Identification of past and present gobies: distinguishing *Gobius* and *Pomatoschistus* (Teleostei: Gobioidae) species using characters of otoliths, meristics and body morphometry

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Abstract

Gobies (Gobiidae + Oxudercidae) are among the largest groups of extant marine fishes. Fossils of gobies are abundant since the Miocene, and many species have been reported so far. However, delimitation of fossil goby species is challenging because molecular markers and diagnostic traits such as the disposition of sensory head papillae are lost. This study provides, for the first time, an actualistic framework for the identification of fossil goby species. We focus on characters that can in principle be recognized in fossils, and evaluate their ability to discriminate between extant goby species based on statistical analyses. Using 14 extant species of *Gobius* and seven species of *Pomatoschistus*, we conducted otolith morphometry, elliptic Fourier shape analysis of otoliths using the package 'Momocs', conventional fish morphometry, and

meristic counts. In addition, the otoliths of all species are depicted based on SEM images and briefly described. Otolith Fourier shape analysis proved to be most efficient in discrimination of species within both genera, *Gobius* and *Pomatoschistus*. Several characters used in the other approaches also worked well, but the results were variable, and the relative taxonomic significance of particular variables tended to change depending on the species under consideration. We propose otolith shape analysis as a powerful tool to explore ancient goby species diversity when samples with abundant fossil otoliths are present. Overall, the herein presented data will greatly facilitate delimitation of fossil goby species in future studies, and will consequently shed new light on the evolution of goby diversity and biogeography through time.

Keywords

Actualistic study – Gobioidae – morphology – otoliths – species discrimination

Introduction

Gobiidae and Oxudercidae (“gobies” hereafter) are generally small fishes (length <25 cm), and together they constitute one of the most species-rich groups within the modern bony fishes (Teleostei) (Nelson et al., 2016), with >1,250 species in the Gobiidae and >650 species in the Oxudercidae (Fricke et al., 2019). Gobies are globally distributed in diverse marine, brackish and freshwater habitats, and also play important roles in reef ecology (Patzner et al., 2011; Tornabene et al., 2015; Brandl et al., 2018). Many species are highly specialized including close associations with crustaceans (Karplus, 1987; Karplus et al., 2011; Kovačić et al., 2016) or as part of the cryptobenthic community (Glavičić et al., 2016; Brandl et al., 2018). The European Gobiidae and Oxudercidae comprise three distinct lineages, which were named *Aphia*-lineage, *Gobius*-lineage, and *Pomatoschistus*-lineage (Agorreta et al., 2013; Thacker, 2015). In the present work, we focus on the name giving genera of the latter two lineages, i.e., *Gobius* Linnaeus, 1758 and *Pomatoschistus* Gill, 1863, which are the most species-rich genera of European gobies (Miller, 1986; Patzner et al.,

2011). Their lifestyle is usually benthic or cryptobenthic, with a preference for sandy and rocky substrates (Macpherson & Duarte, 1994; Miller, 2004; Patzner et al., 2011). The genus *Gobius* currently comprises 28 recognized species, which are mainly distributed in the NE Atlantic, the Mediterranean and the Black Sea (Miller, 1986; Patzner et al., 2011; Froese & Pauly, 2019). Notably, two of these species are recent discoveries (Iglésias et al., 2016; Kovačić & Šanda, 2016). Fourteen species are traditionally attributed to the genus *Pomatoschistus*, including three that were only recognized in the last decade (Miller & Šanda, 2008; Engin & Innal, 2017; Engin & Seyhan, 2017; Froese & Pauly, 2019); however, the genus is not monophyletic according to recent phylogenetic studies (Thacker et al., 2019). Species of *Pomatoschistus* mostly occur in coastal habitats along the NE Atlantic, the Baltic Sea, and in some areas of the Mediterranean and the Black Sea (Miller, 1986; Patzner et al., 2011). Identification of the individual species in both genera, *Gobius* and *Pomatoschistus*, relies on coloration patterns, the arrangement of the head sensory organs (papillae), and combinations of various other characteristics such as squamation, morphometric and

meristic traits; species determination often turns out to be very difficult, especially between closely related species (Miller, 1974, 1986, 2004; Kovačić & Šanda, 2016; Engin & Seyhan, 2017).

Fossils are the only direct source of information on ancient species diversity and biogeography, and thus provide essential insights into the evolutionary history of taxa. Fossils of gobies are abundant since the early Miocene (23 Ma) and comprise both articulated skeletons (sometimes with otoliths in situ) and isolated otoliths (e.g., Weiler, 1963; Radwańska, 1992; Bratishko et al., 2015). Records of fossil otoliths are much more common than discoveries of fossil skeletons, and this explains why about two-thirds of the known fossil goby species have been described solely on the basis of otoliths (Gierl et al., 2018). But the delimitation of a fossil goby species is often problematic because information on the range of variation in skeletal and otolith traits within a respective species and between related taxa is restricted. Furthermore, the characters of the skeleton may vary little between extant goby species (Miller, 1986; Kovačić & Šanda, 2016). This, together with the loss of delicate characters in fossil skeletal material due to taphonomic processes, can lead to underestimation of the number of fossil species (see Gaudant & Quayle, 1988 vs. Gierl & Reichenbacher, 2017; Gaudant, 1998 vs. Bradić-Milinović et al., 2019). Otoliths of gobies have the advantage that they are quite robust and do not experience major loss of informative characters during the fossilisation process. On the other hand, they usually display a high degree of morphological variability, which can make their taxonomic interpretation challenging (Malz, 1978; Nolf, 1985; Jost et al., 2015). In some cases, subtle differences in otolith morphology may allow the identification of several species (Schwarzhans, 2014; Gierl & Reichenbacher, 2015;

Reichenbacher et al., 2019), while in others clear differences in otolith morphology may reflect variation in a single species living in different environments (Vasil'eva et al., 2016). The objective of this study is to provide, for the first time, an actualistic framework for the taxonomic value of characters that can in principle be recognized in fossil gobies, i.e., otoliths, body morphometry and meristics. We used 14 extant species of *Gobius* and 7 extant species of *Pomatoschistus* to test the suitability of our approach.

Materials and methods

Samples and datasets

Fourteen species of *Gobius* and seven species of *Pomatoschistus* were collected from sites along the coasts of the Mediterranean and the Baltic Sea. Details of sites and numbers of individuals are provided in table 1, collection numbers are available in the supplementary table S1. Specimens were collected with hand nets during scuba diving or with seine nets from the shore and euthanized with an overdose of benzocaine prior to fixation in ethanol. Species identification was based on morphological characters (patterns of sensory papillae and pores, coloration, squamation) using the identification keys of Miller (1986) and Kovačić (2008) as well as the original descriptions for more recently described species (i.e., Kovačić & Šanda, 2016 for *G. incognitus*; Miller & Šanda, 2008 for *P. montenegrensis*). The sampling complied with protocols approved by the responsible governmental authorities (according to the countries where the sampling was conducted). The number of specimens obtained varied from species to species, owing to differences in population sizes or difficulty of access. It is generally recommended that at least 30 specimens per category should be used in a statistical

TABLE 1 Details of the sample sets of the *Gobius* and *Pomatoschistus* species studied

Species	Fish specimens		Otoliths	Locality (N)	Region
	N	SL ranges (means±SD)	N (left/right)		
<i>Gobius auratus</i> Risso, 1810	11	26.1–60.2 (37.2±11.9)	17 (11/6)	Croatia: Selce (9); Krk Island, Kamenjak (2)	Adriatic Sea
<i>G. bucchichi</i> Steindachner, 1870	10	44.4–67.3 (48.9±15.1)	20 (10/10)	Croatia: Kraljevica (1); Selce (9)	
<i>G. cobitis</i> Pallas, 1814	11	33.4–80.0 (54.8±7.6)	21 (11/10)	Montenegro: Boka Kotorska Bay, Kostanjica (10); Croatia: Selce (1)	
<i>G. couchi</i> Miller & El-Tawil, 1974	4	32.9–44.3 (38.8±5.2)	8 (4/4)	Croatia: Krk Island, Omišalj (4)	
<i>G. cruentatus</i> Gmelin, 1789	11	78.3–117.0 (101.0±14.1)	21 (11/10)	Croatia: Selce (9), Krk Island, Kamenjak (2)	
<i>G. fallax</i> Sarato, 1889	2	46.0–50.0 (48.0±2.0)	4 (2/2)	Croatia: Unije Island, Cape Maračol (2)	
<i>G. gasteveni</i> Miller, 1974	1	68.0	2 (1/1)	Spain: Galicia (1)	Atlantic Sea
<i>G. geniporus</i> Valenciennes, 1837	10	63.6–111.6 (90.8±16.6)	14 (10/4)	Montenegro: Boka Kotorska Bay, Kostanjica (4); Croatia: Selce (6)	Adriatic Sea
<i>G. incognitus</i> Kovačić & Šanda, 2016	9	44.0–79.0 (63.0±13.0)	15 (6/9)	Croatia: Pelješac peninsula (9)	
<i>G. kolombatovici</i> Kovačić & Miller, 2000	2	70.7–80.1 (75.4±6.6)	4 (2/2)	Croatia: Krk, Tenki (2)	
<i>G. niger</i> Linnaeus, 1758	10*	40.7–59.3 (49.5±6.6)	20 (10/10)	England: Pilsey Island (10)	Atlantic Sea
<i>G. paganelus</i> Linnaeus, 1758	10	46.8–69.1 (56.2±6.3)	19 (9/10)	Portugal: Ria Formosa lagoons, Faro (10)	
<i>G. roulei</i> de Buen, 1928	10	43.8–57.5 (51.0±4.6)	20 (10/10)	Croatia: Selce (10)	Adriatic Sea
<i>G. vittatus</i> Vinciguerra, 1883	11	17.3–43.2 (30.1±10.4)	21 (10/11)	Croatia: Selce (9); Krk, Kamenjak (2)	
<i>Pomatoschistus knerii</i> (Steindachner, 1861)	10	21.6–27.0 (24.8±1.8)	20 (10/10)	Croatia: Krk Island, Plovanov Stol (10)	
<i>P. marmoratus</i> (Risso, 1810)	9	24.8–37.1 (36.9±2.4)	14 (9/5)	Croatia: Selce (7); Albania: Vilunit Lagoon (2)	

TABLE 1 Details of the sample sets of the *Gobius* and *Pomatoschistus* species studied (Cont.)

Species	Fish specimens		Otoliths	Locality (N)	Region
	N	SL ranges (means±SD)	N (left/right)		
<i>P. microps</i> (Krøyer, 1838)	15	21.7–31.6 (27.9±2.4)	30 (15/15)	Germany: Stralsund (15)	Baltic Sea
<i>P. minutus</i> (Pallas, 1770)	2	48–51.7 (49.9±2.6)	4 (2/2)		
<i>P. montenegrensis</i> Miller & Šanda, 2008	10	10.3–23.7 (20.2±3.9)	19 (10/9)	Albania: Skadar lake, Shegan spring (10)	Adriatic Sea
<i>P. pictus</i> (Malm, 1865)	2	28.4, no data	4 (2/2)	Norway: 4 km S of Brattvåg (2)	Atlantic Sea
<i>P. quagga</i> (Heckel, 1837)	10	26.3–29.9 (28.1±0.9)	20 (10/10)	Croatia: Krk, Kamenjak (10)	Adriatic Sea

Abbreviations: SL, standard length; N, number of fish specimens and otoliths; *ten specimens were used for Fourier analysis, for the other analyses seven specimens were used (see supplementary tables S1–S5).

analysis (Kar & Ramalingam, 2013). However, this criterion is an arbitrary one and cannot always be fulfilled in organismic studies, as in the present instance. In such a case, a minimum of seven specimens per species is often considered to be sufficient for a statistical analysis in fish biology. Here, we compiled for each genus a *complete dataset* containing all collected species, and a *reduced dataset* including only those species for which at least seven specimens were available. This resulted in four datasets (two for each genus), with 14 and ten species, respectively, in the case of *Gobius*, and seven and five species, respectively, in the case of *Pomatoschistus*. Each dataset was subjected to univariate statistical analyses and assessment of Fourier descriptors. For Linear Discriminant Analysis (LDA), only the reduced datasets were used (see below).

Methods

Otolith preparation and SEM imaging

Fish skulls were opened dorsally under a microscope, and the left and right saccular

otoliths (“otolith” hereafter) were removed. Otoliths were incubated in 5% KOH solution for 1 h to clean off adherent organic matter, immersed in distilled water for 1–2 h, then washed several times with distilled water, and stored in the dry state. All otoliths were mounted and gold-coated for scanning electron microscopy (with either a SEM LEO 1430 VP at ZSM Munich, or a HITACHI SU 5000 Schottky FE-SEM at LMU Munich). All otoliths are kept in the Bavarian State collection Palaeontology and Geology under the inventory number 2020 LIV.

