



An investigation of otolith shape analysis as a tool to determine stock structure of ling (*Genypterus blacodes*)

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EXECUTIVE SUMMARY

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The viability of using otolith contour shape analyses to help interpret the stock structure of ling in New Zealand was investigated. Two comparisons were completed: one between LIN 4 (Chatham Rise) and the presumed Sub-Antarctic biological stock (LIN 5 and LIN 6 combined), the other between southern (LIN 6) and northern (LIN 5) parts of the Sub-Antarctic area. Images of each otolith were analysed to produce sets of 17 physical characteristic descriptors (initial descriptors) along with 50 elliptical Fourier descriptors (eFd's). Principal Component Analyses and Descriptive Analyses were applied to these two groups of parameters, and ultimately a Linear Descriptive Analysis was chosen as the final analytical model. For the Chatham Rise vs. Sub-Antarctic comparison the average success rate was 77.4%, a level indicative of a differentiation between ling from these two areas. For the north-south Sub-Antarctic comparison, the success rate was 50–55%, strongly indicative of no differentiation. The stock structure indicated by these two results is the same as that derived from other sets of biological characteristics. It is concluded, therefore, that otolith shape analysis would usefully inform the stock structure of ling.

1. INTRODUCTION

It has been standard practice in New Zealand and elsewhere to subdivide commercial marine fishes into stocks or management units which exhibit some degree of isolation. There is no universal definition of a 'stock', although most definitions include spatial and temporal isolation, and, often, reproductive isolation. Techniques to distinguish fish stocks have included genetics, morphometrics and meristics, parasite faunas, biochemical analyses, geographical distributions and discontinuities, and comparisons of biological characteristics (such as growth rates, spawning times and areas, and patterns of year class strengths) (Begg & Waldman 1999, Cadrin et al. 2005). Discrimination of fish stocks using shape analysis of otolith contours was developed as a branch of the morphometric techniques, but with recent advances in digital image analysis software it has become a powerful and increasingly popular tool. Otolith shape analysis (i.e., using morphometrics, derived shape factors and contour analysis), is frequently being used to discriminate between fish stocks (Agüera & Brophy 2011, Neves et al. 2011, Yu et al. 2014), spawning group and species (Cardinale et al. 2004, Jónsdóttir et al. 2006, Pinkerton et al. 2015, Tuset et al. 2003), and also to apportion age class (Brito et al. 2008, Doering-Arjes et al. 2008, Petursdottir et al. 2006). An advantage of otolith shape analysis is its cheapness relative to other techniques. Otoliths (and associated biological data) are routinely collected as part of standard fisheries sampling to enable the monitoring of the age structure of catches and populations, so the only additional work required is the creation of digital images and their subsequent analysis.

Ling (*Genypterus blacodes*) is one of New Zealand's most important commercial finfish species. It is distributed throughout the EEZ, though is most abundant south of latitude 40° S (Anderson et al. 1998). It is managed as eight administrative fishstocks (Figure 1). A review of all available data pertaining to stock discrimination (Horn 2005) indicated that there were at least five biological stocks: west coast South Island (most of LIN 7), Sub-Antarctic (LIN 5 and the Campbell Plateau section of LIN 6), Bounty Plateau (the eastern section of LIN 6), Chatham Rise (LIN 4 and most of LIN 3), and Cook Strait (parts of LIN 7 and LIN 2). Subsequent stock assessments of ling were completed using these biological stocks, rather than the administrative stocks (Ministry for Primary Industries 2015), although there is some correlation between the two classifications (e.g., the Chatham Rise biological stock is approximated by a combination of LIN 3 and LIN 4).

Although the postulated biological stock structure for ling was based on some significant biological differences between areas, and are logical relative to the bottom topography (i.e., stock boundaries are generally situated at locations where the continental shelf is narrow), it was apparent, however, that questions about stock structure of ling still remained. First, the stock structure and affinity of ling around North Island is essentially unknown. Second, the validity and extent of the 'Cook Strait' biological stock is questionable — these fish appear quite distinct from those on Chatham Rise, but have some similar characteristics to west coast South Island ling, or could also easily be part of a continuous stock from Cook Strait extending up the Wairarapa coast. Third, there have been suggestions that the postulated Sub-Antarctic stock actually comprises two distinct biological stocks, based on the presence of at least two spawning areas (see Horn 2005) and on some reported biochemical differences between ling from LIN 5 and LIN 6 (Ashoka 2009). Subsequent analyses of estimated research survey biomass distribution, by season (Appendix C of Horn et al. 2013) and across years (Appendix B of Roberts 2016), have supported the hypothesis that the Sub-Antarctic area holds a single stock.

The work reported here aimed to examine the viability of using otolith contour shape analyses to inform the stock structure of ling in New Zealand waters. Two comparisons were completed. One compared otoliths from the northern (LIN 5) and southern (LIN 6) areas of the postulated Sub-Antarctic biological stock, with the expectation that no significant differences would be found. The other comparison was between Sub-Antarctic ling and those from the Chatham Rise (LIN 4). Available biological data strongly indicated a stock boundary between these two areas, so a significant difference in otolith shapes between these two areas would be necessary to support the otolith shape contour analysis as being a useful stock discrimination tool for ling.

