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

1. 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

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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).

Fish stock identification can be achieved using different methods that include tagging and acoustic telemetry, demography and meristic data, otolith shape analysis, otolith chemistry and genetics (Hawkins et al., 2016). Otoliths have been among the most used structures as stock discrimination tools in fisheries science, by analysing their morphological and chemical properties (Campana and Casselman, 1993; Stransky et al., 2008; Milano et al., 2014; Westgaard et al., 2017; Leone et al., 2019; Morales-Nin et al., 2022). These structures are composed of calcium carbonate and are the first calcified structures formed in the ontogenic process. The three pairs of otoliths (sagittae, lapilli and asterisci) are found in the membranous labyrinth of the inner ear in fishes and play a key role postural equilibrium and hearing of fishes (Popper et al., 2005). The wide use of these structures is associated with the fact that they are metabolically inert, record fish growth and the chemical elements of the surrounding water (Campana, 1999).

Otolith shape analysis has been vastly used as a stock identification tool for many marine fishes (e.g., *Sebastes* spp (Stransky, 2005); *Trachurus trachurus* (Stransky et al., 2008); *Engraulis encrasicolus* (Bacha et al., 2014; Jemaa et al., 2015); *Sardina pilchardus* (Jemaa et al., 2015); *Clupea harengus* (Libungan and Pálson, 2015); *Trachurus picturatus* (Vasconcelos et al., 2018); *Genidens barbatus* (Maciel et al., 2021)). The use of this technique is possible given that otolith shape is species-specific and can be influenced by feeding behavior (Simoneau et al., 2000) and environmental conditions such as water temperature (Cardinale et al., 2004), depth (Gauldie and Crampton, 2002), and type of substrate (Mérigot et al., 2007), which can be stock specific (Aguëra and Brophy, 2011). Additionally, this methodology is economical and time-efficient compared with others, such as artificial tagging and tracking, genetics, and otolith chemistry, since in many cases otoliths are readily available from routine data collection sampling programs requiring no extra acquisition cost and effort.

The blackbelly rosefish *Helicolenus dactylopterus* (Delaroche, 1809) and European hake *Merluccius merluccius* (Linnaeus, 1758) are two sympatric commercially important species in European waters. The blackbelly rosefish is a benthopelagic slow-growing and long-lived marine fish species (Kelly et al., 1999; Massuti et al., 2000; Sequeira et al., 2009) that belongs to the family *Sebastidae*. It is commonly found at depths between 200 and 1000 m and presents a wide distribution from the Northeastern Atlantic to the Mediterranean (Froese and Pauly, 2023). Its slow-growth, late fecundity and high longevity in combination with a typical sit-and-wait predator behavior (Uiblein et al., 2003), make the blackbelly rosefish particularly vulnerable to overfishing (Pirrer et al., 2009) even when usually caught as a by-catch (Deval et al., 2018). In the Northeastern Atlantic Ocean, the blackbelly rosefish stock structure is still unknown (Neves et al., 2010) and there are currently no specific management measures or landing regulations in EC waters or in the NEAFC Regulatory Area, although this species is being assessed and managed among other deep-sea fish to the present (ICES, 2022; NEAFC, 2023). The European hake is a demersal species widely distributed in the Northeastern Atlantic Ocean and throughout the Mediterranean and Black Sea (Murua, 2010) between 30 and 1075 m depth, with adults found in a wider depth range from the shelf to the upper slope (Cartes et al., 2009). The European hake is a highly exploited species across multiple regions of the Northeast Atlantic (FAO, 2020; GFCM, 2012). Since 1978, the ICES Working Group of Southern Demersal Stocks (WGSSDS) distinguished two different stocks for the European hake - the northern and southern stocks, divided at the Cap Breton Canyon (Anon, 2004). Still, most stock identification studies have focused on the Northeast Atlantic, North Sea, and Baltic, while few have included the entire geographic range of the species distribution, i. e., including archipelagos in the middle of the Atlantic Ocean, such as the Azores, Madeira and the Faroe Islands, or the Mediterranean Sea and transition areas with the Atlantic Ocean (e.g., Neves et al., 2010; Tanner

et al., 2012, 2014; Morales-Nin et al., 2022). As such, the objectives of the present study were (1) to describe otolith shape variations of blackbelly rosefish and European hake in several areas of the Northeast Atlantic and Mediterranean, and (2) to assess the discrimination ability of otolith shape as a tool to delineate stock boundaries of these species. Here, we tested the hypothesis that differences in population connectivity due to oceanic boundaries are translated into distinct otolith shape, which allows discrimination between adjacent population units along the species' geographical distribution gradients.

