

# Evaluation of removal of the size effect using data scaling and elliptic Fourier descriptors in otolith shape analysis, exemplified by the discrimination of two yellow croaker stocks along the Chinese coast\*

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**Abstract** Removal of the length effect in otolith shape analysis for stock identification using length scaling is an important issue; however, few studies have attempted to investigate the effectiveness or weakness of this methodology in application. The aim of this study was to evaluate whether commonly used size scaling methods and normalized elliptic Fourier descriptors (NEFDs) could effectively remove the size effect of fish in stock discrimination. To achieve this goal, length groups from two known geographical stocks of yellow croaker, *Larimichthys polyactis*, along the Chinese coast (five groups from the Changjiang River estuary of the East China Sea and three groups from the Bohai Sea) were subjected to otolith shape analysis. The results indicated that the variation of otolith shape caused by intra-stock fish length might exceed that due to inter-stock geographical separation, even when otolith shape variables are standardized with length scaling methods. This variation could easily result in misleading stock discrimination through otolith shape analysis. Therefore, conclusions about fish stock structure should be carefully drawn from otolith shape analysis because the observed discrimination may primarily be due to length effects, rather than differences among stocks. The application of multiple methods, such as otoliths shape analysis combined with elemental fingerprinting, tagging or genetic analysis, is recommended for stock identification.

**Keyword:** otolith shape analysis; data scaling for fish length; stock discrimination; removal of length effect

## 1 INTRODUCTION

An understanding of stock structure is essential for effective management of fisheries that contain multiple stocks. Studies on fish biology or population dynamics and most estimates of yield are conducted on the basis of a well-established population structure (Campana and Casselman, 1993). Techniques including genetic analysis, otolith elemental fingerprinting and otolith shape analysis or combinations thereof are widely applied for stock discrimination (Bentzen et al., 1996; Begg and Waldman, 1999; Campana, 1999; Thresher, 1999; Longmore et al., 2010; Smith and Campana,

2010). Among these techniques, otolith shape analysis has shown promise as a potential technique for stock identification due to the rapid development of digital photographic technology and statistical tools and their application to shape analysis (Begg and Brown, 2000). Otoliths grow throughout the life of teleosts and are metabolically inert and species specific. The external

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characteristics of otoliths vary among geographic stocks due to differences in the ontogeny, genetics and environmental history of fish (Castonguay et al., 1991; Lombarte and Lleonart, 1993; Begg and Brown, 2000); thus, otolith morphology is a potential indicator of discrete stocks. Moreover, shape analysis is a relatively inexpensive and time-efficient method compared with both genetic and microchemical techniques (Tracey et al., 2006). As an ideal marker of fish population affiliation, the use of otolith shape to discriminate among stocks has been investigated in many fishes, such as herring *Clupea harengus* (Burke et al., 2008), Atlantic cod *Gadus morhua* (Campana and Casselman, 1993; Cardinale et al., 2004), haddock *Melanogrammus aeglefinus* (Begg and Brown, 2000), and southern blue whiting *Micromesistius australis* (Legua et al., 2013).

In the analysis of otolith shape, fish size is a potential source of variability in morphometric measures because size is associated with individual ontogeny (Lleonart et al., 2000). Some statistical procedures have been used to eliminate the size effect of fish individuals. The linear model  $Y=a+bX$  is widely used to scale variables correlated with fish size, where the coefficient  $b$  is equal to the common within-group slope. In this method, the product of the slope ( $b$ ) and fish length is subtracted from the observed variables to obtain size-free variables (i.e., standardized or scaled variables; Bolles and Begg, 2000; Cardinale et al., 2004). Another method for removal of the size effect is based on an allometric growth model,  $Y=aX^b$  (Lleonart et al., 2000). However, the accuracy of these two models in otolith morphology studies and the extent to which the two models remove the size effect on scaled variables have rarely been investigated. Although otolith shape analysis is widely used for stock discrimination, very few studies have attempted to identify weaknesses of this method. For example, variation in fish size (e.g., length range or distribution), even at a small scale, could cause significant discrepancies in stock discrimination; these potential discrepancies have rarely been investigated thus far. To address these questions, we used two known stocks of yellow croaker, *Larimichthys polyactis*, from Chinese coastal waters as a case study to evaluate and compare the effectiveness of the two scaling models in removing the size effect in otolith shape analysis.

Yellow croaker is an important commercially exploited fish distributed in the seas of China (Guo et al., 2006; Xiao et al., 2009; Xu and Chen, 2010). Both morphological and genetic analyses have shown that

the two sample groups of yellow croaker used in this study come from different geographic stocks (Ye, 1991; Jin, 1996; Meng et al., 2003). Using known stock samples for this otolith shape analysis allowed us to effectively evaluate the results of stock discrimination and compare the adjusting effect in a reliable way. This case study was primarily aimed at determining whether frequently applied scaling methods used in otolith shape analysis and normalized elliptic Fourier descriptors (NEFDs) can effectively remove the size effect as expected, and if these methods do not remove the size effect, what measures should be taken to minimize discrepancies during otolith sampling and morphological data analysis.

