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PHENOTYPIC DIVERGENCE DESPITE HIGH GENE FLOW IN CHOKKA SQUID *LOLIGO REYNAUDII* (CEPHALOPODA: LOLIGINIDAE): IMPLICATIONS FOR FISHERY MANAGEMENT

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ABSTRACT

The commercially important chokka squid Loligo reynaudii *occurring in South African waters is currently managed on a single-unit stock hypothesis. We tested this assumption through a spatial comparison of the morphology throughout the distributional range of the species. Forty three morphometric characters were measured from 1079 chokka collected off the south coast of South Africa, the west coast of South Africa, and southern Angola. While no significant differences were found in the hard body parts, results from classification analysis showed that though all four types of morphometric attributes (soft body parts, beaks, statoliths, sucker rings) resulted in some separation, the most consistent separation of samples from the three regions was based on soft body part morphometric characters. On average, though dependant on the model, the overall correct classification rate ranged from 0.68 – 0.99 for males and 0.7 – 0.99 for females in all three regions. Previous DNA analysis had revealed some genetic differences between west coast and south coast samples, suggesting the confluence of the cold Benguela and warm Agulhas current may act as the approximate point of a phenotypic and possible genetic breakpoint. Finer scale genetic analysis of samples collected across the Benguela-Agulhas confluence reported no significant genetic structuring in this area suggesting environmental heterogeneity and not restriction of genetic flow/isolation as the primary driver of the observed phenotypic divergence.*

INTRODUCTION

The marine environment off the coast of southern Africa is one of the most diverse, complex and highly variable in the world (Lutjeharms *et al.*, 2001). The distribution of the cape hope squid *Loligo reynaudii* (locally known as chokka) along this coastline is largely influenced by the warm Angola current and the cold Benguela current upwelling system along the West African coast and the warm Agulhas current system along the south east coast (Figure 1). *L. reynaudii* inhabits these three different environments (south coast of South Africa, west coast of South Africa, and southern Angola) with an apparent break in its distribution off the coast of Namibia (Shaw *et al.* 2010). In South Africa, two-thirds of the adult biomass is concentrated on the eastern Agulhas Bank shelf where it has become an important fishery resource, targeted by a major commercial hand-line jig fishery (6000–13,000t caught

annually) since the mid-1980's (Augustyn 1989, 1991; Augustyn *et al.*, 1993, Arkhipkin *et al.* 2015). In addition, 200–500t is caught annually as a by-catch in the demersal trawl fisheries (Augustyn & Roel, 1998; Arkhipkin *et al.* 2015). In southern Angola artisanal fishers catch *L. reynaudii* close to shore from rafts, using homemade jigs and hand-lines (Sauer *et al.* 2013).

Although a number of studies into the stock structure of *L. reynaudii* have been attempted in the last decade (using various biological and genetic techniques, e.g. Olyott, 2002; Olyott *et al.*, 2006, 2007; Shaw *et al.*, 2010; Stonier, 2012), its demography still remains unclear. Due to lengthy planktonic paralarval stages (40-day passive period) with the potential for high dispersal rates (Roberts & Mullon, 2010), highly migratory adult stages (Sauer, 1995; Sauer *et al.*, 2000) and a lack of obvious physical geographic barriers to movement along the coastline, genetic homogeneity of the South African stock was previously assumed. This assumption was questioned by Olyott *et al.* (2007), who suggested that juveniles growing under different environmental conditions on the western Agulhas Bank could result in discrete subpopulations with different biological characteristics such as slower growth rates and larger size at maturity. The influence of water temperature on the growth of other cephalopod species is well known (Forsythe *et al.*, 1994; Carvalho & Nigmatullin, 1998; Forsythe, 2004).

A subsequent molecular study by Shaw *et al.* (2010) indicated small but statistically significant genetic differences among some *L. reynaudii* samples, suggesting a more complicated stock structure. Although no significant differences were found between genetic samples of different spawning aggregations across the main spawning range on the eastern Agulhas Bank, subtle differences were found in geographically more distant samples from the western Agulhas Bank (Shaw *et al.*, 2010). Such differences may necessitate a rethink of the current management strategy. A finer scale study of this region was therefore suggested to further investigate the possibility of geographically fragmented stocks and stock boundaries.

Although studies of geographic variation has been an accepted method in fish stock discrimination for over a century (Ihssen *et al.*, 1981), and has been extended to cephalopods such as octopods (Voight 2002; Lefkaditou & Bekas, 2004) and sepiids (Guerra *et al.*, 2001; Kassahn *et al.*, 2003; Neige, 2006), the use of morphometric data has not yet been attempted for *L. reynaudii*. Morphometric studies has been widely used to distinguish between species of squid (Haefner, 1964; Lipinski, 1981; Augustyn & Grant, 1988; Pierce *et al.*, 1994b; Sanchez *et al.*, 1996; Bonnaud *et al.*, 1998; Pineda *et al.*, 2002) and to study the geographic variation of population units and fishery stocks within species of squid (Kashiwada & Recksiek, 1978; Kristensen, 1982; Brunetti & Ivanovic, 1991; Boyle & Ngoile, 1993; Pierce *et al.*, 1994a; Borges, 1995; Zecchini *et al.*, 1996; Carvalho & Nigmatullin, 1998; Hernandez-Garcia & Castro, 1998; Vega *et al.*, 2002; Liao *et al.*, 2010). It is important to note, however, that unlike molecular markers, phenotypic variation in body parts is markedly influenced by environmental factors (Carvalho & Nigmatullin, 1998) and does not always result from genetic divergence (Cadrin, 2000). Therefore, phenotypic variation may only provide indirect indication of stock structure (Begg *et al.*, 1999). Although they do not provide direct evidence of genetic isolation between stocks, they can indicate separation of post-larval stocks living in different environmental regimes (Begg *et al.*, 1999). Phenotypic markers may therefore be more useful for studying short-term, environmentally-induced variation, as opposed to long-term genetic variation.

In an attempt to better understand the stock structure of *L. reynaudii* in South African waters this study undertook morphometric analysis across the distributional range of the species, and a more geographically concentrated genetic study of samples from across the Agulhas Bank region.

MATERIALS AND METHODS

Collection of samples

L. reynaudii samples from South Africa were collected utilising various commercial jig vessels and a demersal trawl research vessel (Figure 1). The south coast demersal research survey (08/04/2011 – 13/05/2011) covered the shelf between 20°E (Cape Agulhas) and 27°E (Port Alfred) and the west coast survey (09/01/2012 – 13/02/2012) between 20°E (Cape Agulhas) and 29°S (Orange River). These demersal surveys provided for the collection of random samples over a range of shallow and deep areas on the south and west coast of South Africa. Between June and November 2011 additional samples were collected on board various commercial jig vessels fishing in the main inshore spawning areas which were not covered during the south coast demersal trawl survey. Samples caught by the artisanal jig fishery in southern Angola (in the coastal waters between 15° and 17°S) were collected from a single hawker at a fish market in Namibe, the species' northern-most known geographical limit (Roberts *et al.*, 2012). Only adult squid were sampled, ranging in length from 180 – 420 mm dorsal mantle length (DML) for males and 150 – 260 mm DML for females.

All squid were frozen and transported to Rhodes University, South Africa, where they were kept at -20°C until analysis. Genetic material in the form of tentacle clippings were collected from subsamples of squid caught in the Agulhas Bank and West Coast regions. The clippings were taken immediately after capture and stored in 70% ethanol until processed.

Selection of individuals

A total of 544 male and 535 female individuals from the three regions were used in the classification analyses. The average DML length of males (279.3 mm Angola, 299.8 mm south coast, 250.6 mm west coast) and females (185.8 mm Angola, 207.4 mm south coast, 190.8 mm west coast) from each region differed only slightly (see Appendix A for the descriptive statistics of all character measurements taken). For both males and females the south coast subsample size was by far the largest.

All samples used in the classification analysis were classified as adults with maturity stages of 3 (preparatory), 4 (maturing) and 5 (mature), according to Lipinski's universal maturity scale for commercially-important squid (Lipinski, 1979; Lipinski & Underhill 1995). No samples were classified as belonging to stages 1 (juvenile) and 2 (immature).

Morphometric measurements

Forty three morphometric characters (Table 1) of the soft body parts (body, head, arms, tentacles) and hard structures (gladius, sucker rings, lower beak, statolith) were measured from each sample. Beak morphometric characteristics were modified from Clarke (1986), statolith morphometric characters from Clarke & Maddock (1988) and, gladius, sucker rings and soft parts were selected and modified from Lipinski (1981). Detailed specifics on the measurements taken for each soft part and hard structure can be seen in Table 1 and Figure 2

- 4. In order to prevent any warping of morphological characteristics, which can happen with repeated freezing and thawing (Lipinski, 1981), each specimen was defrosted only once at room temperature before morphometric measurements were taken. No measurements were made on soft parts or hard structures which appeared to be damaged or to have suffered previous damage (e.g. missing arm and tentacle tips; re-grown arms and tentacles; damaged gladius, lower beaks, sucker rings and statoliths). All morphometric measurements were made by the senior author and under standardised conditions to avoid unnecessary variation in measurements.

All soft part morphometric data were measured to the nearest millimetre according to recommendations by Roper & Voss (1983), using a single set of vernier callipers. Measurements on the gladius and sucker rings were made after removing the structures from the squid. Gladius measurements were made to the nearest mm using vernier callipers and sucker ring diameter was measured using a low-powered microscope with an eyepiece micrometer. Beaks were carefully extracted from the buccal mass following the method described by Clarke (1986) and immediately frozen until further analysis. After defrosting at room temperature at a later stage, lower beaks were measured in profile to the nearest 0.01 mm using a single set of digital callipers. Statoliths were removed from the head with a small pair of tweezers and stored in empty vials until further analysis of only one statolith per pair (either left or right) under a low-powered microscope with an eyepiece micrometer.