2. Materials and methods

2.1. Sampling

Blackbelly rosefish and European hake adult samples were obtained from research surveys or commercial fisheries. The sampling areas were selected in accordance with the geographical distribution of each species, habitat, and oceanographic characteristics. Samples from both species were collected in the Northeastern Atlantic Ocean and Mediterranean Sea from 2017 to 2020, despite the blackbelly rosefish having a much more extensive distribution. To minimize possible interannual differences in otolith shape and the analysed total length, size ranges were limited (blackbelly rosefish from 10 to 40 cm; European hake from 20 to 65 cm) to minimize confounding morphometric effects of largely different otolith shapes between very small and very large fish (Table 1, Fig. 1). Collected fish were stored frozen until further analysis. Fish length (cm) was recorded (Table 1), and the sagittal otoliths were removed, air-dried, and stored in Eppendorf vials until further analysis, as described in the European sampling protocol of otoliths (Vitale et al., 2019).

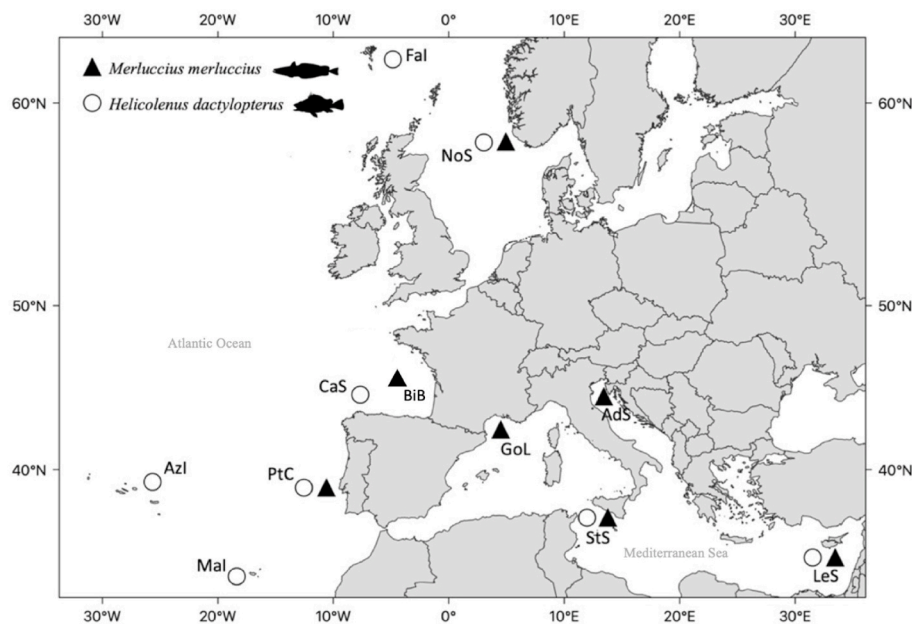
2.2. Otolith shape analysis

The first step was to carefully analyze 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, 2018; 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).

Otolith rotations were arranged horizontally along the longest axis and the areas were set equal to one, then, by drawing a polar axis, from the centroid to the 0° angle of the outline, the coordinates were collected and the radials were retracted with equidistant angles, from 0° to 360° angle (Libungan and Pálson, 2015). The deviation between the original outline and the reconstructed Wavelet otolith outline was used to determine the number of Wavelet coefficients. To visualize differences in the shape among the different sampling areas, a plot with the mean shape of each population was built through the reconstructed outlines of the normalized Wavelet coefficients. To estimate which otolith areas contributed the most to differentiate populations, Wavelet coefficients means, and respective standard deviation were plotted for all the

Table 1Number of individuals, mean length (cm) and corresponding standard deviation per sampling area of *H. dactylopterus* and *M. merluccius* used for shape analysis.

<i>Helicolenus dactylopterus</i>					<i>Merluccius merluccius</i>					
Basin	Sampling area	Area Code	N° of otoliths	Mean length (cm)	Basin	Sampling area	Area Code	N° 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 Portuguese Coast	Bay of Biscay	BiB	116	44.2 ± 14.9
	Cantabrian Sea	CaS	35	20.9 ± 4.7			Portuguese Coast	PtC	79	40.7 ± 7.5
	Azores Islands	AzI	36	30.7 ± 1.8			Mediterranean Sea	Golf of Lion	GoL	47
	Madeira Islands	Mal	44	38.4 ± 2.4		Adriatic Sea		AdS	48	23.6 ± 3.8
Mediterranean Sea	Portuguese Coast	PtC	57	26.5 ± 3.9	Mediterranean Sea	Strait of Sicily	StS	49	19.6 ± 1.4	
	Strait of Sicily	StS	40	18.7 ± 4.9		Levantine Sea	LeS	24	31.3 ± 2.6	
	Levantine Sea	LeS	26	18.0 ± 5.1						

**Fig. 1.** Map of sampling locations for *H. dactylopterus* (circles) and *M. merluccius* (triangles) in the Northeastern Atlantic Ocean and Mediterranean Sea. See Table I for area codes.

otoliths in the analysis (Libungan and Pálson, 2015).

