Microsatellite and Morphometric analysis of chokka squid (*Loligo reynaudi*) from different spawning aggregations around the South African coast.

A thesis submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

at

RHODES UNIVERSITY

by

Terence Anthony Stonier

Abstract

Accurate information on stock structure is very important to the effective management of any commercially exploited species (Angel *et al*. 1994), particularly in annual species like *Loligo reynaudi*. Previous molecular work on a number of fish and cephalopod species has shown that stock structuring may be more complex than originally believed and while much scientific work has been conducted on *Loligo reynaudi* to date, molecular work has been lacking and this species' fishery is currently managed as a single stock. The primary aim of this project was to examine the population genetics of *Loligo reynaudi* on a molecular level, by looking at the levels of genetic variation between different spawning aggregations along the inshore distribution of the chokka squid, with particular attention being paid to any variation between Eastern Cape and Agulhas Bank groups. The secondary aim was to conduct a morphological analysis on samples from the same major areas in order to see if any genetic variation observed would be complemented by phenotypic variation.

Two separate sample sets were collected; Genetic samples were collected from 6 different spawning sites along the South African Coast between April and July, 2006 and whole individuals for morphometric analysis were collected from 4 spawning sites between April and July 2007. Samples were screened for genetic variation between different spawning aggregations along the inshore distribution of chokka squid, from Port Alfred in the Eastern Cape, to the western Agulhas Bank. After this a morphometric analysis on samples from the same major areas, Eastern Cape, Agulhas Bank and Angola, was carried out. Genetic results showed significant variation between some of the sample groups. As expected, the Angolan outgroup consistently showed significant variation

from other samples, while there was evidence of differentiation between the South African samples themselves. These results could have implications for the previously documented life cycle model of *Loligo reynaudi* and provide a basis for further study at a finer resolution into where exactly the boundaries of these different groupings can be found. This stock structuring has implications for the management of the species and warrants further genetic research with microsatellites proving to be a powerful tool in the explanation of stock structuring. Unfortunately, due to possible errors in taking measurements, morphometric analysis did not yield useful results which can be described and interpreted in this study. It is felt that further genetic study conducted on a finer scale, should be accompanied by a repeat of the morphometric analysis.

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Acknowledgements

I would like to thank the following:

- The National Research Foundation and SASMIA for providing the funding for this research. Additional thanks go to SASMIA for their logistical support and provision of samples.
- My supervisors: Professor Warwick Sauer, for all of your guidance and support and for just not giving up on me or this project. Without you, this project would not have been possible. Dr. Paul Shaw, for having me in your lab and all your help and support with the genetics section. Dr. Marek Lipinski, for your guidance and encouragement for the morphometrics section.
- My parents for your love and support and guidance. Mum for your endless encouragement and determination for me to conclude this research and all your help.
- My wife, for your love and enthusiastic support during the final stages of this thesis.

Declaration

The following thesis has not been submitted to a university other than Rhodes University, Grahamstown, South Africa. The work presented here is that of the author.

CHAPTER 1

INTRODUCTION AND OBJECTIVES

1.1 Introduction

In 2011 the total global production of marine capture fisheries was estimated at 78.9 million tonnes (F.A.O. 2012). Of this total, it is estimated that cephalopods accounted for around 3% of marine fishery production, with over 3.3 million tonnes being landed every year around the world (Mazurek 2006). Over the last 30 years, the global exploitation of cephalopods has greatly increased in terms of both total production and the number of different species being exploited. Between 1984 and 1995, total cephalopod production grew by 46% (Sauer *et al*. 2002) and today up to 100 different species are targeted worldwide (which is 14% of total number of species in the class). Of these, the migrating oceanic ommastrephid squids and the inshore, coastal loliginid squids form the basis of the major fisheries with 2 species *Todarodes pacificus* and *Illex argentinus* accounting for a large proportion of the total catch (Boyle and Boletzky 1996).

According to Boyle and Rodhouse (2005) this growth in cephalopod fisheries and particularly those for squid, can be attributed mainly to shifts in fishing effort due to decreasing catches in other fisheries, often described as fishing down the food chain, in addition to an increasing global demand for protein. Initial attention was focused by the apparent abundance of cephalopods worldwide, with the estimated amount of cephalopods annually consumed by predators (sperm whales, seals, seabirds etc.) being larger than the total catch by all global fisheries combined (Clarke 1983) and possibly even up to a hundred-fold higher (Boyle 2002).

Increasing cephalopod abundance has also been linked to the exploitation of other species and the decrease in cephalopod predator populations, which has allowed cephalopods, with their short life cycles and fast growth, to expand under reduced predation pressure and competition to fill other ecological niches (Caddy 1995, Boyle and Rodhouse 2005).

As cephalopod fisheries have grown, so has scientific interest in cephalopods (Clarke 1996) and the level of understanding of many species, their reproduction, life-cycles, growth patterns, distributions and feeding patterns has increased a great deal. While cephalopod systematics are regarded as lagging behind other areas of research (Caddy 1983, Roeleveld 1998, Vecchione *et al*. 2000), the current classification divides cephalopods into at least 9 orders, comprising 47 families, all marine and very widely distributed. Many cephalopod families contain only a single species (monotypic) and a few families; in fact *Sepiidae* (cuttlefish), *Octopodidae*, *Ommastrephidae* and *Loliginidae* contain a high proportion of the total number of species described to date. There seems to be a pattern of distribution relating to the species richness of different genera, with the species rich groups tending to be found in shallow coastal waters or deep sea benthic habitats (Boyle and Rodhouse 2005). Due to this distribution pattern, some of these genera are more susceptible to commercial exploitation. Cephalopods, particularly squid, have a number of important characteristics that make them both desirable as commercial targets, but also difficult to manage as a fishery (Lipinski et al., 1998).

1.2 Stock assessment and management of cephalopod fisheries

May (1984) defines a population as a large group of individuals of the same species maintaining itself by reproduction in a describable geographical area or habitat. This term is regarded by many as sharing the same meaning as a "stock"; however, in terms of marine fisheries, a stock may mean a portion of the overall population that is available for fishing at certain times, or in a certain area. Understanding the population dynamics of a fished resource is very important for its management and successful management requires knowledge of at least the basic biological properties of a population or stock, as well as the dynamics of the population with regard to the exploitation. As yet, the assessment and management of cephalopod fisheries worldwide is at an early stage of development (Boyle and Rodhouse 2005). This has been attributed to the relatively recent development of large scale commercial cephalopod fisheries as well as the controversy over whether or not cephalopods can be assessed and managed successfully as fish or whether a totally new approach is required (Pauly 1998). Most assessment and management practices used so far for cephalopods are the same as those used on marine finfish and no standard practices for cephalopods have been developed. According to Boyle and Rodhouse (2005), one of the most common assessment methods currently used is the Leslie-DeLury method, using the depletion of the stock over a fishing season, whereby catch per unit effort (CPUE) changes through the season, allowing the calculation of the size of the stock at the beginning of the season. As for the management of cephalopod stocks, methods tend to vary depending on species and geographic area. However, the limitation of effort through various means, such as restrictions on fishing time, vessel numbers, vessel sizes and the type of gear used, occurs in many fisheries.

As with all marine species, information is the key for any assessment or management to be successful (Pierce and Guerra 1994, Boyle and Rodhouse 2005) and these include biological parameters such as growth rate, lifespan, life cycle, reproduction and recruitment. Environmental factors and their influences on these biological parameters of a population also need to be known as they can have a large impact on a population. Understanding the effect of fishing on the stock is important and this is of particular concern in certain species where spawning individuals are targeted.

A number of different assessment methods have been used for cephalopods in the past and the data required in order to do this has been collected by a number of methods. Boyle and Rodhouse (2005) cite examples, such as direct fishing surveys (Pierce *et al.* 1998), acoustic surveys (Goss *et al*. 2001), egg and larvae surveys (Sauer and Smale 1993) and in some cases tag-recapture surveys, conducted on *Loligo reynaudi* by Lipinski (1998) and Sauer *et al*. (2000). Biomass is then calculated using an established stock assessment model and Pierce and Guerra (1994) cite a couple of different modelling methods that have been used on cephalopods. These include the surplus production model, cohort analysis, empirical modelling; whereby abundance and biomass are linked to environmental conditions, as well as an ecological approach; where abundance is estimated through studying the diets of cephalopod predators, as mentioned previously. Cephalopods, in particular squid, have generally high growth rates and short life cycles, usually annual, where each generation of squid depends entirely on the generation before it.

These characteristics create a large degree of natural variation within populations from year to year and this can make stock assessment by established methods difficult (Boyle and Rodhouse 2005).

The overall aim of stock assessment is to provide information on a resource, to allow the effective management of that resource in order to attain the maximum possible yield, while preventing its overexploitation and decline. These assessment and management practices mainly focus on the estimation and protection of the biomass of a fished population; however another potentially very important factor in a commercially fished resource is that of stock structuring within the population. Stocks are often defined for the purpose of exploiting a resource and for the convenience of its management, rather than on the actual characteristics of the resource (Boyle and Boletzky 1996). Defining separate stocks within a population on a biological basis can be difficult, but is important for effective management of those stocks (Carvalho and Hauser 1994). Being predominantly annual, with no or limited overlap between generations, the formation of sub-populations in cephalopod stocks can theoretically occur relatively quickly and easily and what may appear as a single large population may comprise a number of small, differentiated sub-populations. Knowledge of stock structuring is not only important for management, but also for e.g. taxonomy (Angel *et al.* 1994). Stock structuring within cephalopod populations has been investigated by a number of methods in a number of studies. Caddy (1991) used demographic data in stock definition, looking at size frequency and age structure of a sampled population.

Distributional patterns have also been used as indicators of possible stock structuring, though some species have been shown to be capable of large migrations. Morphological studies have been used for a number of population and taxonomic studies. Lipinski (1981) used a morphometric approach with all *Illex* species taking detailed measurements of morphological features and analysing these using multivariate statistics. This method is, however, known to be quite subjective on soft bodied cephalopods (Pierce *et al*. 1994) and can fail to show any subtle differences in population structuring. Cephalopod hard structures, namely beaks and statoliths have also been used for taxonomic, ecological and biological purposes and are regarded as being useful and reliable (Pierce and Guerra 1994). Statoliths have primarily been used to examine the ageing and growth rates of cephalopods (Lipinski and Durholtz 1996, Thomas and Moltschaniwskyj 1998, Durholtz *et al*. 2001). Beaks have been used in identifying different prey species in the stomachs of cephalopod predators such as large fish, sperm whales, seals and sea birds. More recently, ecological studies, some using stable isotope analysis, have also used beaks and statoliths (Santos et al. 2001, Peristeraki *et al*. 2005, Hobson and Cherel 2006).

The increase in scientific work on cephalopods has resulted in a growing body of work being done on stock structuring at the molecular level. The potential of molecular markers has been recognized for some time now and as they are not defined or influenced by the environment, but are inherent in an individual, they can be accurate and powerful tools in defining population structure (Goldstein and Schlotterer 1999).

The techniques for looking at stock structuring within cephalopods have evolved through a number of stages since molecular work first began, as more has become known about cephalopod genetic structure and protocols have been modified (O'Connell and Wright 1997). Early molecular studies used blood group polymorphism to differentiate between different groups of individuals (Ward and Grewe 1994). However, this method proved to be difficult to interpret. This then led to the use of allozyme analysis. Allozymes are enzymes from different alleles of the same gene, which differ in amino acid structure but perform the same function (Goldstein and Schlotterer 1999). Through specific staining techniques and starch gel electrophoresis, variation in allozymes can be identified (O'Connell and Wright 1997). This method has proved to be useful in defining broader stock structuring. A number of different allozyme studies have been done on cephalopods (Garthwaite *et al*. 1989, Adams *et al*. 2004, Brierley *et al*. 1995, Carvalho *et al*. 1992, Perez-Losada *et al*. 2002, Triantafillos and Adams 2001, Triantafillos *et al.* 2004) as well as on other marine species. According to O'Connell and Wright (1997), the study of the DNA sequence itself in cephalopods led to the development of microsatellites for use in population genetics studies. These have seen increasing use during the last decade, and in some cases have shown levels of population differentiation that have not been shown by other methods (Shaw *et al.* 1999). Microsatellites are currently regarded as one of the most powerful tools in investigating stock structuring on a molecular level (Bentzen *et al*. 1996, O'Connell and Wright 1997, Goldstein and Schlotterer 1999).

1.3 Microsatellites

Microsatellites, otherwise known as simple sequence repeats (SSR's), consist of short segments of DNA that have a repeated sequence of nucleotides, usually between 1 and 6 base pairs long (Hancock 1999).

These simple sequence repeats are found throughout the DNA, often in neutral, non-coding regions and as such are subject to a relatively high rate of mutation (Armour *et al*. 1999). These mutations, when they occur, lead to changes in the size of the segments over time, with the microsatellite regions changing in length through the insertion or deletion of single bases (Vogler *et al*. 1997). This process has been attributed to slippage, whereby the DNA strand dissociates from the template strand during transcription and while recombining, misaligns itself, either missing a base and deleting it from the resulting DNA, or copying a base again, thus adding one to the resulting DNA strand. This process is believed to be the most common form of mutation in microsatellites (Hancock 1999, O'Connell and Wright 1997). Through meiosis, these resulting microsatellites are passed on through to any offspring an individual may have and over time a number of different microsatellites will be developed and maintained in a population that are unique to that particular group. This makes them useful in population studies, as the level of genetic diversity or similarity between groups of individuals can be can be ascertained by looking at the frequency of shared alleles between the groups, compared to the number of alleles in each group and the population as a whole.

Microsatellites can be isolated relatively easily, (O'Connell and Wright 1997) and a common technique uses primers that are complementary to the sequences on either side of the repeat unit and a polymerase chain reaction (PCR) that amplifies the repeat unit.

The microsatellites can then be sequenced by screening the PCR products in an automated fluorescent sequencer, the primers being radioactively labelled to fluoresce. The repeat units are scored by size in base pairs and thus different alleles are identified. Microsatellites show a high level of variability and the potential for a large number of loci and can be processed quite quickly, making them very useful tools for population genetics and paternity studies, as well as genome mapping (Goldstein and Schlotterer 1999). Microsatellites have been used on a number of fish species from brown trout (Estoup *et al.* 1998) to Atlantic cod (Bentzen *et al.* 1996) and have been increasingly used on cephalopods. Studies include *loliginids*; *L. forbesi* (Shaw *et al*. 1998), *L. opalescens* (Reichow and Smith 2001), *L. gahi* (Shaw *et al.* 2004) and *L. pealei* (Buresch *et al.* 2006) as well as some cuttlefish species; recently *Sepia officinalis* (Perez-Losada *et al*. 2002). These studies have had various results, with some showing stock structuring (Buresch *et al.* 2006, Perez-Losada *et al*. 2002, Shaw *et al*. 1998) and others which do not (Garoia *et al*. 2004, Shaw *et al.* 2004) and they have shown that in some cases what may be regarded as a single stock can in fact be made up of a number of genetically distinct stocks. For *Loligo reynaudi*, microsatellites have previously been used to investigate the link between mating behaviour and paternity (Shaw and Sauer 2004). However, no molecular study on stock structuring has been conducted on this economically important species.