Otolith morphometry

Ten measurements were taken from the SEM images of the left otolith of each specimen following Gierl et al. (2018) and using ImageJ2 (Rueden et al., 2017). The parameters chosen were maximum length, maximum height, perimeter length, areas of both the whole otolith and the sulcus, as well as the vertical distances from the anterior and posterior ends of the sulcus to the ventral margin of the otolith (fig. 1c). The measurements were used to calculate 23 otolith variables (according to Gierl

et al., 2018), which were multiplied by 1000 for subsequent statistical analyses. The original measurements and the otolith variables derived from them are tabulated for all specimens in supplementary tables S1 and S2.

Otolith shape analysis

On the basis of the SEM images of both right and left otoliths, each otolith image was transformed into a black object on white background using Photoshop CS6 and saved as a JPG file. To conduct elliptic Fourier shape analysis, JPG-files were imported into R using the Momocs software package (Bonhomme et al., 2014). After Momocs had extracted the outlines of the otoliths, the appropriate sequence of commands was implemented to normalize the perimeter outlines in terms of size and orientation, and to estimate the number of harmonics required to attain a cumulative power that corresponded to 99.9% of the shape (24 harmonics in *Gobius* and 20 harmonics in *Pomatoschistus*). Subsequently, an elliptic Fourier analysis was performed, which resulted in the Fourier descriptors (supplementary table S3). As no significant intraspecific differences in shape were detected between left and right otoliths (MANOVA, $P < 0.05$), SEM images of both were included in the subsequent analyses, as has been done in previous work (Davoodi & Rahimian, 2016).

Body morphometry

Digital X-ray images of all fish specimens were acquired prior to otolith dissection, using a Faxitron Bioptics instrument (LLC-Vision NDT version 2.2.5, 45 k.v. and 30 sec) at the SNSB Bavarian State Collection of Zoology, Munich (ZSM). On the basis of these images, standard measurements relating to head-, body- and fin-base proportions (fig. 1a) were obtained with ImageJ (Rueden et al., 2017), following Liu et al. (2009) and Larson et al. (2001). Measurements included standard

length (SL), distances from snout to origins of the first and second dorsal fins (SN/D1, SN/D2) and anal fin (SN/A); distance from end of second dorsal fin to first dorsal (procurent) ray of caudal fin (D2C); length of caudal peduncle (CP); body depth at origin of first dorsal fin (B); lengths of the bases of the second dorsal fin (D2b) and anal fin (Ab). All measurements were recorded to the nearest 0.1 mm. Eight variables were calculated by standardizing the measurements based on SL. Raw data and derived variables of all specimens are available in the supplementary table S4.

Meristic counts

Meristic characters were counted based on the X-ray images. They include number of abdominal, caudal, and total vertebrae (last terminal vertebral element was included); number of fin elements in second dorsal fin (D2) and anal fin (A) (the two rays attached to the last pterygiophore were counted as one); number of anal fin pterygiophores inserting in front of haemal spine of first caudal vertebra (AP, Birdsong et al., 1988); and number of dorsal and ventral procurent rays (fig. 1b). The number of fin elements (spinous rays) in the first dorsal fin (D1) and the number of branched caudal rays did not contain useful information as no differences were found in either the *Gobius* or the *Pomatoschistus* dataset. Each meristic count was used as a single variable in the statistical analyses. Counts of all specimens are provided in the supplementary table S5.

Statistical analysis

Variables resulting from otolith morphometry and Fourier shape analyses, body morphometry, and meristic characters each served as input for statistical analyses in R (R Core Team, 2019), using the packages Momocs, car, stats, PMCMRplus, MVN and heplot, as well as PAST

FIGURE 1 a–b, Schematic drawing of a goby skeleton depicting the standard morphometric (1a) and meristic (1b) characters used in this study; c–d, Schematic drawing (c) and SEM image (d) of a right goby otolith (based on *G. buccichi*) showing the measured distances and areas (after Gierl et al., 2018) and the established otolith terminology. Colors in a: red, horizontal measurements; blue, measurements along fin bases; green vertical measurements. Abbreviations: (a) Ab, length of anal fin base; B, body depth at origin of first dorsal fin; CP, length of caudal peduncle; D2b, length of second dorsal fin base; D2C, distance between end of second dorsal fin and first dorsal (procurent) ray of caudal fin; SL, standard length (from snout to begin of caudal fin); SN/A, distance from snout to origin of anal fin; SN/D1, distance from snout to origin of first dorsal fin; SN/D2, distance from snout to origin of second dorsal fin; TL, total length; (b) AbVert, abdominal vertebrae; AP, anal fin pterygiophores inserting in front of haemal spine of first caudal vertebra; Arays, rays of anal fin; CaudVert, caudal vertebrae; D2rays, rays of second dorsal fin; DProCur, dorsal procurent rays; VProCur, ventral procurent rays; (c) 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, vertical distance from posterior end of sulcus to ventral margin of otolith; SuTipV, vertical distance from anterior end of sulcus to ventral margin of otolith.

vers. 3.22 (Hammer et al., 2001). Normal distribution was tested based on the Shapiro-Wilk test ($P > 0.05$ if normal distribution is satisfied). Homogeneity of variances was tested using Levene's test ($P > 0.05$ if homogeneity is satisfied) for the morphometric variables, and the Fligner-Killeen test ($P > 0.05$) for the meristic variables. Normality and homogeneity of the Fourier descriptors were examined with Mardia's test (skewness and kurtosis, $P > 0.05$). One-way analysis of variance (ANOVA, $P < 0.05$) was used to evaluate whether any morphometric variable was significant for a species. Some otolith morphometric variables revealed covariance with OL (Spearman, $P < 0.05$); in this case one-way analysis of covariance (ANCOVA, $P < 0.05$), with otolith length as covariable was applied. If homogeneity was fulfilled, Tukey's HSD post-hoc test ($P < 0.05$) was conducted; if homogeneity was not given we carried out Dunnett's T3 post-hoc test ($P < 0.05$). A Kruskal-Wallis test ($P < 0.05$) with Dunn's post-hoc test ($P < 0.05$) was performed to ascertain whether a given meristic value was significant for a species. Furthermore, the otolith and body morphometric datasets, and the meristic variables were each subjected to Principal Component Analysis (based on the individual values per specimen and species). The resulting first four principal components (PC1–4) were inspected for their significance at species level using ANOVA with Tukey's HSD post-hoc test ($P < 0.05$). The first two PCs of the Fourier descriptors were used for a multivariate analysis of variance (MANOVA) ($P < 0.05$).

Based on the reduced datasets for *Gobius* and *Pomatoschistus*, respectively, a LDA was executed based on PC1–4 of the different arrays of variables derived from the otolith and body morphometry (Box's M Test, $P > 0.05$). The maximal separation power of the Fourier descriptors without overfitting the model

yielded 37 PCs in case of *Gobius* and 14 PCs in case of *Pomatoschistus*. Accordingly, the LDA was conducted based on PC1–37 (*Gobius*), and PC1–14 (*Pomatoschistus*) of the Fourier descriptors (Box's M Test, $P > 0.05$). The classification success of each LDA is given based on jack-knifed cross-validation.

Evaluation of characters to discriminate between extant goby species

For our quantitative approach, we evaluated the taxonomic value of each variable by calculating its *separation success* for each species. In other words, we measured from how many of its congeners a given species could be separated based on a certain variable. Thus, 100% separation success means that the variable concerned could separate a species from all of its congeners (included in the respective dataset) and 0% indicates that it could not separate that species from any of its congeners. Moreover, to minimize the impact of potentially coincidental results, we applied the following constraint: *A given variable was considered to be taxonomically significant if it discriminated between at least two species, and could also differentiate each of those two species from at least 50% of the congeners (in the respective dataset)*. Variables that fulfilled this constraint are depicted against a green background in the relevant tables (see below). Variables that failed this test, but were indicative for one species with a success rate $\geq 50\%$ are indicated in italics.

Otolith descriptions

High-quality SEM images of otoliths obtained from extant goby species are rarely found in previous publications. Therefore, in addition to the morphometric approaches, SEM images of representative otoliths of each species included in our study are presented and brief descriptions for the otoliths of each species are provided. Otolith terminology follows

Nolf (1985) and Lombarte et al. (2018) (see here fig. 1d).

Abbreviations

For otolith morphometry: 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, vertical distance from posterior end of sulcus to ventral margin of otolith; SuTipV, vertical distance from anterior end of sulcus to ventral margin of otolith

For body morphometry: Ab, length of anal fin base; B, body depth at origin of first dorsal fin; CP, length of caudal peduncle; D2b, length of second dorsal fin base; D2C, distance between end of second dorsal fin and first dorsal (procurrent) ray of caudal fin; SL, standard length (from snout to begin of branched caudal fin rays); SN/A, distance from snout to origin of anal fin; SN/D1, distance from snout to origin of first dorsal fin; SN/D2, distance from snout to origin of second dorsal fin; TL, total length. All morphometric measurements are indicated in fig. 1a.

For meristics: AbVert, abdominal vertebrae; AP, anal fin pterygiophores inserting in front of haemal spine of first caudal vertebra; Arays, rays of anal fin; CaudVert, caudal vertebrae; D2rays, rays of second dorsal fin; DProCur, dorsal procurrent rays; TotVert, total vertebrae; VProCur, ventral procurrent rays. All meristic characters are depicted in fig. 1b.

Results

Species separation in *Gobius*

Otolith morphometry

Using the complete dataset (N , 14 species), ten otolith variables (out of 23) were found to be useful for species separation. These variables were significant for a total of eight species

(ANCOVA/ANOVA, table 2a1). The highest separation success was achieved with the variables SuL/SuH (77% for *G. geniporus*) and SuP/SuEndV (77% for *G. paganellus*) (table 2a1). The five most successful variables were OL/OH, SuL/OP, SuL/SuEndV, SuH/SuEndV and SuP/SuEndV, but each of these separated no more than three of the 14 included species (table 2a1). ANOVA based on PC1–4 calculated from the otolith variables successfully discriminated between five species (table 2b1). *Gobius vittatus* was separated from 69%, the others from 54% of the congeners. Notably, *G. cruentatus*, *G. gasteveni* and *G. roulei*, which had not been separated when the individual otolith variables were used, could now be distinguished from each other (compare table 2b1 vs. 2a1).

When the reduced dataset (N , 10 species) was used, 17 otolith variables (out of 23) were successful in species separation (ANCOVA/ANOVA, table 2a2). The same two variables as in the complete dataset (SuL/SuH, SuP/SuEndV) had maximum separation success and also discriminated the same species (*G. geniporus*, *G. paganellus*), each with increased success (89% vs. 77%). Six further variables were also taxonomically indicative, as each discriminated one or two species from all but two of the congeners (78% success). These variables were OL/OH (*G. incognitus*), OP/OL (*G. cobitis*), SuP/OP (*G. vittatus*), SuL/OP (*G. auratus*, *G. vittatus*), SuL/OL (*G. auratus*), SuL/OH (*G. incognitus*), and SuL/SuEndV (*G. auratus*) (table 2a2). With respect to the power of a given variable to discriminate between many species, the two most efficient variables were SuL/SuEndV and SuH/SuEndV; each of these could discriminate between six species (vs. three species when the complete dataset was used). In total, all species could be discriminated in the reduced dataset from $\geq 50\%$ of the congeners (table 2a2). Four species were separated by eight to ten

TABLE 2 Results of the statistical analyses based on the *Gobius* datasets and the indicated variables. The success of a certain variable in separating a species is indicated in % (100% means that the species is separated from all congeners by this variable). Light / dark green color denotes that at least two species were separated by the given variable, and each of those two species from $\geq 50\%$ / $\geq 80\%$ of the congeners (in the respective dataset)