Analysis of morphological data

Prior to analysis, morphometric data were screened for errors using bivariate plots and regression analyses to identify outliers. Unfortunately soft part measurements could not be retaken as specimens were discarded after measurements. Errors in soft part data were therefore corrected by reference to the original data sheets, alternatively data from those samples were deleted. Some hard structures such as beaks and statoliths were re-measured where necessary.

Morphometric studies, whereby multivariate measurements of different body parts are used to characterize the average shape of the sampled population, need to take into account/adjust the effect of body size as most of the variations are the result of changes in body size (Lleonart *et. al.* 2000). There are slightly different ways of removing the effect of body size. In this study, each morphometric character was log-transformed and standardized using the following allometric formula (Liao *et al.*, 2010):

$$M_{std} = log(M) - \beta\left(log(Ml) - log(\bar{Ml})\right)$$

Where M_{std} is the standardized morphometric measurement, $log(M)$ is the log of the morphometric measurement, β is the slope of regression of the morphometric measurement to the dorsal mantle length, *Ml*, $log(Ml)$ is the log of the dorsal mantle length, and $log(\bar{Ml})$ is the mean of the log of the dorsal mantle length. Although this is the commonly used approach to remove the effect of size we have also considered another method that uses a size variable of each morphometric group, such as c for beaks and TSL for statoliths. The classification models were then applied to data of both approaches to remove the effect of size. Results of the classification, using the three models, were comparable (Appendix B).

Sexual dimorphism

To test for sexual dimorphism Multivariate Analysis of Variance (MANOVA) was applied on the standardized measurements of soft body parts, beaks, statoliths and sucker rings. The assumption of normality and homogeneity of variance were checked visually using qqplot and plot of residuals vs. fitted values respectively. In addition, we have also compared the slopes of the regression of different morphometric measurements vs. dorsal mantle length for males and females in all three regions.

Classification of samples

Prior to assessing the distinction between samples collected from the three different regions an exploratory analysis was conducted. This included a graphical summary of the morphometrics by sex and region, and a principal component analysis of the morphometrics. A number of statistical techniques are commonly used in the classification of samples. Based on sets of variables/attributes, these include: Discriminant Function Analysis (e.g. linear and quadratic), Classification Tree Analysis (CTA), Artificial Neural Network (ANN), Random Forest (RF), Support Vector Machine (SVM),...etc. In this study we have used Linear Discriminant Function Analysis (LDA), CTA, and RF. Classification studies on patagonian squid *Doryteuthis gahi* samples from different regions has shown samples to be less distinct when combining soft parts and hard structures (Vega *et al.*, 2002). Thus all three statistical techniques were applied to the combination of all soft parts, and then to each of the different hard structures (lower beak, gladius, sucker rings, and statolith) separately. In addition, data for males and females were analysed separately due to the apparent sexual dimorphism.

Linear Discriminant Analysis (LDA)

In principle, Discriminant Analysis is similar to Principal Component Analysis (PCA) in that they both aim to find the optimal rotation when projecting observations. The main difference is in the way they extract major axis of variation. In PCA the objective is to extract a series of orthogonal axes that cumulatively extract most of the variation in the data, whereas in LDA we are interested in extracting sets of axes (usually two) that maximize the separation of apriori known groups (Zuur *et al.*, 2007). Unlike PCA there are a number of assumptions to be met for the results of LDA to be valid. These include variables to be on a continuous scale (categorical variable to be avoided as much as possible); the number of observations per group to be at least 2 but preferably 4 to 5 times the number of variables; the variables considered in the analysis to be linearly related but not with sets of variables with correlation coefficient of 1/-1; homogeneity of variance across groups (variance/variability of each of the variables considered to be roughly comparable across groups); as the method is based on the calculation of a covariance matrix that is pooled across groups, relationship among variables considered should be the same across groups; assumes independency of observations and multivariate normality (hence variables in each group are expected to be normally distributed). The most important assumptions are homogeneity of variance (required for the validity of the method itself due to the use of pooled covariance) and the normality of variables (required for hypothesis testing) (Zuur *et al.*, 2007).

Classification Tree Analysis (CTA)

CTA is a non-parametric technique using recursive partitioning algorithm. The technique is widely used in fields ranging from social to medical sciences and has only relatively recently been recognized as a powerful method in ecology (De'ath & Fabricus, 2000), mainly because it naturally deals with non-linearity and higher-order interactions among predictors, which is the characteristics of most ecological data. The technique is also invariant to monotonic transformation of predictors, able to handle missing values in both response and predictor variable(s), making it is easy for interpretation (James *et al.*, 2014). CTA explains the

variation in the response variable by repeatedly partitioning the data into more homogenous groups using a combination of predictor variables. The algorithms repeatedly split data into a nested series of groups that are as homogenous as possible with respect to the response variable considered. In the context of classification problems (in this case classification of samples from three regions) the model starts with the whole data set, the root node, and formulates splitting rule for each value of the predictor variables to create candidate splits. The algorithm then selects the candidate splits that results in the smallest misclassification error rate using it to split data into two sub-groups. The algorithm then continues to recursively split the sub-groups until no split leads to substantial reduction in misclassification error rate or the number of observations in the sub-groups are too small for further splitting. When the algorithm cannot split a node further then it has reached the terminal/leaf node. CTA tends to over-fit which can usually be avoided by running tree-based sets of criteria in this using cross validate error rate on the training data. Determination of the optimal size of tree was done via cross validation. The optimal size of the tree was selected based on the complexity parameter *cp*, and the complexity parameter that gave the lowest cross-validation error (and hence size of the tree) was selected. By applying the one standard deviation rule the value of the *cp* (optimal tree size) was selected. Details of the method, implementation of the algorithm and examples are given in Horton & Kleinman, 2010, James *et al.*, 2014 and Zuur *et al.*, 2007.

Random Forest (RF)

Random forest is one of the developments in the area of predictive statistics together with bagging and boosting meant to improve classical tree models. Bagging (bootstrap aggregation) involves the construction of multiple trees based on the training set and finally aggregating the results to produce the final outcome. Although dissimilar, boosting involves creating many trees, not as an independent one but by repeatedly growing/updating the existing tree, and finally predicting the outcome. Random forest on the other hand improves both classical trees and bagging more so on classical trees. Similar to bagging, random forest creates multiple trees from the training data but does so by building the trees based on a random subset of the predictors that de-correlates the multiple tree created. This tends to make the trees less variable and more reliable (Hastie *et al.*, 2009; James *et al.*, 2014). Random forest is important in classification problems when there are large numbers of correlated predictors. Results from random forest is equivalent to that from bagging when the size of the number of variables selected for splitting equals the total numbers of predictors.

Model selection and performance

Mode selection was achieved using the step-wise selection approach. We specifically applied selection in both-direction for all three model types. A combination of variables that lead to an increase in the overall classification rate with a cut-off threshold of 1% was selected to make the variable sets in the final model. The relative importance of predictors were assessed by the improvement in the overall classification rate achieved as a result of the addition of predictors.

Model predictive performance was assessed using re-sampling. There are different re-sampling techniques, specifically we have used the 'Leave Group out Cross Validation' LGOCV (also known as Monte Carlo cross validation) whereby each model is repeatedly trained on a subset of data to evaluate the remaining subset (Kuhn & Johnson, 2013). Predictive performance was then summarized as the mean +/- 1 standard deviation of the predictive accuracy from the LGOCV.

Genetic analysis

Genomic DNA was extracted from tentacle tips using a CTAB- chloroform/IAA method (Winnepenninckx *et al.*, 1993). Individuals were then genotyped at four microsatellite loci (Lfor1, Lfor 3, Lrey44 and Lrey 48,) following Shaw *et al.*, (2010).

Genetic variability was assessed using numbers of alleles (N_A), observed heterozygosity (H_O), and expected heterozygosity (H_E) (Nei 1978), all calculated using FSTAT 2.9.3.2 (Goudet, 1995). Genotype frequency conformance to Hardy-Weinberg equilibrium (HWE) expectations and genotypic linkage equilibrium between pairs of loci were tested using exact tests incorporating a Markov chain method (Guo & Thompson, 1992) with default parameters in GENEPOP 3.3 (Raymond & Rousset, 1995). Locus-by-sample combinations were tested for the presence of null alleles using MICROCHECKER (van Oosterhout *et al.*, 2004) with significant effects adjusted for using the van Oosterhout algorithm. Tests of genetic differentiation were then performed with and without null allele correction (NAC). Using FSTAT, genetic differentiation was quantified using the unbiased F_{ST} estimator, θ (Weir & Cockerham, 1984), calculated globally (over all samples) and between pairs of samples, with significance of estimates inferred following 10 000 permutations (Goudet *et al*., 1996) of genotypes among samples. Global and pairwise exact tests of allele frequency homogeneity were performed for each locus in GENEPOP with default Markov chain parameters. Multilocus values of significance were obtained by Fischer's method.

