Otolith shape analysis as a tool for stock identification of two commercially important marine fishes: *Helicolenus dactylopterus* and *Merluccius*

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Abstract :

Stock identification studies are essential to understanding fish population structure and connectivity across wide geographical areas, and thus contribute to efficient fisheries management. The blackbelly rosefish, Helicolenus dactylopterus, and European hake, Merluccius, are two economically important marine fishes, but there are still gaps in knowledge regarding their present stock structure. Our objective was to assess the ability of otolith shape to define stock structure for the two species along the Northeastern Atlantic Ocean and the Mediterranean Sea, based on samples from eight and seven areas, for blackbelly rosefish and European hake, respectively. Shape analysis was obtained through Wavelet

analysis. Canonical analysis of principal coordinates provided significant evidence for different population units with a clear separation between the Atlantic and Mediterranean populations for both species. However, random forest procedures indicated that the discrimination power varied with species and locations. For blackbelly rosefish, various Atlantic populations were more evident than for European hake. Overall, the usefulness of otolith shape to delineate stock structure of two species with distinct life history traits across a broad spatial region from the mid-Atlantic isles to the polar region, as well as the Mediterranean was demonstrated. Moving forward, it will be key to align our growing understanding of population structure with our increasing knowledge on species' biological traits to ensure management units reflect population structure.

Graphical abstract
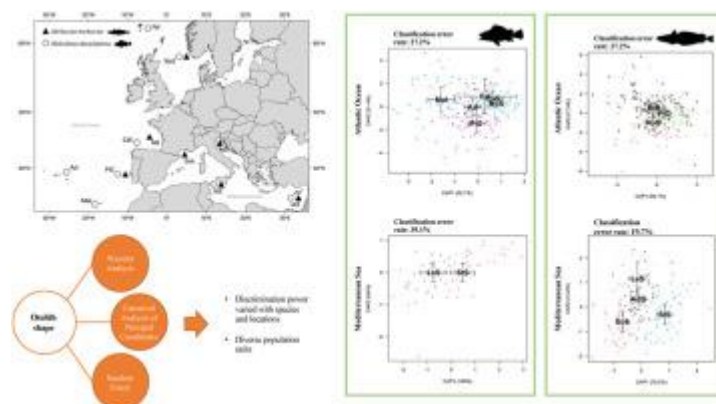
Highlights

► *Helicolenus dactylopterus* and *Merluccius* are fishery exploited species. ► We used otolith shape to define their stock structure in the Atlantic and Mediterranean. ► Wavelet analysis and canonical analysis of principal coordinates revealed diverse population units. ► Random forests showed that the discrimination power varied with species and locations. ► Results suggest a higher degree of connectivity of *M. merluccius* in the Atlantic.

Keywords : Population structure, Blackbelly rosefish, European hake, Atlantic ocean, Mediterranean sea, Connectivity

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Introduction

Stocks are described as self-recruiting groups of fish within a species that share similar growth, natural and non-natural mortality rates, and show relative independent reactions to harvesting (Cadrin et al., 2013). Due to the increasing pressure induced by the higher demands for seafood, studies on stock structure are fundamental to support more sustainable fisheries management strategies (Jackson et al., 2001; Smith et al., 2010; Taillebois et al., 2017), in particular as accurate stock management relies on comprehensive knowledge of stock evaluation and configuration (Cadrin et al., 2013).

80   Fish stock identification can be achieved using different methods that include

81   tagging and acoustic telemetry, demography and meristic data, otolith shape analysis,

82   otolith chemistry and genetics (Hawkins et al., 2016). Otoliths have been among the most

83   used structures as stock discrimination tools in fisheries science, by analysing their

84   morphological and chemical properties (Campana and Casselman, 1993; Stransky et al.,

85   2008; Milano et al., 2014; Westgaard et al., 2017; Leone et al., 2019; Morales-Nin et al.,

86   2022). These structures are composed of calcium carbonate and are the first calcified

87   structures formed in the ontogenic process. The three pairs of otoliths (sagittae, lapilli and

88   asterisci) are found in the membranous labyrinth of the inner ear in fishes and play a key

89   role postural equilibrium and hearing of fishes (Popper et al., 2005). The wide use of these

90   structures is associated with the fact that they are metabolically inert, record fish growth

91   and the chemical elements of the surrounding water (Campana, 1999).

92   Otolith shape analysis has been vastly used as a stock identification tool for many

93   marine fishes (e.g., *Sebastes* spp (Stransky, 2005); *Trachurus trachurus* (Stransky et al.,

94   2008); *Engraulis encrasicolus* (Bacha et al., 2014; Jemaa et al., 2015); *Sardina*

95   *pilchardus* (Jemaa et al., 2015); *Clupea harengus* (Libungan and Pálson, 2015);

96   *Trachurus picturatus* (Vasconcelos et al., 2018); *Genidens barbus* (Maciel et al., 2021).

97   The use of this technique is possible given that otolith shape is species-specific and can

98   be influenced by feeding behavior (Simoneau et al., 2000) and environmental conditions

99   such as water temperature (Cardinale et al., 2004), depth (Gauldie and Crampton, 2002),

100   and type of substrate (Mérigot et al., 2007), which can be stock specific (Aguëra and

101   Brophy, 2011). Additionally, this methodology is economical and time-efficient

102   compared with others, such as artificial tagging and tracking, genetics, and otolith

103   chemistry, since in many cases otoliths are readily available from routine data collection

104   sampling programs requiring no extra acquisition cost and effort.

