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Comparative Analysis of Otolith Micro-Characteristic in *Schizothorax grahami* and *Spinibarbus sinensis*

Qun Lu[1,4], Yang Zhou[1], Hangyu Lin[1,4], Zhong tang He[1], Feifan Ma[1], Yonghua Zheng[1,2], Hongyu Tang[1,2] and Tao He[1,2,3]*

[1]*College of Fisheries, Southwest University, Chongqing, China, 400715.*
[2]*Key Laboratory of Freshwater Fish Reproduction and Development (Ministry of Education), Key Laboratory of Aquatic Science of Chongqing, China, 400715.*
[3]*Guangdong Provincial Key Laboratory of Luminescence from Molecular Aggregates, South China University of Technology, Guangdong 510640, China.*
[4]*Siwo Ecological Environment Technology Co., Ltd, Chongqing, China, 400715*

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TH, YHZ and HYT conceptualized the study. QL, HYL, ZTH, FFM collected the data from field. QL and YZ analyzed the data and drafted the manuscript. TH reviewed and improved the manuscript

Key words
***Schizothorax grahami, Spinibarbus sinensis*, Sagittal otolith, Micro-characteristic**

ABSTRACT

In this paper, the micro-features of sagittal otoliths of *Schizothorax grahami* and *Spinibarbus sinensis* were analyzed in the upper reaches of the Yangtze River. The results of age identification showed that *Sc. grahami* and *Sp. sinensis* did not reach the age of sexual maturity. The linear discriminant analysis of otolith shape-index analysis can effectively distinguish the otolith shape of the two kinds of fish. The highest content of five chemical elements in otolith is Ca, and the other four elements are Mg, Sr, Ba, and Mn in turn. The results of one-way ANOVA show that, the molar ratio of each element to calcium (Mg/Ca, Ba/Ca, Sr/Ca and Mn/Ca) showed that there was significant difference between *Sc. grahami* and *Sp. sinensis*, which could be used to distinguish two species of fish belonging to different genera of the same family. This research could also provide data reference for the study of the migration route between *Sc. grahami* and *Sp. sinensis*.

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INTRODUCTION

Otoliths are formed in the embryonic stage and mainly play the role of hearing and balance. Otolith are composed of calcium carbonate crystals suspended in protein matrix. Calcium carbonate is usually deposited as aragonite in the otolith of balloon (sagittal body) and utricle (lapillus) and as vaterite in the otolith of the lagena (asteriscus) (Falini *et al*., 2005; Oliveira *et al*., 1996). In addition, due to the closed nature of the otolith environment, the otolith continues to grow even if the fish's body stops growing. Therefore, the otolith can be used as a reliable carrier of fish growth information. In the past a few decades, geometric morphometry of body, otolith and scale has been used for analyzing morphological characteristics and discriminating between fish stocks or populations, especially using otolith morphology (Adams *et al*., 2004; Begg *et al*., 1999). Otoliths of fish include micro otoliths, sagittal otoliths and stellate otoliths. Among these otoliths, the sagittal otoliths have the most prominent feature because it shows a species-specific morphology, but changes less within a species (Campana, 2004). The morphological changes of sagittal otoliths may be affected by age, genetic factors and environmental conditions (Vignon and Morat, 2010), as well as by growth rate, feeding history (Gagliano and McCormick, 2004) and living habitat (Lombarte and Lleonart, 1993). Due to these complex factors, the analysis of the size and shape of sagittal otoliths has become a useful tool to distinguish fish species and geographical stocks (Tuset *et al*., 2003).

Schizothorax grahami and *Spinibarbus sinensis* are endemic fish in the upper reaches of the Yangtze River. Their natural resources have reduced due to overfishing, environmental pollution and the construction of water conservancy facilities. Since the discovery of *Sc. grahami*, the related studies have mainly involved morphology, physiology and biochemistry, genetic diversity, phylogenetic evolution, etc. (Hai-tao *et al*., 2012; Miao, 2009; Si-yu *et al*., 2007), while the studies of *S. sinensis* have involved physiology and biochemistry, disease,

* Corresponding author: hh1985@swu.edu.cn
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toxicology, juvenile behavior and metabolism, etc., without involving otoliths microstructure and microchemical characteristics.

