

Original Article

The multifaceted diversification of the sagitta otolith across the fish tree of life

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ABSTRACT

Otoliths of actinopterygians are calcified structures playing a key role in hearing and equilibrium functions. To understand their morphological diversification, we quantified the shape of otoliths in both lateral and dorsal view from 697 and 323 species, respectively, using geometric morphometrics. We then combined form (i.e. size and shape) information with ecological data and phylogenetically informed comparative methods to test our hypotheses. Initially, the exploration of morphospaces revealed that the main variations are related to sulcus acusticus shape, elongation and lateral curvature. We also found strong integration between otolith and sulcus shape, suggesting that they are closely mirroring each other, reinforcing a shape-dependent mechanism crucial for otolith motion relative to its epithelium and validating the functional significance of otolith morphology in auditory and vestibular processes. After revealing that otolith shape and size retained a low phylogenetic signal, we showed that the disparity of otolith size and shape is decoupled from order age and from the level of functional diversity across clades. Finally, some traits in otolith disparity are correlated with their morphological evolutionary rate and the order speciation rate. Overall, we observed that the pattern of diversification of otoliths across the fish tree of life is highly complex and likely to be multifactorial.

Keywords: ray-finned fishes; Actinopterygii; inner ear; otolith; audition; vestibular function; morphology; geometric morphometrics; disparity; evolutionary dynamics

INTRODUCTION

The vertebrate inner ear is a multifunctional mechanosensory organ with two primary functions. First, the inner ear performs a hearing function by detecting sounds that are crucial for communication and environmental awareness. Second, it ensures a vestibular function, essential for detecting motion, coordinating movements, and maintaining stability and balance across various positions and activities (Torres and Giraldez 1998, Kasumyan 2004, Popper and Fay 2011, Pfaff *et al.* 2019). The structure of the fish inner ear is remarkably conserved across taxa (Platt and Popper 1981, Ladich and Schulz-Mirbach 2016).

From an anatomical point of view, it can be divided into two main parts. First, the pars superior is characterized by three semicircular canals oriented in the three different perpendicular planes along with the otolithic organ utricle, which is associated with the otolith lapillus. Second, the pars inferior consists of two otolithic organs, the saccule and the lagena. They are, respectively, associated with the otoliths sagitta and asteriscus, formed by aggregated calcium carbonate crystals (aragonite or vaterite) and a mucoprotein (Platt and Popper 1981, Morales-Nin 1987, Popper *et al.* 2005, Schulz-Mirbach *et al.* 2019). About three times denser than the fish and the surrounding water, these

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inertial masses move relative to a sensory epithelium found within the sac, the macula, when stimulated by the components of an acoustic field (Schulz-Mirbach *et al.* 2019). For most fish species, the sagitta is the largest otolith, except in otophysans, in which the asteriscus holds that distinction (Popper and Platt 1983, Ladich and Schulz-Mirbach 2016). As first emphasized by Koken (1884), the sagitta shows the largest morphological diversity, recognized as species specific, supporting its wide use for identification purposes (Nolf 1985, Härkönen 1986, Smale *et al.* 1995, Rivaton and Bourret 1999, Schwarzhans 2003, 2007, 2018, Campana 2004, Furlani *et al.* 2007, Tuset *et al.* 2008, Baremore and Bethea 2010, Lin and Chang 2012, Sadighzadeh *et al.* 2012, Rossi-Wongtschowski *et al.* 2014, Volpedo *et al.* 2018). A distinctive feature of the sagitta is the sulcus acusticus, an imprint of the sensory macula attachment located on the mesial face (Platt and Popper 1981, Gauldie 1988, Lombarte and Fortuño 1992). Many studies have associated this otolith with hearing functions (Parker 1910, Coombs and Popper 1979, Platt and Popper 1981, Saidel and Popper 1983, Lu and Xu 2002, Lu *et al.* 2002, Ramcharitar *et al.* 2004, Braun and Grande 2008, Deng *et al.* 2013), although it might also play a key role in balance and swimming abilities (Popper *et al.* 2005).

As with all functional structures in an organism, the stato-acoustic system is shaped and structured to balance various needs and functions (Thomas 1979, Bock 1991, Parmentier *et al.* 2001). The shape and size of fish sagittae undergo changes during ontogeny, acquiring species-specific features and functions at a relatively early age (Nolf 1985, Lombarte and Castellón 1991, Paxton 2000, Campana 2004). The absolute size of adult fish sagittae ranges from 0.1 (pipefishes, Syngnathidae) to 31.4 mm (drums, Sciaenidae) (Paxton 2000, Lombarte *et al.* 2006). Although it is tempting to think that the development of acoustic communication is the main driver of their disparity, other factors can influence the morphological diversity of sagittae. For instance, the sound-producing Sciaenidae, Myripristinae, and Gadiformes have large and thick otoliths with modified morphology (Nolf 1993, Paxton 2000). The sciaenids all have enlarged, thick sagittae, whereas the silver perch (*Bairdiella chrysoura*) is unique in having a triangular sagitta closely associated with a large asteriscus, along with the broadest hearing bandwidth known in Sciaenidae (Ramcharitar *et al.* 2002, Ramcharitar *et al.* 2004). Consistently, this overall triangular shape is convergent in the most acoustically specialized holocentrid subfamily, Myripristinae, differing from the typical percomorph morphology (Coombs and Popper 1979, Cooney and Thomas 2021, Andrews *et al.* 2023). Additional lifestyle traits also seem to support the acoustic influence on sagitta morphology. For instance, otolith size has been positively correlated with depth and suggested to compensate for light reduction (Wilson 1985, Lombarte and Cruz 2007). Moreover, bioluminescent species, which typically thrive in low-light environments, have also been found to have relatively larger otoliths in comparison to their non-bioluminescent counterparts (Paxton 2000). Finally, fish species harbouring bright colours and using visual cues for communication, such as wrasses (Labridae), have relatively small otoliths, supporting the idea of a sensory organ trade-off (Cruz and Lombarte 2004). Depth (Wilson 1985, Schwarzhans and Geringer 2023), habitat, and lifestyle (Nolf 1993, Parmentier *et al.* 2001, Volpedo and Echeverría 2003, Volpedo *et al.* 2008,

Kéver *et al.* 2014), in addition to feeding habits (Lombarte *et al.* 2010, Tuset *et al.* 2015, Assis *et al.* 2020), are additional examples of factors reported to influence the form of the sagitta.

With ~34 190 valid species, actinopterygian fishes include >50% of the vertebrate diversity (Betancur-R *et al.* 2017, Dornburg and Near 2021). They are characterized by a tremendous morphological disparity, with a large diversity of body plans and adult body sizes (Price *et al.* 2019, Friedman 2022). Over the last 20 years, many studies have tested various hypotheses about the factors driving their phenotypic diversity, including body shape (Friedman *et al.* 2020, Rincon-Sandoval *et al.* 2020, Martinez *et al.* 2021, Corn *et al.* 2022, Miller *et al.* 2022), fin shape (Wainwright *et al.* 2002, Collar *et al.* 2007), skull morphology (Collar *et al.* 2007, Evans *et al.* 2023), jaw apparatus (Wainwright and Bellwood 2002, Burress 2016), and sensory structures, such as eye size (Schmitz and Wainwright 2011, Caves *et al.* 2017). Surprisingly, macroevolutionary studies exploring the tempo and the mode of fish otolith diversification at a large phylogenetic scale are currently lacking (Popper *et al.* 2005, Ladich and Schulz-Mirbach 2016, Schulz-Mirbach *et al.* 2019). Schwarzhans *et al.* (2017) presented the first attempt to link the past (from the Mesozoic) and present otolith diversity in relationship to major events of teleost radiations. However, few studies have compared otolith morphology at the family or order levels (Schwarzhans 1978, Cruz and Lombarte 2004) while considering phylogeny (Lombarte *et al.* 2010, Tuset *et al.* 2016a, Teimori *et al.* 2019), and none has used modern phylogenetic comparative analyses.