105   The blackbelly rosefish *Helicolenus dactylopterus* (Delaroche, 1809) and

106   European hake *Merluccius merluccius* (Linnaeus, 1758) are two sympatric commercially

107   important species in European waters. The blackbelly rosefish is a benthopelagic slow-

108   growing and long-lived marine fish species (Kelly et al., 1999; Massutí et al., 2000;

109   Sequeira et al., 2009) that belongs to the family *Sebastidae*. It is commonly found at

110   depths between 200 and 1000 m and presents a wide distribution from the Northeastern

111   Atlantic to the Mediterranean (Froese and Pauly, 2023). Its slow-growth, late fecundity

112   and high longevity in combination with a typical sit-and-wait predator behaviour (Uiblein

113   et al., 2003), make the blackbelly rosefish particularly vulnerable to overfishing (Pirrera

114   et al., 2009) even when usually caught as a by-catch (Deval et al., 2018). In the

115   Northeastern Atlantic Ocean, the blackbelly rosefish stock structure is still unknown

116   (Neves et al., 2010) and there are currently no specific management measures or landing

117   regulations in EC waters or in the NEAFC Regulatory Area, although this species is being

118   assessed and managed among other deep-sea fish to the present (ICES, 2022; NEAFC,

119   2023). The European hake is a demersal species widely distributed in the Northeastern

120   Atlantic Ocean and throughout the Mediterranean and Black Sea (Murua, 2010) between

121   30 and 1075 m depth, with adults found in a wider depth range from the shelf to the upper

122   slope (Cartes et al., 2009). The European hake is a highly exploited species across

123   multiple regions of the Northeast Atlantic (FAO, 2020; GFCM, 2012). Since 1978, the

124   ICES Working Group of Southern Demersal Stocks (WGSSDS) distinguished two

125   different stocks for the European hake - the northern and southern stocks, divided at the

126   Cap Breton Canyon (Anon, 2004). Still, most stock identification studies have focused

127   on the Northeast Atlantic, North Sea, and Baltic, while few have included the entire

128   geographic range of the species distribution, i.e., including archipelagos in the middle of

129   the Atlantic Ocean, such as the Azores, Madeira and the Faroe Islands, or the

130   Mediterranean Sea and transition areas with the Atlantic Ocean (e.g., Neves et al., 2010;

131   Tanner et al., 2012; 2014; Morales-Nin et al., 2022). As such, the objectives of the present

132   study were (1) to describe otolith shape variations of blackbelly rosefish and European

133   hake in several areas of the Northeast Atlantic and Mediterranean, and (2) to assess the

134   discrimination ability of otolith shape as a tool to delineate stock boundaries of these

135   species. Here, we tested the hypothesis that differences in population connectivity due to

136   oceanic boundaries are translated into distinct otolith shape, which allows discrimination

137   between adjacent population units along the species' geographical distribution gradients.

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140   **Materials and Methods**

141   **Sampling**

142   Blackbelly rosefish and European hake adult samples were obtained from research

143   surveys or commercial fisheries. The sampling areas were selected in accordance with

144   the geographical distribution of each species, habitat, and oceanographic characteristics.

145   Samples from both species were collected in the Northeastern Atlantic Ocean and

146   Mediterranean Sea from 2017 to 2020, despite the blackbelly rosefish having a much

147   more extensive distribution. To minimize possible interannual differences in otolith shape

148   and the analysed total length, size ranges were limited (blackbelly rosefish from 10 to

149   40cm; European hake from 20 to 65cm) to minimize confounding morphometric effects

150   of largely different otolith shapes between very small and very large fish (Table I, Fig.

151   1). Collected fish were stored frozen until further analysis. Fish length (cm) was recorded

152   (Table I), and the sagittal otoliths were removed, air-dried, and stored in Eppendorf vials

153   until further analysis, as described in the European sampling protocol of otoliths (Vitale

154   et al., 2019).
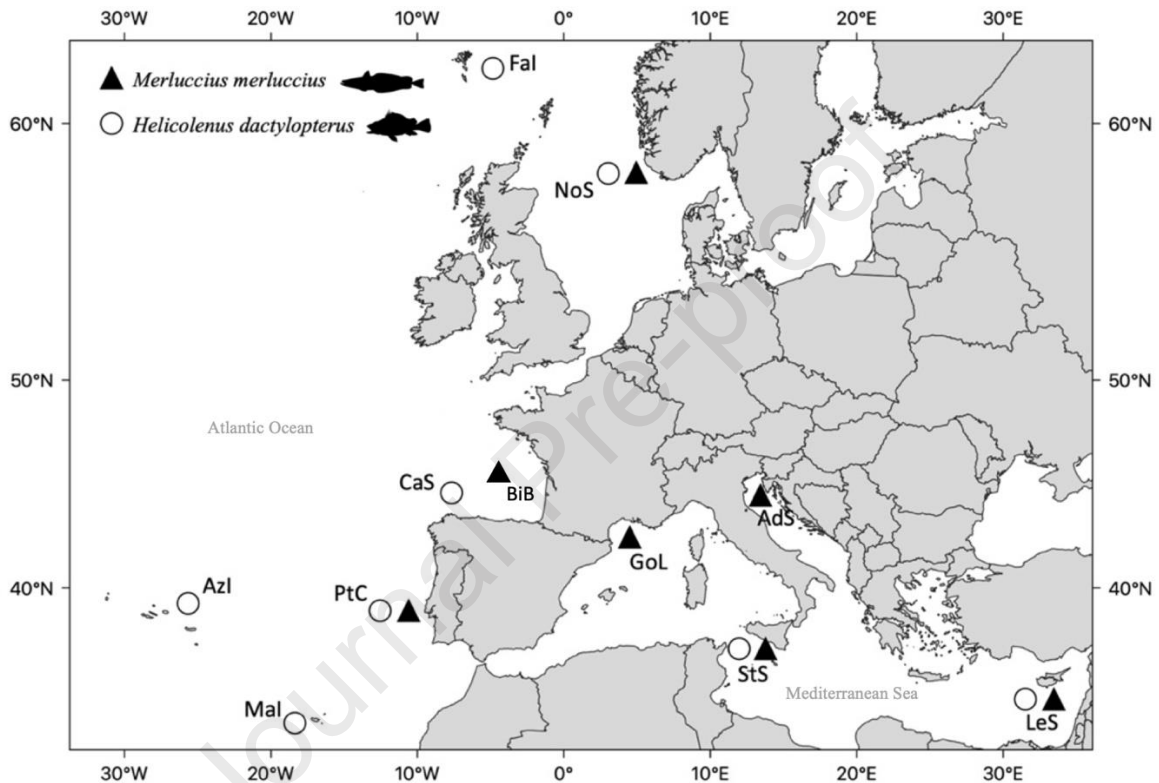
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156

157   **Figure 1:** Map of sampling locations for *Helicolenus dactylopterus* (circles) and *Merluccius merluccius*

158   (triangles) in the Northeastern Atlantic Ocean and Mediterranean Sea. See Table I for area codes.