## 2 MATERIAL AND METHOD

### 2.1 Sample collection

The samples used in this study came from the otolith collection of the Institute of Oceanology of the Chinese Academy of Sciences and were collected during fishery research surveys in Chinese coastal waters, as described in Dou et al. (2012). This collection includes yellow croaker samples from the Bohai Sea (BH) and the Changjiang River estuary of the East China Sea (CJ; Fig.1). Immediately after capture, the fish were labelled and frozen for subsequent biological analysis. In the laboratory, the total length (TL,  $\pm 1$  mm), weight ( $\pm 0.1$  g), sex, reproductive maturity stage and other biological characteristics of the fish were routinely determined and recorded. The sagittal otoliths on both the left and right sides were removed from each fish, cleaned of adhering tissues in distilled water, and stored dry in sealed glass vials until morphological analysis.

A total of 282 (185 males and 97 females) and 472 (263 males and 209 females) pairs of otoliths from the BH stock and the CJ stock, respectively, were analysed morphologically to test the inter- or intra-stock differences in shape characters between sexes or body sides. Because the objective of the study was to test the effectiveness of the two models in removing the size effect in otolith shape analysis, the otolith sampling programme was designed to include 3 length groups from the BH stock (BH1–BH3) and 5 length groups from the CJ stock (CJ1–CJ5), including 30 specimens in each group with a relatively uniform individual length distribution (the maximum individual length difference was controlled to be less than 1 cm). A total of 8 groups ( $n=30 \times 8$ ) with different length ranges were used for otolith morphology analysis (Table 1).

## 2.2 Otolith imaging and shape analysis

Prior to imaging, the otoliths were cleaned in distilled water with an ultrasonic cleaner, then air-dried and weighed to the nearest 0.1 mg using an electronic balance. Digital images of both the left and right sagittal otoliths of each fish were captured using a video camera linked to a binocular microscope (ACT-2, Nikon SMZ1000; Tokyo, Japan). The otoliths were positioned on a dark background with the sulcus acusticus facing down and the anterior side

pointing to the left for the left otolith, or to the right for the right otolith (Fig.2).

The otolith size descriptors (SDs: area, length, width, perimeter, minimum diameter, maximum diameter, mean diameter, minimum radius, maximum radius and the radius ratio) were obtained with Image-Pro Plus (Legua et al., 2013). The shape indices (SIs: circularity, form factor, rectangularity, roundness and ellipticity) were obtained using the specified mathematical equations (Tuset et al., 2003; Table 2).

The digitizing program SHAPE 1.2 software was used to extract the elliptic Fourier descriptors (EFDs)

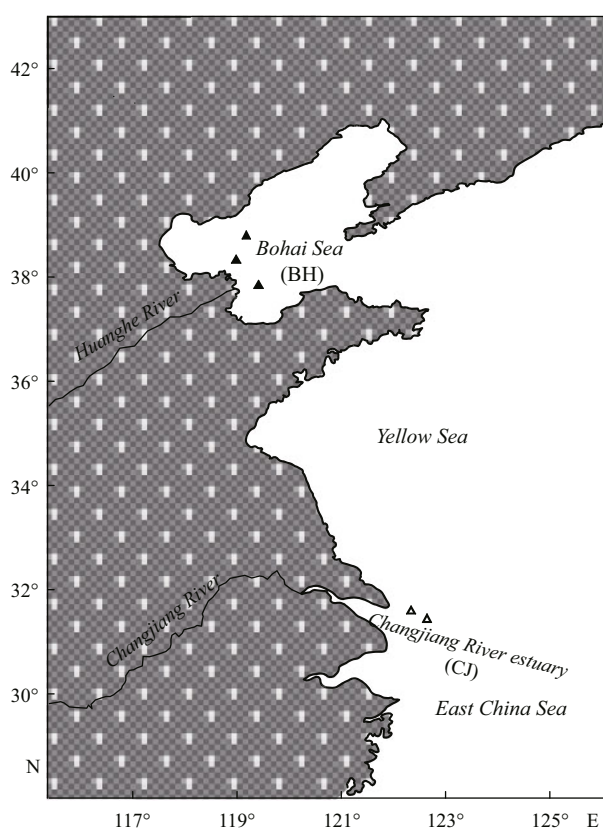


Fig.1 Sampling sites of the two yellow croaker stocks: BH, the Bohai Sea stock; CJ, the Changjiang River estuary stock

Table 1 Basic information on the fish length groups from the Bohai Sea stock (BH) and the Changjiang River estuary stock (CJ)

Sampling site	Group	Sample size	Fish total length (mm)	
			Range	Mean±SD
Changjiang River estuary	CJ1	30	105–110	107.6±1.81
	CJ2	30	118–121	119.3±1.06
	CJ3	30	136–140	138.3±1.99
	CJ4	30	152–160	155.9±2.64
	CJ5	30	167–172	169.1±1.73
Bohai Sea	BH1	30	119–125	122.6±2.13
	BH2	30	136–140	138.5±1.38
	BH3	30	155–165	160.4±4.00

Table 2 Size descriptors and shape indices used for stock discrimination

Size descriptor (SDs)	Shape indices (SIs)
Area (A)	Form-factor= $(4\pi \times A)/P^2$
Perimeter (P)	Rectangularity= $A/(OL \times OW)$
Otolith length (OL)	Roundness= $(4A)/(\pi \times OL^2)$
Otolith width (OW)	Ellipticity= $(OL-OW)/(OL+OW)$
	Aspect-ratio= $OL/OW$

