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BRIEF COMMUNICATION

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First documentation of the otoliths of the species of *Gouania* (Teleostei: Gobiesocidae) in the Mediterranean Sea

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Abstract

Otolith morphology is a widely accepted tool for species identification in teleost fish, but whether this holds true for very small species remains to be explored. Here, the saccular otoliths of the cryptobenthic Mediterranean clingfish *Gouania* (Gobiesocidae) are described for the first time. The new data, although preliminary, indicate that otolith morphology and morphometry support the recognition of the recently differentiated five species of *Gouania* in the Mediterranean Sea. Furthermore, otoliths of phylogenetically closely related *Gouania* species resemble each other more than do those of the more distantly related species.

KEYWORDS
clingfish, ecomorphotypes, Mediterranean, otolith morphology

The name "clingfish" collectively refers to small, cosmopolitan species of the family Gobiesocidae found in intertidal (and freshwater) environments. The term itself derives from the fact that they attach themselves to the substrate by means of a ventrally located adhesive disc (Briggs, 1955; Conway *et al*., 2017, 2019). Their unusual lifestyle and small body size explain why they are generally considered as cryptobenthic, which in turn suggests that clingfish biodiversity has been underestimated (Brandl *et al*., 2018; Wagner *et al*., 2019, 2020). Within the Gobiesocidae, this applies in particular to the genus *Gouania* Risso 1810, which originally included only the species *Gouania willdenowi*. Nevertheless, recent results from molecular and morphometric analyses suggested that this endemic Mediterranean genus comprises four additional species (Wagner *et al*., 2019) and led to the taxonomic revision of the genus (Wagner *et al*., 2020). Accordingly, (a) the species name *G. willdenowi* Risso 1810 should be reserved for clingfish inhabiting the western Mediterranean coasts, (b) two further species are present in the Adriatic (*Gouania pigra* Nardo 1827 and *Gouania adriatica* Wagner *et al*., 2020) and (c) two additional species occur in the eastern Mediterranean (*Gouania hofrichteri* Wagner *et al*., 2020, and *Gouania orientalis* Wagner *et al*., 2020). Notably, in both the latter regions, the two species are congruent with two morphotypes – one slender bodied with a small head and the other stout bodied with a larger head – which suggests convergent evolution (Wagner *et al*., 2019).

Otoliths form three pairs of calcium carbonate structures in the inner ear of teleosts (Popper *et al*., 2005). The saccular otoliths, usually the largest of the three pairs, are widely used for the identification of species, as the morphology of most saccular otoliths has been shown to be species specific (Nolf, 1985, 2013; Reichenbacher & Reichard, 2014; Tuset *et al*., 2008). Hitherto, the otoliths of only a few species of Gobiesocidae – fossil or extant – have been studied, mainly from the genus *Lepadogaster* Goüan 1770 (Schwarzhans *et al*., 2017; Smale *et al*., 1995; Tuset *et al*., 2008). Here, the saccular otoliths of *Gouania* are described for the first time and compared between the five species to examine the congruence between genetic data, morphotypes and overall otolith morphology.

In total, 22 saccular otoliths were extracted from 12 specimens – 2 representatives of *Lepadogaster lepadogaster* Bonnaterre 1788 (from St. Baska, Croatia, and Agni Beach, Corfu, Greece), 2 specimens of

G. willdenowi from the western Mediterranean (Messina, Italy), 2 specimens of each species from the Adriatic Sea (*G. pigra* and *G. adriatica*, Glavotok, Otok Krk, Croatia) and 2 specimens of each species from the eastern Mediterranean Sea (*G. hofrichteri* from Kapsali, Greece, and *G. orientalis* from the Gulf of Corinth, Greece). See Supporting Information for details on specimens and sites. The same set of specimens was used in the molecular study published by Wagner *et al.* (2019, 2020).

Ethical statement: Fish collection and euthanasia were carried out with the approval of the Ethics Committee of the University of Graz (permit number: GZ. 39/54/63 ex 2019/20) and in accordance with EU Directive 2010/63/EU, Annex IV, and the Austrian Animal Experimentation Ordinance, §20.