159

160   **Table I:** Number of individuals, mean length (cm) and corresponding standard deviation per sampling area

161   of *H. dactylopterus* and *M. merluccius* used for shape analysis.

162

	Helicolenus dactylopterus					*Merluccius merluccius*			
Basin	**Sampling area**	**Area Code**	**Nº of otoliths**	**Mean length (cm)**	**Basin**	**Sampling area**	**Area Code**	**Nº of otoliths**	**Mean length (cm)**
Atlantic Ocean	**Faroe Islands**	FaI	15	26.6±4.0	Atlantic Ocean	**North Sea**	NoS	61	57.3±14.7
	North Sea	NoS	67	14.6±3.1		**Bay of Biscay**	BiB	116	44.2±14.9
	Cantabrian Sea	CaS	35	20.9±4.7					
	Azores Islands	AzI	36	30.7±1.8		**Portuguese Coast**	PtC	79	40.7±7.5
	Madeira Islands	MaI	44	38.4±2.4	Mediterranean Sea	**Golf of Lion**	GoL	47	32.9±3.8
	Portuguese Coast	PtC	57	26.5±3.9		**Adriatic Sea**	AdS	48	23.6±3.8
Mediterranean Sea	**Strait of Sicily**	StS	40	18.7±4.9		**Strait of Sicily**	StS	49	19.6±1.4
	Levantine Sea	LeS	26	18.0±5.1		**Levantine Sea**	LeS	24	31.3±2.6

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Otolith shape analysis

The first step was to carefully analyse each otolith under a Leica M80 stereomicroscope to ensure that only left otoliths in perfect conditions were used (i.e., with no cracks and with a good crystallization). Then, images of the otoliths were captured with Leica LAS X software using a Leica DFC450 color camera with 5-megapixel CCD sensor, connected via Firewire to Windows 10 PC, and converted to monochrome in Adobe Photoshop. All otoliths were positioned on a microscope slide with the *sulcus* down and *rostrum* to the left in a horizontal line to minimize distortion errors and obtained high-contrast images with transmitted light, allowing a clear visualization of the otolith outlines. To ensure a high resolution, the microscope magnification was adjusted to the otolith size. Through the software package ShapeR (Libungan and Pálson, 2015) that runs on the R platform (R Core Team; www.r-project.org), the otolith outlines were extracted. The shape of each otolith was recorded as a matrix of x and y coordinates and the otoliths were subsequently normalized to remove any possible size-induced bias. Shape coefficients and mean otolith shape for each population were obtained through a Wavelet analysis, since it is a more powerful and robustness approach (Libungan and Pálson, 2015), and does not require as much data as Fourier analysis (Baradad et al., 2005; Libungan and Pálson, 2015).

183　　　　　Otolith rotations were arranged horizontally along the longest axis and the areas

184　　were set equal to one, then, by drawing a polar axis, from the centroid to the 0º angle of

185　　the outline, the coordinates were collected and the radials were retracted with equidistant

186　　angles, from 0º to 360º angle (Libungan and Pálson, 2015). The deviation between the

187　　original outline and the reconstructed Wavelet otolith outline was used to determine the

188　　number of Wavelet coefficients. To visualize differences in the shape among the different

189　　sampling areas, a plot with the mean shape of each population was built through the

190　　reconstructed outlines of the normalized Wavelet coefficients. To estimate which otolith

191　　areas contributed the most to differentiate populations, Wavelet coefficients means, and

192　　respective standard deviation were plotted for all the otoliths in the analysis (Libungan

193　　and Pálson, 2015).

194　　　　　An ANOVA-like permutation test and a Canonical Analysis of Principal

195　　Coordinates (CAP) was performed using the vegan package (Oksanen et al., 2022), and

196　　the capscale function to analyze the shape variation among the different sampling sites

197　　for each species separately (following Libungan and Pálson, 2015). Classification of

198　　individuals to their sampling area based on Wavelet coefficients was performed using

199　　random forest classification (Breiman, 2001) implemented in the randomForest package

200　　(Liaw and Wiener, 2002). Average out-of-bag (OOB) classification error was determined

201　　using 2000 trees and the number of variables tried at each split was 11.

202

203　　**Results**

204　　　　　The average shape outline reconstruction plot based on all otoliths showed clear

205　　differences among populations for both species (Figure 2A; D), as reflected by the high

206　　degree of variation in the Wavelet coefficients between populations. For better

207　　visualization, a separate analysis was performed for the Atlantic (Fig. 2B; E) and the

208　　Mediterranean populations (Fig. 2C; F). Further analysis showed that for blackbelly

209　　rosefish, the populations from Madeira and Azores differed the most from the other

210　　sampling areas, with the otolith's outlines moving inwards to the otolith centroid between

211　　180 and 270º at the *excisura* (Figure 2A). For European hake, the North Sea and

212　　Portuguese populations showed a higher degree of differentiation from the rest, with an

213　　inward shift of the otolith contour at around 270º (Fig. 2D). More detailed examination

214　　with separated Atlantic Ocean and Mediterranean Sea populations showed more subtle