In the present work, we examined the sagittae (hereafter referred to as otoliths) from 697 fish species, encompassing all major actinopterygian lineages, to unravel major trends in the morphological evolution of otoliths at a broad phylogenetic scale. We quantified their morphology using landmark-based geometric morphometrics on lateral (sulcus acusticus side) and dorsal views. After exploring the otolith morphospace, we examined how size, the shape of the sensory-associated sulcus, and species phylogenetic relatedness are correlated with their shape. We also combined size and shape data with a set of diverse phylogenetically informed methods to test how much the evolutionary dynamics of otoliths have remained constant through time. We assessed the evolutionary dynamics of otolith diversification at the order level by testing linear relationships among order age, speciation rate, otolith disparity, and morphological evolutionary rate. Lastly, suspecting a positive relationship, we tested the hypothesis of a link between the functional diversity in each fish order and their disparity in otolith morphology. Overall, we found that the morphological evolution of otoliths was more complex than expected and non-random, a pattern likely to be driven by clade-specific morphofunctional constraints.

MATERIALS AND METHODS

Assembling the comparative dataset

Pictures of left otoliths in lateral view (sulcus facing) (Supporting Information, Fig. S1A) of 697 species of actinopterygians (ray-finned fishes) were gathered from the AFORO (Anàlisi de FORMes d'Otòlits) repository (607 species) (Lombarte *et al.* 2006) and from the work by Nolf (2013) (90 species).

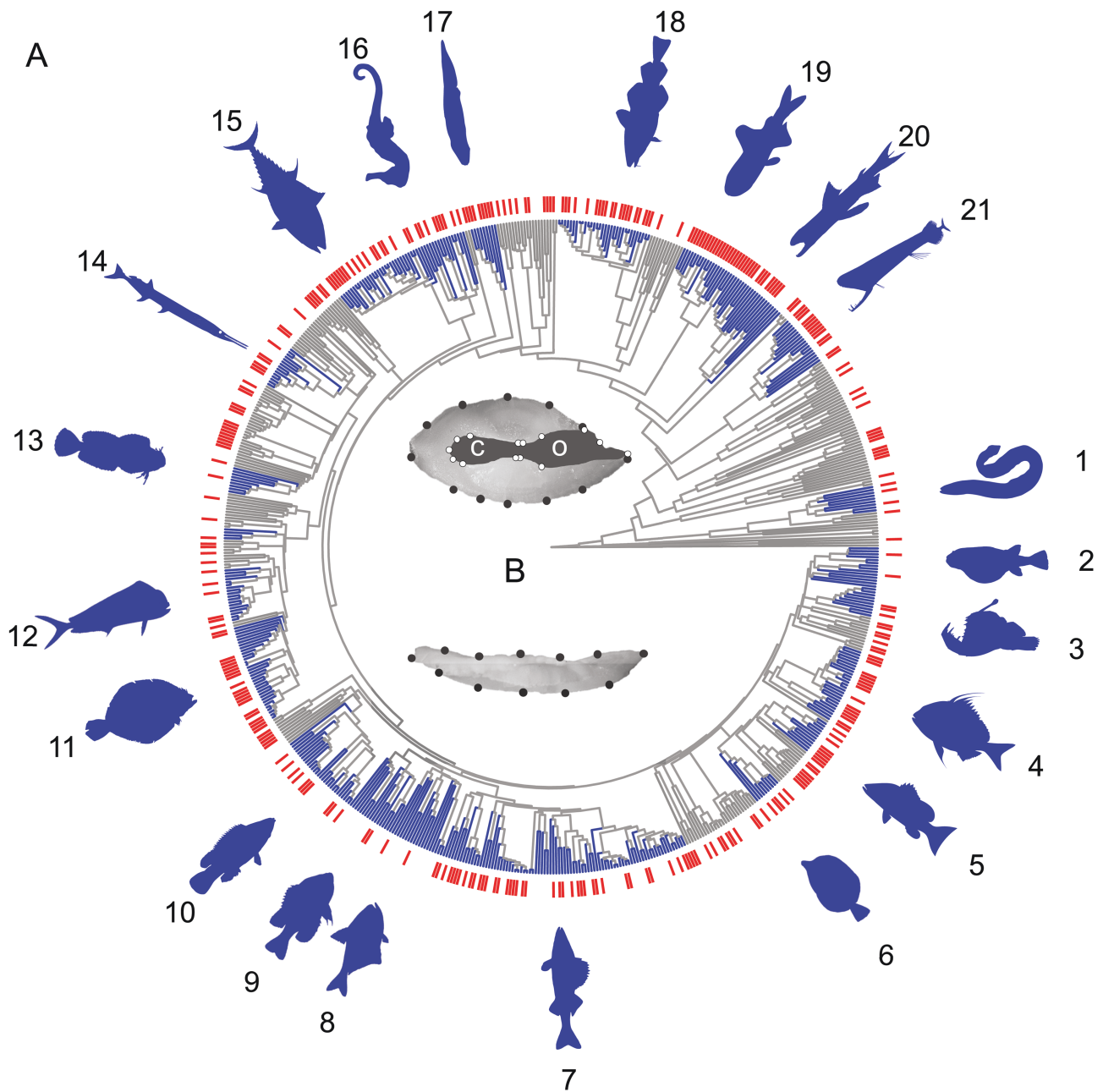


Figure 1. Morphology of otoliths studied at a large phylogenetic scale. A, phylogenetic tree of species sampled for the present study, highlighting the 21 orders studied in the context of evolutionary dynamics (in blue) and the specimens covered in dorsal view (in red) (for complete dataset description, see [Supporting Information, Tables S1 and S2](#)). 1, Anguilliformes; 2, Tetraodontiformes; 3, Lophiiformes; 4, Spariformes; 5, Lutjaniformes; 6, Acanthuriformes; 7, Perciformes; 8, Pempheeriformes; 9, Centrarchiformes; 10, Labriformes; 11, Pleuronectiformes; 12, Carangiformes; 13, Blenniiformes; 14, Beloniformes; 15, Scombriformes; 16, Syngnathiformes; 17, Ophidiiformes; 18, Gadiformes; 19, Myctophiformes; 20, Aulopiformes; 21, Stomiiformes. Silhouettes come from www.phylopic.org and are used under a Creative Commons CC0 1.0 licence. B, example of quantification of otolith shape for *Pontinus furcirhinus*, showing the landmark data for left lateral (26) and dorsal (12) views. The sulcus is highlighted in dark grey (14 white landmarks), with its two distinct areas, the ostium (O) and the cauda (C) (for additional examples of otoliths used in this study and a detailed description of shape quantification, see [Supporting Information, Fig. S1](#)).