Two kinds of fish sagitta otoliths microstructure are observed, measured and translated into eight otoliths parameters shape index, through the analysis of the shape index of the two kinds of fish in the different genus of the distinguish. The size-dependent variables of sagitta morphology include the anteroposterior length, maximal Feret's diameter, dorsoventral width, distances from the center to margins, the area of the 2-dimensional (2-D) sagittal projection, and the otolith perimeter (Ponton, 2006). From these variables, different shape indices that give indications of roundness, circularity, rectangularity, ellipticity, and eccentricity of otoliths have been used to morphometric analysis (Tuset *et al*., 2003). As the elements come from water needed in the otolith growth, fish living in different geographical environments will show differences in the micro chemical characteristics of otoliths. Therefore, the micro chemical characteristics of otolith could be used as a means to distinguish fish populations. Qin (2019) found that there were significant differences in the ratio of metal elements to calcium in the otoliths of *Coilia mystus* with different habitat resume types. Shuo-zeng *et al*. (2011) determined and analyzed the elements in the otolith nucleus of different *Coilia nasus* populations by multi measuring point LA-ICP-MS technology, and mainly studied the population identification by Sr/Ca and Ba/Ca ratio (Shuo-Zeng *et al*., 2011). Combined with the actual situation, the contents of strontium (Sr), calcium (Ca), manganese (Mn), barium (Ba) and magnesium (Mg) in sagittal otoliths were determined, and the differences of chemical elements in two species of sagittal otoliths were analyzed. In this paper, the micro-structure and micro-chemical characteristics of otolith of *Sc. grahami* and *Sp. sinensis* were studied to provide a reference for the protection and utilization of the unique fish in the upper reaches of the Yangtze River.

MATERIALS AND METHODS

Sample collection and measurement

In this study, the *Sc. grahami* was fished from the Shuitian section of Chishui River (27.75664602°N, 105.21669513°E), and the *Sp. sinensis* was purchased from Chongqing Dongping aquaculture company. Head length, body length, and weight were measured in the laboratory (the results were retained to two decimal place). The measurement results are shown in Table I.

Otolith shape observation and morphological measurement

The left sagittal otoliths were taken out from the auditory sac by dissecting the head with an anatomical needle, surface impurities and tissues were washed with 5% NaOH, and store them in a numbered 1.5ml centrifuge tube for standby. The Moticam S6 digital camera connected to Nikon SM171 dissecting mirror was used to take pictures of otolith towards the inner side of the fish body. The morphological parameters such as otolith area (A), perimeter (P), feret length (FL), feret width (FW), maximum feret length (Fmax), minimum feret length (Fmin), maximum radius (Rmax) and minimum radius (Rmin) of otoliths were measured by using the micro image analysis software Image-Pro Plus 6.0. The calculation formula of the index is as follows: roundness=4 A/πFL^2, format factor=4πA/P^2, circularity= P^2/A, rectangularity= A/(FL×FW), ellipticity=(FL-FW)/(FL+FW), radius ratio=Rmax/Rmin, feret ratio=Fmax/Fmin, aspect ratio= FL/FW. And the measurement method is shown in Figure 1A, B (Tuset *et al*., 2003). By comparing and analyzing the otolith shapes of samples with different body lengths, it is considered that the otolith shapes of each group have tended to be stable and could be used for morphological comparative analysis.

Table I. Basic biological parameters (Mean±SD) of *Schizothorax grahami* and *Spinibarbus sinensis*.