215　　differences within these two areas (Fig. 2E; F).
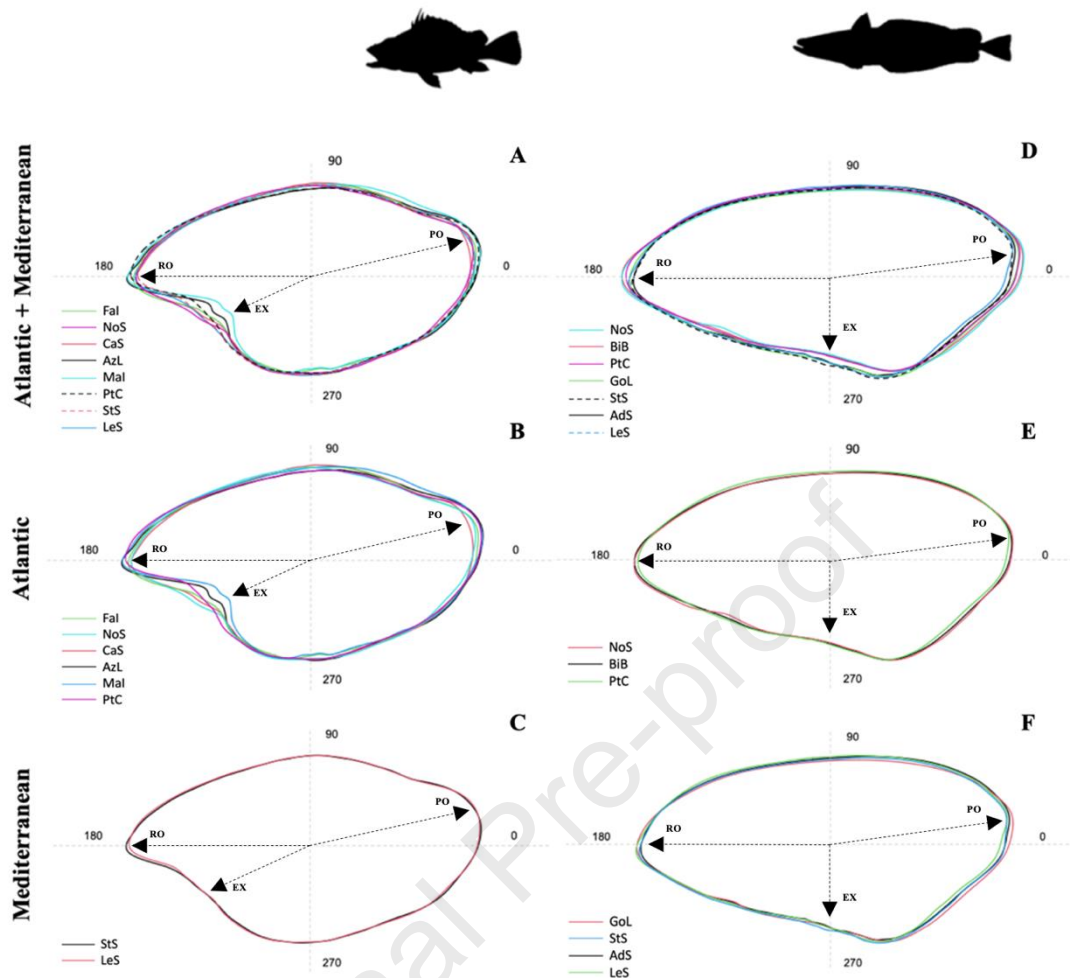
Figure 2: Otoliths average shapes obtained by Wavelet descriptors for *H. dactylopterus* (left panels) and *M. merluccius* (right panels) for all sampled populations (A, D), Atlantic Ocean (B, E) and Mediterranean Sea (C, F). The *excisura* (EX), *rostrum* (RO) and *postrostrum* (PO) are highlighted in each average shape.

The ANOVA-like permutations test showed significant differences in the otolith shape among populations for each species, both in the combined analysis (p<0.001 for both species), and when separated into Atlantic Ocean (p<0.001 for both species) and Mediterranean Sea (p<0.004 for blackbelly rosefish; p<0.001 for European hake). The Canonical Analysis of Principal Coordinates demonstrated differentiation among sampling areas based on otolith shape for both species (Fig. 3). With all areas pooled together, the first discriminating axis for the blackbelly rosefish explained 44.1% of the variance while the second axis explained 35% (Fig. 3A). In this case, despite some overlap three clusters are visible: Atlantic islands (Madeira and Azores), northern areas (Faroe, North Sea and Cantabrian Sea) and southern areas (Portuguese coast, Strait of Sicily and Levantine Sea). Analysing the results separately, the first axis explained 52.1% and the second 31.4% in the Atlantic CAP (Fig. 3B), while in the Mediterranean 100%