Classification success based on otolith variables (AN(C)OVA, $P<0.05$)																									
a1 complete dataset															a2 reduced dataset										
Species	aur	buc	cob	cou	cru	fal	gas	gen	inc	kol	nig	pag	rou	vit	aur	buc	cob	cru	gen	inc	nig	pag	rou	vit	
N	10	10	10	11	4	11	2	1	10	6	2	7	9	10	10	10	10	11	11	10	6	7	9	10	10
OL/OH	31	31	38	54	23	15	8	54	62	46	38	46	38	46	33	33	44	33	67	78	44	56	33	56	
OP/OL	15	15	62	23	15	0	15	31	38	31	0	15	15	54	22	22	78	44	44	44	11	22	22	67	
OP/OH	31	31	31	62	38	15	0	38	23	23	23	46	31	38	33	44	33	44	56	33	33	56	56	56	
SuP/OP	38	15	15	8	8	0	15	23	62	0	8	46	23	62	44	22	22	11	22	67	11	22	33	78	
SuL/OP	62	15	15	8	8	0	23	38	62	0	15	23	23	62	78	22	33	11	56	67	22	22	33	78	
SuH/OP	23	23	0	0	15	0	0	15	23	0	0	23	0	0	33	33	0	33	33	33	0	33	0	0	
SuTipV/OP	46	15	23	54	38	23	8	31	23	8	23	38	31	54	56	33	22	44	33	22	33	44	44	67	
SuEndV/OP	38	38	15	15	23	23	15	46	23	15	46	54	38	23	56	56	33	33	44	33	44	67	67	33	
SuA/OA	38	15	15	8	23	0	15	15	54	0	0	0	0	31	44	22	22	22	22	56	0	0	0	33	
SuL/OL	54	8	23	31	0	0	0	23	23	0	0	15	8	38	78	11	22	11	22	22	0	22	11	56	
SuL/OH	38	8	0	38	0	0	0	0	69	0	0	0	0	38	44	33	33	44	67	78	67	44	67	44	
SuL/SuH	15	15	15	31	15	0	8	77	8	15	23	54	15	31	22	22	22	22	89	22	22	78	22	33	
SuL/SuP	0	15	8	15	0	0	0	23	0	0	0	31	0	15	0	22	22	0	33	0	0	33	0	22	
SuL/SuTipV	38	23	23	62	15	23	23	46	38	15	46	46	46	38	56	22	22	33	56	44	67	56	67	44	
SuL/SuEndV	54	23	46	23	23	15	23	54	46	15	23	54	31	38	78	33	56	22	56	56	22	56	44	56	
SuH/OL	15	15	23	0	23	0	0	23	0	0	8	23	0	0	22	22	33	33	33	0	11	33	0	0	
SuH/OH	31	15	8	0	23	0	0	0	23	0	15	0	0	15	44	33	22	33	0	33	33	0	0	33	
SuH/SuP	15	15	15	38	15	0	0	54	15	15	15	46	15	46	22	22	22	22	67	11	11	56	22	56	
SuH/SuTipV	38	15	15	8	46	0	8	15	23	0	15	8	8	31	56	22	22	56	22	33	22	11	22	44	
SuH/SuEndV	54	31	54	38	38	0	31	31	62	0	46	31	46	8	67	44	56	56	44	67	56	44	56	11	
SuP/SuTipV	54	31	23	62	23	23	23	38	38	0	38	38	46	38	56	33	22	33	44	44	67	44	67	44	
SuP/SuEndV	31	62	62	15	23	23	23	0	23	15	23	77	38	46	44	67	67	33	33	44	33	89	44	56	
SuTipV/SuEndV	8	8	8	8	8	15	0	0	8	0	54	0	0	8	11	11	11	11	11	11	100	11	11	11	

TABLE 2 Results of the statistical analyses based on the *Gobius* datasets and the indicated variables. The success of a certain variable in separating (Cont.)

Classification success based on PC1-4 of otolith variables (ANOVA, $P<0.05$)																									
b1 complete dataset															b2 reduced dataset										
Species	aur	buc	cob	cou	cru	fal	gas	gen	inc	kol	nig	pag	rou	vit	aur	buc	cob	cru	gen	inc	nig	pag	rou	vit	
PC1	31	46	38	8	38	8	54	23	46	0	31	38	46	69	89	33	33	44	67	44	56	33	67	89	
PC2	15	23	23	15	54	0	8	54	15	0	15	15	31	38	0	33	22	22	67	22	11	67	22	22	
PC3	23	23	8	23	54	0	23	54	38	0	8	23	54	23	33	56	22	33	33	22	67	89	56	33	
PC4	8	23	54	0	23	0	8	8	23	0	8	23	0	23	44	33	67	22	33	22	33	56	11	11	
Classification success based on Fourier otolith descriptors (MANOVA, $P<0.05$)																									
c1 complete dataset															c2 reduced dataset										
Species	aur	buc	cob	cou	cru	fal	gas	gen	inc	kol	nig	pag	rou	vit	aur	buc	cob	cru	gen	inc	nig	pag	rou	vit	
N	17	20	20	8	21	4	2	14	15	4	20	19	20	21	17	20	20	8	21	14	15	20	19	20	21
Fourier descr.	85	92	100	85	100	77	85	100	77	85	85	100	92	85	90	100	80	80	80	100	90	100	90	80	80
Classification success based on fish morphometric variables (ANOVA, $P<0.05$)																									
d1 complete dataset															d2 reduced dataset										
Species	aur	buc	cob	cou	cru	fal	gas	gen	inc	kol	nig	pag	rou	vit	aur	buc	cob	cru	gen	inc	nig	pag	rou	vit	
N	11	10	10	4	11	2	1	10	9	2	7	10	10	11	11	10	10	11	10	9	7	10	10	11	
SN/D1*	38	54	31	38	38	46	77	23	54	0	8	23	0	38	22	67	22	22	22	67	0	22	0	22	
SN/D2*	15	46	31	0	31	38	54	31	0	54	23	31	0	31	22	78	22	22	22	78	22	22	0	22	
SN/A	8	31	31	0	31	0	46	23	0	31	0	23	0	23	11	44	33	44	22	44	0	22	0	22	
D2C	8	15	23	0	23	0	0	15	23	0	8	8	54	8	11	22	33	33	22	33	11	11	78	11	
CP	8	8	8	8	8	8	8	8	8	8	8	8	8	100	11	11	11	11	11	11	11	11	11	100	
B	8	0	8	8	15	0	0	46	0	0	8	15	0	15	11	11	11	22	56	0	11	22	0	33	
D2b*	23	69	46	38	38	0	62	38	23	46	54	38	0	62	22	78	56	44	44	56	56	44	0	78	
Ab*	38	62	62	31	38	77	31	0	46	31	31	8	54	56	44	67	67	44	33	67	33	33	0	56	

TABLE 2 Results of the statistical analyses based on the *Gobius* datasets and the indicated variables. The success of a certain variable in separating a species (Cont.)

Classification success based on PC1-4 of fish morphometric variables (ANOVA, $P<0.05$)															
e1 complete dataset															
e2 reduced dataset															
Species	aur	buc	cob	cru	cou	cru	fal	gas	gen	inc	kol	nig	pag	rou	vit
PC1	31	54	46	0	31	0	31	0	23	54	0	31	15	23	46
PC2	31	46	31	8	23	8	31	15	54	0	31	31	31	46	62
PC3	23	23	23	15	54	0	0	62	15	0	15	15	15	23	38
PC4	23	23	8	23	46	0	0	46	38	0	8	23	23	46	23

Classification success based on meristic variables (Kruskal-Wallis, $P<0.05$)															
f1 complete dataset															
f2 reduced dataset															
Species	aur	buc	cob	cru	cou	cru	fal	gas	gen	inc	kol	nig	pag	rou	vit
N	11	10	10	4	11	2	2	1	10	9	2	7	10	10	11
TotVert	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CaudVert	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AbVert	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dzrays	8	15	15	8	8	0	0	15	54	0	0	8	8	0	8
Arays	8	8	0	0	0	0	0	15	38	8	0	8	8	0	8
AP	8	8	69	15	8	0	0	15	8	0	8	8	8	8	8
DProCur	0	8	8	0	0	0	0	23	38	0	8	8	8	8	8
VProCur	8	8	8	0	0	0	0	31	38	8	8	8	8	0	8

Classification success based on PC1-4 of meristic variables (ANOVA, $P<0.05$)															
g1 complete dataset															
g2 reduced dataset															
Species	aur	buc	cob	cru	cou	cru	fal	gas	gen	inc	kol	nig	pag	rou	vit
PC1	31	23	46	15	23	23	77	15	62	92	23	15	23	15	31
PC2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PC3	0	0	8	0	0	0	15	0	0	0	0	0	8	0	0
PC4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Abbreviations: N, numbers of otoliths or fish specimens; * refers to Tamhane post-hoc test. See methods for abbreviations of variables and details of analyses. AN(C)OVA means that ANOVA or, if covariance was detected, ANCOVA was used.

variables (*G. auratus*, *G. geniporus*, *G. paganellus*, *G. vittatus*), four more species were discriminated by four to six variables (*G. cobitis*, *G. incognitus*, *G. niger*, *G. roulei*), and two species (*G. buccichichi*, *G. cruentatus*) by two variables (table 2a2). Furthermore, the results of ANOVA using PC1–4 calculated from all otolith variables enabled the discrimination of eight species (table 2b2), vs. five when the complete dataset was analysed in the same way (table 2b1). Four of these (*G. auratus*, *G. buccichichi*, *G. niger*, *G. paganellus*) had not been separated when the complete dataset was tested. On the other hand, *G. cruentatus* was not separated when PC1–4 were used, although it had been discriminated when the entire dataset was used. Overall, the approach based on PC1–4 was less effective than the analysis of individual otolith variables because it was unable to distinguish between *G. cobitis*, *G. cruentatus* and *G. incognitus* (compare table 2b2 and 2a2).

Fourier shape analysis of the otoliths

Based on PC1 and PC2 of the Fourier descriptors, species separation success was 77–100% and 80–100%, respectively, in the complete and reduced datasets (MANOVA; table 2c1, c2). *Gobius paganellus* was the only species that could be discriminated from all others with 100% separation success in both datasets. *Gobius cobitis*, *G. cruentatus* and *G. geniporus* were differentiated with 100% success when the complete dataset was used (80% in the reduced dataset), whereas *G. buccichichi* and *G. incognitus* were distinguished with 100% success when the reduced data was analysed (92% and 77% in the complete dataset) (table 2c1, c2).

Body morphometry

When the complete dataset was analysed, four (out of eight) morphometric variables

were taxonomically indicative, i.e., SN/D1, SN/D2, D2b, Ab (ANOVA; table 2d1). The highest success rates (77%) were achieved with SN/D1 (for *G. gasteveni*) and Ab (for *G. fallax*). The most powerful single variable (with respect to the number of species that can be separated) was Ab, which resolved five species, followed by D2b (four species) (table 2d1). In total, nine species could be separated based on one, two or three morphometric variables, while five species could not be separated (*G. auratus*, *G. couchi*, *G. cruentatus*, *G. geniporus*, *G. paganellus* (table 2d1). Using PC1–4 of the variables derived from body morphometry in the ANOVA analysis was less efficient than when the individual variables were used (table 2e1). Only five species were separated in this case and separation success for a given species varied from 54 to 62% (table 2e1). *Gobius cruentatus* and *G. geniporus*, which could not be distinguished in the preceding analysis, were now separated. The other three species that could be discriminated were *G. buccichichi*, *G. incognitus* and *G. vittatus*.

The results based on the reduced dataset (table 2d2, e2) were very similar to those obtained from the complete dataset. The same four morphometric variables could separate a species (SN/D1, SN/D2, D2b, Ab), and the individual species were separated with the same success (56–78%; see table 2d2). As before, most powerful in relation to the number of species that could be separated were Ab (four species) and D2b (five species). With the single exception of *G. roulei* (not separated), the same species as in the complete data were separated (five in total), each based on one to four variables (table 2d2). When PC1–4 of the morphometric variables were used in the ANOVA, a total of seven species could be separated (table 2e2). Three of those (*G. roulei*, *G. cruentatus*, *G. geniporus*) had not been separated based on

the approach using single morphometric variables (table 2d2). In contrast, *G. niger* could not be discriminated based on PC1–4 of the morphometric variables (table 2e2), whereas it was distinguished on the basis of D2b alone (table 2d2).

Meristic counts

None of the individual meristic characters met our criteria for species separation when we used the complete dataset (Kruskal-Wallis; table 2fi). On the other hand, ANOVA on the basis of PC1–4 of the meristic values distinguished *G. incognitus* (with 92% success), *G. fallax* (77%) and *G. geniporus* (62%; see table 2g1). A similar outcome was obtained based on the reduced dataset. A single character (ventral procurrent rays) separated with 56% success *G. geniporus* and *G. incognitus* from the others (table 2f2). ANOVA based on PC1–4 of the meristic variables also separated *G. geniporus* and *G. incognitus* from all others, now with 89% and 100% success, respectively; in addition, *G. cobitis* was now separated (table 2g2).

Discriminant analyses

Each LDA was based on the reduced dataset and the variables used were (i) PC1–4 of the otolith morphometric variables, (ii) PC1–23 of the otolith Fourier descriptors, and (iii) PC1–4 of the body morphometric variables (fig. 2, table 3).