	***Sc. grahami* (n=30)**	***Sp. sinensis* (n=25)**
Head length (cm)	2.93±0.59	6.63±0.45
Body length (cm)	13.36±3.21	35.49±1.01
Weight (g)	60.03±56.63	1058.70±119.90

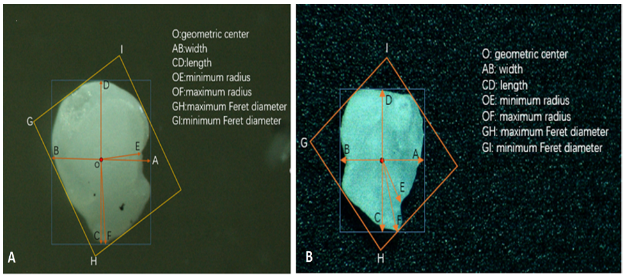

Fig. 1. Measurement illustration of sagitta in *Sc. grahami* (A), and *Sp. sinensis* (B).

Otolith chemistry measurement

The washed otolith was dissolved in 50 ml of 5% dilute hydrochloric acid, and then filtered through 0.45 μm filter head combined with syringe filtration. Finally, the contents of strontium (Sr), calcium (Ca), manganese (Mn), barium (Ba), and magnesium (Mg) were tested by inductively coupled plasma mass spectrometry (ICP-MS).

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Statistical analysis

Microsoft excel 2021 is used to sort out the data, and spss 26.0 statistical software is used for one-way ANOVA, principal component analysis and discriminant function analysis. The results are recorded as mean ± SD. There was no significant difference when $p > 0.05$, $p < 0.05$ is significant difference, and $p < 0.01$ is a very significant difference.

RESULTS

Morphological description of sagitta otolith

Morphological description and calculation results of sagitta shape index of *Sc. grahami* and *Sp. sinensis* are shown Table II.

Table II. Morphological measurements and sagitta shape indices of Otolithis of *Sc. grahami* and *Sp. sinensis.* The values are Mean±SD (ranges).

Parameter	*Sc. grahami*	*Sp. sinensis*
Otolith area (A)	0.798±0.252[a] (0.624-2.096)	0.744±0.060[a] (0.618-0.899)
Perimeter (P)	0.941±0.291[a] (0.791-2.473)	0.878±0.043[a] (0.802-0.949)
Feret length (FL)	12.866±1.759[a] (5.019-15.880)	14.336±0.716[a] (13.237-15.668)
Width (FW)	0.818±0.263[a] (0.721-2.210)	0.752±0.043[a] (0.659-0.818)
Feret length maximum (F_{max})	0.133±0.033[a] (0.066-0.201)	0.126±0.034[a] (0.0614-0.201)
Minimum (F_{min})	1.767±0.294[a] (1.458-2.742)	1.631±0.157[a] (1.346-1.963)
Radius maximum (R_{max})	1.340±0.083[a] (1.151-1.507)	1.332±0.081[a] (1.205-1.534)
Minimum (Rmin)	1.310±0.087[a] (1.141-1.502)	1.293±0.092[a] (1.131-1.504)

Same superscripts mean that differences are not significant.

In *Sc. grahami*, the front and rear ends of the sagittal otolith are nearly circular. The front end is small with no notch, while the rear end is larger than the front with a notch. The inner surface edge has three small notches, and the outer side edge has two notches: One is larger than the other one. The larger notch makes the outer side edge concave. In addition, the dorsal part of sagitta is convex and the ventral part is concave.

In *Sp. sinensis*, the front end of otolith is approximately square, and the rear end, with nearly circular, is larger than the front. The edge of the inner side is smooth and arc-shaped, while there is a notch on the edge of the outer side, making the edge concave. In addition, the dorsal part of sagitta is convex and the ventral part is concave.

Shape index analysis

The one-way ANOVA of 8 otolith shape indices at the level of significant difference of P = 0.05 shows that there was significant difference in radius ratio between *Sc. grahami* and *Sp. sinensis*, and the other seven shape indices were not significant by different from each other.