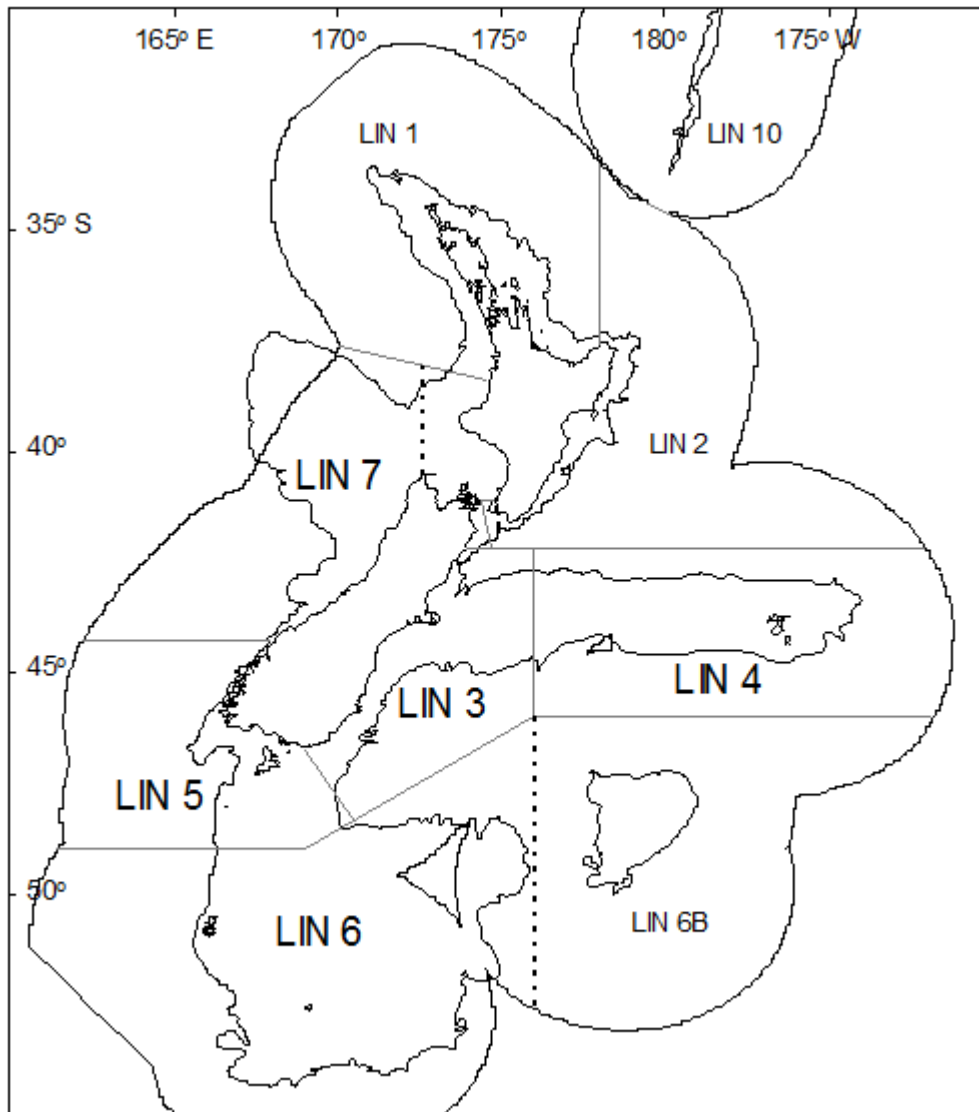


Figure 1: Ling administrative fishstock boundaries. The boundaries used to separate biological stock LIN 6B from the rest of LIN 6, and the west coast South Island section of LIN 7 from the rest of LIN 7, are shown as dashed lines.

2. METHODS

Otoliths were obtained from three distinct geographical areas: Chatham Rise (FMA 4), Sub-Antarctic North (FMA 5), and Sub-Antarctic South (FMA 6) (Figure 1). Sample size for each area was 140, split equally between males and females. Complete, left-side otoliths were selected from a truncated length range of fish (69–82 cm TL) to minimise any length- or age-related influences between areas on the subsequent shape analysis. The samples were also selected from either December 2012 (research trip TAN1215 in the Sub-Antarctic) or January 2013 (trip TAN1301 on Chatham Rise), to minimise any possible seasonal effects. Otoliths were also weighed to the nearest 0.1 mg and their depth (thickness) at the primordium recorded. Fulton's condition factors (CF) were calculated for each sampled fish: $CF = 100(W/L^3)$, where W is fish weight (g) and L is fish length (cm).

Digital images of otoliths were all captured in bmp format under a stereomicroscope at the same magnification ($160.67 \text{ pixels mm}^{-1}$), using transmitted dark field illumination to highlight otolith edges. Illumination intensity was a uniform 22% for all images captured, using a Schott KL2500 LED light source. Otoliths were positioned in the centre of the image field using an interactive crosshair overlay, orientated with the anterior portion of the otolith to the left, distal above. All captured images were processed with standard ImageJ routines (Abramoff et al. 2004): conversion to binary with holes filled, edges improved, and noise removal. Subsequently, calibrated images were analysed in ImageJ giving data fields of initial size descriptors: perimeter, surface area, bounding box dimensions, fit-an-ellipse, maximum and minimum feret diameters (Figure 2). Standard derived otolith shape factors were also calculated: aspect ratio, roundness, solidity, circularity, rectangularity and ellipticity (Abramoff et al. 2004, Keating et al. 2014), as well as otolith depth and weight as noted previously (Table 1). The otolith shape indices and the physical characteristic descriptors are subsequently referred to as the initial descriptors.

Using captured images, 50 elliptic Fourier harmonics were calculated for each otolith from their chain coded contours, using the software SHAPEv1.3 (Iwata & Ukai 2002, Agüera & Brophy 2011, Tracey et al. 2006). Each k^{th} harmonic is composed of four coefficients [a_k , b_k , c_k , d_k] (elliptical Fourier descriptors or eFd's), giving 200 coefficients per individual. In the software, eFd's were normalised for size and orientation, which caused the degeneration of the first three coefficients to fixed values: $a_1 = 1$; $b_1 = c_1 = 0$. This resulted in 197 eFd's per otolith being generated. The remainder of the analysis was conducted using a standard suite of Matlab statistical routines.

The images captured for analysis with the SHAPEv1.3 software (as described above) were transformed to jpg format and their contours analysed using the wavelet transformation methodology in the shapeR package (Libungan & Pálsson 2015).

Table 1: Size parameters and size based shape indices with calculation formulas.