The first two functions of the LDA based on PC1–4 of the otolith morphometric variables captured 50.5% and 33.5% of the variation, respectively (fig. 2a). Overall classification success (jack-knifed) was 45.4%; the highest classification success was 60% for both *G. buccichi* and *G. geniporus*, and the lowest was 18% for *G. cruentatus*, 30% for *G. roulei* and 33% for *G. incognitus* (table 3a). The scatter plot revealed overlap of all species

(fig. 2a). Three “pairs” of species are tentatively recognizable: *G. vittatus* and *G. auratus*, *G. geniporus* and *G. paganellus*, as well as *G. cruentatus* and *G. cobitis* (fig. 2a). The members of each pair show some overlap, but only relatively moderate overlap with the other species considered.

The first two functions of the LDA based on PC1–23 of the otolith Fourier descriptors captured 39.1% and 19.1% of the variation, respectively (fig. 2b). Overall classification success (jack-knifed) of this LDA was 85% (table 3b). 100% separation success was achieved for *G. cruentatus* and *G. geniporus* (vs. 18% and 60% in the previous LDA), and 95% was obtained for *G. cobitis* (vs. 50% in the previous LDA) (Table 3b). The scatter plot delineates three groups: *Gobius cobitis*, which had broadly overlapped with *G. cruentatus* in the preceding LDA, is now distinct (fig. 2b). The second group includes *G. geniporus*, *G. niger* and *G. roulei*, and the third one comprises the remaining species (fig. 2b).

The first two functions of the LDA based on the body morphometric variables accounted for 66.3% and 18.6% of the variation, respectively. Overall classification success (jack-knifed) was 54.6% (table 3c). Classification success was highest for *Gobius vittatus* and *G. buccichi*, with 81.8% and 70%, respectively, while the success rates were lowest for *G. auratus*, *G. cobitis* and *G. roulei* (27–30%) (table 3c). The scatter plot revealed overlap of almost all species, except *G. vittatus*, which is comparatively distinct, and *G. buccichi* and *G. incognitus*, which overlap with each other, but not or not very much with the remainder (fig. 2c).

Species separation in Pomatoschistus

Otolith morphometry

Five otolith variables (out of 23) were successful in species separation when the complete dataset was used (*N*, 7 species; ANCOVA/

FIGURE 2 Plots of the discriminant function scores derived from the different arrays of variables (as indicated in the figure) of the studied species of *Gobius* (a–c) and *Pomatoschistus* (d–f). The reduced datasets for *Gobius* and *Pomatoschistus* were used for this analysis. LD1, 2, linear discriminant functions 1 and 2. See tables 3 and 5 for details and values.

TABLE 3 Results of the Linear Discriminant Analysis (LDA) using the reduced dataset of *Gobius* and the indicated variables. The numbers in rows are percentages that denote the classification into the species given in columns (correctly classified species are highlighted in grey)

a Classification success (jack-knifed) based on LDA using PC1-4 of otolith variables												
N	Species	<i>G. auratus</i>	<i>G. bucchichi</i>	<i>G. cobitis</i>	<i>G. cruentatus</i>	<i>G. geniporus</i>	<i>G. incognitus</i>	<i>G. niger</i>	<i>G. paganellus</i>	<i>G. roulei</i>	<i>G. vittatus</i>	
10	<i>G. auratus</i>	54.5	18.2	0.0	0.0	0.0	0.0	9.1	0.0	0.0	18.2	
10	<i>G. bucchichi</i>	0.0	60.0	0.0	20.0	0.0	20.0	0.0	0.0	0.0	0.0	
11	<i>G. cobitis</i>	0.0	10.0	50.0	20.0	0.0	0.0	20.0	0.0	0.0	0.0	
11	<i>G. cruentatus</i>	0.0	9.1	27.3	18.2	0.0	9.1	27.3	9.1	0.0	0.0	
10	<i>G. geniporus</i>	0.0	0.0	0.0	0.0	60.0	10.0	20.0	10.0	0.0	0.0	
6	<i>G. incognitus</i>	0.0	0.0	0.0	11.1	11.1	33.3	44.4	0.0	0.0	0.0	
7	<i>G. niger</i>	14.3	0.0	14.3	0.0	0.0	0.0	42.9	14.3	14.3	0.0	
9	<i>G. paganellus</i>	0.0	0.0	0.0	11.1	22.2	0.0	11.1	55.6	0.0	0.0	
10	<i>G. roulei</i>	0.0	20.0	0.0	0.0	0.0	10.0	20.0	0.0	30.0	20.0	
10	<i>G. vittatus</i>	30.0	10.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0	50.0	

Overall classification success (jack-knifed): 45.4%

b Classification success (jack-knifed) based on LDA using PC1-23 of Fourier otolith descriptors												
N	Species	<i>G. auratus</i>	<i>G. bucchichi</i>	<i>G. cobitis</i>	<i>G. couchi</i>	<i>G. cruentatus</i>	<i>G. geniporus</i>	<i>G. incognitus</i>	<i>G. niger</i>	<i>G. paganellus</i>	<i>G. roulei</i>	<i>G. vittatus</i>
17	<i>G. auratus</i>	88.2	5.9	0.0	0.0	5.9	0.0	0.0	0.0	0.0	0.0	0.0
20	<i>G. bucchichi</i>	0.0	75.0	0.0	0.0	0.0	0.0	25.0	0.0	0.0	0.0	0.0
20	<i>G. cobitis</i>	0.0	0.0	95.0	0.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0
8	<i>G. couchi</i>	0.0	0.0	0.0	75.0	0.0	0.0	0.0	0.0	0.0	0.0	25.0
21	<i>G. cruentatus</i>	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0
14	<i>G. geniporus</i>	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0
15	<i>G. incognitus</i>	0.0	0.0	0.0	0.0	0.0	0.0	86.7	0.0	13.3	0.0	0.0
20	<i>G. niger</i>	5.0	0.0	0.0	0.0	0.0	0.0	85.0	0.0	0.0	10.0	0.0
19	<i>G. paganellus</i>	0.0	5.3	0.0	0.0	0.0	0.0	0.0	89.5	0.0	0.0	0.0
20	<i>G. roulei</i>	0.0	4.5	0.0	13.6	9.1	9.1	0.0	0.0	72.7	0.0	0.0
21	<i>G. vittatus</i>	4.8	4.8	0.0	19.0	0.0	0.0	0.0	0.0	0.0	0.0	71.4

Overall classification success (jack-knifed): 85.0%

TABLE 3 Results of the Linear Discriminant Analysis (LDA) using the reduced dataset of *Gobius* and the indicated variables. The numbers in rows are percentages (cont.)

c Classification success (jack-knifed) based on LDA using PC1-4 of fish morphometric variables												
N	Species	<i>G. auratus</i>	<i>G. bucchichi</i>	<i>G. cobitis</i>	<i>G. cruentatus</i>	<i>G. geniporus</i>	<i>G. incognitus</i>	<i>G. niger</i>	<i>G. paganellus</i>	<i>G. roulei</i>	<i>G. vittatus</i>	
11	<i>G. auratus</i>	27.3	0.0	18.2	27.3	0.0	9.1	18.2	0.0	0.0	0.0	0.0
10	<i>G. bucchichi</i>	0.0	70.0	0.0	0.0	10.0	20.0	0.0	0.0	0.0	0.0	0.0
10	<i>G. cobitis</i>	10.0	0.0	30.0	10.0	0.0	0.0	40.0	0.0	10.0	0.0	0.0
11	<i>G. cruentatus</i>	18.2	0.0	0.0	63.6	18.2	0.0	0.0	0.0	0.0	0.0	0.0
10	<i>G. geniporus</i>	10.0	10.0	0.0	30.0	50.0	0.0	0.0	0.0	0.0	0.0	0.0
9	<i>G. incognitus</i>	0.0	33.3	0.0	0.0	11.1	55.6	0.0	0.0	0.0	0.0	0.0
7	<i>G. niger</i>	28.6	0.0	28.6	0.0	0.0	0.0	42.9	0.0	0.0	0.0	0.0
10	<i>G. paganellus</i>	20.0	0.0	0.0	10.0	0.0	10.0	0.0	60.0	0.0	0.0	0.0
10	<i>G. roulei</i>	10.0	20.0	10.0	10.0	0.0	10.0	10.0	0.0	30.0	0.0	0.0
11	<i>G. vittatus</i>	18.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	81.8	0.0

Overall classification success (jack-knifed): 51.5%
Abbreviations: N, numbers of otoliths (a, b) and fish specimens (c) included in the LDA.

ANOVA; table 4a1). The maximum classification success for a given species was 67%. OL/OH and OP/OH were the best variables with respect to the number of species that could be discriminated; OL/OH separated all species except *P. knerii*, and OP/OH discriminated four species (table 4a1). In total, all species could be separated from one another; *P. marmoratus*, *P. microps* and *P. montenegrensis* each by four variables, and the remainder by a single variable each (table 4a1). When PC1–4 calculated from the otolith morphometric variables was analysed, the success in species separation declined in the case of *P. microps* (33% vs. 67% in the preceding analysis) and *P. pictus* (0 vs. 50%), but was improved in the case of *P. montenegrensis* (83% vs. 67%; table 4b1).

When the reduced dataset (N, 5 species) was analysed in the same way, most of the otolith morphometric variables contributed to species separation (table 4a2). Four variables achieved 100% separation success for a given species: SuEndV/OP for *P. marmoratus* and *P. microps*, and SuH/OL, SuH/SuTipV and SuP/SuTipV each for *P. montenegrensis* (table 4a2). Overall, all species could be separated from ≥50% of their congeners, and 13–17 variables contributed in each case (table 4a2). As observed in the complete dataset, the results of ANOVA on the basis of PC1–4 of the otolith morphometric variables discriminated the species less efficiently than when the individual otolith variables were used (compare table 4b2 vs. 4a2).

Fourier shape analysis of the otoliths

When the complete dataset was used, PC1 and PC2 of the Fourier descriptors attained success rates in species separation ranging from 67% (*P. microps*, *P. pictus*) to 100% (*P. knerii*, *P. montenegrensis*, *P. quagga*) (MANOVA; table 4c1). The values increased to 83–100% per species when the reduced dataset was used for the same analysis (table 4c2).

TABLE 4 Results of the statistical analyses based on the *Pomatoschistus* datasets and the indicated variables. The success of a certain variable in separating a species is indicated in % (100% means that the species is separated from all congeners by this variable). Light / dark green color denotes that at least two species were separated by the given variable, and each of those two species from $\geq 50\%$ / $\geq 80\%$ of the congeners (in the respective dataset)

Classification success based on otolith variables (AN(C)OVA, $P < 0.05$)

a1 complete dataset								a2 reduced dataset				
Species	kne	mar	mic	min	mon	pic	qua	kne	mar	mic	mon	qua
<i>N</i>	10	9	15	2	10	2	9	10	9	15	10	9
OL/OH	33	67	67	50	67	50	50	50	75	75	0	50
OP/OL	33	67	33	17	50	0	33	50	75	50	75	0
OP/OH	50	67	67	33	50	33	33	50	75	75	50	50
SuP/OP	17	33	33	0	50	0	33	25	50	50	75	50
SuL/OP	0	33	33	0	33	0	33	0	50	50	50	50
SuH/OP	17	17	17	0	67	0	17	25	25	25	100	25
SuTipV/OP	17	17	17	0	67	0	17	25	25	25	100	25
SuEndV/OP	33	33	83	17	17	0	17	50	100	100	50	50
SuA/OA	17	17	17	0	50	0	0	25	25	25	75	0
SuL/OL	0	0	0	0	0	0	0	0	50	50	50	50
SuL/OH	0	33	33	0	33	0	33	50	75	75	50	50
SuL/SuH	33	17	33	0	33	0	50	75	50	50	50	75
SuL/SuP	17	0	17	0	50	0	17	25	0	25	75	25
SuL/SuTipV	17	33	17	0	50	0	17	50	75	50	75	50
SuL/SuEndV	33	50	67	17	33	0	33	50	75	75	50	50
SuH/OL	17	17	17	0	67	0	17	50	25	50	100	25
SuH/OH	17	17	17	0	83	17	17	25	25	25	100	25
SuH/SuP	33	17	33	0	33	0	50	75	50	50	50	75
SuH/SuTipV	17	17	17	0	67	0	17	25	50	50	100	25
SuH/SuEndV	17	17	17	0	67	0	17	25	25	25	100	25
SuP/SuTipV	17	33	17	0	50	0	17	50	75	50	100	75
SuP/SuEndV	17	33	67	17	33	0	33	50	75	75	50	50
SuTipV/SuEndV	33	33	50	0	50	0	33	50	50	75	75	50

Classification success based on PC1-4 of otolith variables (ANOVA, $P < 0.05$)

b1 complete dataset								b2 reduced dataset				
Species	kne	mar	mic	min	mon	pic	qua	kne	mar	mic	mon	qua
PC1	50	50	33	50	83	0	67	33	50	33	67	50
PC2	67	33	33	33	33	0	67	50	33	33	33	50
PC3	17	0	17	50	17	0	0	0	0	0	0	0
PC4	17	17	33	0	0	0	0	0	17	17	0	0

Classification success based on Fourier otolith descriptors (MANOVA, $P < 0.05$)

c1 complete dataset								c2 reduced dataset				
Species	kne	mar	mic	min	mon	pic	qua	kne	mar	mic	mon	qua
<i>N</i>	20	14	30	4	19	4	20	20	14	30	19	20
MANOVA	100	83	67	83	100	67	100	100	83	100	100	83

TABLE 4 Results of the statistical analyses based on the *Pomatoschistus* datasets (Cont.)