1.4 *Loligo reynaudi*

Loligo reynaudi (D'Orbigny 1845) is an annual, semalparous species, and is found in the shelf waters around the South African Coast, ranging to the Orange river on the west

coast and as far north as East London on the east coast.

Loligo reynaudi, previously known as *Loligo vulgaris reynaudii*, was established as a subspecies of the European *Loligo vulgaris* by Augustyn and Grant (1988) and is believed to be separated from the northern *L. vulgaris* by a gap in distribution along the Namibian coastline (Augustyn *et al*. 1992).

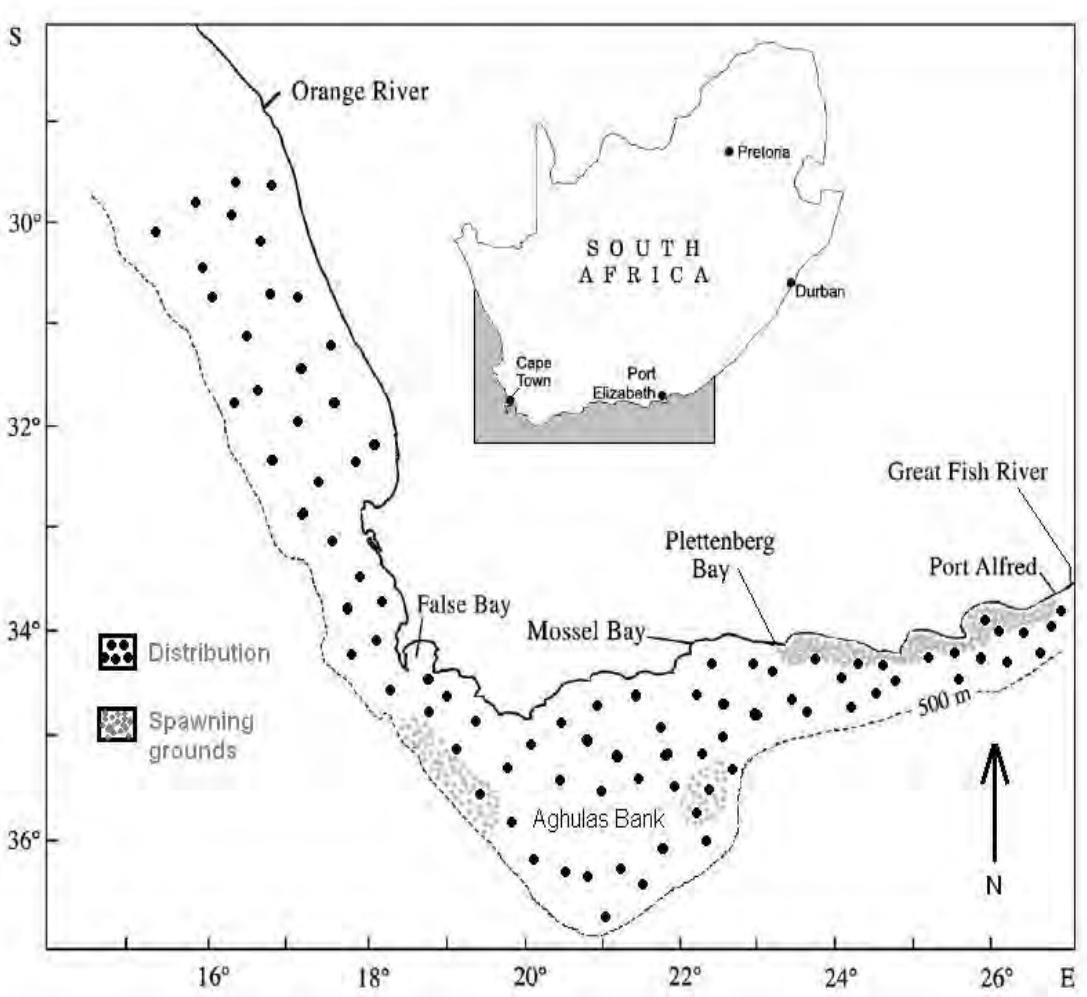


Fig. 1 Distribution and spawning areas of *Loligo reynaudi* around the shelf waters of South Africa (after Roberts 2005).

Extensive scientific research has been conducted on *Loligo reynaudi* (Table A1.1), and the currently accepted life cycle and migration patterns are reviewed up by Olyott *et al.* (2007).

Under the current model, squid spawn and hatch in the inshore shelf waters between Plettenberg Bay and Port Alfred, where Sauer *et al*. (1992) identified more than 39 different spawning locations. The squid form large, dense spawning aggregations at these various geographic locations along the coast at depths of less than 50m (Sauer *et al*. 1992), forming an egg bed on suitable substrate. Male and female individuals pair up in the water column above the egg bed, where spermatophore transfer takes place and the female then descends to the egg bed to attach the egg strand to the substrate (Augustyn 1989, Sauer *et al.* 1992). The selection of spawning sites is currently assumed to be strongly influenced by environmental parameters, with water clarity and substrate type playing important roles (Roberts 2005) because turbid water may prevent or hinder communication between spawning individuals.

After hatching, it is believed that the squid migrate offshore and westwards on to the Agulhas Bank to feed and mature, evidence for which is provided by the distribution patterns of different age groups of squid (Olyott *et al.* 2007). This process of paralarval movement was once believed to be passive, due to westward flowing currents, however newer evidence suggests that this may not be the case in the Eastern Cape (Roberts and Van den Berg 2002). Some paralarvae may even drift to the West Coast of South Africa. Once mature, adult squid then return to the spawning grounds (Augustyn 1989; Sauer et al. 1992; Augustyn et al. 1994) with spawning groups appearing in consistent locations from year to year.

These locations fall predominantly between Port Alfred and Plettenberg Bay, although the individuals using the spawning sites are different, being an annual species with a lifespan of only up to 14 months (Lipinski and Durholtz, 1994).

Spawning occurs throughout the year though commercial catches indicate that there may be a distinct spawning pulse in spring and early summer, when the largest aggregations occur (Augustyn 1990).

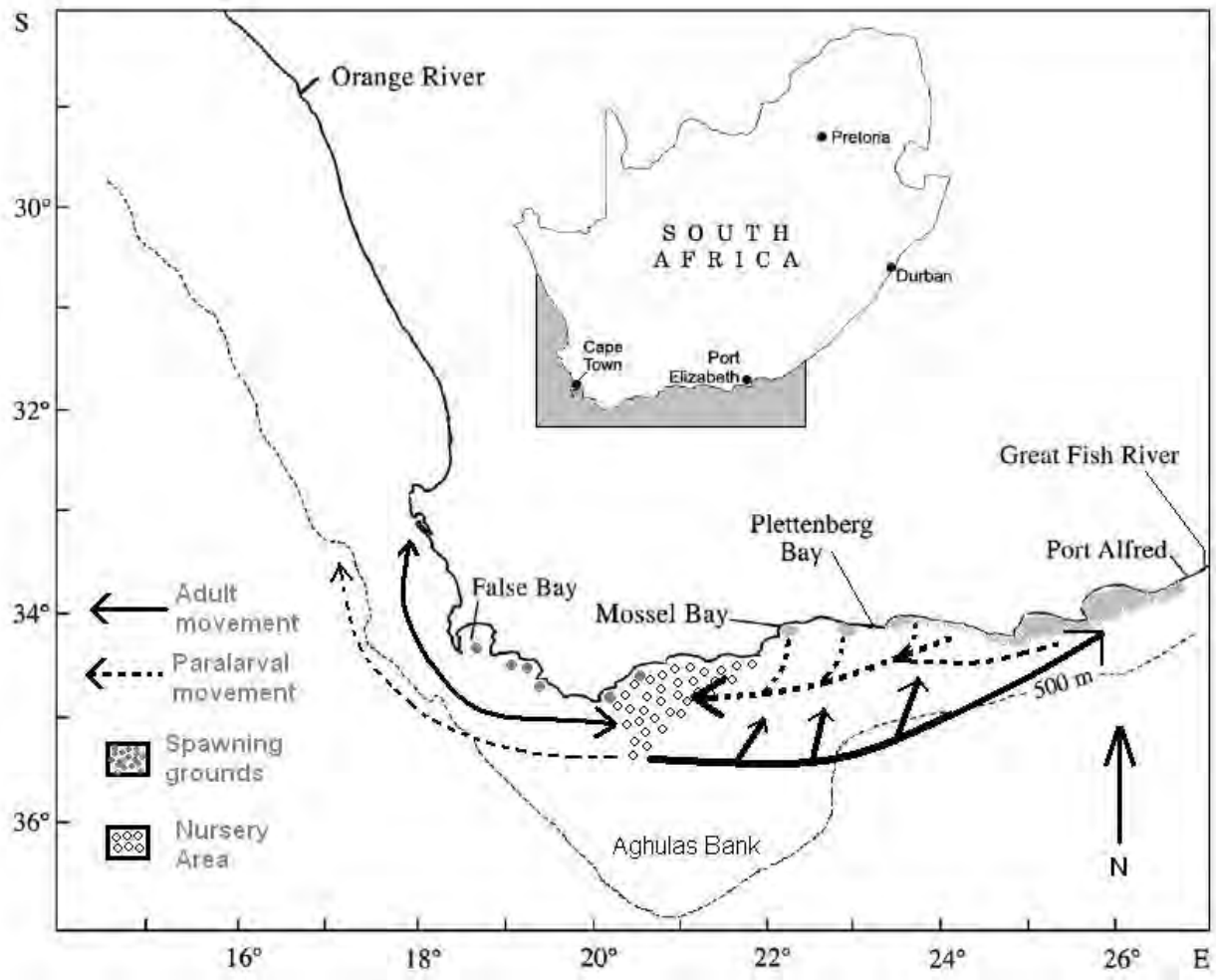


Fig. 2. Simple life cycle model. Showing paralarval and adult movement with relation to spawning sites and nursery area, limited spawning occurring on the Agulhas Bank and paralarval drift to the west coast (after Olyott *et al.* 2007)

Assuming that environmental conditions influence spawning locations, *L. reynaudi* at first appears to consist of a single population, moving freely throughout its range, with individuals from different areas of its distribution freely interbreeding, this simple model being well illustrated by Olyott *et al.* (2007). There is, however, evidence that squid regularly use deep water spawning sites, both along the Eastern Cape and Agulhas Bank as well as inshore on the Agulhas bank itself (Roberts *et al.* 2002, Roberts 2005). This means that squid spawning is occurring over an area that is much larger than that which is currently utilised by the fishery, and, according to Olyott *et al*. (2007) these different spawning groups could possibly result in the formation of sub-populations of squid, where juveniles and paralarvae grow up under differing environmental conditions and thus develop different biological characteristics.

The commercial chokka fishery as it exists today was formed around 1985 and chokka squid quickly became the most important harvested cephalopod in South Africa (Augustyn 1990). Up until 1985, *Loligo reynaudi* had been caught for export on a small scale as bycatch mainly by foreign trawlers targeting other species and by ski-boat fishermen for bait. However, in the early 1980's, foreign trawlers were gradually excluded from South African waters and a specific squid industry formed. The current fishery is a jig fishery, where squid are caught with coloured jigs on a handline (typically, 2 jigs per line) from inshore and, to a lesser degree, offshore spawning aggregations, where the squid are found in sufficient concentrations for harvesting. The current jig fishery is based mainly in the Eastern Cape, between Plettenberg Bay and Port Alfred, where squid are caught at depths of between 6 and 50 metres to the bottom (Augustyn 1989, 1990).

Squid are also caught in the Western Cape, between False Bay and Cape Agulhas, although this forms only a small part of the fishery as a whole. Total catch fluctuates widely from year to year (Sauer *et al*. 1991), which is a typical feature of a short-lived cephalopod, as well as from month to month, this having been correlated to changes in environmental conditions which affect distribution, spawning activity and recruitment (Roberts, 2005). Augustyn (1990) recognized that catches generally peaked between April and June and between September and December, although smaller catches continued throughout the year; a trend which holds true today. The chokka fishery comprises a medium sized industry, comprising about 145 boats and employing more than 2500 people. Total catch per year fluctuates around 8000 metric tonnes.

Cephalopods as a group are considered to be very difficult to manage effectively due to the highly fluctuating biomass levels and catches from year to year. Therefore management in the form of total allowable catch, in the case of *Loligo reynaudi*, has not been recommended. With biomass and recruitment from year to year being very hard to predict, the fishery is thus managed on the basis of effort limitation (Lipinski *et al.* 1998) coupled with a closed season strategy. In 1988, limits were imposed on the number of boat permits issued for the fishery and a closed season over October-November was implemented, with the aim of protecting spawning aggregations at the peak spawning time. A total allowable effort (TAE) was also set for the industry at 2500 men. These strategies, coupled with the phasing out of inefficient vessels by setting a minimum allowed catch, have meant that the fishing fleet has shrunk from 240 boats in 1998, to the current level of about 145, with a shift towards larger boats carrying more fishers.

Since its inception in 1985, the industry has also become increasingly modern, currently using decked vessels with on board blast freezers, generators and powerful lights, capable of staying out at sea for 21 days at a time. This management on the basis of total allowable effort, versus total allowable catch, allows the fishery to benefit from the good years and survive the poor years (Augustyn and Roel 1998). The fishery is also managed on the assumption that it is a single stock, with all commercial fishing throughout the range of *Loligo reynaudi* falling under the same management plan. This single stock approach assumes that squid do not return to their natal site to spawn, but rather select spawning sites along the coast based on environmental suitability; thus individuals freely interbreed, forming a large, single stock.

Of the research on *Loligo reynaudi* to date, work on a molecular level has been limited and is mostly confined to biochemical systematics (Augustyn and Grant 1988), paternity studies and more recently a study of mating patterns and paternity on a genetic level utilising microsatellites (Shaw and Sauer 2004). As recent work on other cephalopod species and specifically squid, has shown, population structuring on a genetic level may be more complex than it first appears and it is important to investigate this for *Loligo reynaudi*.

1.5 Objectives

Accurate information on stock structuring is very important to the effective management of any commercially exploited species (Angel *et al*. 1994). Specifically, knowledge of any structuring on a genetic level (Allendorf *et al*. 1987) is important for successful

management, particularly in annual species where each generation depends entirely on the one before it. The over exploitation of potentially localised groups can cause large changes in the genetic structure and diversity of a population, which in turn can greatly affect reproductive success, affecting biomass and, in turn, the fishery. These changes in the genetic structure of a population may be irreversible in the long term and would negatively affect any fishery associated with it

While much scientific research has been conducted on *Loligo reynaudi*, no studies have yet focused on any potential genetic diversity and stock differentiation in South African waters.