Left and right saccular otoliths (termed "otoliths" in the following) were extracted dorsally, and residual tissues were removed by immersion in 1% KOH solution for 3 h. The otoliths were then rinsed in distilled water for 4 h; if necessary, the procedure was repeated, and the otoliths were stored in distilled water overnight. Scanning electron microscopy (SEM) images of all otoliths were obtained using a HITACHI SU 5000 Schottky FE-SEM at the Department of Earth and Environmental Sciences (Ludwig-Maximilians- Universität München, Munich). Otoliths were mounted on aluminium pin stubs (12.5 mm in diameter, 3.2 × 8 mm), to which adhesive tabs had already been applied. A thin (20 nm) coating of gold was applied to the stubs (sputter coating) in a high-vacuum coater. The pin stubs were then inserted into the imaging system, and current (15 kV) was applied.

Morphological descriptions and otolith morphometry were based on SEM images of all otoliths. The images were processed using Adobe Photoshop. Figure 1A,B shows the otolith terminology and morphometry used in this study. SEM images were oriented so that the ventral margin of the otolith was essentially horizontal (Figure 1A, B). For otolith morphometry, eight distances were measured from the otolith images using Image J (Schneider *et al.*, 2012): otolith length (OL), otolith height (OH), sulcus length (SuL), sulcus height (SuH), ostium length (OstL), cauda length (CaudL), rostrum length (RoL) and rostrum height (RoH) (Figure 1B). All distances were measured to the nearest 0.001 mm. In addition, the perimeter (*P*) and area (*A*) of each otolith were determined (in mm and mm^2, respectively). To measure the lengths and heights of the otolith and sulcus, rectangles enclosing the dorsal-most, ventral-most, anterior-most and posterior-most points of the two structures were drawn (Figure 1B). The horizontal and vertical edges of these rectangles were then taken to represent the dimensions of interest. Ten otolith variables were calculated according to Tuset *et al.* (2003), Reichenbacher *et al.* (2007) and Gierl *et al.* (2018) (Figure 2; Table 1, Supporting Information). The outcome of the morphometric measurements was then transformed into descriptive statistics using Past (Hammer *et al.*, 2001).

A summary of the otolith characters and morphometric results of all studied otoliths is provided in Table 1. The general otolith outline and sulcus traits are largely similar among the otoliths of *G. willdenowi* and those of the four recently (re)described species from the Adriatic and the eastern Mediterranean Sea (Figure 1C). The otolith outline is oval to elliptical to slightly triangular; the anterior region is usually blunt, and the posterior region is round. The sulcus acusticus has a median to slightly supramedian position and is adjoined by well-developed thickened ("swollen") cristae that cover the entire (or almost the entire) inner portion of the sulcus. The ostium extends to the anterior margin (=heterosulcoid opening according to Tuset *et al.*, 2008), and it is separated from the cauda by a prominent structure, the collum. The ostium is tubular in shape; the cauda is slightly shorter than the ostium and round to oval in outline. The cauda is straight to slightly inclining and ends far from the posterior margin. The rostrum is mostly short, round and broad, whereas the antirostrum is usually absent or poorly defined. The excisura is narrow and shallow. All otoliths are thick and robust and exhibit a thicker posterior region when viewed from the ventral side (not shown). Most of the otolith variables examined indicate overlapping ranges among the five *Gouania* species, although there are some exceptions (see following text and Table 1).

The otoliths of *L. lepadogaster* are elliptical to trapezoid in outline (Figure 1D). The rostrum is well developed. The main morphological differences compared to the *Gouania* species are the (relatively) longer sulcus (in % of OL, see Table 1), the reduced RoH (in % of OH, see Table 1; Figure 2d) and the absence of "swollen" cristae on the inner portion of the sulcus (see Figure 1D). In addition, the ranges of the otolith variable "circularity" differ between *Lepadogaster* and all other groups (Table 1; Figure 2a).

