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Otolith shape analysis supports three cryptic species in the *Stellifer punctatissimus* complex (Acanthuriformes: Sciaenidae)

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Sciaenidae species (croakers and drums) are known for specializations in their sensory apparatus, such as otoliths and lateral line. Within this family, some Stelliferinae members remain taxonomically unresolved due to their cryptic aspects and low phenotypic disparities. *Stellifer punctatissimus* comprises a species complex putatively formed by three species on morphological grounds, but molecular data have indicated only two evolutionary lineages. Herein, we tested the hypothesis that this complex is composed of *Stellifer gomezi* (GM), *S. menezesi* (MN), and *S. punctatissimus* (PC), using the otolith shape and morphology to differentiate them. Seventy-seven sagittal otoliths (GM = 30, MN = 30, PC = 17) were photographed to outline the otolith contour by Elliptical Fourier descriptors. Ten otoliths for each group were used to detect shape variation in the *sulcus acusticus* through semilandmarks. Differences in otolith shape contour were recorded among the species by PERMANOVA (Pseudo-F = 4.02, df = 2, $p < 0.001$) and corroborated by the Linear Discriminant Analysis, which demonstrated three partially segregated groups with satisfactory re-classification rates. A larger *sulcus acusticus* (GM and MN), rounded projection (PC), and distinct rectangularity pattern were also recorded. Our results support the morphological hypothesis and thus contribute to narrowing the taxonomic gaps in Sciaenidae.

Keywords: Croakers, Cryptic species, Otolith morphology, Speciation, Taxonomy.

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As espécies de Sciaenidae (corvinas e pescadas) são conhecidas por especializações nos aparatos sensoriais, como otólitos e linha lateral. Nesta família, alguns membros de Stelliferinae permanecem com sua taxonomia não resolvida devido às características crípticas e baixas disparidades fenotípicas. *Stellifer punctatissimus* supostamente compreende um complexo formado por três espécies, mas os dados moleculares têm indicado apenas duas linhagens evolutivas. Aqui, testamos a hipótese de que esse complexo é composto por *Stellifer gomezi* (GM), *S. menezesi* (MN), e *S. punctatissimus* (PC), usando a forma e morfologia do otólito para diferenciá-las. Setenta e sete otólitos *sagita* (GM = 30, MN = 30, PC = 17) foram fotografados para delimitar o seu contorno pelos descritores Elípticos de Fourier. Dez otólitos, por grupo, foram usados para detectar a variação de forma no *sulcus acusticus* através dos *semilandmarks*. Foram registradas diferenças na forma do otólito entre as espécies através da PERMANOVA (Pseudo-F = 4,02, df = 2, p < 0,001) e pela análise discriminante linear, demonstrando três grupos parcialmente segregados com taxas de reclassificação satisfatórias. Também foram registrados um *sulcus acusticus* mais largo (GM e MN), projeção arredondada (PC) e padrões alométricos distintos no índice de retangularidade. Nossos resultados apoiam a hipótese morfológica; assim, contribuem na redução das lacunas taxonômicas em Sciaenidae.

Palavras-chave: Especiação, Espécies crípticas, Morfologia do otólito, Pescadinhas, Taxonomia.

INTRODUCTION

Sciaenidae is one of the most speciose families within Acanthuriformes (nearly 300 species), and members of this family are widely known as ‘croakers’ or ‘drums’ (*i.e.*, they produce sound) (Chao *et al*., 2015; Fricke *et al*., 2022). The diversity of morphological traits in the family has enabled the adaptation of the species to a variety of habitats, such as estuaries, sand bottoms, and surf zones, with few species being strictly freshwater or reef-associated (Chao *et al*., 2015). Although recent evidence based on morphological and molecular approaches has supported the monophyly of this family, evolutionary relationships in less inclusive taxonomic ranks remain uncertain (Chao, 1978; Lo *et al*., 2015). As an example, the status of some genera of the monophyletic subfamily Stelliferinae (also known as ‘*Stellifer*-group’ *sensu* Chao, 1978) is still unsolved due to their great morphological similarity or even the lack of diagnostic characters to distinguish them (Chao *et al*., 2015; Silva *et al*., 2018).

The paucity of informative characters has led to shifts in the generic positioning of morphologically similar species, such some originally described in *Stellifer* Oken, 1817 and later changed to *Ophioscion* Gill, 1863, or vice versa (Chao *et al*., 2021). Species of both genera are common or abundant, particularly in coastal waters of North-Northeastern Brazil, frequently caught as bycatch in shrimp trawls. Despite their abundance, the taxonomic status of several species remains undefined (Chao *et al*., 2015; Silva *et al*., 2018). A particular case of uncertainty is *Stellifer punctatissimus* (Meek & Hildebrand, 1925), initially described in *Ophioscion* by Meek, Hildebrand (1925). Later, a species complex with two additional species was recognized by Chao (2002) using a morphological approach. Recent molecular data, however, have pointed out only two evolutionary lineages within this complex (Barbosa *et al*., 2014; Silva *et al*., 2018). Finally, one of these species was described and another redescribed, both now included in *Stellifer*, which is considered a senior synonym of *Ophioscion* (Chao *et al*., 2021).

Otoliths are calcified structures located in the inner ear of bony fishes (Teleostei) disposed in the three semicircular canals (utricle, saccule and lagena), which correspond to the lapillus, sagitta and asteriscus pairs (Schulz-Mirbach *et al*., 2019). They are surrounded by endolymph and connected to the macula (which holds the sensory hair cells and nerves) by the otolithic membrane, which lies on the *sulcus acusticus* (Popper *et al*., 2005; Schulz-Mirbach *et al*., 2019). Due to their higher density when compared to the fish body, otoliths exhibit a different response (*i.e*., distinct amplitudes and phases) to sound- or motion-induced movements (*e.g*., angular acceleration). Thus, variations in otolith shape, as well as differences in the features of their sensory cells (*e.g*., stereocilia number, cell orientation), may be correlated with directional sound sensitivity (Ramcharitar *et al*., 2001; Popper *et al*., 2005). Furthermore, otolith shape is influenced by extrinsic factors, such as salinity and temperature, and intrinsic, such as physiology and ontogeny (Campana, Thorrold, 2001; Clark *et al*., 2021).