Species included in the study spanned 309 families and 78 fish orders, ranging from early-diverging actinopterygian fishes (e.g. Polypteriformes and Acipenseriformes) to more derived teleosts (e.g. Tetraodontiformes and Pleuronectiformes) ([Supporting Information, Table S1](#)). Additionally, a subset of 323 specimens was photographed in dorsal view ([Fig. 1A](#);

[Supporting Information, Fig. S1B](#)) with a UI-3880LE-C-HQ digital camera (IDS Imaging Development Systems, Obersulm, Germany) mounted on a binocular microscope. Acknowledging the presence of allometric variation of otoliths in fish species ([Lombarte 1992](#)), only adult specimens were included in our dataset.

A set of 10 species traits was used to estimate functional diversity by taxonomic order (Supporting Information, Table S1). Eight of these corresponded to key functions, such as feeding (diet and mouth position), habitat use (position in the water column and depth occurrence), and behaviour (social behaviour and area of activity), in addition to more complex, yet ecologically relevant traits, such as body size and overall shape. Those traits were sourced from the work of Quimbayo *et al.* (2021) and completed with FishBase.org (Froese and Pauly 2000, 2024). Additionally, two major ecological traits related to the inner ear were considered: fish hearing and sound production abilities. First, enhanced hearing abilities were inferred based on the presence of morphological specializations known to enhance hearing (Braun and Grande 2008: tables S1–S4, Popper and Fay 2011). Second, we identified sound-producing species using the review of family-level evidence of sound production by Rice *et al.* (2022).

The evolutionary relationships among studied taxa were retrieved by pruning the time-calibrated phylogeny of Betancur-R *et al.* (2013, 2017). Among the 697 studied species, the phylogenetic position of 179 species was lacking in the time tree and was thus approximated by congeneric relatives.

Otolith shape and size

We quantified the diversity of otolith form, i.e. shape and size, using landmark (LM)-based geometric morphometric methods (Fig. 1B). Landmark coordinates were recorded by using the software TpsDIG v.2.31 (Rohlf 2017). We followed the protocol of Tuset *et al.* (2016b) to quantify the otolith shape in lateral view, where a total of 26 LMs were used. Eight type II LMs (homologous LM following the definition of Bookstein 1992) and four (sliding) semi-LMs were used to describe the otolith outline, and 11 LMs (six of type I; five of type II; Bookstein 1992) and three semi-LMs were used to capture the outline of the sulcus acusticus (Supporting Information, Fig. S1C). The diversity of otolith shapes was also examined in dorsal view, for which four type II LMs and eight semi-LMs were used (Supporting Information, Fig. S1D).

For each view (dorsal and lateral), LM configurations were superimposed optimally using a generalized Procrustes analysis with the 'gpgen' function from the R package 'geomorph' v.4.0.5 (Adams *et al.* 2023). This method rotates, scales, and translates LM configurations to isolate shape information from all other components of variation. This allows the recovery and separate treatment of the shape (Procrustes coordinates) and the size [i.e. centroid size (CS)] of otoliths (Rohlf and Slice 1990, Adams *et al.* 2004, Mitteroecker and Gunz 2009). Centroid size is the overall otolith size measured from the LM dispersion around the centroid, i.e. the centre of the landmarks. It is calculated as the square root of the sum of squared distances from each landmark to the centroid (Bookstein 1992). We used Procrustes coordinates as shape variables and log-transformed CS (logCS) as size data in subsequent analyses.

Otolith disparity, allometry, integration, and phylogenetic signal

Initially, we performed a principal component analysis (PCA) on shape data to summarize and illustrate the major axes of

morphological diversification in otoliths. Shape spaces were built by using the first two principal components (PCs). Next, we estimated whether size variation across taxa (i.e. interspecific allometry) explains a portion of otolith shape variation. To do so, we applied phylogenetic generalized least squares regression (PGLS) of shape on logCS by using the function 'procD.PGLS' from the R package 'geomorph' with 1000 iterations. This function applies multivariate regression models in a phylogenetic context under a Brownian motion model of evolution, efficiently considering all dimensions of shape variation while accounting for phylogenetic non-independence (Adams 2014a).

Beyond size effects, we also aimed to test the interdependence between different features of the otoliths. The otolith and its sulcus are moving simultaneously relative to the sensory area, and we can expect that maintaining such conserved and important functions as hearing and balance would imply evolutionary covariation. Accordingly, we assessed the degree of evolutionary integration between the lateral view of the otolith and the sulcus outline and between the lateral and the dorsal shape of the otolith by using the function 'phylo.integration' from 'geomorph' with 1000 iterations. This function quantifies morphological covariation between different sets of variables while accounting for phylogeny using partial least squares and under a Brownian motion model of evolution (Adams and Felice 2014, Adams *et al.* 2023). Analogous to PCA, orthogonal axes of linear combinations of variables are created, but two sets are compared simultaneously by calculating linear combinations maximizing covariance. A correlation coefficient, r_{PLS} , is recovered and represents the strength of the covariation between entities (Adams and Collyer 2016).

In order to assess the dependence of otolith diversity on phylogeny, we quantified the phylogenetic signal of shape variables and the logCS using the function 'physignal' from 'geomorph' with 1000 iterations (Adams *et al.* 2023). This function uses a mathematical generalization of the Kappa statistic (K_{mult}) appropriate for highly multivariate datasets to estimate the degree of phylogenetic signal expected under a Brownian motion model assumption. Theoretically, K ranges from zero to infinity. A value of $K \approx 1.0$ indicates a trait perfectly follows a Brownian motion model of evolution, and values of <1.0 or >1.0 describe data with less or more phylogenetic signal, respectively, than expected under the null model (Blomberg and Ives 2003, Adams 2014c).

Evolutionary dynamics of otolith diversification

To assess whether otolith evolutionary dynamics have remained constant through time, we evaluated the potential relationships between the disparity level of otoliths in each fish order, the age of the order, and the mean rate of lineage and morphological diversification observed in each order. To do so, we computed for each studied actinopterygian order: (i) the ages of the most recent ancestor; (ii) levels of morphological disparity; (iii) mean rates of morphological evolution; and (iv) mean rates of speciation. We then successively tested the links between (i) and (ii), between (ii) and (iii), and between (iii) and (iv).

Studied orders included between 1 (e.g. Albuliformes, Gobiesociformes, most of Ostariophysan orders) and 85 (Perciformes) species. For this part of the study, we selected well-resolved, monophyletic orders represented by at least nine

species in the dataset (21 for lateral and 10 for dorsal shape) (Fig. 1A; Supporting Information, Table S2).

First, we identified most recent ancestor age of each order, expressed in millions of years, by measuring the total length of the tree from the root to the highest tip by using the functions ‘max’ and ‘nodeHeights’ from the R package ‘phytools’ v.2.1-1 (Revell 2012, 2023). Second, we used the functions ‘morphol.disparity’ and ‘compare.evol.rates’ from the R package ‘geomorph’ with 1000 iterations to calculate the level of morphological disparity and the mean rate of shape and size evolution in each order. Morphological disparity was estimated as the Procrustes variance of the group, i.e. the sum of the diagonal elements of the group covariance matrix divided by the number of observations in the group, computed on Procrustes shape variables and logCS (Adams and Otárola-Castillo 2013). Comparing rates of shape and size evolution among groups is based on the net multivariate rate of phenotypic change (σ^2), computed on the outer-product matrix of between-species differences in morphospace after phylogenetic transformation assuming a Brownian motion model of evolution (Adams and Otárola-Castillo 2013, Adams 2014b). Next, we retrieved mean speciation rate (λ_{BAMM} , SR) of each order from the paper by Rabosky *et al.* (2018), who used the Bayesian analysis of macroevolutionary mixtures (BAMM) approach to estimate the tempo of lineage diversification. Regular linear model fits were performed by using the ‘lm’ function from the R package ‘stats’ v.2.2.2 to test the relationships between the level of morphological disparity, the ages of orders, the rate of speciation, and the rates of morphological evolution.