An ANOVA-like permutation test and a Canonical Analysis of Principal Coordinates (CAP) was performed using the vegan package (Oksanen et al., 2022), and the capscale function to analyze the shape variation among the different sampling sites for each species separately (following Libungan and Pálson, 2015). Classification of individuals to their sampling area based on Wavelet coefficients was performed using random forest classification (Breiman, 2001) implemented in the randomForest package (Liaw and Wiener, 2002). Average out-of-bag (OOB) classification error was determined using 2000 trees and the number of variables tried at each split was 11.

3. Results

The average shape outline reconstruction plot based on all otoliths showed clear differences among populations for both species (Fig. 2A; D), as reflected by the high degree of variation in the Wavelet coefficients between populations. For better visualization, a separate analysis was performed for the Atlantic (Fig. 2B; E) and the Mediterranean populations (Fig. 2C; F). Further analysis showed that for blackbelly rosefish, the populations from Madeira and Azores differed the most from the other sampling areas, with the otolith's outlines moving inwards to the otolith centroid between 180 and 270° at the *excisura*

(Fig. 2A). For European hake, the North Sea and Portuguese populations showed a higher degree of differentiation from the rest, with an inward shift of the otolith contour at around 270° (Fig. 2D). More detailed examination with separated Atlantic Ocean and Mediterranean Sea populations showed more subtle differences within these two areas (Fig. 2E; F).