By growing throughout the fish life cycle, otoliths also seem to respond to other factors, such as ontogenetic shifts and those linked to environmental gradients (Schulz-Mirbach *et al*., 2019). Likewise, as they carry a signal of those traits, surveys that use otolith shape provide an indirect and effective way to investigate the living habits of species and to seek diagnostic details in cryptic species. Such analyses contrast with traditional taxonomic approaches, which generally show more overlap than otolith shape analyses (Lombarte *et al*., 2006; Capoccioni *et al*., 2011). Many techniques have been used to delineate the otolith shape contour (*e.g*., shape indices, wavelet, Fourier descriptors). Shape indices appear to be powerless to differentiate cryptic species due to their low resolution. However, they provide valuable biological information by shedding light on the association of shape patterns with habits such as feeding and water depth (Wong *et al*., 2016; Assis *et al*., 2020). Fourier descriptors are definitely more efficient in distinguishing slight differences in cryptic- and similar-species than other methods, particularly because they decompose the otolith's contour into several harmonics, which encompass almost the entire variation across that border (Wong *et al*., 2016; Avigliano *et al*., 2018). In contrast, the application of geometric morphometric methods (GMM) is impaired by methodological constraints. In body shape, this method likely achieves a fine resolution of cryptic species or species-rich groups (Anjos *et al*., 2020; Argolo *et al*., 2020). However, due to the lack of homologous points, GMM's use in otolith analysis is mostly restricted to contour descriptions with semi-landmarks (Tuset *et al*., 2016). However, it provides additional data when applied to structures such as the *sulcus acusticus* (*i.e*., *ostium* and *cauda*); as a result, such information could be applied to discuss hearing capabilities, taxonomy, and prey identification (Byrd *et al*., 2020; Granados-Amores *et al.,* 2020).

This study aimed to test the following hypothesis: *Stellifer punctatissimus* species complex is formed by three distinct cryptic species that can be distinguished on morphological grounds by complementary tools such as otolith shape analysis.

MATERIAL AND METHODS

Sampling sites and otolith preparation. The fishes were sampled along the coast of Bahia state, and 77 right sagittal otoliths of three *Stellifer* species were sampled as follows: *Stellifer gomezi* (Cervigón, 2011) (GM) – 30; *Stellifer menezesi* Chao, Carvalho-Filho & Andrade Santos, 2021 (MN) – 30; and *Stellifer punctatissimus* (Meek & Hildebrand, 1925) (PC) – 17. The sampling was carried out at four sites: Itaparica Island, Porto Seguro (#2), and Caravelas (Tab. 1; Fig. 1). The surveys were conducted in April 2018 and February 2019 on Itaparica Island, May and March 2016 in Porto Seguro and Caravelas, respectively. The vouchers were deposited at the fish collection of Museu de Zoologia da Universidade Estadual de Feira de Santana, Feira de Santana, Bahia, Brazil (MZFS) under the numbers 17694, 18115, 18116, 18117, 18119, 18124, 18125, 18126, 18127, 18128, 18129, 18131, 18133, 18135. Five trawls were deployed parallel to the coast (totaling about 250 m) using a manual beach-seine net (9 m in length, 1.7 m in height, 13 mm mesh on the lateral, and 5 mm mesh in the center). At the Caravelas site, fish sampling was performed in shrimp trawling, and specimens were caught as bycatch.

Sagittal otoliths were extracted preferentially by cutting through the upper end of the right gill cover. Otoliths were then manually cleaned, washed in distilled water, dried, and stored in individually labeled Eppendorf® tubes. Right otoliths were photographed using a stereomicroscope Leica EZ4 HD (for contour analysis) and Leica M205 A (for description and geometric morphometrics). All pictures had their contrast improved in Adobe Photoshop CC 2019 and were digitally cleaned to avoid noise in contour analysis. Otolith's descriptive nomenclature (Fig. 2) was based on Chao (1978), Schwarzhans (1993), and Aguilera *et al.* (2016).

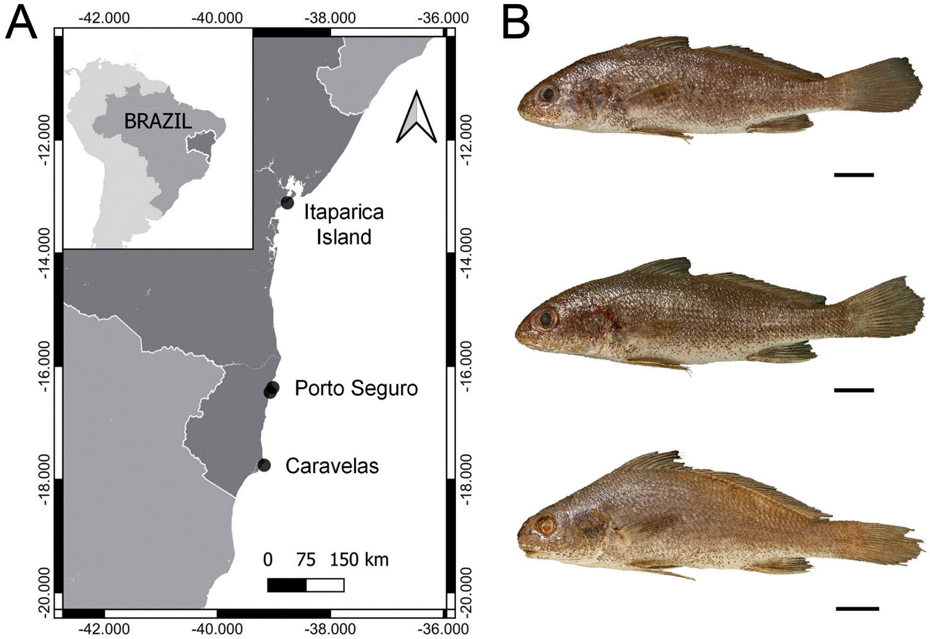

FIGURE 1 | Map of the study area highlighting the sampling sites in the Bahia State, Brazil. **B.** Representative specimens of the species studied. *Stellifer gomezi* (top); *S. menezesi* (middle); *S. punctatissimus* (bottom). Scale bars = 10 mm.