Link between otolith disparity and functional diversity

Finally, we tested the prediction that the level of morphological disparity in otoliths within a given group of fishes is related to its level of functional diversity. To assess the association between otolith disparity and the functional diversity among fish orders, we computed their functional richness (FRic) as a measure of their functional diversity. The FRic index quantifies the amount of functional space occupied by a group of species and can be measured as the minimum convex hull volume of the multidimensional space (Mason *et al.* 2005, Cornwell *et al.* 2006, Villéger *et al.* 2008). The FRic computation was performed with the R package ‘mFD’ v.1.0.7, following three major steps (Magneville *et al.* 2023). First, the between-species functional distance was computed using the ‘funct.dist’ function based on the Gower distance, which is suited for our categorical morphofunctional traits. Second, we applied a principal coordinate analysis to the between-species functional distances, and functional spaces were built from the principal coordinates using the ‘quality.fspaces’ function. The optimal functional spaces (i.e. the first six principal coordinates for the lateral view and the first four for the dorsal view) were determined by minimizing the mean absolute deviation between trait-based distance and distance in the principal coordinate analysis-based space. Third, we extracted the FRic index from species coordinates in the functional spaces using fish orders as the grouping factor and the ‘alpha.fd.multidim’ function. Then we fitted a linear model to test the relationship between the level of otolith morphological disparity and the functional richness of each order. The *P*-values obtained for our model fits were adjusted with the Benjamini–Hochberg

correction to control for the false discovery rate (Benjamini and Hochberg 1995).

RESULTS

Otolith disparity

The lateral view shape variation summarized in the first two principal components (PC1 and PC2) accounts for 46.4% of total shape variation (Fig. 2A). The morphospace occupation of the 21 orders selected for studying evolutionary dynamics can be found in Supporting Information, Fig. S2. Ninety per cent of cumulative proportion of variance is reached at the 13th PC, suggesting high and complex shape disparity that cannot be summarized totally in a few dimensions. PC1 mainly shows ventral and dorsal margins of otoliths shifting ventrally and dorsally towards the transversal plane while the ostial section of the sulcus enlarges and opens rostrally. As a result, it differentiates otoliths having a more triangular–elliptic shape with mesial, archeo- to homosulcoid sulcus (negative values) from those that are more rhomboidal–hexagonal with an ostially-opened heterosulcoid sulcus and prominent rostrum (positive values). Variation along PC1 suggests the presence of two major groups: (i) otoliths being, on average, more elongated, with proportionally narrower ventral and dorsal area and ostially-closed sulcus (negative values, Fig. 2A, C), mainly represented by species of Ostariophysi, Gadiformes, Ophidiiformes, Lophiiformes, and Pleuronectiformes (Supporting Information, Fig. S2); and (ii) the remaining otoliths clustering around the centre of the morphospace and having elliptic or rhomboid shapes with an ostially-opened heterosulcoid sulcus (Fig. 2A, C). In contrast, PC2 is associated with shortening and lengthening of otoliths and their sulcus along the rostrocaudal axis for both groups identified by PC1. Rostral and caudal sections of the otoliths, and particularly the sulcus, extend horizontally, while ventral and dorsal outlines are perpendicularly enlarged. Therefore, PC2 differentiates between elongate, rectangular otoliths with a conspicuous rostrum, heterosulcoid sulcus, and slightly to strongly curved cauda (negatives values), and discoid otoliths with a mesial sulcus and a small or absent rostrum (positive values) (Fig. 2A, C).

The morphospace of the dorsal view of otoliths for 323 specimens is shown in Figure 2B and the morphospace occupation of the 10 orders selected for studying evolutionary dynamics can be found in Supporting Information, Fig. S3. PC1 accounts for 42.2% and PC2 for 33.1%, and 92% of the cumulative variance is reached with PC3. Along PC1, the lateral outline radiates inwards while compressing the structure, and the rostrum lengthens, becoming sharper. This axis distinguishes rostrally thick, rounded, medially flattened, and laterally convex otoliths with a forward-shifted centre of mass (negative values) from those that are thinner, rostrally pointed, medially convex, and laterally concave, and also appear almost symmetrical on the transverse plane (positive values) (Fig. 2B, C). Regarding PC2, towards positive values the laterocaudal outline radiates inwards, narrowing the otolith, while the posterior part expands caudally forming a sharp postrostrum. PC2 separates otoliths that are rostrally acuminate, laterally convex, and caudally rounded, with a backward-shifted centre of mass (negative values) from those

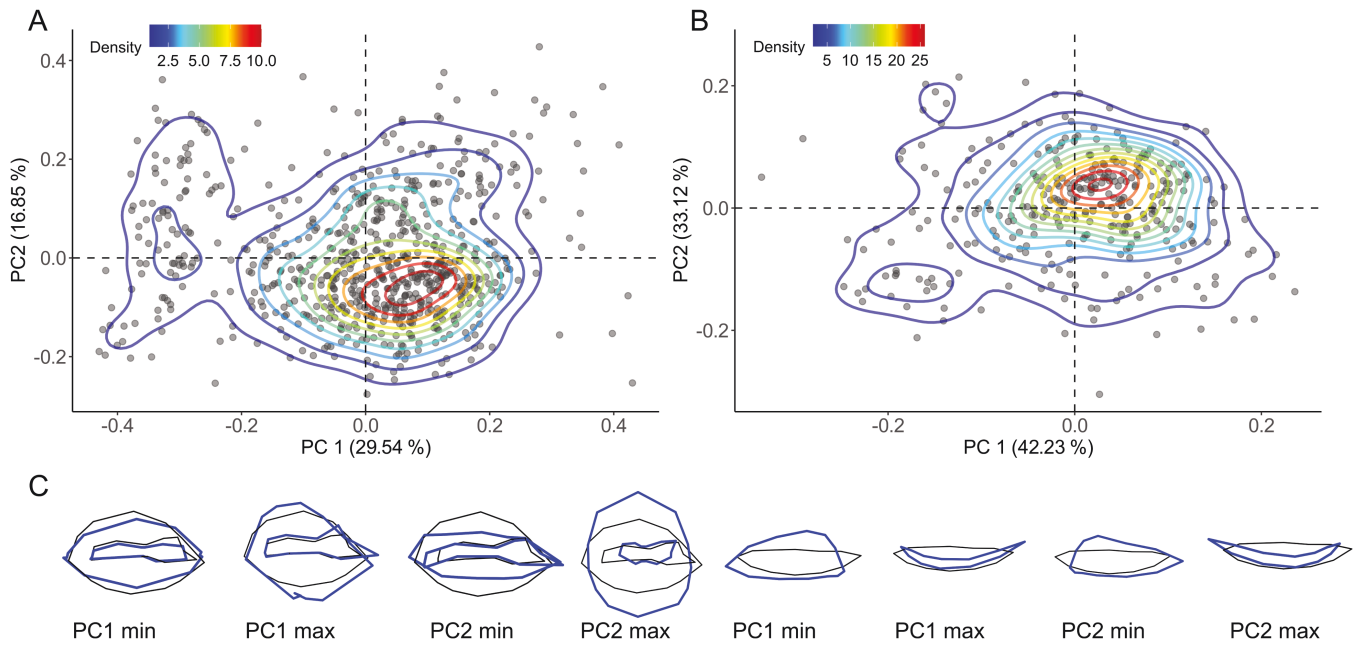


Figure 2. Morphospace of otoliths in lateral and dorsal views described by principal components (PC1 and PC2). A, lateral morphospace for 697 species. B, dorsal morphospace for 323 species. The colour hues of lines represent smoothed specimen kernel density. C, major changes in otolith shape (rostrum on the right) along PC1–PC2 axes are illustrated with configuration shifts between the consensus (black) and predicted minimal and maximal PC deformations (blue).

that are medially convex, laterally concave and caudally pointed, which are also more symmetrical on the transversal plane (positive values). Taken together, the dorsal morphospace of otoliths shows a trend from thicker, heavy-built otoliths (negative values) to increasingly thin, curved otoliths (positive values). Most otoliths are symmetrical, relatively straight, and medially slightly convex in their dorsal shape and are gathered near the centre (Fig. 2B, C).