of the variation was explained by the first axis (Fig. 3C). Hence, the Atlantic cluster
consistently differentiated the more northern areas (Faroe Islands, North Sea, and
Cantabrian Sea), the Portuguese coast, and the Madeira and Azores Islands. There was
also a clear separation between samples from the Strait of Sicily and the Levantine Sea
in the Mediterranean Sea.
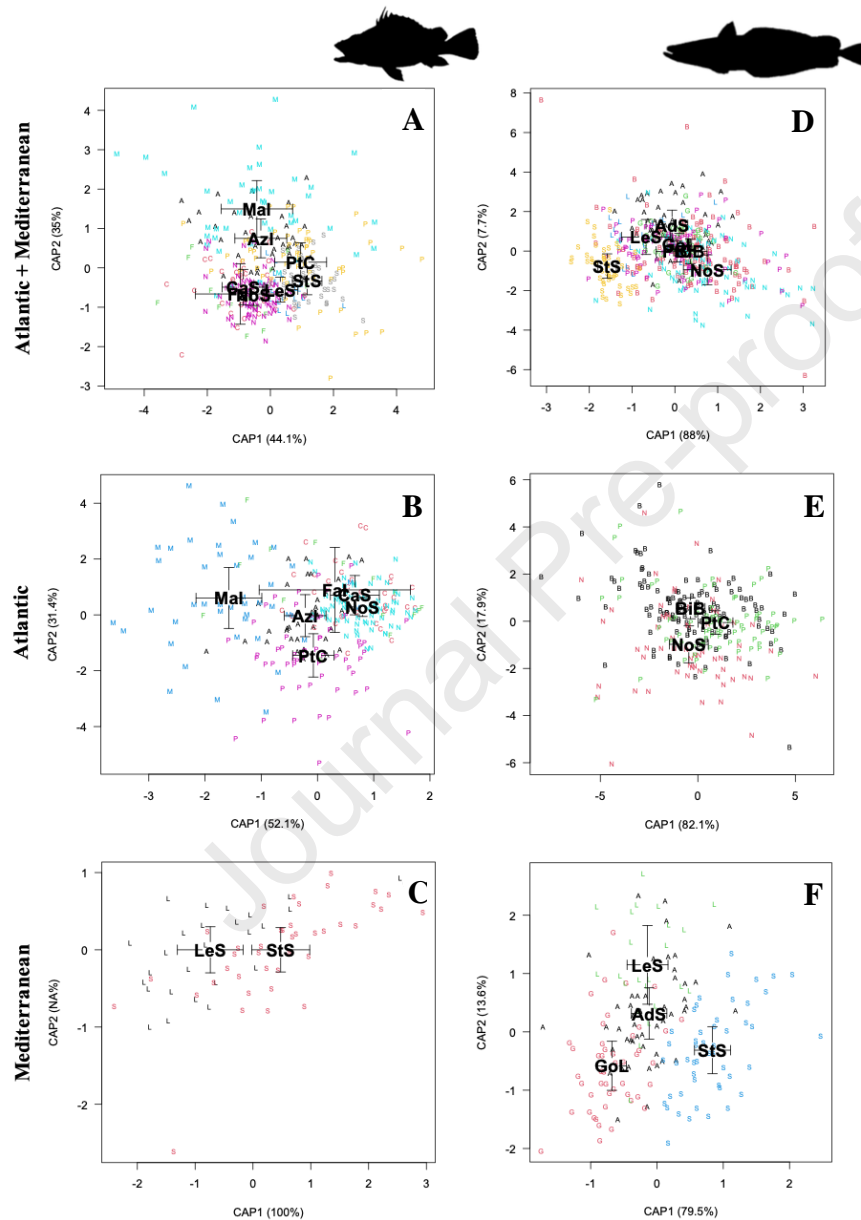
Figure 3: Canonical Analysis of Principal Coordinates of normalized Wavelet descriptors of the otolith
shape of *H. dactylopterus* (left panels) and *M. merluccius* (right panels) from all sampled populations (A,
D), Atlantic Ocean (B, E) and Mediterranean Sea (C, F).

For European hake, the first axis of the general CAP explained 88% and the
second axis represented 7.7% of the variation. There was a high degree of overlap and

245   only the population from the Strait of Sicily was clearly distinguished (Fig. 3D). When

246   analysing the Atlantic Ocean and Mediterranean Sea populations separately, five clusters

247   were established: an indistinct Atlantic cluster, composed of North Sea, Bay of Biscay,

248   and Portuguese samples (Fig. 3E), and each of the four Mediterranean areas: Gulf of Lion,

249   Strait of Sicily, Adriatic Sea, and Levantine Sea (Fig. 3F).

250   For blackbelly rosefish, random forest classification produced an error rate of

251   36.6%, with the Faroe Islands and Levantine Sea populations showing the highest error

252   rates (87% and 81%, respectively), and Madeira Islands and North Sea showing the

253   lowest error rates (16% and 5%, respectively) (Table II). When separating sampling areas

254   into Atlantic Ocean and Mediterranean Sea, a reduction in classification error was

255   observed (27.2% and 24.2%, respectively), but both the Faroe Islands and Levantine Sea

256   remained the areas with the highest classification error (87% and 46%, respectively)

257   (Table III). For European hake, the overall analysis error was 37.7%, with the highest

258   misclassification rates in the Levantine Sea (83%), Portugal (64%) and Gulf of Lion

259   (57%) and the lowest in the Adriatic (8%), Sicily (16%) and Bay of Biscay (18%) (Table

260   IV). No improvement was observed when only Atlantic Ocean samples were analyzed

261   together (39.1%), but a clear differentiation within the Mediterranean region was possible

262   (19.7%) (Table V).

263

264   **Table II:** Random Forest confusion matrix for all populations in the study of *H. dactylopterus*. Overall

265   classification error rate was 36.6% for all populations. The horizontal lines show the number of otoliths

266   from each population assigned to one or more areas. See Table I for area codes.

			Predicted areas								
			FaI	**NoS**	**CaS**	**PtC**	**AzI**	**MaI**	**StS**	**LeS**	**Total**	**Classification Error**
		FaI	2	0	6	5	2	0	0	0	15	87%
		NoS	0	64	2	0	0	0	1	0	67	5%
	Atlantic Ocean	**CaS**	0	6	21	2	2	0	4	0	35	40%
Actual areas		**PtC**	0	0	0	39	9	5	4	0	57	32%
		AzI	0	1	1	13	17	4	0	0	36	53%
		MaI	0	0	1	1	5	37	0	0	44	16%
	Mediterranean Sea	**StS**	0	8	3	8	0	1	18	2	40	55%
		LeS	0	4	3	7	0	0	7	5	26	81%

267

268   **Table III:** Random Forest confusion matrix for the Atlantic Ocean and Mediterranean Sea populations of

269   *H. dactylopterus*. Overall classification error rate of all populations was 27.2% and 24.2%, respectively.