TABLE 1 | The number of specimens analyzed per site, their mean body size (in mm) with standard deviation (SD) and length range by species.

Species	Sites			Body length	
	Itaparica	Porto Seguro	Caravelas	Mean ± SD	Range
Stellifer gomezi	21	9	–	67.24±14.31	46.43-111 mm
Stellifer menezesi	15	15	–	65.50±15.05	38.57-99.98 mm
Stellifer punctatissimus	4	12	1	60.45±20.32	35.33-109 mm

Statistical analyses. Each image was processed by ImageJ software (Schneider *et al*., 2012) to obtain its pixel-cm ratio. Initially, the following shape indices were calculated: aspect ratio, circularity, ellipticity, form factor, rectangularity, and roundness. An ANOVA test was used to determine differences among species using species as a fixed factor (3 levels: GM, MN, and PC).

We created an analytical framework for assessing the degree of differentiation of the three putative species of the *Stellifer punctatissimus* complex, inferred from the otolith shape (adapted from Tuset *et al*., 2020). Firstly, Elliptical Fourier descriptors (EFDs) were used to quantify the otolith shape contour with 'shapeR' package (Libungan, Pálsson, 2015) in the R platform (R Development Core Team, 2020). Allometry was examined and controlled by the standard length (SL, in mm) of each fish using *stdCoefs* function ('ShapeR' package). Six EFDs exhibited significant allometric effects and were then omitted (45 – 6 = 39 EFDs). Next, a covariance-based Principal Component Analysis was applied with 39 EFDs for extracting the most informative principal components (as retained for eigenvalues > 1) and thus creating an orthogonal matrix (uncorrelated variables) that avoids multicollinearity (Jolliffe, 1986). This procedure shrunk 39 EFDs into 16 components that explained 94.6% of the total variation of data. Lastly, the normality for each component was examined by Shapiro-Wilk test, with three of them excluded ($p < 0.001$) (16 – 3 = 13 components), and a Bartlett's test demonstrated homogeneity of covariance matrix across species (Bartlett's K-squared = 0.44, df = 2, $p = 0.80$).

A Linear Discriminant Analysis (LDA) was performed to identify the degree of separation of the species (response variable) based on 13 components of otolith shape contour (predictors). LDA is an adequate technique as it reduces the dimensionality of the data and works also as a classification method. The predictive accuracy of the model was estimated by the percentage of correctly individuals ascribed to their species, using the leave-one-out re-classification method (Venables, Ripley, 2002). Complementarily, a PERMANOVA was performed for testing multivariate differences in the otolith shape contour (13 components) among species, with permutational pairwise comparisons (nperm = 999) used as a post-hoc test ('RVAideMemoire' package; Hervé, 2022).

For the geometric morphometric methods (GMM), ten photographs of each species were analyzed using the Tps series (Rohlf, 2017a, 2021). To account for the variation within the *sulcus acusticus*, a curve was drawn on its entire contour, with landmarks on the initial and final points of that curve. The original curve was resampled to 35 points (by length), which were later assigned as landmarks (LM), by using the function "append tps curves to landmarks". Semilandmarks (sLM) at the initial and final points of the curve were removed from the dataset, remaining only the 33 points (sLM) from the curve plus the two landmarks (LM) (Fig. 2). In the sequence, the sliding step was performed on those curves based on the minimum bending energy method. All data were initially subjected to a Generalized Procrustes Analysis (GPA) using tpsRelw (Rohlf, 2017b). To describe *sulcus acusticus* morphology, a Principal Component Analysis (PCA) was performed using the Procrustes residuals. As the dataset had much more variables (33 sLM + 2 LM) than specimens (10 by species), the statistical power was impaired. Thus, in all GMM analyses, we rather consider it as a descriptive approach for such analysis than its statistical significance in regard to distinction among groups. Although the otoliths present an angled inner face in the anteroposterior axis, we disregard such variation in our results once it was viewed by the same tangent space approximation. Thus, by comparing species with similar angled sagittal otoliths, the rules of Kendall's shape space were not broken (Klingenberg, 2020). It would be a case in a comparison with "flat-otolith" species (*e.g.*, *Macrodon* spp.) because in this scenario, for instance, the proportions between *ostium* and *cauda* would present a bigger disparity.