As the sample available for each species was small (and the body sizes of specimens varied within and among groups), no conclusions could be drawn with regard to within-species variability of the otoliths such as sexual dimorphism (Teimori *et al.*, 2020; Vaux *et al.*, 2019), ontogenetic variation (Vignon, 2012; Więcaszek *et al.*, 2020) or asymmetry between right and left otoliths (Lord *et al.*, 2012; Lychakov *et al.*, 2008; Panfili *et al.*, 2005). Nonetheless, some preliminary remarks can be made based on the comparison between otolith groups. It was observed that the otoliths of *G. pigra* and those of *G. willdenowi* exhibit greater resemblance to each other than to the otoliths from the other species with respect to circularity (Figure 2a), RoL (% OL) and RoH (% OH) (Figure 2c,d) and also based on overall comparison of their SEM images [Figure 1C(a–d)]. Only the ratio of OstL to CaudL indicated non-overlapping ranges (Table 1). This high similarity is compatible with their sister relationship according to molecular data (Wagner *et al.*, 2019; see Figure 2f). The otoliths of the two stout morphotypes (*G. adriatica* and *G. orientalis*) also exhibit close similarity with each other with regard to the aforementioned otolith variables (circularity, RoL and height) and little overlap with the other groups (Table 1; Figure 2a,c,d), which is again consistent with their sister relation based on molecular data (Figure 2f). In the case of the two slender morphotypes (*G. pigra* and *G. hofrichteri*), the otolith variables circularity, rectangularity, RoL (% OL) and RoH (% OH) display no overlap in range (Table 1; Figure 2a–d). This implies possible differentiation between their otolith morphologies, in spite of their similarity in body shape, and thus supports the notion that *G. pigra* and *G. hofrichteri* are not closely related and that their slender body shapes result from convergent evolution (see Figure 2f). On the contrary, "rectangularity" was the only variable that separated the otoliths of *G. hofrichteri*, the eastern Mediterranean slender type, from almost all other otolith groups (the