270 The horizontal lines show the number of otoliths from each population assigned to one or more areas. See

271 Table I for area codes.

		Predicted areas										
		Atlantic Ocean								Mediterranean Sea		
		FaI	NoS	CaS	PtC	AzI	MaI	Total	Classification Error	StS	LeS	Classification Error
Actual areas	**FaI**	2	0	5	6	2	0	15	87%			
	NoS	0	64	2	1	0	0	67	5%	StS	12	14	10%
	CaS	0	8	21	4	2	0	35	40%			
	PtC	0	0	1	42	9	5	57	26%			
	AzI	0	1	2	12	17	4	36	53%	LeS	36	4	46%
	MaI	0	0	1	1	3	39	44	11%			

272

273 **Table IV:** Random Forest confusion matrix for all populations in the study of *M. merluccius*. Overall

274 classification error rate of all populations was 37.7% for all populations. The horizontal lines show the

275 number of otoliths from each population assigned to one or more areas. See Table I for area codes.

		Predicted areas								
		NoS	BiB	PtC	GoL	AdS	StS	LeS	Total	Classification Error
Actual areas · Atlantic Ocean	**NoS**	32	21	3	4	1	0	0	61	48%
	BiB	5	95	12	2	0	2	0	116	18%
	PtC	5	36	28	3	1	5	1	79	64%
Mediterranean Sea	**GoL**	2	16	1	20	6	2	0	47	57%
	AdS	0	0	2	0	44	0	2	48	8%
	StS	0	1	4	3	0	41	0	49	16%
	LeS	0	3	6	0	3	8	4	24	83%

291

292

293 **Table V:** Random Forest confusion matrix for the Atlantic Ocean and Mediterranean Sea populations of

294 *M. merluccius*. Overall classification error rate of all populations was 39.1% and 19.7%, respectively. The

295 horizontal lines show the number of otoliths from each population assigned to one or more areas. See Table

296 I for area codes.

297

298

		Predicted areas											
		Atlantic Ocean					Mediterranean Sea						
		NoS	BiB	PtC	Total	Classification Error		GoL	AdS	StS	LeS	Total	Classification Error
Actual areas	NoS	37	19	5	61	39%	GoL	38	5	4	0	47	20%
	BiB	6	88	22	116	24%	AdS	2	44	0	2	48	8%
							StS	4	0	44	1	49	10%
	PtC	8	40	31	79	61%	LeS	2	3	10	9	24	63%

299

300

Discussion

301   Otolith shape analysis of blackbelly rosefish and European hake successfully
302 distinguished different populations in the Northeast Atlantic Ocean and Mediterranean
303 Sea, confirming it is a useful technique in the stock identification toolbox. Using the
304 Wavelet approach, we detected morphological differences in the otolith outline that
305 contributed to delineate shape variations among populations. Obtained results indicated
306 population differentiation for each species, however, separate analyses of Atlantic Ocean
307 and Mediterranean Sea were necessary for better discrimination at smaller spatial scale,
308 since a strong separation between these two regions was visualized but more subtle
309 among them.

310   The differences in relation to the most variable areas in the otolith shape found
311 between the large geographical areas (Atlantic Ocean and Mediterranean Sea) can be
312 explained by genetic factors (Cardinal et al., 2004), since different deposition of the
313 otolith increments due to different growth rates can affect the otolith structure (Geffen,
314 1982; Folkvord et al., 2000; Feet et al., 2002; Fox et al., 2003), and may also reflect
315 different environmental conditions (Campana and Neilson, 1985; Lombarte and Lleonart,
316 1993; Cardinale et al., 2004; Vignon, 2012). Indeed, the sampling areas in this study
317 present a latitudinal cline in sea water temperature from the more northern areas (Faroe
318 Islands for blackbelly rosefish; North Sea for European hake) to the southern ones
319 (Mediterranean Sea), as well as in salinity (the Mediterranean is characterized by higher
320 salinity than the Atlantic) and continental shelf and slope dynamics. Distinct otolith
321 shapes have mostly evolved in association with how species not only process ambient
322 sound, but also how they balance and manoeuvre (reviewed in Schulz-Mirbach et al.,
323 2018), and are thus related with species life history and habitat use. Overall, the European
324 hake has longer otoliths, characteristic of fast-swimming species (Tuset et al., 2015),

326   when compared to blackbelly rosefish, a sedentary long-lived benthopelagic species

327   whose otolith shape had more intricate structure.

328

329   **Case study: *Helicolenus dactylopterus***

330   For the blackbelly rosefish, the *excisura* presented the highest morphological

331   differences between populations, especially for Azores and Madeira islands, while the

332   *rostrum* and *postrostrum* presented more subtle differences. Overall, six major groups

333   were identified: four in the Atlantic Ocean that included Madeira and Azores islands,

334   Portugal and Northern areas (Faroe, North Sea and Cantabrian Sea), and two in the

335   Mediterranean Sea: Strait of Sicily and Levantine Sea. A moderate degree of overlap was

336   found inside the North group, which is in agreement with the misclassification results,

337   where a relative connectivity among Faroe Island, North Sea and Cantabrian Sea

338   individuals was found, which could indicate a possible common population for this

339   species in those three areas, mostly possibly favoured by a continuity in the NE Atlantic

340   continental shelf and slope. The shape of otoliths from the Portuguese coast is also more

341   similar to the Mediterranean (more rounded profiles) than to the nearest northern

342   population in the Cantabrian Sea, which is consistent with previous findings based on

343   otolith chemistry (Swan et al., 2006) and age and growth studies (Sequeira et al., 2009).

344   Our results also suggest a clear separation between fish from the Portuguese

345   islands (Azores and Madeira) and mainland, in agreement with previous stock

346   identification studies by means of macroparasites (Sequeira et al., 2010), body geometric

347   morphometrics (Sequeira et al., 2011), genetics (Aboim et al., 2005), otolith shape

348   analysis (Neves et al., 2010) and otolith chemistry (Swan et al., 2006). In the

349   Mediterranean Sea, the overall classification error was low (24%) and though there was

350   a segregation between the two sampling locations (Strait of Sicily and Levantine Sea),

351   many samples from the Levantine Sea were misclassified. In part, this can be explained

352   by the low number of samples collected in this area, which may complicate otolith shape

353   comparisons between adjacent sites, but also by a high degree of connectivity between

354   these two Mediterranean populations, convergent evolution or even by a relatively

355   homogenous environment.