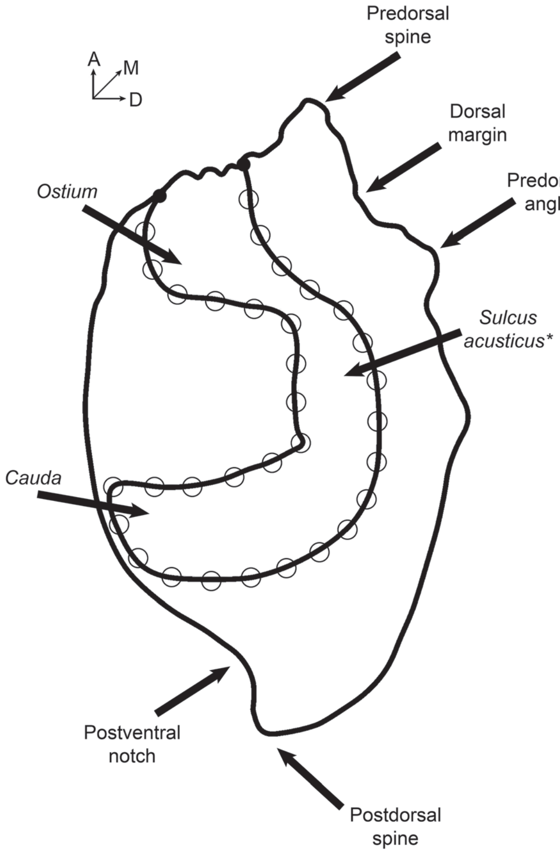

FIGURE 2 | Illustration of representative sagitta otolith with key anatomical features, based on an average shape specimen. Filled dots = landmarks, empty circles = semilandmarks; A = anterior, M = medial, D = dorsal (asterisk: *sulcus acusticus* = *ostium* + *cauda*).

RESULTS

General otoliths description. Sagittal otoliths' contour somewhat rectangular. Inner face strongly convex. A well-marked dorsal furrow. Postdorsal spine broad, relatively short. Dorsal depression not well-defined, smooth, and shallow; a curve follows between predorsal spine and predorsal angle. The ontogenetic variation shows a more rounded otolith in juveniles (*vs.* more rectangular in adults), a smoother ostium in juveniles, and postdorsal spine shorter in juveniles (Fig. 3).

Stellifer gomezi: Predorsal spine relatively long, straight to slightly curved outwards in the dorsal profile. Dorsal margin smooth, slightly concave; forming an angle of approximately 130° with predorsal angle. Predorsal angle rounded, occasionally projecting posteriorly. Postventral notch smooth, with an angle of approximately 130°. Ostium short, shallow, and wide; narrowed close to the ostial-caudal joint. A spout-like groove between the ostium and dorsal depression, bent at approximately 100°. *Cauda* deep, widened, strongly bent; rounded on its tip. Outer face rather straight, except for the posterior margin, slightly convex. A well-defined mid-dorsal projection, rounded to slightly sharp and oriented backward. The ontogenetic variation shows the *cauda* tip slightly pointed in juveniles, and the projection on the outer face less pronounced in juveniles (Fig. 3A).

Stellifer menezesi: Predorsal spine relatively long, straight, also nearly straight in dorsal profile. Dorsal margin smooth, slightly concave; forming an angle of approximately 140° with predorsal angle. Predorsal angle rounded, occasionally projecting outwards. Postventral notch smooth, with an angle of approximately 130°. Ostium short, generally shallow (except for a depression on its mid-posterior section), slightly ventrally widened, narrowed close to the ostial-caudal joint. A spout-like groove between the ostium and dorsal depression, bent at approximately 130°. Deep, widened, strongly bent *cauda*, slightly forward-pointed on its tip. Outer face rather straight, except for the posterior margin slightly convex. A well-defined projection on the mid-dorsal section; its margin squarish to rhomboidal and backward oriented. The ontogenetic variation shows the *cauda* slightly less bent in adults and its tip somewhat pointed in juveniles, predorsal angle less pronounced in juveniles, and the projection on the outer face less pronounced in juveniles (Fig. 3B).

Stellifer punctatissimus: Predorsal spine relatively long, straight, sharp to slightly rounded, nearly outwards directed in dorsal profile. Dorsal margin rather smooth or slightly sinuate forming an angle of approximately 135° with predorsal angle. Predorsal angle rounded, occasionally projecting outwards. Postventral notch smooth, with an angle of approximately 135°. Ostium short, surface irregular (a depression on its mid-posterior section), slightly widened ventrally, narrowed close to the ostial-caudal joint. A shallow, rather inconspicuous spout-like groove between the ostium and dorsal depression, bent at approximately 130°. *Cauda* deep, widened, strongly bent, slightly forward-pointed on its tip. Outer face flat to somewhat irregular, posterior margin slightly convex. A well-defined projection on the mid-dorsal section; its margin C-shaped to somewhat backward oriented. The ontogenetic variation shows the *cauda* tip somewhat pointed in juveniles, predorsal spine more rounded in adults, predorsal angle less pronounced in juveniles, projection on the outer face with its margin squarish to rhomboidal and backward oriented in adults (Fig. 3C).