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

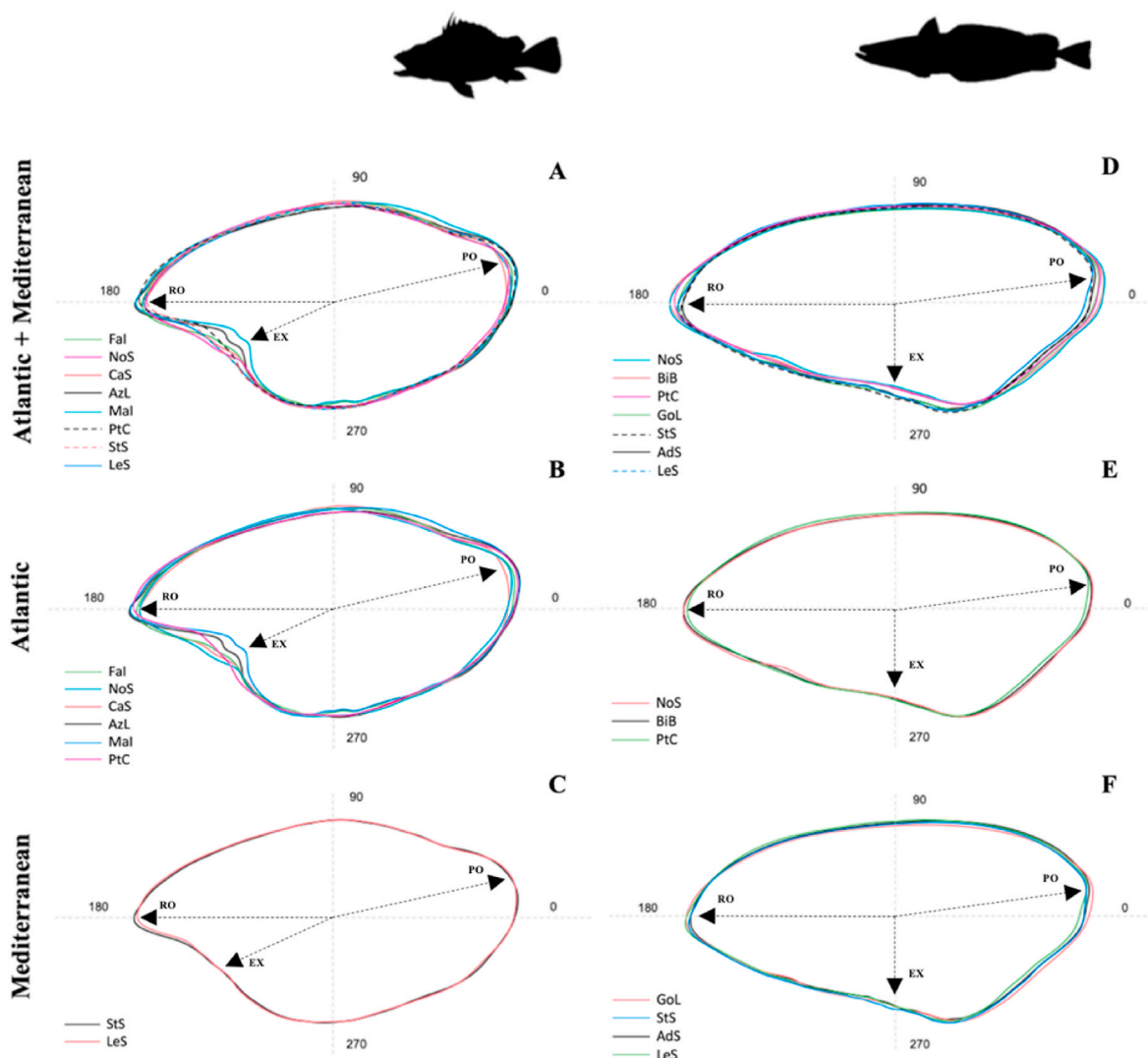


Fig. 2. Otoliths average shapes obtained by Wavelet descriptors for *H. dactylopterus* (left panels) and *M. merluccius* (right panels) for all sampled populations (A, D), Atlantic Ocean (B, E) and Mediterranean Sea (C, F). The excisura (EX), rostrum (RO) and postrostrum (PO) are highlighted in each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

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.

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 only the population from the Strait of Sicily was clearly distinguished (Fig. 3D). When analysing the Atlantic Ocean and Mediterranean Sea populations separately, five clusters were established: an indistinct Atlantic cluster, composed of North Sea, Bay of Biscay, and Portuguese samples (Fig. 3E), and each of the four Mediterranean areas: Gulf of Lion, Strait of Sicily, Adriatic Sea, and Levantine Sea (Fig. 3F).

For blackbelly rosefish, random forest classification produced an error rate of 36.6%, with the Faroe Islands and Levantine Sea populations showing the highest error rates (87% and 81%, respectively), and Madeira Islands and North Sea showing the lowest error rates (16% and 5%, respectively) (Table II). When separating sampling areas into Atlantic Ocean and Mediterranean Sea, a reduction in classification error was observed (27.2% and 24.2%, respectively), but both the Faroe Islands and Levantine Sea remained the areas with the highest classification error (87% and 46%, respectively) (Table III). For European hake, the overall analysis error was 37.7%, with the highest misclassification rates in the Levantine Sea (83%), Portugal (64%) and Gulf of Lion (57%)

and the lowest in the Adriatic (8%), Sicily (16%) and Bay of Biscay (18%) (Table IV). No improvement was observed when only Atlantic Ocean samples were analysed together (39.1%), but a clear differentiation within the Mediterranean region was possible (19.7%) (Table V).

4. Discussion

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

The differences in relation to the most variable areas in the otolith shape found between the large geographical areas (Atlantic Ocean and Mediterranean Sea) can be explained by genetic factors (Cardinale et al., 2004), since different deposition of the otolith increments due to different growth rates can affect the otolith structure (Geffen, 1982;