As other international studies have shown, geographically distinct spawning sites may possibly result in the formation of a number of genetically distinct groups within a population. In the case of *Loligo reynaudi*, a tagging study (Sauer *et al.* 2000) has shown that chokka squid do move between spawning aggregations within the Eastern Cape. In order to aid the successful management of the species, examining dynamics of the *L. reynaudi* population is crucial, as any stock structuring would necessitate a change in the current management strategy. Microsatellite markers have shown to be useful tools in examining population dynamics in a number of commercially exploited species (O'Connell and Wright 1997, Golstein and Schlotterer 1999, Garoia *et al.* 2004,); therefore the aim of this project is to examine the population genetics of *Loligo reynaudi* on a molecular level, using microsatellite markers previously developed for this species. The aim of this project was to investigate two different aspects of the *L. reynaudi* stock.

This project will firstly to look at the levels of genetic variation between different spawning aggregations along the inshore distribution of the chokka squid, from as far north as Port Alfred in the Eastern Cape, to the western Agulhas Bank, with particular attention being paid to any variation between the Eastern Cape and Agulhas Bank groups. Once these relationships were better understood in molecular terms, a morphological analysis on samples from the same major areas, Inshore Eastern Cape, Offshore Eastern Cape, Agulhas Bank and Angola, was carried out in order to complement any levels of genetic variation observed with phenotypic variation.

CHAPTER 2

METHODS AND MATERIALS

2.1 Sampling

Two separate sample sets were collected over the course of the project. Genetic samples were collected from 6 sites between April and July, 2006 and morphological samples were collected from 4 sites between April and July 2007. Sample sites from which both genetic and morphological samples were collected are shown in Figure 3.

2.1.1 Genetic samples

Samples were collected from 5 sites along the South African coast, as shown in Figure 3. A 6th sample set from Angola and northern Namibia, previously collected by Dr. P. Shaw in 2005 was also included in the study as an outgroup for comparison to the South African sample set. Sample sizes per site are indicated in Figure 3. The four inshore Eastern Cape samples were taken off commercial squid vessels, each set being collected from a single spawning aggregation, while the western Agulhas bank and Angolan samples were taken by demersal trawl by the R.V. Nansen in January 2006. Sexually mature adults of both sexes were sampled with maturity assessed according to Lipinski's (1979) maturity scale and a 2cm tentacle clipping taken from each individual and stored in 80% ethanol until use. A total of 456 individuals were sample; 80 from Angola, 54 from the Agulhas bank, 100 from Tsitsikamma, 72 from Kromme, 50 from Algoa Bay and 100 from Kleinemonde. Sample sizes were limited by the specific need for sexually mature individuals.

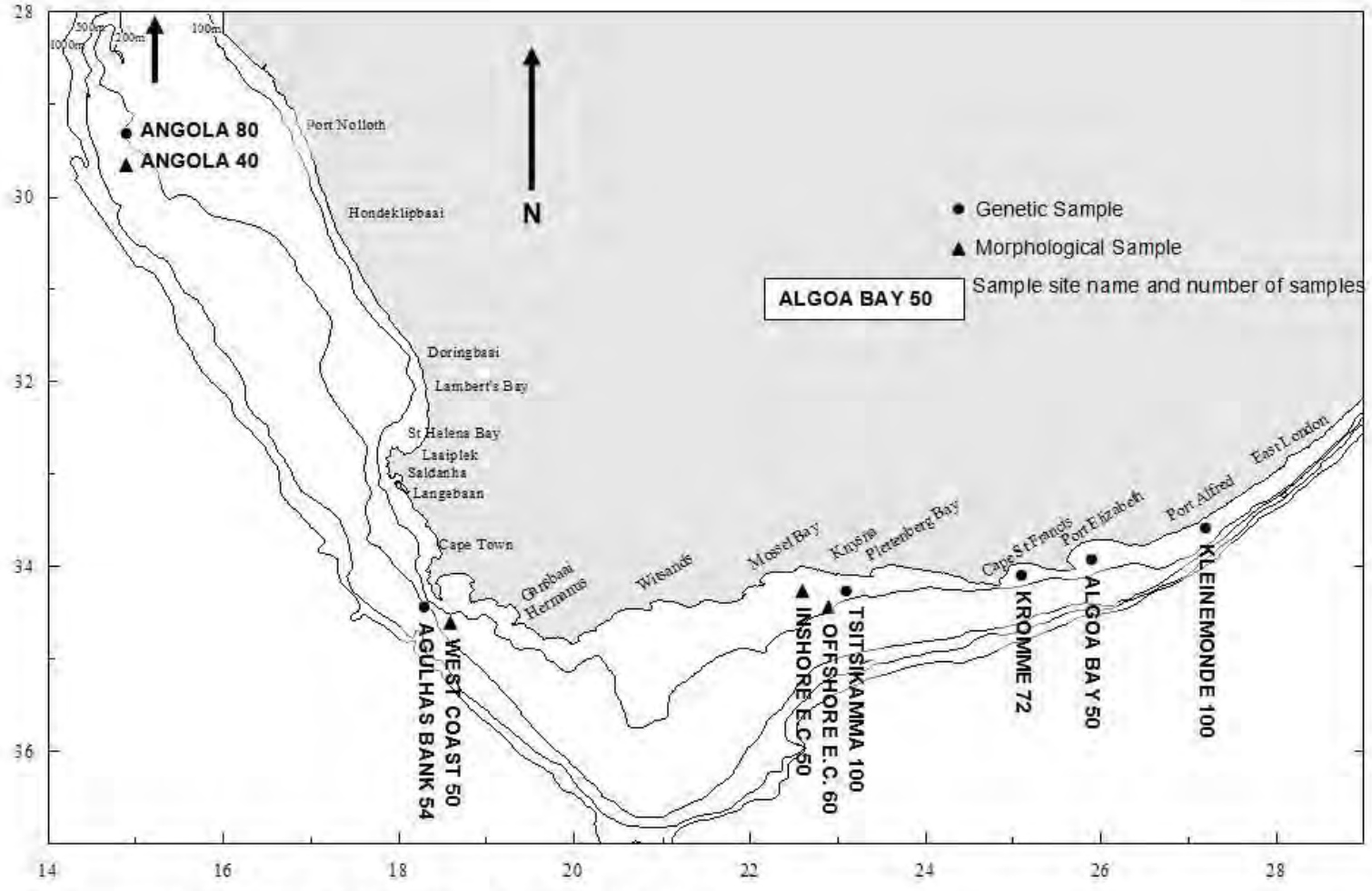


Fig .3 Sample locations for genetic and morphological samples 2006 - 2007

2.1.2 Morphological samples

Whole individuals were collected from spawning aggregations for morphological analysis from 3 sites along the South African Coast (Fig. 3.), an inshore Eastern Cape spawning aggregation, an offshore Eastern Cape aggregation, a west coast/Agulhas Bank site and a 4th sample set from Angola. The aim was to collect 60 mature individuals from each site, consisting of 30 male and 30 female individuals. The individuals were blast-frozen on collection, packed loosely, and kept deep frozen until use.

2.2 Microsatellite analysis

2.2.1 Laboratory work

Initial laboratory work was conducted at the Royal Holloway College of the University of London between July and September 2006. DNA was extracted from all genetic samples using a modified CTAB extraction protocol developed by Shaw and Boyle (1997). Initially, 48 samples from each sample site were extracted, and PCR reactions then set up. Microsatellite markers had previously been developed for *Loligo reynaudi* by Naud and Shaw (2008), and the similar *Loligo forbesi* by Shaw (1997) and from these, 5 loci were initially selected for use, LF1, LF3, Lr21, Lr27 and Lr34. Further laboratory work conducted in 2007 used an additional 3 loci, Lr 43, Lr44, and Lr48, also previously developed by Naud and Shaw (2008). All of these Loci are shown below in Table 4.

For all of the PCR reactions, a 10µl reaction mixture was used consisting 5µl template DNA, 1µl 10x buffer, 1µl of 1/20 dNTP, 0.2µl 1MM MgCl, 0.4µl of each primer. 0.05µl *Taq* polymerase (supplied by Bioline UK), with ddH_2O making up the remainder of the 10µl.

Reactions were carried out on a Hybaid Omnigene thermal cycler, using cycling conditions modified from Shaw and Sauer (2004), with a specific annealing temperature of 55ºC. All PCR products were checked on a 1% Agarose Gel, stained with ethidium bromide, and viewed under ultraviolet light. Successfully amplified products were then run on a 6% polyacrylamide gel, using an ALFexpressII automated sequencer (Pharmacia Biotech). Fragment Manager 1.2 (Pharmacia Biotech) was then used to compare PCR product to standard size markers developed in the lab and specific product sizes were determined and scored.

Table 1: Microsatellite loci developed for *Loligo forbesi* and *Loligo reyaudi.* T_a is optimal annealing temperature for each primer pair, n is number of alleles observed, H_E and H_O are levels of expected and observed heterozygosity (after Naud and Shaw, 2008 and Shaw, 1997).

Locus	**Repeat motif**	**Primer sequences**	**T_a (^oC)**	**n**	**Size of product (base pairs)**	**No. of alleles**	**H_E**	**H_O**
Lfor1	$(AAG)_{13}$	F: TTCAACCCAACAACAAAGAG R: CCCTGCTAATTCACAAAAAG	56	25	145	15	0.64	-
Lfor3	$(AAT)_{22}$	F: GTCATGTCATTAACAGAGTAGCA R: ACATTTATCCATTAACAGTAGCA	56	18	139	15	0.89	-
Lrey21	$(AAT)_{10}(GAA)_{11}$	F: CCTCAGATTCCAGTGAAAGAC R: GGGATCTCCATCAGATTATTAA	55	38	144-186	14	0.86	0.50
Lrey27	$(ATT)_{19}$	F: GCAGTGGCGATAGTTCTTTG R: CACTGATGTGAAAAACTTACG	55	38	125-185	17	0.92	0.58
Lrey34	$(TTG)_5$ $(TTA)_{14}$	F: ATCCTTGAAAGAGAATTAGG R: ACTAGGTCTGGTCTTCTGTC	50	47	112-169	28	0.94	0.85
Lrey43	$(GATA)_{15}$	F: GACCTGGGAGTAATGACGAC R: AACAGTGCTTATCTGGCTGC	55	48	190-234	12	0.78	0.81
Lrey44	$(GTT)_5(TAT)_{21}$	F: TCCCCGTATCTATAAGCATTAC R: TTTGAAACGAATTTGCCATATC	50	47	145-205	19	0.90	0.92
Lrey48	$(CTT)_{10}(ATT)_{13}$ $(ATT)_{13}$	F: CCATCAAAATAGTTTCCTTCA R: GCTAGCATAGACGAGAAGAC	55	45	167-266	30	0.97	0.89

2.2.2 Statistical analysis

Data from screened samples was analysed using two programs, GENEPOP (v. 4.0) and FSTAT. The screened loci were first examined for Hardy Weinberg equilibrium to check for technical errors within the data, and then the data was tested for any genotypic linkage between the loci. Deviations from Hardy Weinberg equilibrium were tested using a chi-squared exact test, with the level of significance being determined by Markov chain parameters (Raymond and Rousset 1995). The six sample sets were then analysed for genetic diversity, represented as Dst (Nei's estimates, Nei 1978), and finally a frequency based analysis was performed using FSTAT. In this, and overall and pairwise genetic differentiation between samples and between loci were tested using F_{ST} which shows genetic variation distributed between samples. F_{ST} was selected for use in this project over R_{ST} as results tends to show less variability at low numbers of loci (Shaw *et al.* 2004). A further temporal comparison was run using FSTAT, using 50 individuals from the original Kromme group and 50 individuals collected the next year in Kromme. Bonferroni corrections were also applied and further tests for isolation by distance were also performed using GENEPOP and FSTAT software.

2.3 Morphometrics

2.3.1 Morphological analysis

Individual samples needed to be defrosted before measurements were taken. In order to prevent any unnecessary warping of morphological characteristics, which can happen with repeated freezing and thawing (Lipinski, personal communication), individuals were only defrosted once before measuring. A single set of vernier callipers were used on all

samples, and all measurements were conducted by the author to avoid unnecessary variation. The variables used were adapted from Lipinski (1979). Sucker diameter (ST) was measured using a stereo microscope with an eyepiece micrometer.

Table 2: Morphometric measurements and their definition.

Abbreviation	Character	Definition
DML	Dorsal mantle length	Measured from most anterior to most posterior point of mantle on the dorsal side
VML	Ventral mantle length	Same along the midline on the ventral side
HL	Head length	Taken from anterior neck groove(dorsal side) to V-junction between first pair of arms
HW	Head width	Taken between the eyes
FL1	Fin length 1	Total length including anterior fin lobe
FL2	Fin length 2	same excluding fin lobe
FW	Fin width	At widest point of both fins
MW1	Mantle width 1	Width of the anterior end of mantle
MW2	Mantle width 2	Width of mantle at the base of fin lobes
MW3	Mantle width 3	Width at the widest point of fins
CL	Club length	Taken from 1st carpal sucker to tip
AF	Funnel length	Ventral side, taken along central line of funnel
A1	Length of arm 1	From 1st sucker at the base to tip of the arm
A2	Length of arm 2	Same
A3	Length of arm 3	Same
A4	Length of arm 4	Same
TL	Length of left tentacle	Taken from base to club tip
GLA	Gladius length	Anterior to posterior tip
ST	Diameter of largest sucker on club	Measured inside of a sucker

2.3.2 Beak analysis

After morphological measurements were taken, beaks were carefully extracted from samples following the method described by Clarke (1986). Beaks were fixed in 10% formalin for 48 hours, and then transferred to 20% alcohol for storage. Weak concentrations were specifically used to prevent warping of the beak structure. Measurements were taken from upper and lower beaks in profile using a stereo microscope with eyepiece micrometer. Figure 4. shows upper and lower beak structure of a typical cephalopod showing the measurements that were taken for this project.