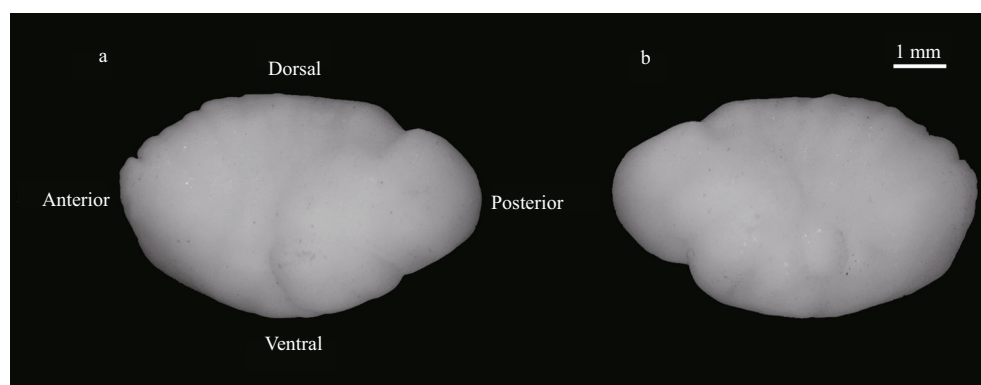


Fig.2 The right (a) and the left (b) otoliths of a yellow croaker

from the images of the otoliths. The coefficients were normalized with the program such that they were invariant to otolith size and orientation. The Fourier power (FP) spectrum was calculated to determine the optimal number of harmonics for the best reconstruction of the otolith outline. For the  $n^{\text{th}}$  harmonic, the Fourier power ( $FP_n$ ) was calculated according to the following formula (Crampton, 1995):

$$FP_n = \frac{a_n^2 + b_n^2 + c_n^2 + d_n^2}{2},$$

where  $a_n$ ,  $b_n$ ,  $c_n$  and  $d_n$  are the Fourier coefficients of the  $n^{\text{th}}$  harmonic. The cumulative variance ( $FP_c$ ) was calculated as follows:

$$FP_c = \sum_1^n FP_n.$$

Because the first eight harmonics described more than 99% of the cumulative power, otolith shape was summarized using 8 Fourier harmonics (i.e.,  $8 \times 4 = 32$  Fourier coefficients). However, normalization of the EFDs (NEFDs) resulted in degeneration of the first three coefficients to fixed values:  $c_1=1$ , and  $c_2=c_3=0$ ; thus, the number of Fourier coefficients was reduced to 29 ( $32-3$ ) in the present study (Tracey et al., 2006).

### 2.3 Data analysis

For each analysis, the otolith shape variables (NEFDs, SDs and SIs) were first examined for normality and homogeneity using Kolmogorov-Smirnov (KS) and Levene's tests, respectively. Variables that did not meet the normality or homogeneity test were excluded from the statistical analysis.

A multivariate analysis of covariance (MANCOVA) was employed to test the differences in otolith morphology between sexes or body sides. Fish length was used as a covariate, and the shape variables were used as independent variables. Because no significant difference in otolith morphology were found between the sexes or between the left and right otoliths (MANCOVA,  $P > 0.05$ , in all cases), the images of the left otoliths of both male and female samples were used in the statistical analyses.

#### 2.3.1 Fish length scaling

An analysis of covariance (ANCOVA) was performed to test the magnitude of the fish length effect on each otolith shape variable and NEFD (dependent variable). Fish length was used as a covariate, and the length group was used as the independent variable. Variables were removed from analysis when adjustment for fish size was

unsuccessful in MANOVA to compare otolith morphology between regions (Begg and Brown, 2000). When a significant interaction between the length group and fish length was detected ( $P < 0.05$ , groups with unequal slopes), the dependent variable was excluded from subsequent analyses because the variable could not be adjusted or corrected for fish length in a consistent manner. Otherwise, the differences that were detected could exclusively be related to fish length, and not to any other stock or environmental effect (Cardinale et al., 2004). When the remaining variables were significantly correlated with fish length, they were standardized or scaled for the length effect using two data scaling methods. One of these approaches is the most widely used common within-group slope method (Campana and Casselman, 1993; Bolles and Begg, 2000; DeVries et al., 2002; Tracey et al., 2006; Burke et al., 2008; Zhang et al., 2014). This method is based on the linear model of  $Y = a + bX$ , where the coefficient  $b$  is equal to the common within-group slope. Standardization was conducted using the following formula (Bolles and Begg, 2000; Cardinale et al., 2004):

$$V_{i(\text{std})} = V_i - bX_i,$$

where  $V_i$  is the original variable;  $V_{i(\text{std})}$  is the standardized variable; and  $X_i$  is the fish length of the  $i^{\text{th}}$  individual.

The second data scaling method is based on an allometric growth model,  $Y = aX^b$ , where  $a$  and  $b$  are constants. After a series of transformations and substitutions, the following data standardization equation was obtained (Leonart et al., 2000):

$$Y_i^* = Y_i \left[ \frac{X_0}{X_i} \right]^b,$$

where  $X_0$  is the average length of the fish samples.

Therefore, two sets of standardized data were scaled using the two models. To avoid the effect of multicollinearity among the variables and decrease the dimensions of the variables in the subsequent discriminant analysis, principal component analysis (PCA) was performed on the scaled SDs and SIs based on the correlation matrix (Thalib et al., 1999; Yu et al., 2014). Additionally, PCA was conducted on the scaled NEFDs based on the variance-covariance matrix of the coefficients rather than the correlation matrix because the coefficients with low variance and covariance values were of little importance in explaining the observed morphological variation. Several sets of scored variables were produced from the PCA results for the subsequent discriminant analysis.