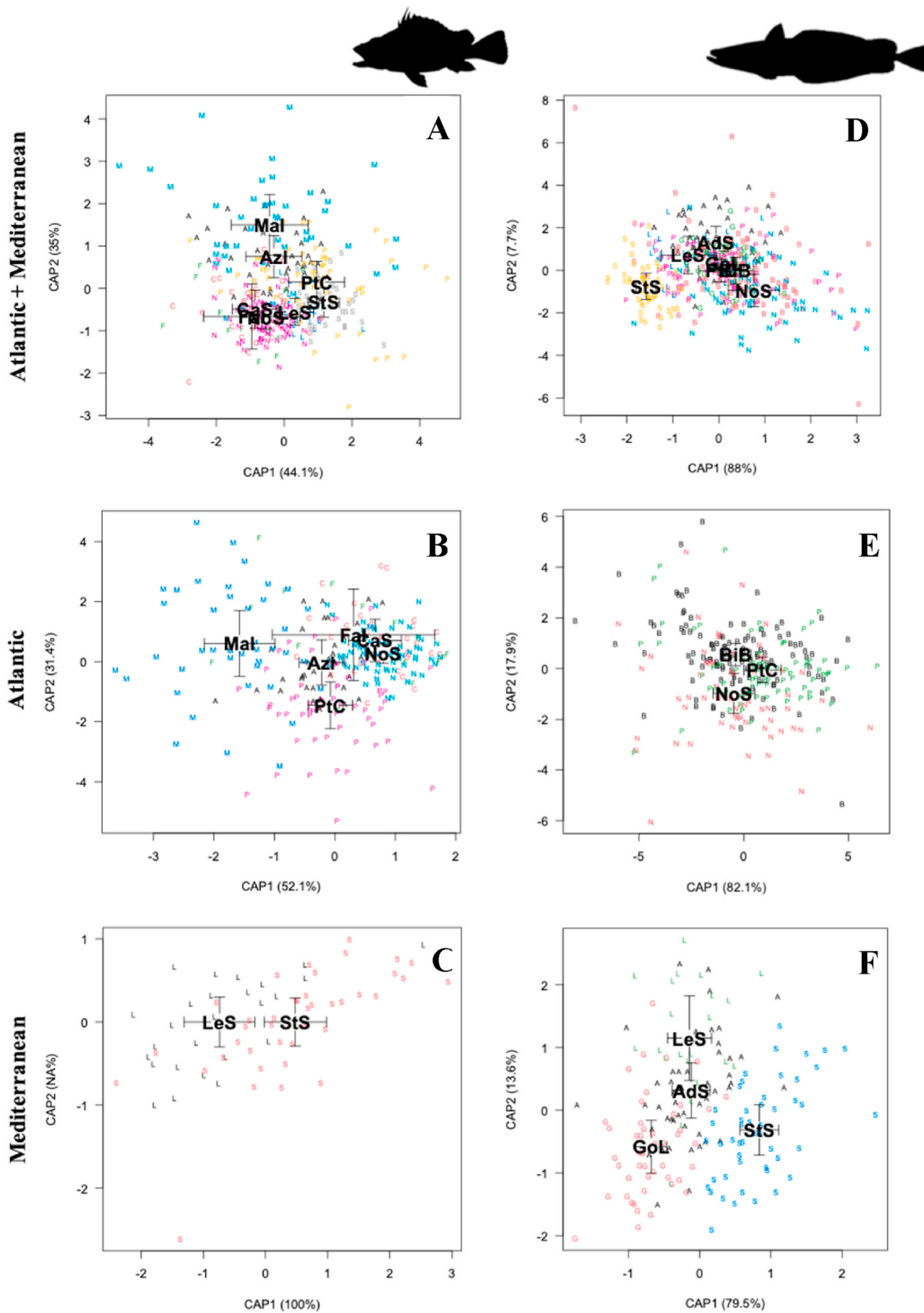


Fig. 3. Canonical Analysis of Principal Coordinates of normalized Wavelet descriptors of the otolith shape of *H. dactylopterus* (left panels) and *M. merluccius* (right panels) from all sampled populations (A, D), Atlantic Ocean (B, E) and Mediterranean Sea (C, F).

Table 2

Random Forest confusion matrix for all populations in the study of *H. dactylopterus*. Overall classification error rate was 36.6% for all populations. The horizontal lines show the number of otoliths from each population assigned to one or more areas. See Table I for area codes.

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

Table 3

Random Forest confusion matrix for the Atlantic Ocean and Mediterranean Sea populations of *H. dactylopterus*. Overall classification error rate of all populations was 27.2% and 24.2%, respectively. The horizontal lines show the number of otoliths from each population assigned to one or more areas. See 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%	StS	12	14	10%
	NoS	0	64	2	1	0	0	67	5%				
	CaS	0	8	21	4	2	0	35	40%				
	PtC	0	0	1	42	9	5	57	26%	LeS	36	4	46%
	AzI	0	1	2	12	17	4	36	53%				
	MaI	0	0	1	1	3	39	44	11%				

Table 4

Random Forest confusion matrix for all populations in the study of *M. merluccius*. Overall classification error rate of all populations was 37.7% for all populations. The horizontal lines show the number of otoliths from each population assigned to one or more areas. See Table I for area codes.

		Predicted areas										Total	Classification Error
		NoS	BiB	PtC	GoL	AdS	StS	LeS					
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%		

Table 5

Random Forest confusion matrix for the Atlantic Ocean and Mediterranean Sea populations of *M. merluccius*. Overall classification error rate of all populations was 39.1% and 19.7%, respectively. The horizontal lines show the number of otoliths from each population assigned to one or more areas. See Table I for area codes.