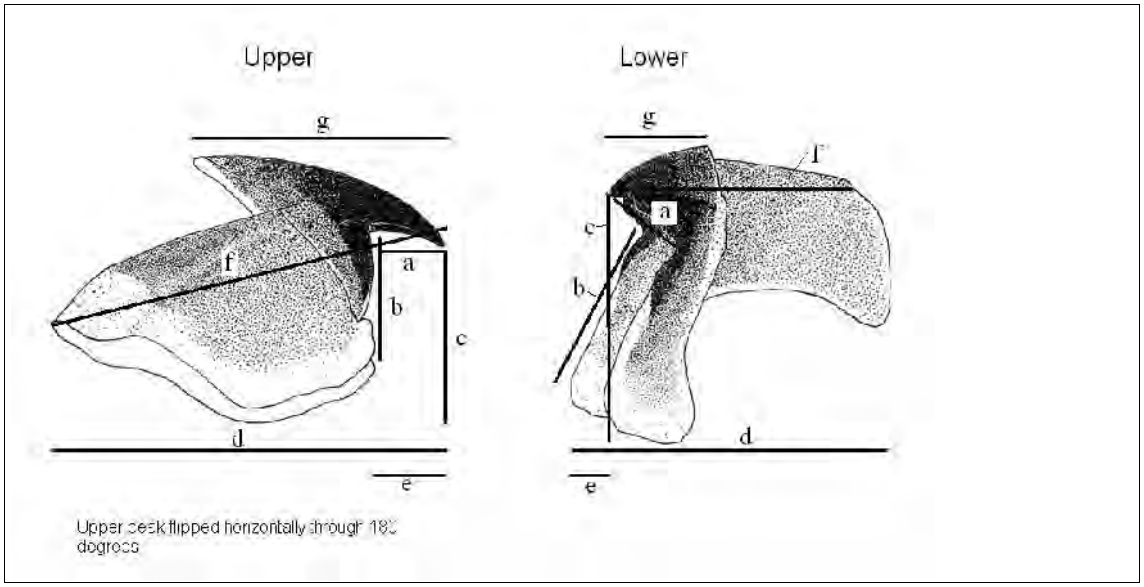


Fig. 4: Upper and lower beak dimensions taken for morphological analysis (after Clarke 1986).

In order to help with sample comparison, five ratios adapted from Clarke's (1986) handbook were also calculated. These were; edge to wing, height to base, rostral protrusion, hood to crest, crest to base and crest to rostral edge ratios.

Table 3: Beak measurement ratios and notes (after Clarke 1986).

Ratio notation	Description	Notes
$\frac{b}{a}$	Edge to wing ratio	Measured in profile, edge from rostral tip to wing fold
$\frac{c}{d}$	Height to base ratio	Length of baseline measured in profile, height is to rostral tip
$\frac{d}{e}$	Rostral protrusion	Distance between rostral tip and front edge of wing, divided into baseline
$\frac{f}{g}$	Hood to crest ratio	Measured along midline of beak, in profile
$\frac{d}{f}$	Crest to base ratio	Both measured with beak in profile
$\frac{g}{a}$	Crest to rostral edge ratio	Measured in profile

2.3.3 Statistical analyses

For the purpose of this study, multiple multivariate statistical tools were used: DFA, nMDS, cluster analysis using agglomerative hierarchical clustering based on group average linkage, and Analysis of Similarity (ANOSIM).

Two statistical programs were used to apply the above mentioned statistical techniques: R (for DFA), and PRIMER-E (for nMDS, cluster analysis, and ANOSIM) (Clarke and Warwick 2002, Clarke and Gorley 2006). Because all morphometric measurements are in the same unit there was no prior standardization or normalization of the data.

Discriminant Function Analysis is related to Principal Component Analysis (PCA) in that it extracts axes which are linear combination of the original variables. The main point of difference between DFA and PCA is in the latter parameter estimation and axes extraction is optimized in such a way that the maximum variation is observed on the first axes followed by subsequent axes. In DFA the main interest is in discriminating between the apriori defined groups. Despite similarity in the form of the underlying equation used for both PCA and DFA, unlike PCA for the results from DFA (linear DFA) to be useful a number of important assumptions have to be met. Some of the most important assumptions include: homogeneity of variance, existence of sufficient sample size, and normality. Without these there are two recommended alternatives: multinomial logistic regression or quadratic discriminant analysis (Zuur *et al.* 2007).

After an initial graphical exploration, using dotchart and box plots, spread of variables among the two major regions (West coast and South coast) was found to be substantially different, thus a quadratic discriminant function analysis was conducted. Quadratic discriminant function analysis was conducted, in R, using the package "MASS" (Venables and Ripley 2002). The performance of the quadratic discriminant function was assessed by looking at its overall misclassification error rate.

Following the DFA analysis the multivariate morphometric data was displayed in two dimensional ordination plot, to summarize the relationship among individuals from the four sub-regions in terms of the various morphometric measurements. For this purpose non-metric multidimensional scaling (nMDS) was used. Prior to displaying the data in the ordination plot the data between sample similarity/resemblance was expressed using Euclidean distance, which was based on the normalized values of the various morphometric measurements. The resulting resemblance matrix was then projected into two dimensional nMDS ordination plot. Cluster analysis was then conducted, using the resemblance matrix, based on group average linkage (Clarke and Gorley, 2006).

Based on the resemblance matrix created above, the significance of the apriori defined groups was then tested using ANOSIM (Analysis of Similarity). ANOSIM can be thought of as a non-parametric counterpart of the parametric ANOVA (Analysis of Variance). ANOSIM, works by comparing the within group resemblance with among group resemblance. ANOSIM in its current formulation can only test for main effect of factors/treatment. It allows for post-hoc comparison of differences among levels in each factor (Clarke and Gorley 2006, Clarke and Warwick 2002).

CHAPTER 3

MICROSATELLITE ANALYSIS OF STOCK STRUCTURE OF *LOLIGO REYNAUDI*

3.1 Results, preliminary analysis, 2006

3.1.1 General Properties

The first screening of the samples was conducted at Royal Holloway University of London during the June-September period 2006. Initial PCRs were run on the DNA extracted from 48 individuals from each sample. Although more samples were available from some of the sample locations, for the preliminary analysis it was decided to use an equal number of each. Five loci were used; Lfor1, Lfor3, Lr21, Lr27 and Lr34 and data analysis was carried out using both FSTAT and GENEPOP (v4.0).

Table 4: Allelic richness per locus and population, preliminary runs (based on minimum sample size of 48 diploid individuals).

Locus	Sample area						
	Agulhas Bank	Tsitsikamma	Kromme	Algoa Bay	Kleinemonde	Angola	Overall
Lfor1	18.20	15.30	15.12	16.57	15.70	16.54	16.72
Lfor3	12.79	13.59	12.00	13.81	13.72	11.80	13.05
Lr21	13.05	12.37	9.22	11.44	13.82	12.17	12.62
Lr27	12.73	12.49	12.31	12.43	11.59	11.97	12.97
Lr34	16.40	17.99	16.16	15.99	14.85	15.44	16.20

Overall allelic richness, while reasonably low, was similar over all of the sample populations with 2 loci, Lfor1 and Lr34 amplifying better than the rest. The lowest allelic richness of all the loci was shown by Lr21; however Lfor3 and Lr27 did not amplify much better and had similarly low richness. The numbers of alleles (N_a), observed values of heterozygosity (H_o) and expected values of heterozygosity (H_e) for each of the 5 loci over all of the populations were recorded (Table 5). H_e was calculated using Nei's (1987) unbiased estimator in FSTAT. Over all loci mean levels of genetic variability were high (0.898), with observed heterozygosity, although lower (0.774), not differing significantly from that expected under conditions of random mating. Two individual loci did exhibit significant departure from expected heterozygosity, Lr21, and Lr27, however after Bonferroni corrections were applied, only one, Lr27 remained significant. This low level of observed heterozygosity could be an indication of null alleles being present.

Table 5: Genetic variability at 5 loci over all populations. Number of alleles (N_a), unbiased expected heterozygosity (H_e) observed heterozygosity (H_o) and genetic diversity among samples (D_{st}) shown. Significant departure from expected heterozygosity in bold, underlined values remained significant after Bonferroni correction.

	Lfor1	Lfor3	Lr21	Lr27	Lr34	Overall
N_a	28	19	22	22	24	115
H_e	0.928	0.861	0.87	0.904	0.925	0.898
H_o	0.911	0.874	**0.623**	**<u>0.566</u>**	0.904	0.774
D_{ST}	0.004	0.012	-0.001	0.008	0.002	0.005

Overall D_{ST} was low (0.005) and genetic diversity among samples for each locus was uniformly low for 4 of the 5 loci. Locus Lfor3 showed the highest D_{ST} value (0.012), however this locus also had the lowest number of alleles ($N_a = 19$) overall and this would have affected the perceived level of genetic diversity between samples.

3.1.2 Linkage disequilibrium and Hardy Weinberg equilibrium

Before a frequency based analysis could be performed, the data was checked for Hardy Weinberg equilibrium. GENEPOP (v4.0) was used for this analysis, using standard Markov Chain Parameters (dememorization steps 1000 over 100 batches with 1000 iterations per batch). Hardy Weinberg equilibrium describes a population that has gene and allele frequencies that do not change over generations and assumes 5 conditions within a population; equal sex ratios, random mating, a large population from which individuals are randomly drawn, no microsatellite mutation between generations and little immigration into or emigration from the population. These conditions are not realistic for natural populations, but allow the comparison of observed gene frequencies with those predicted by the Hardy Weinberg equilibrium. All of the loci used in the preliminary runs were found to be in agreement with Hardy Weinberg equilibrium.

The data were then tested for genotypic linkage disequilibrium using Fisher's (1922) exact test for each locus pair over all of the sample populations and standard Markov Chain Parameters were used. The results of this test are shown below (Table 6).

Table 6: Fisher's exact test for genotypic disequilibrium, p-values for pairs of Loci across all sample areas. (Adjusted p-value, $p < 0.002381$ significant).

Locus	Lfor3	Lr21	Lr27	Lr34
Lfor1	0.757	0.031	0.024	0.986
Lfor3		0.280	0.514	1.000
Lr21			0.068	0.978
Lr27				0.979

Linkage disequilibrium is a situation where alleles at two loci may be non-randomly associated with each other (Hedrick 2005) and these combinations occur in a population either more or less than they would due to random formation. In the preliminary analysis no loci were found to be in disequilibrium with each other. This means that the observed allele frequencies for the different loci in this data set agree with allele frequencies that would be expected for natural populations and thus will not affect the analysis of the data.

3.1.3 F-statistics and exact tests

Once the data was shown to be in Hardy Weinberg equilibrium, and showed no linkage disequilibrium, a frequency-based analysis was conducted. By this, allele frequencies at the different loci are compared between the different groups. The premise of this method is that, with a largely homologous population, a number of shared alleles will be represented fairly equally throughout the population and thus all of the samples. Using both FSTAT and GENEPOP (V4.0), FST values were generated from the data using Weir and Cockerham (1984) estimates, and their significance from 0 determined using permutation testing.

FST is an estimate of level of genetic variance between samples and Wright (1978) provided some guidelines to analysing FST values, these being used in the initial interpretation of the data. By this an FST value of 0 - 0.005 shows little or no genetic differentiation, 0.005 - 0.01 denotes moderate genetic differentiation and 0.01 - 0.1 and above shows a high level of genetic differentiation.

However, in the case of microsatellites, which have many alleles, FST values as low as 0.005 can denote significant differences, but for interpreting how different populations are, Wright's (1978) values are still useful. As the FST value increases from 0, so does the perceived level of genetic differentiation between the groups, meaning that the allele frequencies of groups are increasingly different from each other. Alleles are interchanged between groups through breeding, and genetic differentiation between groups shows a lack of interbreeding and gene flow taking place between them. These populations are thus assumed to be separate groups with their own genetic characteristics.

Table 7: FST values for all loci and all sample groups, first runs (GENEPOP, after Cockerham 1973 and Weir and Cockerham 1984, significant values bold, underlined stayed significant after Bonferroni corrections, negative values changed to 0).

Sample Areas	Tsitsikamma	Kromme	Algoa Bay	Kleinemonde	Angola
Agulhas Bank	0.0028	**0.0094**	**0.0091**	0.0031	0.0034
Tsitsikamma		0.0038	0.0028	0.0004	**<u>0.0110</u>**
Kromme			**0.0052**	0.0033	**<u>0.0173</u>**
Algoa Bay				0.001	**<u>0.0153</u>**
Kleinemonde					**<u>0.0114</u>**

Table 7 shows genetic differentiation between some of the sampled populations. The first noticeable trend from this table is the highly significant level of differentiation between the Angolan sample and all 4 of the Eastern Cape samples. No significant differentiation is shown between Angola and the Agulhas Bank (FST = 0.0034), while the Agulhas Bank shows differentiation from both Kromme and Algoa Bay, although it is less different to these populations than they are to the Angola group. A difference is also seen between Kromme and Algoa Bay.

After Bonferroni corrections, all of the results for Angola remained significant, while differences between Kromme, Algoa Bay and the Agulhas Bank did not. These results indicate that the Angolan population is the most genetically different from the rest, with the South African populations being more genetically similar to each other, albeit with some differences.

An exact Chi^2 test was then carried out on the same data using FSTAT. By this test, a p-value is generated which indicates the significance of any differences in allele frequencies between samples. FST and exact tests are complementary, and show similar trends between them, however exact tests are more sensitive to rare alleles, while FST tests are more sensitive to common alleles. Table 8 below shows the results of the Chi^2 test.

Table 8: First study, Chi^2 test over all loci and Populations. (From FSTAT, bold denotes significant differentiation. ($p < 0.05$), underlined values remained significant after Bonferroni correction).

Sample Areas	Tsitsikamma	Kromme	Algoa Bay	Kleinemonde	Angola
Agulhas Bank	**0.01333**	**<u>0.00333</u>**	**<u>0.00333</u>**	0.30000	0.16333
Tsitsikamma		0.09667	0.47000	0.39000	**0.00667**
Kromme			0.20333	0.16333	**<u>0.00333</u>**
Algoa Bay				0.76333	**0.00667**
Kleinemonde					0.07333

The results show two main trends in the data. Firstly, as shown in the FST tests, the Angolan sample shows significant allele frequency differences from 3 of the Eastern Cape samples, Tsitsikamma, Kromme and Algoa Bay. The second noticeable trend is the significant difference of allele frequencies between the Agulhas Bank and the same three Eastern Cape samples. Differences between the allele frequencies of Angola and the Agulhas Bank are not significant, as are those of Kleinemonde and both the Agulhas Bank and Angola. The exact test supports the results of the FST test, while also indicating that some more subtle genetic differences between the populations exist.

3.2 Results, second run, 2007

3.2.1 General properties

After looking at the data from the first 5 loci, it was decided to re-run the laboratory work using larger sample sizes to provide more accurate results. DNA was extracted from more samples, new PCRs were set up and, for this analysis, 2 new loci were included, Lr44 and Lr48.

Table 9: Allelic richness per locus and population, re-runs. Based on minimum sample size of 34 diploid individuals.