Classification success based on fish morphometric variables (ANOVA, $P<0.05$)												
d1 complete dataset								d2 reduced dataset				
Species, N	kne	mar	mic	min	mon	pic	qua	kne	mar	mic	mon	qua
Variables	10	9	15	2	10	2	10	10	9	15	10	10
SN/D1*	50	17	33	0	50	83	67	50	0	50	25	75
SN/D2	17	33	33	83	33	0	67	0	25	25	25	75
SN/A	50	33	67	67	50	50	50	50	25	50	50	75
D2C	50	67	67	50	50	0	50	50	100	100	50	50
CP	33	33	50	100	33	17	67	25	25	50	25	75
B*	50	17	17	0	33	33	83	50	25	25	50	100
D2b	50	50	33	83	83	83	50	50	25	25	75	25
Ab	67	50	67	83	100	83	50	50	25	50	100	25

Classification success based on PC1-4 of fish morph. variables (ANOVA, $P<0.05$)

e1 complete dataset								e2 reduced dataset				
Species	kne	mar	mic	min	mon	pic	qua	kne	mar	mic	mon	qua
PC1	67	50	83	83	100	67	50	75	25	50	50	100
PC2	50	33	50	50	67	17	100	50	50	25	100	75
PC3	50	0	33	33	0	50	33	25	25	100	25	25
PC4	33	33	33	67	67	0	33	0	0	0	0	0

Classification success based on meristic variables (Kruskal-Wallis, $P<0.05$)

f1 complete dataset								f2 reduced dataset				
Species	kne	mar	mic	min	mon	pic	qua	kne	mar	mic	mon	qua
N	10	9	15	2	10	2	10	10	9	15	10	10
TotVert	17	17	17	17	67	67	50	50	25	75	75	75
CaudVert	17	17	17	17	67	0	33	50	25	25	100	50
AbVert	33	17	67	0	33	17	67	50	50	100	50	100
D2rays	17	17	0	33	33	0	0	50	25	0	50	25
Arays	17	0	0	17	50	0	17	50	0	25	50	25
AP	17	0	33	0	17	0	33	50	0	50	50	50
DProCur	0	17	17	0	33	0	33	0	25	25	50	50
VProCur	0	0	0	17	33	0	17	0	0	0	25	25

Classification success based on PC1-4 of meristic variables (ANOVA, $P<0.05$)

g1 complete dataset								g2 reduced dataset				
Species	kne	mar	mic	min	mon	pic	qua	kne	mar	mic	mon	qua
PC1	67	50	67	83	83	33	83	75	75	100	100	100
PC2	33	17	17	0	0	0	0	75	25	25	0	25
PC3	0	17	0	50	17	0	17	0	0	0	0	0
PC4	0	0	17	0	0	0	17	25	25	75	50	25

Abbreviations: N , numbers of otoliths or fish specimens; * refers to Tamhane post-hoc test. See methods for abbreviations of variables and details of analyses. AN(C)OVA means that ANOVA or, if covariance was detected, ANCOVA was used.

Body morphometry

Each of the eight morphometric variables contributed to species separation and each species was separated from all others when the complete dataset was used (ANOVA; table 4d1). 100% separation success was achieved by CP (for *P. minutus*) and Ab (for *P. montenegrensis*). The most efficient variables in relation to the number of species discriminated were Ab (all species), and D2b, D2C and SN/A (six species). *Pomatoschistus quagga* was the only species that could be separated by each morphometric variable from 50–83% of its congeners; the remaining species were separated by three to six variables from their congeners (table 4d1). Furthermore, each species was separated when PC1–4 of the morphometric variables were analysed (ANOVA; table 4e1). 100% separation success was obtained for *P. montenegrensis* and *P. quagga* (table 4e1).

Overall, similar results were obtained with the reduced dataset, but some differences are noteworthy (table 4d2). With the exception of SN/D2, every morphometric variable could be used to separate some species. The reason that SN/D2 was now disqualified lies in the exclusion of *P. minutus* (which had been separated by this variable) from the reduced dataset. Three variables (vs. one in the complete dataset) showed 100% separation success for at least one species: D2C (for *P. marmoratus*, *P. microps*), B (for *P. quagga*) and Ab (for *P. montenegrensis*). Most effective concerning the number of species that could be separated were D2C (all species) and SN/A (four species) (table 4d2). Apart from *P. marmoratus* (separated by a single variable), each of the included species could be discriminated by five or six variables from their congeners (table 4d2). When ANOVA was done based on PC1–4 of the morphometric body variables, the separation success was generally similar to that of the previous analysis and all species could be separated (table 4e2).

Meristic characters

Numbers of total and abdominal vertebrae contributed to species separation with moderate success (50–67%) when the complete dataset was employed (table 4fi). *Pomatoschistus microps*, *P. montenegrensis* and *P. pictus* could be separated by either the total or the abdominal vertebrae count, while *P. quagga* was discriminated by both of these counts (table 4fi). ANOVA using PC1–4 of the meristic counts showed improved results, as PC1 separated all species apart from *P. pictus*, with highest separation success (83%) for *P. minutus*, *P. montenegrensis*, and *P. quagga* (table 4gi).

In the reduced dataset, seven meristic characters contributed to species separation (vs. two in the complete dataset), with an improved overall separation success (50–100% vs. 50–67%) (table 4f2). The caudal and the abdominal vertebrae count separated *P. montenegrensis* and *P. microps*/*P. quagga*, respectively, from all other species. The latter was most efficient in relation to the number of species that could be separated (all five species) (table 4f2). In total, all five species could be separated: *P. knerii*, *P. montenegrensis* and *P. quagga* by six, seven and five variables, respectively, and *P. marmoratus* and *P. microps* by one and three variables, respectively (table 4f2). ANOVA using PC1–4 of the meristic values separated each species from 75–100% of its congeners, with 100% success for *P. microps*, *P. montenegrensis* and *P. quagga* (table 4g2).

Discriminant analyses

As for *Gobius*, each LDA was based on the reduced dataset. The variables used as input were (i) PC1–4 of the otolith morphometric variables, (ii) PC1–14 of the Fourier descriptors, and (iii) PC1–4 of the body morphometric variables (table 5).

The first two functions of the LDA based on PC1–4 of the otolith morphometric variables

explained 62.0% and 33.2% of the variation, respectively (fig. 2d). Overall classification success (jack-knifed) of the LDA was 71.1%. The highest classification success was achieved for *P. montenegrensis* (90%) and *P. microps* (87%), while the success rates were between 56 and 60% for *P. knerii*, *P. marmoratus*, and *P. quagga* (table 5a). The scatter plot depicts three groups (fig. 2d). The first consists of *P. montenegrensis* and is completely distinct. The second and third groups include *P. microps*/*P. marmoratus* and *P. knerii*/*P. quagga*; the members of each group overlap with each other, but very little or not at all with the others (fig. 2d).

The first two functions of the LDA based on PC1–14 of the Fourier descriptors captured 57.6% and 21.6% of the variance, respectively. Overall classification success (jack-knifed) was 96%, with 100% success for *P. microps* and *P. quagga*, >90% success for the remainder (table 5b). The corresponding scatter plot depicts *P. knerii*, *P. marmoratus*, and *P. montenegrensis* as separate groups, while *P. microps* and *P. quagga* overlap (fig. 2e).

The first two functions of the LDA based on the body morphometric variables account for 71.7% and 23% of the variation, respectively. Overall classification success (jackknifed) was 75.9%, with 90% success for *P. montenegrensis* and *P. quagga*, and 67–70% success for the remainder (table 5c). The success in separation is also seen in the scatter plot, which reveals that each species is relatively well separated, although some overlap is seen (fig. 2f).

Summary of the results based on the statistical analyses

A comparison of the results based on the four methodological approaches (otolith morphometry, otolith Fourier shape analysis, body morphometry, meristic counts) reveals that Fourier shape analysis of otoliths is the most powerful species discriminator of all the methods tested here (table 6). A plot of

the relative discriminatory power of the individual variables used in the other three approaches indicates that rates of success in species separation vary, and implies that some characters have more taxonomic significance than others (table 7). For example, none of the individual otolith variables had the power to separate all of the species of *Gobius* or *Pomatoschistus* with good success (i.e., with $\geq 75\%$; table 7). While some variables (SuTipV/OP, SuH/SuEndV) were capable (albeit with moderate success) of separating some of the species of *Gobius* from the others, these same variables failed to discriminate between species of *Pomatoschistus* spp., and *vice versa* (table 7). Furthermore, a single variable could be indicative for a certain species, but be unable to reliably discriminate its congeners. An example is SuL/SuH, which achieved good separation success only for *G. geniporus* (89%) and *G. paganellus* (78%) (reduced dataset, table 2a2).

A further outcome was that the number of species that could be separated from their congeners with a success of $\geq 50\%$ declined when PC1–4 of the otolith or the morphometric variables were analysed instead of the individual variables (table 6, complete datasets).

Moreover, the *number of specimens* employed in the statistical analyses did not necessarily influence their separation success. Examples are *G. fallax* (represented with only two specimens) and *G. gasteveni* (one specimen), which were well separated from their congeners using body morphometry, whereas *G. paganellus* and *G. roulei* (ten specimens each) could not be separated based on the same approach (table 2d1). On the other hand, as already pointed out by Lombarte et al. (2018), the *number of species* used in the statistical analysis could have an influence on the results, insofar as species separation becomes more successful when the number of species considered decreases, and less successful when the number of species increases. This

TABLE 5 Results of the Linear Discriminant Analysis (LDA) using the reduced dataset of *Pomatoschistus* and the indicated variables. The numbers in rows are percentages that denote the classification into the species given in columns (correctly classified species are highlighted in grey)

a Classification success (jack-knifed) based on LDA using PC1-4 of otolith variables						
<i>N</i>	Species	<i>P. knerii</i>	<i>P. marmoratus</i>	<i>P. microps</i>	<i>P. montenegrensis</i>	<i>P. quagga</i>
10	<i>P. knerii</i>	60.0	10.0	0.0	0.0	30.0
9	<i>P. marmoratus</i>	11.0	56.0	33.0	0.0	0.0
15	<i>P. microps</i>	0.0	13.0	87.0	0.0	0.0
10	<i>P. montenegrensis</i>	10.0	0.0	0.0	90.0	0.0
9	<i>P. quagga</i>	33.0	0.0	0.0	11.0	56.0

Overall classification success (jack-knifed): 71.1%

b Classification success (jack-knifed) based on LDA using PC1-4 of Fourier otolith descriptors						
<i>N</i>	Species	<i>P. knerii</i>	<i>P. marmoratus</i>	<i>P. microps</i>	<i>P. montenegrensis</i>	<i>P. quagga</i>
20	<i>P. knerii</i>	90.0	0.0	0.0	0.0	10.0
14	<i>P. marmoratus</i>	0.0	92.9	7.1	0.0	0.0
30	<i>P. microps</i>	0.0	0.0	100.0	0.0	0.0
19	<i>P. montenegrensis</i>	5.3	0.0	0.0	94.7	0.0
20	<i>P. quagga</i>	0.0	0.0	0.0	0.0	100.0

Overall classification success (jack-knifed): 96.0%

c Classification success (jack-knifed) based on LDA using PC1-4 of fish morph. variables						
<i>N</i>	Species	<i>P. knerii</i>	<i>P. marmoratus</i>	<i>P. microps</i>	<i>P. montenegrensis</i>	<i>P. quagga</i>
10	<i>P. knerii</i>	70.0	20.0	0.0	0.0	10.0
9	<i>P. marmoratus</i>	11.1	66.7	0.0	11.1	11.1
15	<i>P. microps</i>	0.0	33.3	66.7	0.0	0.0
10	<i>P. montenegrensis</i>	0.0	10.0	0.0	90.0	0.0
10	<i>P. quagga</i>	10.0	0.0	0.0	0.0	90.0

Overall classification success (jack-knifed): 75.9%

Abbreviations: *N*, numbers of otoliths (a, b) and fish specimens (c) included in the LDA.