		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%
	PtC	8	40	31	79	61%	StS	4	0	44	1	49	10%
							LeS	2	3	10	9	24	63%

Folkvord et al., 2000; Feet et al., 2002; Fox et al., 2003), and may also reflect different environmental conditions (Campana and Neilson, 1985; Lombarte and Leonart, 1993; Cardinale et al., 2004; Vignon, 2012). Indeed, the sampling areas in this study present a latitudinal cline in sea water temperature from the more northern areas (Faroe Islands for blackbelly rosefish; North Sea for European hake) to the southern ones (Mediterranean Sea), as well as in salinity (the Mediterranean is characterized by higher salinity than the Atlantic) and continental shelf and slope dynamics. Distinct otolith shapes have mostly evolved in association with how species not only process ambient sound, but also how they balance and manoeuvre (reviewed in Schulz-Mirbach et al., 2019),

and are thus related with species life history and habitat use. Overall, the European hake has longer otoliths, characteristic of fast-swimming species (Tuset et al., 2015), when compared to blackbelly rosefish, a sedentary long-lived benthopelagic species whose otolith shape had more intricate structure.

5. Case study: *Helicolenus dactylopterus*

For the blackbelly rosefish, the *excisura* presented the highest morphological differences between populations, especially for Azores and Madeira islands, while the *rostrum* and *postrostrum* presented more

subtle differences. Overall, six major groups were identified: four in the Atlantic Ocean that included Madeira and Azores islands, Portugal and Northern areas (Faroe, North Sea and Cantabrian Sea), and two in the Mediterranean Sea: Strait of Sicily and Levantine Sea. A moderate degree of overlap was found inside the North group, which is in agreement with the misclassification results, where a relative connectivity among Faroe Island, North Sea and Cantabrian Sea individuals was found, which could indicate a possible common population for this species in those three areas, mostly possibly favoured by a continuity in the NE Atlantic continental shelf and slope. The shape of otoliths from the Portuguese coast is also more similar to the Mediterranean (more rounded profiles) than to the nearest northern population in the Cantabrian Sea, which is consistent with previous findings based on otolith chemistry (Swan et al., 2006) and age and growth studies (Sequeira et al., 2009).

Our results also suggest a clear separation between fish from the Portuguese islands (Azores and Madeira) and mainland, in agreement with previous stock identification studies by means of macroparasites (Sequeira et al., 2010), body geometric morphometrics (Sequeira et al., 2011), genetics (Aboim et al., 2005), otolith shape analysis (Neves et al., 2010) and otolith chemistry (Swan et al., 2006). In the Mediterranean Sea, the overall classification error was low (24%) and though there was a segregation between the two sampling locations (Strait of Sicily and Levantine Sea), many samples from the Levantine Sea were misclassified. In part, this can be explained by the low number of samples collected in this area, which may complicate otolith shape comparisons between adjacent sites, but also by a high degree of connectivity between these two Mediterranean populations, convergent evolution or even by a relatively homogenous environment.

The broad scale morphological differences and the number of different units found for blackbelly rosefish may be related with different environmental conditions (Sequeira et al., 2012) and consequently different feeding ecology. This species uses a variety of habitats with different characteristics (e.g., deep coral reefs, in association with burrows or anemones) (Rodríguez-Mendoza et al., 2019) and feeds on different food types depending on the habitat (Serrano et al., 2003), which influence the otolith shape (Simoneau et al., 2000; Gaudie and Crampton, 2002; Cardinale et al., 2004). The fact that the blackbelly rosefish is a rockfish with a sedentary nature, attached to the bottom and mostly inactive (Uiblein et al., 2003; Aboim et al., 2005), with a typical sit-and-wait predatory strategy (Uiblein et al., 2003) supports our findings. In fact, tagging studies have recaptured the marked individuals at the exact same place after more than a year (Aboim et al., 2005), leading to the conclusion that they present a high site fidelity. This may lead to population isolation, reinforcing the idea that the surrounding habitat and water circulation may contribute to the retention of larvae (Rogers, 1994), suggesting a semi-enclosed habitat where each population lives and breeds.