Table III. Loadings and eigenvalues of the first 2 principal components of shape indices of sagittae of *Sc. grahami* and *Sp. sinensis.*

Otolith shape indices	Principal components	
	1	2
Otolith area (A)	0.996	0.030
Perimeter (P)	0.989	0.104
Feret length (FL)	0.976	-0.153
Feret width (FW)	-0.962	0.109
Maximum feret length (F_{max})	-0.017	0.978
Minimum feret length (F_{min})	-0.009	0.974
Maximum radius (R_{max})	-0.071	0.974
Minimum radius (Rmin)	-0.018	0.752
Eigenvalue	3.961	3.360
Variance explained	49.516	41.995
Cumulative percentage	49.516	91.512

Table IV. Parameters of discriminant functions for *Sc. grahami* and *Sp. sinensis.*

Shape index	*Sc. grahami*	*Sp. sinensis*
Otolith area (A)	2629.165	2610.208
Perimeter (P)	3348.292	3410.833
Feret length (FL)	329.499	336.900
Feret width (FW)	-3942.835	-3946.431
Minimum feret length (F_{min})	-551.739	-566.384
Maximum radius (R_{max})	2351.774	2355.107
Constant	-4383.705	-4507.079

Principal components analysis

Principal component analysis was carried out for otolith shape indices. The eigenvalues of the two indices were greater than 1, and they were taken as the first two principal components respectively (Table III). The Table shows that the cumulative contribution rate of the first two principal components is 91.512% while indicating that the otolith morphological differences between *Sc. grahami*

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and *Sp. sinensis* could be summarized with a few indices.

In the first principal component, the loadings of shape index roundness, format factor, circularity, and rectangularity are larger, which mainly reflects the difference of external contour regularity of otolith. The loading of ellipticity, radius ratio, feret ratio, and X_8-aspect ratio is larger, which mainly reflects the difference between long and short axes of the otolith. Figure 2 is the scatter diagram of the first two principal components. The two species amostly distributed near the origin and mixed with each other.

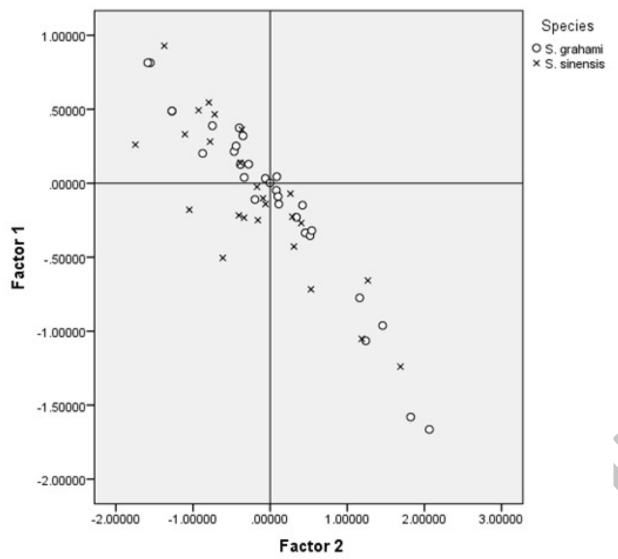

Fig. 2. Scatter plot of principal component analysis for sagittal shape indices of *Sc. grahami* and *Sp. sinensis*.

Discriminant analysis

Using the general discriminant analysis program in SPSS, the discriminant equation (the canonical discriminant function) with 6 otolith shape indices (roundness, format factor, circularity, rectangularity, radius ratio, feret ratio) as independent variables is obtained. Parameters of discriminant functions for *Sc. grahami and Sp. sinensis* are shown in Table IV.

The otolith shape indices of each sample are substituted into the above two discriminant equations, and each sample obtains two function values. The group corresponding to the discriminant function with the largest function value is the group to which the sample belongs. The discriminant results are shown in Table V. The comprehensive discrimination accuracy is 85.5%, and the two populations in the table could be discriminated effectively. Figure 3 is a scatter diagram made according to the canonical discriminant function and its calculated value. From the figure, the *Sc. grahami* population and *Sp. sinensis* population could be distinguished.