An important consideration when sampling highly mobile adults is the possible effect of sampling migrants on estimates of population structure derived from comparisons between admixed samples. To address this potential issue population structure was also investigated using the Bayesian clustering analysis implemented in the program STRUCTURE (Pritchard *et al*., 2000) which permits estimations of the most probable number of populations represented by the data without *a priori* sample definition. Both the 'no admixture model' (as recommended for low F_{ST}; Pritchard *et al*., 2000) and 'admixture model with correlated allele frequencies' were used independently to identify the number of clusters, *K* (from a range of 1-5), with the highest posterior probability. Each MCMC run consisted of a burn in of 10^6 steps followed by 5 X 10^6 steps. 3 replicates were conducted for each *K* to assess consistency. The *K* value best fitting the data set was estimated by the log probability of data [Pr(X/K)].

RESULTS

Morphology

Removal of the effect of size

Figure 5 shows the slope of the regression of soft body part variables vs. DML that was used to remove the effect of size on the different morphometric measurements. The figure mainly highlights the difference between males and females suggesting sexual dimorphism. Similar patterns were observed for beaks, statoliths, and sucker ring variables (not shown here due to space limitation but given in Appendix C). Table 2 shows the results of the MANOVA applied to all morphometric measurements. It shows that in all cases the shape, as characterized jointly by all variables, of the soft body parts, beaks, statoliths, and sucker rings differed among the three regions between sexes. In addition, for both soft body parts and beaks there was interaction between the region and sex suggesting the presence of not only sexual dimorphism but also variance among the three different regions.

Classification of samples

Result of the exploratory analysis applied to the morphometric measurements are shown in Figures 6a, 6b, 7, 8, and 9. The figures show distinct differences between males and females and to some extent among the three regions, more so in the soft body part measurements. For the sake of comparison we presented Figure 10. This figure shows the results of the PCA and LDA applied to the same morphometric data for the soft body part measurements. The LDA plots suggest distinction in the body shape of individuals sampled from the three regions. In contrast, the PCA ordination of the same data did not show such distinction among individuals collected from the different regions. This is largely a reflection of the way PCA extracts different PC axes: orthogonal axes that maximize the variance whereas LDA extracts axes that maximizes the separation among groups that are apriori defined (in this case the regions where individual squids were sampled). Similar exploratory comparative multivariate analysis of beak, statolith, and sucker ring data are shown in Appendix D (not shown here due to space limitation).

Model selection and performance

The best model was selected based on the step-wise approach. As shown in Table 3, for each of the four type of morphometric measurements (soft body parts, statoliths, beaks, and sucker rings) the three models appear to mostly select fewer numbers of variables given the sets of variables available (more so for the soft body parts). In addition, the type and number of variables selected by the different models appear to differ. For the soft body parts the FL was the most commonly selected variable by the three variables and for both sexes. The number and types of variables selected for the beak measurements differed among models. The same was observed for sucker ring measurements. For statolith measurements the type of variables selected differed among models but CTA and RF appear to select at least the same types of variables.

Figures 11 – 14 show comparative performance of the three models for males and females separately and when combined. With the exception of the soft body part measurements (Figure 11), where the three models performed relatively well, both the overall accuracy of the models and their relative performance was different (Figures 12 – 14). The most accurate and higher classification of samples from the three regions were achieved using soft body parts (Figure 11). For males and females, and for both sexes combined, LDA and CTA performed very well followed by RF. The comparative performance of the three models based on variables from the beaks shown in Figure 12 highlights the low classification accuracy of the models and indicate lower predictive power of beak variables in the classification of samples from the three regions. A similar pattern was observed in the classification of samples based on statolith and sucker ring variables (Figures 13 and 14 respectively). Figure 15 shows how the soft body part phenotypic differences were more accentuated between samples from the Angola-Benguela Frontal zone (southern Angola) and the southern Benguela Current system (West Coast and Western Agulhas Bank), than between samples from the latter and the Agulhas Current system (central and eastern Agulhas Bank).

Genetics

The total number of alleles per locus ranged from 18 to 28 (mean 23.5, SD 4.1) and levels of genetic variability were similar across samples (Table 4). All tests of linkage disequilibrium were non-significant indicating that the microsatellite loci are unlinked and thus provide independent estimates of population diversity. Lrey44 and Lrey48 exhibited significant

deviations from HWE due to heterozygote deficits in all samples. A significant heterozygote deficit was also reported at locus Lfor1 in the Cape St. Francis (inshore) sample. All remaining locus/sample tests conformed to HWE expectations.

Global differentiation was significant when tested by exact test without NAC (P = 0.0027) but the corresponding test was not significant when performed with NAC (P = 0.1099). Global differentiation as measured by θ_{ST} was not significant for both null allele corrected (θ_{ST} = -0.004; P = 0.9990) and uncorrected data (θ_{ST} = 0; P = 0.9110). Estimates of θ_{ST} between pairs of samples were also low with no comparison significant when tested by permutation for either uncorrected or with NAC data (Table 5). Pairwise exact tests yielded a number of significant outcomes, with five out six significant results (for uncorrected data) involving the Plettenberg Bay (offshore) sample (Table 6). With NAC, only 2 pairwise comparisons were significant, both involving the Plettenberg Bay (offshore) sample. The Bayesian clustering method did not detect any significant population structure with unanimous support for models of K = 1 in all runs.

DISCUSSION

A number of methodological concerns encountered in the course of the study should be considered. For comparative morphometric studies, Pierce *et al.* (1994 a, b) recommended simultaneous sampling to minimize mixed-stock samples. In this case, samples for all three regions should ideally have been collected at least in the same season. However, due to the cost of sampling and large sampling area covered, it was not possible to collect all samples in the entire geographic range simultaneously or even during the same season. As squid are believed to be highly mobile (Boyle, 1990), this may have had a temporal effect on the results of the morphometric analyses and should be kept in mind when interpreting results.

A difference in morphological characteristics between *L. reynaudii* sampled in South Africa and Angola is perhaps not surprising, with the exception of soft body part phenotypic differences being more accentuated between samples from the Angola-Benguela Frontal zone and the southern Benguela Current system, than between samples from the latter and the Agulhas Current system. Given the break in distribution of *L. reynaudii* in Namibian waters, a much higher degree of mixing between individuals from the Agulhas Current and the southern Benguela Current than between the latter and southern Angola would be assumed. However, given the highly mobile nature of both larvae and adults, one would intuitively expect a single stock along the South African coast. Populations occurring on the West Coast and western Agulhas Bank vs. those occurring on the central and eastern Agulhas Bank however may also be phenotypically distinct from each other due to the different environmental conditions found on either side of Cape Agulhas. The corresponding genetic data support high gene flow throughout this region and suggest that the subtle differentiation reported by Shaw *et al.* (2010) does not reflect temporally stable population sub-structuring but rather temporary random variation within a single population. This implicates environmental heterogeneity and not population isolation as a driver of the phenotypic divergence.

Temperature regimes can have a significant influence on the growth and development of cuttlefish and squid, and growth at different temperatures can result in squid of markedly different size and growth-related parameters (Forsythe *et al.*, 1994; Carvalho & Nigmatullin, 1998; Forsythe *et al.*, 2001). According to Portner & Zielinski (1998), oxygen availability can also limit performance levels in squid. Some squid may be able to operate at their

functional and environmental limits, revealing a trade-off between oxygen availability, temperature, performance level, growth, and possibly body size (Portner & Zielinski, 1998). Conditions on the west coast (southern Namibia, west coast of South Africa and the western Agulhas Bank, 29° - 35°S) are influenced by the cold equatorward flowing Benguela current and associated with much colder bottom water temperatures fluctuating between 5° and 11°C with an average of 10°C, and low bottom dissolved oxygen (BDO) of 1.5 – 4.5 ml/1 (Augustyn, 1991; Roberts, 2005). It is likely that the Lüderitz upwelling cell off the coast of southern Namibia acts as a partial environmental barrier to movement of squid. On the south coast (central and eastern Agulhas Bank, 20° - 26°E) of South Africa, conditions are influenced by the warm south-westward flowing Agulhas current and associated with moderate water temperatures fluctuating between 9° and 24°C, and well oxygenated bottom waters (Augustyn *et al.*, 1994). Therefore, given that water temperature and bottom dissolved oxygen considerably differ in each region, they may act as the main drivers of phenotypic variation found in chokka *L. reynaudii* from the different regions. However, better defined and substantiated relations need to be further researched.

In contrast to the findings of Borges (1995), Vega *et al.* (2002), and Martinez *et al.* (2002), soft parts in this study proved to be more effective than hard structures (gladius, lower beaks, sucker rings, statoliths) in discriminating between squid populations from different geographical regions. This is surprising as soft body parts are generally accepted as being less reliable than hard structures due to their plasticity and warping response to freezing and thawing (Carvalho & Nigmatullin, 1998). Nevertheless, as pointed out earlier, the geographic variation found in *L. reynaudii* soft body parts may be related to phenotypic responses derived from region-bound environmental conditions (Shea & Vecchione, 2002). This is an evolutionary phenomenon that has been identified in other species of squid occurring in different habitats across large geographical areas (Carvalho & Pitcher, 1989; Hernandez-Garcia & Castro, 1998; Vega *et al.*, 2002).

