy sub-

Table IV. – Otolith variables that were significantly different between the studied groups ($p < 0.05$, ANOVA, Dunnett T3 post-hoc test for normally distributed variables, Mann-Whitney for non-normally ones). To facilitate comparison, the otolith variables are arranged in each cell in the same order. The symbol -- indicates that the respective variable was not significantly different. For complete species names see Table I, for abbreviations of otolith variables, see table II. Since the content of the table shows a symmetric matrix, we shaded the upper part.

	Non sand-goby		Recent species of sand-gobies				
	Buenia affinis	*Deltentosteus quadrimac.*	*Gobiusculus flavescens*	*Knipow. croatica*	*Pomatosch. marmoratus*	*Pomatosch. quagga*	†*Pomatosch.* sp.
Buenia affinis	X	OL/OH -- -- -- SuH/SuP -- SuP/SuEndV --	OL/OH SuL/OP SuL/OH SuL/SuEndV -- SuP/SuTipV SuP/SuEndV --	OL/OH SuL/OP SuL/OH SuL/SuEndV -- -- SuP/SuEndV --	OL/OH -- -- -- SuH/SuP -- -- --	OL/OH SuL/OP SuL/OH SuL/SuEndV SuH/SuP -- SuP/SuEndV --	-- SuL/OP SuL/OH SuL/SuEndV -- SuP/SuTipV SuP/SuEndV SuA/OA
Deltentosteus quadrimac.	OL/OH -- -- -- SuH/SuP -- SuP/SuEndV --	X	OL/OH SuL/OP SuL/OH SuL/SuEndV SuH/SuP SuP/SuTipV SuP/SuEndV --	OL/OH SuL/OP SuL/OH SuL/SuEndV SuH/SuP SuP/SuTipV SuP/SuEndV --	OL/OH -- -- -- -- -- SuP/SuEndV --	OL/OH SuL/OP SuL/OH SuL/SuEndV -- SuP/SuTipV SuP/SuEndV SuA/OA	OL/OH SuL/OP SuL/OH SuL/SuEndV SuH/SuP SuP/SuTipV SuP/SuEndV SuA/OA
Gobiusculus flavescens	OL/OH SuL/OP SuL/OH SuL/SuEndV -- SuP/SuTipV SuP/SuEndV --	OL/OH SuL/OP SuL/OH SuL/SuEndV SuH/SuP SuP/SuTipV SuP/SuEndV --	X	-- -- -- -- -- -- -- SuA/OA	OL/OH SuL/OP -- SuL/SuEndV SuH/SuP SuP/SuTipV SuP/SuEndV --	-- -- -- -- SuH/SuP -- -- SuA/OA	-- -- -- -- -- -- -- SuA/OA
Knipow. croatica	OL/OH SuL/OP SuL/OH SuL/SuEndV -- -- SuP/SuEndV --	OL/OH SuL/OP SuL/OH SuL/SuEndV SuH/SuP SuP/SuTipV SuP/SuEndV --	-- -- -- -- -- -- -- SuA/OA	X	OL/OH SuL/OP SuL/OH SuL/SuEndV SuH/SuP SuP/SuTipV SuP/SuEndV --	-- -- -- -- SuH/SuP -- -- --	OL/OH -- -- -- -- -- -- --
Pomatosch. marmoratus	OL/OH -- -- -- SuH/SuP -- -- --	OL/OH -- -- -- -- -- SuP/SuEndV --	OL/OH SuL/OP -- SuL/SuEndV SuH/SuP SuP/SuTipV SuP/SuEndV --	OL/OH SuL/OP SuL/OH SuL/SuEndV SuH/SuP SuP/SuTipV SuP/SuEndV --	X	OL/OH SuL/OP SuL/OH SuL/SuEndV -- -- SuP/SuEndV --	-- SuL/OP SuL/OH SuL/SuEndV SuH/SuP SuP/SuTipV SuP/SuEndV --
Pomatosch. quagga	OL/OH SuL/OP SuL/OH SuL/SuEndV SuH/SuP -- SuP/SuEndV --	OL/OH SuL/OP SuL/OH SuL/SuEndV -- SuP/SuTipV SuP/SuEndV SuA/OA	-- -- -- -- SuH/SuP -- -- SuA/OA	-- -- -- -- SuH/SuP -- -- --	OL/OH SuL/OP SuL/OH SuL/SuEndV -- -- SuP/SuEndV --	X	OL/OH -- -- -- SuH/SuP -- -- --
†*Pomatosch.* sp.	-- SuL/OP SuL/OH SuL/SuEndV -- SuP/SuTipV SuP/SuEndV SuA/OA	OL/OH SuL/OP SuL/OH SuL/SuEndV SuH/SuP SuP/SuTipV SuP/SuEndV SuA/OA	-- -- -- -- -- -- -- SuA/OA	OL/OH -- -- -- -- -- -- --	-- SuL/OP SuL/OH SuL/SuEndV -- -- -- --	OL/OH -- -- -- SuH/SuP -- -- --	X