**Table 3** The included groups, fish length ranges and objectives of each CDA trial conducted

CDA	Trial 1		Trial 2–3*		Trial 4–5*		Trial 6–7*		Trial 8–9*	
	Group	FL (mm)	Group	FL (mm)	Group	FL (mm)	Group	FL (mm)	Group	FL (mm)
Datasets	CJ3	136–140	CJ1	105–110	BH1	119–125	CJ1	105–110	BH1	119–125
	BH2	136–140	CJ3	136–140	BH2	136–140	CJ2	118–121	BH2	136–140
			CJ5	167–172	BH3	155–165	CJ3	136–140	BH3	155–165
							CJ4	152–160	CJ3	136–140
							CJ5	167–172		
							BH2	136–140		
Objective	The first objective described above		The second objective described above		The third objective described above					

\* Two CDA trials were conducted on each dataset, in respective to the variables scaled using the two methods.

### 2.3.2 Canonical discriminant analysis (CDA)

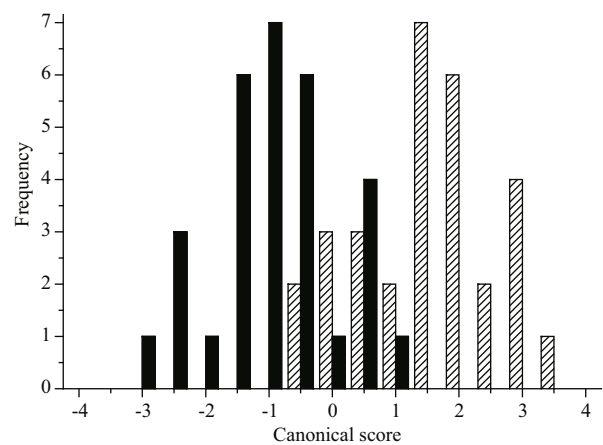
As the most commonly employed method for discriminating among groups using multiple observed variables, CDA produces linear combinations that maximize the ratio of ‘inter-groups’ to ‘pooled within-group’ sample variances. A series of CDA trials were conducted on the scaled datasets to test whether the size effect was effectively removed by the two length scaling methods and the NEFDs (Table 3). These trials were performed to realize three specific objectives.

The first objective was to determine whether the samples from the different locations could be discriminated on the basis of otolith shape. Samples from the BH2 and CJ3 groups, with identical ranges of fish length and a similar size distribution, were used to realize this goal.

The second objective was to test the effectiveness of the two fish length scaling methods and the NEFDs in removing the size effect. CDA was applied separately to the BH1, BH2 and BH3 groups and the CJ1, CJ3 and CJ5 groups to test the discrimination among the different fish length groups within a single stock, following the scaling of the shape variables with the two methods.

The third objective was to illustrate that otolith shape analysis could produce misleading results in discriminating among stocks, particularly when the size effect was not effectively removed using the two data scaling methods and the NEFDs. Two CDA trials were conducted to discriminate between two inter-stock length groups. The first trial included the CJ3 and BH1–BH3 groups, whereas the second trial included the BH2 and CJ1–CJ5 groups, among which some groups exhibit the same size but come from different fish stocks (i.e., CJ3 and BH2).

In each CDA, the stepwise method was used to identify the suitable variables for inclusion in the discriminant functions, followed by a jackknife cross-



**Fig.3** Frequency distribution of canonical scores derived from CDA for the BH2 (the solid bars) and CJ3 (the slashed bars) groups

validation to produce an unbiased estimation of classification success.

All statistical tests were conducted at a significance level of  $P < 0.05$  using SPSS 22 for Macintosh computers.

## 3 RESULT

### 3.1 Inter-stock otolith shape variation

No significant difference was found in either fish length ( $t$ -test,  $P > 0.50$ ) or the length distribution (KS test,  $P > 0.50$ ) between the BH2 and CJ3 groups. Otolith shape variation caused by fish length can be counteracted to a great extent using samples with similar size distributions. Moreover, the two groups showed an identical length range of 136–140 mm. Therefore, the data were not scaled for between-stock discriminant analysis.

PCA was conducted on the SDs, SIs and NEFDs, and the principle scores were combined and used for CDA. Otolith shape discriminated between the two geographic stocks with an overall classification success rate of 83.3% (Fig.3). Significant inter-stock

**Table 4 Results of CDA run on the scaled otolith shape variables of the intra-stock length groups\***

Region	Group	Classification success (%)							
		Linear model scaled				Allometric model scaled			
Changjiang River estuary		CJ1	CJ3	CJ5	Total	CJ1	CJ3	CJ5	Total
	CJ1	76.7 (23)	16.7 (5)	6.7 (2)		80 (24)	16.7 (5)	3.3(1)	
	CJ3	20 (6)	63.3 (19)	16.7 (5)	76.7	16.7 (5)	50 (15)	33.3 (10)	72.2
	CJ5	3.3 (1)	6.7 (2)	90 (27)		3.3(1)	10 (3)	86.7 (26)	
Bohai Sea		BH1	BH2	BH3		BH1	BH2	BH3	
	BH1	83.3 (25)	16.7 (5)	0		86.7 (24)	13.3 (4)	0	
	BH2	16.7 (5)	73.3 (22)	10 (3)	83.3	20 (6)	70 (21)	10 (3)	82.2
	BH3	0	6.7 (2)	93.3 (28)		0	10 (3)	90 (27)	

\* The number of fishes is given in brackets.

otolith variation existed in the two yellow croaker stocks ( $P<0.05$ ), which provided a theoretical basis for the follow-up analyses.