Size parameters	Size base shape indices
Area (A)	Circularity (Cir) = P/A^2
Perimeter (P)	Rectangularity (Rec) = $A/(OL \times OW)$
Otolith Length (OL)	Form-Factor (FF) = $(4\pi A)/P^2$
Otolith Width (OW)	Roundness (Rnd) = $(4A)/(\pi OL^2)$
	Ellipticity (Ell) = $(OL - OW)/(OL + OW)$
	Aspect Ratio (AR) = OL/OW

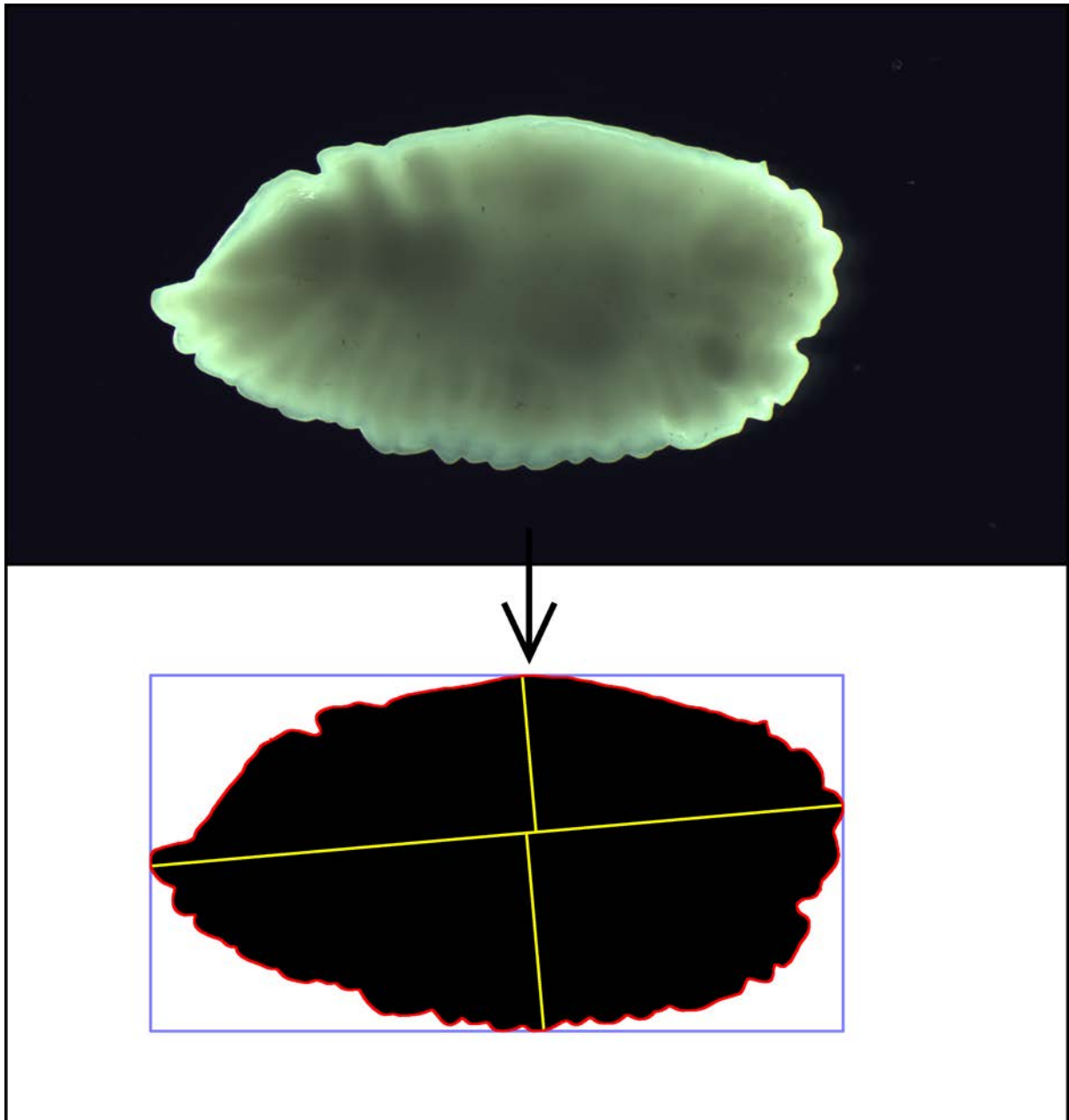


Figure 2: Ling otolith image processing and analysis, showing original image above and the resultant binary image with major descriptors: bounding box (blue); perimeter (red); surface area (black); maximum and minimum feret diameters (yellow).

3. RESULTS

3.1 Pre-analysis

An analysis of Fulton's fish condition factor (CF), shows a close similarity between mean CFs for the two Sub-Antarctic areas, while the Chatham Rise area appears different, with CF means of 0.464, 0.463 and 0.441 respectively (Figure 3). Mean values for all initial descriptors show a similar disparity between Sub-Antarctic and Chatham Rise areas, and a similarity between the two Sub-Antarctic areas (Table 2). A matrix scatter plot of basic otolith shape indices indicates some of the standard initial descriptors appear visually well correlated (Figure 4), and this is analysed below.