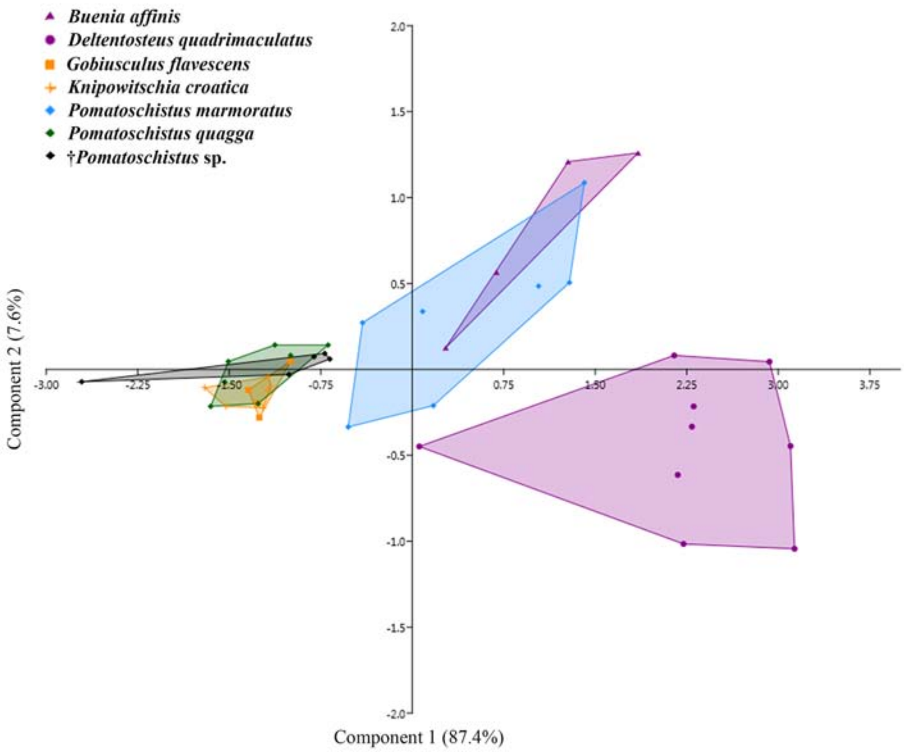

Figure 7. – PCA of the studied *Pomatoschistus* species and genera and †*Pomatoschistus* sp. based on all 23 otolith variables.

strates (see Gierl and Reichenbacher, 2017) and it appears that this particular lifestyle has had an impact on their sensory system and otoliths, as discussed above for the mudskippers. Moreover, otolith variables proved useful for species classification within the *Pomatoschistus* lineage, as all cases were separated (Tab. IV).

Another interesting outcome of our study is the similarity observed between the otoliths of the sand goby *P. marmoratus* and those of the two non-sand goby species (*D. quadrimaculatus*, *B. affinis*). It could be speculated that this might reflect a more basal phylogenetic position of *P. marmoratus* among the sand-gobies, but such a placement is not supported by molecular studies (Vanhove *et al.*, 2012; Agorreta *et al.*, 2013). *Pomatoschistus marmoratus* is one of the most abundant benthic oxudercid species in the estuaries and sandy coastal lagoons of the Mediterranean Sea (see Mejri *et al.*, 2011). It is also widespread throughout the eastern Atlantic, the Black Sea and the Sea of Azov (Miller, 1986). Accordingly, *P. marmoratus* appears to be a generalist rather than a specialist, and this might explain its 'basal' otolith morphology and similarity to the two non-sand goby species, respectively.