In conclusion the study demonstrated some phenotypic population sub-structuring of *Loligo reynaudii*. LDA demonstrated that morphologically there is some evidence that squid from the south coast (central and eastern Agulhas Bank), west coast (west coast and western Agulhas Bank), and southern Angola are different. The diverse marine environment was postulated to be one reason for this variation. Molecular analysis did not support the existence of a genetic breakpoint allowing a geographical reference point for separating stocks. While the potential disconnect between genetic and demographic connectivity (i.e. low migration rates may be sufficient to homogenise genetic variation but be insufficient to prevent independent reaction of populations/stocks) is an important consideration for management, data indicate that the regional patterns of morphological divergence are occurring against a background of high gene flow. This pattern confirms the influence of environmental heterogeneity and not restriction of genetic flow/isolation as the primary driver of the phenotypic divergence. The observed phenotypic heterogeneity probably reflects the interplay between genetic adaptation and short term plasticity, which may vary through the studied range. Although cephalopods are renowned for their phenotypic plasticity, the phenotypic divergence may reflect adaptive differences which may be important for future sustainability and management of this resource. Further targeted experimental investigations will be needed to determine the exact underlying drivers of the phenotypic divergence. Furthermore, recent advances in molecular techniques may also help to link phenotypic and genomic variation and improve understanding of the roles of adaptation and plasticity (Allendorf *et al.*, 2010). Discovered phenotypic differences may signal the beginning of the evolutionary divergence between various geographic groupings (eventually resulting in

differences between hard parts, and genetic splits), but they are insufficient at this stage to revise the current management strategy of the chokka squid resource.

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FIGURE LEGENDS

Figure 1. The current known distribution of *Loligo reynaudii*, with major oceanographic features of the different regions indicated (modified from Henriques *et al.*, 2012). Morphological and genetic sampling locations of *Loligo reynaudii* on the south and west coast of Southern Africa are shown. PN) Port Nolloth; YZ) Yzerfontein; CT) Cape Town; CA) Cape Agulhas; MB) Mossel Bay; PB) Plettenbergbay; SF) Cape St. Francis; PE) Port Elizabeth; PA) Port Alfred.

Figure 2. Soft part morphometric measurements recorded for *Loligo reynaudii* in this study, based on the work by Lipinski (1981). A) soft part dimensions (taken from Pierce *et al.*, 1994a), B) fin angle dimension (taken from Pierce *et al.*, 1994a).

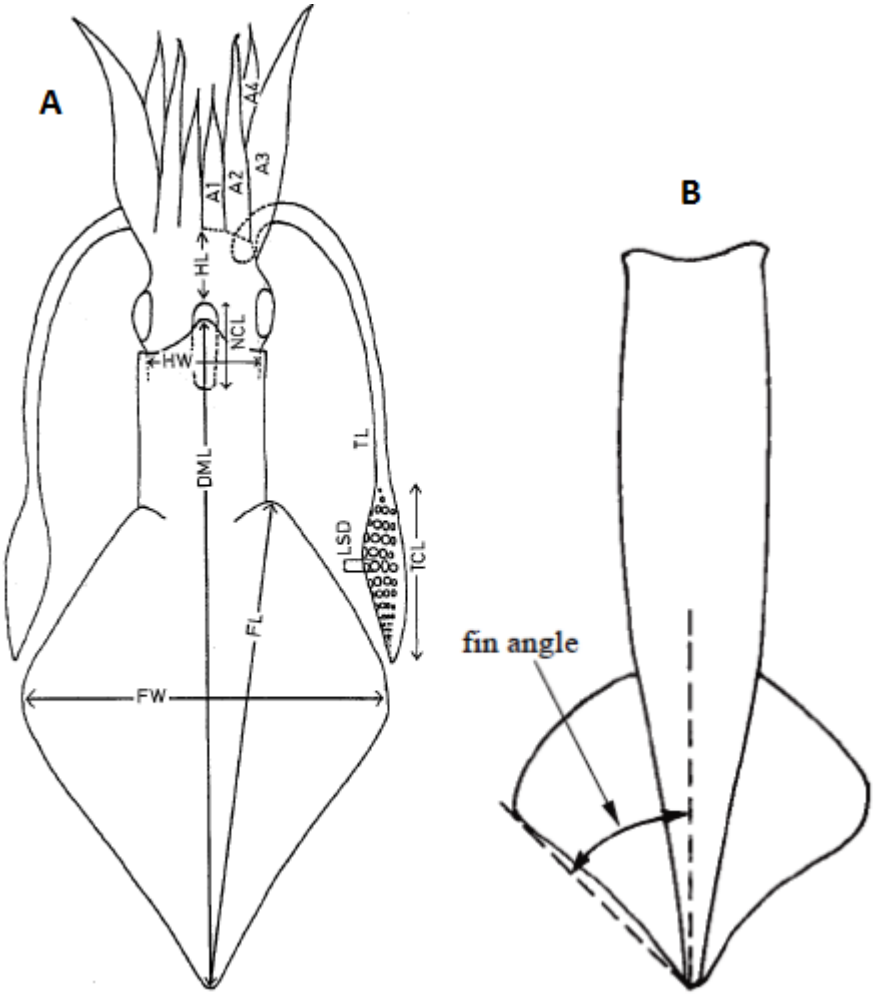

Figure 3. Hard structure morphometric measurements recorded for *Loligo reynaudii* in this study, based mainly on the work by Clarke (1986). A) lower beak dimensions (taken from Ogden *et al.*, 1998), B) Gladius dimensions (taken from Baron & Re, 2002)

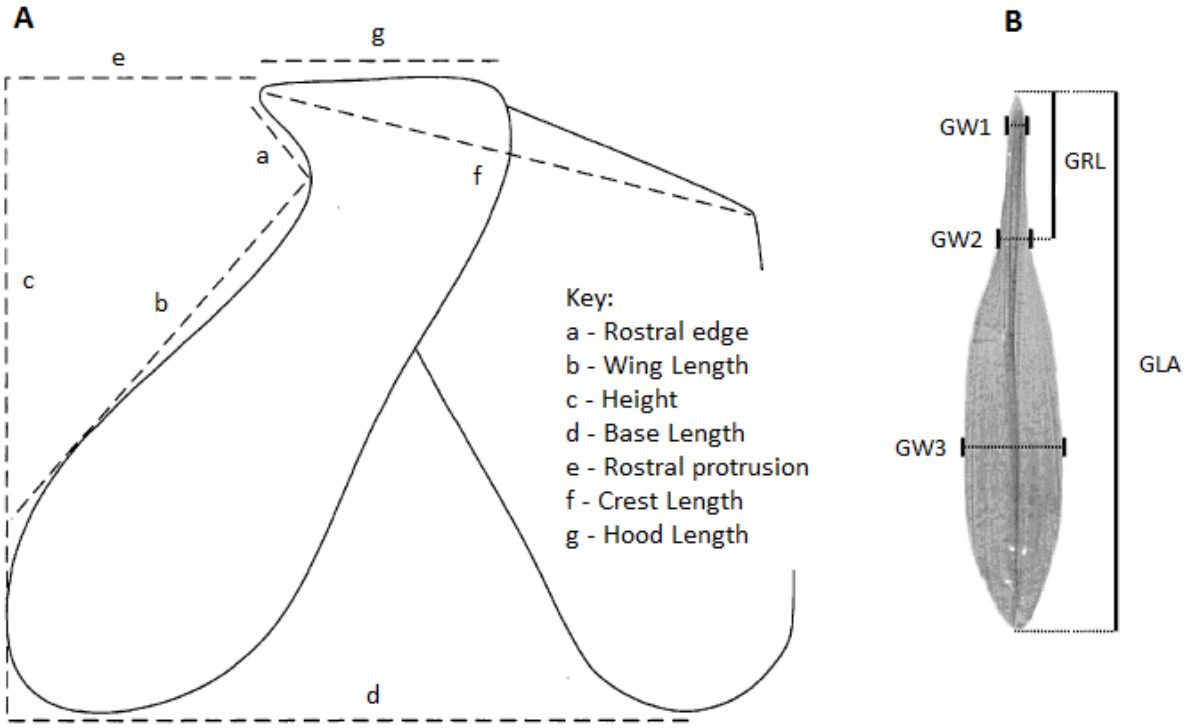

Figure 4. Diagram of a *Loligo reynaudii* statolith. A) basic terms of a *L. reynaudii* statolith (after Jereb & Roper 2010), B) *L. reynaudii* statolith dimensions measured in this study (modified from the work by Clarke & Maddock 1988; Lipinski *et al.* 1993).

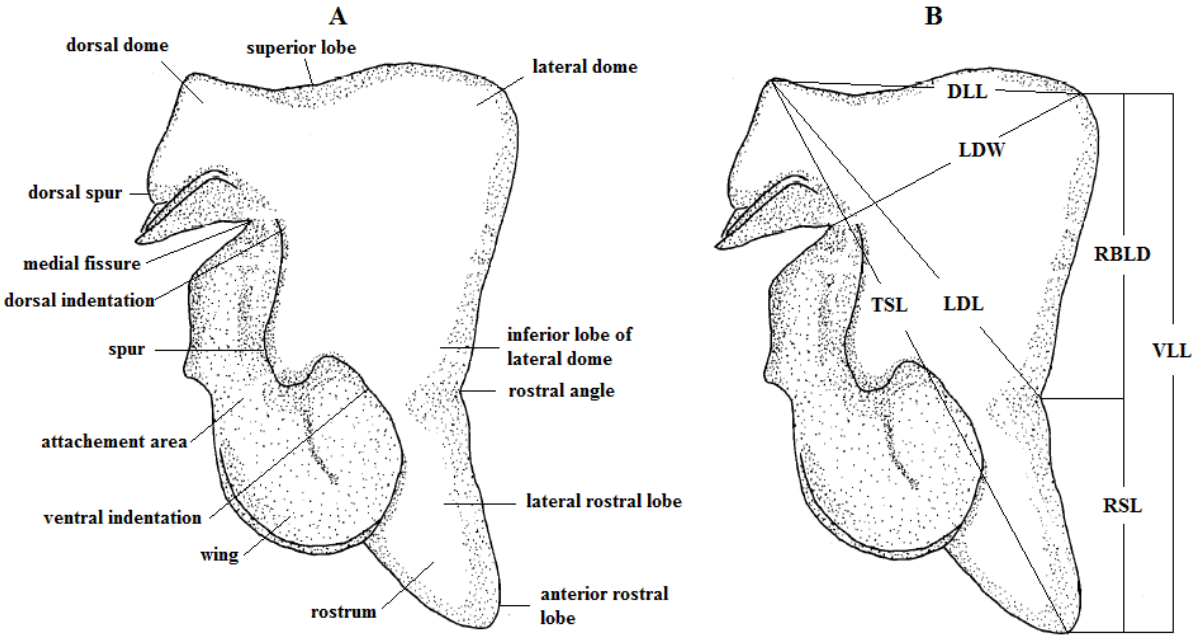

Figure 5. Plot of the slopes and the 95% confidence interval of the regression of *Loligo reynaudii* soft part measurements vs. DML.