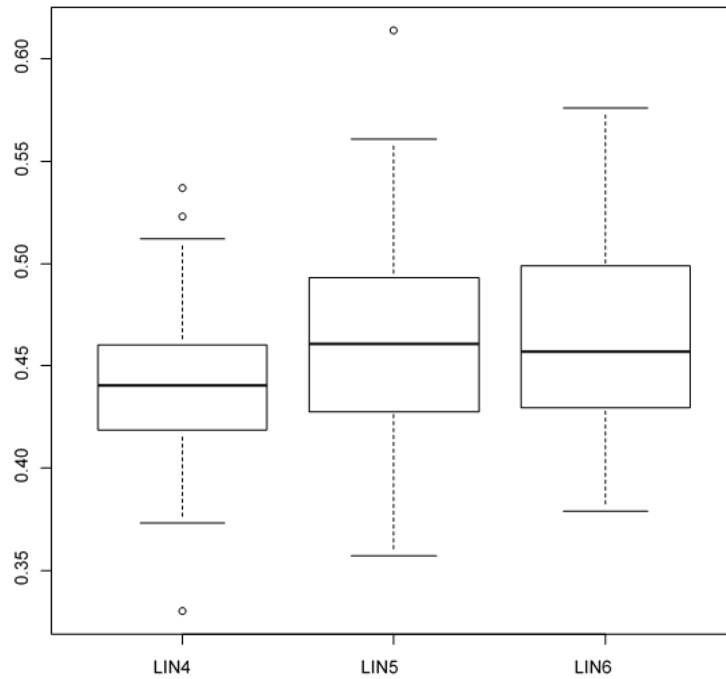


Figure 3: Boxplot showing distribution of Fulton condition factors for selected ling in the three areas: LIN 4 (Chatham Rise), LIN 5 (Sub-Antarctic North) and LIN 6 (Sub-Antarctic South).

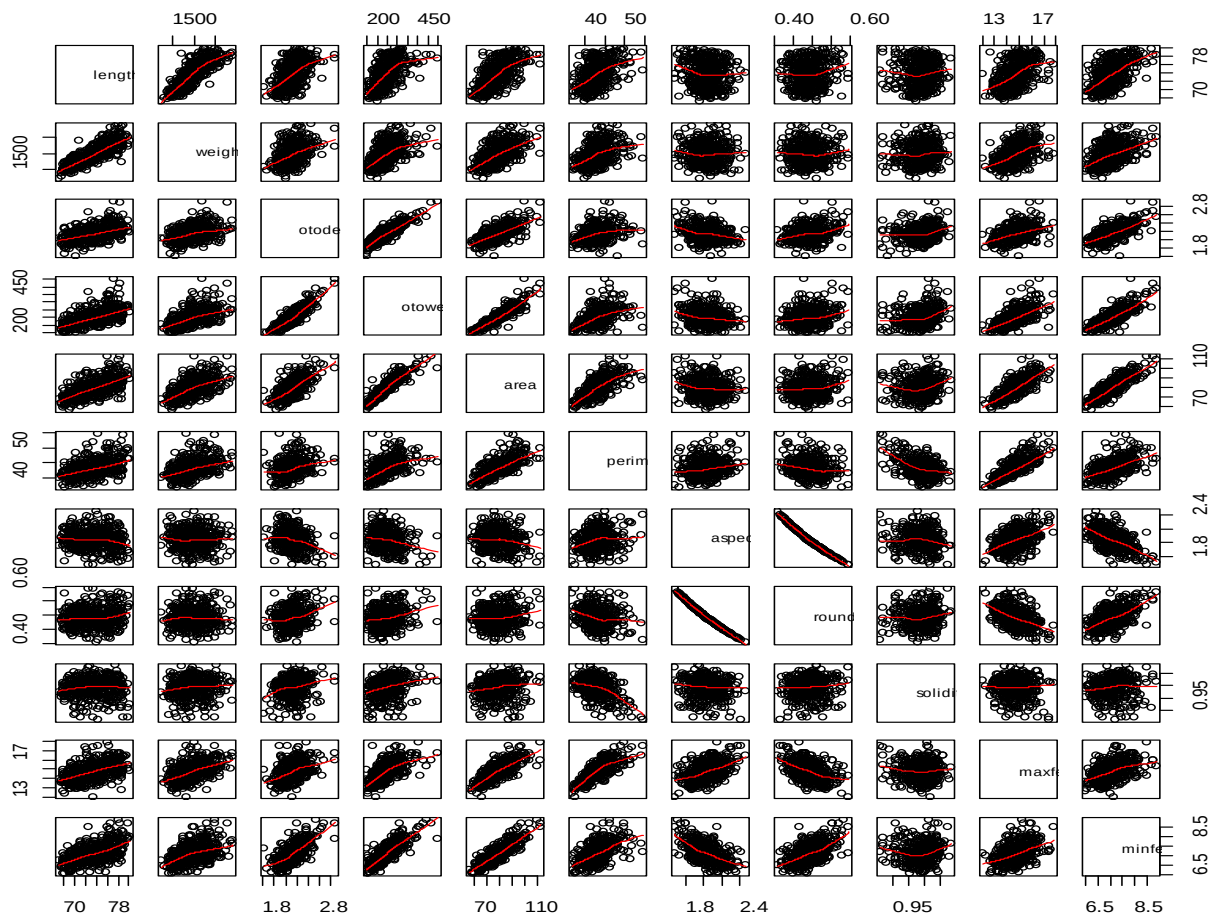


Figure 4: Scatter matrix of main otolith size descriptors including fish length and weight, with a lowess smoothed line (overlaid in red).

Table 2: A summary of mean values (by FMA), for all initial descriptors (i.e., size variables and shape factors) in the sample.

Size & Shape Factors	Ling FMA		
	Sub-Antarctic South LIN 6	Sub-Antarctic North LIN 5	Chatham Rise LIN 4
fish_length (cm)	75.8	75.6	75.5
fish_weight (kg)	2.030	2.010	1.910
otolith_weight (g)	0.295	0.296	0.306
otolith_depth (mm)	2.293	2.322	2.393
perimeter (mm)	42.885	43.044	43.793
surface area (mm ²)	89.075	88.940	90.050
Fulton_CF	0.464	0.463	0.441
aspect ratio	2.058	2.076	1.992
Roundness	0.488	0.484	0.504
Solidity	0.970	0.969	0.965
Circularity	0.00540	0.00544	0.00540
Ellipticity	0.349	0.352	0.334
form-factor	0.6086	0.6032	0.5900
max_feret (mm)	15.802	15.848	15.677
min_feret (mm)	7.547	7.531	7.733
otolith_length (mm)	15.785	15.829	15.657
otolith_width (mm)	7.612	7.593	7.823

The initial descriptors and eFd's were first tested for normality and homogeneity of variance across both areas using a one-sample Kolmogorov-Smirnov test. All indices were found to be normally distributed ($P < 0.01$) and were kept for further analysis. ANCOVA was then used to study the effect of otolith size on initial descriptors and normalize them by the within-group slope (Radhakrishnan et al. 2012) (see Table 3). All descriptors were strongly correlated with otolith length ($P < 0.001$) apart from otolith solidity ($P = 0.159$). Also, significant interactions between regions and otolith length were found for the indices solidity ($P = 0.0003$) and form-factor ($P = 0.0093$), and those indices were removed from further analysis.