Locus	Sample area						
	Agulhas Bank	Tsitsikamma	Kromme	Algoa Bay	Kleinemonde	Angola	Overall
Lfor1	20.72	22.83	17.92	19.18	18.82	18.70	20.12
Lfor3	13.61	14.94	14.66	14.81	15.90	15.35	15.12
Lr21	14.47	13.45	12.62	12.89	14.21	13.64	14.17
Lr27	13.96	13.78	13.21	14.06	12.98	13.17	14.01
Lr34	18.49	17.73	18.19	18.00	17.67	18.45	18.31
Lr44	19.21	17.45	17.78	17.82	15.77	17.90	17.61
Lr48	24.65	27.01	29.39	29.39	27.89	24.23	26.44

Allelic richness was higher than that of the preliminary runs, this being due to the larger number of samples used from each population. Allelic richness for 3 loci, Lfor3, Lr21 and Lr27 was again lower than the other 4, with Lr27 showing the lowest overall. The two new loci, Lr44 and Lr48 showed higher allelic richness than the 5 original loci. Again, richness was relatively uniform for each locus over all of the sample populations.

As before, the number of alleles (N_a), observed values of heterozygosity (H_o) and expected values of heterozygosity (H_e) for the 7 loci over all of the populations were calculated using FSTAT (Table 10). Overall expected and observed heterozygosity were higher than the preliminary runs, again mostly due to the larger sample sizes, with the overall H_o, while being lower, not differing significantly from H_e. Again, as in the previous run, H_o for Lr21 and Lr27 showed significant departure from H_e, with only the value for Lr27 remaining significant after Bonferroni correction.

Table 10: Genetic variation at 7 loci over all populations. Number of alleles (N_a), unbiased expected heterozygosity (H_e) observed heterozygosity (H_o) and genetic diversity among samples (D_{st}) shown. Significant departure from expected heterozygosity in bold, underlined values remained significant after Bonferroni correction.

	Lfor1	Lfor3	Lr21	Lr27	Lr34	Lr44	Lr48	Overall
Na	34	23	22	22	26	24	39	190
He	0.934	0.876	0.869	0.896	0.928	0.925	0.961	0.913
Ho	0.924	0.897	**0.605**	**0.543**	0.892	0.866	0.892	0.803
Dst	0.004	0.003	0.001	0.012	0.001	-0.001	-0.001	0.003

The two new loci, Lr44 and Lr48 exhibited similar characteristics to loci Lfor1, Lfor3 and Lr34, with uniformly high H_o and H_e. Overall D_{st} was low (0.003) with one locus, Lr27 being the exception.

3.2.2 Linkage disequilibrium and Hardy Weinberg equilibrium

Table 11: Fisher's exact test for genotypic disequilibrium, p-values for pairs of loci across all sample areas. (Adjusted p-value, $p < 0.002381$ significant, shown in bold).

Locus	Lfor3	Lr21	Lr27	Lr34	Lr44	Lr48
Lfor1	0.173	0.126	0.326	0.003	0.016	0.015
Lfor3		0.028	0.674	0.906	0.029	0.282
Lr21			0.004	0.998	0.237	0.072
Lr27				0.910	0.003	0.602
Lr34					0.161	0.154
Lr44						0.036

As in the previous runs, the data were then tested for genotypic linkage disequilibrium and Hardy Weinberg equilibrium. Fisher's exact test (Table 11) showed no evidence for linkage disequilibrium between any of the loci used. Some low values were returned, however these were due to comparisons between pairs of samples with p-values of 0, bringing the overall p-value down. Replacing the 0.000 value for 0.001 increased the overall p-value. Upon testing for Hardy Weinberg equilibrium, 6 of the 7 loci were found to be in equilibrium with Lr27 being the exception.

3.2.3 F-statistics per locus

Lfor1

Locus Lfor1 indicates significant differentiation between Kromme and both the Agulhas Bank (FST = 0.0144) and Angola (FST = 0.0142) samples. A lesser degree of differentiation is shown between Tsitsikamma and Kromme (FST = 0.0085).

Angola shows no differentiation from the Agulhas bank and Tsitsikamma, but then a high

level of segregation from Kromme and Algoa Bay, which in turn show no significant difference between each other.

Table 12: FST values for Lfor1, pairwise tests for all populations (GENEPOP, after Cockerham 1973 and Weir and Cockerham 1984, significant values bold, negative values changed to 0).

Sample Areas	Tsitsikamma	Kromme	Algoa Bay	Kleinemonde	Angola
Agulhas Bank	0.0014	**0.0144**	**0.0074**	0.0000	0.0010
Tsitsikamma		**0.0085**	0.0026	0.0038	0.0005
Kromme			0.0016	**0.0065**	**0.0142**
Algoa Bay				0.0021	**0.0074**
Kleinemonde					0.0000

The results from this locus follow some of the trends indicated by the initial study, with the most differentiation occurring between Angola and Agulhas Bank and the Eastern Cape groups. Kleinemonde shows significant differentiation from the Kromme, but no significant differentiation from Angola and the Agulhas bank.

Lfor3

Locus Lfor3 shows the Agulhas Bank as the most genetically different group, differing significantly from all but one of the other groups. This locus shows less variation between the Angolan and Eastern Cape groups than Lfor1 or the first study with the Kleinemonde group, unlike the other Eastern Cape Groups, and Agulhas Bank differing significantly from Angola (FST = 0.0147 and 0.0086 respectively). The Kleinemonde

sample site is geographically the furthest away from the Agulhas and Angolan sites (Table A2.2.) and a level of genetic differentiation could reasonably be expected to be between these sites. This difference is not evident in Lfor1 or the first analysis, however.

Table 13: FST values for Lfor3, pairwise tests for all populations. (GENEPOP, after Cockerham 1973 and Weir and Cockerham 1984, significant values bold, negative values changed to 0).

Sample Areas	Tsitsikamma	Kromme	Algoa Bay	Kleinemonde	Angola
Agulhas Bank	0.0000	**0.0080**	**0.0056**	**0.0098**	**0.0086**
Tsitsikamma		0.0011	0.0000	**0.0096**	0.0000
Kromme			0.0000	0.0011	0.0026
Algoa Bay				0.0000	0.0000
Kleinemonde					**0.0147**

Lr21

Locus Lr21 returned only 3 significant values, showing the highest differentiation between the Kleinemonde and the Agulhas Bank (FST= 0.0092). This locus did not show any differentiation between the Agulhas Bank and the other Eastern Cape groups, unlike the previous loci. Other significant differentiation observed was between Angola and Algoa Bay (FST = 0.0085), and Kleinemonde (FST = 0.0076).

Table 14: FST values for Lr21, pairwise tests for all populations (GENEPOP, after Cockerham 1973 and Weir and Cockerham 1984, significant values bold, negative values

changed to 0).

Sample Areas	Tsitsikamma	Kromme	Algoa Bay	Kleinemonde	Angola
Agulhas Bank	0.0000	0.0000	0.0000	**0.0092**	0.0000
Tsitsikamma		0.0000	0.0000	0.0012	0.0000
Kromme			0.0000	0.0000	0.0011
Algoa Bay				0.0001	**0.0085**
Kleinemonde					**0.0076**

This locus indicates the most genetic difference occurring between Angola and the two Eastern Cape sites furthest away from it and between the Agulhas Bank and Kleinemonde.

Lr27

Lr27 returned a large number of very significant results, unlike all of the other loci used. At the beginning of this study, Lr27 was found not to be in Hardy Weinberg equilibrium, and so needed to be looked at in more detail before it could be included in any of the final analysis of the data. An exact test, shown below in table 16, also produced the same number of significant results. According to both tests, Kromme, Algoa Bay and Kleinemonde all show high levels of differentiation between each other, as well as a slightly lower level of differentiation from the Tsitsikamma group.

Table 15: FST values for Lr27, pairwise tests for all populations (GENEPOP, after Cockerham 1973 and Weir and Cockerham 1984, significant values bold, negative values changed to 0).

Sample Areas	Tsitsikamma	Kromme	Algoa Bay	Kleinemonde	Angola
Agulhas Bank	0.0000	0.0000	**0.0275**	**0.0216**	0.0000
Tsitsikamma		0.0000	**0.0137**	**0.0178**	0.0000
Kromme			**0.0279**	**0.0146**	**0.0052**
Algoa Bay				**0.0392**	**0.0177**
Kleinemonde					**0.0157**

Table 16: Chi^2 test for Lr27, pairwise tests for all populations (FSTAT, bold denotes significance, $p < 0.05$, underlined values remained significant after Bonferroni corrections).

Sample Areas	Tsitsikamma	Kromme	Algoa Bay	Kleinemonde	Angola
Agulhas Bank	0.75000	0.61333	**0.00667**	**0.01333**	0.62000
Tsitsikamma		0.52667	**0.03667**	**0.02000**	0.5776
Kromme			**<u>0.00333</u>**	**<u>0.00333</u>**	**0.05333**
Algoa Bay				**<u>0.00333</u>**	**0.02000**
Kleinemonde					**0.00667**

When taking into account the locus not being in Hardy Weinberg equilibrium, as well as

the significant departure from expected heterozygosity, it could be seen that there was a problem with it.

Lr34

This locus returned only 1 significant value, indicating significant differentiation between Algoa Bay and Kleinemonde (FST = 0.0062). Some trends can be seen in the smaller values, with difference of Angola from other samples increasing along the Eastern Cape, with the largest value for Kleinemonde.

No differentiation is shown between the Tsitsikamma, Kromme and Algoa Bay samples.

Table 17: FST values for Lr34, pairwise tests for all populations (GENEPOP, after Cockerham 1973 and Weir and Cockerham 1984, significant values bold, negative values changed to 0).

Sample Areas	Tsitsikamma	Kromme	Algoa Bay	Kleinemonde	Angola
Agulhas Bank	0.0028	0.0020	0.0047	0.0036	0.0000
Tsitsikamma		0.0013	0.0000	0.0038	0.0000
Kromme			0.0000	0.0000	0.0005
Algoa Bay				**0.0062**	0.0010
Kleinemonde					0.0022

Lr44

Lr44 returned 2 significant values, indicating genetic differentiation between Kleinemonde and both Algoa Bay (FST = 0.0053) and Angola (FST= 0.0055).

A high number of negative values were returned for this locus, these being interpreted as showing no differentiation and equated to 0. Both this locus and Lr34 showed no unusual properties in heterozygosity, allelic richness and Hardy Weinberg.

Table 18: FST values for Lr44, pairwise tests for all populations (GENEPOP, after Cockerham 1973 and Weir and Cockerham 1984, significant values bold, underlined stayed significant after Bonferroni corrections, negative values changed to 0).

Sample Areas	Tsitsikamma	Kromme	Algoa Bay	Kleinemonde	Angola
Agulhas Bank	0.0000	0.0001	0.0000	0.0000	0.0022
Tsitsikamma		0.0000	0.0000	0.0000	0.0000
Kromme			0.0038	0.0000	0.0001
Algoa Bay				**0.0053**	0.0000
Kleinemonde					**0.0055**

Lr48

Lr48 showed 2 significant values, with significant differentiation between Kleinemonde and both Algoa Bay (FST = 0.0053) and Angola (FST = 0.0055). This locus supports the results from Lr44. No significant differentiation can be seen between any of the other Eastern Cape samples. Some trends can be seen in the lower values, with the Kromme group differing slightly more from the Agulhas bank than Tsitsikamma.

Table 19: FST values for Lr48, pairwise tests for all populations (GENEPOP, after Cockerham 1973 and Weir and Cockerham 1984, significant values bold, underlined stayed significant after Bonferroni corrections, negative values changed to 0).

Sample Areas	Tsitsikamma	Kromme	Algoa Bay	Kleinemonde	Angola
Agulhas Bank	0.0041	0.0045	0.0000	0.0000	0.0000
Tsitsikamma		0.0000	0.0000	0.0000	0.0027
Kromme			0.0038	0.0000	0.0001
Algoa Bay				**0.0053**	0.0000
Kleinemonde					**0.0055**

3.2.4 F-statistics and exact tests over all samples

Including Lr27

For an overall picture, all the loci and population data were combined in a single analysis. As mentioned previously, the overall pairwise tests were conducted first including Lr27, and then excluding it to show how this locus affected the final results. Table 20 below shows overall FST for all the loci over all the populations. Overall FST for all loci returned 4 significant values. These indicate significant genetic differentiation between both the Agulhas Bank and Angola and the Algoa Bay and Kleinemonde samples. The Agulhas Bank shows no difference to Angola, or Tsitsikamma, but as one moves along the Eastern Cape, FST values increase and the groups appear to become more different from each other showing some difference from Kromme (FST = 0.0037) and then significant differentiation to Algoa Bay and Kleinemonde (FST = 0.0056 for both).

Table 20: FST values for all loci and sample groups (GENEPOP, weighted analysis of variance, after Cockerham 1973 and Weir and Cockerham 1984, significant values in bold, negative values changed to 0).

Sample Areas	Tsitsikamma	Kromme	Algoa Bay	Kleinemonde	Angola
Agulhas Bank	0.0000	0.0037	**0.0056**	**0.0056**	0.0005
Tsitsikamma		0.0006	0.0004	0.0048	0.0001
Kromme			0.0025	0.0020	0.0035
Algoa Bay				**0.0062**	0.0042
Kleinemonde					**0.0063**

The Angolan sample shows a similar pattern, showing increasing differentiation as the distance between sample sites increases, being the most different from the Kleinemonde group, the furthest away. Tsitsikamma, Kromme and Algoa Bay show no difference between them. Kleinemonde appears to be more different to the Agulhas Bank than it is to Tsitsikamma, while its highest levels of differentiation are from Angola (FST = 0.0063) and Algoa Bay. (FST = 0.0062). Angola is the furthest away sample site, however the high level of differentiation from Algoa Bay is interesting. An exact test was also carried out, the results of which appear to support the initial indications of the first run, shown below in Table 21.

The exact test shows significant allele frequency differences between the Agulhas Bank and 3 of the Eastern Cape samples, Kromme, Algoa Bay and Kleinemonde, as well as between Angola and both Kromme and Kleinemonde. No significant differences in allele frequencies are shown between any of the Eastern Cape samples.

Table 21: Chi^2 test over all loci and sample groups (FSTAT, bold denotes significance. $p < 0.05$, underlined values remained significant after Bonferroni corrections $p < 0.003$).

Sample Areas	Tsitsikamma	Kromme	Algoa Bay	Kleinemonde	Angola
Agulhas Bank	0.3333	**<u>0.0033</u>**	**<u>0.0033</u>**	**0.0333**	0.4266
Tsitsikamma		0.1600	0.5133	0.0833	0.1633
Kromme			0.4633	0.1233	**<u>0.0033</u>**
Algoa Bay				0.3667	0.0500
Kleinemonde					**0.0266**

After Bonferroni corrections were applied, 3 of the values remained significant, and these are underlined above. A borderline significant value indicates possible differentiation between Algoa Bay and Angola ($p = 0.0500$)

Excluding Lr27

As shown previously, FST values for locus Lr27 on its own showed a high number of significant results throughout all 6 populations which can affect compiled FST results. Compiled FST for all populations, excluding Lr27 is shown below.