Interspecific allometry

We found a significant effect of otolith size on lateral (PGLS: $R^2 = .0160$, $Z = 5.98$, $F = 11.2$; $P = .001$) and dorsal (PGLS: $R^2 = .0698$, $Z = 5.25$, $F = 24.1$, $P = .001$) shape variation. This indicates that, with a substantial size effect (Z), otolith size explains between 1.6% and 7% of the variation in their lateral and dorsal shape, respectively. The relationships between lateral shape variation described by PC1, PC2, and logCS reveal that larger otoliths are, on average, more elongated, with a marked rostrum, and presenting a heterosulcoid sulcus with an ostial opening (Supporting Information, Fig. S4A, B, E). Regarding dorsal shape, larger otoliths are thinner, elongated, and curved (Supporting Information, Fig. S4C, D, E).

Outlines and sulcus acusticus integration

We find a very strong morphological covariation between lateral and sulcus shapes ($r_{PLS} = .867$, $Z = 12.5$, $P = .001$). This covariation is stronger than the one observed between lateral and dorsal shapes ($r_{PLS} = .677$, $Z = 6.10$, $P = .001$), while accounting for phylogenetic non-independence.

Phylogenetic signal

We find low phylogenetic signal for shape and size traits (lateral shape: $K_{mult} = 0.262$, $P = .001$; lateral size: $K_{mult} = 0.154$,

$P = .006$; dorsal shape: $K_{mult} = 0.183$, $P = .001$; dorsal size: $K_{mult} = 0.277$, $P = .001$). These values indicate that, on average, two related lineages have otolith shapes and sizes that are more dissimilar than would be expected under a Brownian motion model, in comparison to two randomly selected taxa.

Evolutionary dynamics of otolith diversification

The ages of the studied orders range from 114 (Aulopiformes) to 44 Myr (Scombriformes). We detect no linear relationship between the level of morphological disparity observed in a fish order and its age (lateral shape: $F = 1.68$, $P = .913$, Fig. 3A; lateral size: $F = 0.206$, $P = .803$, Fig. 3B; dorsal shape: $F = 0.00142$, $P = .971$, Fig. 3C; dorsal size: $F = 0.0242$, $P = .941$, Fig. 3D). Orders can present various levels of otolith morphological disparity (MD), independently of their divergence times. For instance, Scombriformes and Gadiformes show a high lateral and dorsal shape MD (Supporting Information, Figs S2, S3), whereas Spariformes have both low lateral and dorsal MD (Supporting Information, Figs S2, S3, S5, S6). Interestingly, lineages that emerged between 65 and 90 Mya present a highly diverse range of otolith MD, with Lophiiformes–Lutjaniformes (Fig. 3A; Supporting Information, Fig. S2), Anguilliformes–Acanthuriformes (Fig. 3B; Supporting Information, Fig. S5), and Syngnathiformes–Pemppheriformes (Fig. 3C, D; Supporting Information, Figs S3, S6) having extremes values.

Concerning mean evolutionary rates (ER), the only link we recover between the rate of morphological evolution and the level of MD accumulated in fish orders is for lateral shape ($F = 10.7$, $P = .0156$, $R^2 = .326$, Fig. 3E). Such a relationship is not observed for lateral size ($F = 1.62$, $P = .246$, Fig. 3F) or for dorsal shape ($F = 0.225$, $P = .810$, Fig. 3G) and size ($F = 5.78$, $P = .107$, Fig. 3H). Some orders (e.g. Aulopiformes and Syngnathiformes) seem to deviate from this trend, having a

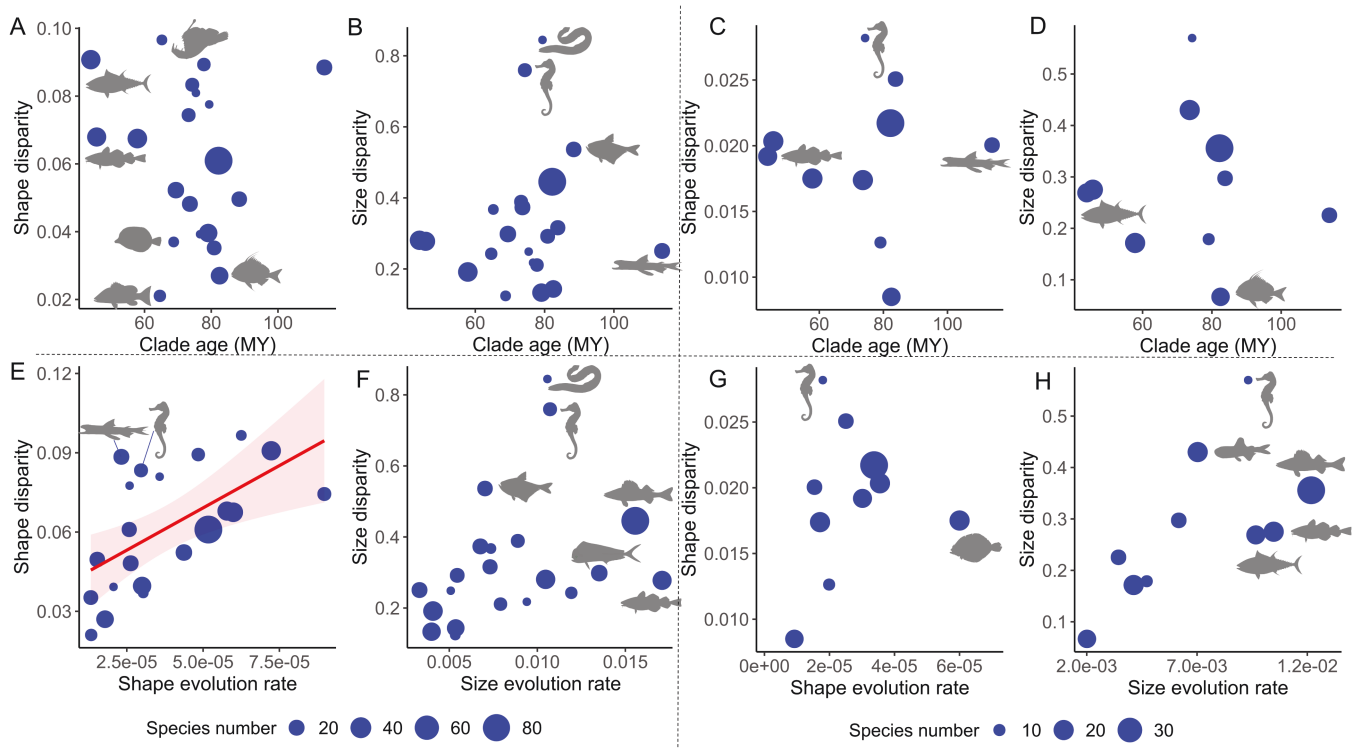


Figure 3. Relationships between otolith morphological disparity, measured as Procrustes variance, and age of the most recent ancestor (A–D; top) and rate of morphological evolution (E–H; bottom) for the 21 lateral (A, B, E, F; left) and 10 dorsal (C, D, G, H; right) orders well represented in our dataset. Point size corresponds to the number of species included from the orders in the analysis. When significant, linear regression fits are represented with a red line and 95% confidence interval. Silhouettes come from www.phylopic.org and are used under a Creative Commons CC0 1.0 licence.