TABLE 6 Summary of the taxonomic significance of the different groups of variables (indicated by colors). Numbers and percentages in rows indicate how many species were separated from $\geq 50\%$ of their congeners in the dataset given in the columns

Statistical analyses	Gobius datasets		Pomatoschistus datasets	
	complete (<i>N</i> , 14)	reduced (<i>N</i> , 10)	complete (<i>N</i> , 7)	reduced (<i>N</i> , 5)
Otolith variables (AN(C)OVA)	8 (57%)	All	All	All
PC1-4 of otolith variables (ANOVA)	5 (36%)	7 (70%)	5 (71%)	4 (80%)
PC1-4 of otolith variables (LDA)	–	6 (60%)	–	All
Fourier otolith shape analysis (MANOVA)	All	All	All	All
Fourier otolith shape analysis (LDA)	–	All	–	All
Fish morph. variables (ANOVA)	9 (64%)	5 (50%)	All	All
PC1-4 of fish morph. variables (ANOVA)	5 (36%)	7 (70%)	All	All
PC1-4 of fish morph. variables (LDA)	–	6 (60%)	–	All
Meristic variables (Kruskal-Wallis)	0	2 (20%)	4 (57%)	All
PC1-4 of meristic variables (ANOVA)	3 (21%)	3 (30%)	6 (86%)	All

Abbreviations: All, all species were separated; *N*, numbers of species; –, not applicable. See tables 2–5 for details.

AN(C)OVA means that ANOVA or, if covariance was detected, ANCOVA was used; LDA, Linear Discriminant

Analysis.

TABLE 7 Summary of the taxonomic significance of the individual variables (ANOVA or, if covariance was detected, ANCOVA with post-hoc tests, $P < 0.5$; see tables 2 and 4 for details)

Type of variables	Variables	<i>Gobius</i> spp.		<i>Pomatoschistus</i> spp.	
		complete dataset	reduced dataset	complete dataset	reduced dataset
Otolith morphometry	OL/OH	△	△	▲	▲
	OP/OL	△	△	△	▲
	OP/OH		△	▲	▲
	SuP/OP	△	△		▲
	SuL/OP	△	△		▲
	SuH/OP				
	SuTipV/OP	△	△		
	SuEndV/OP		△		▲
	SuA/OA				
	SuL/OL		△		▲
	SuL/OH		△		▲
	SuL/SuH	△	△		▲
	SuL/SuP				
	SuL/SuTipV		△		▲
	SuL/SuEndV	△	△	△	▲
	SuH/OL				▲
	SuH/OH				
	SuH/SuP		△		▲
	SuH/SuTipV		△		▲
	SuH/SuEndV	△	△		▲
	SuP/SuTipV	△	△		▲
	SuP/SuEndV	△	△		▲
	SuTipV/SuEndV			△	▲
Fish morphometry	SN/D1	△	△	▲	▲
	SN/D2	△	△	△	
	SN/A			▲	▲
	D2C			▲	▲
	CP			▲	△
	B			△	▲
	D2b	△	△	▲	△
	Ab	△	△	▲	▲
Meristics	TotVert			△	▲
	CaudVert				▲
	AbVert			△	▲
	D2rays				△
	Arays				△
	AP				▲
	DProCur				△
	VProCur		△		

△, at least two species were separated from ≥50% of their congeners; ▲, ≥50% of the species included were separated from ≥50% of the congeners. See methods for abbreviations of variables.

phenomenon appears to be related to growing spread (variance) after inserting values of additional species. In other words, the additional values increased the overall variance of the variables, which finally blurs the information of a certain variable that would otherwise

be characteristic for an individual species. This likely explains why in our study the success of a given variable in species separation generally increased when the reduced datasets of *Pomatoschistus* and *Gobius* were used (tables 6, 7).

Descriptions of the otoliths

For the detailed descriptions of the shapes of all sagittal otoliths, we have divided our dataset in four groups, i.e., (i) the otoliths of the *G. auratus* species complex (sensu Miller & El-Tawil, 1974; Herler et al., 2005) (fig. 3), (ii) the otoliths of *Gobius* species that could be identified based on otolith variables (fig. 4), (iii) the otoliths of *Gobius* species that could not be identified based on otolith variables (fig. 5), and (iv) the otoliths of the species of *Pomatoschistus* (fig. 6). The descriptions refer to the mesial side of the otoliths, which, as in all saccular otoliths of teleost fish, bears the sulcus and most relevant characters.

In all studied otoliths, the sulcus acusticus is slightly inclined and reveals the 'shoe sole-like' shape that is typical for most Gobioidae (Nolf, 1985, 2013; Gierl et al., 2018; Lombarte et al., 2018). The cauda is slightly or clearly shorter than the ostium and the ostium is anteriorly tapering or pointed (figs. 3–6). The sulcus of all species is covered by a microcrystalline texture that is usually smooth, with exceptions in *G. niger* and *G. roulei* (presence of some flat bumps; fig. 5g–l), and *G. cobitis*, *G. incognitus* and *G. paganellus* (lack of this texture along the dorsal part of the ostium; fig. 4a–c, h–k). Some irregularities of the sulcus shape can occur within a species: one specimen of *G. couchi* showed a triangular extension of the dorsal ostial margin (fig. 3f), and one specimen of *G. fallax* revealed an irregular shape of the ostium (fig. 3h). In the otoliths of *Pomatoschistus*, the shape of the sulcus is generally somewhat variable (fig. 6). Additionally, the size of the cauda may be reduced in *P. kneri* (fig. 6a–d), *P. marmoratus* (fig. 6e–h) and *P. microps* (fig. 6i–l), and is always reduced in *P. montenegrensis* (fig. 6n–p) and *P. quagga* (fig. 6r–t). A well defined, rounded or elongate, thick crista inferior is visible at the beginning of the cauda in almost all species of *Gobius* and *Pomatoschistus*

('subcaudal iugum' sensu Schwarzhans, 2014). The shape and strength of the crista inferior can vary between and also within a species (figs. 3–6). In otoliths of *Pomatoschistus* that possess a reduced cauda, the crista inferior reaches almost the end of the cauda (*P. kneri*, *P. marmoratus*, *P. microps*; fig. 6a–l), or even continues beyond (*P. montenegrensis*, *P. quagga*; fig. 6n–p, r–t).

The Gobius auratus species complex (fig. 3)

The otolith shape in all species of the *G. auratus* species complex is approximately rhomboidal, with the highest shape seen in *G. couchi* (fig. 3d–f), and the longest in *G. kolombatovici* (fig. 3k, l). All species display a prominent posterodorsal projection at the posterior margin and slight differences in the shape of this projection occur between the species: it is blunt in *G. auratus* (fig. 3a–c) and *G. fallax* (fig. 3g, h), slightly tapering in *G. couchi* (fig. 3d–l), short and truncated in *G. gasteveni* (fig. 3i, j), and broadly rounded in *G. kolombatovici* (fig. 3k, l). The further posterior margin is separated from the posterodorsal projection by a clear incision or notch; it is slightly rounded in *G. auratus* and *G. fallax*, and relatively straight in the remainders. The ventral margin is slightly curved in *G. couchi* and *G. gasteveni*, in the others it is relatively straight. A well developed preventral projection occurs in *G. fallax*, *G. gasteveni*, *G. kolombatovici* and, to a lesser extent, also in *G. couchi*. In *G. auratus* no clear preventral projection is present, but the ventral margin ends in a sharp point. The anterior margin is usually concave or slightly incised in the middle in all species. A small predorsal projection can be developed, but its size and shape is variable within and between the species. It seems that otoliths of smaller specimens tend to have such a projection, while those of larger specimens do not (see fig. 3). The dorsal margin is rounded and can bear some lobes or crenulations; only

FIGURE 3 Otoliths (mesial view) of the species of the *Gobius auratus* complex, i.e. *G. auratus* (a: Selce, 6l; b: Selce, 5l; c: Krk, 2r mirrored), *G. couchi* (d–f: Krk, 2l, 3r mirrored, 1l), *G. fallax* (g, h: Unije Island, 2r mirrored, 1l), *G. gasteveni* (i, j: Galicia, 1l, 1r mirrored) and *G. kolombatovici* (k, l: Krk, 1l, 2l). Numbers following the localities refer to the fish specimen from which the otolith was extracted; l, left otolith; r, right otolith, mirrored for better comparison. SL denotes the standard length (in mm) of the corresponding fish specimen. Scale bars: 0.5 mm. All figured otoliths are kept in the Bavarian State Collection (collection number SNSB-BSPG 2020 LIV).

FIGURE 4 Otoliths (mesial view) of *Gobius cobitis* (a–c: Montenegro, 7l, 4l, 1l), *G. geniporus* (d: Montenegro, 3l; e, f: Selce, 2l, 'medium'), *G. incognitus* (g–i: Pelješac Peninsula, J1914l, J1910r mirrored, J1906l), *G. paganellus* (j–l: Galicia, 1l, 6l, 8l) and *G. vittatus* (m, Selce, 2l; n, o, Krk, Krk, 1l; 2l; p: Selce 2l). Numbers following the localities refer to the fish specimen from which the otolith was extracted; l, left otolith; r, right otolith, mirrored for better comparison. SL denotes the standard length (in mm) of the corresponding fish specimen. Scale bars: 0.5 mm. All figured otoliths are kept in the Bavarian State Collection (collection number SNSB-BSPG 2020 LIV).

FIGURE 5 Otoliths (mesial view) of *Gobius bucchichi* (a–c: Selce, 3l, 1l, 4l), *G. cruentatus* (d–f: Selce, 2l, 3l, 8l), *G. niger* (g–i: Pilsey Island, 2l, 6l, 5l) and *G. roulei* (j–l: Selce, 2l, 1l, 3l). Numbers following the localities refer to the fish specimen from which the otolith was extracted; l, left otolith. SL denotes the standard length (in mm) of the corresponding fish specimen. Scale bars: 0.5 mm. All figured otoliths are kept in the Bavarian State Collection (collection number SNSB-BSPG 2020 LIV).

FIGURE 6 Otoliths (mesial view) of *Pomatoschistus knerii* (a–d: Krk, 1r, 2r, each mirrored, 9l, 4r mirrored), *P. marmoratus* (e–h: Selce, 1r, 2r, 3r, each mirrored, 4l), *P. microps* (i–l: Stralsund, 8l, 5l, 6l, 14l), *P. minutus* (m: Stralsund, 1l), *P. montenegrensis* (n–p: Skadar lake, 8l, 9l, 6l), *P. pictus* (m: Norway, 2r mirrored), and *P. quagga* (r–t: Krk, 2l, 8r mirrored, 4l). Numbers following the localities refer to the fish specimen from which the otolith was extracted; l, left otolith; r, right otolith, mirrored for better comparison. SL denotes the standard length (in mm) of the corresponding fish specimen. Scale bars: 0.5 mm. All figured otoliths are kept in the Bavarian State Collection (collection number SNSB-BSPG 2020 LIV).

in *G. kolombatovici* it is relatively flat. A median tip of the dorsal margin is visible in *G. auratus* and *G. couchi*.

Gobius species that could be identified based on otolith variables (fig. 4)

Six species could be recognized with good success based on their otolith variables (table 2a), i.e., *G. auratus* (described above), *G. cobitis*, *G. geniporus*, *G. incognitus*, *G. paganellus* and *G. vittatus*. Of those, the otoliths of *G. incognitus* (fig. 4g–i) and *G. vittatus* (fig. 4m–p) display a rhomboidal shape, while the otoliths of *G. cobitis* (fig. 4a–c), *G. geniporus* (fig. 4d–f), and *G. paganellus* (fig. 4j–l) are more rectangular. The otoliths of all five species are usually longer than high except some otoliths of *G. vittatus*, which are approximately quadratic. All five species possess a prominent posterodorsal projection on the posterior margin, which differs slightly between them: it is blunt, slightly to distinctly crenulated and mostly facing upwards (dorsally) in *G. cobitis* (fig. 4a–c) and *G. paganellus* (fig. 4j–l); it is slightly pointed and mostly facing upwards in *G. incognitus* (fig. 4g–i); it is of triangular-rounded shape in *G. vittatus* (fig. 4m–p); and it is broadly rounded in *G. geniporus* (fig. 4d–f). The posterior margin beneath the projection is relatively straight in all five species and can bear slight crenulations, only in *G. vittatus* it is always smooth. The ventral margin is faintly curved in *G. incognitus* and *G. vittatus*, and relatively straight in *G. cobitis*, *G. geniporus*, and *G. paganellus*. A clear preventral projection occurs only in some otoliths of *G. cobitis* (fig. 4c) and *G. paganellus* (fig. 4k, l). In both *G. incognitus* and *G. vittatus* the anterior end of the ventral margin is relatively sharp and pointed. The anterior margin is straight in *G. cobitis*, concave in *G. geniporus*, oblique in *G. incognitus* and *G. paganellus*, and straight with a clear incision in the middle in *G. vittatus*. A predorsal

projection occurs only in *G. geniporus* and *G. vittatus*, it is sharp in *G. geniporus*, while it is rounded in *G. vittatus*. A relatively flat dorsal margin occurs in *G. incognitus* and *G. paganellus*; the dorsal margin is regularly rounded in the other three species. A median dorsal tip is usually visible in *G. vittatus* and in some otoliths of *G. incognitus*. A smooth dorsal margin occurs in *G. vittatus*, in the other species the dorsal margin can bear lobes or crenulations.