### 3.2 Intra-stock discriminant analysis

CDA was applied to the BH (BH1, BH2 and BH3) group and the CJ (CJ1, CJ3 and CJ5) group, respectively. In each analysis, the shape variables were scaled for fish length using the two models.

CDA results indicated that the length groups within the BH or CJ group were well discriminated from each other using both scaling methods (Table 4; Fig.4).

In the BH group, CDA of the linearly and allometrically standardized data achieved overall classification success rates of 83.3% (73.3%–93.3%) and 82.2% (70%–90%), respectively (Table 4). For the linearly scaled data, function 1 explained 98.1% of the between-group variance, which was significant for the differentiation of the length groups (Wilks' Lambda=0.152,  $P<0.05$ ). Among the 30 individuals in each length group, four BH1 individuals were misclassified into the BH2 group, while five and three BH2 individuals were incorrectly assigned to the BH1 and BH3 groups, respectively. Similarly, using the allometrically scaled data, function 1 explained 98.1% of the between-group variance, which was also significant for the differentiation of the groups (Wilks' Lambda=0.174,  $P<0.05$ ). Among the 30 individuals in each length group, four BH1 individuals were incorrectly assigned to the BH2 group; six BH2 individuals to the BH1 group; one BH2 individual to the BH3 group; and three BH3 individuals to the BH2 group.

In the CJ group, CDA of the linearly and allometrically standardized data achieved overall classification success rates of 76.7% (63.3%–90%) and 72.2% (50%–86.7%), respectively (Table 4). For the linearly scaled data, function 1 and function 2

explained 66.6% and 33.4% of the between-group variance, respectively, which was significant for the differentiation of the groups (Wilks' Lambda=0.303,  $P<0.05$  for function 1; Wilks' Lambda=0.641,  $P<0.05$  for function 2). Among the 30 individuals in each length group, five and two CJ1 individuals were incorrectly assigned to the CJ3 and CJ5 groups, respectively; six and five CJ3 individuals to the CJ1 and CJ5 groups; one and two CJ5 individuals to the CJ1 and CJ3 groups. Similarly, for the allometrically scaled data, function 1 and function 2 explained 92.4% and 7.6% of the between-group variance, respectively, with both discriminant functions being significant for the differentiation of the groups (Wilks' Lambda=0.321,  $P<0.05$  for function 1; Wilks' Lambda=0.876,  $P<0.05$  for function 2). Among the 30 individuals in each length group, five CJ1 individuals were incorrectly assigned to the CJ3 group; three CJ5 individuals to the CJ3 group; and one and three CJ3 individuals to the CJ1 and CJ5 groups, respectively.

### 3.3 Inter-stock otolith shape variation across different fish length groups

Two CDA trials were conducted to discriminate among inter-stock length groups. The BH2 group was pooled with the CJ1–CJ5 groups, and the CJ3 group was pooled with the BH1–BH3 groups. Prior to CDA, data scaling and PCA were conducted as described above. The standardized canonical discriminant functions and main components of each principle score used in each CDA trial are listed in Supplementary Table 1.

In the BH2 and CJ1–CJ5 groups, the length groups could be separated from each other with modest classification success rates of 55.6% and 67.8% using the linear and allometric scaling methods, respectively, although CJ1–CJ5 were from the same stock (Fig.5; Table 5). The variation in otolith shape between