Table 3: ANCOVA of the initial descriptors with the otolith length as the covariate.

Initial descriptor	ANCOVA results			Slope
	<i>F</i>	<i>P</i>	'r*o'	
Solidity	1.983	0.159	0.0003	-0.0011
Depth	106.122	<0.001	0.4783	0.0780
Weight	299.075	<0.001	0.6014	35.4704
Roundness	117.707	<0.001	0.2307	-0.0149
Aspect-ratio	122.619	<0.001	0.1456	0.0618
Ellipticity	120.286	<0.001	0.2168	0.0131
Rectangularity	194.259	<0.001	0.9491	0.1334
Form-factor	20.256	<0.001	0.0093	-0.0145
Circularity	360.607	<0.001	0.3185	-0.0007

'r*o' is the 'region*otolith length' interaction term in the ANCOVA.

Slope is used to normalise each variable for otolith length.

Remaining initial descriptors and the 197 eFd's for each otolith were submitted to a one-way analysis of variance (ANOVA) to determine if they can be used efficiently later in a Principal Component analysis (PCA). The ANOVA tests the null hypothesis that samples in the two groups are drawn from populations with the same mean values, allowing the selection of relevant parameters, and giving a metric for their usefulness in a classification. Nineteen key parameters with $P < 0.01$ were selected: four of the initial descriptors (otolith depth, roundness, aspect-ratio, ellipticity), and 15 eFd's [d_1 ; a_2 ; b_3 ; c_3 ; d_3 ; b_4 ; b_5 ; b_7 ; c_7 ; a_8 ; b_9 ; a_{11} ; c_{11} ; c_{12} ; d_{13}]. Note that the ANOVA analysis removed all high order eFd's, showing that the variability between regions was mainly described in the first 13 harmonics, at which 95% of the shape is explained by the eFd's (see Figure 5).

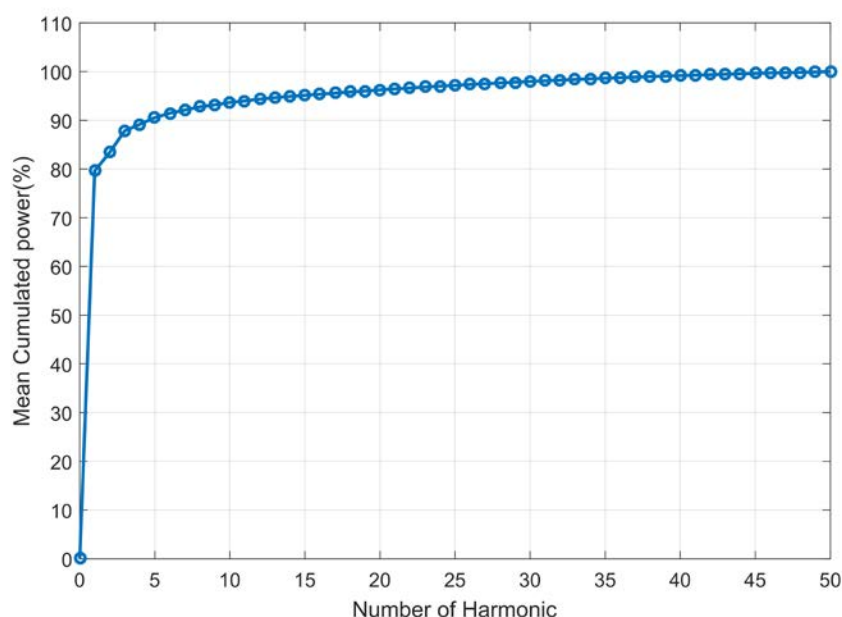


Figure 5: Mean cumulated power percentage of Fourier power variation with the number of harmonics describing the otolith shape. More than 90% of the shape is described at the 5th harmonic.

A Principal Component Analysis and Discriminant Analysis (PCA) was then applied separately to both those groups of parameters (i.e., the remaining initial descriptors and eFd's). An appropriate discriminant analysis was then applied to the relevant Principal Components (PCs) and the average success rate was calculated for each area using a jackknife cross-validation method.

The first two PCs calculated from the four remaining initial descriptors were kept. Those two components explain more than 99.99% of the variance. A Box's M test showed that the covariance matrices are homogeneous ($P = 0.286$), which indicates that a Linear Discriminant Analysis (LDA) can be used, being careful to set a uniform prior on the classification as our dataset distribution would give a biased one (Chatham: $\frac{1}{3}$, Sub-Antarctic: $\frac{2}{3}$). A jackknife cross-validation showed that an average success rate of 62.1% was achieved (Table 4).

The first four PCs from the 15 calculated from the remaining eFd's were kept. A Box's M test showed that the covariance matrices are homogeneous only up to three PCs ($P = 0.095$), and that this criterion is no longer respected for four PCs ($P = 0.0006$). Since three PCs explain only 86.3% of the variance, it would be better to include at least one other PC and get to 91.3%. This indicates that it would be best to use a Quadratic Discriminant Analysis (QDA),

still with a uniform prior on the regions. Jackknife cross-validation, however, achieved an average success rate of 74.3% using QDA and 75.7% using LDA (Table 4). Also the QDA gives worse classification results on Chatham (65.3%) compared to LDA (73.6%) (Table 4), which is the reason for the choice of an LDA in the final analysis grouping the selected PCs.