Removing the Lr27 locus from the analysis did change overall FST values, specifically regarding the Kleinemonde group, with this sample site showing little differentiation from the other South African sites.

Table 22: FST values for 6 loci over all sample groups (GENEPOP, after Cockerham 1973 and Weir and Cockerham 1984, significant values bold, underlined, stayed significant after Bonferroni corrections, negative values changed to 0).

Sample Areas	Tsitsikamma	Kromme	Algoa Bay	Kleinemonde	Angola
Agulhas Bank	0.0003	0.0046	0.0018	0.0032	0.0041
Tsitsikamma		0.0009	0.0000	0.0034	0.0031
Kromme			0.0000	0.0002	**0.0076**
Algoa Bay				0.0010	**0.0078**
Kleinemonde					**0.0122**

These results show strong evidence for a geographic effect on genetic differentiation with the first clear trend being the significant level of differentiation between Angola and the 3 Eastern Cape samples furthest away from it. It can also be seen that Kleinemonde is more different to the Agulhas Bank and Tsitsikamma than it is to Kromme and Algoa Bay, with significant differentiation from Angola. The largest difference seen is between Angola and Kleinemonde (FST = 0.0122), the two sites that are the furthest apart.

Angola showed the highest level of differentiation from all of the other sites while all of the South African sites, Agulhas Bank, Tsitsikamma, Kromme, Algoa Bay and Kleinemonde show no significant differentiation between them. An exact test was then carried out on the same data set, and the results from this are shown below.

Table 23: Chi^2 test for 6 loci over all sample groups. (FSTAT, bold denotes significance $p < 0.05$, underlined values remained significant after Bonferroni corrections $p < 0.003$).

Sample Areas	Tsitsikamma	Kromme	Algoa Bay	Kleinemonde	Angola
Agulhas Bank	0.1966	**<u>0.0033</u>**	**0.0410**	**0.0433**	0.2666
Tsitsikamma		0.1033	0.9033	0.0833	0.1325
Kromme			0.9333	0.3566	**0.0135**
Algoa Bay				0.6646	0.1466
Kleinemonde					**0.0500**

Two of the sample sites stand out in these results, both Angola and the Agulhas Bank having significant differences in allele frequencies from some of the Eastern Cape sites. The Agulhas Bank shows no significant difference from either Angola or Tsitsikamma, and significant differences from the other 3 sites. Angola shows no significant difference in allele frequency from Agulhas Bank, Tsitsikamma or Algoa Bay, while differing from Kleinemonde and Kromme. The results of this test show a similar pattern to those from the first study and support the initial impression that some more subtle genetic structuring exists between the sample sites.

3.2.5. Kromme samples, temporal analysis.

While completing the laboratory work for the second runs, a further set of samples, gathered in the Kromme Bay in 2003 by Dr. P. Shaw, were extracted. For this study, a 2006 Kromme set of samples had already been collected and a preliminary temporal comparison between the two was done. This would provide some indication as to whether the basic genetic characteristics of individuals at a sample site changed over time.

As an annual species, the individuals returning from year to year would be different, however if spawning is relatively site specific, with limited gene flow between one site and others, then allele frequencies within the group as a whole would stay relatively uniform. The two Kromme samples were processed just as in the rest of this study, using the same loci.

Table 24: Allelic richness per locus and sample, Kromme 2003 and 2006. Based on minimum sample size of 38 diploid individuals>

Locus	Sample		
	Kromme 2006	Kromme 2003	Overall
Lfor1	18.50	21.82	20.38
Lfor3	15.33	15.70	15.19
Lr21	12.97	14.00	14.19
Lr27	13.38	17.00	15.44
Lr34	18.64	18.06	18.18
Lr44	18.19	17.00	17.76
Lr48	28.38	24.59	26.68

No significant differences were found in the allelic richness of the two samples.

While some small differences between the two samples can be seen, the same trends in richness seen in both the preliminary and secondary studies were seen for all of the loci, with loci Lr21 Lr27 and Lfor3 having the similarly low richness. Genetic variability was compiled for the two samples over all 7 loci (Table 25, below) and showed no significant differences between the two samples.

Table 25: Genetic variability over all loci for Kromme 2003 and 2006. Number of alleles (N_a), unbiased expected heterozygosity (H_e) observed heterozygosity (H_o) are shown.

Sample	Kromme 2006	Kromme 2003	Overall
N_a	128	125	157
H_e	0.913	0.906	0.913
H_o	0.807	0.789	0.797

The overall expected genetic variability was higher than in the previous studies, and H_o, while lower, showed no significant departure from H_e. The overall genetic diversity among samples (D_{ST}, not shown in table) was very low. A pairwise FST test was conducted, returning an FST value of 0.0010, indicating no significant genetic differentiation between the two samples. An exact test was also conducted and returned a non-significant value. These results indicate that the genetic characteristics of individuals from this one sample site stay relatively stable over time.

3.2.6 Isolation by distance

Results from both sets of data appeared to indicate a geographical effect on genetic differentiation between samples.

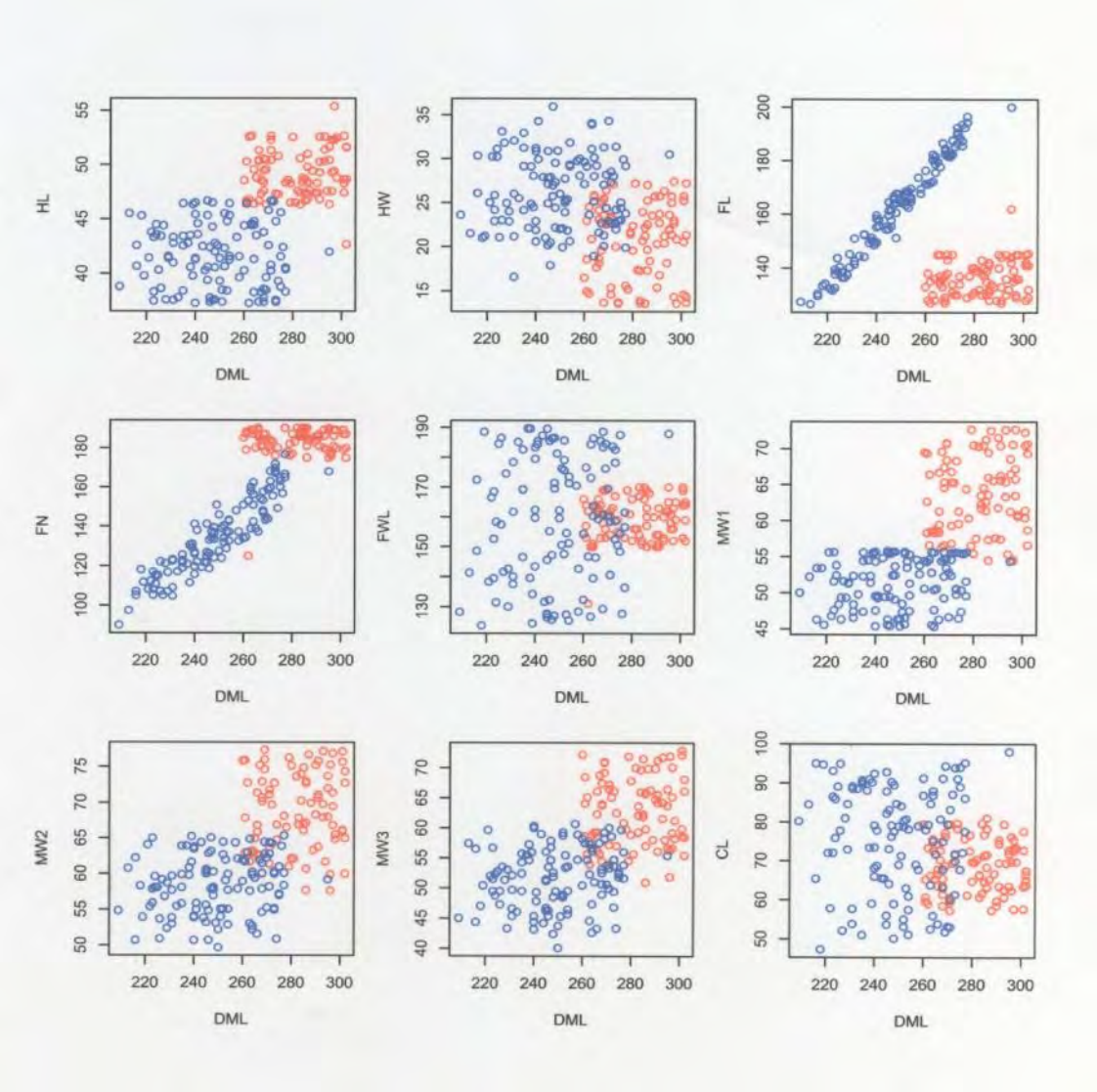
A partial Mantel test was conducted in FSTAT, using compiled FST values from Table 26, and geographic distances between sample sites, measured as the shortest distance by sea. Under an isolation-by-distance effect, the genetic differentiation between sites would be affected by the distance between them, and thus FST, representing the degree of genetic differentiation was designated as the dependant variable.

The results of the partial Mantel test were as follows: R^2 68.90, correlation 0.8301 and sum of squares 0.001. This shows a definite positive correlation between FST and the distance between sample sites.

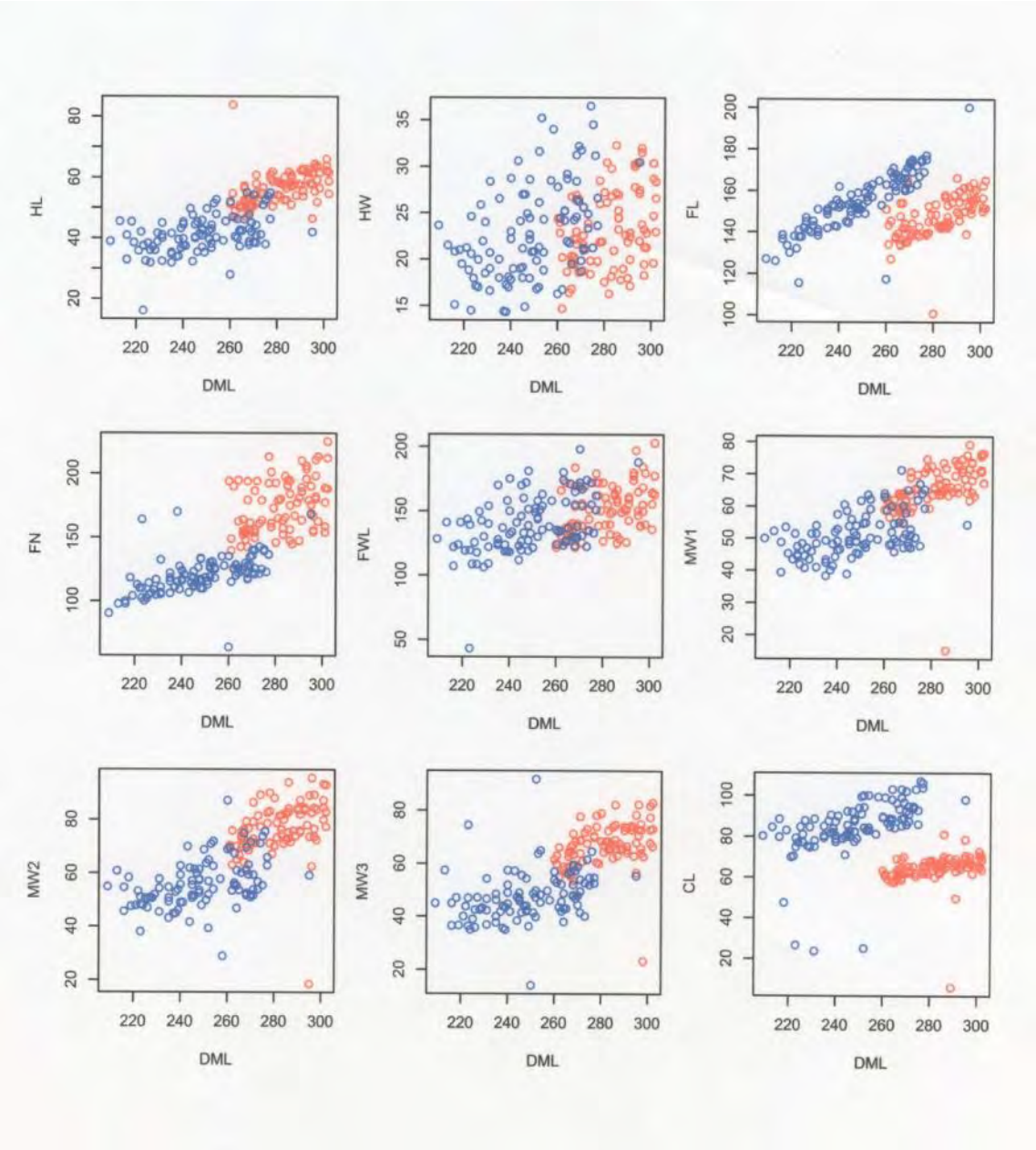
MULTIVARIATE ANALYSIS OF MORPHOMETRIC MEASUREMENTS

3.3 Results of initial statistical analysis

After analyses were conducted, it was found that data show random variation and not directional change and also do not demonstrate allometric effects. Therefore, significant differences between regions were predominantly an effect of body size differences. This may indicate a serious problem with the method of taking measurements. As it was not possible to re-measure the squid samples, these results will not be suitable for publication. Results shown below are for illustrative purposes only, to demonstrate the techniques used. Simple plots to illustrate these problems mentioned above are provided below. The first is before data were scanned and corrections were made, the second is after the corrections. It is apparent in the second data set that difficulties encountered were not limited to data punching, but also were related to apparent random, large variability. This results in the predominance of Mantle Length (ML) over other factors in determination of differences between regions (Large squid along the West coast – red circles; Smaller squid along the South coast – blue circles).



1. Before corrections. (Large squid along the West coast – red circles; Smaller squid along the South coast – blue circles)



3. After corrections. (Large squid along the West coast – red circles; Smaller squid along the South coast – blue circles)

It is clear, that even after corrections the significant differences between regions were predominantly an effect of body size differences.

3.3.1 Comparison of distribution of morphometric measurements across the four sub-regions

The distribution of morphometric measurements for the 4 sample sites (Angola, West coast, East coast inshore, and East coast offshore) was compared based on kernel density which shows the density of observation along discrete class of the variable of interest.