relatively high shape MD/ER ratio (Fig. 3E). Despite no global influence of size MD on ER, two different evolutionary trends can be described depending on the order MD/ER ratio. Some orders (e.g. Anguilliformes, Syngnathiformes, and Pempheriformes) exhibit a high MD/ER ratio with relatively fast MD accumulation, whereas others (e.g. Gadiformes, Carangiformes, and Perciformes) show the opposite pattern, displaying a rather low MD/ER (Fig. 3F, H; Supporting Information, Figs S5, S6). Specifically, for the dorsal size MD/ER ratio, Myctophiformes exhibit a higher ratio, whereas Scombriformes show a lower ratio (Fig. 3H). The dorsal shape MD ~ ER plot shows that ER is a poor predictor of MD (Fig. 3G). For example, Syngnathiformes otoliths have twice the MD of Pleuronectiformes despite the otoliths of the latter evolving three times faster (Supporting Information, Fig. S3).

Finally, we found a significant positive relationship between the rate of lateral size evolution and the rate of speciation observed across fish orders ($F = 10.7$, $P = .0156$, $R^2 = .327$, Fig. 4B). At the extremes, Aulopiformes show a relatively low rate of both speciation and otolith size evolution, whereas Perciformes and Gadiformes exhibit relatively high rates for both factors. However, some orders, including Anguilliformes, Carangiformes, Stomatiformes, and Tetraodontiformes deviate from this linear relationship between the tempo of size evolution and lineage diversification (Fig. 4B). Dorsal size ($F = 6.26$, $P = .105$, Fig. 4D) and rates of shape evolution (lateral: $F = 4.24$, $P = .119$, Fig. 4A; dorsal: $F = 1.64$, $P = .431$, Fig. 4C) are decoupled from the tempo of lineage diversification.

Link between otolith disparity and functional diversity

There is no significant relationship between the level of otolith MD and the Fric present in fish clades (lateral shape: $F = 0.0182$, $P = .894$, Fig. 5A; lateral size: $F = 0.460$, $P = .506$, Fig. 5B; dorsal shape: $F = 0.676$, $P = .435$, Fig. 5C; dorsal size: $F = 0.507$, $P = .497$, Fig. 5D). For example, the Lophiiformes, an order with the highest otolith lateral shape MD, present very low FRic (Fig. 5A). Remarkably, the Myctophiformes exhibit the lowest FRic despite having the second highest size MD (Fig. 5D). Overall, the Perciformes show very high FRic in comparison to other orders despite having intermediate otolith MD. Some orders, such as Centrarchiformes, Pempheriformes, Gadiformes, and Scombriformes (cited with increasing FRic), are distinguished from the remaining 16 orders (lateral FRic < 0.005; Fig. 5A, B) by being slightly more functionally diverse.

DISCUSSION

Here, we present an analysis of a comprehensive otolith morphological dataset that includes ecological and phylogenetic information, covering all major actinopterygian groups, providing a basis for a deeper understanding of their diversification. Major variations involve the sulcus acusticus shape, rostrocaudal elongation, forward–backward motion of the centre of mass and curvature. As clearly illustrated by morphospace occupation and suggested by the tests of phylogenetic signal, morphological convergence characterizes the morphological evolution of otoliths. Interestingly, the lateral shape and sulcus morphology appear to