Finally, the PCs obtained separately from the initial descriptors and the eFd's were grouped to create one unique set of parameters. Those parameters were then used in a LDA which obtained an average success rate of 77.4% jackknife cross-validation (Table 4).

Table 4: Average success rate of Discriminant Analysis computed using jackknife cross-validation.

	Nb_c	P. Thr.	% var	% classified	Average success rate		
					Chatham	Sub-Antarctic	Total
Initial descriptors LDA	2	0.5	99.9%	100%	62.9%	61.8%	62.1%
eFd's QDA	4	0.5	91.3%	100%	65.3%	78.9%	74.3%
eFd's LDA	4	0.5	91.3%	100%	73.6%	76.8%	75.7%
Initial descriptors + eFd's LDA	6	0.5	–	100%	75.7%	78.2%	77.4%
Initial descriptors + eFd's LDA	6	0.8	–	39.3%	81.5%	92.8%	89.1%

Nb_c : number of principal components used in discriminant analysis.

P. Thr.: threshold applied on score calculated in DA to accept an attribution to a class.

It is interesting to note that if the classifier is constrained to accept only results with a higher probability (0.8 instead of 0.5 in Table 3), the percentage of classified otoliths drops to 39.3%, but the success rate is increased from 77.4% to 89.1%, giving a much better certainty on the classification. Figure 6 illustrates this classification result.

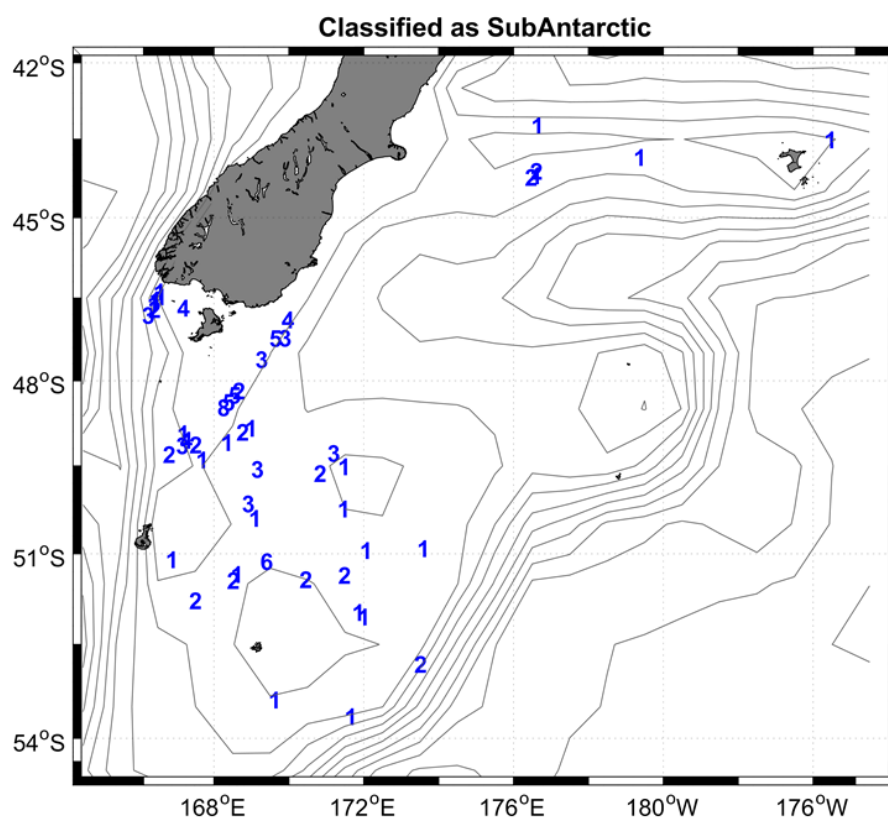
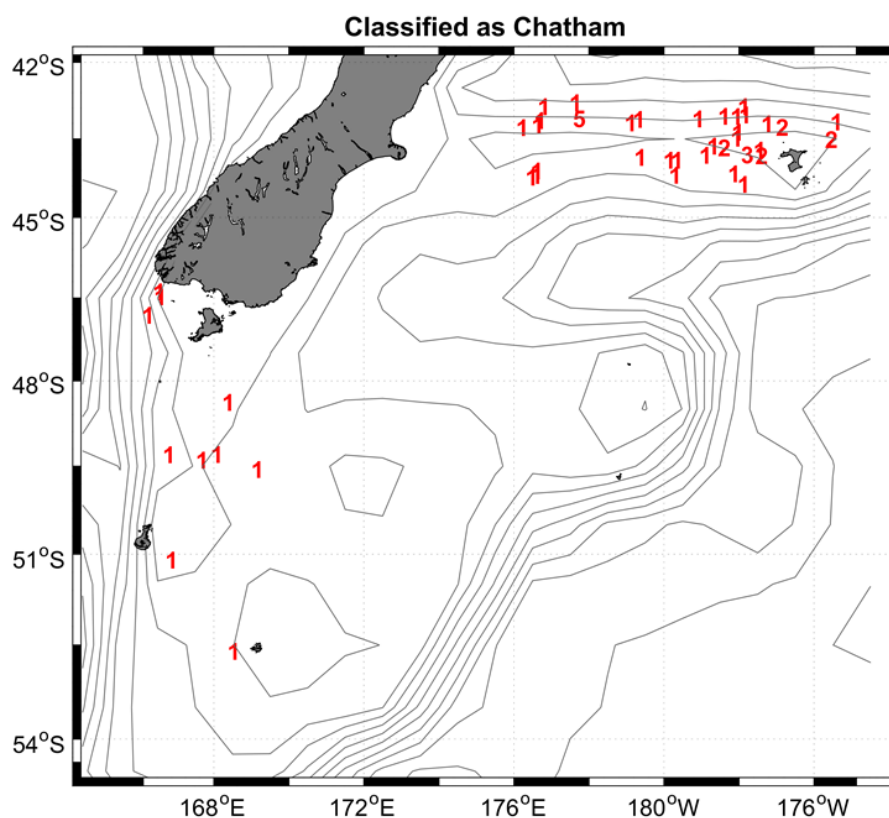


Figure 6: Map of otoliths classified to an area with a confidence threshold of 0.8. Coloured numbers indicate total otoliths allocated to within-area actual capture points. Red is Chatham Rise, blue is Sub-Antarctic.