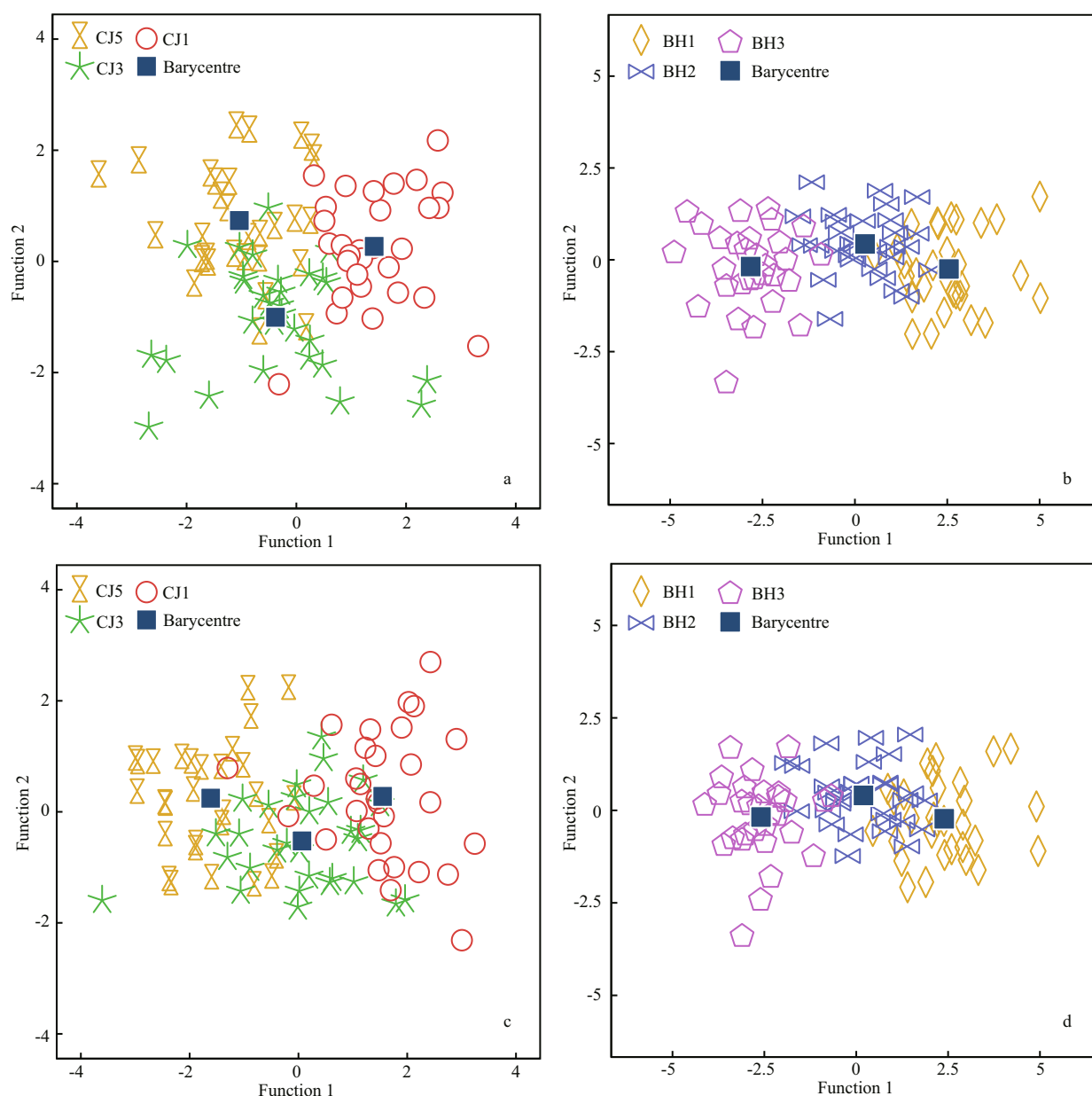
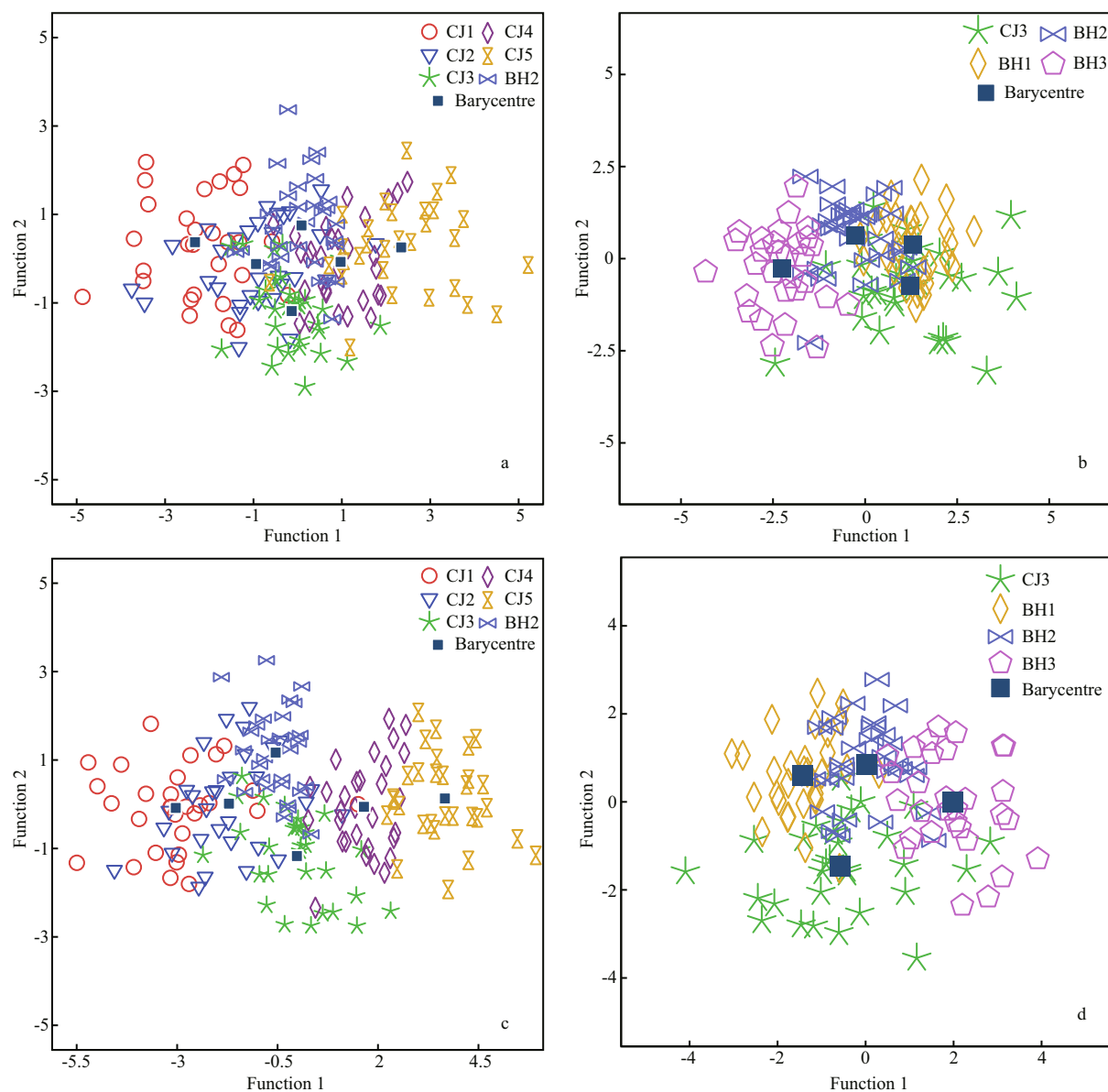