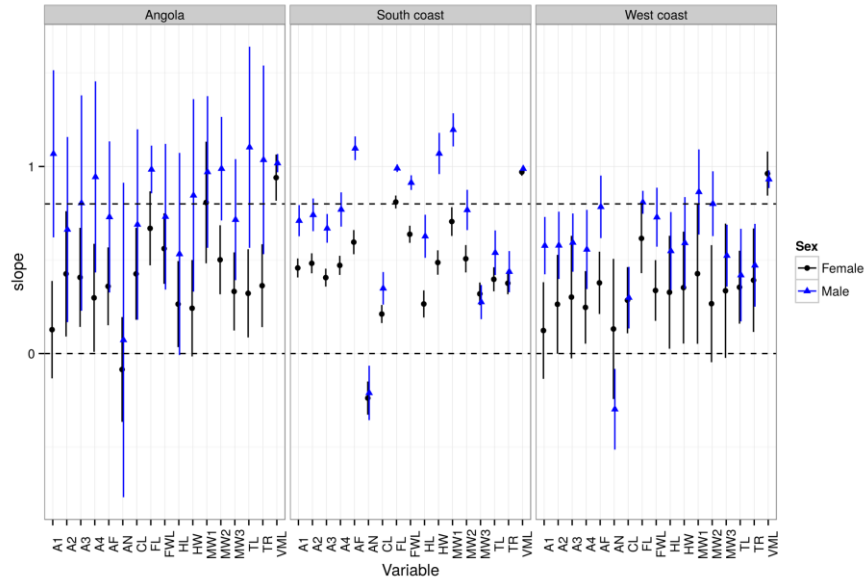

Figure 6a. Box-Whisker plots of standardized values of attributes for the soft body parts of *Loligo reynaudii* males and females from the three regions.

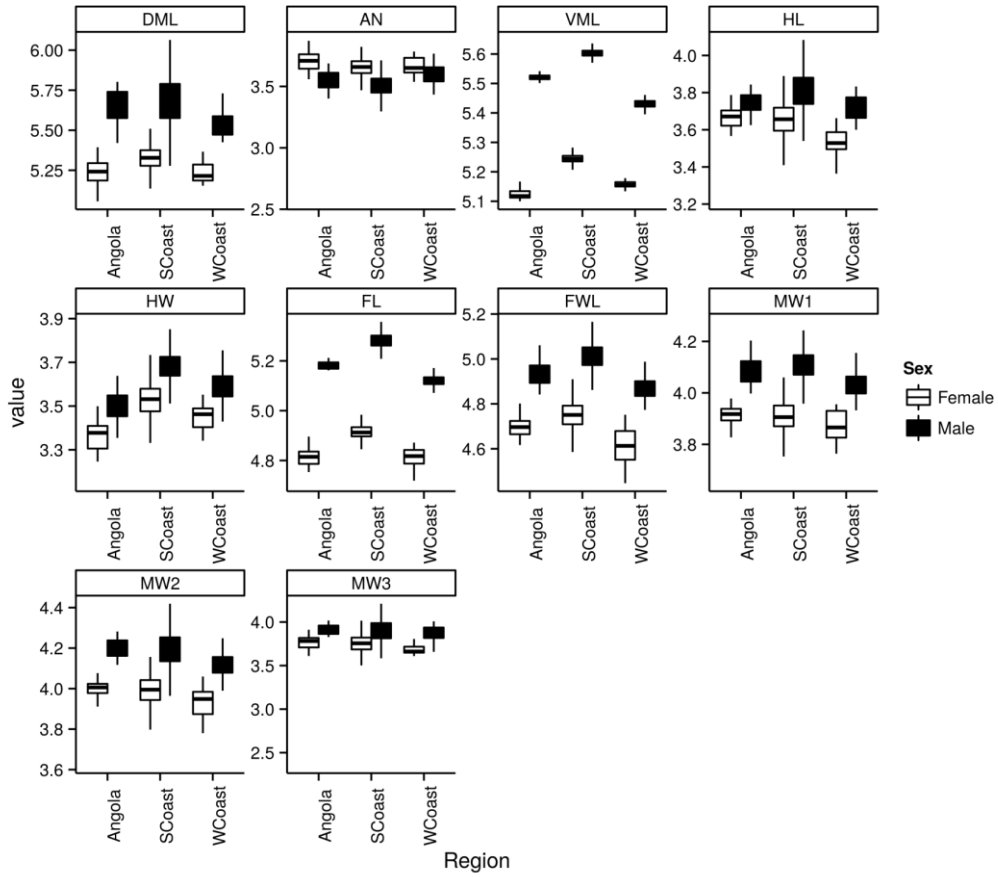

Figure 6b. Box-Whisker plots of standardized values of attributes for the soft body parts of *Loligo reynaudii* males and females from the three regions cont.

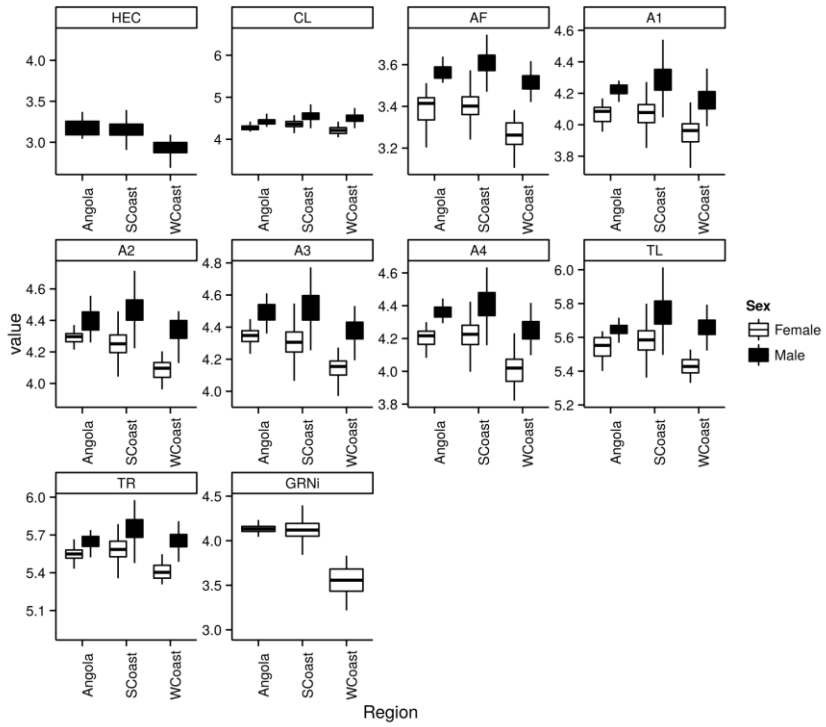

Figure 7. Box-Whisker plots of standardized values of attributes for the beaks of *Loligo reynaudii* males and females from the three regions.

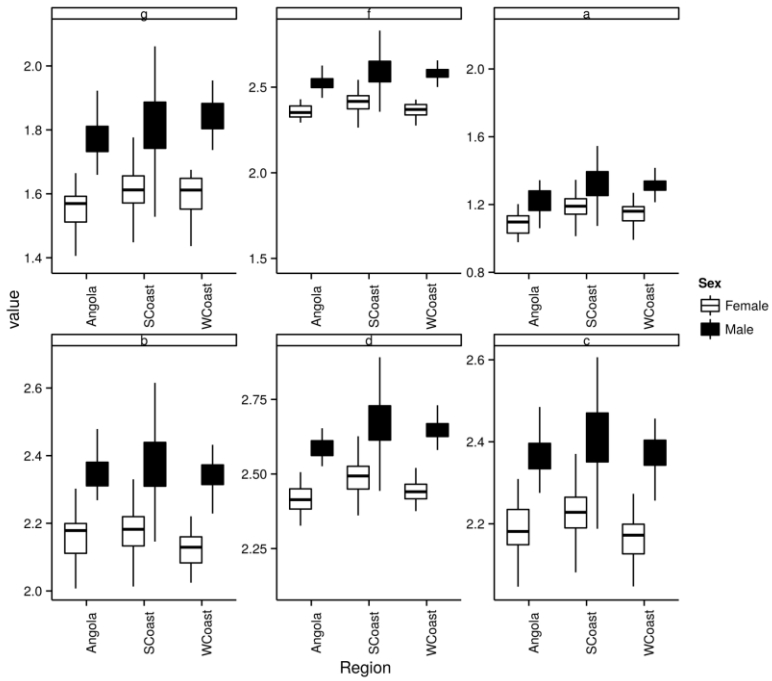

Figure 8. Box-Whisker plots of standardized values of attributes for the statoliths of *Loligo reynaudii* males and females from the three regions.