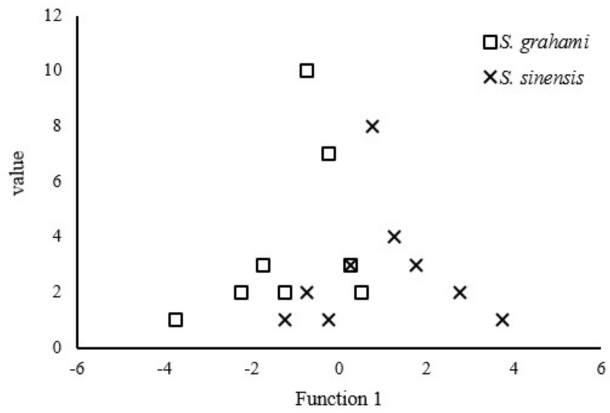

Fig. 3. Scatter plot of scores based on the first one canonical discriminant functions.

Table V. Discriminant results for sagitta shape indices of *Sc. grahami* and *Sp. sinensis.*

Species	Number	Accuracy %	Predicted species membership	
			Sc. grahami	***Sp. sinensis***
S. grahami	30	86.7	26	4
S. sinensis	25	84.0	4	21

Microchemistry of the otolith

The contents of Ca, Mg, Ba, Sr, and Mn in sagittal otoliths of *Sc. grahami* and *Sp. sinensis* are shown in Table VI. According to the determination results of chemical elements, Ca is the most abundant on the sagittal otolith of the two kinds of fish, followed by Mg, Sr, Ba, and Mn.

After converting each element into the molar ratio to Ca (mmol/mol), one-way ANOVA was performed at the significance level of $P < 0.05$. The results of one-way ANOVA are shown in Table VII. The results showed that there were significant differences in Mg/Ca, Ba/Ca, Sr/Ca, and Mn/Ca on the sagittal otoliths of *Sc. grahami* and *Sp. sinensis*, indicating that there were significant differences in the content of chemical elements in the otoliths of the two kinds of fish.

DISCUSSION

Microstructure of otolith

Otolith shape is unstable in the early stage of fish life history. It needs to go through a process of formation, growth, and stability, which is usually affected by genetic information, geographical environment, and other factors (Gagliano *et al*., 2004; Vignon and Morat, 2010). Therefore, the population division of fish could be carried out through the microstructure of otolith. At present, the main methods

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Table VI. Contents of major chemical elements in sagittal otoliths of *Sc. grahami* and *Sp. sinensis* (g/L).

Species		Ca (10^{-6})	Mg (10^{-6})	Ba (10^{-6})	Sr (10^{-6})	Mn (10^{-6})
Sc. grahami	Mean±SD	60.38±2.56	13.32±0.26	0.054±0.003	0.42±0.011	0.068±0.018
	(Ranges)	(55.90-67.97)	(12.67-13.83)	(0.046-0.06)	(0.39-0.43)	(0.019-0.11)
Sp. sinensis	Mean±SD	83.77±4.75	13.03±0.26	0.044±0.027	0.44±0.078	0.070±0.006
	(Ranges)	(75.93-92.49)	(12.12-13.44)	(0.036-0.049)	(0.042-0.49)	(0.049-0.075)

Table VII. The result of one-way ANOVA on the molar ratio of each element to calcium (mmol/mol).

The molar ratio of each element to calcium			Sum of squares	df	Mean square	F	Signifi-cance
Mg/Ca	Between groups	(Combination)	1.75×10^{11}	1	1.75×10^{11}	812.054	0.000
	Within groups		1.25×10^{11}	58	2.15×10^{8}		0.000
	Total		1.87×10^{11}	59			
Ba/Ca	Between groups	(Combination)	1.71×10^{5}	1	1.71×10^{5}	733.814	0.000
	Within groups		1.35×10^{4}	58	232.412		0.000
	Total		1.84×10^{5}	59			
Sr/Ca	Between groups	(Combination)	8.07×10^{6}	1	8.07×10^{6}	82.729	0.000
	Within groups		5.66×10^{6}	58	9.76×10^{4}		0.000
	Total		1.37×10^{7}	59			
Mn/Ca	Between groups	(Combination)	6.76×10^{5}	1	6.76×10^{5}	24.879	0.000
	Within groups		1.58×10^{6}	58	2.72×10^{4}		0.000
	Total		2.25×10^{6}	59			