Natural boundaries and oceanic fronts are important factors explaining population isolation in this species. For instance, for horse mackerel (*T. trachurus*), the northwestern tip of the Iberian Peninsula (Cape Finisterre), between the Cantabrian Sea and the Portuguese coast was evidenced as a geographical boundary between two Atlantic stocks (Abaunza et al., 2008), and the northern stock also included areas from the Cantabrian Sea to Faroe Islands. This is consistent with our results, where a clear separation from the populations north of the Cantabrian Sea (Faroe Islands, North Sea, and Cantabrian Sea) was found in relation to mainland Portugal. Furthermore, Sequeira et al. (2011) obtained similar results using geometric morphometric methods, where samples from Galicia (Spain) and Peniche (Portugal) were distinct despite their relatively close geographical distance. This can be explained by the existence of distinctive conditions (e.g., predation risk, food resources, sediment type, water depth and temperature) that can affect fish growth (Hayes et al., 1996). The Sicilian Channel has also been described as a physical barrier between the western and eastern Mediterranean (Skliaris, 2014), which may also help explain the population structuring found

for this area. The separation between Atlantic and Mediterranean shape profiles is most probably the result of the Strait of Gibraltar acting as a barrier to both adult and larval connectivity, as has been demonstrated for other deep-sea fishes in the region (e.g., Catarino et al., 2017).

6. Case study: *Merluccius merluccius*

In the case of European hake, once again, the *excisura* presented the greatest morphological variation, but both the *postrostrum* and *rostrum* showed some degree of differentiation, with the North Sea population showing a more elongated shape. A detailed analysis with Atlantic and Mediterranean Sea separately demonstrated a more cropped outline at the *excisura* for the Mediterranean Sea populations. When all the seven sampled areas were analysed together, otolith shape could only unambiguously identify fish populations from the Strait of Sicily, with a generally high overlap between the remaining sites. Separating the Atlantic Ocean and Mediterranean Sea allowed for a better understanding of the European hake population structure. Yet, there was still a high overlap between the three areas sampled in the Atlantic (North Sea, Bay of Biscay, and Portuguese coast), which suggests a high degree of connectivity between them. On the other hand, all Mediterranean populations were easily distinguished. Only samples from the Levantine Sea showed elevated error rates (63%), which again may result not only from the lower number of samples collected, but also from convergent evolution or relatively homogenous environment that led to similar otolith shapes between geographically distant populations. Another possible explanation can be related with the high connectivity with the Strait of Sicily population, since 8 individuals from a total of 24 from the Levantine Sea were misclassified in this population.

Several studies (e.g., Lundy et al., 1999; Castillo et al., 2004; Leone et al., 2019) suggested a subdivision of the two established hake stocks in the Atlantic, divided at the Cap Breton Canyon (Anon, 2004). In fact, significant genetic differences between Norwegian and more southern samples in the Celtic Sea (Lundy et al., 1999) and eastern Bay of Biscay (Leone et al., 2019) were found, as well as between the Bay of Biscay and southern Portugal (Lundy et al., 1999), which can also be seen in this study by the weak separation of North Sea from the Bay of Biscay and Portuguese samples (Fig. 3E). However, Pita et al. (2011) found systematic grouping of Porcupine Bank (Irish shelf) and Iberian Atlantic samples, suggesting the occurrence of gene flow, which contradicts the idea that Cap Breton and ocean dynamics in the Bay of Biscay act as a connectivity barrier, also reported for horse mackerel *T. trachurus* (Kasapidis and Magoulas, 2008). Most recently, Westgaard et al. (2017) and Leone et al. (2019) described a distinction between the Bay of Biscay and northern samples using SNP markers, which implied a clear differentiation between the Norwegian Sea and southern Europe waters but not between Bay of Biscay and northwestern Iberian Peninsula. This suggests that all specimens are part of a single population with gene flow between them, which represents a panmictic scenario. Similarly, our otolith shape analysis results support these findings, with an overlap between hake samples from the Bay of Biscay and Portugal, and a slight differentiation in the North Sea. Migration and consistent dynamics throughout its biogeographical area (Pita et al., 2011) explain high levels of genetic homogeneity of marine species over large ocean distances (Lessios et al., 1998), as is the case for European hake in the North Atlantic, suggesting that Cap Breton Canyon may not represent an effective barrier (Lundy et al., 1999; Pita et al., 2011) for this species.