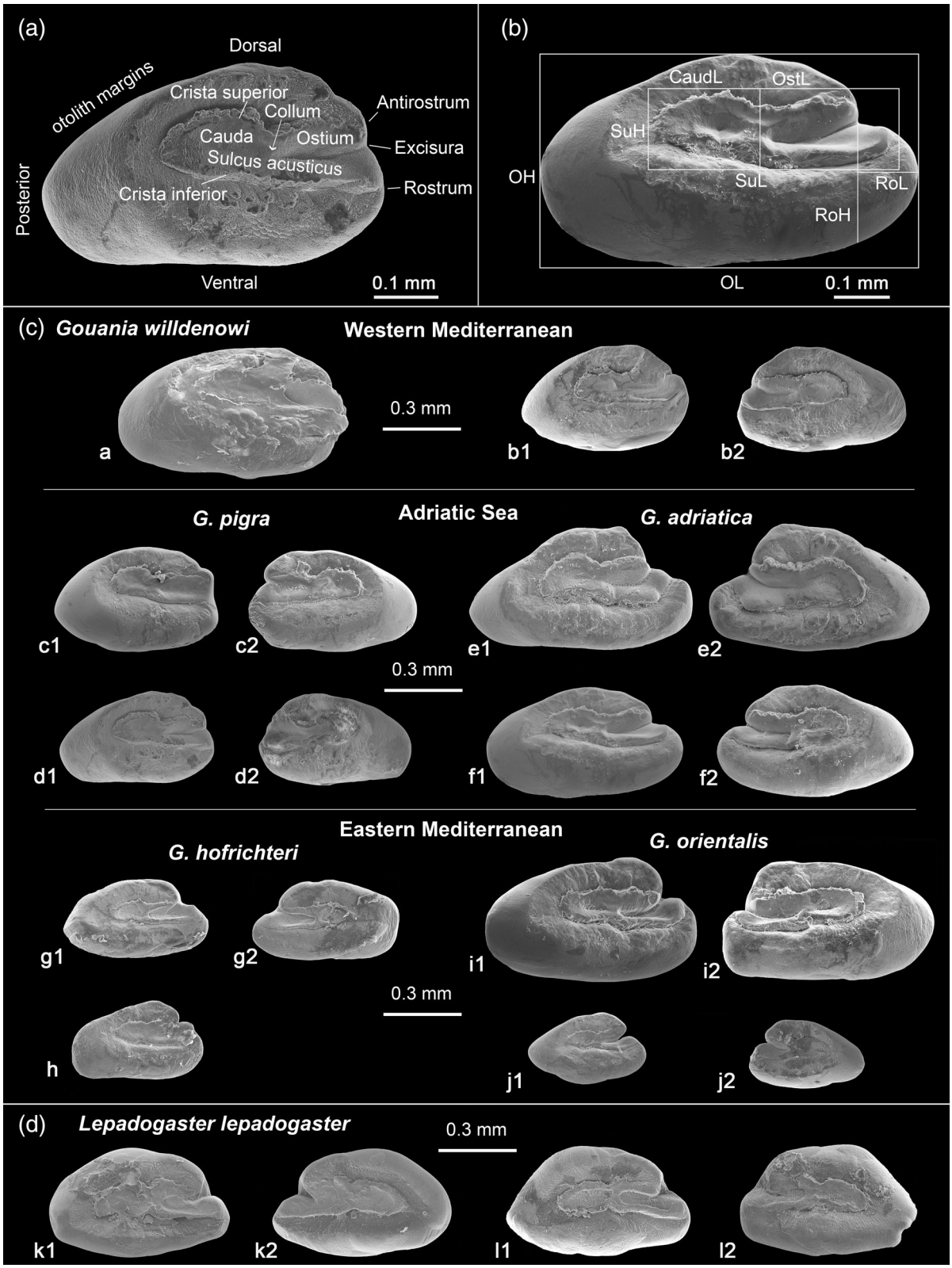

FIGURE 1 Otolith morphology of the clingfish species studied here (left and right sagittal otoliths, inner face). (A) Left otolith of *Gouania pigra* (GWK_03) with otolith nomenclature used in this study. (B) Left otolith of *Gouania adriatica* (GWK_05) with measurements according to Reichenbacher *et al.* (2007) and Gierl *et al.* (2018). (C) Otoliths of *Gouania* species; (a, b) *Gouania willdenowi*, specimen numbers GWM_06, GWM_05; (c, d) *Gouania pigra* (=slender ecomorphotype from the Adriatic), specimen numbers GWK_13, GWK_03; (e, f) *Gouania adriatica* (=stout ecomorphotype from the Adriatic), specimen numbers GWK-01, GWK_05; (g, h) *Gouania hofrichteri* (=slender morphotype from the eastern Mediterranean), specimen numbers KYT_22, KYT_23; (i, j) *Gouania orientalis* (=stout morphotype from the eastern Mediterranean), specimen numbers GOK_38, GOK_37. (D) Otoliths of *Lepadogaster lepadogaster*; (k, l) specimen numbers LepKrk7, LGCorf_21. Abbreviations: CaudL, caudal length; OH, otolith height; OL, otolith length; OstL, ostium length; RoH, rostrum height; RoL, rostrum length; SuH, sulcus height; SuL, sulcus length

single exception being the stout eastern Mediterranean morphotype, *G. orientalis*) (Table 1; Figure 2b). Based on the genetic analyses reported by Wagner *et al*. (2019), a higher degree of divergence of *G. hofrichteri* from all other groups would be expected.

The results of this study, although preliminary, are largely in accordance with the genetic results in Wagner *et al*. (2019). It is suggested that further exploration of otolith morphology from the five *Gouania* species could provide additional support for species differentiation within *Gouania*. Finally, this study contributes to the expansion of the hitherto-limited clingfish otolith record and offers new insights into the otolith morphology of the group.