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to study otolith morphology are the shape index method and the Fourier analysis method (Xiao-zhe and Tian-xiang, 2010). Both methods use otolith contour variables to study otolith morphological differences, so as to identify fish species, different geographical groups of the same fish species, fish genetic relationships. The shape index method is the deepening of the measurable character comparison method, that is, the measurement results could be combined into shape indexes that represent more shape information, and then the otolith morphology is compared and analyzed. In recent years, scholars have used the otolith shape index to identify fish species and achieved good results (Hoff *et al*., 2020; Saygin *et al*., 2020).

In this study, the shape index method was used to analyze the sagittal otolith shape of *Sc. grahami* and *Sp. sinensis*. In the shape index, the shape factor represents the regularity of the otolith contour. The smaller the value, the more irregular the contour is. Ellipticity indicates whether the distance from the outer edge point to the major and minor axes is proportional. Roundness and circularity represent the difference between otolith contour and equal area circle. The larger the roundness value or the smaller the circularity value, the closer it is to circle. The rectangularity describes the relationship between the otolith contour and the minimum circumscribed rectangle of the otolith. Radius ratio, feret ratio, and aspect ratio represent the difference between long and short axes of otoliths (Ponton, 2006). These indexes are ratios, which could eliminate the influence of different otolith sizes and otolith placement on the image analysis results (Xiao-zhe and Tian-xiang, 2010). The results of one-way ANOVA of shape index analysis showed that there was an only significant difference in the shape index of radius ratio at the significance level of $P < 0.05$, but there was no significant difference in the other seven shape indexes, indicating that one-way ANOVA could not effectively distinguish the sagittal otoliths of *Sc. grahami* and *Sp. sinensis*. The results of the principal component analysis show that the cumulative contribution rate of the first principal component and the second principal component is 91.512%, which could explain 91.512% of the total variation, indicating that a small amount of otolith shape index could be used to summarize the morphological differences of sagittal otoliths between *Sc. grahami* and *Sp. sinensis*. The shape index of otolith in the first principal component is roundness, shape factor, and ring rate, which mainly reflects the differences of otolith long axis, otolith short axis, and otolith contour. In the first principal component, the loadings of shape index roundness, format factor, circularity and rectangularity is larger than the second principal component, which mainly reflects the difference of external contour regularity of otolith. The loading of -ellipticity, -radius ratio, -feret ratio, and -aspect ratio is larger, which mainly reflects the difference between long and short axes of the otolith. From the scatter diagram of principal components, *Sc. grahami* and *Sp. sinensis* overlap each other and could not be completely distinguished. Consequently, the principal component analysis of the otolith shape index could not distinguish the two kinds of fish.

One-way ANOVA and principal component analysis of shape index could not effectively distinguish the two kinds of fish, so discriminant analysis was carried out. The results of the discriminant analysis showed that the comprehensive discriminant rate was 85.5%, and four tails of the two species have identified incorrect. This analysis obtains the canonical discriminant function. Through the scatter diagram made by the canonical discriminant function and its calculated value, we could intuitively see that although the two populations are partially crossed, they could also effectively distinguish the two populations. This is similar to Jiao's research results (Jiao, 2013). Jiao used the shape index method to compare the sagittal otoliths of *Scomber japonicus*, *Scomber australasicus* and *Scomber scombrus*. The principal component analysis of the shape index could not effectively distinguish Japanese mackerel from Atlantic mackerel, and linear discriminant analysis could better distinguish them. It shows that the shape index method has limitations, so other scholars use the Fourier method to analyze it deeply at the same time in the silver carp, bighead carp, and grass carp by elliptic Fourier analysis (Xiangbo and Guohua, 2012). The results show that with the growth and development of fish, the accuracy of identifying fish species by otolith morphology gradually increases. Yingjun made a Fourier analysis on otolith morphology of 255 species of marine fish in 83 families of 16 orders (Yingjun, 2010). The results showed that the accuracy of otolith morphology for genera and species was higher than that of families. Therefore, through more perfect research on the otolith morphology of various fish, it could be used as an auxiliary means of fish classification.