Gobius species that could not be identified based on otolith variables (Fig. 5)

The otoliths of *G. buccichi*, *G. cruentatus*, *G. niger*, and *G. roulei* could not be separated based on the individual otolith variables (table 2a). Their otoliths are longer than high, with a clear rhomboidal shape in *G. buccichi* (fig. 5a–c), and a rectangular to rhomboidal shape in *G. cruentatus* (fig. 5d–f), *G. niger* (fig. 5g–i), and *G. roulei* (fig. 5j–l). The otoliths of all four species possess a clear posterodorsal projection, which reveals slight differences between the species: it is rounded to slightly pointed in *G. buccichi*; it is rounded-to-squared in *G. cruentatus*; and it is broad, mostly rounded and sometimes slightly lobed in *G. niger* and *G. roulei*. The posterior margin beneath the projection and also the posteroventral angle are clearly (*G. buccichi*) or slightly (*G. roulei*) rounded. In *G. cruentatus* the posterior margin is straight and can be weakly crenulated, in *G. niger* it is straight or curved; the posteroventral angle is mostly angular in these two species. The ventral margin is straight in *G. cruentatus*, and faintly curved in the other three species. A weak preventral projection occurs in some otoliths of *G. cruentatus* (fig. 5d) and *G. roulei* (fig. 5j–l). The anterior margin is mostly concave, with an incision in the middle part. An angular predorsal angle is typical for *G. buccichi*, *G. cruentatus* and *G. niger*; in *G. roulei* a rounded predorsal “angle” can occur (fig. 5k, l). The dorsal margin

is mostly slightly undulated; it is gently inclined in *G. buccichi*, slightly rounded in *G. cruentatus*, clearly rounded in *G. roulei*, and strongly rounded in *G. niger*.

The otoliths of the Pomatoschistus species (fig. 6)

The otoliths of *Pomatoschistus* are usually rounded to square, but reveal a relatively high intraspecific variation regarding the details of their contours (fig. 6). The otoliths of *P. knerii* (fig. 6a–d), *P. montenegrensis* (fig. 6n–p) and *P. quagga* (fig. 6r–t) are slightly higher than long, the otoliths of *P. minutus* reveal a relatively rectangular shape (fig. 6m), and those of *P. marmoratus* (fig. 6e–h) and *P. microps* (fig. 6i–l) are nearly quadratic. A posterodorsal projection can be present or not in *P. marmoratus*, *P. microps* and *P. minutus*, it is lacking in *P. knerii*, *P. microps* and *P. pictus*, and it is usually not present or replaced by a broad bulge in *P. montenegrensis* and *P. quagga*. The further posterior margin is relatively straight and slightly or clearly incised approximately in the middle; the posteroventral angle is rounded, angular or oblique. The ventral margin is straight or faintly curved in all species, no preventral projection is present. The anterior margin is straight, oblique or rounded, an incision in the middle may be present or not. An angular predorsal angle may occur in all species, except in *P. knerii*. The rounded dorsal margin is slightly lobed or crenulated in *P. marmoratus*, *P. microps* and *P. minutus*. It is smooth in *P. knerii*, *P. pictus*, *P. montenegrensis*, and *P. quagga*; a clear median tip can occur in the latter two (fig. 6n, p, r).

Discussion

The objective of this study was to evaluate the ability of characters of otoliths, body morphometric traits and meristic counts, all of which

can be preserved in fossils, to discriminate species from their congeners.

Evaluation of otolith morphometry and otolith shape analysis

The morphology of the sagittal otolith is generally considered species-specific. This view goes back to the pioneering work of Koken (1884, 1891) and has since been demonstrated in numerous studies dealing with many different fish groups (Nolf, 1985; Campana, 2004; Tuset et al., 2008). Besides genetic and ontogenetic factors, also environmental parameters can influence the overall otolith shape (Tuset et al., 2003; Volpedo & Echeverría, 2003; Vignon & Morat, 2010).

Regarding otolith morphometry, the varying results from different statistical analysis (table 6), and the fact that the taxonomic significance of a certain otolith variable depends on the species under consideration are consistent with previous work on the taxonomic value of otolith morphometry (e.g., Reichenbacher & Reichard, 2014; Avigliano et al., 2016; Gierl et al., 2018). In the context of samples of fossil goby otoliths and attempts to detect the number of species represented, it therefore may be worth considering all otolith variables at the outset of the study.

Otolith shape analysis is an established method for stock or population discrimination in fisheries research (Campana & Casselman, 1993; Mérigot et al., 2007; Stransky et al., 2008; Libungan et al., 2015), but not commonly used for species discrimination. But for gobies, several studies are available that have analysed the differences between species: Elliptic Fourier analysis was conducted by Lord et al. (2012) to analyse three sympatric species of *Sicyopterus*, by Bani et al. (2013) to examine three sympatric Caspian species (one species of *Neogobius*, two of *Ponticola*), by Davoodi & Rahimian (2016) for three sympatric Caspian species of *Neogobius*,

and by Yu et al. (2014) for the study of five species representing five different genera from northern Chinese coastal waters. Moreover, Lombarte et al. (2018) used wavelet analysis to examine 25 Mediterranean goby species distributed in 14 genera (including ten species of *Gobius* and two species of *Pomatoschistus*). In each of these studies, the authors found that the species included could be reliably discriminated based on their otoliths, but that the classification success varied among species. Our study largely confirms these results. In addition, it significantly advances our knowledge of the interspecific differences between goby otoliths within a given genus, as we focused on the discrimination of individual species from their congeners. Moreover, like Bani et al. (2013) and Yu et al. (2014) we found that otolith Fourier shape analysis works more effectively in goby species separation than otolith morphometry. This appears to be related to the circumstance that otolith shape differences between goby species can be very subtle (see figs. 3–6), and in such cases cannot easily (or not at all) be quantified using single otolith variables (see Yu et al., 2014; Bonhomme et al., 2014; Caillon et al., 2018). The high discriminatory power of the otolith Fourier shape analysis makes this method a promising tool to examine ancient species diversity when abundant and well preserved fossil otoliths are present.

Evaluation of body morphometry

Body morphometry in fish can be greatly influenced by the demands of swimming, and associated factors like habitat size or presence/absence of predators and competitors (Gholami et al., 2015 and references therein). Therefore, body morphometry is often not considered to be relevant for species identification. Also, the taxonomic keys to the species of *Gobius* and *Pomatoschistus* provided in Miller (1986) do not include morphometric

characters. On the other hand, body morphometry can be very efficient in detecting differences between populations or stocks (e.g., Haddon & Willis, 1995; Turan, 2004; Cheng et al., 2005; Buj et al., 2008). In our study, some body morphometric variables showed a high degree of separation success among species of both *Gobius* and *Pomatoschistus* (tables 2d, 4d, 7); but only one population per species was available. Addition of further populations could reveal intraspecific differences in some of these variables, and eventually render them unsuitable for species separation. However, fossil finds are usually restricted to a single location, and often to a certain timeframe. Perhaps the most famous example is the locality of the fossil *Lagerstätte* Monte Bolca (c. 50 Ma; Marramà et al., 2016 and references therein). Examples of fossil locations bearing several goby fossils include Illerkirchberg in Germany (c. 17 Ma; Gierl & Reichenbacher, 2015), Klinči in Serbia (c. 16–19 Ma; Bradić-Milinović et al., 2019) and Oran in Algeria (c. 5–6 Ma; Arambourg, 1927). Our results indicate that measurements of SN/D₁, SN/D₂, D_{2b}, and Ab are well suited to evaluating the ancient goby species diversity found in such singular localities.

Evaluation of the meristic counts

Meristic traits are usually estimated to work better than morphometry in species separation, but meristic counts among gobies are often very similar (Birdsong et al., 1988). In our study, the meristic values had little or no discriminatory power within *Gobius*, whereas they worked more efficiently for *Pomatoschistus*, especially when the reduced dataset was used (tables 6, 7). It is possible that the use of only five species in the reduced *Pomatoschistus* dataset contributed to the greater separation success in that case (see above and Lombarte et al., 2018). However, the most conspicuous meristic character that distinguishes

Pomatoschistus from *Gobius* is the elongated vertebral column, usually comprising 30–31 vertebrae (vs. 28 in *Gobius*) (Miller, 1986; supplementary table S3). An elongated body form can, among other things, improve swimming abilities, affect feeding habits, and promote adaptation to specialized microhabitats (Claverie & Wainwright, 2014). An example among the Oxudercidae is *Luciogobius*, whose adaptive radiation into interstitial microhabitats was facilitated by marked body elongation (Yamada et al., 2009). Thus, it is possible that species of *Pomatoschistus* can be more readily separated from each other based on meristic counts than species of *Gobius* because they are adapted to more specific microhabitats. However, as data on microhabitats such as distinctive grain sizes, or abundance of certain algae, or presence of distinct shell sizes for spawning, are relatively limited for species of *Pomatoschistus* and *Gobius* (Miller, 1986; Nellbring, 1993; Wilkins & Myers, 1992; Kovačić & Šanda, 2016), this issue cannot be resolved at present. In the context of fossils, our results indicate that shared meristic characters among fossil gobies need not necessarily indicate the presence of only a single species, as has been proposed in some previous work (e.g., Gaudant & Quayle, 1988; Gaudant, 1998; Reichenbacher et al., 2007).

Comparisons of the otolith morphology of Gobius with previous works

Generally, digital photographs or SEM images of extant species of *Gobius* are rarely provided in the literature and the otoliths of *G. fallax* (fig. 3g, h), *G. gasteveni* (fig. 3i, j), *G. kolombatovici* (fig. 3k, l) and *G. incognitus* (fig. 4g–i) are shown here for the first time. SEM images of *G. niger* and *G. paganellus* were also depicted in Reichenbacher & Cappetta (1999: pl. 6, Fig. 3A–C), and drawings of a series of otoliths of *G. cruentatus* were provided in Nolf (2013:

pl. 317); these otolith images fit well with the otoliths of the same species in our study. Furthermore, Lombarte et al. (2018) presented digital photographs of ten species of *Gobius* of which six (i.e., *G. auratus*, *G. bucchichi*, *G. cruentatus*, *G. geniporus*, *G. roulei*, *G. vittatus*) were from the same region as the corresponding species of our study (northern Adriatic Sea). The photographs of the otoliths of these six species (see Lombarte et al., 2018: Fig. 3) reveal that their shape is almost identical with the otoliths of our material (see figs. 3–5). In contrast, the otoliths of *G. couchi*, *G. niger*, *G. paganellus* and *G. cobitis* displayed in Lombarte et al. (2018: Fig. 3) reveal some differences compared to the otoliths of the same species in our study. The otolith of *G. couchi* in Lombarte et al. (2018: Fig. 3c) shows a more curved dorsal margin and a more rounded posterodorsal projection than our otoliths of this species (fig. 3d–f). As the specimen of *G. couchi* figured in Lombarte et al. (2018) comes from the same area (northern Adriatic Sea) and has a similar size like our specimens, the reason of this variation cannot be explained. The otolith of *G. niger* (Lombarte et al. 2018: Fig. 3i) displays a sharp posteroventral angle and a prominent preentral projection and thus differs from our otoliths of *G. niger* (fig. 5g–i). Also the otolith of *G. paganellus* (Lombarte et al. 2018: Fig. 3e) differs from our material of *G. paganellus* (fig. 4j–l) because the dorsal margin is rounded (vs. relatively flat in our otoliths) and the curvature of its posterodorsal projection is directed posteriorly (vs. dorsally). In both *G. niger* and *G. paganellus*, the differences could be due to ontogenetic variation because Lombarte et al. (2018) used relatively large specimens (*G. niger*: TL 104 mm vs. max. TL 71 mm in our material; *G. paganellus*: TL 125 mm vs. max. TL 84 mm in our material). But it could also be that the observed intraspecific variation results from differences between geographically

distant populations. Lombarte et al. (2018) collected both *G. niger* and *G. paganellus* in the Mediterranean Sea, while our species were assembled from sites in the NE Atlantic (table 1). Moreover, also the otolith of *G. cobitis* (Lombarte et al. 2018: Fig. 3d) differs from our otoliths of this species (fig. 4a–c) in its more rounded posteroventral margin (vs. angular), its smaller posterodorsal projection that is not facing dorsally, and in the relatively smooth margins (vs. crenulated). As the corresponding fish utilized in Lombarte et al. (2018) is within the size range of our specimens, the differences may again result from variation between geographically distant populations: Lombarte et al. (2018) sampled *G. cobitis* from the NW Mediterranean sea (NE of Barcelona, Catalan coast), while our *G. cobitis* specimens originate from the Adriatic Sea (Croatia and Montenegro). Overall, these comparisons confirm that the details of the otolith shape is generally species-specific in *Gobius*, but differences between geographically distant populations may occur and need further study.