The methodology described above was applied to the samples derived from Sub-Antarctic North and South to see if they could also be distinguished. The results showed that the two areas were entirely indistinguishable, with classification success of around 50% to 55%, marginally better than a coin toss. The two populations were so similar that the ANOVA step actually removed most of the parameters from the process, and only kept three eFd's and no shape indices.

When applying the method described above to males and females separately, very similar results were obtained regardless of sex. Total classification success was 65.2% for males only and 68.0% for females only. This indicates that there is no advantage in considering them separately in this case, and that it is better to merge males and females to increase the sample size used by the LDA.

Wavelet Analysis

Running ShapeR on the dataset gave successful classification rates using wavelet transformations of 70.4% (Chatham Rise vs Sub-Antarctic south) and 72.5% (Chatham Rise vs Sub-Antarctic north), which are both less than the total classification success achieved using the combined Fourier descriptors approach described above (77.4%). ShapeR, however, produces a visually useful polar coordinate plot of mean otolith shape using Fourier descriptors. This plot illustrates the close similarities between the two Sub-Antarctic areas, and the main areas of otolith shape differences occurring between the Chatham Rise and Sub-Antarctic (Figure 7). In general, Chatham Rise otoliths are relatively wider, and have a slightly shorter rostral tip, than Sub-Antarctic otoliths. A canonical analysis of principal coordinates viewed as a cluster distribution (Figure 8) shows that the majority of shape variation exists in the first discriminating axis (CAP1) with wavelets describing 92.7% of otolith shape differences, while normalised elliptical Fourier coefficients describe 96.8%.

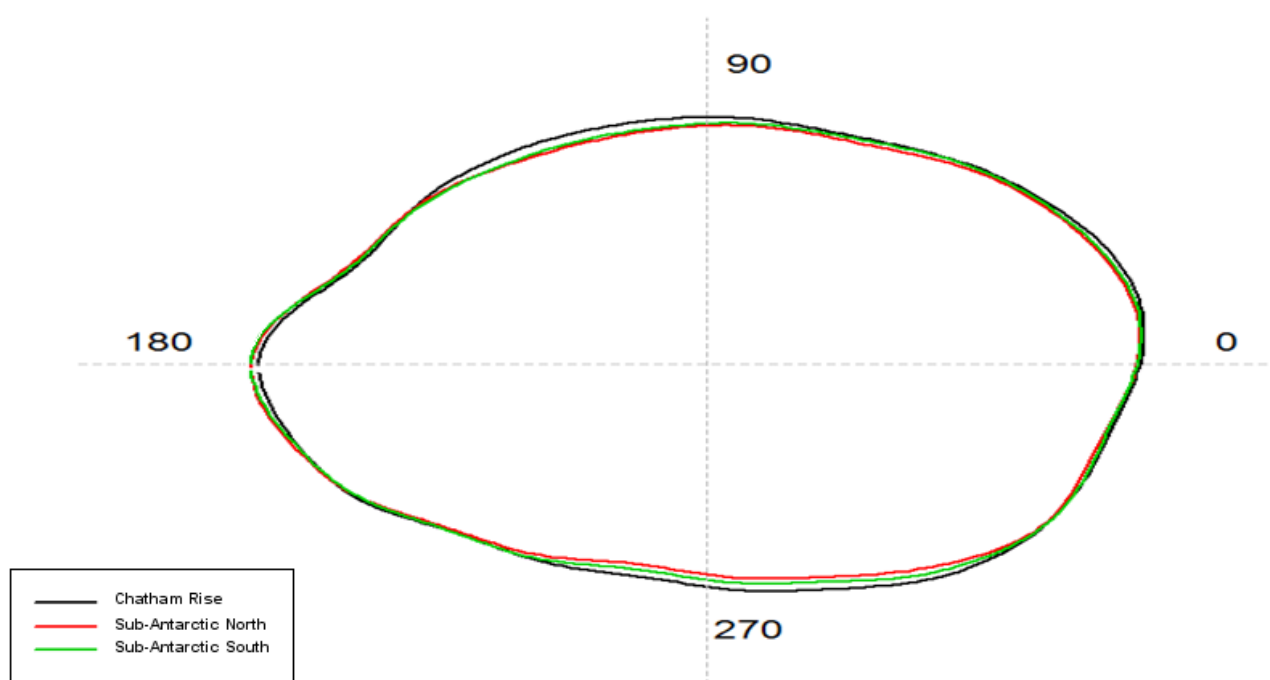


Figure 7: Polar coordinate reconstruction of mean otolith shape, by sample area, using Fourier descriptors.