Fig.4 Scatter plots showing the CDA scores among within-stock groups: (a) and (b), linear model-scaled; (c) and (d), allometric model-scaled

Table 5 Results of CDA run on the scaled otolith shape variables of the BH2 and the CJ1–CJ5 groups\*

Group	Classification success (%)													
	Linear model scaled							Allometric model scaled						
	BH2	CJ1	CJ2	CJ3	CJ4	CJ5	Total	BH2	CJ1	CJ2	CJ3	CJ4	CJ5	Total
BH2	60 (18)	0	13.3 (4)	3.3 (1)	23.3 (7)	0	55.6	76.7 (23)	0	10 (3)	13.3 (4)	0	0	67.8
CJ1	3.3 (1)	70 (21)	20 (6)	6.7 (2)	0	0		3.3 (1)	70 (21)	23.3 (7)	0	3.3 (1)	0	
CJ2	20 (6)	23.3 (7)	30 (9)	16.7 (5)	3.3 (1)	6.7 (2)		23.3 (7)	23.3 (7)	36.7 (11)	10 (3)	6.7 (2)	0	
CJ3	3.3 (1)	10 (3)	13.3 (4)	63.3 (19)	10 (3)	0		10 (3)	3.3 (1)	13.3 (4)	66.7 (20)	6.7 (2)	0	
CJ4	16.7 (5)	0	3.3 (1)	23.3 (7)	36.7 (11)	20 (6)		6.7 (2)	0	0	13.3 (4)	70 (21)	10 (3)	
CJ5	0	0	0	6.7 (2)	20 (6)	73.3 (22)		0	0	0	0	13.3 (4)	86.7 (26)	

\* The number of fish is given in brackets.



**Fig.5** Scatter plots showing the CDA scores across inter-stocks of different fish lengths: (a) and (b), linear model scaled; (c) and (d), allometric model scaled

**Table 6** Results of CDA run on the scaled otolith shape variables of the CJ3 and the BH1–BH3 groups\*

Group	Classification success (%)									
	Linear model scaled					Allometric model scaled				
	CJ3	BH1	BH2	BH3	Total	CJ3	BH1	BH2	BH3	Total
CJ3	50 (15)	33.3 (10)	13.3 (4)	3.3 (1)	62.5	70 (21)	6.7 (2)	13.3 (4)	10 (3)	67.5
BH1	30 (9)	53.3 (16)	16.7 (5)	0		6.7 (2)	63.3 (19)	30 (9)	0	
BH2	6.7 (2)	20 (6)	60 (18)	13.3 (4)		16.7 (5)	10 (3)	60 (18)	13.3 (4)	
BH3	3.3(1)	0	10 (3)	86.7 (26)		6.7 (2)	0	16.7 (5)	76.7 (23)	

\* The number of fish is given in brackets.

geographic stocks or between length groups could be observed from the distance between their barycentres in the scatter plots (Fig.5).

Similar CDA results were obtained for the CJ3 and BH1–BH3 groups (Fig.5; Table 6). Using the linear and allometric scaling methods, the two CDA trials



achieved modest overall classification successes rate of 62.5% and 67.5%, respectively.

The results revealed that inter-stock otolith shape variation might be affected by an interaction with fish length, which led to CDA misclassification when the size effect was not effectively removed. Thus, otolith shape variation caused by fish length could easily result in confusing CDA results.

#### 4 DISCUSSION

Otolith morphology is primarily determined by genetics but is also affected by environmental factors (Begg and Brown, 2000; Cardinale et al., 2004). Therefore, otolith morphometrics are a potential indicator for species or stock discrimination. However, otolith morphology also changes allometrically with fish size, which is one of the decisive factors in the variability in otolith morphometrics, even within a geographic stock (Smith, 1984; Lleonart et al., 2000). The methods for removing the size effect on otolith morphology caused by allometric growth are important and should be cautiously considered when otolith shape analysis is applied to stock discrimination (Cardinale et al., 2004). If the size effect is not taken into account, the validity of the results of morphological analyses of otoliths will be confounded or compromised. The common assumption is that the size effect caused by allometric growth is diminished or even eliminated with application of the appropriate sample selection procedure and statistical analyses (Lleonart et al., 2000). Thus, previous studies have typically attempted to minimize or remove the size effect by restricting fish size to a narrow range in samples evaluated (Neves et al., 2011) and by scaling the variables that are correlated with fish size using either a linear (Bolles and Begg, 2000; Cardinale et al., 2004) or allometric growth model (Lombarte and Lleonart, 1993; Torres et al., 2000).

However, simply minimizing the range of fish sizes in the samples and routinely scaling length data may not always be effective in ensuring that the size effect is removed. A small difference in fish size potentially confounding otolith shape analysis could lead to potential for poor or erroneous stock discrimination. As shown in this study, the intra-stock length groups (with only a 10-mm difference in size) of both the BH and CJ stocks were successfully differentiated from one another based on the otolith shape variables standardized for fish length using both the linear and the allometric scaling methods. This finding suggested

that otolith shape variation might exist among the length groups within a geographic stock.