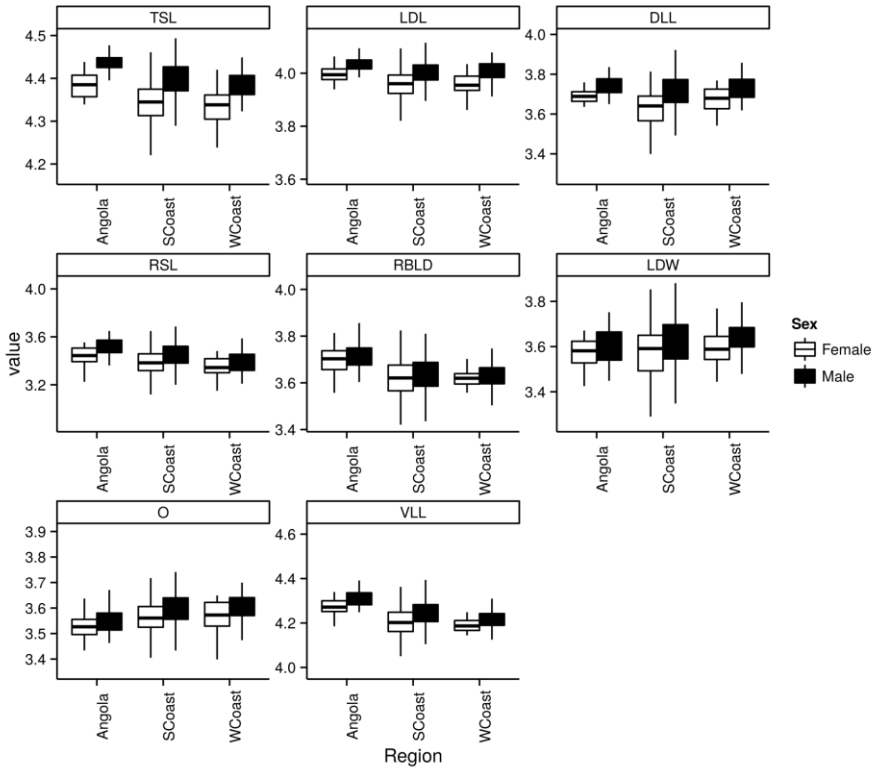

Figure 9. Box-Whisker plots of standardized values of attributes for the sucker rings of *Loligo reynaudii* males and females from the three regions.

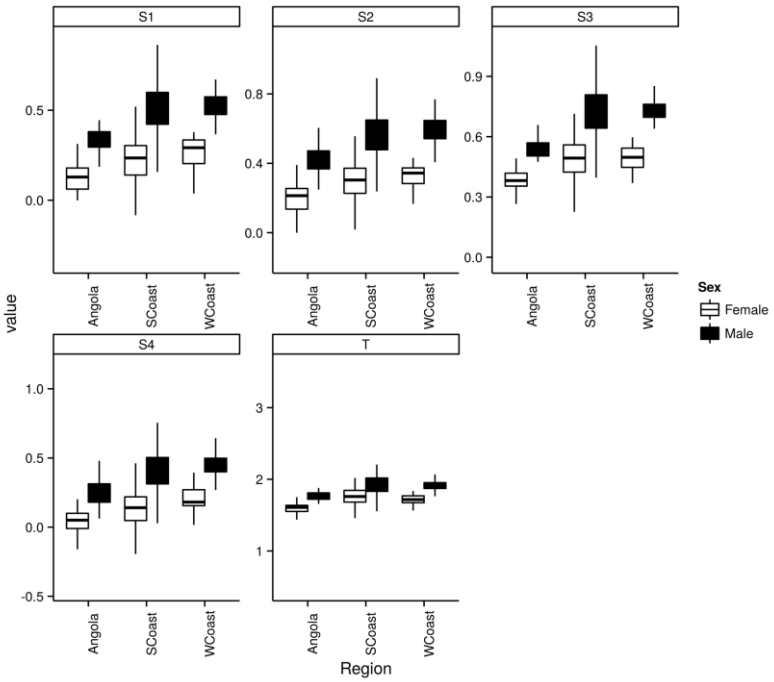

Figure 10. Top: PCA ordination of *Loligo reynaudii* male (left) and female (right) individuals based on soft body part attributes. Bottom: LDA ordination of the same data for males (left) and females (right).

Figure 11. Comparison of the predictive performance of the three models for *Loligo reynaudii* males, females, and combined individuals from the three region based on attributes of soft body parts. The performance of the models on both the training and validation sets are shown.

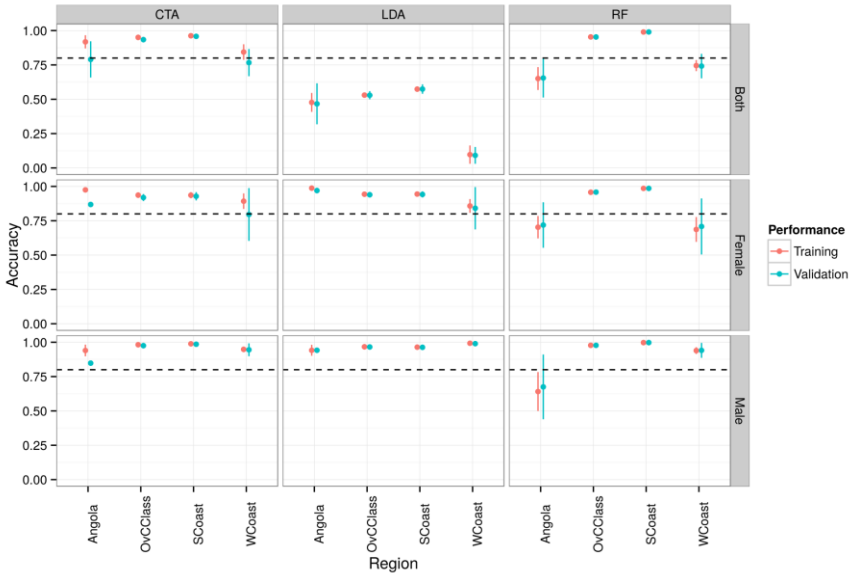

Figure 12. Comparison of the predictive performance of the three models for *Loligo reynaudii* males, females, and combined individuals from the three region based on attributes of beaks. The performance of the models on both the training and validation sets are shown.

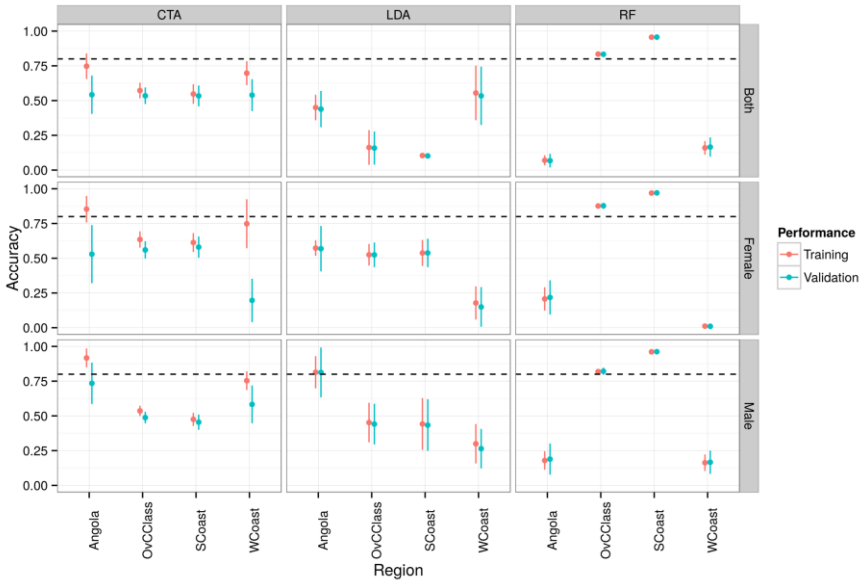

Figure 13. Comparison of the predictive performance of the three models for *Loligo reynaudii* males, females, and combined individuals from the three region based on attributes of statolith. The performance of the models on both the training and validation sets are shown.

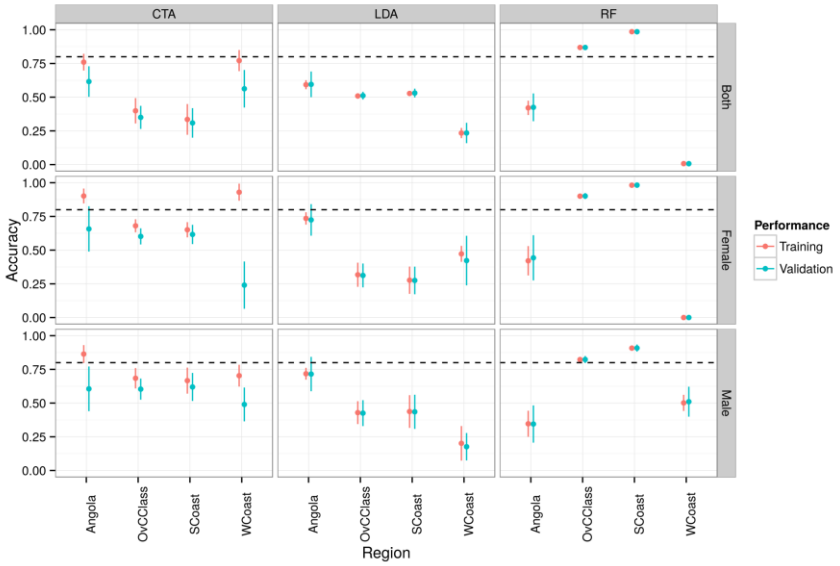

Figure 14. Comparison of the predictive performance of the three models for *Loligo reynaudii* males, females, and combined individuals from the three region based on attributes of sucker ring. The performance of the models on both the training and validation sets are shown.