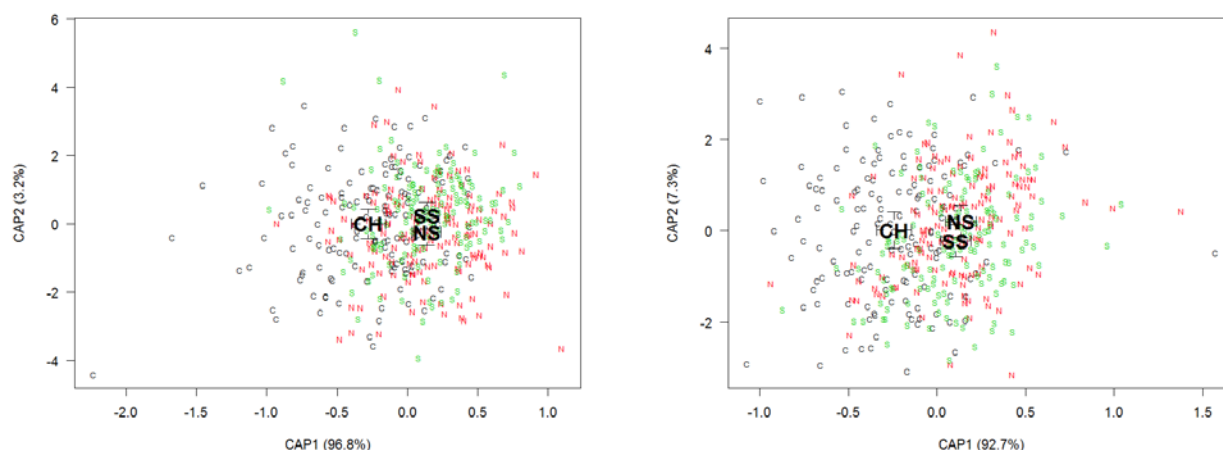


Figure 8: Cluster analysis showing classification distribution by area using Fourier (left plot) and Wavelet (right plot) methodologies. Sample areas in black letters (CH, Chatham Rise; SS, south Sub-Antarctic; NS, north Sub-Antarctic) are located at the mean population values, with surrounding bars representing $\pm$ one standard error. Individual data points are indicated by the letters C (Chatham Rise, black), N (north Sub-Antarctic, red), and S (south Sub-Antarctic, green).

4. DISCUSSION

It is known that the stocks of many marine fish can consist of several spawning components with variable degrees of reproductive segregation. Ling in the New Zealand EEZ are an example of this, being distributed across more than 20 degrees of latitude along both the east and west coasts of the country (Anderson et al. 1998), and with at least six locations where spawning is recorded consistently (Horn 2005). While genetic differentiation should ideally form the basis for any stock distinction, low levels of gene flow between stocks may prevent the detection of genetic differences (Begg & Waldman 1999), so non-genetic means of differentiation must then play an important role in identifying populations. Any available genetic analyses of New Zealand ling were equivocal and are relatively dated (Smith 1979, Smith & Francis 1982), and while subsequent advances in genetic methods may now allow the production of more useful information on stock differentiation the currently accepted stock structure of ling is based largely on differences in life history parameters among areas (Horn 2005).

The work reported here tests the viability of using otolith contour shape analyses to inform the stock structure of ling. Part of the justification for comparing the Sub-Antarctic and Chatham Rise ling stocks was that they are known to have markedly different growth rates and so are strongly believed to comprise different stocks (Horn 2005). Based on a shape analysis of otoliths of Atlantic cod (*Gadus morhua*) from a variety of putative stocks, Campana & Casselman (1993) concluded that otolith shape was strongly related to fish growth rate, and, consequently, that otolith shape might not differentiate well among populations with similar growth rates. This may be an issue for ling stock differentiation using otolith shape analysis as some postulated stocks have quite similar growth curves (e.g., Chatham Rise and west coast South Island).

Otolith shape has been shown, however, to be influenced by an interplay of environmental, ontogenetic, and genetic influences (Cardinale et al. 2004 Hüsey et al. 2016). This strengthens the validity of using otolith shape analyses as a stock differentiation tool — otoliths combine

both phenotypic and genotypic influences. But while otolith shape can differ among stocks of a species, it can also differ among ages, sexes, and year classes within a stock (Campana & Casselman 1993). Consequently, it is necessary to standardise the data to remove the influence of these effects (particularly the size effect), or at least to test their influence (Hüssy et al. 2016). Also, otolith shape does not always vary between groups of a species even when there are strong indications of stock separation based on differences in genetics (Smith et al. 2002) or otolith microchemistry (Longmore et al. 2010).

The analysis presented above for ling attempted to minimise the fish size effect by using otoliths from a relatively narrow size range of fish (i.e., 69–82 cm). The sex effect was also examined in the analysis. The numbers of available otoliths and age data precluded the use of otoliths from a single year class. Where age data were available for the analysed fish ($n = 175$), 73% of them were aged 7–9 years, but the range was 5–15 years. Mean age was 8.2 years in both the Chatham Rise and Sub-Antarctic samples. It is apparent, however, that if fish from a single age class had been used, then the length range of, say, 8-year-old fish would have been greater than the 13 cm range for the multiple age class sample used here.

The results produced here for ling support the currently accepted stock hypothesis that fish in the northern and southern areas of the Sub-Antarctic comprise a single stock, and that Chatham Rise fish are distinct from Sub-Antarctic fish. The percentage of successful classifications (77%) is similar to other studies that have claimed the method to be applicable as a stock differentiation tool (e.g., Tracey et al. 2006, Agüera & Brophy 2011, Leguá et al. 2013). Harbitz & Albert (2015) suggest that a level of better than 70% of successful classifications supports a hypothesis of separate stocks. Consequently, we conclude that the analytical method described here may usefully help to better describe the stock structure of ling in New Zealand waters.

It is interesting to note that most of the Sub-Antarctic otoliths that were classified as being Chatham Rise fish were collected on the Stewart-Snares shelf, i.e., that part of the Sub-Antarctic area that is nearest to the Chatham Rise (see Figure 6). Also, most of the Chatham Rise otoliths that were classified as being Sub-Antarctic fish were collected at the western end of Chatham Rise FMA 4, i.e., that part of FMA 4 that is nearest the Sub-Antarctic. Such a distribution of ‘misclassified’ otoliths may support the hypothesis that there is some movement of ling between the two areas, in both directions, along the lower east coast of South Island.

It must be acknowledged that otolith shape analysis is not the ‘silver bullet’ for fish stock differentiation, but it does appear to be a very useful tool in this regard. Some studies have analysed the results from otolith shape studies in combination with other stock differentiation techniques to provide stronger conclusions, e.g., with parasite loading and composition data (Vignon et al. 2008), or with microchemical analyses (Green et al. 2015). It appears likely that analyses of otolith shape would usefully inform the stock structure of ling in New Zealand waters.

5. ACKNOWLEDGMENTS

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