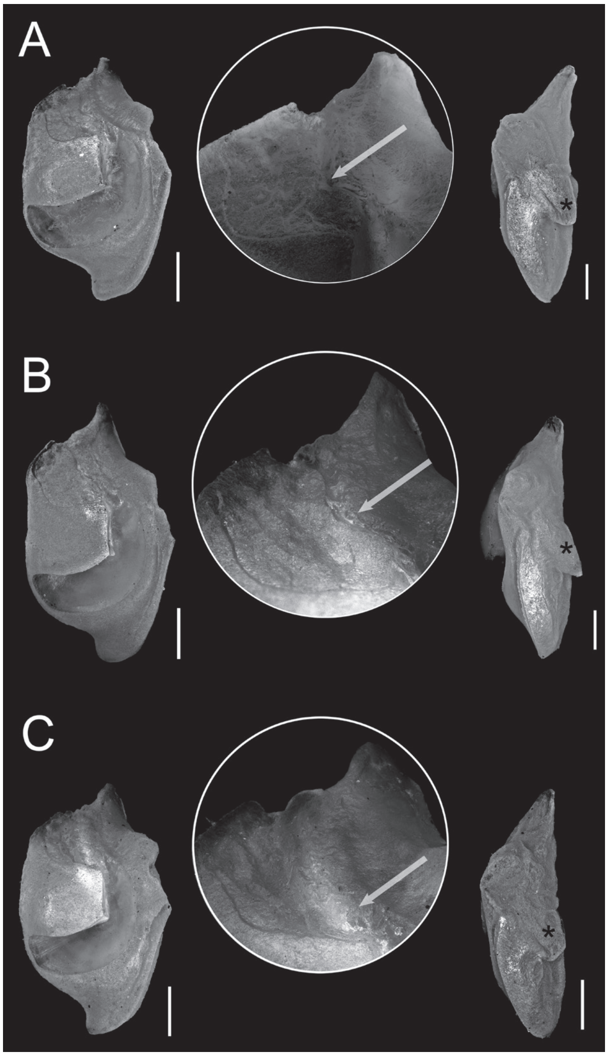

FIGURE 3 | Otoliths of *Stellifer punctatissimus* complex. **A.** *S. gomezi*. **B.** *S. menezesi*. **C.** *S. punctatissimus*. Left: inner face; Middle: inset of the *ostium*; Right: dorsal profile. Arrow indicates spout-like groove; asterisk indicates projection on the outer face. Scale bars = 1 mm.

Shape indices and shape analyses. Only the circularity and form factor exhibited statistically significant differences among species (ANOVA: F = 3.3, $p < 0.05$ and F = 3.1, $p < 0.05$, respectively), which displayed differences in allometry (Tab. 2; Fig. 4). Differences in otolith allometry were found among the species: negative in *Stellifer gomezi* and positive in *S. menezesi* and *S. punctatissimus*, the latter with the highest rectangularity value among all species (Fig. 4).

The shape contour (Fourier) of all otolith sections showed a higher variation at the anterior margin: *S. gomezi* exhibited the most concave *ostium* contour, while *S. menezesi* had a more elongated predorsal spine and a rounded predorsal angle (Fig. 5). Results from LDA showed a clustering pattern across species, with a certain degree of overlap near zero on both axes. There was a slight separation between *S. punctatissimus* (PC) and other species *S. gomezi* (GM) and *S. menezesi* (MN): the former had most of its variation

TABLE 2 | Values of shape indices by species in the *Stellifer punctatissimus* complex. Different letters show significant differences ($p < 0.05$), using ANOVA and Tukey's post hoc tests.

Species	Shape indices					
	Aspect ratio	Circularity	Ellipticity	Form factor	Rectangularity	Roundness
Stellifer gomezi	0.64±0.02	16.17±0.46^{a}	6.44±0.35	0.78±0.02^{a}	4.93±0.44	5.19±0.21
Stellifer menezesi	0.64±0.02	16.00±0.28	6.43±0.33	0.78±0.01	4.92±0.45	5.18±0.17
Stellifer punctatissimus	0.64±0.01	15.90±0.28^{b}	6.37±0.32	0.79±0.01^{b}	4.90±0.47	5.25±0.19

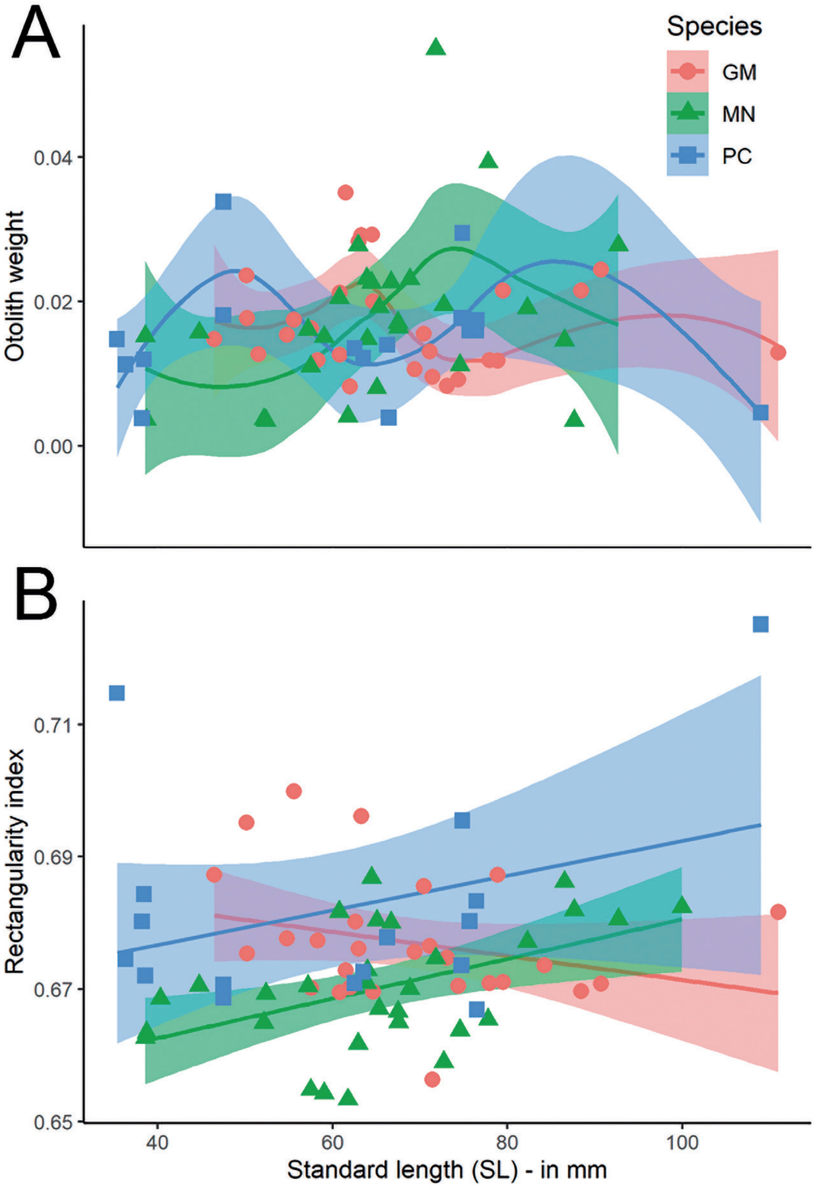

FIGURE 4 | **A.** Scatterplot of otolith weight using a LOESS curve fitting. **B.** Scatterplot of rectangularity index. GM = *Stellifer gomezi*, MN = *S. menezesi*, PC = *S. punctatissimus*.