The Mediterranean Sea European hake populations appeared as a separated cluster, being congruent with the genetic distance found between Portuguese and Mediterranean (Southeast Spain and Ionic Sea) populations in Castillo et al. (2004). Additionally, this discontinuity between Atlantic and Mediterranean can be explained by the Almerian-Oran front (Castillo et al., 2004, 2005). A more detailed analysis of the population structure in the Mediterranean suggests a strong differentiation in three separated units: Gulf of Lion, Strait of Sicily, and Adriatic Sea, despite the Levantine Sea appearing as a fourth

cluster, whose classification error was substantially high, as discussed above. These findings are supported by previous studies based on genetic features (Roldán et al., 1998; Milano et al., 2014), which subdivided the Mediterranean stock into Western, Central and Eastern units, contrary to the findings of Morales-Nin et al. (2022). In addition, the outputs of the present study are in general agreement with Spedicato et al. (2022), who integrated both genetic and otolith shape data for European hake in the Mediterranean Sea. The main difference was characterized by the unique population in Adriatic and Strait of Sicily. A possible explanation for these results may be related to adaptation to local conditions, such as water temperature and salinity (Milano et al., 2014) and the existence of a physical barrier in the Sicily Channel, with relatively shallow depth and particular circulation patterns that separates the western and eastern Mediterranean basins (Skirris, 2014), as well as sample availability. Our results based on otolith shape do not agree with a recent paper by Morales-Nin et al. (2022) that suggests the existence of a continuous longitudinal gradient explained by the evolution of otolith shape from the Western to Eastern parts the Mediterranean. This gradient could be linked to environmental factors such as water temperature or salinity, which are important in the Mediterranean Sea (Spedicato et al., 2022), and that could drive distinct phenotypic responses. Also, the fact that the study by Morales-Nin et al. (2022) sampled throughout the Mediterranean Sea, while in the present study we sampled only a few distinctive points may be the reason for the different results between these two studies.

Some overlap between populations on both species can be explained by several confounding effects such as sex and age (Simoneau et al., 2000). However, in this study, only the fish size was considered, which the Wavelet descriptors were normalized for. The use of otoliths from fish caught in different sampling years is also a common practice in these studies, as the year of capture has a negligible effect on otolith shape (e.g., Campana and Casselman, 1993; Denechaud et al., 2020). In fact, Bergenius et al. (2006) mentioned the use of multiple cohorts obtained through several years as a good practice in otolith shape analysis, to minimize possible confounding spatial variations. Also, samples of European hake from Sicily and blackbelly rosefish from Madeira analysed in the present study are the smallest and the largest (Table I), respectively, which could explain their clear separation from the other samples in the CAP analysis (Fig. 3). Thus, further analysis with complementary techniques such as genetics and otolith chemistry are strongly suggested since they are less size-dependent. Considering that each method presents limitations in its resolving power, two (or more) methods should be used together to evaluate the levels of concordance and/or complement results (Begg and Waldman, 1999; Leonart and Maynou, 2003; Abaunza et al., 2008; Welch et al., 2009, 2015; Tanner et al., 2014; Reis-Santos et al., 2018).

7. Conclusions

In this paper, we demonstrated the differences in otolith mean shape for the selected Atlantic and Mediterranean populations of blackbelly rosefish and European hake, two fish species with high commercial interest. In both species, differentiation between areas was mostly evident at the *excisura*, and more limited at the *rostrum* and *postrostrum*. The blackbelly rosefish from the Azores and Madeira islands were characterized by a more pronounced indentation at the *excisura*, while those from the Mediterranean had a rounder shape. For the European hake, otoliths were longer and narrower in the northernmost Atlantic populations.

Indeed, otolith shape can be regarded as a valid tool to identify population structure in both species. The high level of overlap between some areas was improved by dividing the sampling sites into Atlantic and Mediterranean contingents, assuming that the Strait of Gibraltar is a significant barrier for fish connectivity, enabling a more streamlined distinction of population units in each separate area. A lower dispersion of European hake Atlantic samples when compared with blackbelly

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

CRedit authorship contribution statement

Ana Vaz: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Milene Alexandra Guerreiro:** Writing – review & editing, Methodology, Investigation. **Jorge Landa:** Writing – review & editing, Methodology, Investigation. **Olsen Hannipoula:** Writing – review & editing, Methodology, Investigation. **Ioannis Thasitis:** Writing – review & editing, Methodology, Investigation. **Giuseppe Scarcella:** Writing – review & editing, Methodology, Investigation. **Laura Sabatini:** Writing – review & editing, Methodology, Investigation. **Sergio Vitale:** Writing – review & editing, Methodology, Investigation. **Estanis Mugerza:** Writing – review & editing, Methodology, Investigation. **Kélig Mahé:** Writing – review & editing, Methodology, Investigation. **Patrick Reis-Santos:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Susanne E. Tanner:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Christoph Stransky:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Miguel Pardal:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Filipe Martinho:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

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.

Data availability

Data will be made available on request.

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