In previous studies, researchers have cautiously attempted to address this problem by selecting samples of similar size during the sample selection step (Castonguay et al., 1991; Campana and Casselman, 1993; Begg and Brown, 2000; DeVries et al., 2002). Some researchers have emphasized that conclusions regarding fish stock structure that are based on otolith shape analysis should be carefully addressed because the discrimination that is achieved could be primarily due to age- or year-class effects, which would therefore highlight sample differences, rather than stock discrimination (Castonguay et al., 1991). Begg and Brown (2000) also suggested that for stock discrimination, otolith shape variables should be recalculated each year for each age group.

To scale otolith shape variables, linear or allometric growth models are commonly used to remove the size effect in studies on otolith shape (Lombarte and Lleonart, 1993; Bolles and Begg, 2000; Torres et al., 2000; Cardinale et al., 2004). Contour morphometry (NEFDs) determined through elliptic Fourier analysis of otoliths is widely used to generate variables that are independent of otolith size variables (Yu et al., 2014). However, the present study demonstrated that these data scaling methods might fail to remove the size effect, as previously suggested, which was clearly shown by CDA of the intra-stock length groups of both geographic stocks. CDA of the inter-stock shape variation of the different length groups (the BH2 group pooled with the CJ groups, or the CJ3 group pooled with the BH groups) further indicated the failure of data scaling to remove the size effect and illustrated how this could result in erroneous stock discrimination. The BH2 and CJ3 groups came from two different geographic stocks with identical fish length ranges and distributions, whereas CJ1–CJ5 and BH1–BH3 each came from their own respective geographic stocks with different fish lengths. From either a statistical or an ecological perspective, intra-stock shape variation was reduced when the size effect was effectively removed by length scaling; therefore, CDA should achieve greater classification success in discriminating between inter-stock length groups than among intra-stock groups. However, in both CDA trials, similar or even greater classification success was observed between most of the intra-stock length groups (CJ1–CJ2, CJ4–CJ5; BH1 and BH3) than for the two inter-stock groups with identical fish size (CJ3 and BH2). Additionally, the distance

between group centroids in the CDA function plots led to confusing stock discrimination results, which was inconsistent with the results of stock discrimination in previous studies (Ye, 1991; Jin, 1996). These findings suggested that the intra-stock variation in otolith shape caused by fish length could exceed that of the inter-stock geographical separation, even when the otolith shape variables are standardized for fish length using both scaling methods and NEFDs. In this case, data scaling failed to remove the size effect on CDA, which consequently produced a misleading classification in stock discrimination. This finding implied that the length effect might not be effectively removed simply by scaling the data using the two models and NEFDs in either the intra-stock or the inter-stock length groups, particularly when the length variation of the sample groups exceeds a certain level (for yellow croaker, a 10-mm difference in size could not be effectively scaled in the present study).

Nevertheless, data scaling methods and normalized elliptic Fourier coefficients could play an important role in stock discrimination based on otolith shape analysis. Regressions between otolith shape variables (original data and scaled data) and fish length should be conducted to assess the ability of the two scaling methods to select the appropriate shape variables for the subsequent statistical analysis. In this study, all the original shape variables were significantly correlated with fish length and could therefore not be directly included in the subsequent CDA. The allometric model removed the correlation with fish length for all size descriptors (e.g., area, length, width, perimeter, diameter and radius) and shape indices (e.g., circularity, form factor, rectangularity, roundness and ellipticity). In contrast, the linear model removed the correlation of the size descriptors with fish length but failed to achieve the same goal for the shape indices (Supplementary Table 2). Most of the shape indices were proportionally calculated from size descriptors that are generally correlated with fish length. Thus, the linear model did not effectively remove the correlation between the ratios of the size descriptors and fish lengths, leading to exclusion of the shape indices from subsequent statistical analyses of the linearly scaled data. Therefore, the allometric scaling model tended to retain more effective shape variables and, thus, more shape information for subsequent CDA than the linear scaling model, which may affect the results of stock discrimination.

The results indicated the possibility that stock

discrimination might be primarily biased by the length effect, which highlighted differences in the samples, rather than stock discrimination, particularly when the size variation of the samples was large relative to fish size. In such cases, data scaling using both growth models and NEFDs may fail to remove the size effect. This problem could result in misleading stock discrimination, particularly in fish species of small size.

## 5 CONCLUSION

To help improve the reliability of otolith shape analyses and their ability to discriminate among stocks, a few points should be considered with caution during sample selection and data analysis: (1) even slight variation in length among sample groups can considerably confound the results of otolith shape analysis. Thus, the size variation of samples, both within- and between-groups, must be minimized as much as possible during sample selection; (2) to achieve unbiased discrimination among groups, an equal length distribution of the samples is essential and, to some extent, is more important than a narrow length range or age class. To realize this goal, statistical analysis should be conducted to test the homogeneity of length distributions among sample groups in morphological analyses of otoliths; (3) because many otolith shape variables that are commonly used in otolith shape studies cannot be correctly scaled for the length effect, statistical analysis should be applied to exclude those variables from subsequent statistical analyses for stock discrimination.

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### Electronic supplementary material

Supplementary material (Supplementary Tables 1–2) is available in the online version of this article at <https://doi.org/10.1007/s00343-016-5164-4>.