enclosed in the positive values of the linear dimension 2, whereas its relatives were almost evenly distributed in positive and negative values. This pattern was repeated in the linear dimension 1, which gave a minor separation between GM and MN, respectively in the negative and positive values. This is reinforced by reclassification accuracy rates attained by the leave-one-out method, with relatively high percentages for GM (73.3%) and MN (80.0%) and a moderate one for PC (52.9%). The observed pattern was again corroborated by PERMANOVA, which recorded multivariate differences in the otolith shape contour among species (Pseudo-F = 4.02, df = 2, p < 0.001), with significant differences for all pairwise combinations: GM and MN (p = 0.001), MN and PC (p = 0.002), and GM and PC (p = 0.046) (Fig. 6).

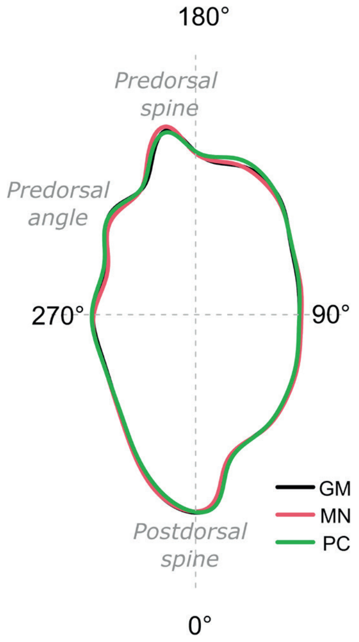

FIGURE 5 | Otolith's contour reconstruction (Fourier). GM = *Stellifer gomezi*, MN = *S. menezesi*, PC = *S. punctatissimus*.

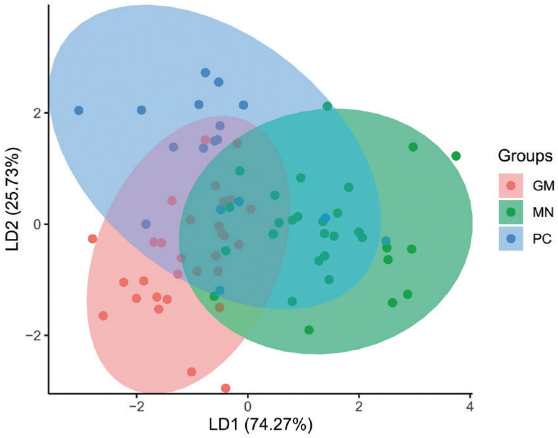

FIGURE 6 | Linear Discriminant Analysis (LDA) of contour shape according to elliptical Fourier descriptors (EFDs). GM = *Stellifer gomezi*, MN = *S. menezesi*, PC = *S. punctatissimus*.

Regarding geometric morphometrics, the PCA explained the major variation of shape in the otolith's *cauda*. The first two components of the PCA accounted for 65.1% of the shape variation in the *sulcus acusticus*; PC1 corresponded to a narrower/larger *cauda*, angle at the *cauda* almost straight, slightly bent *cauda* tip, and a narrower *ostium* (on the dorso-ventral axis). The second component (PC2), in turn, showed a narrower ostial-caudal joint, a wider *ostium* (on the antero-posterior axis), and an expansion on the *cauda*. On the PCA plot, most of the morphospace overlapped among the species. However, slight differences were found among them, especially for *Stellifer gomezi* (GM) and *S. menezesi* (MN). *Stellifer menezesi* (MN) had an almost centered distribution. The GM-MN group was allocated mostly in the positive values of the PC2, while PC exhibited the highest negative values and a more spread distribution. On the pair comparisons, GM-PC displayed the higher variation – linked to the expansion of the *cauda*, *sulcus*, and *ostium* and a less straight caudal curve; followed by MN-PC, with slight expansion on the *ostium* and caudal tip; finally, the pair GM-MN presented a lower distinction, which was restricted to small variations in caudal curve and *ostium* expansion (Fig. 7).