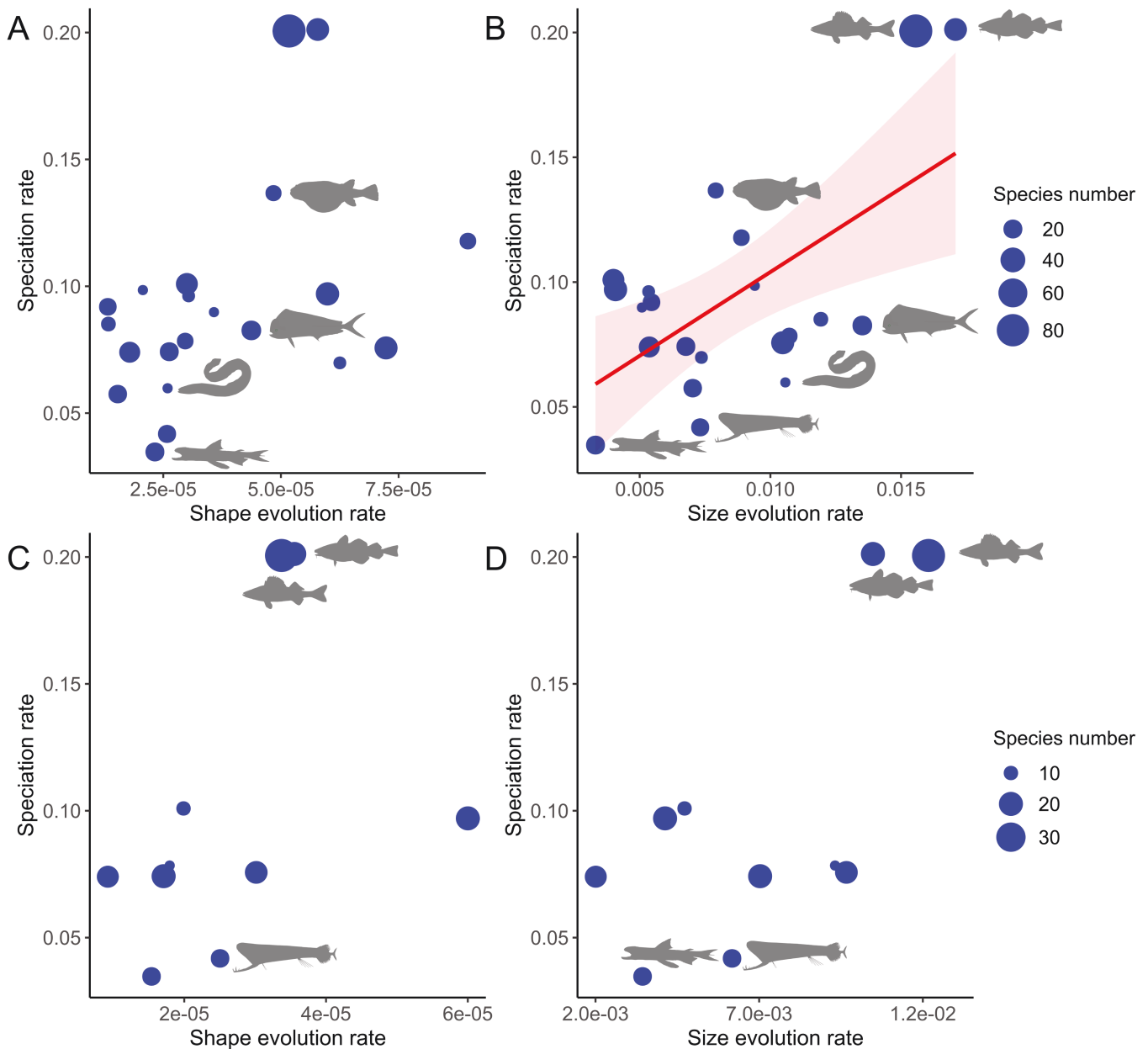


Figure 4. Relationships between rate of speciation (λ_{BAMM}) from Rabosky *et al.* (2018) and rate of otolith shape (A, C; left) and size (B, D; right) evolution for 21 lateral (A, B; top) and 10 dorsal (C, D; bottom) orders well represented in our dataset. Point size corresponds to the number of species included from the orders in the analysis. When significant, linear regression fits are represented with a red line and 95% confidence interval. Silhouettes come from www.phylopic.org and are used under a Creative Commons CC0 1.0 licence.

have evolved as a single unit, suggesting similar responses to the functional constraints. Size explained a small amount of otolith shape variation, with larger otoliths tending to be more elongated and curved, and having a conspicuous rostrum. The evolutionary dynamics ultimately reveal a multifactorial morphological evolution that cannot be attributed simply to time, fish ecology, and mean speciation or morphological evolutionary rates.

Otoliths frequently display distinctive shapes, sizes, and structures specific to a particular fish species. They have diagnostic value and are thus often discussed in taxonomical and systematic studies (Nolf 1975, Schwarzhans 1978, Gaemers 1983, Nolf and Steurbaut 1989, Teimori *et al.* 2019). At shallow phylogenetic scales (e.g. fish families), several otolith aspects, including growth,

sulcus, and outline shapes, appear to reflect strong phylogenetic relatedness (Lombarte and Castellón 1991, Torres *et al.* 2000, Lombarte *et al.* 2003, Vignon and Morat 2010). In contrast, at deeper phylogenetic scales, we show that variation in otolith morphology does not mirror actinopterygian phylogeny. This results in a high overlap across orders of otolith disparity in the morphospace (Supporting Information, Figs S1, S2). Although this might result from evolutionary convergence, the shape redundancy observed in the morphospace is likely to be driven by the large within-clade diversity. Most species examined exhibit a heterosulcoid sulcus with an ostial opening, a characteristic generally considered to be plesiomorphic (Schwarzhans 1978). This could indicate an ancestral otolith shape that performs its basic

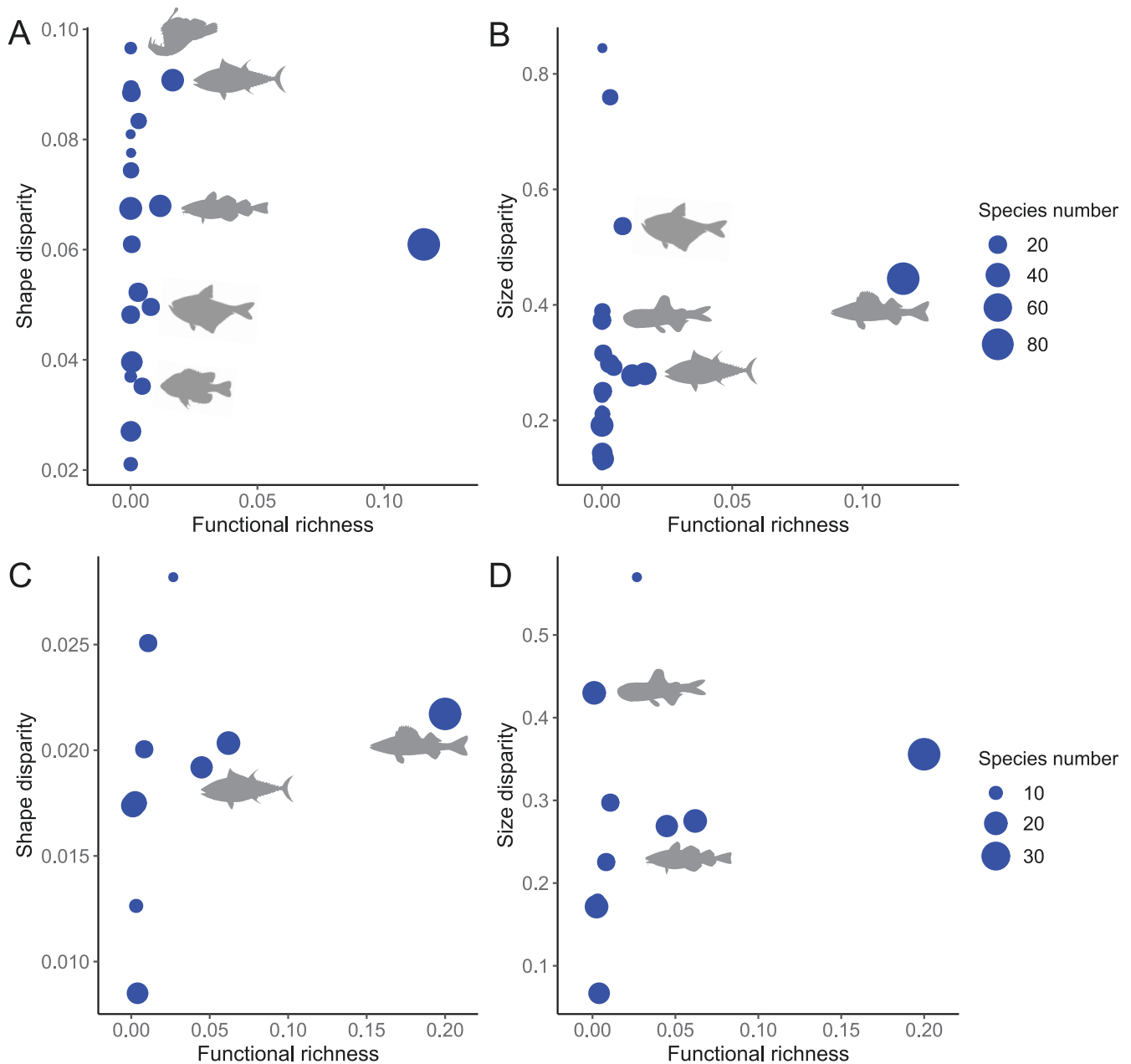


Figure 5. Relationships between otolith shape (A, C; left) and size (B, D; right) disparity, measured as Procrustes variance, and functional diversity, measured as FRic, for 21 lateral (A, B; top) and 10 dorsal (C, D; bottom) orders well represented in our dataset. Point size corresponds to the number of species included from the orders in the analysis. Silhouettes come from www.phylopic.org and are used under a Creative Commons CC0 1.0 licence.

functions efficiently. As highlighted in previous studies, our results support that the sulcus acusticus is an essential feature to consider for accurate otolith shape quantification, diversity assessment, and morphofunctional considerations (Lombarte *et al.* 2010, Tuset *et al.* 2016b, Chollet-Villalpando *et al.* 2019, Verocai *et al.* 2023).