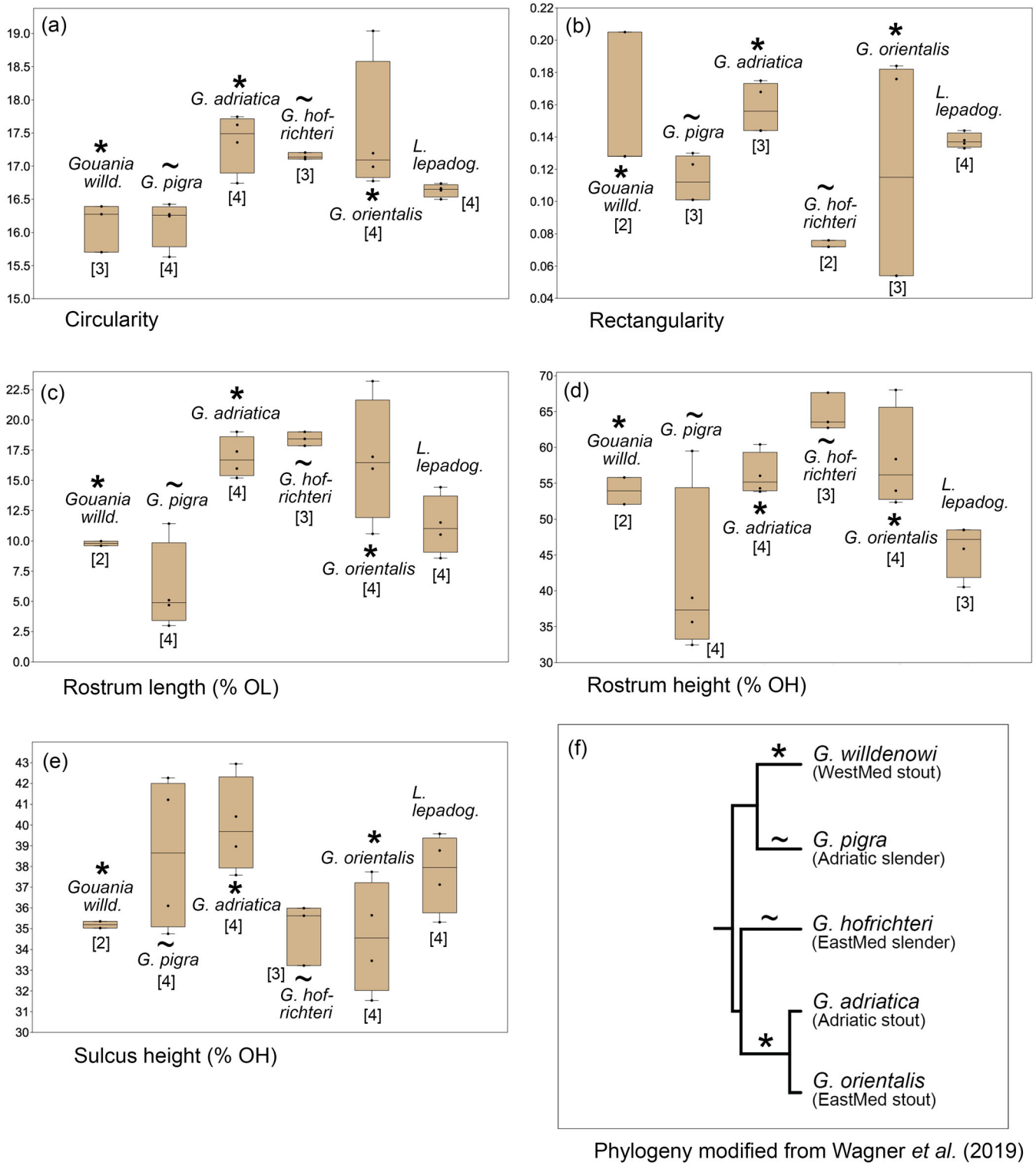

FIGURE 2 (a–e) Summary of the results of otolith morphometry of the studied *Gouania* specimens, [*n*] indicates the number of otoliths that could be used for the measurements; (f) phylogenetic tree modified from Wagner *et al*. (2019). ✱ stout and ~ slender