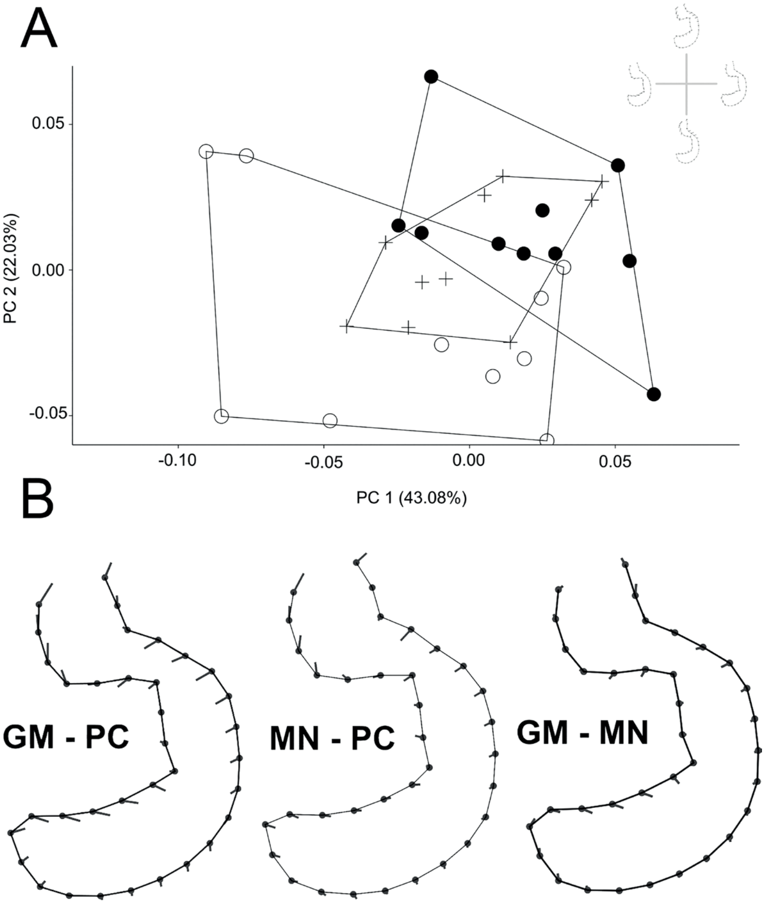

FIGURE 7 | **A.** Principal Component Analysis (PCA) of *sulcus acusticus's* shape variation using Procrustes residual of geometric morphometric method. Shape variation in each principal component (PC1 in the horizontal and PC2 in the vertical) in the upper left corner. **B.** Pairwise shape comparisons; GM = *Stellifer gomezi*, MN = *S. menezesi*, PC = *S. punctatissimus*.

DISCUSSION

Integrative approaches have increasingly been used to distinguish cryptic species of coastal fishes (*e.g.*, Carvalho *et al*., 2020b; Figueiredo-Filho *et al*., 2021). An initial morphological study indicated the existence of a species complex in *Stellifer punctatissimus* (Chao, 2002), but this hypothesis has not been confirmed by molecular evidence, which suggested instead a recent speciation with only two evolutionary lineages in this complex (Silva *et al*., 2018). More recently, all these species were formally recognized through a morphological basis (Chao *et al*., 2021), but complementary studies were still needed to elucidate their status. Herein, elliptic Fourier descriptors (EFDs) provided further evidence to support our initial hypothesis of a complex formed by three species, especially by satisfactory reclassification rates for all species.

Surveys using single species at spatiotemporal or stock levels have shown higher reclassification rates than those found here (Hoff *et al*., 2020; Kikuchi *et al*., 2021). Despite seeming to be rare, rates higher than those found here (52–80%) were already recorded in three (> 90%; Bani *et al*., 2013) and four (> 95%; Zischke *et al*., 2016) sympatric species comparisons. However, closely related species comparisons provide a reclassification range similar to ours (*i.e.*, 52–80%; La Mesa *et al*., 2020). Therefore, these results demonstrate the usefulness of this method to distinguish closely related species. A recent and likely incipient speciation may explain why, despite being a high-resolution method, our LDA showed a partially overlapping among those species. In fact, a previous analysis showed highly segregated groups when the allometry effect was not removed from our data. It might suggest a likely trend in this species complex' differentiation. *Stellifer punctatissimus* species complex' evolutionary history has led to high morphological similarity without clear genetic divergence, where allopatric and sympatric events probably drove the species' divergence (Silva *et al*., 2018). It has been shown that some species might differentiate even though they present non-significant genetic variation (Carvalho-Filho *et al*., 2009; Marceniuk *et al*., 2019).

However, as elliptic Fourier descriptors (EFDs) is a method for outlining a contour, its automated use is unlikely in "inner" structures such as the *sulcus acusticus* (*i.e.*, by being embedded inside another contour), which can be used as a proxy for prey identification (*e.g.*, in dolphins' diet studies – Rodrigues *et al*., 2020; Byrd *et al*., 2020). For instance, the geometric morphometric method (GMM) provided a contrasting result, a partially less overlapped pattern among the species. Interestingly, the observed pattern exhibits a "gradient" from *Stellifer punctatissimus* (PC) to *S. menezesi* and *S. gomezi* (MN, GM), which is in agreement with their external similarity. A convergence between body morphology and otolith shape may explain this pattern; the higher similarity between GM-MN than with PC (most visually distinctive species – Fig. 1B) resulted in its final "gradient" morphospace (Chao *et al*., 2021; unpublished data). In a similar case, La Mesa *et al*. (2020) found that a relatively morphologically distinct icefish species was also the most differentiated from the other two species by otolith shape. Indeed, previous studies have shown that similar species (*i.e.*, external morphology) may occupy a similar place in the morphospace, either when otolith shape or body shape is analyzed, as seen in the genus *Centropomus* (Granados-Amores *et al*., 2020; Figueiredo-Filho *et al*., 2021). Although the actual shape may be affected by the phylogenetic inertia – similar shape among closely related species coming from a lagged rate of evolution of a trait – it could present distinct growth rates that might allow distinguishing such species (Lombarte *et al*., 2010).

Studies on species delimitation must consider a broad source of data to yield a reliable taxonomic recommendation, because the evolutionary origin itself might lead to constraints in phenotypic disparity, such as stasis and niche conservatism, it turns more difficult to identify cryptic species than "regular" ones, thus, finer-scale methods are needed (Riddle *et al*., 2011; Struck, Cerca, 2019). That is, to reject the null hypothesis in species delimitation (*i.e*., two species in the complex – molecular hypothesis), a much higher amount of data should be used in a well-studied species than to describe/record a new species – in doing so, a better understanding of the evolutionary process would be achieved and taxonomic issues would be solved (Carstens *et al*., 2013; Hillis *et al*., 2021). For instance, a recent study has shown that the Bigtooth corvina (*Isopisthus parvipinnis*) in SW Atlantic, has well-marked groups based on otolith shape (*e.g*., populations, years), which probably reflects fishery pressure and changes in population structure toward a metapopulation structure (Hoff *et al*., 2020). Although there might have additional influences like unknown diversity (Guimarães-Costa *et al*., 2020). In such situations, only the combined use of approaches and data review might lead to a resolution; as discussed herein, different growth rates and otolith shapes aid in a well-based result that places certainty on taxonomic status.