Nevertheless, we expect that the herein presented SEM images of the otoliths of 14 species of *Gobius*, together with the images shown in Lombarte et al. (2018), will provide a valuable source of information for the detection of extant species of *Gobius* in the fossil record. As a first step, we compared our dataset with some previously published fossil otoliths of which SEM images had been presented (Lin et al., 2015; Agiadi et al., 2013, 2018, 2019). On this basis, we found the interpretation of otoliths as *G. aff. paganellus* from the Upper Miocene (Tortonian) of northern Italy in Lin et al. (2015: Fig. 7: 8, 9) reliable because the fossil otoliths are similar to the extant ones, but differ in the rounded posteroventral angle (vs. angular) and the broader posterodorsal projection. Also the identification of Pleistocene otoliths as *G. niger* in Agiadi et al.

(2018: Fig. 5:68) and as *G. vittatus* in Agiadi et al. (2019: Fig. 4I, J) appears plausible; these fossil otoliths correspond largely with the otoliths of *G. niger* and *G. vittatus* of our data sets and that of Lombarte et al. (2018). On the other hand, the otolith from the Pleistocene that was classified as *G. geniporus* in Agiadi et al. (2019: Fig. 4F) is eroded and in our view not identifiable with certainty. Also the documentation of *G. cf. paganellus* from Pliocene sediments (Agiadi et al., 2013) and *G. paganellus* and *G. cobitis* from Pleistocene deposits (Agiadi et al., 2019) appear doubtful, as these otoliths clearly do not possess the typical shape of the respective species. However, based on the new comparative material presented here, they can be re-classified. The Pliocene otolith documented as *G. cf. paganellus* (Agiadi et al. 2013: Fig. 8:14) and the two otoliths from the Pleistocene named as *G. cobitis* (Agiadi et al., 2019: Fig. 4D, E) could well represent *G. fallax*, with which they share the general outline, inclination of the dorsal margin and blunt posterodorsal projection (see here fig. 3g, h); they would be the first fossil record of *G. fallax*. The two otoliths from the Pleistocene that were depicted as *G. paganellus* in Agiadi et al. (2019: Fig. 4G, H) are relatively high and have a conspicuously rounded anterodorsal margin, together with their pronounced and slightly pointed posterior projection they are very similar to *G. incognitus* and would be the first fossil record of this species.

Comparisons of the otolith morphology of Pomatoschistus with previous works

As said for *Gobius*, digital photographs or SEM images of extant species of *Pomatoschistus* are rarely provided in the literature and the otoliths of *P. knerii* (fig. 6a–d), *P. microps* (fig. 6i–l), and *P. montenegrensis* (fig. 6n–p) are presented here for the first time. Photographs of an otolith of *P. marmoratus* and *P. pictus* were also depicted in Lombarte et al.

(2018: Fig. 1) and SEM images of *P. marmoratus* and *P. quagga* were shown in Gierl et al. (2018: Fig. 3); these species originated from the same region as our material, and their otolith morphology conforms well with our otoliths. Additionally, drawings of a series of otoliths of *P. minutus* were given in Nolf (2013: pl. 318), and also these images fit entirely with our otoliths of this species. Although the otoliths of a *Pomatoschistus* species can bear considerable intraspecific variability (see fig. 6), the here presented SEM images of their otoliths will greatly help to identify possible fossil representatives of extant species of *Pomatoschistus* in future studies.

Why can some goby species be more easily recognized than others?

Gobius auratus, *G. geniporus*, *G. paganellus* and *G. vittatus* could be separated by many otolith variables (table 8) and revealed also characteristic shapes based on the SEM images (figs. 3a–c, 4d–f, j–p). *Gobius vittatus* could also be separated from its congeners based on two body morphometric variables, but no body morphometric variable separated the other three species from one another. Previous studies have demonstrated clear links between otolith shape and environmental parameters (e.g., Volpedo & Echeverría, 2003; Lombarte et al., 2010; Vignon & Morat, 2010; Avigliano et al., 2016). Based on these works it can be hypothesised that the specific otolith morphometry of *G. auratus*, *G. geniporus*, *G. paganellus* and *G. vittatus* reflects particular habitat preferences, e.g., for a certain water depth or temperature or a specific type of substrate and diet. A review of the literature on the four species under discussion provides some support for such a relationship.

(i) Herler et al. (2005) investigated the habitat use of *G. auratus* and *G. fallax* in the northern Adriatic Sea. The authors

found that both species prefer water depths greater than 8 m, but that *G. auratus* clearly differed from *G. fallax* in its preference for more inclined substrates ($>30\text{--}90^\circ$).

(ii) *Gobius geniporus* inhabits solely sandy bottoms and does not consume algae; both traits are unusual for *Gobius* species (Zander, 2011).

(iii) The rock goby *G. paganellus* is an intertidal-to-inshore, epibenthic species that lives under stones or on sheltered rocky shores with abundant algal cover (Miller, 1986; Zander, 2011). In their study on the composition of fish assemblages in six different macrohabitats in the Gulf of Trieste, Bonaca & Lipej (2005) found *G. paganellus* to be associated with a specific assemblage of algae (*Wrangelia* and *Padina*), while *G. cobitis*, *G. cruentatus* and *G. fallax* each occurred in all or almost all of the macrohabitats studied. It thus appears that *G. paganellus* has specific habitat preferences.

(iv) The striped goby *G. vittatus* differs phenotypically from its congeners in having a broad, black, longitudinal band that extends along its head and body (Miller, 1986). It is found offshore on coralline substrates at depths of 5 to 34 (exceptionally 42) m, with a preference for moderately to steeply inclined bottoms composed of both rock and sand (Miller, 1986; Kovačić, 2007; Kovačić & Arko Pijevac, 2008). Its characteristic broad stripe may reflect an adaptation to some additional aspect(s) of its habitat.

In the *Pomatoschistus* datasets, otolith morphometry was most significant for *P. montenegrensis* (table 9). In this case too, a relationship to environmental factors could be proposed, as *P. montenegrensis* is one of the few freshwater species in the genus *Pomatoschistus* (Šanda & Kovačić, 2009). Alternatively,

phylogenetic factors may play a role as there is some evidence that *P. montenegrensis* actually might belong to a different genus, i.e. *Nin-nigobius* (see Miller & Šanda, 2008; Freyhof, 2011; Thacker et al., 2019).

Regarding fossil otoliths, it is obvious that some otolith-based species of *Gobius* are easier to recognize than others. Examples are *G. rostratus* from the lower Miocene of the Mainz Basin, Germany (easily recognizable by its prolonged preventral projection; see Weiler, 1963: Figs. 190–198), *Ponticola zosimovichi* from the middle Miocene of Kazakhstan (well identifiable due to its strongly bulged anterodorsal margin; see Bratishko et al., 2015: Figs. 9–21), and several others. The results of

our studies tentatively indicate that such distinctive otolith shapes may be related to specific environmental niches that these ancient species occupied. A further factor that could potentially influence otolith shape but cannot be evaluated yet is intraspecific communication based on acoustic signals. Experimental studies have revealed that gobies are capable of producing multiple sounds (Malavasi et al., 2008; Polgar et al., 2011; Parmentier et al., 2013; Horvatić et al., 2016), and acoustic courtship signals were demonstrated to be relevant for mating success in the painted goby *Pomatoschistus pictus* (Amorim et al., 2013). However, additional studies will be necessary before such a link between acoustic communication

TABLE 8 Comparison of the species classification success in the reduced dataset of *Gobius* based on the different groups of variables (indicated by colors) and different statistical analyses. Bold numbers in rows indicate how many individual variables had separated the species given in the columns from $\geq 50\%$ of the congeners (see table 2a2, d2, f2 for details). Percentages in rows represent the success of the respective multivariate analyses in separating the species given in the columns (100% means that the species is separated from all congeners by this variable; see table 2c2 and table 3 for details). Species are sorted by the maximum number of otolith variables that account for their separation from $\geq 50\%$ of the congeners

<i>G. vittatus</i>	<i>G. auratus</i>	<i>G. paganellus</i>	<i>G. geniporus</i>	<i>G. incognitus</i>	<i>G. cobitis</i>	<i>G. roulei</i>	<i>G. niger</i>	<i>G. bucchichi</i>	<i>G. cruentatus</i>	
10**	9***	8**	8*	6**	4*	6	4	2	2	Individual otolith variables (AN(C)OVA)
50%	54%	56%	60%	33%	50%	30%	43%	60%	18%	PC1–4 of otolith variables (LDA)
80%	90%	100%	80%	100%	80%	90%	90%	100%	80%	Fourier otolith descriptors (MANOVA)
71%	88%	89%	100%	87%	95%	73%	85%	75%	100%	PC1–23 of Fourier otolith descriptors (LDA)
2*	0	0	0	4*	2	0	1	4**	0	Individual fish morphometric variables (ANOVA)
82%	27%	60%	50%	56%	30%	30%	43%	70%	64%	PC 1–4 of fish morphometric variables (LDA)
0	0	0	1	1	0	0	0	0	0	Meristic variables (Kruskal-Wallis)

Abbreviations: *, **, *** indicates that one, two or three singular variables separated the species given in the columns from 78–89% of the congeners. AN(C)OVA means that ANOVA or, if covariance was detected, ANCOVA was used; LDA, Linear Discriminant Analysis.

TABLE 9 Comparison of the species classification success in the reduced dataset of *Pomatoschistus* based on the different groups of variables (indicated by colors) and different statistical analyses. Bold numbers in rows indicate how many individual variables had separated the species given in the columns from $\geq 50\%$ of the congeners (see table 4a2, d2, f2 for details). Percentages in rows represent the success of the respective multivariate analyses in separating the species given in the columns (100% means that the species is separated from all congeners by this variable; see table 4c2 and table 5 for details). Species are sorted by the maximum number of otolith variables that account for their separation from $\geq 50\%$ of the congeners

<i>P. montenegrensis</i>	<i>P. microps</i>	<i>P. kneri</i>	<i>P. quagga</i>	<i>P. marmoratus</i>	
16***	17*	13	14	16*	Individual otolith variables (AN(C)OVA)
90%	87%	60%	56%	56%	PC1–4 of otolith variables(LDA)
100%	100%	100%	83%	83%	Fourier otolith descriptors (MANOVA)
95%	100%	90%	100%	93%	PC1–14 of Fourier otolith descriptors (LDA)
5*	5*	6	5*	1*	Individual fish morphometric variables (ANOVA)
90%	67%	70%	90%	67%	PC 1–4 of fish morphometric variables (LDA)
7*	3*	6	5*	1	Meristic variables Kruskal-Wallis

Abbreviations: *, **, *** indicates that one, two or three singular variables separated the species given in the columns from 78–89% of the congeners. AN(C)OVA means that ANOVA or, if covariance was detected, ANCOVA was used.

and otolith morphology can be seriously considered (see Popper et al., 2005).

Conclusions

The outcome of this study significantly advances our knowledge of the interspecific differences between goby otoliths within a given genus. In agreement with previous work, we have shown that goby otoliths show a high species-specificity and are useful for taxonomic studies concerning this taxon. As the interspecific differences can be very delicate, the otolith shape analysis is a particular valuable tool for species identification. Studies of past goby species diversity using fossil otoliths may greatly benefit from the use of both singular otolith variables and elliptic Fourier shape analysis, but also traditional comparative morphology remains

useful. Moreover, we uncovered certain body and fin proportions, and in the case of *Pomatoschistus* also meristic traits suitable to unravel species diversity when fossil skeletons are preserved. Notably, shared meristic characters among fossil gobies do not necessarily indicate that only a single species is present, as has been proposed in some previous works. The outcome of our study provides a new basis for the delimitation of fossil goby species in future studies, and will consequently help to better understand the evolution of goby diversity and biogeography through time.

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Supplementary material

Supplementary material is available online at: <https://doi.org/10.6084/m9.figshare.11994000>

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