Moreover, the otolith variables permit a surprisingly clear degree of distinction between *P. marmoratus* and *P. quagga* (five differing variables, see Table IV). In this case, the otolith data are fully confirmed by the molecular study of the sand gobies by Vanhove *et al.* (2012). These authors showed that *Pomatoschistus* splits into two main clades (one so far containing mainly *P. marmoratus*) and is, in addition, paraphyletic, because *P. quagga* is not a member of the two main clades, but falls within a *Knipowitschia* clade. The presence of two main clades within *Pomatoschistus*, and a basal position for *P. marmoratus* in one of those, has now been further confirmed by Engin and Seyhan (2017).

In addition, we found few differences between *P. quagga* and *G. flavescens* (two differing variables). According to Vanhove *et al.* (2012), *G. flavescens* belongs to the second of the two *Pomatoschistus* clades (which does not contain *P. marmoratus*), a conclusion that is consistent with its '*Pomatoschistus*-like' otoliths. This is further supported by Thacker *et al.* (2018), who place "*flavescens*" within the genus *Pomatoschistus*. Furthermore, both *G. flavescens* and *P. quagga* share a 'hyperbenthic' lifestyle, which means that they remain within 1 m above the sea bottom (Kovačić, 2003). So the two species could also be a further example of otolith similarity because of a shared specialisation in lifestyle.

Another interesting point is that we found only one otolith variable that differed between *P. quagga* and *K. croatica*. In this case, related lifestyles cannot be responsible for this close similarity in otolith morphology, because *P. quagga* is marine, whereas *K. croatica* is a freshwater species. In the molecular tree of Vanhove *et al.* (2012), *P. quagga* is a member of a *Knipowitschia* clade, but *K. croatica* was not included in this tree. In the DNA sequence-based study by Geiger *et al.* (2014), a comprehensive molecular data set from *Knipowitschia* was used, and *K. croatica* did not fall in the *Knipowitschia* clade in that study. Also Vanhove *et al.* (2016) found two main clades within the *Knipowitschia* species. One clade comprised *K. croatica* + *K. punctatissima* and these two were sister to *Pomatoschistus canestrinii*. This group in turn was sister to the second clade of *Knipowitschia*. So more work is necessary to resolve the relationships between *P. quagga* and *K. croatica* and to evaluate whether their otolith similarity is a phylogenetic signal.

The fossil otoliths of †*Pomatoschistus* sp. were clearly similar to those of the sand gobies, but not to *P. marmoratus* (Fig. 7, Tab. IV). This, however, concurs precisely with the aforementioned results of the molecular studies. It cannot be definitively decided whether the fossil otoliths of †*Pomatoschistus* sp. represent a 'true' *Pomatoschistus* species. But this genus is certainly a member of the sand gobies and, with its middle Miocene age, represents the oldest record of a sand goby species to date. Since fossil otoliths have a much higher chance of preservation, they usually precede the fossil skeletal record. The oldest skeleton-based *Pomatoschistus* is †*Pomatoschistus* sp. from the Middle Miocene of Russia (Carnevale *et al.*, 2006).

Concluding remarks

The ratio of otolith length to otolith height (OL/OH) has long been considered as a useful parameter for species separation in fossil otoliths (*e.g.* Weiler, 1963). The ratio of the sulcus area to overall otolith area (SuA/OA) has previously been used mainly to study the ecomorphological patterns of otoliths of recent species (Volpedo *et al.*, 2008). Other already established otolith variables mainly relate to the rostrum and antirostrum (Reichenbacher *et al.*, 2007; Volpedo *et al.*, 2008; Vignon and Morat, 2010). They cannot be used for the otoliths of the Gobiidae and Oxudercidae because these do not possess such structures.

Separation of otoliths at different taxonomic levels may require different otolith variables (see Reichenbacher *et al.*, 2007). This was also the case in our study. While the SuL/OL was involved in all cases of successful separation at the level of lineages (see Tab. III), it was not meaningful at the level of species (see Tab. IV). This implies that the size of the sulcus may bear a phylogenetic signal at higher taxonomic levels. On the other hand, OL/OH was among the most useful variables at the species level, but was of little import in the separation of the lineages. So OL/OH can be considered as an autapomorphy for an individual species. However, further work based on additional species is needed to verify the possible phylogenetic value of the individual otolith variables.

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