Microchemistry of the otolith

The deposition of trace elements in otolith is a complex product of multiple factors. The deposition process of trace elements in otoliths is mainly controlled by the changes of physical (water temperature) and chemical (salinity, element concentration, etc.) environment in the water environment (Campana, 1999; Izzo *et al*., 2018). The change of deposition has the ability to reflect the real-time living environment. Generally, the concentration level of an element in the water environment determines the deposition level in otolith. After years of geographical isolation, the same species will appear in reproductive isolation and then differentiate into two stocks. For fish, different geographical environments could lead to different deposition of one or more elements in otoliths. Therefore, trace elements in otolith could be used to verify the results of otolith morphological analysis.

Combined with the actual situation, this study selects the trace elements of Ca, Mg, Sr, Ba, and Mn in otoliths. In terms of element content alone, the measurement results show that the most chemical element content on the otoliths of *Sc. grahami* and *Sp. sinensis* is Ca, which is consistent with the results of *Todarodes pacificus*, *Coregonus ussuriensis*, and *Electrona carlsbergi* (Ji-Long *et al*., 2019; Jin, 2010; Lian, 2018). The contents of the remaining four elements are Mg, Sr, Ba, and Mn in turn, which is consistent with Shanshan's measurement results on the otoliths of Northwest autumn knife fish, but inconsistent with Lian's results (Lian, 2018; Shanshan, 2018). The chemical elements in otolith are affected by many factors, such as water area, geology, fish species, fish physiological and biochemical activities. After measuring the element content, it was standardized. Through analysis, it was found that there was a significant difference in the trace element content between *Sc. grahami* and *Sp. sinensis*, which could prove that the geographical environment of the two kinds of fish was different. The research results are similar to other scholars. For example, Jing measured the chemical elements on the otoliths of larval silver carp collected from the two waters in three periods (Jing *et al*., 2018). The results show that there are significant differences in Si/Ca and Sr/Ca values in the otoliths of larval silver carp in the two waters, which could be used to determine the population of larval silver carp in the two waters, the discrimination accuracy is more than 80% for the samples collected in each period. Yukun measured the chemical elements in the otoliths of small yellow croakers collected from the Yellow Sea and the Bohai Sea (Yukun *et al*., 2016). It was found that there were significant differences in the content of chemical elements in the otoliths of small yellow croakers in the two waters. They could be divided into two geographical groups through discriminant analysis. Hao collected small yellow croakers in five sea areas near the Yellow Sea and the East China Sea, and determined and separated the chemical elements on its otolith (Hao, 2015). The results showed that there were significant differences in chemical elements in the otoliths of small yellow croakers in five sea areas. It showed that small yellow croakers in the Yellow Sea and the East China Sea could be distinguished by

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discriminant analysis. Xin (2014) study found that Sr/Ca and Ba/Ca could effectively distinguish Coilia populations in five different waters. The above results show that otolith chemical elements could be used as a basis for fish species identification.

CONCLUSION

In conclusion, three mathematical methods were used to analyze the sagittal otolith shape index of *Sc. grahami* and *Sp. sinensis* in this study. The analysis results showed that the principal component analysis of otolith shape index analysis could not effectively distinguish the two kinds of fish, and the comprehensive discrimination accuracy of linear discriminant analysis is 85.5%. The results of otolith microchemical measurement showed that calcium is the most abundant in the sagittal otolith of the two kinds of fish, followed by Mg, Sr, Ba, and Mn, respectively. There are significant differences in Mg/Ca, Ba/Ca, Sr/Ca, and Mn/Ca, indicating that there were significant differences in the content of chemical elements in sagittal otolith. The above two methods could be used to distinguish the two kinds of fish.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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