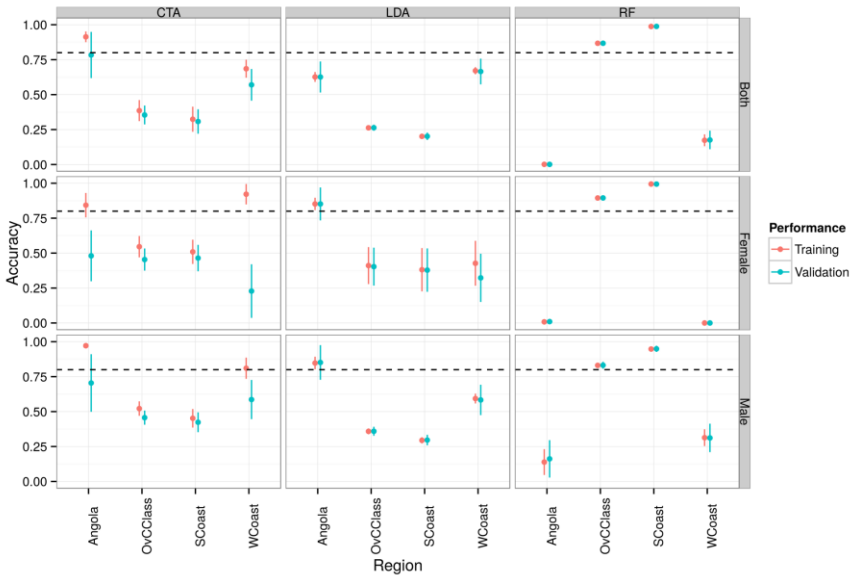

Figure 15. The multivariate distance among samples of all *Loligo reynaudii* variables between each of the three regions.

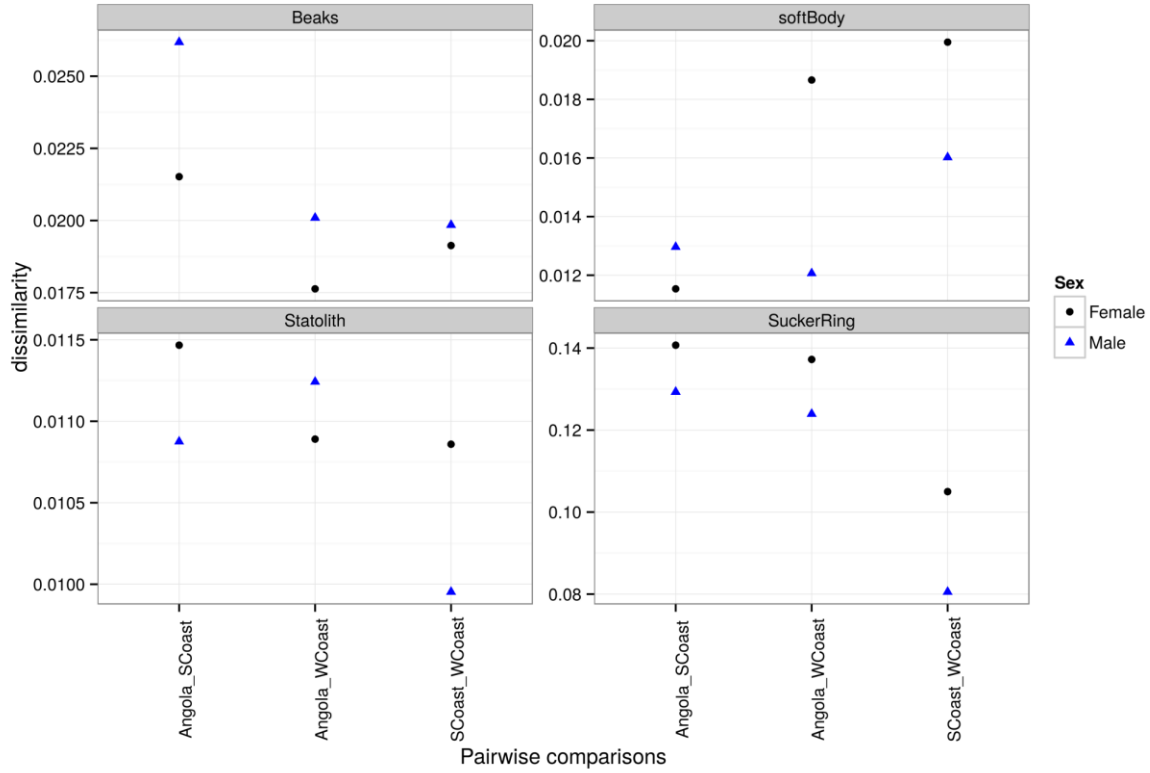

Table 1. *Loligo reynaudii* soft part and hard structure morphometric characters measured in this study.

Abbreviation	Character	Description
Soft parts		
Body		
AN	Fin angle	Angle of fin to body on ventral side
DML	Dorsal mantle length	Anterior to most posterior point of mantle, along midline (dorsal side)
VML	Ventral mantle length	Anterior to most posterior point of mantle, along midline (ventral side)
FL	Fin length	Total length of a fin including the anterior fin lobe
FWL	Fin width length	Between widest points of fin lobes
MW1	Mantle width 1	Width of mantle at the anterior end of mantle (mantle opening width)
MW2	Mantle width 2	Width of mantle at the base of fin lobes
MW3	Mantle width 3	Width of mantle at the widest points between fin lobes
AF	Funnel cartilage length	Along central line of funnel to opening of funnel tube (ventral side)
GRNI	Nidamental gland length	Nidamental gland length along central line of gland
Head		
HL	Head length	Anterior neck groove (dorsal side) to V-junction between 1st arm pair
HW	Head width	Taken between eyes
Arms		

A1	First arm length	1st arm of 1st pair (1st sucker at the base of arm to tip of arm)
A2	Second arm length	1st arm of 2nd pair (1st sucker at the base of arm to tip of arm)
A3	Third arm length	1st arm of 3rd pair (1st sucker at the base of arm to tip of arm)
A4	Fourth arm length	1st arm of 4th pair (1st sucker at the base of arm to tip of arm)
Tentacles		
TL	Left tentacle lenght	Base of the tentacle to the tip of club
TR	Right tentacle length	Base of the tentacle to the tip of club
HEC	Hectocotylus arm length	3rd arm (left side), 1st sucker (nearest to tip of arm) to arm tip
CL	Club length	1st carpal sucker to club tip
Hard structures		
Gladius		
GLA	Gladius length	Taken from anterior to posterior tip
GW1	Gladius width 1	Free rachis width
GW2	Gladius width 2	Rachis width at origin of gladuis wings
GW3	Gladius width 3	Width taken at widest point of gladius
GRL	Free gladius length	Taken from anterior tip of gladius to rachis
Sucker rings		
S1	Sucker diameter 1	Diameter of largest sucker on first arm (inside sucker measurement)
S2	Sucker diameter 2	Diameter of largest sucker on 2nd arm (inside sucker measurement)
S3	Sucker diameter 3	Diameter of largest sucker on 3rd arm (inside sucker measurement)
S4	Sucker diameter 4	Diameter of largest sucker on 4th arm (inside sucker measurement)
T	Tentacle sucker diameter	Diameter of largest left tentacle sucker (inside sucker measurement)
Lower beak		
g	Hood length	Measured along midline of the beak, in profile
f	Crest length	Measured along midline of the beak, in profile
a	Rostral length	Distance between rostral tip and front edge of wing
b	Wing length	Taken from front edge of wing to base of wing
d	Baseline length	Taken from base of wing to base of crest, in profile
c	Rostral height to base	Taken from rostral tip to base of beak platform, in profile
Statolith		
TSL	Total statolith length	Taken from apex of dorsal dome to tip of rostrum
LDL	Lateral + dorsal dome length	Taken from rostral angle to apex of dorsal dome
DLL	Dorso-lateral length	Taken from apex of dorsal dome to lateral tip of lateral dome
RSL	Rostral length	Taken from angle to tip of rostrum
RBLD	RB to LT of lateral dome	Taken from the base of rostrum to lateral tip of lateral dome
LDW	Lateral dome width	Taken from lateral tip of lateral dome to medial fissure
VLL	Ventro-lateral length	Taken from tip of rostrum to lateral tip of lateral dome

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Table 2. Results of the MANOVA applied to all morphometric measurements taken for *Loligo reynaudii* in this study.

	Df	Pillai	approx F	num Df	den Df	Pr(>F)
	Soft body parts					
(Intercept)	1	0.999993	6709289	18	882	0
Region	2	0.960129	45.29375	36	1766	2.40E-221
factor(Sex)	1	0.992621	6591.065	18	882	0
Region:factor(Sex)	2	0.381343	11.55711	36	1766	1.91E-58
Residuals	899	NA	NA	NA	NA	NA
	Beaks					
(Intercept)	1	0.999445	315821.9	6	1052	0
Region	2	0.231147	22.93373	12	2106	1.60E-48
factor(Sex)	1	0.674416	363.1854	6	1052	2.91E-252
Region:factor(Sex)	2	0.022919	2.034454	12	2106	0.018334
Residuals	1057	NA	NA	NA	NA	NA
	Statolith					
(Intercept)	1	0.999962	3429103	8	1056	0
Region	2	0.252959	19.13077	16	2114	1.37E-51
factor(Sex)	1	0.340773	68.23443	8	1056	2.80E-90
Region:factor(Sex)	2	0.009544	0.633514	16	2114	0.858923
Residuals	1063	NA	NA	NA	NA	NA
	Sucker rings					
(Intercept)	1	0.994619	37965.93	5	1027	0
Region	2	0.162106	18.13437	10	2056	3.88E-32
factor(Sex)	1	0.603219	312.2665	5	1027	2.96E-203
Region:factor(Sex)	2	0.009738	1.005982	10	2056	0.435699
Residuals	1031	NA	NA	NA	NA	NA

Table 3. Selection of *Loligo reynaudii* soft and hard part variables based on the stepwise-selection procedure.