356   The broad scale morphological differences and the number of different units found

357   for blackbelly rosefish may be related with different environmental conditions (Sequeira

358   et al., 2012) and consequently different feeding ecology. This species uses a variety of

359   habitats with different characteristics (e.g., deep coral reefs, in association with burrows

360 or anemones) (Rodríguez-Mendoza et al., 2019) and feeds on different food types

361 depending on the habitat (Serrano et al., 2003), which influence the otolith shape

362 (Simoneau et al., 2000; Gauldie and Crampton, 2002; Cardinale et al., 2004). The fact

363 that the blackbelly rosefish is a rockfish with a sedentary nature, attached to the bottom

364 and mostly inactive (Uiblein et al., 2003; Aboim et al., 2005), with a typical sit-and-wait

365 predatory strategy (Uiblein et al., 2003) supports our findings. In fact, tagging studies

366 have recaptured the marked individuals at the exact same place after more than a year

367 (Aboim et al., 2005), leading to the conclusion that they present a high site fidelity. This

368 may lead to population isolation, reinforcing the idea that the surrounding habitat and

369 water circulation may contribute to the retention of larvae (Rogers, 1994), suggesting a

370 semi-enclosed habitat where each population lives and breeds.

371 Natural boundaries and oceanic fronts are important factors explaining population

372 isolation in this species. For instance, for horse mackerel (*T. trachurus*), the northwestern

373 tip of the Iberian Peninsula (Cape Finisterre), between the Cantabrian Sea and the

374 Portuguese coast was evidenced as a geographical boundary between two Atlantic stocks

375 (Abaunza et al., 2008), and the northern stock also included areas from the Cantabrian

376 Sea to Faroe Islands. This is consistent with our results, where a clear separation from the

377 populations north of the Cantabrian Sea (Faroe Islands, North Sea, and Cantabrian Sea)

378 was found in relation to mainland Portugal. Furthermore, Sequeira et al. (2011) obtained

379 similar results using geometric morphometric methods, where samples from Galicia

380 (Spain) and Peniche (Portugal) were distinct despite their relatively close geographical

381 distance. This can be explained by the existence of distinctive conditions (e.g., predation

382 risk, food resources, sediment type, water depth and temperature) that can affect fish

383 growth (Hayes et al., 1996). The Sicilian Channel has also been described as a physical

384 barrier between the western and eastern Mediterranean (Skliris, 2014), which may also

385 help explain the population structuring found for this area. The separation between

386 Atlantic and Mediterranean shape profiles is most probably the result of the Strait of

387 Gibraltar acting as a barrier to both adult and larval connectivity, as has been

388 demonstrated for other deep-sea fishes in the region (e.g., Catarino et al., 2017).

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394 **Case study:** *Merluccius merluccius*

395   In the case of European hake, once again, the *excisura* presented the greatest

396 morphological variation, but both the *postrostrum* and *rostrum* showed some degree of

397 differentiation, with the North Sea population showing a more elongated shape. A

398 detailed analysis with Atlantic and Mediterranean Sea separately demonstrated a more

399 cropped outline at the *excisura* for the Mediterranean Sea populations. When all the seven

400 sampled areas were analysed together, otolith shape could only unambiguously identify

401 fish populations from the Strait of Sicily, with a generally high overlap between the

402 remaining sites. Separating the Atlantic Ocean and Mediterranean Sea allowed for a better

403 understanding of the European hake population structure. Yet, there was still a high

404 overlap between the three areas sampled in the Atlantic (North Sea, Bay of Biscay, and

405 Portuguese coast), which suggests a high degree of connectivity between them. On the

406 other hand, all Mediterranean populations were easily distinguished. Only samples from

407 the Levantine Sea showed elevated error rates (63%), which again may result not only

408 from the lower number of samples collected, but also from convergent evolution or

409 relatively homogenous environment that led to similar otolith shapes between

410 geographically distant populations. Another possible explanation can be related with the

411 high connectivity with the Strait of Sicily population, since 8 individuals from a total of

412 24 from the Levantine Sea were misclassified in this population.

413   Several studies (e.g., Lundy et al., 1999; Castillo et al., 2004; Leone et al., 2019)

414 suggested a subdivision of the two established hake stocks in the Atlantic, divided at the

415 Cap Breton Canyon (Anon, 2004). In fact, significant genetic differences between

416 Norwegian and more southern samples in the Celtic Sea (Lundy et al., 1999) and eastern

417 Bay of Biscay (Leone et al., 2019) were found, as well as between the Bay of Biscay and

418 southern Portugal (Lundy et al., 1999), which can also be seen in this study by the weak

419 separation of North Sea from the Bay of Biscay and Portuguese samples (Fig. 3E).

420 However, Pita et al. (2011) found systematic grouping of Porcupine Bank (Irish shelf)

421 and Iberian Atlantic samples, suggesting the occurrence of gene flow, which contradicts

422 the idea that Cap Breton and ocean dynamics in the Bay of Biscay act as a connectivity

423 barrier, also reported for horse mackerel *T. trachurus* (Kasapidis and Magoulas, 2008).

424 Most recently, Westgaard et al. (2017) and Leone et al. (2019) described a distinction

425 between the Bay of Biscay and northern samples using SNP markers, which implied a

426 clear differentiation between the Norwegian Sea and southern Europe waters but not

427 between Bay of Biscay and northwestern Iberian Peninsula. This suggests that all

428   specimens are part of a single population with gene flow between them, which represents

429   a panmictic scenario. Similarly, our otolith shape analysis results support these findings,

430   with an overlap between hake samples from the Bay of Biscay and Portugal, and a slight

431   differentiation in the North Sea. Migration and consistent dynamics throughout its

432   biogeographical area (Pita et al., 2011) explain high levels of genetic homogeneity of

433   marine species over large ocean distances (Lessios et al., 1998), as is the case for

434   European hake in the North Atlantic, suggesting that Cap Breton Canyon may not

435   represent an effective barrier (Lundy et al., 1999; Pita et al., 2011) for this species.