We observed a less complex shape variation in the dorsal view compared with the lateral view of the otoliths. Nonetheless, the variation in curvature and rostrocaudal thickness affects the position of the centre of mass and, consequently, the radius from the otolith centre of gravity to the macula, impacting movements of the otolith and its functional sensitivity (Lyachkov and Rebane

2000, Schulz-Mirbach *et al.* 2019). Thicker otoliths might, therefore, be more readily detected by the sensory cells, potentially leading to an improved ability to perceive sound or detect movements (Gauldie 1988, Lyachkov and Rebane 2000, Parmentier *et al.* 2001, 2002, Schulz-Mirbach *et al.* 2019).

Larger otoliths were frequently more elongated, laterally compressed, and curved, with an ostially opened sulcus. The biological significance of this result remains unclear. Given that we were limited to using absolute otolith size, caution is needed in interpreting these results. Because fish and their otolith sizes usually covary strongly (Gauldie 1988, Campana 1990, Morales-Nin *et al.* 1998), variations in otolith size might simply reflect

variations in fish body size. Using standardized otolith size (for example, $\log CS$ divided by body/head length) would therefore provide a better measure of the relative sensory allocation within the otolithic organ. In addition, a thorough examination of the associations with the brain and the skeletal apparatus could pinpoint the physical constraints that modulate inner ear evolution (Parmentier *et al.* 2001). A large trend observed in interspecific shape and size covariation for otoliths is that generally, smaller otoliths from deeper-living species are structurally less complex, with smoother margins, and are more rounded and flattened (Wilson 1985, Schwarzahns and Geringer 2023). The PC2 axis of lateral view morphospace (Supporting Information, Fig. S4B, E) particularly supports this observation, with smaller otoliths being circular–discoidal and having a closed, mesial sulcus. The amount of otolith shape variation explained by size in this study is comparable to what is reported for the contribution of fish size to body shape. However, stronger allometric signals might be present at shallower phylogenetic scales (Friedman *et al.* 2019).

Integration is a key aspect of variation that describes the magnitude and the pattern of interdependence between different traits (Olson and Miller 1999). It significantly influences the evolvability of the structure, either supporting or hindering it (Wagner and Altenberg 1996, Goswami and Polly 2010). The strong integration between the sulcus and the lateral outline of the otolith enhances our understanding of its functional anatomy. The outline of the otolith reflects the shape of the sulcus, effectively reinforcing the lever arm to support the axes along which the sensory cells are most sensitive. In other words, the sulcal surface projection amplifies the forces that stimulate the stereocilia and kinocilia and contributes to the efficient transmission of vibrations to the sensory cells (Platt and Popper 1981, Gaudie 1988, Lombarte 1992, Lombarte and Fortuño 1992). This strong relationship indicates that the shape of the otolith is not merely a passive structural feature, but contributes actively to the efficiency of the sensory system. Our findings provide a more integrated view of the evolutionary and functional significance of the otolithic organ from a macroevolutionary perspective, opening new avenues for investigating how the physical attributes of otoliths influence auditory and balance functions. Integration is often seen as a constraint on evolution, favouring the stabilization of a given function and limiting the ability of traits to vary and evolve autonomously in a fluctuating environment (Albertson *et al.* 2005, Evans *et al.* 2017). However, highly integrated structures have also been reported to promote phenotypic diversification and facilitate evolvability (Randau and Goswami 2017, Du *et al.* 2019, Evans *et al.* 2021). Considering otolith variability, it is tempting to think that the observed integration did not necessarily slow down their morphological diversification but rather maintained the functionality of the sensory organ and fine-tuned its performance.

Phenotypic disparity, evolutionary rate, and speciation rate are often considered to be positively correlated aspects of evolution; rapid changes and diversification in phenotype can lead to increased opportunities for speciation, thereby accelerating the evolutionary process (Simpson 1953, Gould and Eldredge 1977, Ricklefs 2006, Adams *et al.* 2009, Cooney and Thomas 2021). Overall, we found that otolith evolutionary dynamics were not constant through time. The age of fish orders is unrelated to

the levels of disparity in otolith morphology within each order. Lateral shape was the only morphological variable related to the evolutionary tempo of otoliths. At macroevolutionary scales, disparity has been reported to increase over time (Adams *et al.* 2009) and is not necessarily correlated with the rate of morphological evolution (Michaud *et al.* 2018, Friedman *et al.* 2019, Bibi and Tyler 2022, Morinaga *et al.* 2023). The relationship between otolith lateral shape diversity and the rate of evolution probably implies that otolith lateral shape has relevant functional implications and evolves under a controlled process. The highly integrated sulcus and otolith shapes discussed above support this assumption. Otherwise, evolutionary rates differ greatly among orders such that the accumulation of variation over time is likely to be masked by lineage-specific evolutionary constraints.

Speciation rate tends to be positively associated with morphological evolution (Cooney and Thomas 2021). In this study, cladogenesis was associated only with the rate of otolith lateral size evolution. Lateral otolith size diversification might be favoured during speciation events, suggesting that, as new lineages evolve, distinct otolith sizes can emerge as adaptive traits. In other vertebrate groups, the derived inner ear features have been strongly related to phylogeny and habitat preferences, and found to be correlated with ecological diversification (Costeur *et al.* 2018, Mennecart *et al.* 2022). In the context of this study, absolute otolith size complicates the discrimination between variations coming from intraspecific fish body size and interspecific otolith size variation.

Adaptation through natural selection frequently results in correlations between the ecology of an organism and its morphology, thereby enabling inferences about its ecological role or habitat based on its physical traits (Norton *et al.* 1995). If otolith morphology is related to fish lifestyle and habitat use, we can expect that fish orders diversifying along various ecological axes would show significant disparity in their otolith morphology. The many dimensions of our functional dataset might weaken the expected covariation between fish eco-functional and otolith morphological diversities. Similar to other functional structures, otolith morphology is shaped by a range of constraints associated with life histories and ecologies. Specifically, for the inner ear, constraints related to acoustic communication and balance, including swimming style and manoeuvrability, would be of major importance (Lyckakov and Rebane 2000, Parmentier *et al.* 2001, Popper *et al.* 2005, Kéver *et al.* 2014). The resulting ecology of a species and its interactions with its environment are multimodal, and inner ear function is only one aspect of how a fish can interact and adapt to its environment. In response to similar ecological and functional demands, evolution might have promoted shape convergence in otolith morphology across taxonomic groups.

CONCLUSION

The exploration of otolith morphospaces emphasized the significance of the sulcus and provided insights into the functional relevance of otolith curvature and variations in thickness. Together, the low phylogenetic signal, morphospace occupation, and the tendency for larger otoliths to be more elongated, curved, and to have a conspicuous rostrum imply some level of convergence

in shape. We provided evidence that the otolith outline might contribute to the fine-tuning of the motion of the otolith relative to its macula. Comparative analyses across fish orders revealed a complex morphological evolution that cannot be simplified to the basic evolutionary aspects tested here. Overall, our macro-evolutionary analysis of otolith diversification paves the way for a more integrative understanding of the role they played in fish evolution as part of the inner ear sensory organ. The in-depth elucidation of the intrinsic and extrinsic evolutionary constraints at play in otolith diversification is likely to rely on systematic studies using state-of-the-art phylogenetically informed analyses for highly diversified fish families.

SUPPLEMENTARY DATA

Supplementary data is available at *Biological Journal of the Linnean Society* online.

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CONFLICT OF INTERESTS

None declared.

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DATA AVAILABILITY

The data underlying this article are available in the article and in its online [Supplementary material](#).

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