Despite our data came from a single source (otoliths), they were not appraised through a single perspective. For instance, its description reveals some agreement with the current phylogeny, namely, a closer relationship between the *Stellifer punctatissimus* species complex and *S. scierus*/*S. strabo* (both from the Pacific Ocean). That is, they shared a well-marked spout-like groove, with almost a right angle, and shared with fossil species a backward oriented projection on its otolith's outer face (Aguilera *et al*., 2016; Silva *et al*., 2018). In Sciaenidae, a family with an evolutionary perspective of specialization on the sensory apparatus, those characters could help solve incongruencies throughout their phylogeny by being explored in an ancestral state reconstruction (Trewavas, 1977; Schwarzhans, 1993). Additionally, a few differences, such as *sulcus acusticus* shape and contour angles, add evidence to refute our null hypothesis. Therefore, it agrees with the other methods' results that indicate the occurrence of three species on its complex.

Conversely, variations in otolith shape of the *Stellifer punctatissimus* complex can also result from factors not measured herein, such as the metabolic and growth rates (Alewijnse *et al*., 2021; Jónsson *et al*., 2021). As Geller (1999) argues, these aspects could help to evaluate cryptic species, as they reveal possible confounding effects in species complexes. This seems to be achieved in our results, which show each species with a specific rate of otolith weight (Fig. 4B), which could suggest a distinct size at first maturity for each species, and in agreement with our initial hypothesis. Additionally, by showing a distinct allometric pattern in the rectangularity index, at least to one of the species (GM), our results further support the hypothesis of three distinct species in the *Stellifer punctatissimus* species complex. It has been proven that either accretion rates or growth rates in otolith shape can be used as a proxy for differentiating species or even stocks, due to the assumed distinct intrinsic factors by species (Pavlov, 2016; Kikuchi *et al*., 2021). In the same way, shape indices can be linked to traits such as depth preference, habitat use, and feeding habits (Assis *et al*., 2020). In doing so, we could apply the differences in ecological niches into a taxonomic approach in order to achieve a better resolution of taxonomic issues, as discussed here. Our data show values above 64% of the aspect-ratio, which may classify all these species as associated with demersal habit over unconsolidated substrata, but do not suggest strictly benthic habit, such as that occurring in *Menticirrhus* (Jaramillo *et al*., 2014; Carvalho *et al*., 2020a). These findings are consistent with data on this species complex, which have changed an incorrect assignment from zoobenthivorous to zooplanktivorous guild (Santos *et al*., 2021).

Furthermore, of all shape indices, only circularity and form factor were statistically significant. This low contribution to differentiation among the species is not unexpected, because these indices present an unsuitable resolution to detect small differences at the species level (Pavlov, 2016; Wong *et al*., 2016; Avigliano *et al*., 2018). Although shape indices rarely support species-delimitation studies, otolith-based approach in *Macrodon* (Cardoso *et al*., 2012) had validated previous molecular and morphological hypotheses (Santos *et al*., 2006; Carvalho-Filho *et al*., 2010). As discussed by Tuset *et al.* (2021), the shape indices only provide good results if clearly distinct species are compared – which is not the case for cryptic species. We suggest therefore that Ellipitical Fourier descriptors perform better than shape indices to distinguish cryptic and congeneric species. On the other hand, once the application of homologous landmarks in otoliths is unlikely, we needed to apply several semilandmarks on the *sulcus acusticus*, as a result, we lost statistical power. Thus, our GMM results would be improved by adding specimens, which could give a balance between variables (semilandmarks) and specimens (otoliths) (Gunz, Mitteroecker, 2013).

As the otolith shape is known to be species-specific, it accurately responds to environmental gradients; thus, we could indicate habitat use through otoliths. We cannot disregard the likelihood of those otolith distinctions holding a signal of a putatively different coastal zone occupation; this could retrieve a recent speciation process likely by ecological divergence (Carvalho-Filho *et al*., 2009; Silva *et al*., 2018; Caires *et al*., 2019). As these shapes vary (*e.g.*, contour, *sulcus acusticus*) among species, they can be applied for taxonomic purposes to fulfill the gaps within Sciaenidae and in ecological surveys, once otoliths are one of the most informative remains to identify bony fishes in diet studies (Rodrigues *et al*., 2020; Byrd *et al*., 2020).

In regard to conservation, both of them – prey and predators – are caught as bycatch in shrimp fisheries, with the former (*S. punctatissimus*) being classified as Least concern (LC) and Data deficient (DD) according to IUCN and MMA/IBAMA, respectively (ICMBio, 2018; Aguilera Socorro, 2020). To date, *S. menezesi* has not been evaluated either by IUCN or ICMBIO, and *S. gomezi* was classified as LC by the last available IUCN's assessment (Chao, 2020). Despite their lack of commercial value, we could not dismiss the ecological importance of these species because they need better monitoring and some effects of fisheries pressure on marine food webs remain unclear (Márquez-Velásquez *et al*., 2021; Lira *et al*., 2022). Due to the possibility of differences in habitat use among those species, such as discussed for the Tonkin weakfish (Caires *et al*., 2019), the existence of distinct conservation statuses by species in a future assessment is presumable due to the likely specific threats upon each species. For this reason, it is essential to continue pursuing additional data that could elucidate if those species truly have a degree of distinct habitat use throughout their life cycle, which would also help to solve uncertainties in their recent speciation process. Therefore, our results provide significant data that might aid to species' conservation status assessment, as taxonomic uncertainties are one of the main issues that usually hamper these evaluations.

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COMPETING INTERESTS

The authors declare no competing interests.

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