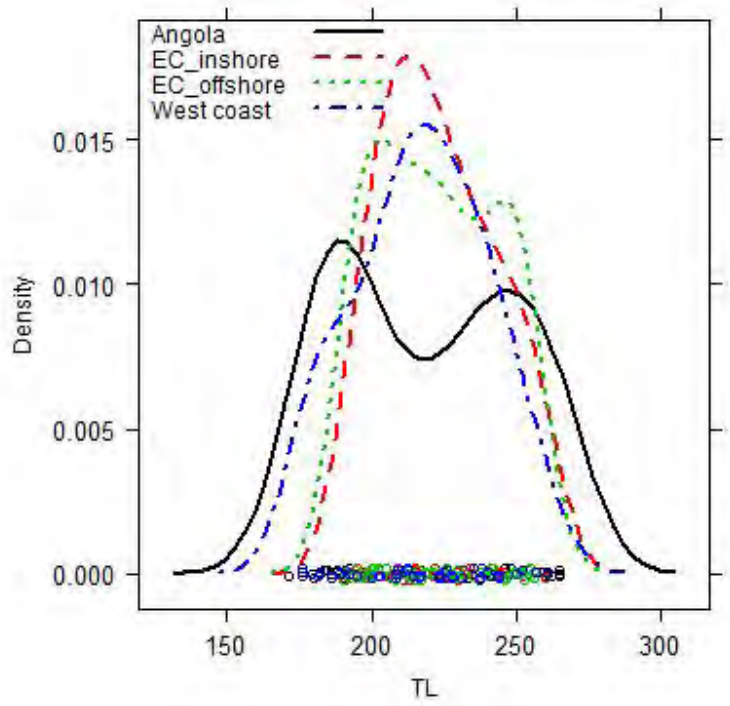


Fig. 5 Distribution of “TL” across the four sample sites.

In most of the morphometric variables there appeared to be a distinct difference in distribution, mainly between the western samples (Angola and West coast) and the Eastern samples (East Cape inshore and offshore). Fig. 5 shows this using the TL measurement, where a clear differentiation can be seen between the Eastern and Western samples.

3.3.2 Discriminant Function Analysis (DFA)

Initially a quadratic DFA was applied to the data of the four sample sites. The overall accuracy of this model was not satisfactory as the total proportion of correct classification (total accuracy) was 53%. This is not far from a random result. A quadratic DFA was then used to classify individuals, based on the whole body morphometric measurements, into the two clear groupings (Western and Eastern), the resulting classification ability of the DFA was more than 99%, which indicate that this is an efficient classifier that can be used in the future to predict where an individual collected from either of the two major regions came from.

3.3.3 Non-Metric multidimensional Scaling (nMDS)

The result of the nMDS based on whole body measurement (Fig. 6) appeared to show the existence of clear separation in body size and possibly shape between individuals from the two major regions (Western and Eastern)

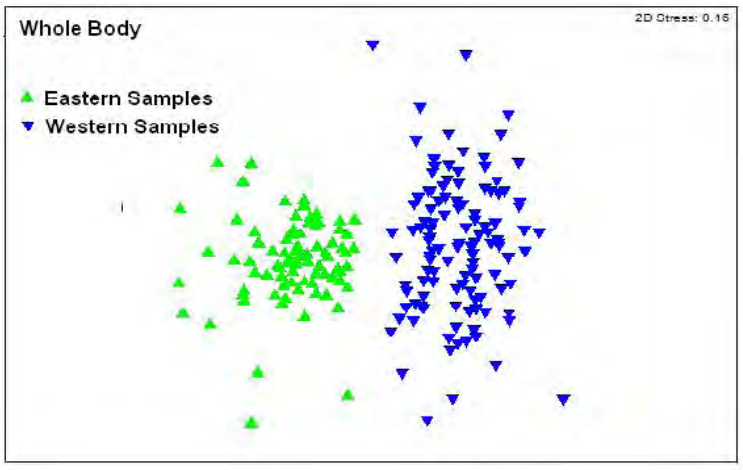


Fig. 6 nMDS ordination plot, based on whole body morphometrics, of individuals from the two groups. (Normalise resemblance: D1 Eucidean distance)

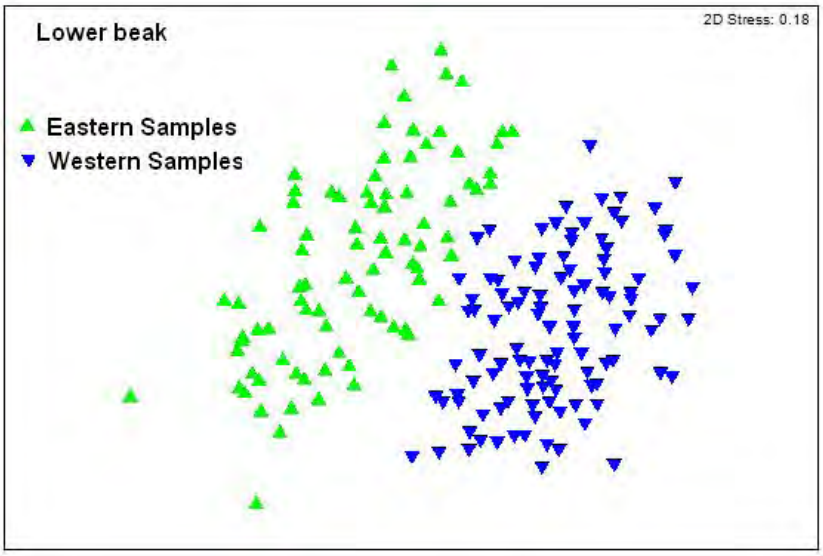


Fig.7 nMDS ordination plot, based on lower beak measurments, of individuals from the two groups. (Normalise resemblance: D1 Eucidean distance)

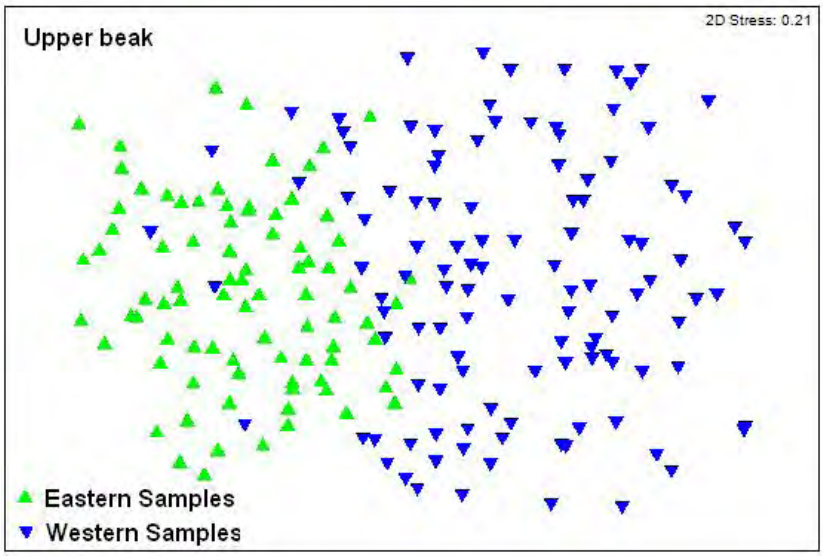


Fig. 8 nMDS ordination plot, based on upper beak measurments, of individuals from the two groups. (Normalise resemblance: D1 Eucidean distance)

Lower beak measurements showed similar results (Fig. 7) to the whole body results while upper beak measurements showed less distinct results (Fig.8)

3.3.4 ANOSIM

The result of ANOSIM also appeared to confirm what was seen on the nMDS ordination plot. ANOSIM was conducted to compare difference among individuals collected from the four sample sites. Based on the whole body morphometrics the result of ANOSIM shows the existence of difference ($R= 0.495$, $p=0.001$). The pair-wise comparison showed the existence of a statistically significant difference between the two main regions but not among the sub regions.

Similarly ANOSIM was conducted based on lower beak measurements. The results also confirms the existence of differences in lower beak size and shape ($R=0.443$, $p=0.001$). The pair-wise comparison showed a statistically significant difference between the two major regions and lack of difference between sub-regions.

CHAPTER 4

DISCUSSION

4.1 Microsatellite analysis

The results of the genetic section of this project showed significant genetic variation between some sample groups. As expected, the Angolan outgroup consistently showed significant variation from the other samples, accounting for a large proportion of the variability, while there was evidence of differentiation between the South African samples themselves.

Table 26: Frequency of significant differences between sample sites over all loci except Lr27. Expressed as proportion of overall significant differences.

Sample Areas	Tsitsikamma	Kromme	Algoa Bay	Kleinemonde	Angola
Agulhas Bank	0	0.1	0.1	0.1	0.05
Tsitsikamma		0.05	0	0.05	0
Kromme			0	0.05	0.05
Algoa Bay				0.15	0.1
Kleinemonde					0.2

In the South African sample sets, results pointed to isolation by distance effect occurring with the Agulhas Bank and Kleinemonde samples accounting for most of the remaining variation. Of these two, the Agulhas Bank was the most genetically different from the Eastern Cape sites while showing proportionately less differentiation from the Angolan group and no difference from the Tsitsikamma group.

At the other end of the coast the Kleinemonde group showed similar differentiation from the other sites, being most different from Angola, from which it is geographically most distant. Interestingly, Kleinemonde showed significant differentiation from Algoa Bay, the closest spawning area to it. The cause of this is not clear and needs further investigation. When compiled, the genetic results point roughly to three genetically similar groupings along the South African coast; the Agulhas bank, the East Cape coast, and a splinter group around Kleinemonde. Genetic variability between groups of individuals can be caused either by isolation by distance, or by selection (Laurent 2007). The isolation by distance effect, first described in detail by Wright (1943), works through the gradual changing of gene frequencies over time and increasing distance. In this study, this effect was tested for, using a partial Mantel test, which returned a significant result (R^2 68.90, correlation 0.8301, SS 0.001), indicating an isolation by distance effect occurring. On closer examination of the data, it was seen that the Angolan sample accounted for a lot of this differentiation, as was expected, and when removed from the Mantel test, a weaker, but still significant result was returned. This result indicates that distance is definitely playing a part in the level of genetic flow between the sampled spawning aggregations, but that some (unquantified at present) genetic flow between all of the groups is still occurring. The Sauer *et al.* (2000) tagging study showed that individuals are capable of moving between spawning sites along the coast between Cape St Francis and Algoa Bay. This shows that such movement and thus genetic exchange over their whole range is possible, however the overall study area in this experiment is much larger, and the genetic results may point to a more complex pattern of migration than documented previously.

Finding genetic variation between the different spawning areas means that squids do not all necessarily move very large distances. Under a simple isolation by distance effect, a larger proportion of the population will return to natal spawning area to spawn and a smaller proportion will move between spawning areas, with the level of movement and thus genetic exchange decreasing stepwise as distance increases.

The temporal analysis of the Kromme samples was only conducted once these trends in the data became apparent and due to limited availability of samples only two sample sets were used, the results showing no evidence of genetic variability between 2003 and 2006. *Loligo reynaudi* is an annual species, so any genetic drift within a population will theoretically occur quite rapidly. This result could indicate a genetically stable population which supports the hypothesis that enough squid spawned in this area must be returning here to offset any variability that would be introduced by "non-local" squid. More data from more sample sites over more years would of course be required in order to confirm this, but it is an interesting start for such a study, given the long time span between the samples and the chokka squid's short life cycle.

4.2 Morphometric analysis

The results of the morphological study appeared initially to support the genetic results in principle. Any evidence of phenotypic differentiation for west coast and east coast samples was however found mainly because individuals from different sampling sites were, predictably, of different size (ML). Due to the low correlation coefficients found in the data and lack of allometric relationship, the result returned could not be relied upon and further study is currently underway.

Lipinski (1981) hypothesized that evolutionary considerations and conclusions can be reached on the basis of morphometric analysis in squid (*Illex*), as the overall shape in fast-swimming squid is a critical parameter for survival and likely to play a role in fitness in the population. The genetic evidence found in this study would indicate that there may be phenotypic evidence to support this hypothesis, but further study and larger data sets are required to confirm any phenotypic variation between the different samples.

4.3 Conclusions

BASED ONLY ON THE RESULTS OF THE GENETIC STUDY

The results of this study could have some implications for the previously documented life cycle model. In this model paralarvae from the Eastern Cape spawning grounds move alongshore West to the Agulhas bank and, joining with paralarvae resulting from incidental spawning on the Agulhas Bank itself, form a common larval pool from which adults are recruited. These recruits fall into three categories, non migratory, short and long distance migrants. The non migrants stay on and around the Agulhas bank, forming small, fragmented spawning aggregations. The long distance migrants, which in this model form the bulk of the stock, move up the coast back to the Eastern Cape spawning grounds, while the short range migrants end up contributing to the incidental spawning along the coast between the Agulhas bank and the Eastern Cape, including deep spawning squid.