	Model	OvAccrV	OvAccrT	Sex	ModelName
1	Region ~ FL	0.972736	0.974872	Male	LDA
2	Region ~ FL+HEC	0.983421	0.983385	Male	LDA
3	Region ~ FL	0.978422	0.98224	Male	CTA
4	Region ~ FL	0.956731	0.960835	Male	RF
5	Region ~ FL+HW	0.982582	0.978945	Male	RF
6	Region ~ FL	0.929807	0.937562	Female	LDA
7	Region ~ FL+GRNi	0.958632	0.961109	Female	LDA
8	Region ~ FL+GRNi+HW	0.977486	0.974765	Female	LDA
9	Region ~ FL	0.922649	0.943812	Female	CTA
10	Region ~ FL+GRNi	0.963789	0.96585	Female	CTA
11	Region ~ FL	0.915103	0.899236	Female	RF
12	Region ~ FL+GRNi	0.959006	0.959307	Female	RF
13	Region ~ FL+GRNi+MW2	0.970582	0.952156	Female	RF

14	Region ~ TL	0.875358	0.868852	Both	LDA
15	Region ~ FL	0.950635	0.962828	Both	CTA
16	Region ~ FL+CL	0.961895	0.965939	Both	CTA
17	Region ~ FL	0.927671	0.932133	Both	RF
18	Region ~ FL+HW	0.961052	0.952769	Both	RF
1	Region ~ a	0.826653	0.817667	Male	LDA
2	Region ~ d	0.821953	0.819211	Male	CTA
3	Region ~ f	0.726924	0.746993	Male	RF
4	Region ~ f+g	0.791064	0.785388	Male	RF
5	Region ~ f+g+a	0.834482	0.813699	Male	RF
6	Region ~ g	0.900177	0.882498	Female	LDA
7	Region ~ a	0.897086	0.899083	Female	CTA
8	Region ~ d	0.840307	0.812855	Female	RF
9	Region ~ d+b	0.884472	0.873216	Female	RF
10	Region ~ b	0.85368	0.8545	Both	LDA
11	Region ~ d	0.85906	0.85239	Both	CTA
12	Region ~ d	0.76721	0.769905	Both	RF
13	Region ~ d+g	0.846436	0.828848	Both	RF
1	Region ~ LDL	0.829299	0.809633	Male	LDA
2	Region ~ RBLD	0.827076	0.867412	Male	CTA
3	Region ~ RBLD	0.826484	0.815737	Male	RF
4	Region ~ RBLD	0.893317	0.886019	Female	LDA
5	Region ~ DLL	0.903557	0.906456	Female	CTA
6	Region ~ O	0.886845	0.875116	Female	RF
7	Region ~ O+LDL	0.910871	0.897092	Female	RF
8	Region ~ LDL	0.854268	0.850043	Both	LDA
9	Region ~ O	0.852171	0.857034	Both	CTA
10	Region ~ O	0.793629	0.795937	Both	RF
11	Region ~ O+LDL	0.847152	0.849723	Both	RF
12	Region ~ O+LDL+DLL	0.85993	0.863688	Both	RF
13	Region ~ O+LDL+DLL+RBLD	0.874048	0.866686	Both	RF
1	Region ~ S2	0.835826	0.822593	Male	LDA
2	Region ~ T	0.836729	0.825475	Male	CTA
3	Region ~ S3	0.754735	0.74514	Male	RF
4	Region ~ S3+S2	0.837839	0.825333	Male	RF
5	Region ~ S3	0.902349	0.885816	Female	LDA
6	Region ~ S2	0.912471	0.893976	Female	CTA
7	Region ~ S2	0.861399	0.83951	Female	RF
8	Region ~ S2+S4	0.898989	0.892141	Female	RF
9	Region ~ S2+S4+S1	0.908573	0.886782	Female	RF
10	Region ~ S1	0.872031	0.860549	Both	LDA
11	Region ~ S3	0.867532	0.863662	Both	CTA
12	Region ~ S2	0.804356	0.795946	Both	RF
13	Region ~ S2+S3	0.867833	0.858623	Both	RF
14	Region ~ S2+S3+S4	0.8767	0.863881	Both	RF

872 **Table 4.** Indices of genetic variation within *Loligo reynaudii* samples genotyped at four
873 microsatellite loci.
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Sample site	Sampling method	N	H_E	H_O	NA
West Coast	Trawl	48	0.9138	0.6714	16.25
Western Agulhas Bank	Trawl	43	0.9057	0.7362	16.25
Mossel Bay (offshore)	Trawl	32	0.9227	0.7962	16.75
Mossel Bay (inshore)	Trawl	48	0.9155	0.7533	17.5
Plettenberbay (offshore)	Trawl	41	0.9161	0.6619	17.5
St. Francis (inshore)	Trawl	49	0.9245	0.6921	18.25
Port Elizabeth (offshore)	Jig	48	0.9153	0.7061	15.25

N, sample size; HE, multilocus expected heterozygosities; HO, multilocus observed heterozygosities; NA, mean number of alleles per locus.

875
876 **Table 5.** Pairwise estimates of genetic differentiation (Θ_{ST}) between *Loligo reynaudii*
877 samples, without (above diagonal) and with (below diagonal) null allele correction. No Θ_{ST}
878 was significant when tested by permutation.
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Sampling site	WC	WAB	MB (offshore)	MB (inshore)	PB	SF	PE
WC		0.007	-0.004	-0.004	0.004	-0.003	0.007
WAB	0.003		0.001	0.001	0.007	0	0.001
MB (offshore)	0.005	0.003		-0.001	0.003	-0.004	0.004
MB (inshore)	0.005	0.002	-0.002		-0.001	-0.001	0.004
PB	0.003	0.002	-0.001	-0.003		0.001	0.002
SF	0.006	0.004	-0.004	-0.002	-0.003		0.005
PE	0.004	0.005	-0.005	-0.003	-0.002	-0.007	

WC, West Coast; WAB, Western Agulhas Bank; MB, Mossel Bay; PB, Plettenberg Bay; SF, St. Francis; PE, Port Elizabeth.

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Table 6. P values from exact tests of genetic differentiation between *Loligo reynaudii* samples, without (above diagonal) and with (below diagonal) null allele correction. Significant values in bold, and values that remain significant after Bonferroni correction (underlined).

Sampling site	WC	WAB	MB (offshore)	MB (inshore)	PB	SF	PE
WC	-	**0.006**	0.236	0.453	**0.003**	0.429	0.199
WAB	0.22	-	0.258	0.056	**<u>0.001</u>**	0.22	0.165
MB (offshore)	0.446	0.402	-	0.355	**0.021**	0.626	0.716
MB (inshore)	0.769	0.125	0.376	-	0.259	0.195	0.069
PB	**0.033**	**0.003**	0.056	0.396	-	**0.049**	0.082
SF	0.78	0.364	0.684	0.304	0.122	-	0.6
PE	0.54	0.436	0.792	0.093	0.176	0.732	-

WC, West Coast; WAB, Western Agulhas Bank; MB, Mossel Bay; PB, Plettenberg Bay; SF, St. Francis; PE, Port Elizabeth.

APPENDICES

Appendix A

Table A1. Descriptive statistics of male *Loligo reynaudii* character measurements from each of the three regions (Angola, south coast and west coast).

Table A2. Descriptive statistics of female *Loligo reynaudii* character measurements from each of the three regions (Angola, south coast and west coast).

Appendix B

Figure B1. Comparison of the removal of the effect of size using *Loligo reynaudii* DML or C for the beak measurement.

Figure B2. Comparison of the removal of the effect of size using *Loligo reynaudii* DML or TSL for the statolith measurement.

Figure B3. Comparing the performance of the three models after removing the effects of size using *Loligo reynaudii* DML or C for the beak measurement.

Figure B4. Comparing the performance of the three models after removing the effects of size using *Loligo reynaudii* DML or TSL for the statolith measurement.

Appendix C

Figure C1. Plot of the slopes and the 95% confidence interval of the regression of *Loligo reynaudii* beak attributes vs. DML.

Figure C2. Plot of the slopes and the 95% confidence interval of the regression of *Loligo reynaudii* statolith attributes vs. DML.

Figure C3. Plot of the slopes and the 95% confidence interval of the regression of *Loligo reynaudii* sucker ring attributes vs. DML.

Appendix D

Figure D1. Top: PCA ordination of *Loligo reynaudii* male (left) and female (right) individuals based on beak attributes. Bottom: LDA ordination of the same data for males (left) and females (right).

Figure D2. Top: PCA ordination of *Loligo reynaudii* male (left) and female (right) individuals based on statolith attributes. Bottom: LDA ordination of the same data for males (left) and females (right).

Figure D3. Top: PCA ordination of *Loligo reynaudii* male (left) and female (right) individuals based sucker ring attributes. Bottom: LDA ordination of the same data for males (left) and females (right).