TABLE 1 Description of otolith morphology based on specific otolith characters and ranges of the measured otolith variables among the five species of *Gouania* and *Lepadogaster lepadogaster*

	Gouania willdenowi, *n* = 3	*Gouania pigra*, *n* = 4	*Gouania adriatica*, *n* = 4	*Gouania hofrichteri*, *n* = 3	*Gouania orientalis*, *n* = 4	*Lepadogaster lepadogaster*, *n* = 4
Shape outline	Oval to elliptic	Oval to elliptic	Elliptic to triangular	Elliptic squared	Elliptic	Elliptic to trapezoid
Sulcus position	Median	Median	Median	Median	Median	Median
Sulcus type	Ostial	Ostial	Ostial	Ostial	Ostial	Ostial
Sulcus opening	Heterosulcoid	Heterosulcoid	Heterosulcoid	Heterosulcoid	Heterosulcoid	Heterosulcoid
Ostium shape	Tubular	Tubular	Tubular, slightly wider towards the opening	Tubular	Tubular	Tubular
Cauda shape	Round oval	Round oval	Round oval	Round oval	Round oval	Round oval
Cauda position	Ending far from the posterior margin	Ending far from the posterior margin	Ending far from the posterior margin	Ending far from the posterior margin	Ending far from the posterior margin	Ending far from the posterior margin
Cauda curvature	Straight	Straight	Straight	Straight	Straight	Straight
Collum	Solid bridge	Solid bridge	Solid bridge	Solid bridge	Solid bridge	Solid bridge
Excisura	Very narrow, shallow	Very narrow, shallow	Narrow, shallow	Narrow, shallow notched	Slightly deep notched, narrow	Narrow, shallow notched
Rostrum	Very short, round, broad	Very short, round, broad	Long, round, broad	Long, round, broad	Short, round, broad	Long, round to pointed
Antirostrum	Absent	Poorly defined	Poorly defined	Poorly defined or absent	Short, round, broad	Absent
Otolith margins	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Posterior region	Round	Round	Round to oblique	Round	Round	Round to oblique
Cristae	Well developed	Well developed	Well developed	Well developed	Well developed	Less well developed
Otolith height (in % OL)	57.383–63.594	59.565–64.465	55.556–57.876	54.577–60.000	56.438–60.521	59.861–63.808
Rostrum height (in % OH)	52.058–55.797	32.439–59.500	53.831–60.412	62.739–67.647	52.330–68.024	40.515–48.519
Rostrum length (in % OL)	9.604–9.985	2.987–11.433	15.195–19.015	17.863–19.014	10.590–23.210	8.576–14.429
Sulcus height (in % OH)	35.024–35.351	34.750–42.265	37.585–42.944	33.226–35.987	31.541–37.740	35.308–39.578
Sulcus length (in % OL)	62.980–64.318	60.220–67.835	58.534–62.468	60.196–62.852	59.806–61.733	68.895–75.000
Ostium length (in % SuL)	55.854–56.490	45.550–54.831	53.620–56.531	49.837–58.523	50.903–57.505	49.798–58.857
Caudal length (in % SuL)	43.510–48.000	45.169–54.450	43.469–46.380	41.477–50.163	42.495–49.097	41.143–50.202
Ratio ostium length/cauda length	1.265–1.298	0.837–1.214	1.156–1.300	0.994–1.411	1.037–1.353	0.992–1.431
Circularity	15.703–16.391	15.632–16.423	16.742–17.746	17.106–17.205	16.774–19.039	16.499–16.737
Rectangularity	0.128–0.205	0.101–0.130	0.144–0.175	0.072–0.076	0.054–0.184	0.133–0.144

Note: Each species was represented by two specimens, of which both the right and left otoliths (sagitta) were extracted, except in the case of one specimen of *G. willdenowi* and *G. hofrichteri* (one otolith was lost). Bold font indicates character differences between at least two species; circularity was calculated as (P^2/A) (P, perimeter; A, area), and rectangularity was calculated as [A/(OL × OH)]. *n*: number of otoliths; OH: otolith height; OL: otolith length; SuL: sulcus length.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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