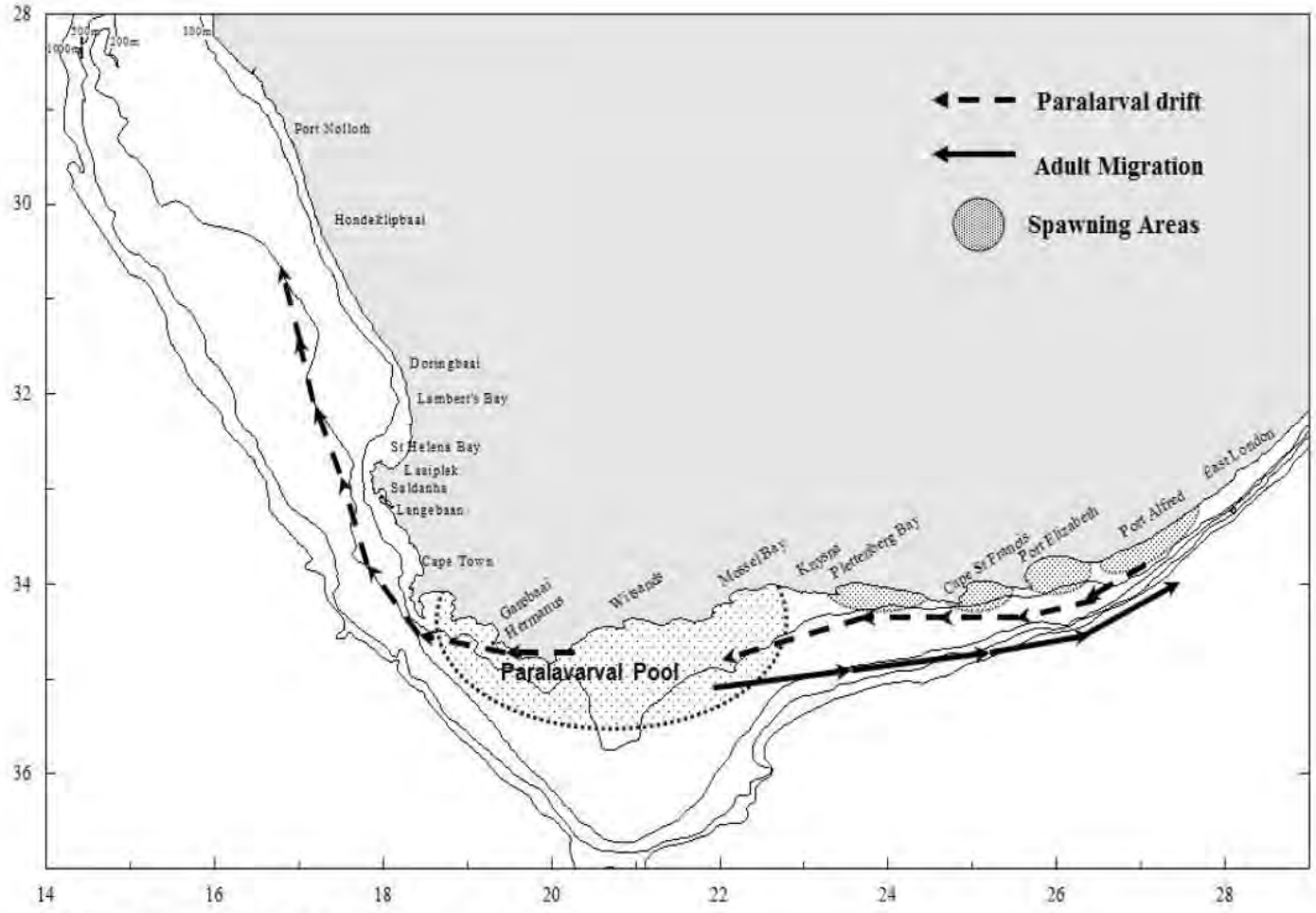


Fig. 9 Simple Life cycle model, *Loligo reynaudi*, East Coast

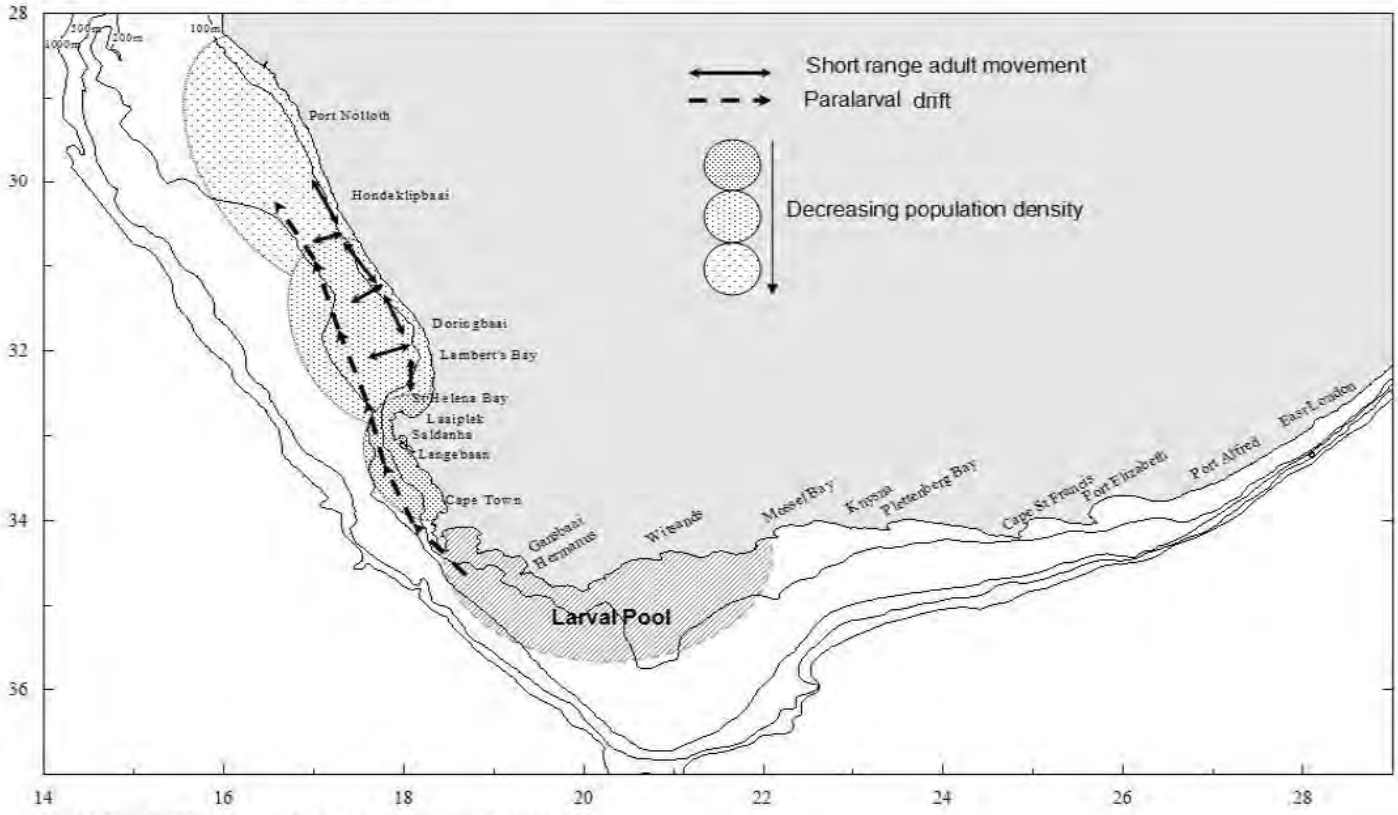


Fig. 10 Simple life cycle model of *Loligo reynaudi* West Coast

Along the West Coast paralarval drift from the deep water spawning on the Agulhas bank is believed to feed the west coast population (Roberts and van den Berg 2002). The population on the West coast is smaller and more variable than that of the Eastern Cape, consisting of smaller, slower growing adults due to the cold temperatures, indicating that they simply live where they end up. Further north, paralarval drift and short range movements along the West coast have in time created a population further north-west than *L. reynaudi*'s established range up to the Orange River. This model is illustrated in Fig. 9 (East coast) and Fig. 10 (West coast)

The results of this study show some evidence for a genetic split between East Coast and Agulhas Bank groups with a Port Alfred fragment group. A stepwise change in genetic characteristics along the coast was found, with genetic flow occurring mainly between adjacent groups and an apparent gap in genetic flow somewhere between the Kromme and Tsitsikamma areas. These results indicate a more complex population structure consisting of 2, possibly 3 paralarval pools, from which recruitment occurs, with the non migratory and short range migratory groups forming the bulk of the spawning population while the long distance migrants form a smaller proportion than previously thought. A hypothesis of what this might look like is illustrated in Fig 11. Under this model, paralarvae and juveniles on the East Cape coast keep to a more limited range within the Eastern Cape, while the squid found on the Agulhas bank are those resulting from the spawning that occurs inshore and offshore there.

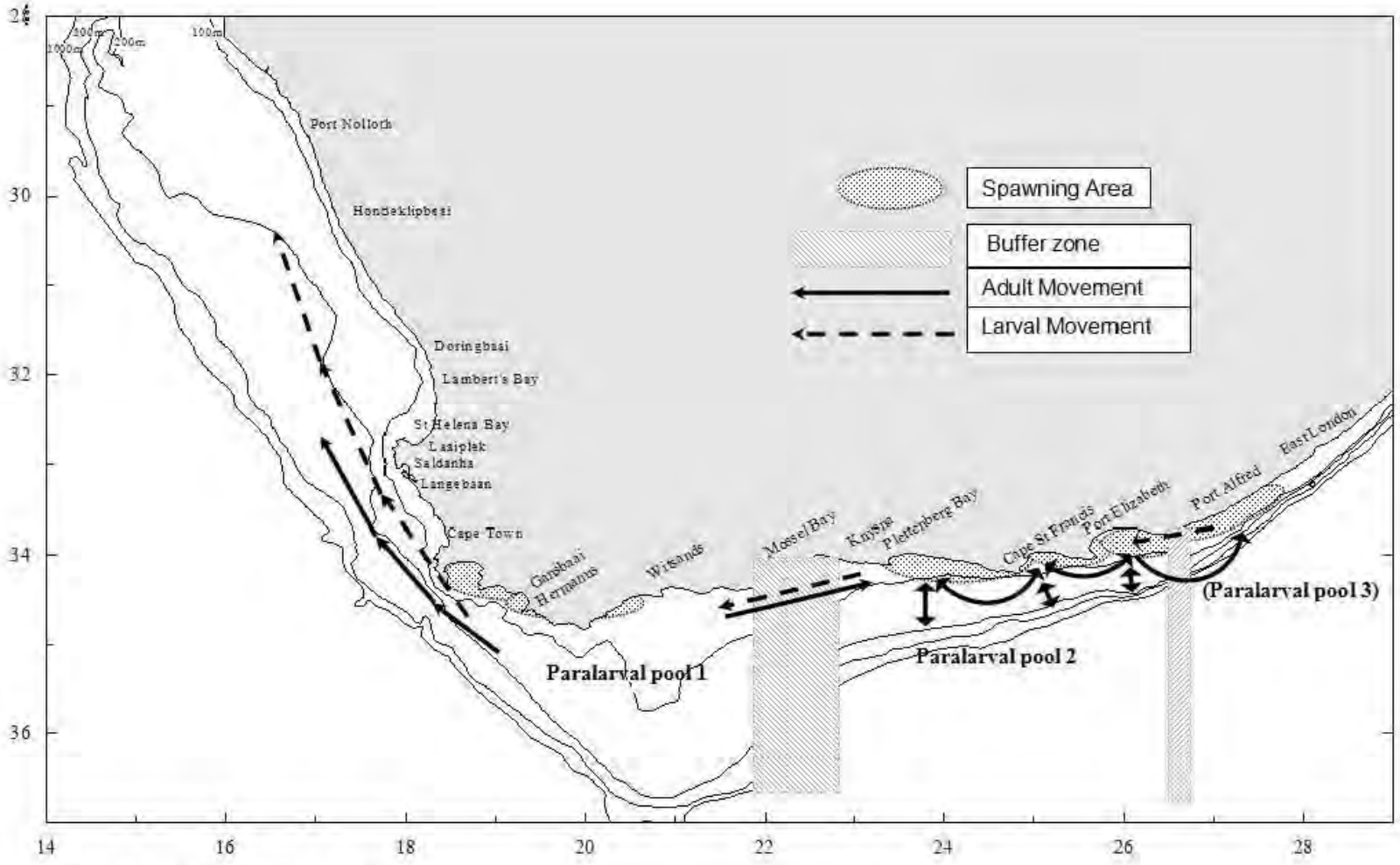


Fig. 11 Complex stock hypothesis

The possibility of subpopulations of *L. reynaudi* existing has previously been proposed by OIyott *et al. (*2007), based on the evidence of different spawning areas which could result in paralarvae that grow up under different environmental conditions. The results of this study support this, and add new ideas concerning identity, size and locations of these groups, and interactions between them. This provides a basis for further study at a finer resolution into where exactly the boundaries of these different groupings can be found. This stock structuring has implications for the management of the species and warrants further genetic research.

Lessons for future research learned from this study, were the need for a large number of available primers, as some loci can prove unsuitable for this kind of analysis, as well as the need for the inclusion of more intermediate sampling sites in order to improve the resolution.

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APPENDIX ONE

RESEARCH SUMMARY

Table A1.1: Principal research to date on *Loligo reynaudi* by category

Loligo reynaudi				
Life History	**Age and Growth**	**Fishery and Stock assessment**	**Genetics**	**Morphology**
Badenhorst 1974 Lipinski 1987 Augustyn 1990 Lipinski 1990 Lipinski and David 1990 Sauer and Lipinski 1990 Sauer 1991 Sauer and Lipinski 1991 Lipinski 1992 Sauer *et al.* 1992 Sauer *et al.* 1993 Augustyn *et al.* 1994 Lipinski 1994 Smale *et al.* 1995 Vecchione and Lipinski 1995 Peredo *et al.* 1996 Augustyn and Roberts 1997 Melo and Sauer 1997 Lipinski *et al.* 1998a Sauer *et al.* 2000 Schon 2000 Hanlon *et al*. 2002 Roberts 2005 Olyott *et al.* 2006	Lipinski 1991 Lipinski 1993 Lipinski and Durholtz 1994 Durholtz *et al*. 1995 Gerneke *et al.* 1995 Lipinski and Durholtz 1996 Durholtz *et al.* 1997 Durholtz and Lipinski 1997 Lipinski *et al.* 1997	Hatanaka *et al.* 1983 Uozumi *et al.* 1984 Wallace *et al.* 1984 Uozomi *et al.* 1985 Sauer *et al.* 1991 Augustyn *et al*. 1992 Augustyn *et al.* 1993 Augustyn *et al.* 1994 Roberts and Sauer 1994 Sauer 1995a Sauer 1995b Roberts *et al.* 1996 Booth *et al*. 1997 Augustyn and Roel 1998 Lipinski *et al.* 1998b Roel *et al.*1998 Roel *et al.* 2000 Schon 2000 Glazer and Butterworth 2006 Roel and Lipinski and Soule 2007	Durholtz *et al.* 1997 Shaw and Sauer 2004	Augustyn and Grant 1988 Sauer and Lipinski 1990 Vecchione and Lipinski 1995

APPENDIX TWO

SAMPLE LOCATIONS AND DISTANCES BETWEEN SAMPLE SITES

Table A2.1: Site coordinates for genetic samples, June-September 2006.

Sample Area	Mark	
	Latitide	Longtitude
Agulhas Bank	34° 23'25.43"S	18° 03'59.99"E
Tsitsikamma	34° 07'54.80"S	23° 50'02.44"E
Kromme	34° 09'04.48"S	24° 53'40.49"E
Algoa Bay	33° 53'45.22"S	25° 47'41.19"E
Kleinemonde	33° 33'29.58"S	27° 05'08.31"E
Angola	15° 09'53.48"S	12° 02'48.67"E

Table A2.2: Distances (km) between genetic sample sites, measured as shortest distance by sea.

Sample Location	Agulhas Bank	Tsitsikamma	Kromme	Algoa Bay	Kleinemonde
Tsitsikamma	546.06				
Kromme	648.85	102.79			
Algoa Bay	742.46	196.40	93.61		
Kleinemonde	868.68	322.62	219.83	126.22	
Angola	2244.64	2790.70	2893.49	2987.10	3113.32

Table A2.3: Site coordinates for morphological samples, June-September 2007

Sample Area	Mark	
	Latitide	Longtitude
Agulhas Bank	34° 27'29.51"S	18° 04'02.92"E
Eastern Cape	34° 07'56.09"S	23° 50'40.15"E
Offshore	34° 08'48.27"S	23° 50'09.46"E
Angola	15° 09'53.48"S	12° 02'48.67"E

Table A2.4: PCR reaction mixtures

Ingredients	Working Conditions (µl)	
	Lr21, 27, 34, 44, 48	Lfor1, 3
Reaction Buffer / dNTP	1/1	1/1
$MgCl_2$	0.2	0.25
Primer (x2)	0.5	0.4
Biotaq	0.05	0.05
DNA	2	5
ddH_2O	4.75	1.9