436   The Mediterranean Sea European hake populations appeared as a separated

437   cluster, being congruent with the genetic distance found between Portuguese and

438   Mediterranean (Southeast Spain and Ionic Sea) populations in Castillo et al. (2004).

439   Additionally, this discontinuity between Atlantic and Mediterranean can be explained by

440   the Almerian-Oran front (Castillo et al., 2004; 2005). A more detailed analysis of the

441   population structure in the Mediterranean suggests a strong differentiation in three

442   separated units: Gulf of Lion, Strait of Sicily, and Adriatic Sea, despite the Levantine Sea

443   appearing as a fourth cluster, whose classification error was substantially high, as

444   discussed above. These findings are supported by previous studies based on genetic

445   features (Roldán et al., 1998; Milano et al., 2014), which subdivided the Mediterranean

446   stock into Western, Central and Eastern units, contrary to the findings of Morales-Nin et

447   al., (2022). In addition, the outputs of the present study are in general agreement with

448   Spedicato et al. (2022), who integrated both genetic and otolith shape data for European

449   hake in the Mediterranean Sea. The main difference was characterized by the unique

450   population in Adriatic and Strait of Sicily. A possible explanation for these results may

451   be related to adaptation to local conditions, such as water temperature and salinity

452   (Milano et al., 2014) and the existence of a physical barrier in the Sicily Channel, with

453   relatively shallow depth and particular circulation patterns that separates the western and

454   eastern Mediterranean basins (Skliris, 2014), as well as sample availability. Our results

455   based on otolith shape do not agree with a recent paper by Morales-Nin et al. (2022) that

456   suggests the existence of a continuous longitudinal gradient explained by the evolution

457   of otolith shape from the Western to Eastern parts the Mediterranean. This gradient could

458   be linked to environmental factors such as water temperature or salinity, which are

459   important in the Mediterranean Sea (Spedicato et al., 2022), and that could drive distinct

460   phenotypic responses. Also, the fact that the study by Morales-Nin et al., (2022) sampled

461 throughout the Mediterranean Sea, while in the present study we sampled only a few

462 distinctive points may be the reason for the different results between these two studies.

463   Some overlap between populations on both species can be explained by several

464 confounding effects such as sex and age (Simoneau et al.; 2000). However, in this study,

465 only the fish size was considered, which the Wavelet descriptors were normalized for.

466 The use of otoliths from fish caught in different sampling years is also a common practice

467 in these studies, as the year of capture has a negligible effect on otolith shape (e.g.,

468 Campana and Casselman, 1993; Denechaud et al., 2020). In fact, Bergenius et al. (2006)

469 mentioned the use of multiple cohorts obtained through several years as a good practice

470 in otolith shape analysis, to minimize possible confounding spatial variations. Also,

471 samples of European hake from Sicily and blackbelly rosefish from Madeira analysed in

472 the present study are the smallest and the largest (Table I), respectively, which could

473 explain their clear separation from the other samples in the CAP analysis (Fig. 3). Thus,

474 further analysis with complementary techniques such as genetics and otolith chemistry

475 are strongly suggested since they are less size-dependent. Considering that each method

476 presents limitations in its resolving power, two (or more) methods should be used together

477 to evaluate the levels of concordance and/or complement results (Begg and Waldman,

478 1999; Lleonart and Maynou, 2003; Abaunza et al., 2008; Welch et al., 2009; Tanner et

479 al., 2014; Welch et al., 2015; Reis-Santos et al., 2018).

480

481 **Conclusions**

482   In this paper, we demonstrated the differences in otolith mean shape for the

483 selected Atlantic and Mediterranean populations of blackbelly rosefish and European

484 hake, two fish species with high commercial interest. In both species, differentiation

485 between areas was mostly evident at the *excisura*, and more limited at the *rostrum* and

486 *postrostrum*. The blackbelly rosefish from the Azores and Madeira islands were

487 characterized by a more pronounced indentation at the *excisura*, while those from the

488 Mediterranean had a rounder shape. For the European hake, otoliths were longer and

489 narrower in the northernmost Atlantic populations.

490   Indeed, otolith shape can be regarded as a valid tool to identify population

491 structure in both species. The high level of overlap between some areas was improved by

492 dividing the sampling sites into Atlantic and Mediterranean contingents, assuming that

493 the Strait of Gibraltar is a significant barrier for fish connectivity, enabling a more

494 streamlined distinction of population units in each separate area. A lower dispersion of

495 European hake Atlantic samples when compared with blackbelly rosefish suggests a
496 higher degree of connectivity between these populations. The use of more samples with
497 smaller size range is strongly recommended to achieve the highest precision possible in
498 stock identification of these two species. Future approaches should integrate other
499 complementary natural markers such as genetics and/or otolith chemistry to further
500 improve the identification of marine fish populations and to reconcile the discrepancies
501 between biological and management units. Our results are fundamentally applicable in
502 fisheries management and contribute to a spatially explicit discrimination of marine fish
503 stock structure, with important benefits to researchers and stakeholders in the fisheries
504 sector.

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Highlights

- *Helicolenus dactylopterus* and *Merluccius merluccius* are fishery exploited species.

- We used otolith shape to define their stock structure in the Atlantic and Mediterranean.

- Wavelet analysis and canonical analysis of principal coordinates revealed diverse population units.

- Random forests showed that the discrimination power varied with species and locations.

- Results suggest a higher degree of connectivity of *M. merluccius* in the Atlantic.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: