UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Genetic variation within Cape stumpnose, *Rhabdosargus holubi* Steindachner (Teleostei: Sparidae)

By

Carel Jakobus Oosthuizen

<u>Supervisors</u>
Prof. Paulette Bloomer
Dr. Paul Cowley

Submitted in partial fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

in the Faculty of Natural and Agricultural Sciences
University of Pretoria
Pretoria

© University of Pretoria

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

July 2006

Genetic variation within Cape stumpnose, *Rhabdosargus holubi* Steindachner (Teleostei: Sparidae)

By

Carel Jakobus Oosthuizen

Supervisor: Prof. P. Bloomer
Molecular Ecology and Evolution Programme
Department of Genetics
University of Pretoria
Pretoria

Co-supervisor: Dr. P.D. Cowley
South African Institute for Aquatic Biodiversity
Grahamstown

Department of Genetics

Magister Scientiae

Thesis abstract

Due to the nature of the marine environment genetic studies allow insight into behaviour and natural history that is difficult or impossible to identify by direct field observation. Current as well as historical population demography and gene flow can be detected by using molecular techniques. Genetic studies on only a few commercially important marine species along the South African coast have been conducted, although many marine fish species utilize estuaries as nursery areas and little attention has been afforded to studying larval distribution and recruitment

of these species from a molecular point of view. Many of these estuarine associated species, especially in the South African milieu, are important for recreational and subsistence use. Associated with southern African estuaries are 13 species of the family Sparidae of which Cape stumpnose *Rhabdosargus holubi* is the most abundant. Juveniles are mostly confined to estuaries while the adults are strictly marine. *Rhabdosargus holubi* are serial spawners but temporally separated spawning peaks have been recorded along the South African coastline.

Within the first part of this dissertation, the general characteristics of marine fish populations and the marine environment along the South African coast are being discussed. The main aim of this study was to determine the population genetic structure from estimates of nuclear and mitochondrial genetic variation across the distributional range of *Rhabdosargus holubi*. Samples were collected from 13 geographic localities along the South African coastline from St Lucia in the northeast to Klein River in the southwest. Juveniles were sampled in estuaries and adults were collected in the marine intertidal zone. Mitochondrial DNA control region fragments of 368 bp in length were obtained from a total of 214 individuals from all sampling localities. A total of 36 alleles were identified from 34 polymorphic sites. Following an allele homogeneity test, samples from different localities were lumped to represent six distinct geographical regions. Mitochondrial DNA control region analyses of juveniles showed high haplotype diversity and low nucleotide diversity with no divergent maternal lineages. No pattern between haplotype genealogy and geographic locality was evident.

Population genetic analyses using heterologous microsatellite amplification have been successfully completed for a number of studies, including numerous studies of variation within marine fish species. Microsatellite studies have proven to be more sensitive in detecting subtle population structure than mtDNA and/or protein polymorphisms in high gene flow species. A total of 113 microsatellite loci previously isolated from phylogenetically closely related marine fish species were tested for amplification. The success rate of heterologous microsatellite amplification was extremely low (0.02%), with only two polymorphic loci amplifying consistently for analysing 133 individuals sampled from six localities along the distributional range of *R. holubi*. Results from these two loci were insufficient to

draw conclusions about the population genetic structure of *R. holubi* along the South African coast. Possible reasons for the low rate of amplification success and future research recommendations are discussed. The findings from this study suggest that *R. holubi* is not geographically restricted, has high gene flow among localities and likely exist as a single stock.

Declaration

I declare that this dissertation, which I hereby submit for the degree Magister Scientiae at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Date: …………………   Signature: …………………

Vir ma en Oom Chris

Acknowledgements

I am especially indebted to my main supervisor Prof. Paulette Bloomer for giving me encouragement, hours of advice, guidance and funding during this study. I would also like to thank Dr. Paul Cowley for sharing his ichthyological knowledge. He together with Prof. Bloomer, provided invaluable advice as well as countless suggestions and their critical review of this dissertation contributed to enormous improvement.

I would also like to thank Dr. Paul Cowley for collecting specimens and professors: J Gold, T Turner, A Magoulas, P Smith and R Chapman, who generously provided our research group with microsatellite primers to test. Special thanks to the National Research Foundation, University of Pretoria and the South African Institute for Aquatic Biodiversity for funding of this project. Without your support this work would not have been possible.

I would like to thank all the MEEPers for fun, help and support in the lab. To Sandrina Dos Santos, Dr. Wayne Delport, Arrie Klopper, Illona Pelser, Lorraine Harris, Dr. Kelley Whitaker, Dr. Sarita Maree, Lucille Hermann, Marina Alais, Dr. Ernst Swartz, Jonathan van Alphen-Stahl and Isa-Rita Russo.

To all my friends, Eugene, Kristien, Tina, Pierre, Freddie, Adelle, Stefan, and especially Amanda Joubert for constantly reminding me of my thesis, thanks for your support and understanding. Thanks to Aret Meyer for all the support when I needed it, the wine, sushi and shopping, it was awesome! Thanks Jeanne and Marsha for all the nice food evenings and your support!

Sandrina, thanks for all your support and help. You won't know how much it means to me. Thanks for dealing with, and joining me for toast, and thanks for all the snacks, lunches and Lays®.

To my family, Jannie, Danie, Marina and almost-family (Petro), thanks for the support. Lastly and with enormous gratitude, I would like to thank my mother and father for all their support throughout my studies and making all of it possible.

No human being, however great, or

powerful, was ever so free as a

FISH.

<>< <>< <>< <>< <>< <><

John Ruskin (1819–1900)

English art critic.

Table of contents

Title page I
Thesis abstract II
Declaration V
Acknowledgements VII
Table of contents IX
List of tables XII
List of figures XIV

Chapter 1: 1

General introduction 1

1.1 The marine environment 1
1.2 Marine management and conservation in South Africa 3
1.3 The South African coastline 4
1.4 South African estuaries 8
1.5 *Rhabdosargus holubi* 9
1.6 Gene flow in the marine environment 12
1.7 The use of genetic markers in stock delineation studies 13
1.7.1 Mitochondrial DNA 14
1.7.2 Nuclear markers 15
Microsatellites 15
1.8 Combining mtDNA and microsatellite markers 17
1.9 Research questions 18
Expectations 18

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Chapter 2: 19

Mitochondrial DNA variation reveals a lack of geographic differentiation within the estuarine dependant Cape stumpnose *Rhabdosargus holubi* (Teleostei: Sparidae) from South Africa

Abstract 19
2.1 Introduction 20
2.2 Materials and Methods 24
2.2.1 Sample collection 24
2.2.2 DNA extraction 26
2.2.3 Primer design, mtDNA amplification and sequencing 26
2.2.4 Sequence analysis 28
2.2.5 Homoplasy 28
2.2.6 Homogeneity test 28
2.2.7 Genetic diversity 29
2.2.8 Neutrality test and population size history 29
2.2.9 Mantel test 31
2.3 Results 31
2.3.1 DNA extraction 31
2.3.2 Sequence analysis 31
2.3.3 Homogeneity test 36
2.3.4 Genetic diversity 36
2.3.5 Neutrality test and population size history 38
2.3.6 Mantel test 39
2.4 Discussion 39

Chapter 3: 47

A preliminary assessment of nuclear genetic variation in *Rhabdosargus holubi* based on heterologous microsatellite loci

Abstract 47
3.1 Introduction 47
3.2 Materials and methods 50
3.2.1 Sample collection 50
3.2.2 Polymerase chain reaction 52
3.2.3 Linkage disequilibrium 54
3.2.4 Hardy-Weinberg equilibrium 54
3.2.5 Population differentiation 55
3.2.6 Mantel test 55
3.2.7 Principle component analysis 56
3.3 Results 56
3.3.1 Optimisation 56
3.3.2 Microsatellite allele variation 57
3.3.3 Linkage disequilibrium 59
3.3.4 Hardy-Weinberg equilibrium 59
3.3.5 Population differentiation 61
3.3.6 Mantel Test 61
3.3.7 Principle component analysis 61
3.4 Discussion 64

Chapter 4: 69

Conclusion

Future research 71

References 72

Appendices 91

Appendix I 91

List of tables

Table 2.1 Aligned allele nucleotide sequences showing variable nucleotide positions in the 368 non-homoplasic sites of mtDNA control region for *Rhabdosargus holubi*. Allele numbers (1-36) are listed in the left column and polymorphic base pair positions are across the top row. Only nucleotides that are different from allele one are given for all other alleles. Nucleotides identical to allele one are represented by dashes (-). 32

Table 2.2 Number of *Rhabdosargus holubi* samples analysed (N_A) per sampling locality/region (listed in the left column). Alleles are listed across the top row, labelled 1-36, and alleles occurring within each sampling locality/region as well as the number of individuals sharing a specific allele (N_S) are shown. Dashes indicate the absence of alleles. 34

Table 2.3 Intraregional nucleotide sequence diversity (π, given as a percentage) and allele diversity (*h*) at all sampling regions as well as overall nucleotide and allele diversity values for *Rhabdosargus holubi*. 37

Table 2.4 Average number of pairwise differences between sampling regions of *Rhabdosargus holubi* above diagonal. Diagonal elements are average number of pairwise differences within sampling regions and pairwise F_{ST} values are below the diagonal. 37

Table 2.5 Summary of neutrality and population history test statistics for the mtDNA control region of *Rhabdosargus holubi*. 38

Table 3.1 Microsatellite loci identified for determining genetic variation among *Rhabdosargus holubi* sampled from six geographical localities along the South African coast. The Soc abbreviation is used for loci isolated from red drum, *Sciaenops ocellatus* (Sciaenidae) and GA2A was isolated from *Pagrus auratus* (Sparidae). 53

Table 3.2 Microsatellite loci used for determining genotype variation among *Rhabdosargus holubi* sampled from six localities along the South African coast. 57

Table 3.3 Microsatellite results for Soc12 and Soc428 for each sampling locality. For each sampling locality the following are shown: number of samples analysed (N), number of alleles identified (A), most common allele (A_C), frequency of the most common allele (F_C), allele range (A_R), expected heterozygosity (H_E), observed heterozygosity (H_O), p-value for Hardy-Weinberg equilibrium test (p) and the exact p-value for the linkage disequilibrium test (EP). 60

Table 3.4 Pairwise RST (above diagonal) and FST (below diagonal) comparisons between all sampling localities. No significant population differentiation values were obtained. 61

List of figures

Figure 1.1 Map of southern Africa showing the dominant currents (solid arrows) and the three biogeographical regions along the coastline of South Africa. Vertical, slanted and horizontal lines indicate the cool-temperate, warm temperate and subtropical regions, respectively. The area between the open arrows represents *Rhabdosargus holubi* distribution along the coast (Modified from Harrison & Whitfield 2006). 6

Figure 1.2 A picture of *Rhabdosargus holubi* illustrating the oval shaped silvery body and head as well as the characteristic yellow/golden line underneath the lateral line (van der Elst 1988). 10

Figure 2.1 Geographic locations of all sampling localities of *Rhabdosargus holubi* along the South African coast analysed for mtDNA control region variation. Sampling localities (1-13) are given in the legend together with the sample size per locality in parentheses. Localities lumped into regions are illustrated with the sample size per region in parenthesis. The area between the open arrows represents *Rhabdosargus holubi* distribution along the coast. (Map modified from Harrison & Whitfield 2006). 25

Figure 2.2 Sliding window across the 5' end of the mtDNA control region of *Rhabdosargus holubi* indicating the number of nucleotide substitutions per 25 base pair region. 33

Figure 2.3 Minimum spanning allele network summarizing genealogical relationships among 36 mtDNA alleles identified in a sample of 214 *Rhabdosargus holubi*. Each connecting line equals one mutation; perpendicular lines crossing branches indicate additional mutational changes along a path. Information to the side of each circle represents the allele number and the number of individuals, in parenthesis, that share a specific allele. The size of each circle represents the number of individuals sharing a specific allele and colours represent sampling localities/regions. Allele numbers follow Table 2.1. 35

Figure 2.4 Mismatch distribution showing the distribution of pairwise differences for 214 *Rhabdosargus holubi* obtained from mtDNA control region sequence data. Solid bars represent the observed pairwise differences and the curve represents the expected distribution under the sudden expansion model (Rogers & Harpending 1992) (p = 0.032). Theta0 = 0.000 (pre-expansion population size) and Theta1 = 3479 (post-expansion), with τ = 2.36 (time in number of generations, elapsed since the sudden expansion episode). 39

Figure 3.1 Geographic locations for all sampling localities of *Rhabdosargus holubi* along the coast of South Africa analysed for microsatellite variation. All sampling localities are given in the legend, together with the sample size per locality in parenthesis. A more detailed figure of the South African coastline can be seen in Chapter 1, Fig 1.1. (Modified from Harrison & Whitfield 2006). 51

Figure 3.2 Allele frequency distribution at locus Soc12 over all sampling localities for *Rhabdosargus holubi*. A total number of 14 alleles were identified. 58

Figure 3.3 Allele frequency distribution at locus Soc428 over all sampling localities for *Rhabdosargus holubi*. A total number of 21 alleles were identified. 58

Figure 3.4 A scatter plot of the PCA axes scores for all samples (133) from all localities/regions (6) of *Rhabdosargus holubi*. The x-axis of this plot is the first PCA axis, explaining 11.78 % of the variance; the y-axis is the 2nd PCA axis, explaining 11.50 % of the variance. Colour legend labels represents: Richards Bay Harbour (RBH), Mngazana region (MR), East Kleinemonde Estuary (EKE), Tsitsikamma National Park (TNP), Gourits Estuary (GE), Klein River Estuary (KRE). 62

Figure 3.5 A scatter plot of the PCA axes scores for the sampling localities of *Rhabdosargus holubi*. The x-axis of this plot is the first PCA axis, explaining 43.28 % of the variance; the y-axis is the 2nd PCA axis, explaining 21.31 % of the variance. Locality data point labels represents:

Richards Bay Harbour (RBH), Mngazana region (MR), East Kleinemonde Estuary (EKE), Tsitsikamma National Park (TNP), Gourits Estuary (GE), Klein River Estuary (KRE). 63

Figure 3.6 A scatter plot of the PCA axes scores for the three size classes of *Rhabdosargus holubi* from East Kleinemonde Estuary. The x-axis of this plot is the first PCA axis, explaining 64.63 % of the variance; the y-axis is the 2nd PCA axis. Data points represent size class ranges. 63

Figure 4.1 Mitochondrial DNA and microsatellite results indicate that mixing occurs along the coast potentially via larval dispersal after spawning events, however slightly lower genetic diversity was observed towards the southern Cape. 70

Chapter 1

General introduction

1.1 The marine environment

When considering studies that are based on fossils, comparative anatomy, embryology, and genetics, it is evident that fishes predate man's existence by some 400 million years (my) and all other vertebrates by 100 my. This might be due to the fact that, as at present, more than 70% of the earth's surface has been covered by water for millions of years (Lagler *et al.* 1962; Waples 1998). The importance of the sea is indicated by the fact that most of the world's biological diversity at higher levels (32 of the 33 known phyla) is found in this environment, including 15 phyla that are exclusively marine (Agardy 1994). Fishes have for long been the main resource exploited from the marine environment and extraction has caused a number of undesirable results, including the threat of extinction (Hjermann *et al.* 2004; Fromentin & Powers 2005; Mullon *et al.* 2005; Stockley *et al.* 2005). Some examples of overexploitation include the slender armourhead *Pseudopentaceros wheeleri* in the North Pacific, orange roughy *Hoplostethus atlanticus* in the South Pacific, roundnose grenadier *Coryphaenoides rupestris* in the Atlantic (Stockley *et al.* 2005), Peruvian anchoveta *Engraulis ringens*, North Sea herring *Clupea pallasii* and Newfoundland cod *Gadus morhua* (Hauser & Ward 1998). The main factors associated with fish becoming threatened, together with overexploitation, are habitat degradation, disruption of essential ecological processes, hydrological manipulations, environmental pollution and genetic contamination (Whitfield 1998). These threats are becoming increasingly serious as the global human population expands. In 1995, the Food and Agricultural Organization (FAO) reported that 69% of the world's marine fish stocks were either depleted, overexploited or fully to heavily exploited (Lauck *et al.* 1998). More recent estimates have suggested that over 90% of the world's fish stocks are overexploited (Carvalho & Hauser 1998). The exhaustion of fishery resources and reduction in marine biodiversity has been attributed to the lack of abundance data and misconceptions regarding the ecology and dynamics of marine systems and their biodiversity (Agardy 1994; Turpie *et al.* 2000).

A severe decline in coastal fishery resources from the 1950's onwards has also been documented from South Africa (Griffiths 2000). Griffiths *et al.* (1999) discussed a new management protocol for the linefishery of South Africa with the primary objective of

managing resources in such a way as to ensure fair and optimal sustainable utilisation. Specific goals are to recover overexploited linefish stocks and to maintain these stocks at an optimal production level. This is in line with the Convention on Biological Diversity in recognizing that biodiversity should be conserved at the genetic-, species-, and ecosystem level. The economic value of bio-resources, ecosystem services, aesthetics and rights of living organisms to exist are the four main justifications for conserving biodiversity (Frankham 1995). In order to optimally conserve biodiversity, accurate assessment is required.

Assessment methods currently used for stock identification are weakened by various factors that include the absence of sufficient information about the year-to-year recruitment, the age structure of currently identified stocks and also the availability of this information for at least a few years (Attwood 2003; Gotz 2005). The collection of this information is limited to a few economically important and highly abundant species. Other complicating factors include the fact that most fisheries are multi-target industries and exact effort cannot be determined for a specific species alone. For this reason, trends in catch composition are being used which introduces uncertainty to the reliability of assessments. Two models frequently used for assessment are: yield per-recruit and spawner biomass per-recruit models. Both of these models do not reflect stock size. Catch Per Unit Effort (CPUE) information is used as an alternative to these models. This information may well reflect the proportional availability of fish and do allow the comparison of inter-annual information, but do not provide any information on the status of a particular stock. In order to perform this, CPUE data are required that includes information from before exploitation started and this information does not exist for the South African linefishery, as is the case for worldwide fisheries (Attwood 2003; Gotz 2005). There is also doubt regarding the usefulness of data for this kind of comparison since increased efficiency in fishing methods (termed technology creep) may bias these results. These problems led to a number of studies that were unable to deliver results concerning the state of certain stocks and in most cases inaccurate conclusions were drawn (Gotz 2005). Examples include geelbek (*Atractoscion aequidens*, Griffiths & Hecht 1995), white steenbras (*Lithognathus lithognathus*, Bennett 1993a,b), red steenbras (*Petrus rupestris*, Smale & Punt 1991), red roman (*Chrysoblephus laticeps*), dageraad (*C. cristiceps*, Buxton 1987) and galjoen (*Dichistius capensis*, Attwood 2003).

Due to the nature of the marine environment genetic studies allow insight into behaviour and natural history that is difficult or impossible to identify by direct field observation. Current as well as historical population demography and gene flow can be detected by using molecular techniques (Avise 1998; Waples 1998). Genetic studies on only a few commercially important marine species along the South African coast have been conducted, for example

anchovy, pilchard, Cape hake and abalone (reviewed by Davies-Coleman & Cook 2000). Although many marine fish species utilize estuaries as nursery areas, little attention has been afforded to studying larval distribution and recruitment of these species from a molecular ecological point of view (Whitfield 1999; Griffiths 2000; Whitfield *et al.* 2000). Many of these estuarine associated species, especially in the South African milieu, are important for recreational and subsistence use (Wallace *et al.* 1984).

1.2 Marine management and conservation in South Africa

Fish harvesting along the South African coast dates back to the Middle Stone Age, about 70 000 ybp (years before present). Rock art in caves also illustrate that the San hunter-gatherers were utilizing fish (Beckley *et al.* 2002). Fish have become a very important source of protein and with increased demand, overexploitation of certain stocks has been inevitable. The three main directions for protecting depleted stocks or species that are in danger of becoming threatened are: (1) setting daily bag limits, (2) managing commercial fisheries and (3) establishing marine protected areas.

1. The first step taken to protect fish species that are vulnerable to angling and species that have shown a steady decline in catch per unit effort over the last few years, was the setting of daily bag limits to recreational anglers. Daily bag limits were set in 1984 on those species that were caught in high numbers. Restrictions were revised and new daily bag limits were set in 1992. There were cases in which the fishing mortality for certain fish species decreased considerably, but gradually increased again over time. This was mainly due to the fact that the restrictions were not severe enough to influence rehabilitation. Restrictions are now being frequently revised (Attwood & Bennett 1995).

2. It has been estimated that between 17.9 and 39.5 million tons (an average of 27.0 million) of fish are being discarded annually by global commercial fisheries as by-catch, with shrimp trawl fisheries accounting for almost a third of the global total, generating the highest proportion of discards of any fishery (Alverson *et al.* 1996). South African fisheries have a bycatch rate that varies between five and 70% (http://www.scienceinafrica.co.za/3marine.htm). Within South Africa, the two major restrictions imposed on commercial fisheries are size and effort restriction. By imposing mesh size restrictions (currently 75mm stretched mesh), the capturing of small/juvenile fish are considerably reduced (Booth & Buxton 1997).

3. The World Conservation Union defines Marine Protected Areas (MPAs) as: "Any area of intertidal or sub-tidal terrain, together with its overlaying water and associated flora, fauna, historical and cultural features, which has been reserved by law or other effective means to protect part or all of the enclosed environment" (Jarre-Teichmann *et al.* 1998). One of the main goals of marine protected areas (analogous to terrestrial nature reserves, also called Protected Marine Reserves or "no-take" areas) is to conserve genetic diversity of marine species and to restore the populations to such a level that life history traits that were evident before intense exploitation will be restored (Bohnsack 1993; Allison *et al.* 1998; Lauck *et al.* 1998; Trexler & Travis 2000; Gerber *et al.* 2002). If MPAs are selected and managed correctly, they will be able to protect biomass and population structure as well as protect essential life stages of commercial species, limit by-catch of juveniles, protect and enhance productivity, provide a location for marine research and educate the public, protect artisanal and community fisheries and thus protect ocean biodiversity (Bohnsack 1993; Lauck *et al.* 1998). Within South Africa there are currently six MPAs that collectively cover 17% of the total coastline (Attwood *et al.* 1997b). South Africa's three main existing marine protected areas are: De Hoop Marine Reserve in the southern Cape; Tsitsikamma National Park further east and St. Lucia Marine Reserve near the Mozambique border (Turpie *et al.* 2000). The coastline of South Africa displays a long and interesting history.

1.3 The South African coastline

Significant global temperature changes took place within the last five million years and the effect this had on the sea level along the west coast of South Africa was estimated based on paleoclimatic data on sea surface temperatures from the coast of Namibia (Lambeck *et al.* 2002). Three dramatic minimum temperature events were identified: a decrease in temperature was detected at 3.2 million years before present (mybp) that intensified at 2.0 mybp and 0.6 mybp (deMenocal 2004). From the African paleoclimatic records, three periods (2.9-2.4 mybp, 1.8-1.6 mybp and 1.2-0.8 mybp) of drier African conditions are recorded. Marine paleoclimatic records show extended periods (in the order of 1 000-10 000 years) of maximum and minimum variations. These extremes may have played a significant role in speciation events within the marine as well as the terrestrial environment (deMenocal 2004).

During the Cenozoic era (65 mybp – present), South Africa's coast experienced a series of transgressions and regressions caused by continental uplifting and seaward tilting of the subcontinent as well as sea level changes caused by glaciation cycles (Maud & Botha 2000).

During the Pleistocene epoch, the sea surface along the South African coast was 100 - 150 m below the present level (Day 1981; Maud 1990). Later, during the Holocene epoch, sea levels remained relatively stable (Maud 1990). Baxter and Meadows (1999) performed Late Quaternary radiocarbon-dating and palaeoenvironmental studies along the Western Cape at Verlorenvlei, which is a large freshwater coastal lake today. They used pollen analysis, sedimentology and geochemistry that revealed patterns of climate change during the last 20 000 years coupled with sea level fluctuations. It is believed that these sea level fluctuations influenced the ecology and geomorphology of the region. Focusing on the mid-to-late Holocene from 8 000 ybp, Lambeck *et al.* (2002) estimated that there were four distinct Holocene sea level phases: (1) an initial drop in sea level 8 000 ybp that lowered the sea level by approximately two meters; (2) a rapid sea level lowering phase around 6 500 ybp of three to four meters that allowed for sand dune establishment along the southern periphery of Verlorenvlei; (3) a sea level fluctuating phase with a sea level increase around 4 000 ybp; and (4) another sea level regression of approximately 2 metres around 3 500 ybp, followed by a small recovery at around 1 500 ybp. Since 1 500 ybp the sea level has remained relatively stable at the level that we observe today. This fluctuation in sea level along the entire coast of South Africa was also confirmed by studies conducted on the south, south eastern and eastern coast of South Africa (Rogers 1985; Maud 1990; Reddering 1990; Ramsay & Cooper 2002).

South Africa's coastline (Fig 1.1), between Kosi Bay, in the east, and the Orange River in the west, covers a distance of 3 100 km (Heydorn 1989). The continental shelf along the coast from Cape St Lucia to East London is within a 25 km width. Along the coastline between Durban and Richards Bay, also known as the Natal Bight, the continental shelf is much broader at 50 km (Lutjeharms & de Ruijter 1996). The continental shelf broadens towards the south and forms the Agulhas Bank just south of Cape Agulhas. On the west coast the continental shelf broadens from 40km at the Cape Peninsula to 180km off the Orange River (Shannon 1985). The coastline west of Cape Agulhas borders the South Atlantic Ocean and east of Cape Agulhas, the Indian Ocean (Fig 1.1). Along the west coast with water temperature reaching a maximum at 10 °C, the Benguela Current characteristically causes sporadic upwelling of cold, nutrient-rich water (Beckley 1988; Lutjeharms *et al.* 2000; Beckley *et al.* 2002; Lutjeharms *et al.* 2003a,b). Water along the east coast is typically warmer, generally higher than 22 °C with a maximum of 28 °C during late summer, due to the southward flowing Agulhas Current, transporting large quantities of warm water down the narrow continental shelf (Beckley *et al.* 2002).

The Agulhas Current flows at a core speed of 1.4 ms^{-1}, approximately 52 km off Durban at 28° latitude and has a width of 100 km. The Agulhas Current is driven by sub-gyral circulation of the South West Indian Ocean with discontinuous inputs from the Mozambique Current and the East Madagascar Current (Lutjeharms & de Ruijter 1996). The current is relatively stable in its flow. Approximately 20% of the time the current moves inside a 15 km

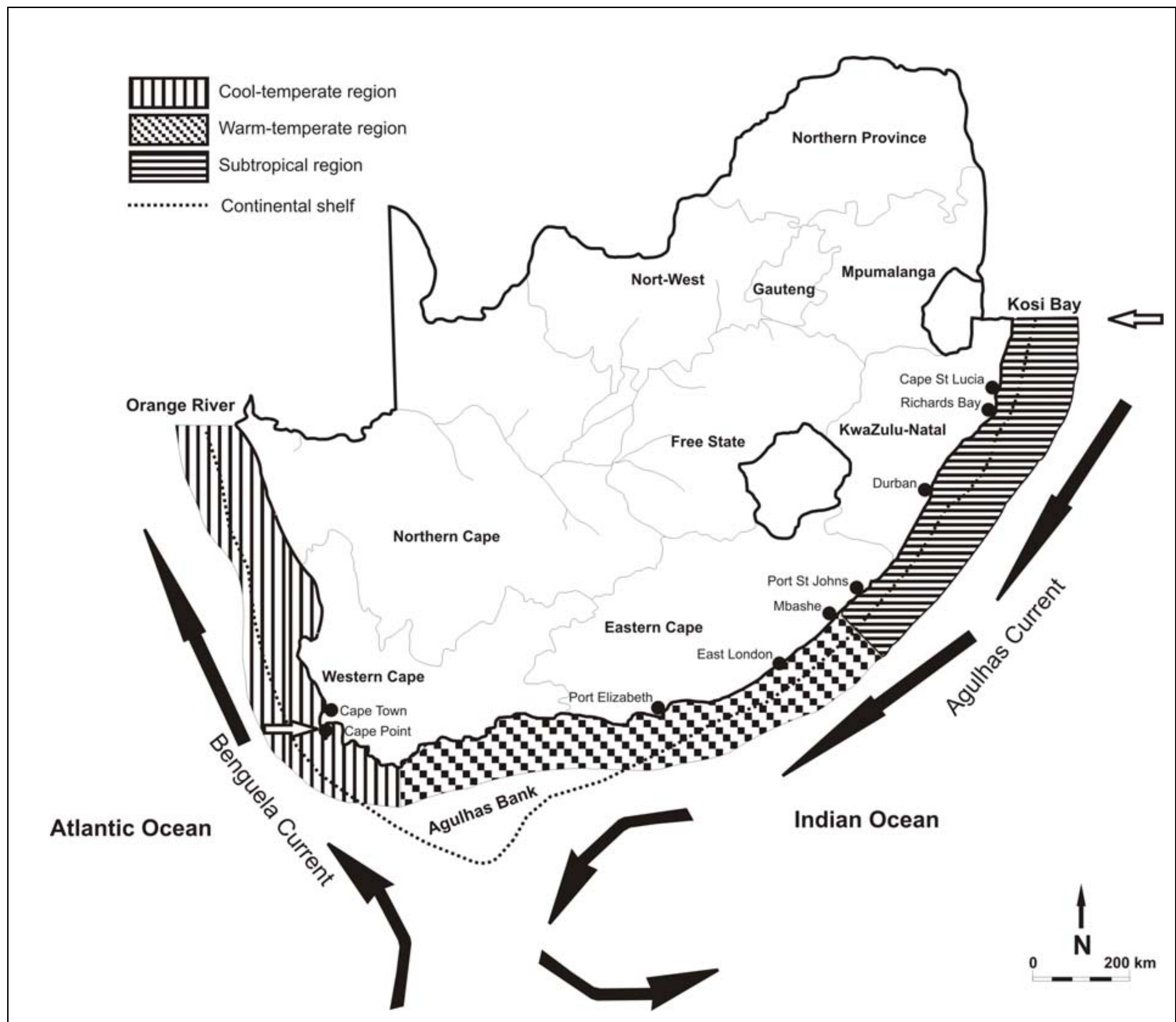

Figure 1.1 Map of southern Africa showing the dominant currents (solid arrows) and the three biogeographical regions along the coastline of South Africa. Vertical, slanted and horizontal lines indicate the cool-temperate, warm temperate and subtropical regions, respectively. The area between the open arrows represents *Rhabdosargus holubi* distribution along the coast (Modified from Harrison & Whitfield 2006).

margin during what is known as the Natal Pulse initiated in the Natal Bight caused by deep-sea eddies. This causes a circulatory movement of water, a coastal eddy, that flows in the opposite direction to the Agulhas Current (Lutjeharms *et al.* 2003b). The Agulhas Current

ends in a loop south of the Agulhas Bank, the Agulhas retroflection, with most of the water returning to the South Indian Ocean (Beckley 1988; Lutjeharms & de Ruijter 1996; Lutjeharms & Ansorge 2001). In addition to the coastal eddies on the east coast, upwelling also occurs along the eastern extreme of the Agulhas Bank as the current flows past East London to Port Elizabeth. Wind driven water upwelling at the edge of the Agulhas Bank causes cold eddies that move eastwards along the coast (Beckley 1988; Lutjeharms *et al.* 2000; Lutjeharms *et al.* 2003b). This has been determined by sea surface temperature studies, hydrographical studies and thermal infrared satellite imagery (Beckley 1988; Lutjeharms & de Ruijter 1996; Lutjeharms *et al.* 2000; Lutjeharms *et al.* 2003a). These processes along with temperature, geology and biological interactions are considered as the main factors affecting the distribution of the 1239 coastal fish species, of which 227 are endemic to southern Africa and 101 species endemic to South Africa (Randall 1998; Turpie *et al.* 2000).

Along the southern African coast, species richness and endemic species diversity decreases from east to west (Harrison & Whitfield 2006) and the number of endemic species is at a maximum in the region of Port Elizabeth (Fig 1.1) (Turpie *et al.* 2000). This observed pattern of decreasing species diversity from east to west is due to gradually cooler water to the west. Endemic marine fish species of southern Africa are dominated mainly by the Clinidae (klipfishes) and Sparidae (seabreams), with clinids mainly occurring between Cape Point and Port Elizabeth, whereas the sparids occur mostly between Cape Point and Durban, with a peak at the KwaZulu-Natal south coast (Turpie *et al.* 2000). Smith & McKay (1986) estimated that 80% of all marine fishes of shallow waters known from southern Africa occur in northern KwaZulu-Natal seas. The Agulhas Current has been identified as the major factor in the distribution of tropical and subtropical estuarine and marine fish species extending southwards along the South African coast due to the warmer water that the current provides to the region and also the dispersal opportunity that it offers since it is connected to the central species rich Indo-Pacific region (Maree *et al.* 2000). Although there is less diversity, in terms of species numbers, along the west coast where frequent upwelling of cold water from the Benguela Current causes the water temperature to stay below 10 °C, it is one of the most productive systems in the world housing several important industrial fisheries (Beckley *et al.* 2002).

Three biogeographical zones have been identified along the SA coastline, namely cool temperate, warm temperate and subtropical (Fig 1.1) (Whitfield 1998; Allanson & Baird 1999). According to Whitfield (1998) the cool temperate region extends westwards of Cape Point, with semi-arid conditions to the west and winter rainfall along the southwest. The

warm-temperate region extends eastwards of Cape Point to the Mbashe River and experiences both winter and summer rainfall. The subtropical region along the south east coast extends north-eastwards of the Mbashe River and experiences predominantly summer rainfall. Other authors have variously defined the boundaries between the biogeographical zones based on studying the patterns shared by marine and coastal associated taxa: rocky shore invertebrates (Emanuel *at al.* 1992); marine molluscs (Kilburn & Rippey 1982); intertidal fishes (Prochazka 1994); water-birds (Siegfried 1981; Hockey & Turpie 1999); rocky shore plants and animals (Stephenson & Stephenson 1972); rock pool fishes (Hockey & Buxton 1989) and estuarine associated fish species (Heydorn 1989; Whitfield 1998; Maree *et al.* 2000; Turpie *et al* 2000; Harrison 2002; James *et al.* 2003; Harrison 2004). These boundaries need to be seen as transition zones from one region to the next, influenced by the Benguela and Agulhas Currents and the role they play in maintaining or changing coastal water temperature, thus affecting species distribution along the coast (Maree *et al.* 2000; Turpie *et al.* 2000). Along the South African coast there are 117 subtropical, 123 warm-temperate, and 10 cool-temperate estuaries with a total surface area estimated at between 500 and 600 km^2, of which the estuaries in KwaZulu-Natal cover an area of approximately 400 km^2 (Heydorn 1989; Whitfield 1998). Estuaries play an important role in supporting high species numbers and diversity. Greater ichthyofaunal diversity is observed within large estuarine systems and is probably due to the greater habitat diversity within these large systems together with the marine interaction (Whitfield 1998).

1.4 South African estuaries

Estuaries can be viewed as transition zones between terrestrial and marine environments where freshwater mixes with salt water from the ocean (Lagler *et al.* 1962). An estuary is defined by Day (1980) as: “a partially enclosed coastal body of water which is either permanently or periodically open to the sea and within which there is measurable variation of salinity due to the mixture of sea water with freshwater derived from land drainage”. Associated with southern African estuaries are 53 fish families of which 50 have a primarily marine origin. Whitfield (1998) categorized these South African marine fish species according to their dependence on estuarine systems during different life history stages. The families that are most common in estuaries in terms of species number are the Gobiidae with 23 species, Mugilidae with 13 species and Sparidae with 13 species. Of these families, ten species of the Gobiidae, eight species of the Sparidae and three species of the Mugilidae are endemic to the southern African coast (Whitfield 1998). Within the Sparidae *Rhabdosargus holubi* is categorized as one of the dominant estuary-associated marine fishes and is

considered to be the dominant species in southeastern and southern Cape estuaries. In a study on the open East Kleinemonde estuary (Cowley & Whitfield 2001), *R. holubi* accounted for between 70- 80% of the total marine fishes within the estuary between 1994 and 1996. Successful entry and settlement of larvae into the estuarine environment is highly dependant on estuarine mouth phase (Cowley *et al.* 2001).

When the estuary mouth is open, recruitment of larvae and juvenile fish can occur actively or passively. Active entrance into estuaries occurs through swimming and the success of this method depends on the restricted swimming ability and limited energy reserves of the larvae (Bell *et al.* 2001). Passive transport is made possible by sea currents and offshore winds (Lagler *et al.* 1962). About 70% of South African estuaries are intermittently open to the sea. Marine overwash events (or overtopping events) provide access opportunities for larvae in the case where sand bars close off intermittently open estuaries. Overwash events occur when, at high tide, waves overtop sand bars that close estuaries from the sea, extending the surf zone over the sand bars to contact estuarine waters (Bell *et al.* 2001). Whitfield & Kok (1992) found this to be the case for *R. holubi* (amongst others) in the Haga-haga Estuary. The post-flexion larvae move directly from the surf zone into the estuary (Cowley *et al.* 2001). Larvae and juveniles utilize certain cues for orientation towards estuaries, including salinity-, temperature- and turbidity gradients as well as olfactory cues. Experimental studies showed that turbidity and olfactory cues play the most important role (Whitfield 1998). Dissolved organic and inorganic olfactory cues orientate estuarine dependent larvae towards estuarine habitats by water seeping through the sand bar of temporarily open/closed estuaries (Cowley *et al.* 2001). In the case of estuarine opening events, Strydom (2003) observed a significant increase in abundance of specifically *R. holubi.*

1.5 *Rhabdosargus holubi*

Rhabdosargus holubi (Fig 1.2) is an endemic sparid along the Southern African coast (Fig 1.1), and has been recorded from the Diep Estuary (around Cape Point) (Millard & Scott 1954) on the west coast to southern Mozambique (including Inhaca Island) (Mann 2000). Recordings of stray individuals have been made further westwards as far as False Bay (Clark *et al.* 1994). This distribution is believed to be mainly influenced by temperature (Blaber 1973a). According to Whitfield (1998), *R. holubi* is categorized as a euryhaline marine species that breeds at sea, with the juveniles being dependant on estuaries as nursery areas (category IIa). The juveniles are always present within estuaries while adults only occur in the sea, from the coast to a depth of 40m (Blaber 1973a,b; Wallace 1975a,b).

Blaber (1973a,b) intensively studied Cape stumpnose for the first time in the 1970's. His studies focused on the salinity and temperature tolerance, population dynamics, growth, food and the feeding ecology of *R. holubi* in the temporarily closed West Kleinemonde Estuary in the Eastern Cape. No detailed investigations on the reproductive biology, age and growth or movement behaviour of *R. holubi* have been undertaken. However, aspects of the biology and life history of this ubiquitous species have been documented in numerous studies (mostly within estuarine environments) conducted at different locations around the SA coastline. The main findings from these studies have been synthesized to provide insight into the life history of this species.

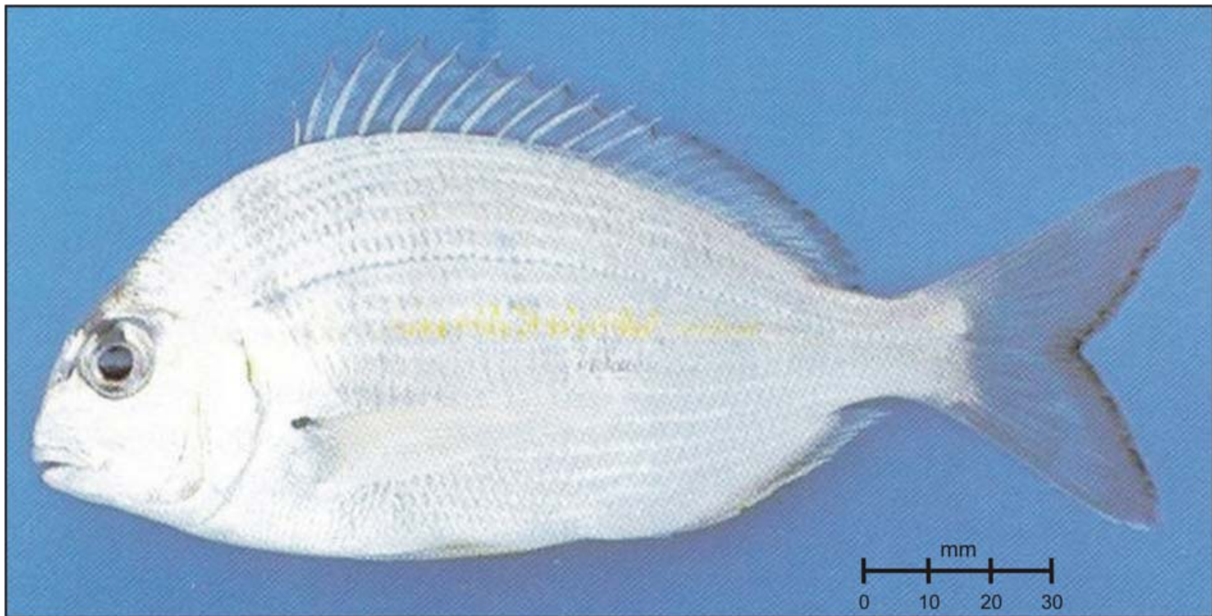

Figure 1.2 A picture of *Rhabdosargus holubi* illustrating the oval shaped silvery body and head as well as the characteristic yellow/golden line underneath the lateral line (van der Elst 1988).

Juveniles enter estuarine environments as postflexion larvae (7-14 mm total length) at an estimated age of one to two months old. It is suggested, in general, that sparid larvae require at least thirty days before they can actively settle (Brownell 1979; Brouwer *et al.* 2003). Recruitment into permanently open estuaries takes place on the flood tide and during open mouth phases or overwash events in temporarily open/closed estuaries (Beckley 1985; Whitfield 1989; Cowley & Whitfield 2001; Cowley *et al.* 2001). Once inside permanently open estuaries juveniles actively move towards the banks in order to prevent them from being swept out of the estuary (Beckley 1985).

Rhabdosargus holubi juveniles are normally found in mixed species shoals of between 10 to 400 individuals alongside macrophyte beds, mainly *Ruppia spiralis, Potamogeton pectinatus* and *Zostera capensis* beds (Blaber 1974a,b; De Wet 1988). Hanekom & Baird (1984) recorded *R. holubi* in significantly higher numbers in *Zostera* beds than in places lacking *Zostera* in the Kromme Estuary, indicating that macrophyte beds form an important habitat for juvenile *R. holubi* within estuaries (De Wet 1988). Not only do these plants serve as refuge areas for predator avoidance but also as a food source. Microorganisms that attach themselves to these plants are digested (Blaber 1974a,c; Blaber 1981). Experimental results showed that there is a correlation between tooth morphology and the type of food that is consumed at different life history stages (Blaber 1974a). As the size of the fish increases, they tend to eat larger prey with an associated change in tooth morphology. Juveniles possess tricuspid teeth that are ideally suited for biting off bits of aquatic macrophytes. Adult *R. holubi* mainly prey on bivalves and large crustaceans (Blaber 1974a; Blaber 1984) that are more abundant in the sea than in estuaries (De Wet 1988). Feeding activity in *R. holubi* in the Swartkops estuary appears to be dependant on the tide cycle rather than the diurnal cycle with peak feeding activity during the incoming tide (De Wet 1988). Juvenile *R. holubi* has a wide range of temperature (10 - 30 °C) and salinity (0.7 – 70$‰_0$) tolerance (Blaber 1973a,b; Blaber 1974a,b).

Male *R. holubi* become sexually mature at a total length of about 26 cm and in female fish, ripe gonads were found at a size of 14 cm (Blaber 1974a,b,c). Observed data indicate that they undergo an annual increase in size of between 60 mm, within the West Kleinemonde (Blaber 1974c), and 100 mm, within the Swartkops and KwaZulu Natal estuaries (Beckley 1983; Beckley 1984). *Rhabdosargus holubi* enters an estuary as fish fry at a size smaller than 20 mm (Blaber 1974c; Beckley 1986), spend at least the first year of their lives in this environment and return to the sea during their second year, before becoming sexually mature (Blaber 1974c; Beckley 1984). They attain a maximum size of 45 cm and the current South African angling record stands at 3.5 kg (Heemstra & Heemstra 2004).

Different spawning peaks are observed throughout the distributional range of *R. holubi* that corresponds to rainfall and temperature along the coast. This implies that temperature plays an important role in determining spawning peaks and that this could be the period of lower waiting times for access to the estuarine environment, resulting in higher survival rates (Bell *et al.* 2001). Estuarine immigration of marine-spawned larvae occurs mainly during late winter and spring when river flow is often at a minimum along the KwaZulu-Natal and northern Eastern Cape coasts (Wallace 1975a). The presence of *R. holubi* in the surf zone adjacent to the East Kleinemonde estuary (Eastern Cape) peaks in August (Bell *et al.* 2001).

Recruitment into estuaries along the southern Cape occurs mainly during spring (Wallace 1973a; Blaber 1974c) and into estuaries found along the southwestern Cape, well into the summer (Beckley 1983).

Results from tagging studies (Sedgewicks/WWF/ORI national tagging programme) provide little insight into the movement behaviour or migrations of adult *R. holubi*. To date 833 fish, mostly adults, have been tagged but yielded only six recaptures (0.72%). Four fish were recaptured at their tagging locality within the same year. The fifth individual was recaptured after ten days at a locality nine kilometres from where it was tagged. The sixth individual was recaptured after two years and had traveled a distance of 56 km. Three potential reasons exist for this low level of tag return data: (1) *R. holubi* suffers from high tagging mortality, (2) a high tag shedding rate exists, or (3) existence of a very large population of adult *R. holubi* (Bullen & Mann 2004).

De Wet (1988) suggested that *R. holubi* is not of much importance or commercial value due to their small size. However, recent estuarine fishery surveys have indicated that it was the dominant catch species in a number of estuaries and is an important subsistence fishery species (Cowley *et al.* 2001; Smith in prep). Genetic information is becoming more crucial in studying marine species. The knowledge of genetic variation within and between populations provides important information of interactions between separate populations since direct observations, especially in the marine realm, are difficult or impossible.

1.6 Gene flow in the marine environment

In the marine environment there is a relative absence of barriers to gene flow, compared to terrestrial and freshwater environments (Avise 1998) and this leads to the fact that over vast distances populations of the same species might still be genetically linked or connected. Additionally, marine organisms tend to have large population sizes and this fact alone will decelerate the process of genetic divergence between isolated populations or stocks (Palumbi 1994). Defining genetically isolated fish populations or stocks of the same species has proven to be much more difficult than expected. Many definitions for stocks currently exist. Within this study a stock will be defined as “an intraspecific group of randomly mating individuals with temporal and spatial integrity”, according to Ihssen *et al.* (1981). Gene flow affects the integrity of stocks and is influenced by various factors (see below).

Compared to freshwater environments, there is a relative absence of barriers to prevent migration and marine subpopulations may exchange between 10 and 100 times more migrants per generation compared to freshwater subpopulations (Ward *et al.* 1994). The degree of differentiation among marine fish stocks (measured by F_{ST}) averages 0.062 in marine fish species compared to freshwater fish at 0.222 (Ward 2000).

The movement behaviour and migrations of fishes are influenced by physical, chemical and biological factors. Physical factors include temperature, turbidity, light intensity, photoperiod, water depth, atmospheric pressure, currents, tide movement and topography (Lagler *et al.* 1962, Lundy *et al.* 1999). Chemical factors include salinity, alkalinity, hydrogen ion concentration, dissolved gasses, odours, tastes, and pollutants. The biological factors include blood pressure, sexual development, phototaxis, social response, predators, competitors, food, memory, physiological clock and endocrine state (Lagler *et al.* 1962). Since greater opportunity for stock intermixing exists compared to freshwater fish, only slight differences between stocks can be expected (Waples 1998). Genetic markers have proven in numerous studies to be capable of detecting these subtle differences.

1.7 The use of genetic markers in stock delineation studies

In the past, non-genetic or phenotypic markers, which can be influenced by the environment, were used to discriminate between different stocks and managing these different stocks. These markers used included scale analyses, parasitic specificity, morphometric and meristic characters, growth and gonadal indices (Garcia de Leon *et al.* 1997). Genetic markers are more sensitive in determining stock structure and studying the patterns of genetic variation within a species provides insight into the reproductive patterns, past geographic structure and dispersal patterns of populations (Arnaud *et al.* 2001; Feral 2002; Gold & Turner 2002; Wakeley 2005). Understanding stock structure contributes to understanding how migration (gene flow), random genetic drift and selection shape populations and divergence among populations (Arnaud *et al.* 2001; Wakeley 2005). This in turn will enhance conservation planning and the optimal use of available resources (Gold & Turner 2002). Knowledge about genetic variation within and between populations also has the potential of revealing the importance of/or identify factors such as physical processes (oceanic currents and circulation), behaviour of adults and juveniles and stable migration routes and how these factors influence the species dynamics or distribution (Gold & Turner 2002), not detected by morphological or observational studies. As an example: Cimmaruta *et al.* (2005) studied hake (*Merluccius merluccius*) in the Atlantic Ocean and Mediterranean Sea using allozyme

markers. They were able to identify salinity and temperature along with local gyres as sufficient barriers to gene flow, maintaining genetic differentiation between Atlantic Ocean and Mediterranean Sea stocks. They also found, as would be expected, that the overexploited Atlantic stock had reduced levels of genetic variability. A mitochondrial DNA (mtDNA) study performed on a widespread estuarine-dwelling sparid *Acanthopagrus butcheri* from southern Australia revealed an interesting pattern of gene flow between adjacent estuaries, in a stepping stone fashion, explained by adult movement (Burridge *et al.* 2004). Frequently used genetic markers for stock determination studies are mtDNA and nuclear markers such as microsatellites.

1.7.1 Mitochondrial DNA

A powerful and direct method of obtaining information on the amount of genetic variation or extent of genetic divergence comes from complete sequencing of homologous DNA fragments from different organisms and comparing them (Harrison 1989b). Homologous DNA sequences are clearly provided by the small, closed-circular mitochondrial genomes of animals, that is exceptionally compact with very few intergenic sequences, in which all of the mtDNA genes are linked to each other but are totally unlinked to nuclear genes (Moritz *et al.* 1987; Harrison 1989b). The genetic content within animal mtDNA is remarkably conservative over different taxa. The mtDNA molecule consists of two ribosomal RNA (rRNA) genes, 22 transfer RNA (tRNA) genes and 13 genes that code for proteins involved in electron transport or ATP synthesis. Each mtDNA molecule also has a control region containing sequences functioning in initiation of replication and transcription. No introns and only a few examples of insertions or duplications can be found, except in the control region of the mtDNA molecule (Avise & Lansman 1983; Avise *et al.* 1987; Harrison 1989b).

Mitochondrial DNA evolution appears to proceed in a much more simplified manner than the evolution of nuclear DNA as there is a great reduction in the number of mechanisms of variation available to mtDNA (Moritz *et al.* 1987). Important characteristics of mtDNA include rare or no recombination, rapid sequence evolution, high copy number and maternal inheritance. Since only females pass on mtDNA to the next generation, the effective population size for mtDNA is smaller than that of nuclear genes (Moritz *et al.* 1987) and this enables the detection of population subdivision more easily (Lee *et al.* 1995). This also implies that stochastic processes will have an important role in determining frequencies of mtDNA alleles and any gene flow mediated by male dispersal will not affect the spatial pattern of variation for mtDNA (Wilson *et al.* 1985).

Within teleost species, the mitochondrial control region shows a high variability in length, even between closely related species due to the presence of tandemly repeated sequences and large insertions. In a study performed by Bernardi *et al.* (2001) on coral reef fish, they concluded that mtDNA control regions within fishes are a molecular marker of choice for studying populations that evolve rapidly. In this study they found that the mtDNA control region in Indo-West Pacific butterfly fish was extremely variable at up to 33-43 times more than the cytochrome *b* region. Because mtDNA shows considerable variation among individuals, within and between populations, it has proved to be an effective marker for studying population structure. Analysing the historical relationships of alleles in a geographic context (phylogeography) enable the inference of patterns of gene flow between geographically separated populations (Avise *et al.* 1987; Hare 2001). Studying population structure and the patterns of intraspecific geographic variation has been applied in numerous studies on marine species (Ferris & Berg 1987; Gold *et al.* 1993; Chow *et al.* 2000; Chow & Takeyama 2000; Escorza-Trevino & Dizon 2000; Gilles *et al.* 2000; Hanel & Strurmbauer 2000; Lundy *et al.* 2000; Pfeiler *et al.* 2005; Triantafyllidis *et al.* 2005). One of these, a study of the genetic stock identification in the bigeye tuna *Thunnus obesus* has shown that there are two distinct stocks within the Atlantic and Indo-Pacific regions with limited gene exchange between these two regions taking place along the South African coast (Chow *et al.* 2000). This result would not have been clear by observational or morphological studies. At the other extreme, Pfeiler *et al.* (2005) studied the population genetic structure (using mtDNA) of the swimming crab *Callinectes bellicosus* from the eastern Pacific Ocean and detected no genetic structure with high dispersal ability.

1.7.2 Nuclear markers

Numerous methods are available for screening variation at nuclear DNA loci. The method most commonly used in fish studies has been allozymes (developed by Hunter & Markert 1957), however, over the past ten years there has been a shift towards utilizing microsatellite markers (Lui & Cordes 2004). Allozyme electrophoresis is used in order to identify different alleles of protein coding genes. This method is relatively inexpensive compared to microsatellite analysis, but the number of variable loci and the level of variation of allozyme markers are generally very low. These problems are overcome by using microsatellite markers in that they are abundant, distributed genome wide and highly variable (Feral 2002).

Microsatellites

Many population studies on fish have been conducted using microsatellite DNA markers (Brooker *et al.* 1994; Batargias *et al.* 1999; Stockley *et al.* 2000; De Innocentiis *et al.* 2001;

Reusch *et al.* 2001; Chiu *et al.* 2002; Laikre *et al.* 2005; Stockley *et al.* 2005). Microsatellites are short and usually 2-6 base pairs long, tandem repeated sequences found in the nuclear genome (Goldstein & Pollock 1997). They occur throughout the genome, on average every 50 000 to 60 000 bp (Moore *et al.* 1991, Lowe *et al.* 2004). Unique primers are designed for amplifying a genomic region that contains the well-defined repeat unit. Microsatellites show a relative high rate of mutation, due to polymerase slippage, where polymerase tends to miscopy repeated units (Levinson & Gutman 1987), and slipped-strand mispairing during replication (Hancock *et al.* 1999). Variation in the repeat unit length between alleles within individuals and between individuals is responsible for the observed variation (Goldstein & Pollock 1997).

Microsatellites are ideal for population genetic studies, because of the ease of DNA extraction from small tissue samples that could, if sampled correctly, cause no or little trauma to subjects, high level of variation within loci and the relative ease of scoring, genome wide distribution and their biparental inheritance (Moore *et al.* 1991; Garcia de Leon *et al.* 1997; Goldstein & Pollock 1997). The fact that they are distributed throughout the genome allows modelling of the divergence of populations as opposed to the genealogical history of particular genomic regions. Microsatellites are biparentally inherited which make them less sensitive to effective population size fluctuations, as is the case with mtDNA (Gold & Turner 2002).

There are however some potential problems associated with using microsatellites: irregular pattern of microsatellite mutation between different loci, causing differing mutational rates between loci (Takezaki & Nei 1996) and the apparent upper limit in the number of repeats (Goldstein & Pollock 1997). This will increase the variance of distance values (Takezaki & Nei 1996).

Another major drawback is the long development time of these markers and the cost involved (Mueller & Wolfenbarger 1999). Due to the high cost and time involved in the identification of species-specific polymorphic microsatellites and the development of primers, one option is to test and make use of loci identified in other related species. Usefulness of these so-called heterologous amplifications are somewhat uncertain, with possible decrease in the level of polymorphism and possible increase in null alleles as the phylogenetic distance increases from the source species (Moore *et al.* 1991; FitzSimmons *et al.* 1995; Angers & Bernatchez 1996). In a study conducted by Rico *et al.* (1996) on marine fish, it was found that some microsatellite regions were conserved for over 470 million between a number of diverse fish species belonging to different suborders. Microsatellites are very

useful for studying closely related populations (Takezaki & Nei 1996). In a study performed on northern pike *Esox lucius* using differences in the distribution of microsatellite allele frequencies Laikre *et al.* (2005) were able to show the existence of isolation by distance within a seemingly random mating, continuously distributed species. In another study on whiting *Merlangius merlangus* by Rico *et al.* (1997), using microsatellite loci isolated from this species as well as microsatellite loci isolated from closely related species, a low level of differentiation between sampling localities were detected.

Inferential methods have been developed based on the mode of microsatellite evolution. Mutations at microsatellite loci are generally believed to result in one of two patterns: (1) A stepwise fashion, changing allelic sizes by one or a very few number of repeats, resulting in allele sizes that could already be present in a population or in new alleles (Goldstein & Pollock 1997), termed the Stepwise Mutation Model (SMM); or (2) A more classical model is the Kimura & Crow's (1964) Infinite Alleles Model (IAM) where each mutation results in a new unique allele. Mathematical models designed for analysing microsatellite data generally assume either the SSM or IAM or generalizations thereof. The strength of the discriminating power of microsatellites in detecting weak population structure is the high level of polymorphism associated with these loci. This generally provides high statistical power (Gold & Turner 2002). Loci conforming to the SMM have higher statistical power since allele size and frequency are taken into account, but the latter lead to a higher variance and increased sensitivity to unequal sample sizes (Carvalho & Hauser 1998).

1.8 Combining mtDNA and microsatellite markers

When populations are in mutation/drift equilibrium and equal male and female dispersal occurs, although rarely observed for most species, one would expect to find that the degree of divergence in mtDNA be four times higher than that in nuclear DNA (Hare 2001; Gold & Turner 2002). Thus, as a result of the mode of mtDNA inheritance, mtDNA is more sensitive to bottlenecks in population size (Wilson *et al.* 1985). Conversely, subtle population structure has been demonstrated with the use of microsatellites in cases where mtDNA and/or protein polymorphisms failed to detect genetic heterogeneity among geographic localities (Gold & Turner 2002; Stockley *et al.* 2005). The joint comparative study of mtDNA and nuclear DNA variability should therefore be advisable in the study of genetic differentiation within marine fish species and can provide important cues about the history of these species and details of current population structure (Wilson *et al.* 1985; Gold & Turner 2002).

1.9 Research questions

Genetic studies on only a few commercially important marine species along the South African coast have been conducted (reviewed by Davies-Coleman & Cook 2000). Although many marine fish species utilize estuaries as nursery areas, little attention has been afforded to studying larval distribution and recruitment of these species from a molecular point of view (Whitfield 1999; Griffiths 2000; Whitfield *et al*. 2000). Many of these estuarine associated species, especially in the South African milieu, are important for recreational and subsistence use (Wallace *et al*. 1984).

The aim of the present study was to answer the following questions:

- How much genetic variation or diversity exists within *R. holubi*?
- How is this variation distributed between the different sampling localities that are largely representative of the distribution of the species? Are samples that are geographically separated also genetically differentiated?
- How do these results compare to completed studies on marine fish with similar life history traits?
- If genetic differentiation is detected, where are the potential phylogeographic barriers to gene flow between these samples, how much gene flow is taking place and how does this compare to other studies performed in the same region?

Expectations

Rhabdosargus holubi is distributed within all three biogeographical regions identified along the South African coast, illustrating that this species is able to withstand a wide range in water temperature. It is expected that adult *R holubi* are able to move over vast distances within the marine environment due to a relative absence of obvious barriers to gene flow. Eggs and larvae are also expected to be widely distributed along the coast after spawning events, mainly by long-shore wind driven currents before larvae and juveniles enter and settle within the estuarine environment. Eggs and larvae spend at least 15 days within the near-shore environment, allowing ample opportunity for passive transport by currents and thus the mixing of larvae from separate spawning events. This high dispersal potential of *R. holubi* will expectantly result in no detectable level of population differentiation.

Chapter 2

Mitochondrial DNA variation reveals a lack of geographic differentiation within the estuarine-dependant Cape stumpnose *Rhabdosargus holubi* (Teleostei: Sparidae) from South Africa

Abstract

There are 13 species of the family Sparidae associated with southern African estuaries of which Cape stumpnose *Rhabdosargus holubi* is the most abundant. Juveniles are mostly confined to estuaries while the adults are strictly marine. *Rhabdosargus holubi* are serial spawners but temporally separated spawning peaks have been recorded along the South African coastline. The aim of this study was to determine whether *R. holubi* in South African waters exist as a single spawning population or several discrete populations by using mitochondrial DNA (mtDNA) analysis. Samples were collected from 13 geographic localities along the South African coastline from St Lucia in the northeast to Klein River in the southwest. Juveniles were sampled in estuaries and adults were collected in the marine intertidal zone. Mitochondrial DNA control region fragments of 368 bp in length were obtained from a total of 214 individuals from all sampling localities. A total of 36 alleles were identified from 34 polymorphic sites. Following an allele homogeneity test, samples from different localities were lumped to represent six distinct geographical regions. Mitochondrial DNA control region analyses of juveniles showed high haplotype diversity and low nucleotide diversity with no divergent maternal lineages. No pattern between haplotype genealogy and geographic locality was evident. The findings from this study suggest that *R. holubi* is not geographically restricted, has high female gene flow among localities and likely exist as a single genetic stock.

2.1 Introduction

The marine environment is characterized by a relative absence of barriers to gene flow when compared to freshwater and terrestrial environments (Avise 1998) and marine species may exchange between 10 and 100 times more migrants per generation (Ward *et al.* 1994). However, physical (bottom materials, water depth, water pressure, atmospheric pressure, current and tide movement, turbidity, topography, thermal gradients, temperature, light intensity and photoperiod), chemical (salinity, alkalinity, hydrogen ion concentration, dissolved gasses, odours, tastes, and pollutants) and biological factors (blood pressure, sexual development, phototaxis, social response, predators, competitors, food, memory and endocrine state) have been identified in influencing fish distributions and may act as barriers to gene flow (Lagler *et al.* 1962, Carvalho & Hauser 1998; Graves 1998; Waples 1998; Lundy *et al.* 1999; Maree *et al.* 2000; Turpie *et al.* 2000; James *et al.* 2002; Briggs *et al.* 2004; Harrison & Whitfield 2006). Since greater dispersal opportunities exist within the marine environment, only subtle differences between spatially or temporally separated populations are expected (Avise 1998; Waples 1998) and genetic markers have proven to be sufficiently sensitive in detecting these subtle differences (see for example Gold *et al.* 1993; Graves 1998; Gold & Richardson 1998; Stockley *et al.* 2005).

In the past, non-genetic or phenotypic markers, which can be influenced by the environment, were used to discriminate between different populations (Garcia de Leon *et al.* 1997). Genetic markers are however more sensitive in detecting population structure. Studying the patterns of genetic variation within a species can reveal reproductive patterns, past geographic structure and dispersal patterns of populations (Arnaud *et al.* 2001; Feral 2002; Gold & Turner 2002; Wakeley 2005). A direct method of obtaining information on the amount of genetic variation or extent of genetic divergence comes from complete sequencing and comparison of homologous DNA fragments from different individuals within and between sampling localities (Harrison 1989b). Such homologous fragments are provided by the mitochondrial genome that contains very few intergenic sequences, is relatively small and is completely unlinked to the nuclear genome (Moritz *et al.* 1987; Harrison 1989b). Further important characteristics for population analysis include maternal inheritance, thus a decreased effective population size, and allele frequencies influenced by stochastic processes, making it possible to track past population changes (Wilson *et al.* 1985; Moritz *et al.* 1987; Lee *et al.* 1995; Hare 2001, Domingues *et al.* 2006). Studying population structure and the patterns of intraspecific geographic variation have been applied in numerous marine studies (Ferris & Berg 1987; Harrison 1989a; Gold *et al.* 1993;

Escorza-Trevino & Dizon 2000; Gilles *et al*. 2000; Hanel & Strurmbauer 2000; Lundy *et al*. 2000; Bernardi *et al*. 2001; Cimmaruta *et al*. 2005; Pfeiler *et al*. 2005; Triantafyllidis *et al*. 2005).

Studies conducted on estuarine associated species showed contrasting results. Bargelloni *et al*. (2003) studied five biologically similar sparid species (*Lithognathus mormyrus*, *Spondyliosoma cantharus*, *Dentex dentex*, *Pagrus pagrus* and *Pagellus bogaraveo*) that share similar life history characteristics as well as the same distributional pattern along the Strait of Gibraltar. Three of the five species (*L. mormyrus*, *S. cantharus* and *D. dentex*) showed differentiation between the two biogeographical regions explained either by an ecological difference between the two regions or as a consequence of historical events. The other two species (*P. pagrus* and *P. bogaraveo*), in contrast, displayed high levels of gene flow. The genetic structure of the marine and estuarine distributed silverside *Odontesthes argentinensis* from southern Brazil showed divergence between different geographical localities and this pattern was explained by homing behaviour and isolation-by-distance (Beheregaray & Sunnucks 2001). A mtDNA study performed on the widespread estuarine-dwelling sparid *Acanthopagrus butcheri* from southern Australia revealed a stepping stone pattern of gene flow between adjacent estuaries whereby adults were dispersed during flooding events (Burridge *et al*. 2004). According to Bilton *et al*. (2002), estuarine species being washed out to sea during flooding events allows for dispersal of larger (adult) individuals, but when recruitment takes place it does not necessarily occur into the original environment. Following spawning events, several biological and physical factors play a role throughout the process of larval recruitment and settlement within estuaries (Brown *et al*. 2005). The duration of this process varies between different species, providing ample opportunities for larvae to be transported long distances by major currents as well as long-shore currents. The southern tip of the African continent, bounded by two different current systems, provide unique opportunities to study the biological and environmental effects influencing larval dispersal.

The South African coastline between Kosi Bay, in the east, and the Orange River in the west, covers a distance of 3 100 km (Heydorn 1989). The coastline west of Cape Agulhas borders the South Atlantic Ocean and east of Cape Agulhas, the Indian Ocean (Fig 2.1). West of Cape Agulhas flows the cold Benguela Current, normally colder than 10 °C, along the west coast of South Africa, causing frequent upwelling of cold nutrient-rich water that is most intense off Luderitz (Beckley 1988; Lutjeharms *et al*. 2000; Lutjeharms *et al*. 2001; Beckley *et al*. 2002; Lutjeharms *et al*. 2003b). Water along the east coast is typically warmer, generally higher than

22 °C with a maximum of 28 °C during late summer, due to the southward flowing Agulhas Current, transporting large quantities of warm water down the narrow continental shelf (Beckley *et al*. 2002). The Agulhas Current flows approximately 52 km off the Durban coast but moves further away from the coast in its southwards flow after reaching Port Elizabeth, and even further from the coast as the continental shelf broadens at what is known as the Agulhas Bank and ends in the Agulhas retroflection (Beckley 1988; Lutjeharms & de Ruijter 1996; Lutjeharms & Ansorge 2001). The flow pattern of the Agulhas Current not only influences the coastal water temperature and dynamics, but also the rainfall pattern along the coast (Lutjeharms & de Ruijter 1996; Lutjeharms *et al*. 2001). Along the KwaZulu-Natal coast there are coastal eddies that moves in the opposite direction of the Agulhas Current, close to the shore. These coastal eddies have been identified in providing passive transport to eggs and larvae of marine species (Lutjeharms *et al*. 2003b).

Rhabdosargus holubi (Fig 1.2) is an endemic sparid along the Southern African coast (Fig 1.1), and has been recorded from the Diep Estuary (around Cape Point) (Millard & Scott 1954) on the west coast to southern Mozambique (including Inhaca Island) (Mann 2000). Recordings of stray individuals have been made further westwards as far as False Bay (Clark *et al*. 1994).

The Cape stumpnose *R. holubi* is a Southern African endemic, recorded from the Diep Estuary (around Cape Point) (Millard & Scott 1954) on the west coast to southern Mozambique in the east (including Inhaca Island) (Blaber 1973a,b; Mann 2000). Recordings of stray individuals have been made further westwards as far as False Bay (Clark *et al*. 1994). According to Whitfield's (1998) categorisation of estuarine associated fishes, *R. holubi* is a euryhaline marine species that usually breeds at sea but the juveniles are dependent on estuaries as nursery areas (category IIa). Year round spawning has been recorded along the coast for *R. holubi*, however spawning peaks have been observed. Harris & Cyrus (1996) recorded peaks in larval abundance in the St Lucia surf zone during June and August. Cowley *et al.* (2001) revealed a significant peak in abundance during late winter (August) in the East Kleinemonde surf zone (Bell *et al*. 2001). Whitfield & Kok (1992) suggested that the major recruitment period into southern Cape estuaries were from August to April. Blaber (1974a,c) suggested a winter/spring spawning peak along the Eastern Cape at West Kleinemonde Estuary. Post larval individuals were most abundant during November in the Sundays Estuary along the south coast (Harrison & Whitfield 1990). In the Swartvlei Bay surf zone, highest abundance was recorded during the summer with a decline in densities during winter (Whitfield 1988). On the southwestern Cape

coast near Fishoek, Bennett (1989) recorded peak recruitment of 14-40 mm individuals between October and April. This implies that temperature plays an important role in determining spawning peaks as has been found to be the case for the yellow seabream *Acanthopagrus latus*, a sparid from Kuwait (Abou-Seedo *et al*. 2003).

Rhabdosargus holubi is thought to spawn in the nearshore zone close to estuary mouths and larvae of size 7-14 mm recruit from spawning waters mainly by passive transport into the estuarine environment (Wallace 1975b). Transport into estuaries takes place mostly during flood tide and in the case of intermittently open/closed estuaries, *R holubi* also utilizes overwash events during periods of mouth closure (Cowley *et al*. 2001). It has been observed that larvae may occur in non-estuarine associated surfzone waters, giving rise to the notion that extensive mixing can take place while the larvae are still in the marine environment, transported by offshore winds and currents (Strydom & d'Hotman 2005). Bilton *et al*. (2002) reported the observation of larvae, after entering an estuary, being swept out to sea during flood tide, transported by offshore winds and currents upon which they enter another nearby estuary, facilitating movement between estuaries.

Upon entering the estuarine environment, larvae settle preferably alongside sea grass beds. These sea grass beds also serve as a food source for the larvae, with small pieces of sea grass being bitten off and the diatoms found on the surface digested (Hanekom & Baird 1984; De Wet 1988). Larvae and juveniles grow at a rate of 60mm per year and reach sexual maturity at an average total length of 15 cm and at an age of approximately two years. *Rhabdosargus holubi* return to the sea before becoming sexually mature and can be found along the coastline up to a depth of 40m (Blaber 1973a; Blaber 1974c; Beckley 1984; Wallace 1975b). *Rhabdosargus holubi* is important as a recreational angling species and serves as a significant protein source for subsistence fisherman (Wallace *et al*. 1984; De Wet 1988). Although many marine species utilize estuaries as nursery areas (Whitfield 1998), little attention has been afforded to studying larval distribution and recruitment of estuarine associated species from a molecular genetic point of view (Whitfield 1999; Griffiths 2000; Whitfield *et al*. 2000). To date, genetic studies on only a few commercially important marine species along the South African coast have been conducted, for example anchovy, pilchard, Cape hake and abalone, on a phylogenetic scale (reviewed by Davies-Coleman & Cook 2000).

The main aim of this study was to determine the level and distribution of genetic variation that exists within *R. holubi* by using mtDNA control region sequences sampled from 13 localities along the South African coast. We expected that the apparently large size of the adult population and the passively transported larval stage might result in a lack of geographic differentiation among sites. The observation of discrete spawning peaks along the South African coast could however indicate the presence of two or more genetically differentiated populations of *R. holubi*. Mixing of larvae and juveniles from spatially/temporally separated spawning events may occur before settling in the estuarine environment, brought about by wind driven long-shore currents. In this paper we explore the influence of these factors on intraspecific geographic genetic differentiation.

2.2 Materials and Methods

2.2.1 Sample collection

Samples were collected during the period December 2000 to March 2003, from 13 geographic localities between St Lucia Estuary along the northeast coast and the Klein River Estuary on the south coast of South Africa (Fig 2.1). In some cases sampling localities were combined prior to analysis for two reasons: first, sample sizes from these localities were small and by combining these localities, the sample size for analysis was increased, and second, the geographic distance between localities combined was relatively small. No important biogeographical barriers or regions (as described in Whitfield 1998) were breached in combining these localities. Prior to combining these localities, an allele frequency homogeneity test among sampling localities were performed by using a randomisation procedure developed by Roff & Bentzen (1989, see paragraph 2.2.6). The localities (with sample size in parenthesis) lumped were: Koppie Alleen (1), De Hoop (3), Breede Estuary (5), Duiwenhoks Estuary (5) and Gourits Estuary (25) into Gourits region (39) which spans a distance of 152 km; East Kleinemonde Estuary (30) and Great Fish Estuary (2) into East Kleinemonde region (32) spanning a distance of 11km; Mngazana Estuary (27) and Mngazi Estuary (41) into Mngazana region (68), spanning a distance of 4 km; Richards Bay Harbour (23) and St Lucia Estuary (1) into the Richards Bay region (24) which covers a distance of 61km. All fish sampled from East Kleinemonde Estuary only (30), could be divided into four groups based on total length: eight individuals within the 65-75mm range, four within 92-100mm, 12 within 107-116mm and six within 122-142mm. Samples

from estuarine environments (sites 1, 4 – 6 & 8 - 13) were collected with a seine-net (50 m x 2 m with a 15 mm bar mesh) fitted with a bag, in a variety of littoral zone habitats (i.e. sandy, muddy and vegetated) within estuaries. Samples collected from the marine environment (site 2, 3 & 7) were caught using standard hook and line techniques. All the samples collected were from juveniles except the samples from Koppie Alleen, De Hoop and Tsitsikamma that originated from adults. Approximately ⅔ of the pelvic fin from each individual sampled was removed and placed in 80% EtOH for preservation. In cases where the individuals were smaller than 10mm, the whole individual was placed in 80% EtOH.

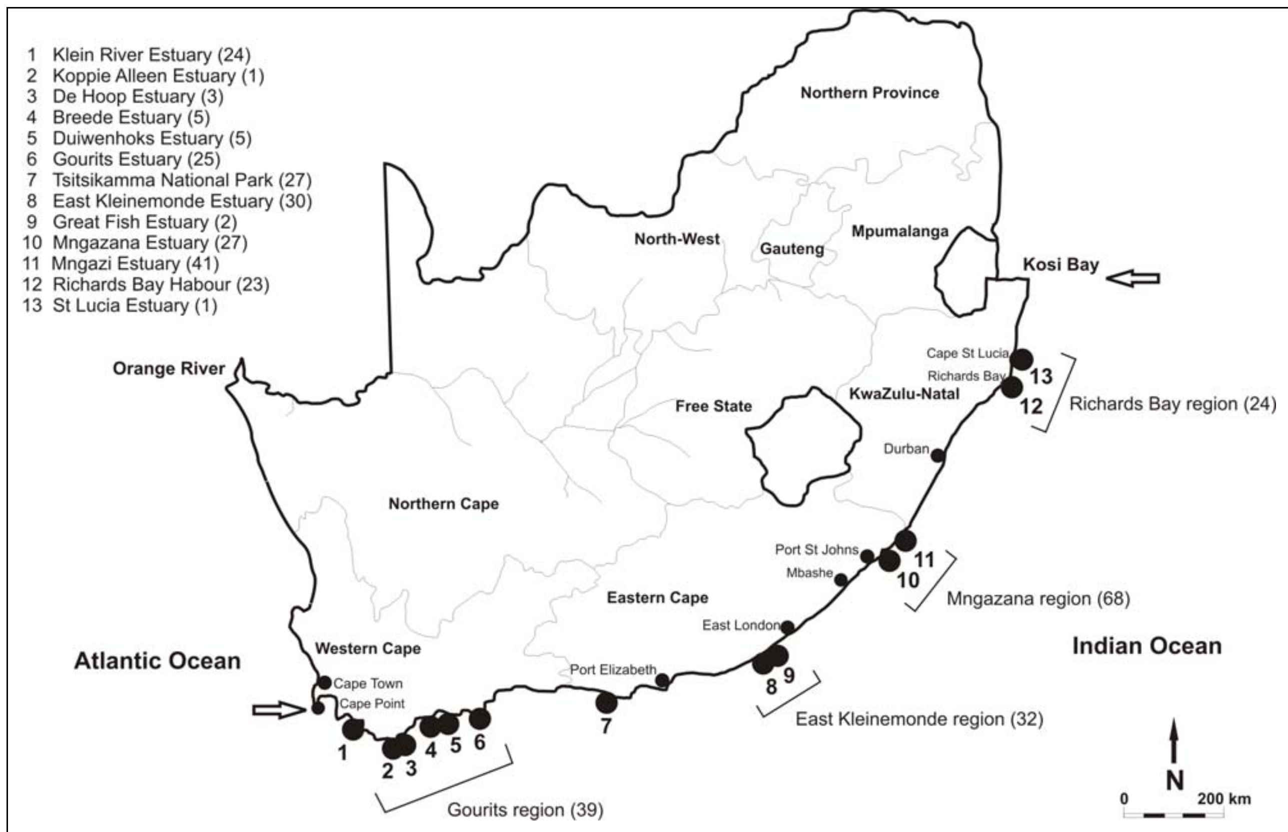

Figure 2.1 Geographic locations of all sampling localities of *Rhabdosargus holubi* along the South African coast analysed for mtDNA control region variation. Sampling localities (1-13) are given in the legend together with the sample size per locality in parentheses. Localities lumped into regions are illustrated with the sample size per region in parenthesis. The area between the open arrows represents *Rhabdosargus holubi* distribution along the coast. (Map modified from Harrison & Whitfield 2006).

2.2.2 DNA extraction

Two methods of total genomic DNA extraction were used: Phenol/chloroform DNA extraction (Sambrook *et al.* 1989) and DNA extraction using Chelex (Estoup *et al.* 1996). Phenol/chloroform DNA extraction was performed as follows: 2 mm X 2 mm fin samples were placed in 500 μl extraction buffer (0.05 M Tris-HCl, 0.5 M EDTA-Na_2, 1.0 M $NaCl_2$ and 10% SDS) to which 0.5 mg Proteinase K (Roche Diagnostics) was added. Sample tissue was digested for 4 hours at 55°C followed by digestion with 0.1 mg RNase A (Roche Diagnostics) for 30 minutes at 37°C. Phenol was used to remove impurities (repeated twice), followed by a phenol removal step using a chloroform: isoamyl alcohol (24:1) solution (Sambrook *et al.* 1989). To this, 2.5 volumes of 99.9% sequencing grade EtOH and 0.1 volumes of 3 M NaAc were added and the DNA was precipitated overnight at –20°C (Sambrook *et al.* 1989). The precipitated DNA was pelleted in a microcentrifuge at 13 000 rpm for 30 minutes and then washed with 70% EtOH. DNA pellets were air-dried and resuspended in 30 μl Sabax® water (Adcock Ingram).

DNA extraction using Chelex was performed as described by Estoup *et al.* (1996). After adding 500 μl (60°C) 10% Chelex solution (Chelex 100 resin/ 100-200 mesh, prepared in sterile de-ionised water and stored at 4°C) to approximately 2 mm X 2 mm fin cutting, the sample was incubated for 15 minutes at 100°C. After incubation, 0.15 mg Proteinase K (Roche Diagnostics) was added and the samples were further incubated for one hour at 55°C. The enzyme was then inactivated by incubating the samples at 100°C for 15 minutes after which the samples were stored at 4°C. Before samples were used in subsequent experimental procedures, they were thoroughly mixed and microcentrifuged for 20 seconds at 13 000 rpm.

2.2.3 Primer design, mtDNA amplification and sequencing

Initial Polymerase Chain Reaction (PCR, Saiki *et al.* 1988) amplification of mtDNA was performed by using a universal vertebrate light strand primer: L14724 (5' TGA YAT GAA AAA YCA TCG TTG 3', Pääbo *et al.* 1988) in combination with H424 (5' AGG AAC CAR ATG CCA GKA ATA 3'), an internal control region primer designed in our laboratory based on cyprinid and salmonid sequence alignments. Primer L14724 anneals to the tRNA-Glu sequence located upstream of the cytochrome *b* gene. Amplified fragments were sequenced (see below) and these sequences were aligned, using Clustal X 1.64b (Thompson *et al.* 1997), to the complete

mitochondrial genome published for Atlantic salmon (*Salmo salar*, GenBank accession number: U12143). Using this sequence alignment, a *R. holubi* specific D-Loop Light strand primer was designed, RDLL (L16547): 5' GCG TCG GTT TTG TAA GCC 3', that anneals to the tRNA-Thr sequence at position 16 547 in *S. salar*, located approximately 100bp upstream of the 5' end of the control region. By using the combination of RDLL and H424, an amplified fragment length of 520bp was obtained consisting of partial tRNA and the 5' end of the control region, previously shown to be the most variable segment of the control region in this study. This primer combination was used for all subsequent sample amplifications. Only the region representing the control region was used in all further analyses.

DNA amplification using PCR was performed in a total reaction volume of 25 μl. Each reaction contained approximately 100 ng of genomic DNA as template and Sabax® water was used in the place of DNA as the negative control. Each reaction contained 1 x reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM of each of the four deoxyribonucleotides (Promega), 10 pmol of each primer and 0.5 U of SuperTherm® DNA polymerase (Southern Cross Biotechnology). Polymerase chain reaction cycles were as follow: initial denaturation step for 3 minutes at 94°C; 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at 58°C for 30 seconds, elongation at 72°C for 30 seconds, and an extended final elongation at 72°C for 5 minutes in a GeneAmp® PCR System 9700 (Applied Biosystems). Polymerase chain reaction fragment amplification was confirmed by electrophoresis through 1% agarose gels (Roche Diagnostics), using 4 μl of the PCR product. Negative controls showed no contamination of the reagents used in PCR reactions were present. Successful PCR amplified fragments were precipitated using 0.08 volumes 3M NaAc, 3 volumes sequencing grade EtOH and 0.4 volumes Sabax® water. Precipitated DNA was eluted in 15-20 μl Sabax® water. Precipitation success was evaluated by electrophoresis through 1% agarose gels.

Precipitated DNA fragments were sequenced in both directions with the primers used in the amplification step. Cycle sequencing was performed using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit Version 3.0 (Applied Biosystems) and a GeneAmp® PCR System 9700. Each cycle sequencing reaction contained the following: 80-100 ng purified PCR product as template, 3.2 pmol primer, and 1μl of the BigDye reaction mix. Cycle sequencing reactions were precipitated by the same precipitation procedure described above. Sequences were separated through an ABI 3100 capillary automated DNA sequencer (Applied Biosystems).

2.2.4 Sequence analysis

Raw sequence data were evaluated using Sequencing Analysis version 3 software (Applied Biosystems) and successful sequences were imported into Sequence Navigator version 1.0.1 software (Applied Biosystems). The heavy and light strand sequences for each individual were aligned and consensus sequences obtained for each individual after thorough inspection of the aligned sequences. All consensus sequences were aligned in Clustal X version 1.64b software (Thompson *et al*. 1997) and then imported into PAUP version 4.0b10 software (Phylogenetic Analysis Using Parsimony, Swofford 2001) to calculate the pairwise distances between the different sequences and to identify unique alleles.

2.2.5 Homoplasy

Due to the high mutation rate of the mitochondrial control region, homoplasy is likely to be observed for sites within this region (Avise & Lansman 1983; Sanderson & Donoghue 1989). Homoplasy (false homology or the independent evolution of the same character state) results in allele networks that do not reflect the actual mutational distances between alleles (Avise & Lansman 1983; Sanderson & Donoghue 1989; Wake 1991). All variable sites were mapped onto the minimum-spanning network by pairwise comparison of allele sequences in order to identify homoplasic characters. These characters were removed for all subsequent analyses.

2.2.6 Homogeneity test

Mitochondrial DNA allele frequency homogeneity among samples was tested by using a randomisation procedure developed by Roff & Bentzen (1989) incorporated into a simulation program, RandoChi, created by W Delport (unpublished). This procedure, specifically developed for small sample sizes, uses a Monte Carlo technique for generating the distribution of expected χ^2 values under the assumption that the null hypothesis of homogeneity cannot be rejected for this data set. Probabilities were estimated from the number of rearrangements that were equal to or greater than the observed χ^2 value. By using this method, no assumptions about the underlying distributions are made, no lumping of data is required and the accuracy of the estimate of α (the probability of rejecting a true null hypothesis) only depends on the number of randomisations of the original data set. A total of 10 000 randomisations were performed.

2.2.7 Genetic diversity

Allele diversity, *h* (Nei & Tajima 1981) and nucleotide diversity, π (Nei 1987), were calculated using Arlequin version 2.000 software (Schneider *et al*. 2000). Allele diversity represents the probability that two randomly drawn alleles from a population will be different. Nucleotide diversity represents the probability that two randomly drawn homologous nucleotides will be different from each other if the assumptions for selective neutrality and no recombination are satisfied. The minimum number of mutational steps between different alleles was illustrated by constructing a minimum spanning allele network using MINSPNET (Excoffier & Smouse 1994) and Arlequin version 2.000 (Schneider *et al*. 2000). Analysis of MOlecular VAriance (AMOVA) was used to generate estimates of genetic variance components (Excoffier *et al*. 1992). This method is in essence the same as standard methods for calculating variance of gene frequencies, but here it takes into account the number of mutations that are found between different alleles. Population differentiation estimates were calculated following the methods of Weir and Cockerham (1984) using Arlequin version 2.000 (Schneider *et al*. 2000). Significance levels for multiple tests done were adjusted using Bonferroni corrections (Rice 1989). By using this test, the chance of making a type I error (true hypothesis being rejected) is reduced. This, however, leads to an increase in the chance of making a type II error, where a false hypothesis is accepted (Rice 1989; Sokal & Rohlf 1994).

2.2.8 Neutrality test and population size history

Past demographic events, such as population growth, leave traces that can be detected from DNA sequence data (Tajima 1989; Slatkin & Hudson 1991; Rogers & Harpending 1992; Harpending 1994). Computer simulations and statistical tests are commonly developed under the coalescent theory (Kingman 1982) that is focused primarily on the neutral evolution of gene trees (Ramos-Onsins & Rozas 2002). Although the control region is a non-coding part of mtDNA, it can, however, be influenced by indirect effects of selection from strong directional selection on many other parts of the mitochondrial genome (Ballard & Whitlock 2004). Therefore it is very important to test for selective neutrality of mtDNA before drawing conclusions about the patterns that are seen from the results. Methods developed for testing selective neutrality and population size history are based on one of three statistics: (1) tests based on mutation frequency distribution, (2) allele distribution and (3) mismatch distribution (distribution of pairwise differences).

1. Statistics using mutation (segregating site) frequency could be used to distinguish mutational neutrality and population growth from constant size populations because population growth results in an excess of mutations in external branches of genealogies, thus an excess of substitutions present in only one sampled sequence (singletons) (Ramos-Onsins & Rozas 2002). Here Tajima's D test (Tajima 1989) and the R_2 test (Ramos-Onsins & Rozas 2002) were used for analyses utilizing polymorphic data within populations. Tajima's D test is based on the difference between two estimates of the mutational parameter theta, (θ): $\theta = 2N\mu$, with N as the effective number of females (for mtDNA) and μ the mutation rate. Theta can be estimated using the average number of variable nucleotide positions among all pairs of sequences (π) and also, using the number of variable nucleotide positions in the aligned sample of sequences (θ_2) (Rand 1996). For a population at mutation-drift equilibrium $\pi = \theta_2$, this is due to the fact that π is sensitive to the frequency of a given type of sequence in the sample and θ_2 is not. When $\pi > \theta_2$, Tajima's D will be positive; balancing selection or admixture of two distinct populations are possible explanations. When $\pi < \theta_2$, Tajima's D is negative; a selective sweep or a population expansion after a recent bottleneck are possible explanations (Fu & Li 1993; Rand 1996). The R_2 statistic is based on the difference between the number of singleton mutations and the average number of nucleotide differences. Under a recent drastic population growth event, low values for R_2 are expected (Ramos-Onsins & Rozas 2002).

2. Fu's F_S test is based on the Ewens' sampling distribution (Ewens 1972) and uses information from allele distribution. F_S tends to be negative when there is an excess of recent mutations and a large negative value is taken as evidence against neutrality of mutations (Fu 1997; Ramos-Onsins & Rozas 2002).

3. A particular signature in the distribution of pairwise sequence differences are expected as a result of a population expansion (Slatkin & Hudson 1991; Rogers & Harpending 1992), mismatch distribution statistics can therefore be used to test for demographic changes, under the assumption of selective neutrality. The raggedness statistic (*RG*), used for analysis, differs among constant size and growing populations and measures the smoothness of the mismatch distribution. Under a population growth model, lower raggedness values (more smoothly peaked distributions, usually unimodal) are expected (Harpending 1994; Ramos-Onsins & Rozas 2002). Higher *RG* values (more ragged) and erratic patterns are indicative of samples from populations that have been stationary for a long time (Harpending 1994).

Neutrality tests were performed using DnaSP version 4.00.5 (Rozas & Rozas 1999). The 95% confidence intervals for each test statistic were obtained after 1 000 permutations. Mismatch analysis of the number of pairwise differences between alleles was performed in Arlequin version 2.000 (Schneider *et al*. 2000).

2.2.9 Mantel test

A Mantel test was used for computing the correlation coefficient (Mantel 1967) between the pairwise matrices of geographical and genetic distances (F_{ST}) between sites. The null hypothesis assumes that there is no correlation between geographical and genetic distances. This test was performed in Arlequin version 2.000 (Schneider *et al*. 2000), using 10 000 random permutations of matrices in order to assess the significance of the correlation coefficient.

2.3 Results

2.3.1 DNA extraction

Compared to phenol/chloroform DNA extractions, Chelex extractions proved to be relatively inexpensive and DNA yield was sufficient. Contamination risk is dramatically reduced as only one tube is used for each sample throughout the extraction procedure and this tube is only opened once after the sample is placed in the Chelex solution during the DNA extraction procedure. Since DNA never needs to be transferred between tubes during this extraction procedure, losing DNA by pipetting error is also not possible. The only potential drawback (Estoup *et al*. 1996) is that DNA may not be as stable as compared to DNA extracted by the phenol/chloroform method, however, no DNA degradation was observed during the period of the study.

2.3.2 Sequence analysis

Mitochondrial DNA fragments of 500 bp in length were obtained from a total of 214 individuals from all sampling localities. No heteroplasmy was detected during sequence analysis as no double peaks were observed. Approximately 100 bp on the 5’ end were removed since only the control region was used in subsequent analyses. The mtDNA control region sequence conformed to a typical A-T-rich region, containing a total of 68% A+T. This observation is consistent with nucleotide composition of non-coding regions that have low functional constraint

Table 2.1 Aligned allele nucleotide sequences showing variable nucleotide positions in the 368 non-homoplasic sites of mtDNA control region for *Rhabdosargus holubi*. Allele numbers (1-36) are listed [illegible]morphic base pair positions are across the top row. Only nucleotides that are different from allele one are given for all other alleles. Nucleotides identical to allele one are represented by dashes (-).

Allele	Nucleotide position																																
	026	066	072	080	082	085	086	112	116	139	158	159	163	177	182	222	223	224	225	226	231	232	238	247	252	273	276	281	296	218	219	242	244	266
1	C	A	A	A	G	C	C	G	C	G	T	T	T	T	A	A	G	A	A	T	T	G	A	A	C	A	T	A	C	T	A	G	G	T
2	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	A	-	-	-	-	A	-	-	-	-	-	-	A	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	A	-	-	-	-	A	-	-	-	-	-	-	A	-	-	C	-	-	G	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	A	-	-	-	-	A	-	-	-	-	-	-	A	-	-	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	G	-	-	A	-	-	-	-	A	-	-	-	-	-	-	A	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	A	-	-	-	-	A	-	-	-	-	-	-	A	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	A	-
8	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	T	-	-	A	-	-
9	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-
10	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	G	-	-	-	-	T	-	-	-	-	-
11	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	A	-	G	-	-	-	-	T	-	-	-	-	-
12	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-
13	T	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-
17	-	-	-	-	A	-	-	-	T	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	A	-	-	-	-	-	-	-	-	C	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	A	-	-	-	-	-	-	-	-	C	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-
20	-	-	-	-	A	-	-	A	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	-	-	-	G	A	-	-	A	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	A	-	-	-	-	-	-	C	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	A	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C
25	-	-	-	-	A	-	T	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26	-	-	-	-	A	-	T	-	-	-	-	-	-	-	-	G	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27	-	-	-	-	A	-	T	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-
28	-	-	-	-	A	-	T	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-
29	-	-	-	-	A	-	T	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-
30	-	-	-	-	A	-	-	-	-	-	-	-	-	-	G	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
31	-	-	-	-	A	-	-	-	-	-	-	-	C	-	G	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32	-	-	-	-	A	-	-	C	-	-	-	-	-	-	G	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
33	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	A	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
34	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	A	-	G	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-
35	-	-	-	-	A	T	-	-	-	-	-	-	-	-	-	-	A	-	G	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-
36	-	-	-	-	A	-	-	-	-	-	C	-	-	-	-	-	A	-	G	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-

(McMillan & Palumbi 1997; Bargelloni *et al*. 2003). Three individuals, two from the Mngazana region and one from the East Kleinemonde region, contained a single 20bp indel (5' TTGTACTTTTATGTACATAT 3'). This insertion sequence was removed for all subsequent analyses. Of the remaining sites, 29 sites showed homoplasy and were removed. Effectively, 368 sites were used for all analyses of which only 34 sites were polymorphic (Table 2.1).

A total of 36 mutations, four transversions (site numbers: 72, 112, 224 and 273) and 32 transitions, were observed. Most changes (five) occurred within the 225-250 base pair region followed by four changes within the 75-100 base pair region (Fig 2.2). Thirty-six alleles were identified (Table 2.1 and 2.2) based on 34 variable sites. A minimum spanning allele network with a total of 37 mutational steps was constructed using MINSPNET (Excoffier & Smouse 1994) and Arlequin version 2.000 (Schneider *et al*. 2000) (Fig 2.3). Only two alternative links were possible between alleles 16 & 34 and 19 & 24, both separated by only one mutational step. Allele three was the most common allele being shared by 42 individuals (19.6%) and was almost twice as frequent as the second most common alleles (alleles 2 and 9). Allele three, together with alleles two, five and nine were the most widespread and present from all sampling regions. This pattern is expected under an expansion model (Rogers & Harpending 1992; Avise 2000).

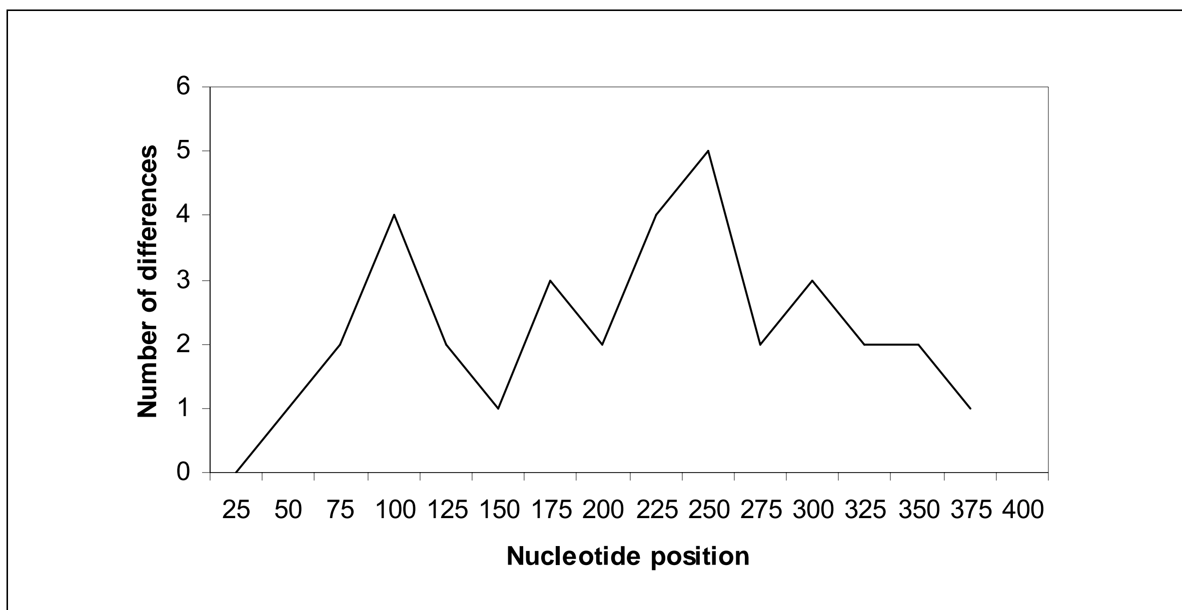

Figure 2.2 Sliding window across the 5' end of the mtDNA control region of *Rhabdosargus holubi* indicating the number of nucleotide substitutions per 25 base pair region.

Table 2.2 Number of *Rhabdosargus holubi* samples analysed (N_A) per sampling locality/region (listed in the left column). Alleles are listed across the top row, labelled 1-36, and alleles occurring within each sampling locality/region as well as the number of individuals sharing a specific allele (N_S) are shown. Dashes indicate the absence of alleles.

Region	N_A	Allele																																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Richards Bay region	24	-	3	4	-	2	1	1	1	2	1	-	-	-	1	-	-	-	-	1	-	1	2	-	-	-	-	-	-	1	1	1	1	-	-	-	-
Mngazana region	68	4	10	16	1	6	4	3	2	7	1	1	6	1	1	1	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
East Kleinemonde region	32	4	5	5	-	4	-	-	-	4	-	1	2	-	-	-	-	-	-	-	-	2	-	1	1	-	1	1	1	-	-	-	-	-	-	-	-
Tsitsikamma National Park	27	-	4	6	-	2	1	1	-	5	-	-	3	-	-	1	-	-	-	-	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Gourits region	39	4	2	4	-	4	-	1	-	8	1	-	7	-	-	2	-	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	1	1
Klein River Estuary	24	2	3	7	-	4	1	1	-	1	-	-	-	-	-	-	1	-	1	1	-	-	-	-	1	1	-	-	-	-	-	-	-	-	-	-	-
N_S		14	27	42	1	22	7	7	3	27	3	2	18	1	2	4	2	1	3	4	1	4	3	2	2	1	1	1	1	1	1	1	1	1	1	1	1
Total	214																																				

Figure 2.3 Minimum spanning allele network summarizing genealogical relationships among 36 mtDNA alleles identified from a sample of 214 *Rhabdosargus holubi*. Each connecting line equals one mutation; perpendicular lines crossing branches indicate additional mutational changes along a path. Information to the side of each circle represents the allele number and the number of individuals, in parenthesis, that share a specific allele. The size of each circle represents the number of individuals sharing a specific allele and colours represent sampling localities/regions. Allele numbers follow Table 2.1.

Allele three is suggested to be the most ancestral allele because (1) it was phyletically central and hence could have given rise to all other alleles, (2) it had the highest frequency in number of individuals and (3) it had a widespread geographical distribution (Avise *et al*. 1987; Templeton *et al*. 1995).

In addition, alleles two and nine also had star-like structure giving rise to some less frequent alleles. Sixteen out of the 36 alleles identified (44%) were alleles found within only one individual. In the current data set the Gourits- and Richards Bay regions had four locality specific alleles followed by the East Kleinemonde- and Mngazana regions with three, and Klein River Estuary and Tsitsikamma National Park localities with one.

2.3.3 Homogeneity test

Mitochondrial DNA allele frequency homogeneity among samples tested by using the randomisation procedure specifically developed for small sample size (Roff & Bentzen 1989) allowed the lumping of: Koppie Alleen, De Hoop, Breede Estuary, Duiwenhoks Estuary & Gourits Estuary into Gourits region (χ^2 = 14.47, P = 0.55); East Kleinemonde Estuary & Great Fish Estuary into East Kleinemonde region (χ^2 = 18.35, P = 0.23); Mngazana Estuary & Mngazi Estuary into Mngazana region (χ^2 = 16.76, P = 0.63); Richards Bay Harbour & St Lucia Estuary into the Richards Bay region (χ^2 = 11.48, P = 0.63).

2.3.4 Genetic diversity

Percentage nucleotide-sequence diversity (π) within sampling regions ranged between 0.48 at the Klein River Estuary and 0.64 at both the Richards Bay- and East Kleinemonde regions. The overall average was 0.59 (Table 2.3). Overall allele diversity (*h*) was 0.91 and ranged between 0.89 for the Klein River Estuary and 0.96 for the Richards Bay region.

Analysis of molecular variance of the mtDNA control region was consistent with the notion of high female gene flow among populations as indicated by a very low overall non-significant F_{ST} value of 0.002 (p = 0.362) following 100 000 permutations. Almost all the observed molecular variation (99.84%) was found to be between individuals within regions with only 0.16% of the variation between regions. Pairwise F_{ST} values were low or zero in most cases (Table 2.4),

suggesting a homogeneous population. Only two pairwise F_{ST} values were significantly greater than zero, these were between the East Kleinemonde- and Gourits regions (0.030), and between the Gourits region and Klein River Estuary (0.040). Consequently, the highest differentiation between populations exists between these regions. Both of these F_{ST} values remained significant after Bonferroni correction. Testing for differentiation between the four size classes identified from East Kleinemonde Estuary all revealed non-significant negative F_{ST} values after pairwise comparisons (results not shown).

Table 2.3 Intraregional nucleotide sequence diversity (π, given as a percentage) and allele diversity (*h*) at all sampling regions as well as overall nucleotide and allele diversity values for *Rhabdosargus holubi*.

Sampling region	Nucleotide Diversity, π (%)	Allele Diversity, *h*
Richards Bay region	0.64	0.96
Mngazana region	0.56	0.90
East Kleinemonde region	0.64	0.92
Tsitsikamma National Park	0.61	0.90
Gourits region	0.61	0.91
Klein River Estuary	0.48	0.89
Overall	0.59	0.91

Table 2.4 Average number of pairwise differences between sampling regions of *Rhabdosargus holubi* above diagonal. Diagonal elements are average number of pairwise differences within sampling regions and pairwise F_{ST} values are below the diagonal.

Sampling region	RB	MR	EKM	TNP	GR	KR
Richards Bay region (RB)	2.355	2.197	2.365	2.253	2.334	2.030
Mngazana region (MR)	-0.005	2.069	2.203	2.115	2.178	1.919
East Kleinemonde region (EKM)	0.004	-0.003	2.355	2.292	2.379	2.051
Tsitsikamma National Park (TNP)	-0.018	-0.017	-0.001	2.234	2.220	2.008
Gourits region (GR)	0.013	0.007	**0.030***	-0.012	2.259	2.105
Klein River Estuary (KR)	-0.016	-0.002	-0.007	0.003	**0.040**	1.768

*Values in bold represents significant values at $p \leq 0.05$

2.3.5 Neutrality test and population size history

Tajima's D test was based on a total number of 34 segregating sites and 36 mutations with the average number of variable nucleotide positions among all pairs of sequences, π = 0.006, and the number of variable nucleotide positions in the aligned sample of sequences, θ_2 = 0.016. After performing Tajima's D test, all the values were negative for all the regions (and also for the samples pooled) with no significant departure from neutrality indicated by the non-significant values for all the estimates of D (Table 2.5). These results suggest that a selective sweep or a population growth event has taken place in the past. In order to rule out the possibility of a selective sweep, another marker will be needed as both loci would be expected to be affected the same in the case of a population event. In the case of selection that is locus specific, only one locus will be affected (Rand 1996). Fu's Fs value being negative, together with the low values for R_2 (Table 2.5) estimated is consistent with the notion of population expansion. Mismatch distribution for pairwise differences was unimodal (Fig 2.4) as expected for an expanding population (mean = 2.168, variance = 1.521) with time since expansion estimated at 2.36 generations (approximately five years) ago (τ). The raggedness statistic (RG), measuring the smoothness of the mismatch distribution, was low for all estimates with no statistically significant values after performing simulations of 10 000 replicates using the computer program 'The Coalescent' incorporated into DnaSP version 4.00.5 (Table 2.5, Rozas and Rozas 1999). In all cases these test statistics indicated that a population expansion took place in the recent past.

Table 2.5 Summary of neutrality and population history test statistics for the mtDNA control region of *Rhabdosargus holubi*.

Sampling region	D (p)	R_2 (p)	Fs (p)	RG (p)
Richards Bay region	-0.08 (0.54)	0.13 (0.56)	-0.04 (0.53)	0.08 (0.64)
Mngazana region	-0.07 (0.57)	0.10 (0.52)	-0.25 (0.55)	0.07 (0.62)
East Kleinemonde region	-0.12 (0.57)	0.12 (0.56)	-0.09 (0.58)	0.09 (0.66)
Tsitsikamma National Park	-0.04 (0.53)	0.12 (0.45)	-0.02 (0.53)	0.10 (0.65)
Gourits region	-0.06 (0.56)	0.16 (0.52)	-0.05 (0.58)	0.09 (0.66)
Klein River Estuary	-0.13 (0.56)	0.13 (0.56)	-0.17 (0.52)	0.12 (0.70)
Overall	-0.11 (0.59)	0.11 (0.53)	-0.72 (0.58)	0.05 (0.69)

D, Tajima's D statistic; R_2, R_2 neutrality statistic; F_S, Fu's F_S; RG, raggedness statistic for mismatch distribution; p, probability values (p > 0.05 are non-significant).

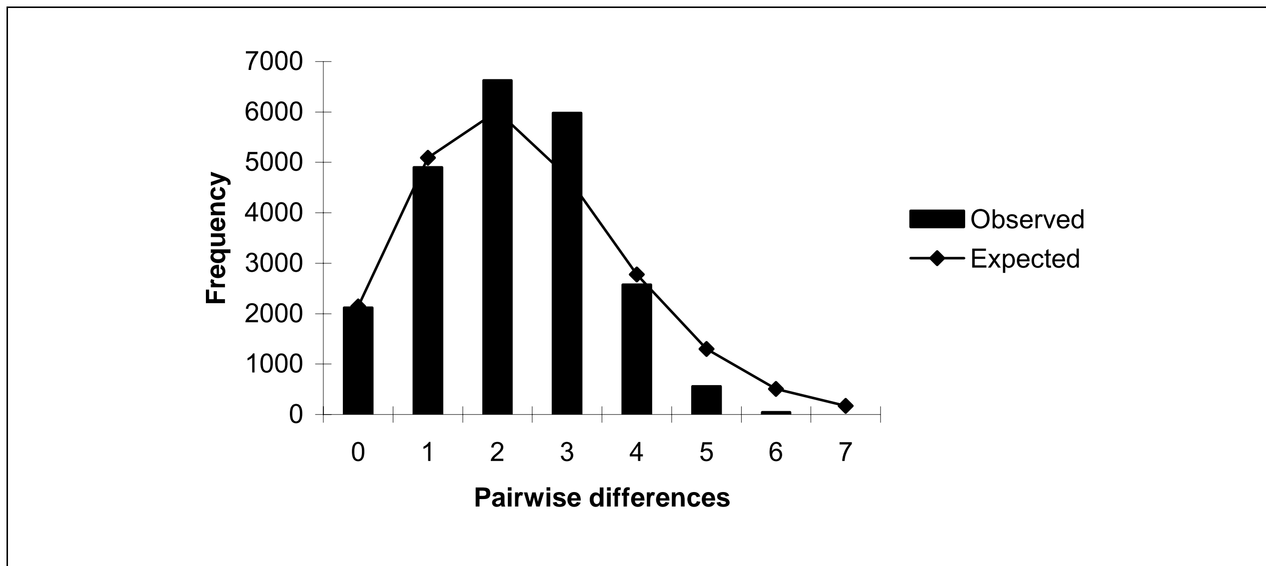

Figure 2.4 Mismatch distribution showing the distribution of pairwise differences for 214 *Rhabdosargus holubi* obtained from mtDNA control region sequence data. Solid bars represent the observed pairwise differences and the curve represents the expected distribution under the sudden expansion model (Rogers & Harpending 1992) (p = 0.032). Theta0 = 0.000 (pre-expansion population size) and Theta1 = 3479 (post-expansion), with τ = 2.36 (time in number of generations, elapsed since the sudden expansion episode).

2.3.6 Mantel test

The Mantel test for isolation-by-distance revealed no association between geographical and genetic distances (r = -0.152, p = 0.68) thereby failing to reject the hypothesis that gene flow in *R. holubi* occurs randomly with respect to geographical distance.

2.4 Discussion

Genetic markers such as mtDNA are used in molecular ecology, evolutionary and conservation genetics to make inferences regarding the demography and evolutionary history of populations (Avise *et al.* 1987). However, conclusions based on these patterns of genetic variation could be deceptive if the marker used is under selection (Rand 1995; Ford 2002). This necessitates the use of statistical tests in order to determine whether the markers used indeed conform to neutrality. Ramos-Onsins & Rozas (2002) showed that R_2 is the most powerful test for small

sample sizes and Fu's F_S was better for large samples. These authors suggested that both of these tests were more powerful than Tajima's D test. The raggedness test showed little power in detecting population growth and performed weaker than both R_2 and Fu's F_S (Ramos-Onsins & Rozas 2002). Within this study, all these tests confirmed the selective neutrality of the mtDNA control region in *R. holubi*. Consequently, the 5' end of the mtDNA control region is a suitable molecular marker to investigate the population demography of this species.

Observed values of π and *h*, with a slight northeast to southeast decrease, fell within the range reported for 32 marine teleost species (category 2: $0.16 < \pi < 0.71$, $0.37 < h < 0.98$) by Grant & Bowen (1998). The high allelic diversity and low nucleotide diversity within the present study are in agreement with the population expansion statistics and also suggest rapid population expansion (Grant & Bowen 1998). This signature was also observed in the topology of the minimum spanning allele network (Fig 2.3). Only one to three mutational steps separated all other alleles detected from the three central alleles (alleles 3, 9 & 12). Under such a situation, where a population is rapidly expanding, new mutations occurring in the population will be maintained (Ramos-Onsins & Rozas 2002) and since the marker is selectively neutral, mutations can only be removed through the process of genetic drift. If exceptionally large population sizes exist, this process will require hundreds of generations to remove mutations (Hauser & Ward 1998; Waples 1998).

Cowley & Whitfield (2001) provided some indication that marine populations of *R. holubi* are extremely large. The East Kleinemonde Estuary is small (< 20 Ha) and one of approximately 250 functional estuaries in South Africa. Assuming equal densities of *R. holubi* occur in all South African estuaries (approximately 70 000 Ha), annual emigration to the marine environment, following their estuarine dependant nursery phase, can equate to millions of individuals. The numerical dominance of *R. holubi* in many South African estuaries (Cowley & Whitfield 2001; Cowley *et al*. 2001; Vorwerk *et al*. 2003; Harrison & Whitfield 2006; and others) can be ascribed to aspects of their life history. They are serial spawners capable of producing batches of eggs over extended periods, supplying vast numbers of recruits that are present within the surfzone (Whitfield 1998; Cowley *et al*. 2001). These larvae are able to recruit into intermittently open estuaries during closed-mouth phases by overtopping events (Cowley *et al*. 2001) and they are able to withstand adverse fluctuations in water salinity (Whitfield *et al*. 1981; Lukey *et al*. 2006).

The reduction of freshwater inflow into estuarine systems due to anthropogenic activities upstream (such as building of dams and agricultural activities) causes increased macrophytes (e.g. *Zostera capensis*) growth within estuaries coupled with fewer flooding events (Heydorn 1989; Whitfield 1989). It has been noted in earlier studies on the Kromme and Swartvlei estuaries that lower levels of abundance of specifically *R. holubi* within permanently open estuaries can be attributed to the relative absence of macrophyte beds compared to closed systems (Hanekom & Baird 1984; Whitfield *et al*. 1989; Vorwerk *et al*. 2003). *Rhabdosargus holubi* recruits utilize these macrophyte beds as a food source as well as shelter from natural predators (De Wet 1988). Increasing abundance and large populations of *R. holubi* within estuaries are plausible because of more shelter provided by the increasing macrophyte growth, a decrease in food competition as well as a decrease in the number of natural overexploited-predators such as kob and leervis present in these systems (Cowley *et al*. 2001).

Large population fluctuations for *R. holubi* within a single estuary have been reported. This would explain the observation that time since expansion was estimated at only approximately two generations ago (Fig 2.4) Using mark-recapture techniques, Cowley & Whitfield (2001) estimated that the population size of *R. holubi* inside the East Kleinemonde Estuary ranged from approximately 13 000 in 1994 to approximately 109 000 in 1995. Enormous population fluctuations may reduce the overall genetic diversity because of a reduction in the long-term effective population size (Hauser & Ward 1998). This, however, was not observed in this study. This could be explained by the fact that population reductions within single estuaries might not be as severe as it appears to be due to the existence of enormous marine populations as well as the fact that extensive mixing of fish from different regions seems to be taking place. This could also contribute to the observed population expansion signature discussed above.

Within high gene flow species the expected population differentiation signal is likely to be weak and more difficult to detect because of various sources of noise within the analysis (Waples 1998). Population differentiation estimates, comparing pairwise F_{ST} values, indicated two statistically significant divisions, between the Gourits region and Klein River Estuary and also between the Gourits- and East Kleinemonde regions. This could be due to the noise within the analysis introduced by unstructured sampling (Waples 1998). Analysis of the distribution of variance indicated that there was no significant division of allelic variation between the sampling regions analysed. Almost all of the variation observed occurred within each sampling locality/region or within the different size classes observed in the East Kleinemonde Estuary.

Common alleles were also found at nearly all of the sampling regions and allele groupings inferred from the minimum spanning allele network were not geographically restricted. These results suggest that extensive female gene flow for *R. holubi* occurs along the South African coast. Annual spawning migrations and aggregations are well documented for a number of fish populations, including elf or bluefish *Pomatomus saltatrix,* sardine *Sardinops sagax,* round herring *Etrumeus teres* and Australian anchovy *Engraulis australis* (Ward *et al.* 2003). Spawning migrations to the east along the South African coast takes place in dusky kob *Argyrosomus japonicus* (Griffiths & Heemstra 1995) and spotted grunter *Pomadasys commersonnii* (Webb 2002). It was proposed that the absence of barriers to movement as well as life history characteristics favourable for dispersal allows for extensive and recent historical gene flow (Avise *et al*. 1987). The effect of this high level of potential gene flow will be a pattern of homogeneity between different regions that are close to each other as well as regions that are geographically separated, as seen from the present study (Avise 1987). It should however also be noted that the possibility exists that recently diverged subpopulations might not have reached drift/migration equilibrium yet and that the genetic homogeneity observed might be the relict of historical rather than present day gene flow (Camper *et al*. 1993). This was reinforced by the estimation of a population expansion of two generations (four to five years) ago.

The observed absence of isolation-by-distance in *R.* holubi together with the observed genetic homogeneity, suggests that dispersal between two geographically extreme localities is as likely as the dispersal between two closely or geographically continuous localities. An example of extensive gene flow over geographically separated localities within sparids is provided by Bargelloni *et al*. (2003) who investigated species that had similar distributional ranges and found that two of the species (*Pagrus pagrus* and *Pagellus bogaraveo*) displayed high levels of gene flow between the Mediterranean Sea and the Northeast Atlantic. In a study on the blue crab *Callinectes sapidus*, along the eastern United States, alleles were geographically widely distributed indicating gene flow over vast distances (McMillen-Jackson & Bert 2004). Similar observations, where closely related mtDNA alleles appear not to be geographically localized, have been made in other species, including the American eel *Anguilla rostrata* (Avise *et al*. 1986), the hardhead sea catfish *Arius felis* and marine gafftopsail catfish *Bagre marinus* (Avise *et al*. 1987). In contrast, Gold & Richardson (1998) studied larval transport in red drum (*Sciaenops ocellatus*) along the coast of the Gulf of Mexico. Spawning occurs in the nearshore region just outside bays and estuaries. Larvae can normally be found inside an 18m-depth range from where they migrate into estuaries and settle within sea grass beds. The young

adults reach sexual maturity inside estuaries and migrate into the sea (Gold & Richardson 1998). Population structure analysis for red drum showed that an isolation-by-distance effect with female philopatry exists and limited larval dispersal between estuaries was noted from mark-recapture studies (Gold & Richardson 1991; Gold & Richardson 1998; Gold *et al.* 1999).

Alternative explanations exist to account for the observed pattern of no population differentiation. The existence of discrete spawning populations cannot be ruled out because when collecting samples at a specific geographical locality at a specific time, the assumption is made that the samples were collected from a randomly spawning population, but this might not always be the case (Waples 1998). Different discrete breeding groups might exist at a particular locality at the same time. Such groups may not necessarily be stable or completely isolated (Scribner & Chesser 2001). Consequently, the observed results for *R. holubi* could also be ascribed to either mixing of discrete stocks during the adult stage or mixing of discrete stocks during larval stages.

Tracking adult behaviour in the marine environment for different species has been successfully performed in the past and tagging data provide a good indication of individual movement in this environment. Examples of extensive tagging studies completed elsewhere include Atlantic cod *Gadus morhua* (Sauer *et al.* 2000) and chokka squid *Loligo vulgaris reynaudii* (Robichaud & Rose 2004). Tagging studies successfully completed along the South African coast include studies on red steenbras *Petrus rupestris* (Brouwer 2002), carpenter *Argyrozona argyrozona* (Brouwer *et al.* 2003) and galjoen *Dichistius capensis* (Attwood & Cowley 2005) to name a few. At this stage, tagging data for *R. holubi* (Bullen & Mann 2004) are not sufficient for any conclusions about adult movement along the South African coast. To date 833 mostly adult fish have been tagged but yielded only six recaptures (0.72%). Four fish were recaptured at their tagging locality within the same year. The fifth individual was recaptured after ten days at a locality nine kilometres from where it was tagged. The sixth individual was recaptured after two years and had traveled a distance of 56 km. Three potential reasons exist for this low level of tag return data: (1) *R. holubi* suffers from high tagging mortality, (2) a high tag shedding rate exists, or (3) existence of an enormously large population of adult *R. holubi* (Bullen & Mann 2004).

Although the dispersal or migration of adults remains inconclusive, evidence for three means of larval dispersal to suitable estuarine habitats (and an opportunity for discrete stocks to undergo

mixing) have been reported: passive transport, active behaviour that can alter larval movement, i.e. utilizing tidal streams, and active swimming (Beckley 1985; Brown *et al.* 2005). Enormous potential exists for the dispersal of larvae along the South African coastline. It has been estimated that larvae along the South African coast has a potential dispersal capability of up to 240 km within one week after spawning by typical inshore currents (Attwood & Cowley 2005). Brouwer *et al.* (2003) studied carpenter *Argyrozona argyrozona* from the Tsitsikamma National Park and determined that larval dispersal is mainly eastwards with an estimated distance of 300 km within 30 days. Clear evidence of passive larval transport into estuarine environments is provided by particle supply experiments where coastal sea levels as well as coastal winds drastically influence transport and retention of particles into and within bays and estuaries. Currents are mostly responsible for transporting larvae into suitable habitats and retaining them in these habitats until settlement (Brown *et al.* 2005).

An interesting study was performed by Strydom & d'Hotman (2005) on a surf zone area not directly associated with an estuary, with the closest intermittently open estuary 15 km from the study site and the closest permanently open estuary 24 km away. This study revealed the potential linkage of estuarine associated surf zones in that most of the larvae caught in this region, including *R. holubi*, were in the recruitment size class of 8-20 mm. Within this region an absence of early developmental stages was observed. Of importance also is the fact that the species composition, species rank, size range and percentage of estuary-dependent species within this region were similar to estuarine associated surf zones, suggesting a continuity in surf zone assemblages along the warm-temperate coast region of South Africa and a potential means for *R. holubi* stock intermixing.

Hutchings *et al.* (2002) suggested that the Agulhas Current, on the east coast of South Africa, flows at speeds greater than $1ms^{-1}$ and plays an important role in the southward migration of marine species. Genetic evidence of such dispersal and hence population mixing is provided by Davis *et al.* (2003). In a study performed on mud crab *Scylla serrata* that occurs along the South African coast it was concluded that a single stock exists with dispersal along the coast brought about by the Agulhas current. Currents play an important role in fish dispersal over vast distances (James *et al.* 2002). It has been suggested in many studies that the Agulhas current causes mixing of early life stages for many species and also the southward dispersal of these early stages (Hutchings *et al.* 2002). The occurrence of a southward dispersal would result in higher genetic diversity along the eastern Cape coast compared to that found along the

KwaZulu-Natal coast. However, inshore counter-currents on the continental shelf are responsible for northward migration/dispersal (Davis *et al*. 2003). Marginally increased diversity towards the north is suggested by the results of this study, indicating that inshore currents may have an influence on the dispersal of larvae from spawning sites.

The results suggest that there are two possibilities for explaining the observed pattern of genetic variation with no differentiation: (1) more than one genetically isolated population of *R. holubi* exist along the South African coast giving rise to three observed temporally separated spawning peaks. Extensive mixing between these genetically isolated populations during the larval phase may occur when larvae can be transported either southward along the coast by the warm Agulhas Current or northwards by retroflections or eddies until they reach a suitable estuarine environment and move into this estuarine habitat; (2) the other extreme is that only one spawning stock exists with migration along the coast, determined or heavily influenced by temperature and spawning at the three regions at three different times during the year. This explanation seems somewhat questionable since no spawning migrations have been observed in the field and since the Agulhas Current may influence the success of a northwards migration.

In order to conclude this study with certainty, there are a few considerations to take into account in planning the collection of samples for maximising the accuracy of the inferences from molecular data. To differentiate between the possible discrete (isolated) breeding populations that are not necessarily spatially separated, it is recommended that adults are sampled during peak spawning periods and are sampled over a few spawning seasons (temporal sampling). This will eliminate the genetic noise brought about by *ad hoc* sampling (Waples 1998). Furthermore, in order to statistically differentiate between different populations, it is recommended that the sample size per locality is increased to > 50 individuals and preferably up to 100 individuals per sample (Balloux & Lugon-Moulin 2002).

The pattern of allele variation observed is consistent with the hypothesis that *R. holubi* comprises a single breeding population or different populations with sufficient gene flow between them to mask any existing differentiated populations. The potential for long distance dispersal of adult *R. holubi*, coupled with the spatially and temporally separated spawning peaks that have been observed along the coast imply that extensive mixing of adult fish might take place before and after spawning events. Evidence exists that eggs and larvae are widely distributed along the coast after spawning events, mainly by long-shore wind driven currents

before larvae and juveniles enter and settle within the estuarine environment, thus giving rise to the observed pattern of variation. By using additional genetic markers, such as microsatellites, subtle genetic heterogeneity among geographic localities might be detected. The joint comparative study of mtDNA and nuclear DNA variability can provide important cues about the history of these species and details of current population structure (Wilson *et al*. 1985; Gold & Turner 2002).

Chapter 3

A preliminary assessment of nuclear genetic variation in *Rhabdosargus holubi* based on heterologous microsatellite loci

Abstract

Population genetic analyses using heterologous microsatellite amplification have been successfully completed for a number of studies, including numerous studies of variation within marine fish species. Microsatellite studies have proven to be more sensitive in detecting subtle population structure than mtDNA and/or protein polymorphisms in high gene flow species. The aim of the present study was to determine the population genetic structure from estimates of nuclear genetic variation across the distributional range of Cape stumpnose *Rhabdosargus holubi*. Distinct spawning peaks observed along the coast, believed to be influenced by developmental and environmental factors, as well as estuarine dependence of juveniles are believed to influence population structure of this South African endemic sparid. A total of 113 microsatellite loci previously isolated from marine fish species were tested for amplification. The success rate of heterologous microsatellite amplification was extremely low (0.02%), with only two polymorphic loci amplifying consistently for analysing 133 individuals sampled from six localities along the distributional range of *R. holubi*. Results from these two loci were insufficient to draw conclusions about the population genetic structure of *R. holubi* along the South African coast. Possible reasons for the low rate of amplification success and future research recommendations are discussed.

3.1 Introduction

Microsatellite DNA markers have been used extensively in fish population genetic studies aimed at detecting patterns of genetic variation (Brooker *et al.* 1994; Batargias *et al.* 1999; Stockley *et al.* 2000; De Innocentiis *et al.* 2001; Reusch *et al.* 2001; Chiu *et al.* 2002; Laikre *et al.* 2005;

Stockley *et al*. 2005). Detecting genetic variation and the distribution thereof can contribute to the understanding of current and historical population demography and processes that influence marine fish populations, i.e. behaviour and life history of the species as well as environmental factors (Avise 1998). Identification of population structure is important for the conservation and protection of existing genetic diversity as the latter determines the evolutionary potential of a species (Lippe *et al*. 2006). In studying marine populations the detection of genetic structure between and within populations becomes more complex due to the relative lack of barriers to gene flow and the large population sizes that characterise these organisms (Avise 1998; Waples 1998). Microsatellite DNA markers exhibit certain characteristics that make them more successful than previously used markers for studying marine fish populations with subtle population structure (Gold & Turner 2002). The most important characteristics include biparental inheritance, high level of variation that exists within loci (due to a relatively high mutation rate), genome wide distribution and the fact that they are normally selectively neutral (Moore *et al*. 1991; Garcia de Leon *et al*. 1997; Goldstein & Pollock 1997; Feral 2002; Gold & Turner 2002).

Microsatellites show a relatively high rate of mutation, on average 1 x 10^{-4} per gamete per generation (Nichols & Freeman 2004). This high mutation rate is explained by polymerase slippage, where polymerase tends to miscopy repeated units (Levinson & Gutman 1987), and slipped-strand mispairing during replication (Hancock *et al*. 1999). Unique primers are designed in the flanking regions for amplifying a genomic region that contains a well-defined repeat unit. Variation in the repeat unit length between alleles within individuals and between individuals is responsible for the observed variation (Goldstein & Pollock 1997). The drawback of using microsatellites is the high costs involved in isolating these repeat units and designing primers for the amplification of these regions (Mueller & Wolfenbarger 1999). Due to the high cost and time involved in the identification of polymorphic microsatellites and the development of primers, another option is to test and make use of loci identified in other related species. Heterologous microsatellite amplifications have been applied to, amongst others, primates, bovids, reptiles, insects, amphibians, cetaceans, marine and freshwater turtles, and fish (Schlotterer *et al*. 1991; Garza *et al*. 1995; Pepin *et al*. 1995: FitzSimmons *et al*. 1995; Rico *et al*. 1996; Doums 1999; Hille *et al*. 2002; Primmer & Merilä 2002). From these selected cases, it appears that microsatellite flanking regions are well conserved and stable in a diverse number of taxa. While Rico *et al.*'s (1996) initial research predicted conservation of flanking regions over 480 million years of marine fish evolution, using heterologous amplifications might cause a decrease in the level of polymorphism detected and a possible increase in null alleles (due to mutations in the

priming sequences) as the phylogenetic distance increases from the source species (Moore *et al.* 1991; FitzSimmons *et al.* 1995; Angers & Bernatchez 1996; Primmer *et al.* 1996; Rico *et al.* 1996). Despite these problems, microsatellites are very useful in studying closely related species, as shown by a study on brook charr, *Salvelinus frontinalis* (Takezaki & Nei 1996; Hille *et al.* 2002), and the use of these loci in studying other *Salvelinus* species (Angers & Bernatchez 1996). In a study on whiting *Merlangius merlangus* by Rico *et al.* (1997), using microsatellite loci isolated from this species as well as microsatellite loci isolated from closely related species, a low level of differentiation between sampling localities was detected. Successful cross species amplification within Sparidae was illustrated by Chen *et al.* (2005), using microsatellite primers isolated from the red sea bream *Chrysophrys major*, tested on black porgy *Sparus macrocephalus*. A success rate of 55 % was reported. Microsatellites isolated within Sparidae show high levels of polymorphism (4-26 alleles) as well as extremely high levels of heterozygosity, compared to other fish, at 0.95 (Batargias *et al.* 1999) and these loci may be applicable across this speciose family.

Within this study we used heterologous amplification of microsatellite markers in order to determine the level of nuclear genetic variation in the Cape stumpnose, *R. holubi*, an endemic sparid from the South African coast. *Rhabdosargus holubi* has a very broad distribution along the coast, and has been recorded from the Berg River on the west coast to southern Mozambique (including Inhaca Island) (Mann 2000). Adults can be found in the offshore environment whereas the juveniles use estuaries as nursery areas for the first two years of their life after which they enter the marine environment as adult fish (Whitfield 1998).

Year round spawning has been recorded along the coast for *R. holubi*, however spawning peaks have been observed. Harris & Cyrus (1996) recorded peaks in larval abundance in the St Lucia surf zone during June and August. Cowley *et al.* (2001) revealed a significant peak in abundance during late winter (August) in the East Kleinemonde surf zone (Bell *et al.* 2001). Whitfield & Kok (1992) suggested that the major recruitment period into southern Cape estuaries were from August to April. Blaber (1974a,c) suggested a winter/spring spawning peak along the Eastern Cape at West Kleinemonde Estuary. Post larval individuals were most abundant during November in the Sundays Estuary along the south coast (Harrison & Whitfield 1990). In the Swartvlei Bay surf zone, highest abundance was recorded during the summer with a decline in

densities during winter (Whitfield 1988). On the southwestern Cape coast near Fishoek, Bennett (1989) recorded peak recruitment of 14-40 mm individuals between October and April. Temperature seems to play an important role in determining spawning peaks, as was the case in yellow seabream, *Acanthopagrus latus* (Sparidae) (Abou-Seedo *et al*. 2003) and may play an important role influencing population structure. Three potential opportunities for population mixing exists, this is during the egg, larval, or adult life stages (Waples 1998). Eggs and larvae are passively transported to estuarine associated environments by offshore currents and winds. Within this environment, wind driven currents and eddies could also cause the transport of these larvae along the coast until larvae recruit at a size of 7-14 mm into estuarine environments (Beckley 1985; Whitfield 1989; Hauser & Ward 1998; Cowley & Whitfield 2001; Cowley *et al*. 2001; Strydom & d'Hotman 2005). Currents and eddies play an important role in fish dispersal, larval retention and mortality along with larval duration before settling (Hauser & Ward 1998; Lemaire *et al*. 2005).

The aim of this study was to determine how life history characteristics such as spawning patterns and juvenile estuarine dependence influence the level of nuclear genetic variation and the distribution of this variation throughout most of the distributional range of *R. holubi*. A total of 113 microsatellite primers were tested for amplification and polymorphism. Nuclear genetic variation at only two polymorphic microsatellite loci could be assessed and these preliminary results indicate high levels of diversity with no genetic differentiation between geographically separated localities.

3.2 Materials and methods

3.2.1 Sample collection

Samples were collected between December 2000 and March 2003, from seven geographic localities between Klein River Estuary on the south coast and Richards Bay Harbour along the east coast of South Africa. Figure 3.1 illustrates the geographical location of the seven sampling localities (interchangeable herein with the term populations) and the sample size for each locality is given in parenthesis. Mngazana (site 5) and Mngazi (site 6) sampling localities were combined into the Mngazana region prior to analysis due to the small geographic distance (4

km) between these two localities. Prior to combining these localities, an allele frequency homogeneity test among sampling localities were performed by using a randomisation procedure developed by Roff & Bentzen (1989, see paragraph 2.2.6). A total of 133 samples were used in this study. Juvenile samples were caught with a seine-net (50 m x 2 m with a 15 mm bar mesh) fitted with a bag, in a variety of littoral zone habitats (i.e. sandy, muddy and vegetated) within estuaries. Samples collected from Tsitsikamma National Park (site 3) originated from adults and were caught using standard hook and line techniques. All other samples collected were from juveniles. Approximately ⅔ of the pelvic fin from each individual sampled was removed and placed in 80% EtOH for preservation. In cases where the individuals were smaller than 10mm, the whole individual was placed in 80% EtOH. Fish sampled from East Kleinemonde Estuary (22), could be divided into three groups based on total length. The size groups and sample sizes were: eight individuals within the 65-75 mm range, 11 individuals within 92-116 mm and three within 130-133 mm.

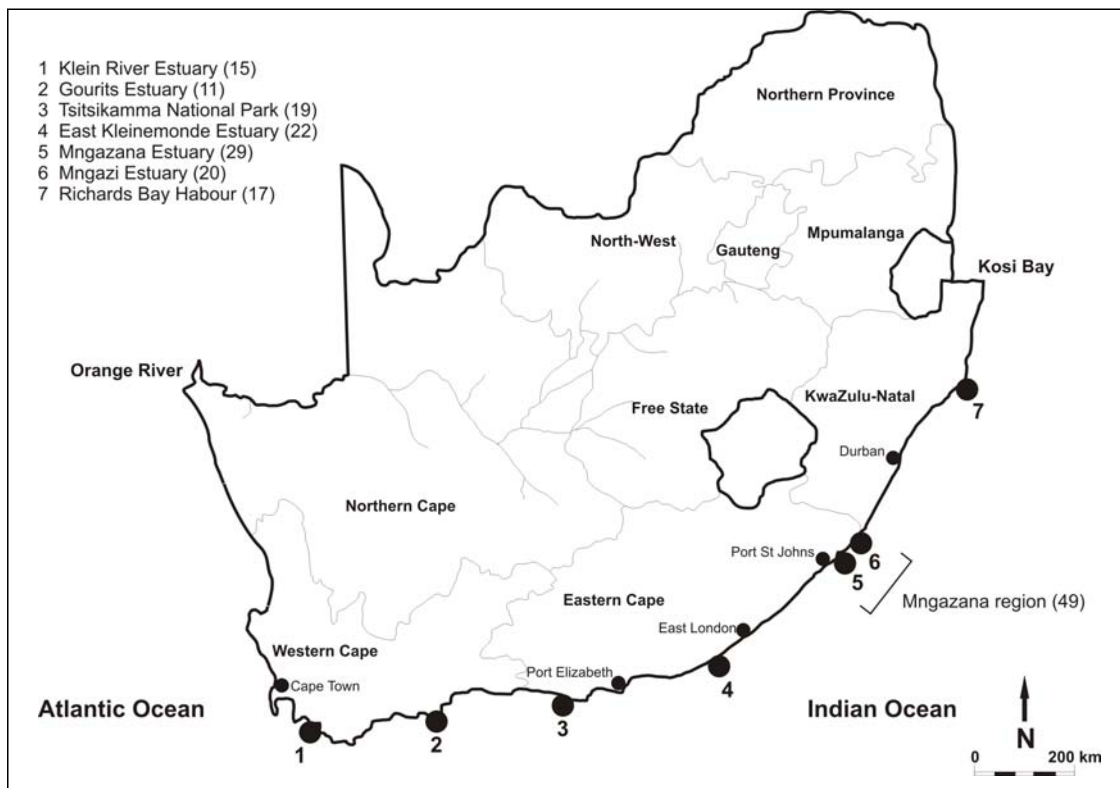

Figure 3.1 Geographic locations for all sampling localities of *Rhabdosargus holubi* along the coast of South Africa analysed for microsatellite variation. All sampling localities are given in the legend, together with the sample size per locality in parenthesis. A more detailed figure of the South African coastline can be seen in Chapter 1, Fig 1.1. (Modified from Harrison & Whitfield 2006).

3.2.2 Polymerase chain reaction

A total number of 113 microsatellite loci, developed in different fish families (Appendix I), were tested for amplification within *R. holubi*: 18 loci isolated from Sparidae, 25 loci isolated from Haemulidae and 70 loci isolated from Sciaenidae. Using the mitochondrial cytochrome *b* gene, sequence divergence between Sciaenidae and Sparidae showed to be 7.81 % (Allegrucci *et al.* 1999) and between Sparidae and Haemulidae, 22.38 % (Orrell *et al.* 2002). Appendix I contains information for all the microsatellites tested. All microsatellite primers were tested using the microsatellite amplification program described below. Annealing temperature used for testing amplification was initially set at 45 ºC and adjusted in the optimisation process for each locus independently. During the optimisation process temperature as well as $MgCl_2$ gradient PCR's were performed with $MgCl_2$ concentrations covering a range of 1.5 - 3.0 mM $MgCl_2$. Different polymerases were also tested in order to increase amplification success.

Success of PCR amplifications was tested by using 2% agarose gel electrophoresis. Loci that amplified successfully were tested for polymorphism using 8% denaturing poly-acrylamide (Whitehead Scientific) gel electrophoresis (Sambrook *et al.* 1989). PCR products were mixed with one volume LIS buffer (consisting of sucrose and bromo-phenol blue), denatured for 3 minutes at 95 ºC and then loaded on poly-acrylamide gels. Gels were run for more than twelve hours in order to ensure clear DNA fragment separation. Poly-acrylamide gel electrophoresis allows the identification of DNA fragments that differ by 1 bp in length. Amplified loci that displayed more than one band within the expected size range were considered polymorphic. These loci were subjected to the optimisation process discussed below. DNA fragment sizes were determined by comparing fragments to a 100 bp DNA ladder (Whitehead Scientific).

Four sets of microsatellite primers (Soc12, Soc406, Soc428 and GA2A) were initially identified as being polymorphic and the PCR reaction was optimised for each of these four loci. A second optimisation series involving an additional locus Soc433, and GA2A, using Expand High Fidelity Polymerase (Roche Diagnostics) was also performed after initial allele scoring failed using SuperTherm® DNA Polymerase (Southern Cross Biotechnologies). One primer from each pair was fluorescently labelled to allow analysis on an ABI 3100 automated DNA sequencer (Applied Biosystems). Primer names, primer sequences and annealing temperatures are given in Table 3.1. PCR amplifications of microsatellites were performed in 7 μl reaction volumes containing 0.2 pmol of each primer, 1 mM $MgCl_2$, 0.2 mM of each of the four nucleotides (Promega), 1 x reaction buffer, 0.2 U SuperTherm® DNA Polymerase (Southern Cross Biotechnologies)/Expand

High Fidelity Polymerase (Roche Diagnostics) and approximately 50 ng DNA as template. The final reaction volume was made up with Sabax® water.

Table 3.1 Microsatellite loci identified for determining genetic variation among *Rhabdosargus holubi* sampled from six geographical localities along the South African coast. The Soc abbreviation is used for loci isolated from red drum, *Sciaenops ocellatus* (Sciaenidae) and GA2A was isolated from *Pagrus auratus* (Sparidae).

Locus	Primer sequence 5'-3'	Repeat unit	Annealing temperature	Reference
Soc12	F: GCACCATCTTGCCACTGATGAATT R: GGGCTCTTACAACTCGTTTCAGAT	$(CA)_{12}$	54°C	Turner *et al.* (1998)
Soc406	F: TAGGGGGTAAGGTAGGATGATG R: GAAGAGCAGTGACGCTATCAAT	$(TG)_{3}$	50°C	O'Malley *et al.* (2003)
Soc428	F: GACATCGCATTTGTCTACAGAGTCG R: AACTCCCAGTCATAATATCCCTTT	$(TG)_{18}$	50°C	O'Malley *et al.* (2003)
Soc433	F: AGTACGCTGACCCTCAAACACA R: TTCTCTTTGCCTCCTTTTTCCCTGA	$(TG)_{16}$	50°C	O'Malley *et al.* (2003)
GA2A	F: ACGGACAGAGAGGGAGTGG R: CATCATCATCAGTCAGAGCTG	$(AG)_{10}$	52°C	Adcock *et al.* (2000)

PCR cycles included an initial denaturing step at 94 °C for 3 minutes; 10 cycles of denaturing at 94 °C for 30 seconds, primer annealing for 30 seconds, elongation at 72 °C for 30 seconds; 25 cycles of denaturing at 89 °C for 30 seconds, primer annealing for 30 seconds, elongation at 72 °C for 30 seconds and an extended final elongation step at 72 °C for 20 minutes in a GeneAmp® PCR System 9700 (Applied Biosystems). All PCR products were tested using a 2% agarose gel, using half the reaction volume. The remaining product was used for allele size determination using GeneScan (Applied Biosystems) on an ABI 3100 automated DNA sequencer (Applied Biosystems) by co-loading all four amplification products (four loci) for one individual. PCR amplification products were diluted as follow after an initial test run: both Soc12 and Soc428 1:12; Soc433 and GA2A 1:20; and Soc406 was not diluted.

Allele sizes were established by comparing fragments with the LIZ size standard (Applied Biosystems) in the software program GeneMapper (Applied Biosystems). Allele sizes were

scored using the default settings. Homozygous individuals for each of the loci were sequenced in order to confirm the presence of the correct microsatellite repeat in each amplified product. Sequencing reactions were performed as described in Chapter 2, paragraph 2.2.3.

3.2.3 Linkage disequilibrium

Genotypic disequilibrium was tested using Arlequin version 2.000 (Schneider *et al.* 2000). This is a test for the non-random association of alleles at different loci. Genotypic disequilibrium between locus pairs was tested using a likelihood-ratio test (Slatkin & Excoffier 1996). First, the likelihood of the data is computed assuming linkage equilibrium (no association between the two loci). For this computation, the allele frequencies are obtained as a product of the allele frequencies observed in the data. Second, the likelihood of the data is computed not assuming linkage equilibrium (genotypes at one locus influences the genotypes at the other locus). For this computation, the allele frequencies are estimated by using the Expectation-Maximization (EM) algorithm. The significance level was set at 0.05 with 100 000 steps in the Markov-chain and 10 000 dememorisation steps. This test of linkage disequilibrium assumes Hardy-Weinberg proportions of genotypes (the assumption of this test and its relevance to marine fish species are addressed in the discussion). The chance of falsely rejecting the null hypothesis (type I error) was reduced by applying Bonferroni corrections to all simultaneous statistical tests (Rice 1989).

3.2.4 Hardy-Weinberg equilibrium

Exact tests were performed in order to test for deviations from Hardy-Weinberg equilibrium, using Fstat version 2.9.3.2 (Goudet 2001). The exact test method is preferred above the goodness of fit methods due to small sample sizes and seeing as there are many alleles per locus, some sample genotype frequencies might be very small or zero since there are many possible genotypes within a population. This test was performed using a Markov-chain algorithm (10 000 dememorisation steps, 1 000 batches and 10 000 iterations per batch) with random mating (taken as the random union of two gametes) as the null hypothesis. This test was performed for each locus separately. Significance levels were calculated globally for each population at all loci and also for each locus in all populations. F_{IS} measures a departure from Hardy-Weinberg within sampling localities and F_{IT} measures such a departure in the species as

a whole. Depending on the F_{IS} results, one of two tests is performed in order to estimate F_{ST}. In the case where F_{IS} is not significantly different from zero, alleles can be considered as independent and F_{ST} is estimated by permuting alleles among samples. In the case where F_{IS} is significantly different from zero, genotypes are permuted among samples, as alleles within individuals are not considered independent (Goudet 1995).

3.2.5 Population differentiation

Fstat, version 2.9.3.2 for windows (Goudet 2001), was used to calculate allele frequencies and estimates of population genetic differentiation. Two models for microsatellite evolution were used for estimating population differentiation due to the fact that different microsatellite loci appear to mainly evolve following either the Infinite Alleles Model (IAM, Kimura & Crow 1964) or the Stepwise Mutation Model (SMM, Ohta & Kimura 1973). Each mutation that occurs within the IAM produces a new allele that is different from all existing alleles for that specific locus. Mutations occurring under the SMM will change the state of an allele only by one step, either by a single step forward or backward, with equal probability. Estimates for population differentiation were determined under both models: F_{ST} (Wright 1951, according to Weir & Cockerham 1984; Nei 1987) that assumes the IAM and R_{ST} (Slatkin 1995, following Rousset 1996) that assumes the SMM. Statistical significance of F_{ST} and R_{ST} values was estimated using 1000 permutations for both sets of calculations.

3.2.6 Mantel test

A Mantel test is an individual-based method that measures the association between elements of two matrices for continuously distributed populations. A Mantel test was used, as incorporated into Arlequin version 2.000 (Schneider *et al.* 2000), in order to measure the association between genetic differentiation (using pairwise F_{ST} values) and geographic distance for detecting an isolation-by-distance pattern between individuals sampled at different localities. A regression value is calculated: significant regression values provide evidence for a correlation between genetic differentiation and geographic distance between individuals sampled at different geographical localities where a large value indicates a strong correlation (Manel *et al.* 2003). A total number of 10 000 permutations were used.

3.2.7 Principle component analysis

The identification of spatial patterns was further examined by spatial clustering according to genetic similarity, using principal component analysis (PCA) within PCAGEN version 1.2.1 (Goudet 1999). PCA is a multivariate analysis method, summarizing all the variation for all loci used in this study and was conducted with all individuals as units as well as individuals sampled at the same locality as a unit. For East Kleinemonde Estuary each size class was defined as a unit (Manel *et al*. 2003). The low-dimensional ordination space created, group similar samples together and dissimilar samples far apart.

3.3 Results

3.3.1 Optimisation

Four microsatellite loci (Soc12, Soc406, Soc428 and GA2A) showed consistent amplification and using poly-acrylamide gel electrophoresis, appeared to be polymorphic during the first round of optimisation. All four loci were subsequently used for amplification of 133 samples. No amplification success was obtained using primers for microsatellite loci isolated from Haemulidae. Following analysis of allele sizes, it became apparent that locus Soc406 was monomorphic and was omitted from further analyses. Loci Soc12 and Soc428 could be successfully scored (Table 3.2). Locus GA2A showed irregular peaks that made the scoring of different alleles difficult and the results unreliable. Since only two loci could be reliably scored, another optimisation cycle was performed using Expand High Fidelity DNA Polymerase (Roche Diagnostics). During this optimisation process, temperature, $MgCl_2$, and DNA concentration gradients were performed. Locus Soc433 was the only locus that could be added to the study from all other potential loci tabulated in Table 3.1. Allele peaks for both loci, GA2A and Soc433, proved to be unreliable. Further optimisation of locus GA2A as well as locus Soc433 failed. Due to time constraints on this study, further attempts for optimisation were terminated. These two loci were subsequently excluded from any further analyses. Homozygous individuals sequenced (results not shown) for the microsatellite loci confirmed the repeat unit and showed no imperfections in the repeat unit.

Table 3.2 Microsatellite loci used for determining allele variation among *Rhabdosargus holubi* sampled from six localities along the South African coast.

Locus	Repeat unit	Annealing temperature	Sample size	Previously reported		Current study	
				Number of alleles	Size range (bp)	Number of alleles	Size range (bp)
Soc12	$(CA)_{12}$	54°C	133	1	187	14	179-207
Soc428	$(TG)_{18}$	48°C	133	8	188-232	21	164-208

3.3.2 Microsatellite allele variation

Both microsatellite markers were di-nucleotide repeats which proved to be highly polymorphic with continuous allele distribution within and across all populations. Similar allele sizes were detected within *R. holubi* individuals as were detected in the original studies on the species for which the loci were developed. All the alleles were coherent in terms of gain and loss of the repeat unit. The number of alleles detected was 14 for Soc12 and 21 for Soc428. Genotypes for both microsatellite loci were obtained for 133 individuals, 118 of which were analysed for mtDNA (Chapter 2). A stepwise process of mutation can be assumed since the allele size distribution is continuous (Fig 3.2 and Fig 3.3). For this reason R_{ST} fixation metrics were also applied (Piertney *et al.* 1998). Allele frequency distributions exhibited some rare alleles at both microsatellite loci. The major modes for these two loci differed in that two major modes were observed for Soc12 (Fig 3.2) and only one major mode was observed for Soc428 (Fig 3.3). The number of alleles (A, Table 3.3) at Soc12 was relatively evenly distributed over all sampling localities (number of alleles per locality ≈ 10). Number of alleles for Soc428 per locality ranged between 11 and 19, with Mngazana region showing the highest diversity.

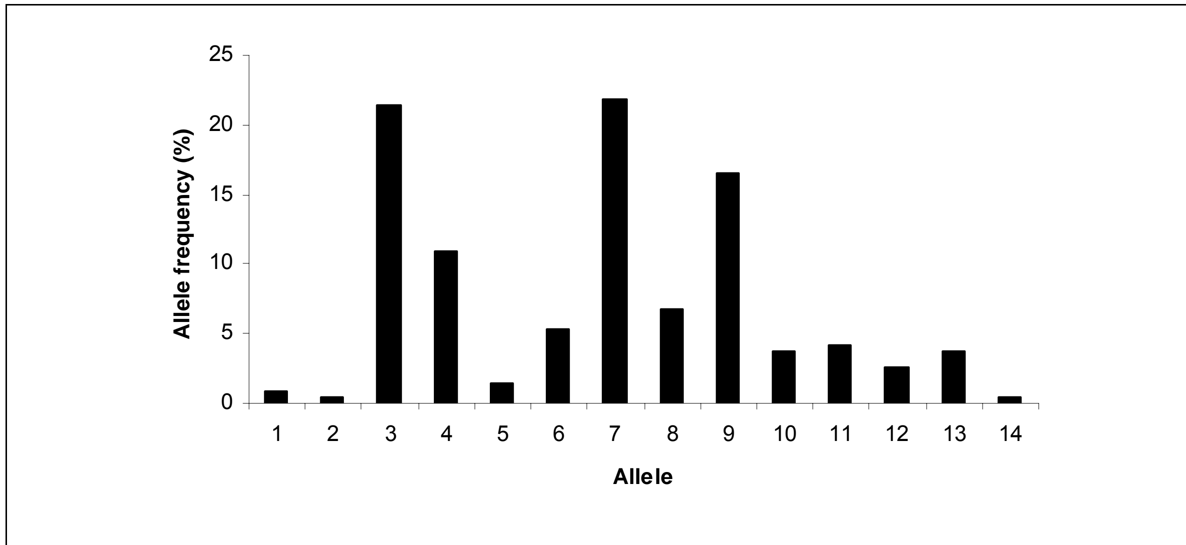

Figure 3.2 Allele frequency distribution at locus Soc12 over all sampling localities for *Rhabdosargus holubi*. A total number of 14 alleles were identified.

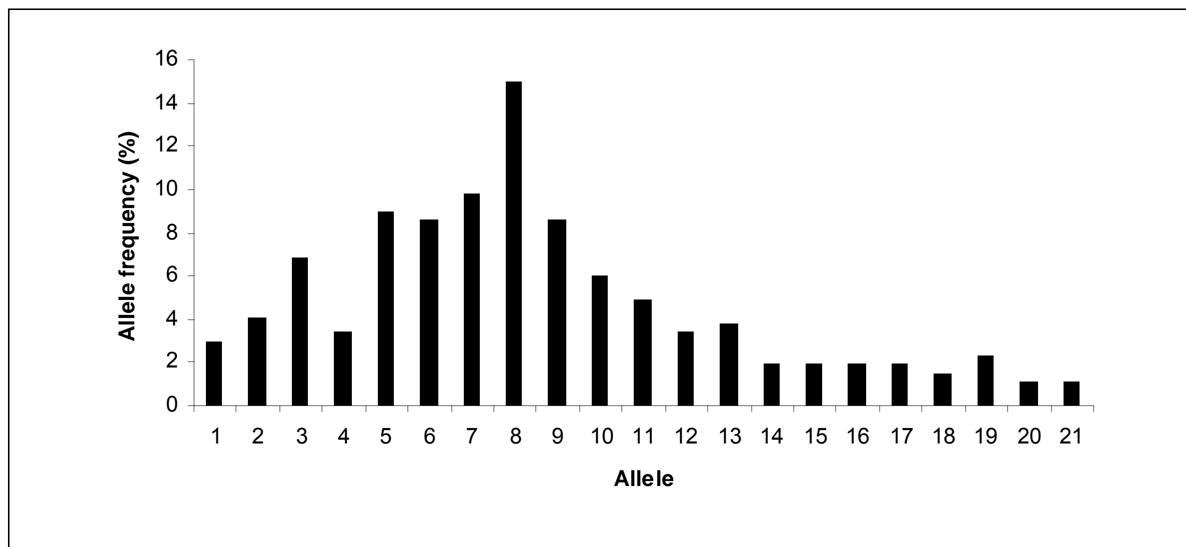

Figure 3.3 Allele frequency distribution at locus Soc428 over all sampling localities for *Rhabdosargus holubi*. A total number of 21 alleles were identified.

Descriptive statistics are presented in Table 3.3. The number of samples analysed per sampling locality ranged between 11 for Gourits River Estuary and 49 for the Mngazana region. The number of alleles identified for each locus within all sampling localities was almost the same for both loci, irrespective of the number of samples analysed, with alleles three and seven for Soc12 being the most common for all localities (corresponding to the overall frequencies for these alleles within the study, Fig 3.2). Allele eight was the most common allele found for Soc428, also corresponding to its overall frequency for all the samples analysed (Fig 3.3). The frequencies for the most common alleles for both loci displayed similar values throughout all sampling localities, but the frequency of the most common alleles at Soc428 was generally lower than for Soc12 due to the higher number of alleles identified for Soc428.

3.3.3 Linkage disequilibrium

Permutation tests indicated that there was no significant linkage disequilibrium, with $p > 0.05$ for all pairwise comparisons and for both loci within each of the sampling localities and overall (Table 3.3).

3.3.4 Hardy-Weinberg equilibrium

No significant departures from Hardy-Weinberg equilibrium were observed for Soc12 (overall $H_O = 0.827$, $H_E = 0.858$, $p > 0.124$) except at Gourits Estuary, where only heterozygous individuals were observed. Significant departure from Hardy-Weinberg equilibrium was observed at five of the sampling localities for Soc428 (overall $H_O = 0.714$, $H_E = 0.929$, $p < 0.001$), only the Mngazana region was in HW equilibrium. This could be attributed to the larger sample size at the Mngazana region in conjunction with the high level of allele diversity displayed by Soc428. Departures from Hardy-Weinberg equilibrium indicated heterozygote deficit within sampled populations and will be discussed later.

Table 3.3 Microsatellite results for Soc12 and Soc428 for each sampling locality. For each sampling locality the following are shown: number of samples analysed (N), number of alleles identified (A), most common allele (A_C), frequency of the most common allele (F_C), allele range (A_R), expected heterozygosity (H_E), observed heterozygosity (H_O), p-value for Hardy-Weinberg equilibrium test (p) and the exact p-value for the linkage disequilibrium test (EP).

Sampling locality		Locus		Sampling locality		Locus	
		Soc12	Soc428			Soc12	Soc428
Richards Bay Harbour	N	17	17	Tsitsikamma National Park	N	19	19
	A	10	16		A	10	16
	A_C	7	8		A_C	3	5/7
	F_C	0.294	0.147		F_C	0.263	0.158
	A_R	2-13	1-20		A_R	1-13	2-21
	H_E	0.881	0.957		H_E	0.824	0.935
	H_O	0.706	0.824		H_O	0.842*	0.684
	p	0.092	**0.001**		p	0.942	**0.001**
	EP	0.196			EP	0.400	
Mngazana region	N	49	49	Gourits Estuary	N	11	11
	A	12	19		A	9	11
	A_C	3	8		A_C	4	2/6/8
	F_C	0.235	0.235		F_C	0.273	0.136
	A_R	1-13	1-21		A_R	3-13	1-18
	H_E	0.877	0.913		H_E	0.870	0.957
	H_O	0.796	0.776		H_O	1.000*	0.636
	p	0.053	0.058		p	**0.032**	**0.015**
	EP	0.500			EP	0.896	
East Kleinemonde Estuary	N	22	22	Klein River Estuary	N	15	15
	A	11	17		A	10	14
	A_C	3/7	8		A_C	7	7/9
	F_C	0.250	0.136		F_C	0.267	0.167
	A_R	3-14	2-19		A_R	3-13	1-20
	H_E	0.843	0.941		H_E	0.867	0.954
	H_O	0.818	0.727		H_O	0.933*	0.467
	p	0.436	**0.000**		p	0.953	**0.000**
	EP	0.185			EP	0.254	

*Observed heterozygosity was higher than the expected heterozygosity under Hardy-Weinberg expectations.

Numbers in bold represent significant p-values after Bonferroni correction with $p \leq 0.004$.

3.3.5 Population differentiation

Overall population differentiation estimation, using the IAM, exhibited a non-significant estimate of F_{ST} = 0.00, with F_{ST} = 0.003 ($p > 0.05$) for Soc12 and F_{ST} = -0.002 ($p > 0.05$) for Soc428. Taking allele size into consideration, using the SMM, an overall non-significant value for R_{ST} = -0.01 was estimated, with R_{ST} = -0.013 ($p > 0.05$) for Soc12 and R_{ST} = -0.007 ($p > 0.05$) for Soc428. Pairwise comparisons of F_{ST} resulted in generally low or negative non-significant values for all pairs (Table 3.4).

Table 3.4 Pairwise R_{ST} (above diagonal) and F_{ST} (below diagonal) comparisons between all sampling localities. No significant population differentiation values were obtained.

Sampling locality	RBH	MR	EKE	TNP	GE	KRE
Richards Bay Harbour (RBH)		-0.001	-0.012	-0.017	0.058	-0.011
Mngazana region (MR)	0.004		-0.020	-0.014	0.081	-0.018
East Kleinemonde Estuary (EKE)	-0.004	-0.002		-0.023	0.056	-0.025
Tsitsikamma National Park (TNP)	0.000	0.011	-0.007		0.032	-0.029
Gourits Estuary (GE)	-0.004	-0.010	-0.014	0.011		0.025
Klein River Estuary (KRE)	-0.002	0.008	-0.008	-0.020	-0.008	

3.3.6 Mantel Test

The Mantel test for isolation-by-distance revealed no association between geographical and genetic distances ($r = -0.0247$, $p = 0.523$) thereby failing to reject the null hypothesis that gene flow in *R. holubi* occurs randomly with respect to geographical distance.

3.3.7 Principle component analysis

All samples analysed were ordinated using principle component analysis (Fig 3.4) with the first Principle Component (PC1) explaining 11.78% ($p = 0.071$) of the total variance and PC2, 11.50% ($p = 0.11$). The first Principle Component (PC1) explained 43.28% ($p = 0.09$) of the total variance and PC2, 21.31% ($p = 0.90$) after ordination of all sampling regions (Fig 3.5). Richards

Bay Harbour was the only locality that displayed positive values for both principle components, separating it from the rest of the sampling localities. The Mngazana region and Gourits Estuary displayed almost similar values for both principle components, grouping them closely together. Tsitsikamma National Park and Klein River Estuary were also relatively closely grouped.

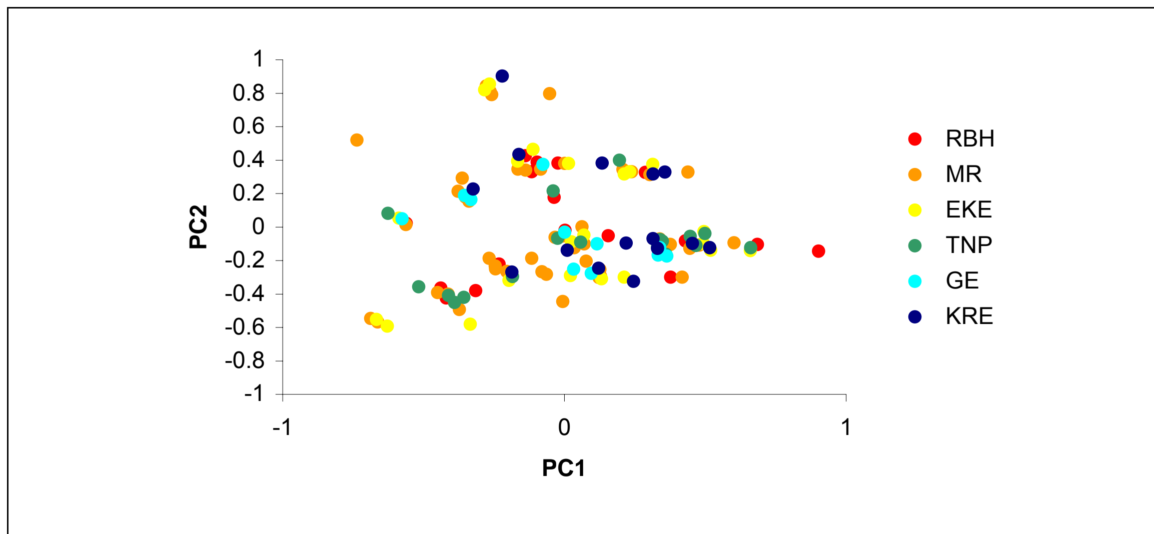

Figure 3.4 A scatter plot of the PCA axes scores for all samples (133) from all localities/regions (6) of *Rhabdosargus holubi*. The x-axis of this plot is the first PCA axis, explaining 11.78 % of the variance; the y-axis is the 2nd PCA axis, explaining 11.50 % of the variance. Colour legend labels represent: Richards Bay Harbour (RBH), Mngazana region (MR), East Kleinemonde Estuary (EKE), Tsitsikamma National Park (TNP), Gourits Estuary (GE), Klein River Estuary (KRE).

Ordination of the three size classes within the East Kleinemonde Estuary sample (Fig 3.6) using principle component analysis showed that the smallest size classes both have a negative PC1 score, thus genetically more similar whereas the largest size class had a much larger positive PC1, with PC1 explaining almost 65% of the variation.

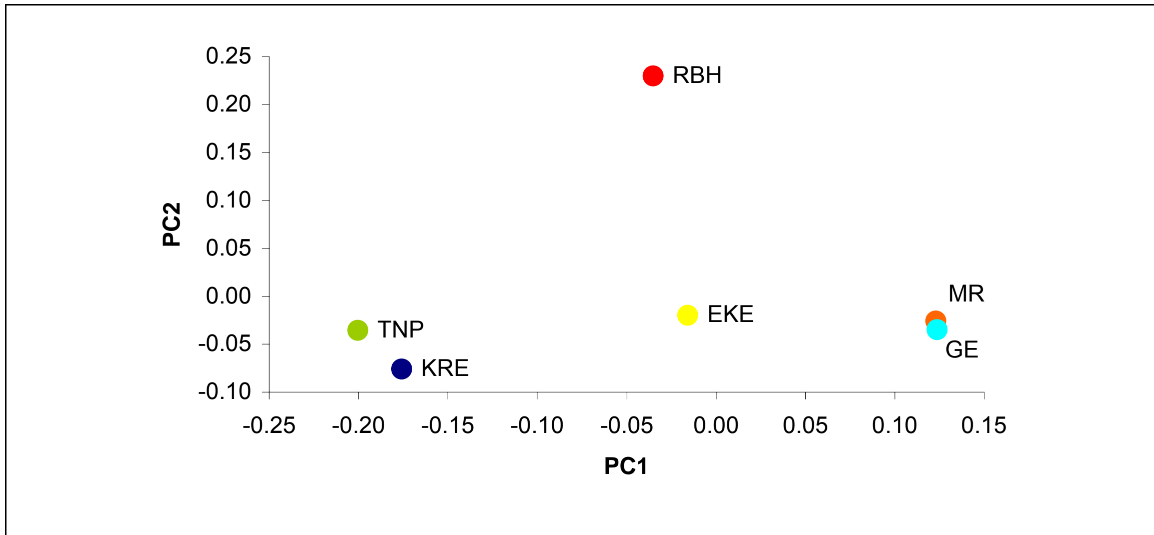

Figure 3.5 A scatter plot of the PCA axes scores for the sampling localities of *Rhabdosargus holubi*. The x-axis of this plot is the first PCA axis, explaining 43.28 % of the variance; the y-axis is the 2nd PCA axis, explaining 21.31 % of the variance. Locality data point labels represent: Richards Bay Harbour (RBH), Mngazana region (MR), East Kleinemonde Estuary (EKE), Tsitsikamma National Park (TNP), Gourits Estuary (GE), Klein River Estuary (KRE).

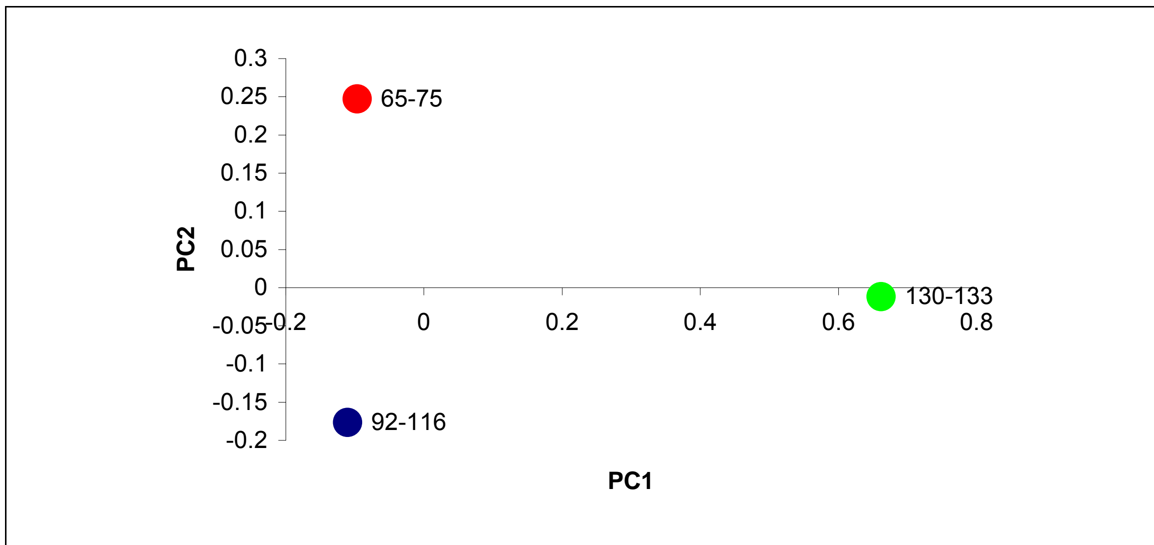

Figure 3.6 A scatter plot of the PCA axes scores for the three size classes of *Rhabdosargus holubi* from East Kleinemonde Estuary. The x-axis of this plot is the first PCA axis, explaining 64.63 % of the variance; the y-axis is the 2nd PCA axis. Data points represent size class ranges.

3.4 Discussion

The power of analysis in this study was low due to the fact that only two microsatellite loci could be used, combined with relatively small sample sizes per locality. Thus, this study should be considered as preliminary and more microsatellite loci and larger sample sizes per sampling locality will be needed in order to draw more reliable conclusions about allele frequency distribution and in the case of more than one population being identified, gene flow within and between *R. holubi* populations.

Microsatellite primers developed for one species have been used successfully for population genetic studies in closely related species (Moore *et al.* 1991; Angers & Bernatchez 1996; Primmer *et al.* 1996; Kirchman *et al.* 2000; Lima-Rosa *et al.* 2000; Hille *et al.* 2002). Microsatellites developed in marine turtles has been reported to be conserved for over 300 million years (FitzSimmons *et al.* 1995). In a study conducted by Rico *et al.* (1996) on marine fish, it was found that some microsatellite regions were conserved for over 470 million years between a number of diverse fish species belonging to different suborders. Microsatellite degradation is believed to occur by the introduction of imperfections into the repeat sequence of the microsatellite brought about by polymerase slippage during the replication process (Garza & Freimer 1996). Mutations within the flanking regions (priming regions) will also cause inconsistent amplification or the absence of amplification. This is also the cause for the frequently observed decrease in the level of polymorphism detected in heterologous amplification studies especially with increased phylogenetic distance between the species for which the microsatellites were isolated and the species studied (FitzSimmons *et al.* 1995). This can cause erroneous results and wrongful interpretation of population dynamics (Rico *et al.* 1996; O'Connell & Wright 1997). Within this study, microsatellite loci isolated from Sparidae, Sciaenidae and Haemulidae were used. Allegrucci *et al.* (1999) studied the phylogenetic relationship between 15 Perciformes families by using the mitochondrial cytochrome *b* gene and estimated a 7.81 % sequence divergence between Sciaenidae and Sparidae. This was relatively low compared to the sequence divergence between the two most divergent families at 26.23 %. Orrell *et al.* (2002) estimated the sequence divergence, within the cytochrome *b* gene, between Sparidae and Haemulidae to be 22.38 %. Mutations within the flanking regions of microsatellite loci are the most likely explanation for the low amplification success (15 out of 113 loci, 13 %). From these, only two loci (0.02%) showed consistent amplification and were also polymorphic, thus could be analysed successfully. Allele diversity for Soc428 was similar to the original study in which it was isolated and analysed in the sciaenid *Sciaenops ocellatus*

(O'Malley *et al*. 2003), indicating that a decrease in the level of polymorphism, observed in some studies that make use of heterologous microsatellites, was not evident for this locus (Takezaki & Nei 1996; Hille *et al*. 2002). Turner *et al*. (1998) have not used locus Soc12 in any population analyses after its isolation but similar to Soc428 it also showed high allelic diversity in *Rhabdosargus*. It is surprising that only one out of 18 microsatellite loci (locus GA2A) isolated from Sparidae could be amplified within this study. Amplification of loci isolated from Haemulidae performed as expected since larger sequence divergence was observed between Sparidae and Haemulidae (22.38%) than between Sparidae and Sciaenidae (7.81%) (Allegrucci *et al*. 1999; Orrell *et al*. 2002).

After testing for Hardy-Weinberg equilibrium, significant heterozygote deficiency was observed for Soc428. Heterozygote deficiency can be explained by the high level of polymorphism (with 21 alleles) combined with the relative small sample sizes that ranged between 11 and 49 with an average of 22 samples per locality, almost matching the number of alleles identified within this study. Another possibility could be the presence of null alleles that cause an excess of homozygotes relative to Hardy-Weinberg equilibrium genotypic proportions (Hedgecock *et al*. 2004). Null alleles frequently occur when heterologous amplifications are performed, this is due to mutations within the annealing positions of primers designed for the original study species, resulting in alleles that do not amplify (FitzSimmons *et al*. 1995). However, in this study, homozygous individuals were detected and the alleles sequenced for these individuals. These sequences corresponded to the alleles identified in the original study. Another reason why the presence of null alleles can be ruled out was the observation that amplification products were obtained for all individuals, in the case of null alleles being present, the expectation would be that some individuals would not have amplification products. Another possible explanation for the observed heterozygote deficiency would be the presence of mating groups (assortative mating) where only a few individuals reproductively contribute to the next generation (Takezaki & Nei 1996; Rico *et al*. 1997).

In the case where sampling is unbalanced, allelic goodness of fit tests have previously been proven to be the most powerful, such as Fisher's exact test of population differentiation (Goodman 1997). Distances that make use of the product of allele frequencies shared between populations are most accurate and also more so when allelic variance, and thus the mutation rate, is high (Takezaki & Nei 1996). Fixation indices might also vary between different loci because of stochastic processes in finite populations. Therefore, single locus data estimates of

fixation indices will have large confidence intervals associated with them and due to this, most studies employing microsatellites normally use four or more loci (Lundy *et al*. 1999). No significant heterogeneity between allele frequencies at different sampling localities for *R. holubi* was detected. Negative F_{ST} values obtained indicate that the level of genetic structuring is so small that it could not be detected (Slatkin & Barton 1989). Homogeneity between different subpopulations has generally been accepted as proof for the existence of a single population where gene flow between different localities is high enough to prevent genetic isolation/divergence. Camper *et al*. (1993) and Gold *et al*. (1994) challenged this view. The reasoning behind their objection is that any null hypothesis is impossible to prove and that homogeneity is only proof that samples were drawn from a population with the same parametric allele frequencies.

With this kept in mind, the observed level of variation may have its origin at one or more of three different dispersal opportunities: (i) at the pelagic stage where larvae are passively dispersed by offshore currents that have their origin from the Agulhas current (Strydom & d'Hotman 2005); (ii) sub-adult movement out of estuaries into neighbouring estuaries or within currents along the coast (Bilton *et al*. 2002); (iii) adult movement offshore into deeper waters where large shoals are formed (Ward *et al*. 2003). Recent results from Gold *et al*. (2001) indicate that passive movement of eggs and/or larvae are taking place, causing a mixing of offspring from different spawning sites. This was also observed by Garcia de Leon *et al.* (1997) working on European sea bass *Dicentrarchus labrax* from Spain and France.

Rhabdosargus holubi shares many life history characteristics with red drum *Sciaenops ocellatus* that occurs in the Western Atlantic Ocean, primarily in the northern Gulf and along the east coast of the United States. Juveniles are also estuarine dependent, spending their sexually immature life cycle stage in bays and estuaries, moving offshore when sexual maturity is reached. Evidence exist that movement between adjacent estuaries or bays can occur at the egg, larval and juvenile stages (Gold & Turner 2002). Population structure identified using mtDNA (θ_{ST} = 0.002) was reinforced with microsatellite data (F_{ST} = 0.003). Isolation-by-distance was detected with neighbourhood size estimated at around 500-700 km. It has been shown that oceanic currents can transport larval red drum, during their planktonic existence (12-16 days), to adjacent localities. Adults that were tagged with ultrasonic transmitters showed strong site-fidelity with 50 out of 75 tagged adults returning to the same shoal where they were tagged

initially (Gold & Turner 2002). It seems likely that similar processes may act to homogenize genetic diversity across the distributional range of the Cape stumpnose.

A negative correlation has been reported between genetic differentiation and dispersal ability for shallow water teleosts (Waples 1998). The detection of genetic differentiation is complicated by the fact that genetically diverged breeding groups can occur in sympatry. In order to identify genetically structured/differentiated populations that occur in sympatry, temporal stability studies need to be performed. Temporal stability has only rarely been addressed in the past due to the fact that most studies performed only had a single year sampling scheme and in cases where sampling was performed over a few years to a few decades, consecutive cohorts were not necessarily sampled. Gold & Turner (2002) performed a study in which they tested for temporal stability over four consecutive cohorts at the same sampling localities. Weak genetic differentiation was detected between the four cohorts, indicating the importance of temporal sampling. Temporal sampling becomes important in the marine environment since genetic divergence is generally much lower than that normally observed for freshwater or terrestrial species and the fact that spatially divergent populations potentially exhibit different or independent population dynamics. Temporal sampling will enable the differentiation between genetic signal and genetic noise that may be caused by opportunistic sampling (Gold & Turner 2002). Principle component analysis performed for all individuals within this study showed no clear ordination, however, the analysis performed for the three size classes observed from East Kleinemonde Estuary (Fig 3.6) suggested that there is separation between the three size classes, with the larger size class (130mm – 133mm) more separated from the two smaller size classes by the first component. The second component separated the two smaller classes. An increased sample size will be needed for conclusive results (Manel *et al.* 2003).

Within this study, a large number of alleles per locus were detected reflecting the same diversity observed in the original studies. When a large number of alleles is detected and in order to accurately reflect allele frequency distributions, large numbers of individuals are needed (Lundy *et al.* 1999). Considering that most of the genetic studies on marine organisms in the past analysed only 25 individuals per sampling locality, Ruzzante (1998) tested the effect of sample size on genetic distance measures and fixation indices at microsatellite loci, and concluded that sample sizes should be larger than 50 individuals in order to estimate divergence with a high probability of accuracy (Lundy *et al.* 1999). Sample size within the current study was not sufficient. Carvalho & Hauser (1998) states that a minimum of 50 individuals from a locality

would be sufficient to be able to show genetic differentiation between localities for loci with 5-10 alleles and that more individuals will be required for loci that show higher diversity. Preferred sample size according to Ward (2000) is a size of 100 samples per locality and ideally temporally separated samples, together with the use of least five loci for reliable estimates of F-statistics (Goudet 1995). A study by Waples (1998) addressed the interaction between detecting genetic signal and minimizing noise within data that is brought about by sample sizes. On average a F_{ST} value of 0.02 is expected for marine fish. Sample sizes of 25 individuals per site introduce sufficient noise to mask any population differentiation signal. An increase in sample size to 100 lowers the noise significantly and for 200 and more individuals, noise within the data will be at a minimum. This supports the notion that sample sizes should at least be close to 100 individuals. Although inconclusive at present, future use of species-specific microsatellite loci of moderate diversity would enable the reliable detection of spatial and/or temporal population differentiation within *R. holubi*, even though it may be expected that such a signal would be weak given the high dispersal potential in this species.

Chapter 4

Conclusion

Incorporating molecular genetics into species biology and the identification of non-biological factors that influence species demography remains one of the biggest challenges to population biologists (DeSalle & Amato 2004). Within this study, I set out to determine the mitochondrial and nuclear genetic variation that exist across the distributional range of *Rhabdosargus holubi* along the South African coast. Because there are no physical links between the mitochondrial and nuclear genomes, different processes may influence loci within these genomes at the same time. It is therefore highly informative to include markers from both genomes to assess intraspecific genetic variability (Lemaire *et al*. 2005). In the present study, both mtDNA and microsatellite loci showed no clear evidence of frequency differences between Cape stumpnose from different sampling localities and there thus appears to be a lack of spatial genetic differentiation in this local endemic sparid.

The results suggest that there are two possibilities for explaining the observed pattern of genetic variation with no geographical differentiation: (1) more than one genetically isolated population of *R. holubi* exist along the South African coast giving rise to observed temporally separated spawning peaks (Fig 4.1). Extensive mixing between these genetically isolated populations during the larval phase may occur when larvae can be transported either southward along the coast by the warm Agulhas Current or northwards by retroflections or eddies until they reach a suitable estuarine environment and move into this estuarine habitat, or (2) the other extreme is that only one spawning stock exists with migration along the coast, determined or heavily influenced by temperature and spawning at the three regions at three different times during the year. This explanation seems somewhat questionable since no spawning migrations have been observed in the field and since the Agulhas current may influence the success of a northwards migration. Although understanding regarding the dispersal or migration of adults remains inconclusive, evidence for three means of larval dispersal to suitable estuarine habitats, and an opportunity for discrete stocks to undergo mixing, have been reported: passive transport, active behaviour that can alter larval movement and active swimming, in a number of other species. This explanation seems probable since no spawning migrations have been observed in the field.

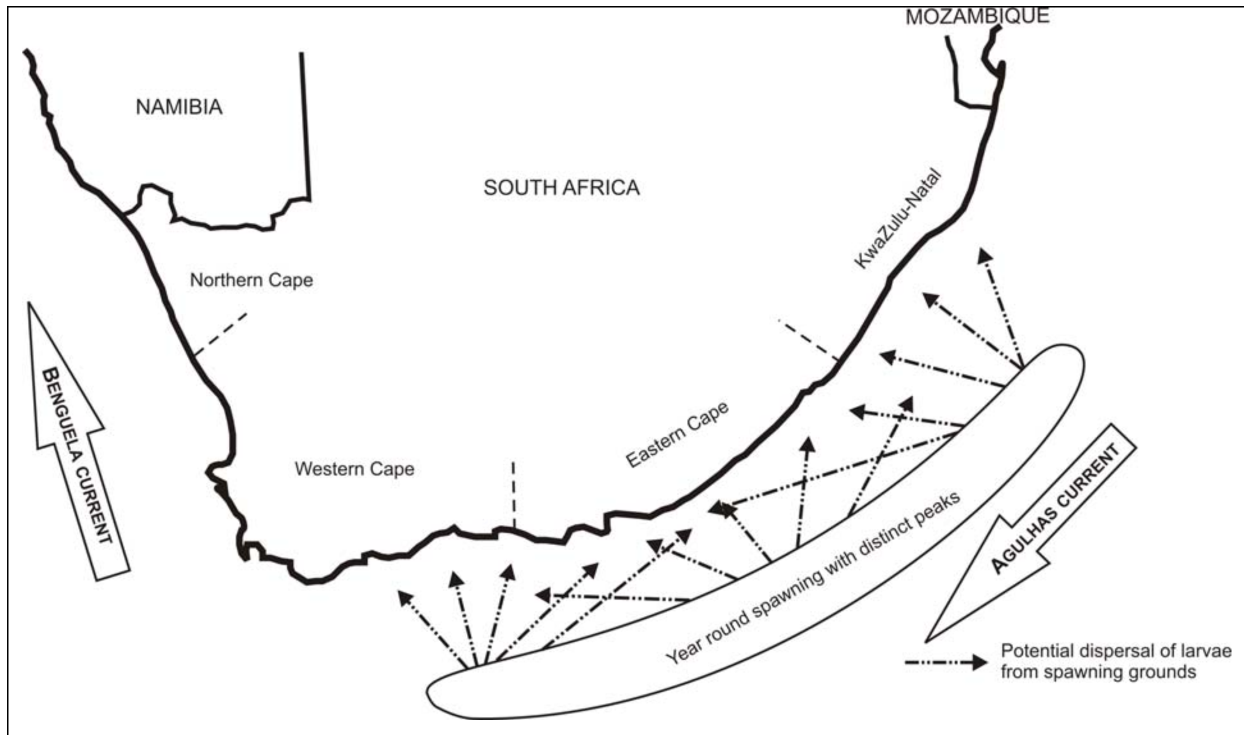

Figure 4.1 Mitochondrial DNA and microsatellite results indicate that mixing occurs along the coast potentially via larval dispersal after spawning events, however slightly lower genetic diversity was observed towards the southern Cape.

The intuitive expectation would be that species associated with estuarine environments for most part of their life cycle (reach maturity and reproduce), would show a higher level of divergence between geographically separated localities along the 3100 km South African coast. Species that only associate with estuaries for a part of their life cycle would have a lower level of divergence between geographically separated localities since there is ample opportunity for mixing between different estuarine localities while they are present in the sea. The third expectation would be that pelagic species would show extremely low to no differentiation between geographically separated localities along the coast since greater opportunity for dispersal of these species exist. These expectations remain to be tested along the South African coast for a range of species representative of different life history strategies with emphasis on endemics and priority species for marine resource management.

Future research

One of the shortcomings of the present study is the fact that an attempt is made to formulate conclusions about the behaviour and distribution of the adult spawning population by sampling the progeny. As discussed above, this can introduce some problems in the reliability of the signal detected. The best way of avoiding this potential problem and to detect whether possible genetic differences exist between localities will be to sample the spawning adults at different localities. Actual migration movement between localities as well as male and/or female mediated gene flow could also be determined using microsatellite loci specifically isolated within the species studied. The use of a structured sampling scheme where samples are collected at the same geographically separated localities over a few years, throughout the distributional range of the species, will allow the testing of spatial and temporal stability of patterns of genetic differentiation.

References

Abou-Seedo FS, Dadzie S, Al-Kanaan KA (2003) Sexuality, sex change and maturation patterns in the yellowfin seabream, *Acanthopagrus latus* (Teleostei: Sparidae) (Houttuyn, 1782). *Journal of Applied Ichthyology* **19**, 65-73.

Adcock GJ, Bernal Ramazrez JH, Hauser L, Smith P, Carvalho GR (2000) Screening of DNA polymorphisms in samples of archived scales from New Zealand snapper. *Journal of Fish Biology* **56**, 1283-1287.

Agardy MT (1994) Advances in marine conservation: the role of marine protected areas. *Trends in Ecology & Evolution* **9**, 267-270.

Allanson BR, Baird D (1999) *Estuaries of South Africa* (eds. Allanson BR, Baird D).

Allegrucci G, Caccone A, Sbordoni V (1999) Cytochrome *b* sequence divergence in the European sea bass (*Dicentrarchus labrax*) and phylogenetic relationships among some Perciformes species. *Journal of Zoological Systematical and Evolutionary Research* **37**, 149-156.

Allison GW, Lubchenco J, Carr MH (1998) Marine reserves are necessary but not sufficient for marine conservation. *Ecological Applications* **8**, S79-S92.

Alverson DL, Freeberg MH, Murawski SA, Pope JG (1996) A global assessment of fisheries bycatch and discards. *FAO Fisheries Technical Paper* **339**.

Angers B, Bernatchez L (1996) Usefulness of heterologous microsatellites obtained from brook charr, Salvelinus fontinalis Mitchill, in other Salvelinus species. *Molecular Ecology* **5**, 317-319.

Arnaud J-F, Madec L, Guiller A, Bellido A (2001) Spatial analysis of allozyme and microsatellite DNA polymorphisms in the land snail Helix aspersa (Gastropoda: Helicidae). *Molecular Ecology* **10**, 1563-1576.

Attwood CG (2003) Dynamics of the fishery for galjoen *Dichistius capensis*, with an assessment of monitoring methods. *African Journal of Marine Science* **25**, 311-330.

Attwood CG, Bennett BA (1995) Modelling the effect of marine reserves on the recreational shore-fishery of the South-Western cape, South Africa. *South African Journal of Marine Science* **16**, 227-240.

Attwood CG, Cowley PD (2005) Alternate explanations of the dispersal pattern of galjoen *Dichistius capensis*. *African Journal of Marine Science* **27**, 141-156.

Attwood CG, Harris JM, Williams AJ (1997a) International experience of marine protected areas and their relevance to South Africa. *South African Journal of Marine Science* **18**, 311-332.

Attwood CG, Mann BQ, Beaumont J, Harris JM (1997b) Review of the state of marine protected areas in South Africa. *South African Journal of Marine Science* **18**, 341-367.

Avise JC (1986) Mitochondrial DNA and the evolutionary genetics of higher animals. *Philosophical Transactions of the Royal Society London B* **312**, 325-342.

Avise JC (1987) Identification and interpretation of mitochondrial DNA stocks in marine species. In: *Proceedings of the Stock Identification Workshop* (eds. Kumpf H, Nakamura EL), pp. 105-136. National Oceanographic and Atmospheric Administration, Panama City.

Avise JC (1998) Conservation genetics in the marine realm. *Journal of Heredity* **89**, 377-382.

Avise JC (2000) Stability, equilibrium and molecular aspects of conservation in marine species. In: *Marine Genetics* (eds. Sole-Cava AM, Russo CAM, Thorpe JP). Kluwer, Dordrecht, The Netherlands.

Avise JC, Arnold J, Ball RM *et al.* (1987) Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annual Review of Ecology and Systematics* **18**, 489-522.

Avise JC, Helfman GS, Saunders NC, Hales LS (1986) Mitochondrial DNA differentiation in North Atlantic eels: population genetic consequences of an unusual life history pattern. *Proceedings of the National Academy of Science USA*, **83**, 4350-4354.

Avise JC, Lansman RA (1983) Polymorphism of mitochondrial DNA in populations of higher animals. In: *Evolution of genes and proteins* (eds. Nei M, Koehn RK), pp. 147- 164. Sinauer Associates INC, Sunderland, Massachusettes.

Ballard JWO, Whitlock MC (2004) The incomplete natural history of mitochondria. *Molecular Ecology* **13**, 729-744.

Balloux F, Lugon-Moulin N (2002) The estimation of population differentiation with microsatellite markers. *Molecular Ecology* **11**, 155-165.

Bargelloni L, Alarcon JA, Alvarez MC *et al.* (2003) Discord in the family Sparidae (Teleostei): divergent phylogeographical patterns across the Atlantic-Mediterranean divide. *Journal of Evolutionary Biology* **16**, 1149-1158.

Batargias C, Dermitzakis E, Magoulas A, Zouros E (1999) Characterization of six polymorphic microsatellite markers in gilthead seabream, *Sparus aurata* (Linnaeus 1758). *Molecular Ecology* **8**, 897-898.

Baxter AJ, Meadows ME (1999) Evidence for Holocene sea level change at Verlorenvlei, Western Cape, South Africa. *Quaternary International* **56**, 65-79.

Beckley LE (1983) The ichtyofauna associated with *Zostera capensis* Setchell in the Swartkops estuary, South Africa. *South African Journal of Zoology* **18**, 15-24.

Beckley LE (1984) Shallow-water trawling off Swartkops estuary, Algoa Bay. *South African Journal of Zoology* **19**, 248-250.

Beckley LE (1985) Tidal exchange of ichthyoplankton in the Swartkops estuary mouth, South Africa. *South African Journal of Zoology* **20**, 15-20.

Beckley LE (1986) The ichthyoplankton assemblage of the Algoa Bay nearshore region in relation to coastal zone utilization by juvenile fish. *South African Journal of Zoology* **21**, 244-252.

Beckley LE (1988) Spatial and temporal variability in sea temperaure in Algoa Bay, South Africa. *South African Journal of Science* **84**, 67-69.

Beckley LE, Hulley PA, Skelton PH (2002) Synoptic overview of marine ichthyology in South Africa. *Marine and Freshwater Research* **53**, 99–105.

Beheregaray LB, Sunnucks P (2001) Fine-scale genetic structure, estuarine colonization and incipient speciation in the marine silverside fish *Odontesthes argentinensis*. *Molecular Ecology* **10**, 2849-2866.

Bell KNI, Cowley PD, Whitfield AK (2001) Seasonality in frequency of marine access to an intermittently open estuary: implications for recruitment strategies. *Estuarine, Coastal and Shelf Science* **52**, 327-337.

Bennett BA (1989) The utilization of littoral and estuarine habitats by fish in the south western Cape. *PhD thesis*, University of Cape Town, Rondebosch.

Bennett BA (1993a) Aspects of the biology and life history of white steenbras *Lithognathus lithognathus* in southern Africa. *South African Journal of Marine Science* **13**, 83-96.

Bennett BA (1993b) The fishery for white steenbras *Lithognathus lithognathus* off the cape coast, South Africa, with some considerations for its management. *South African Journal of Marine Science* **13**, 1-14.

Bernardi G, Holbrook SJ, Schmitt RJ (2001) Gene flow at three spatial scales in a coral reef fish, the three-spot dascyllus, *Dascyllus trimaculatus*. *Marine Biology* **138**, 457-465.

Bilton DT, Paula J, Bishop JDD (2002) Dispersal, genetic differentiation and speciation in estuarine organisms. *Estuarine, Coastal and Shelf Science* **55**, 937-952.

Blaber SJM (1973a) Population size and mortality of juveniles of the marine teleost *Rhabdosargus holubi* (Pisces: Sparidae) in a closed estuary. *Marine Biology* **21**, 219-225.

Blaber SJM (1973b) Temperature and salinity tolerance of juvenile *Rhabdosargus holubi* [Steindachner (Teleostei: Sparidae)]. *Journal of Fish Biology* **5**, 593-598.

Blaber SJM (1974a) Field studies of the diet of *Rhabdosargus holubi* (Pisces: Teleostei: Sparidae). *Journal of Zoology London* **173**, 407-417.

Blaber SJM (1974b) Osmoregulation in juvenile *Rhabdosargus holubi* (Steindachner) (Teleostei: Sparidae). *Journal of Fish Biology* **6**, 797-800.

Blaber SJM (1974c) The population structure and growth of juvenile *Rhabdosargus holubi* (Steindachner) (Teleostei: Sparidae) in a closed estuary. *Journal of Fish Biology* **6**, 455-460.

Blaber SJM (1981) The zoogeographical affinities of estuarine fishes in South-East Africa. *South African Journal of Science* **77**, 305-307.

Blaber SJM (1984) The diet, food selectivity and niche of *Rhabdosargus sarba* (Teleostei: Sparidae) in Natal estuaries. *South African Journal of Zoology* **19**, 241-246.

Bohnsack JA (1993) Marine reserves: they enhance fisheries, reduce conflicts, and protect resources. *Oceanus* **36**, 63-71.

Booth AJ, Buxton CD (1997) Management of the panga *Pterogymnus laniarius* (Pisces: Sparidae) on the Agulhas Bank, South Africa using per-recruit models. *Fisheries Research* **32**, 1-11.

Briggs JC (2004) A marine center of origin: reality and conservation. In: *Frontiers of Biogeography: New directions in the geography of nature* (eds. Lomolino MV Heaney LR) pp. 255-269.

Brooker AL, Cook D, Bentzen P, Wright JM, Doyle RW (1994) Organization of microsatellites differs between mammals and cold-water teleost fishes. *Canadian Journal of Fisheries and Aquatic Sciences* **51**, 1959-1966.

Brouwer SL (2002) Movement patterns of red steenbras *Petrus rupestris* tagged and released in the Tsitsikamma National Park, South Africa. *South African Journal of Science* **24**, 375-378.

Brouwer SL, Griffiths MH, Roberts MJ (2003) Adult movement and larval dispersal of *Argyrozona argyrozona* (Pisces: Sparidae) from a temperate marine protected area. *African Journal of Marine Science* **25**, 395-402.

Brown CA, Holt SA, Jackson GA, Brooks DA, Holt GJ (2004) Simulating larval supply to estuarine nursery areas: how important are physical processes to the supply of larvae to the Aransas Pass Inlet? *Fisheries Oceanography* **13**, 181-196.

Brown CA, Jackson GA, Holt SA, Holt GJ (2005) Spatial and temporal patterns in modeled particle transport to estuarine habitat with comparisons to larval fish settlement patterns. *Estuarine, Coastal and Shelf Science* **64**, 33-46.

Brownell CL (1979) Stages in the early development of 40 marine fish species with pelagic eggs from the Cape of Good Hope. *Ichthyological Bulletin of the J.L.B. Smith Institute of Ichthyology* **40**, 1–84.

Bullen E, Mann BQ (2004) Sedgwick's/ORI/WWF Tagging Programme: summary of Cape stumpnose (*Rhabdosargus holubi*) tagged in South Africa, p. 6. Data Report, Oceanographic Research Institute, 2004/10, Durban.

Burridge CP, Hurt AC, Farrington LW, Coutin PC, Austin CM (2004) Stepping stone gene flow in an estuarine dwelling sparid from south east Australia. *Journal of Fish Biology* **64**, 805-819.

Buxton CD (1987) Life history changes of two reef fish species in exploited and unexploited marine environments in South Africa. *Ph.D. Thesis*, Rhodes University, Grahamstown.

Camper JD, Barber RC, Richardson LR, Gold JR (1993) Mitochondrial DNA variation among red snapper (Lutjanus campechanus) from the Gulf of Mexico. *Molecular Marine Biology and Biotechnology* **2**, 154-61.

Carvalho GR, Hauser L (1998) Advances in the molecular analysis of fish population structure. *Italian Journal of Zoology* **65**, 21-33.

Chen SL, Liu YG, Xu MY, Li J (2005) Isolation and characterization of polymorphic microsatellite loci from an EST-library of red sea bream (Chrysophrys major) and cross-species amplification. *Molecular Ecology Notes* **5**, 215-217.

Chiu T-S, Lee Y-J, Huang S-W, Yu H-T (2002) Polymorphic microsatellite markers for stock identification in Japanese anchovy (*Engraulis japonica*). *Molecular Ecology Notes* **2**, 49-50.

Chow S, Okamoto H, Miyabe N, Hiramatsu K, Barut N (2000) Genetic divergence between Atlantic and Indo-Pacific stocks of bigeye tuna (*Thunnus obesus*) and admixture around South Africa. *Molecular Ecology* **9**, 221-227.

Chow S, Takeyama H (2000) Nuclear and mitochondrial DNA analyses reveal four genetically separated breeding units of the swordfish. *Journal of Fish Biology* **56**,1087-1098.

Cimmaruta R, Bondanelli P, Nascetti G (2005) Genetic structure and environmental heterogeneity in the European hake (*Merluccius merluccius*). *Molecular Ecology* **14**, 2577-2591.

Clark BM, Bennett BA, Lamberth SJ (1994) A comparison of the ichthyofauna of two estuaries and their adjacent surf zones, with an assessment of the effects of beach-seining on the nursery function of estuaries fro fish. *African Journal of Marine Science* **14**, 121-132.

Cowley PD, Whitfield AK (2001) Fish population size estimates from a small intermittently open estuary in South Africa, based on mark-recapture techniques. *Marine and Freshwater Research* **52**, 283-290.

Cowley PD, Whitfield AK, Bell KNI (2001) The surf zone ichthyoplankton adjacent to an intermittently open estuary, with evidence of recruitment during marine overwash events. *Estuarine, Coastal and Shelf Science* **52**, 339-348.

Davies-Coleman M, Cook BD (2000) The status of the genetic and biochemical analysis of marine organisms in South Africa. In: *Summary Marine Biodiversity Status Report for South Africa* (eds. Durham BD, Pauw JC). National Research Foundation, Pretoria.

Davis JA, van Blerk LL, Kirby R, Hecht T (2003) Genetic variation in the mud crab *Scylla serrata* (Forskal, 1775)(Crustacea:Portunidae) in South African estuaries. *African Zoology* **38**, 343-350.

Day JH (1980) What is an estuary? *South African Journal of Science* **76**, 198.

Day JH (1981) The nature, origin and classification of estuaries. In: *Estuarine ecology with particular reference to southern Africa* (ed. Day JH). A.A. Balkema, Cape Town.

De Innocentiis S, Sola L, Cataudella S, Bentzen P (2001) Allozyme and microsatellite loci provide discordant estimates of population differentiation in the endangered dusky grouper (*Epinephelus marginatus*) within the Mediterranean Sea. *Molecular Ecology* **10**, 2163-2175.

De Wet PS (1988) *Aspects of the feeding ecology of Rhabdosargus holubi (Steindachner) (Teleostei: Sparidae) in the Swartkops estuary.* MSc, University of Port Elizabeth.

deMenocal PB (2004) African climate change and faunal evolution during the Pliocene-Pleistocene. *Earth and Planetary Science Letters* **220**, 3-24.

DeSalle R, Amanto G (2004) The expansion of conservation genetics. *Nature Reviews Genetics* **5**, 702-712.

Domingues VS, Santos RS, Brito A, Almada VC (2006) Historical population dynamics and demography of the eastern Atlantic pomacentrid *Chromis limbata* (Valenciennes, 1833). *Molecular Phylogenetics and Evolution* **40**, 139-147.

Doums C (1999) Characterization of microsatellite loci in the queenless Ponerine ant *Diacamma cyaneiventre*. *Molecular Ecology* **8**, 1957–1969.

Emanuel BP, Bustamante RH, Branch GM, Eekhout S, Odendaal FJ (1992) A zoogeographical and functional approach to the selection of marine reserves on the west coast of South Africa. *South African Journal of Marine Science* **12**, 341-354.

Escorza-Trevino S, Dizon AE (2000) Phylogeography, Intraspecific structure and sex-biased dispersal of Dall's porpoise, *Phocoenoides dalli*, revealed by mitochondrial and microsatellite DNA analyses. *Molecular Ecology* **9**, 1049-1060.

Estoup A, Largiader CR, Perrot E, Chourrout D (1996) Rapid one-tube DNA extraction for reliable PCR detection of fish polymorphic markers and transgenes. *Molecular Marine Biology and Biotechnology* **5**, 295-298.

Ewens WJ (1972) The sampling theory of selectively neutral alleles. *Theoretical Population Biology* **3**, 87-112.

Excoffier L, Smouse P (1994) Using allele frequency and geographic subdivision to reconstruct gene genealogies within a species. Molecular variance parsimony. *Genetics* **136**, 343-359.

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131**, 479-491.

Feral J-P (2002) How useful are the genetic markers in attempts to understand and manage marine biodiversity? *Journal of Experimental Marine Biology and Ecology* **268**, 121-145.

Ferris SD, Berg WJ (1987) The utility of mitochondrial DNA in fish genetics and fishery management. In: *Population genetics and fishery management* (eds. Utter F, Ryman N), pp. 277-299. University of Washington Press, Seattle.

FitzSimmons NN, Moritz C, Moore SS (1995) Conservation and Dynamics of Microsatellite Loci over 300 Million Years of Marine Turtle Evolution. *Molecular Biology and Evolution* **12**, 432-440.

Ford MJ (2002) Applications of selective neutrality tests to molecular ecology. *Molecular Ecology* **11**, 1245-1262.

Frankham R (1995) Conservation genetics. *Annual Review of Genetics* **29**, 305-327.

Fromentin J-M, Powers JE (2005) Atlantic bluefin tuna: population dynamics, ecology, fisheries and management. *Fish and Fisheries* **6**, 281-306.

Fu YX (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* **147**, 915-925.

Fu YX, Li WH (1993) Maximum likelihood estimation of population parameters. *Genetics* **134**, 1261-1270.

Garcia De Leon FJ, Chikhi L, Bonhomme F (1997) Microsatellite polymorphism and population subdivision in natural populations of European sea bass *Dicentrarchus labrax* (Linnaeus, 1758). *Molecular Ecology* **6**, 51-62.

Garza JC, Freimer NB (1996) Homoplasy for size at microsatellite loci in humans and chimpanzees. *Genome Research* **6**, 211-217.

Garza JC, Slatkin M, Freimer NB (1995) Microsatellite allele frequencies in humans and chimpanzees with implications for constraints on allele size. *Molecular Biology and Evolution* **12**, 594-603.

Gerber LR, Kareiva PM, Bascompte J (2002) The influence of life history attributes and fishing pressure on the efficacy of marine reserves. *Biological Conservation* **106**, 11-18.

Gilles A, Miquelis A, Quignard J-P, Faure E (2000) Molecular phylogeography of western Mediterranean dusky grouper *Epinephelus marginatus*. *Comptes Rendus de l'Académie des Sciences Paris, Sciences de la vie (of Life Sciences)* **323**, 195-205.

Gold JR, Burridge CP, Turner TF (2001) A modified stepping-stone model of population structure in red drum, *Sciaenops ocellatus* (Sciaenidae) from the northern Gulf of Mexico. *Genetica* **111**, 305-317.

Gold JR, Richardson LR (1991) Genetic studies in marine fishes. IV. An analysis of population structure in the red drum (*Sciaenops ocellatus*) using mitochondrial DNA. *Fisheries Research* **12**, 213-241.

Gold JR, Richardson LR (1998) Mitochondrial DNA diversification and population structure in fishes from the Gulf of Mexico and western Atlantic. *Journal of Heredity* **89**, 404-414.

Gold JR, Richardson LR, Furman C, King TL (1993) Mitochondrial DNA differentiation and population structure in red drum (*Sciaenops ocellatus*) from the Gulf of Mexico and Atlantic Ocean. *Marine Biology* **116**, 175-185.

Gold JR, Richardson LR, Furman C, Sun F (1994) Mitochondrial DNA diversity and population structure in marine fish species from the Gulf of Mexico. *Canadian Journal of Fisheries and Aquatic Sciences* **51**, 205-214.

Gold JR, Richardson LR, Turner TF (1999) Temporal stability and spatial divergence of mitochondrial DNA haplotype frequencies in red drum (*Sciaenops ocellatus*) from coastal regions of the western Atlantic Ocean and Gulf of Mexico. *Marine Biology* **133**, 596-602.

Gold JR, Turner TF (2002) Population structure of red drum (*Sciaenops ocellatus*) in the northern Gulf of Mexico, as inferred from variation in nuclear-encoded microsatellites. *Marine Biology* **140**, 249-265.

Goldstein DB, Pollock DD (1997) Launching microsatellites: a review of mutation processes and methods of phylogenetic inference. *Journal of Heredity* **88**, 335-342.

Goodman SJ (1997) R_{ST} Calc: a collection of computer programs for calculating estimates of genetic differentiation from microsatellite data and determining their significance. *Molecular Ecology* **6**, 881-885.

Gotz A (2005) Assessment of the effect of Goukamma marine protected area on community structure and fishery dynamics. *PhD Thesis*, Rhodes University.

Goudet J (1995) FSTAT (Version 1.2): a computer program to calculate F-satistics. *Journal of Heredity* **86**, 485-486.

Goudet J (1999) An improved procedure for testing key innovations. *American Naturalist* **53**, 549-555.

Goudet J (2001) *FSTAT,* a program to estimate and test gene diversities and fixation indices version 2.9.3.

Grant WS, Bowen BW (1998) Shallow population histories in deep evolutionary lineages of marine fishes: insights from Sardines and Anchovies and lessons for conservation. *Journal of Heredity* **89**, 415-426.

Graves JE (1998) Molecular insights into the population structures of cosmopolitan marine fishes. *Journal of Heredity* **89**, 427-437.

Griffiths MH (2000) Long-term trends in catch and effort of commercial linefish off South Africa's Cape Province: snapshots of the 20th Century. *South African Journal of Marine Science* **22**, 81-110.

Griffiths MH, Attwood CG, Thomson R (1999) New management protocol for the South African linefishery. In: *Proceedings of the Third Southern African Marine Linefish Symposium* (ed. Mann BQ), pp. 145-156. SANCOR Occ. Rep., Arniston.

Griffiths MH, Hecht T (1995) Age and growth of South African dusky kob *Argyrosomus japonicus* (Sciaenidae) based on otoliths. *South African Journal of Marine Science* **16**, 119-128.

Griffiths MH, Heemstra PC (1995) A contribution to the taxonomy of the marine fish genus *Argyrosomus* (Perciformes: Sciaenidae), with descriptions of two new species from southern Africa. *Ichthyological Bulletin of the J.L.B. Smith Institute of Ichthyology* **65**, 1-40.

Hancock JM, Shaw PJ, Bonneton F, Dover GA (1999) High sequence turnover in the regulatory regions of the developmental gene hunchback in insects. *Molecular Biology and Evolution* **16**, 253-265.

Hanekom N, Baird D (1984) Fish community structures in *Zostera* and non-*Zostera* regions of the Kromme estuary, St Francis Bay. *South African Journal of Zoology* **19**, 295-301.

Hanel R, Strumbauer C (2000) Multiple recurrent evolution of trophic types in north-eastern Atlantic and Mediterranean seabreams (Sparidae: Percoidei). *Journal of Molecular Evolution* **50**, 276-283.

Hare MP (2001) Prospects for nuclear gene phylogeography. *Trends in Ecology & Evolution* **16**, 700-706.

Harpending HC (1994) Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. *Human Biology* **66**, 591-600.

Harris SA, Cyrus DP (1996) Larval and juvenile fishes in the surf zone adjacent to the St Lucia Estuary mouth, KwaZulu-Natal, South Africa. *Marine and Freshwater Research* **47**, 465-482.

Harrison JA (1989a) Atlassing as a tool in conservation, with special reference to the southern African Bird Atlas Project. In: *Biotic Diversity in Southern Africa: Concepts and Conservation* (ed. Huntley BJ), pp. 157-169. Oxford University Press Southern Africa, Cape Town.

Harrison RG (1989b) Animal mitochondrial DNA as a genetic marker in population and evolutionary biology. *Trends in Ecology and Evolution* **4**(1), 6-11.

Harrison TD (2002) Preliminary assessment of the biogeography of fishes in South African estuaries. *Marine and Freshwater Research* **53**, 479-490.

Harrison TD (2004) Physico-chemical characteristics of South African estuaries in relation to the zoogeography of the region. *Estuarine, Coastal and Shelf Science* **61**, 73-87.

Harrison TD, Whitfield AK (1990) Composition, distribution and abundance of ichthyoplankton in the Sundays River estuary. *South African Journal of Zoology* **25**, 161-168.

Harrison TD, Whitfield AK (2006) Estuarine typology and the structuring of fish communities in South Africa. *Environmental Biology of Fishes* **75**, 269–293.

Hauser L, Ward RD (1998) Population identification in pelagic fish: the limits of molecular markers. In: *Advances in molecular ecology* (ed. Carvalho GR), pp. 191-224. IOS Press, Amsterdam.

Hedgecock D, Li G, Hubert S, Bucklin K, Ribes V (2004) Widespread null alleles and poor cross-species amplification of microsatellite DNA loci cloned from the Pacific oyster, Crassostrea gigas. *Journal of Shellfisheries Research* **23**, 379-385.

Heemstra PC, Heemstra E (2004) *Coastal fishes of southern Africa.* NISC & SAIAB, Grahamstown.

Heydorn AEF (1989) The conservation status of southern African estuaries. In: *Biotic diversity in southern Africa: concepts and conservation.* (ed. Huntley BJ). Oxford University Press, Oxford.

Hille A, Janssen IAW, Menken SBJ, Schlegel M, Thorpe RS (2002) Heterologous Amplification of Microsatellite Markers From Colubroid Snakes in European Natricines (Serpentes: Natricinae). *Journal of Heredity* **93**, 63-66.

Hjermann DO, Ottersen G, Stenseth NC (2004) Competition among fishermen and fish causes the collapse of Barents Sea capelin. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 11679-11684.

Hockey PAR, Buxton CD (1989) Conserving biotic diversity on southern Africa's coastline. In: *Biotic diversity in southern Africa: concepts and conservation* (ed. Huntley BJ), pp. 298-308. Oxford University Press Southern Africa, Cape Town.

Hockey PAR, Turpie JK (1999) Estuarine birds in South Africa. In: *Estuaries of South Africa* (eds. Allanson BR, Baird D), pp. 235-268. Cambridge University Press, Cambridge.

Hunter RL, Markert CL (1957) Histochemical demonstration of enzymes seperated by zone electrophoresis in starch gels. *Science* **125**, 1294-1295.

Hutchings K, Lamberth SJ, Turpie JK (2002) Socio-economic characteristics of gillnet and beach-seine fisheries in the western Cape, South Africa. *South African Journal of Marine Science* **24**, 243-262.

Ihssen PE, Booke HE, Casselman JM *et al.* (1981) Stock identification: materials and methods. *Canadian Journal of Fisheries and Aquatic Sciences* **38**, 1838-1855.

James MK, Armsworth PR, Mason LB, Bode L (2002) The structure of reef fish metapopulations: modelling larval dispersal and retention patterns. *Proceedings of the Royal Society of London - B* **269**, 2079-2086.

James NC, Mann BQ, Beckley LE, Govender A (2003) Age and growth of the estuarine-dependent sparid Acanthopagrus berda in northern KwaZulu-Natal, South Africa. *African Zoology* **38**, 265–271.

Jarre-Teichmann A, Shannon LJ, Moloney CL., Wickens P (1998) Comparing trophic flows in the Southern Benguela to those in other upwelling ecosystems. In: *Benguela dynamics: impacts of variability on shelf-sea environments and their living resources* (eds. Pillar SC, Moloney CL, Payne AIL, Shillington FA). *South African Journal of Marine Science* **19**, 391–414.

Kilburn R, Rippey E (1982) *Sea shells of southern Africa*. Macmillan South Africa, Johannesburg.

Kimura M, Crow JF (1964). The number of alleles that can be maintained in a finite population. *Genetics* **49**, 725-738.

Kingman JFC (1982) On the genealogy of large populations. In: *Essays in statistical science* (eds. Gani J , Hannan EJ),pp 27-43, Applied Probability Trust.

Kirchman JJ, Whittingham LA, Sheldon FH (2000) Relationships among Cave Swallow Populations (Petrochelidon fulva) Determined by Comparisons of Microsatellite and Cytochrome b Data. *Molecular Phylogenetics and Evolution* **14**, 107-121.

Lagler KF, Bardach JE, Miller RR (1962) *Ichthyology.* USA: John Wiley & Sons, New York.

Laikre L, Miller LM, Palmé A *et al.* (2005) Spatial genetic structure of northern pike (*Esox lucius*) in the Baltic Sea. *Molecular Ecology* **14**, 1955-1964.

Lambeck K, Esat TM, Potter E-K (2002) Links between climate and sea levels for the past three million years. *Nature* **419**, 199-206.

Lauck T, Clark CW, Mangel M, Munro GR (1998) Implementing the precautionary principle in fisheries management through marine reserves. *Ecological Applications* **8**, 72-78.

Lee W-J, Conroy J, Howell WH, Kocher TD (1995) Structure and evolution of teleost mitochondrial control regions. *Journal of Molecular Evolution* **41**, 54-66.

Lemaire C, Versini J-J, Bonhomme F (2005) Maintenance of genetic differentiation across a transition zone in the sea: discordance between nuclear and cytoplasmic markers. *Journal of Evolutionary Biology* **18**, 70-80.

Levinson G, Gutman GA (1987) Slipped-strand mispairing: a major mechanism for DNA sequence evolution. *Molecular Biology and Evolution* **4**, 203-221.

Lima-Rosa CAV, Hutz MH, Oliveira LFB, Andrades-Miranda J, Mattevi MS (2000) Heterologous Amplification of Microsatellite Loci from Mouse and Rat in Oryzomyine and Thomasomyine South American Rodents. *Biochemical Genetics* **38**, 97-107.

Lippe C, Dumont P, Bernatchez L (2006) High genetic diversity and no inbreeding in the endangered copper redhorse, *Moxostoma hubbsi* (Catostomidae, Pisces): the positive sides of a long generation time. *Molecular Ecology* **15**, 1769-1780.

Liu ZJ, Cordes JF (2004) DNA marker technologies and their applications in aquaculture genetics. *Aquaculture* **238**, 1-37.

Lowe AJ, Harris SA, Ashton P (2004) *Ecological genetics: design, analysis and application*. Blackwells, Oxford.

Lukey JR, Booth AJ, Froneman PW (2006) Fish population size and movement patterns in a small intermittently open South African estuary. *Estuarine, Coastal and Shelf Science* **67**, 10-20.

Lundy CJ, Moran P, Rico C, Milner RS, Hewitt GM (1999) Macrogeographical population differentiation in oceanic environments: a case study of European hake (*Merluccius merluccius*), a commercially important fish. *Molecular Ecology* **8**, 1889-1898.

Lundy CJ, Rico C, Hewitt GM (2000) Temporal and spatial genetic variation in spawning grounds of European hake (*Merluccius merluccius*) in the Bay of Biscay. *Molecular Ecology* **9**, 2067-2079.

Lutjeharms JRE, Ansorge IJ (2001) The Agulhas Return Current. *Journal of Marine Systems* **30**, 115-138.

Lutjeharms JRE, Boebel O, Rossby HT (2003) Agulhas cyclones. *Deep-Sea Research Part II* **50**, 13-34.

Lutjeharms JRE, Cooper J, Roberts M (2000) Upwelling at the inshore edge of the Agulhas Current. *Continental Shelf Research* **20**, 737-761.

Lutjeharms JRE, de Ruijter WPM (1996) The influence of the Agulhas Current on the adjacent coastal ocean: possible impacts of climate change. *Journal of Marine Systems* **7**, 321-336.

Lutjeharms JRE, Monteiro PMS, Tyson PD, Obura D (2001) The oceans aound southern Africa and regional effects of global change. *South African Journal of Science* **97**, 119-130.

Lutjeharms JRE, Penvena P, Roy C (2003b) Modelling the shear edge eddies of the southern Agulhas Current. *Continental Shelf Research* **23**, 1099–1115.

Manel Sp, Schwartz MK, Luikart G, Taberlet P (2003) Landscape genetics: combining landscape ecology and population genetics. *Trends in Ecology & Evolution* **18**, 189-197.

Mann BQ (2000) *Rhabdosargus holubi*. In: *Southern African marine linefish status reports* (ed. Mann BQ), pp. 42-44. Special Publication. South African association for marine biological research (7), Durban.

Mantel N (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research* **27**, 209-220.

Maree RC, Whitfield AK, Booth AJ (2000) Effect of water temperature on the biogeography of Southern African estuarine fishes associated with the subtropical/warm temperate subtraction zone. *South African Journal of Science* **96**, 184-188.

Maud RR (1990) Sea-level movements as evidence of late Quaternary environmental changes in southern Africa. In: *Paleoecology of Africa*, volume 21 (ed. Heine K), pp.27-34. A.A. Balkema, Rotterdam.

Maud RR, Botha GA (2000) Deposits of the South Eastern and Southern coasts. In: *The Cenozoic of Southern Africa* (eds. Patridge TC, Maud RR), pp19-32. Oxford University Press, New York.

McMillan WO, Palumbi SR (1997) Rapid rate of control-region evolution in pacific butteflyfishes (Chaetodontidae). *Journal of Molecular Evolution* **45**, 473-484.

McMillen-Jackson AL, Bert TM (2004) Mitochondrial DNA variation and population genetic structure of the blue crab *Callinectes sapidus* in the eastern United States. *Marine Biology* **145**, 769-777.

Millard NAH, Scott KMF (1954) The ecology of South African estuaries, Part VI: Milnerton estuary and the Diep River, Cape. *Transactions of the Royal Society of South Africa* **34**, 279-324.

Moore SS, Sargeant LL, King TJ *et al.* (1991) The conservation of dinucleotide microsatellites among mammalian genomes allows the use of heterologous PCR primer pairs in closely related species. *Genomics* **10**, 661-665.

Moritz C, Dowling TE, Brown WM (1987) Evolution of animal mitochondrial DNA: relevance for population biology and systematics. *Annual Review of Ecology and Systematics* **18**, 269-292.

Mueller UG, Wolfenbarger LL (1999) AFLP genotyping and fingerprinting. *Trends in Ecology and Evolution* **14**, 389-394.

Mullon C, Freon P, Cury P (2005) The dynamics of collapse in world fisheries. *Fish and Fisheries* **6**, 111-120.

Nei M (1987) *Molecular evolutionary genetics.* Columbia University Press, New York, NY, USA.

Nei M, Tajima F (1981) DNA polymorphism detectable by restriction endonucleases. *Genetics* **97**, 145-163.

Nichols RA, Freeman KLM (2004) Using molecular markers with high mutation rates to obtain estimates of relative population size and to distinguish the effects of gene flow and mutation: a demonstration using data from endemic Mauritian skinks. *Molecular Ecology* **13**, 775-787.

O'Connell M, Wright JM (1997) Microsatellite DNA in fishes. *Reviews in Fish Biology and Fisheries* **7**, 331-363.

Ohta T, Kimura M (1973). A model of mutation appropriate to estimate the number of electrophoretically detectable alleles in a finite population. *Genetic Research* **22**, 201-204.

O'Malley KG, Abbey CA, Ross K, Gold JR (2003) Microsatellite DNA markers for kinship analysis and genetic mapping in red drum, *Sciaenops ocellatus* (Sciaenidae, Teleostei). *Molecular Ecology Notes* **3**, 155-158.

Orrell TM, Carpenter KE, Musick JA, Graves JE (2002) A phylogenetic and biogeographic analysis of the Sparidae (Perciformes: Percoidei) based on cytochrome *b* sequences. *Copeia* **3**, 618-631.

Pääbo S, Gifford JA, Wilson AC (1988) Mitochondrial DNA sequences from a 7000-year old brain. *Nucleic Acids Research* **16**, 9775-9787.

Palumbi SR (1994) Genetic divergence, reproductive isolation, and marine speciation. *Annual Review of Ecology and Systematics* **25**, 547-572.

Pepin P, Helbig JA, Laprise R, *et al*. (1995) Variations in the contribution of transport to changes in the planktonic animal abundance: a study of the flux of fish larvae in Conception Bay, Newfoundland. *Canadian Journal of Fisheries and Aquatic Science* **52**, 1475-1486.

Pfeiler E, Hurtado LA, Knowles LL *et al.* (2005) Population genetics of the swimming crab *Callinectes bellicosus* (Brachyura: Portunidae) from the eastern Pacific Ocean. *Marine Biology* **146**, 559-569.

Piertney SB, MacColl ADC, Bacon PJ, Dallas JF (1998) Local genetic structure in red grouse (*Lagopus lagopus*): evidence from microsatellite DNA markers. *Molecular Ecology* **7**, 1645-1654.

Primmer CR, Merilä J (2002) A low rate of cross-species microsatellite amplification success in Ranid frogs. *Conservation Genetics* **3**, 445–449.

Primmer CR, Moller AP, Ellegren H (1996) A wide-range survey of cross-species microsatellite amplification in birds. *Molecular Ecology* **5**, 365-378.

Prochazka K. (1994) Habitat partitioning in shallow-water cryptic ichthyofaunal communities in the western and south-western Cape, South Africa. *Ph.D. thesis*, University of Cape Town.

Ramos-Onsins SE, Rozas J (2002) Statistical properties of new neutrality tests against population growth. *Molecular Biology and Evolution* **19**, 2092-2100.

Ramsay PJ, Cooper JAG (2002) Late Quaternary Sea-Level Change in South Africa. *Quaternary Research* **57**, 82–90.

Rand DM (1996) Neutrality tests of molecular markers and the connection between DNA polymorphism, demography, and conservation biology. *Conservation Biology* **10**, 665-671.

Randall JE (1998) Zoogeography of shore fishes of the Indo-Pacific region. *Zoological Studies* **37**, 227-268.

Reddering JVS (1990) Estuarine history of the south-eastern Cape (South Africa), and the recent influence of man. In: *Paleoecology of Africa*, volume 21 (ed. Heine K), pp.83-88. A.A. Balkema, Rotterdam.

Reusch TBH, Wegner KM, Kalbe M (2001) Rapid genetic divergence in postglacial populations of threespine stickleback (*Gasterosteus aculeatus*): the role of habitat type, drainage and geographical proximity. *Molecular Ecology* **10**, 2435-2445.

Rice WR (1989) Analyzing tables of statistical tests. *Evolution* **43**, 223-225.

Rico C, Ibrahim KM. Rico I, Hewitt GM (1997) Stock composition in North Atlantic populations of whiting using microsatellite markers. *Journal of Fish Biology* **51**, 462-475.

Rico C, Rico I, Hewitt G (1996) 470 million years of conservation of microsatellite loci among fish species. *Proceedings of the Royal Society of London - B* **263**, 549-557.

Robichaud D, Rose GA (2004) Migratory behaviour and range in Atlantic cod: inference from a century of tagging. *Fish and Fisheries* **5**, 185-214.

Roff DA, Bentzen P (1989) The statistical analysis of mitochondrial DNA polymorphisms: X^2 and the problem of small samples. *Molecular Biology and Evolution* **6**, 539-545.

Rogers AR, Harpending H (1992) Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution* **9**, 552-569.

Rogers J (1985) Geomorphology, offshore bathymetry and quaternary lithostratigraphy around the Bot River Estuary. *Transactions of the Royal Society of South Africa* **45**, 211-227.

Rousset F (1996) Equilibrium values of measures of population subdivision for stepwise mutation processes. *Genetics* **142**, 1357-1362.

Rozas J, Rozas R (1999) DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics* **15**, 174-175.

Ruzzante DE (1998) A comparison of several measures of genetic distance and population structure with microsatellite data: bias and sampling variance. *Canadian Journal of Fisheries and Aquatic Science* **55**, 1-14.

Saiki RK, Gelfand DH, Stoffel S *et al.* (1988) Primer-directed enzymatic amplification of DNA with a themostable DNA polymerase. *Science* **239**, 487-491.

Sambrook J, Fritsh EP, Maniatis T (1989) *A laboratory manual*, Second edn. Cold Spring Habor Laboratory Press.

Sanderson MJ, Donoghue MJ (1989) Patterns of variation in levels of homoplasy. *Evolution* **43**.

Sauer WHH, Lipinskib MR, Augustyn CJ (2000) Tag recapture studies of the chokka squid *Loligo vulgaris reynaudii* dOrbigny, 1845 on inshore spawning grounds on the south-east coast of South Africa. *Fisheries Research* **45**, 283-289.

Schlötterer C, Amos W, Tautz D (1991) Conservation of polymorphic simple sequence loci in cetacean species. *Nature*, **354**, 63-65.

Schneider S, Roessli D, Excoffier L (2000) ARLEQUIN version 2.000: a software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland.

Scribner KT, Chesser RK (2001) Group-structured genetic models in analyses of the population and behavioral ecology of poikilothermic vertebrates. *The American Genetic Association* **92**, 180-189.

Shannon LV (1985) The Benguela Ecosystem, I., Evolution of the Benguela, physical features and processes. *Oceanography and Marine Biology* **23**, 105-182.

Siegfried WR (1981) The estuarine avifauna of southern Africa. In: *Estuarine ecology with particular reference to southern Africa* (ed. Day JH), pp. 223-250. A.A. Balkema, Cape Town.

Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics* **139**, 457-462.

Slatkin M, Barton NH (1989) A comparison of three indirect methods for estimating average levels of gene flow. *Evolution* **43**, 1349-1368.

Slatkin M, Excoffier L (1996) Testing for linkage disequilibrium in genotypic data using EM algorithm. *Journal of Heredity* **76**, 377-383.

Slatkin M, Hudson RR (1991) Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. *Genetics* **129**, 555-562.

Smale MJ, Punt AE (1991) Age and growth of the red steenbras *Petrus rupestris* (Pisces: Sparidae) on the south-east coast of South Africa. *South African Journal of Marine Science* **10,** 131-139.

Smith MM, McKay RJ (1986) *Smiths' sea fishes* (ed. Heemstra PC). MacMillan, Johannesburg.

Sokal RR, Rohlf FJ (1994) *Biometry.* Freeman, New York.

Stephenson TA, Stephenson A (1972) *Life between tidemarks on rocky shores*. Freeman, San Francisco.

Stockley B, Menezes G, Pinho MR, Rogers AD (2005) Genetic population structure in the black-spot sea bream (*Pagellus bogaraveo* Brünnich, 1768) from the NE Atlantic. *Marine Biology* **146**, 793-804.

Stockley BM, Rogers AD, Iyengar A *et al.* (2000) Ten microsatellite loci isolated and developed for the blackspot seabream, *Pagellus bogaraveo* (Brünnich 1768). *Molecular Ecology* **9**, 999-1000.

Strydom NA (2003) Occurrance of larval and early juvenile fishes in the surf zone adjacent to two intermittently open estuaries, South Africa. *Environmental Biology of Fishes* **66**, 349-359.

Strydom NA, d'Hotman BD (2005) Estuary-dependance of larval fishes in a non-estuary associated South African surf zone: evidence for continuity of surf assemblages. *Esruarine, Coastal and Shelf Science* **63**, 101-108.

Swofford DL (2001) PAUP*. Phylogenetic Analysis Using Parsimony (and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123**, 585-595.

Takezaki N, Nei M (1996) Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. *Genetics* **144**, 389-399.

Templeton AR, Routman E, Phillips CA (1995) Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinurn*. *Genetics* **140**, 767-782.

Thompson J, Gibson T, Plewniak F, Jeanmougin F, Higgins D (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**, 4876-4882.

Trexler JC, Travis J (2000) Can marine protected areas restore and conserve stock attributes of reef fishes. *Bulletin of Marine Science* **66**, 853-873.

Triantafyllidis A, Apostolidis AP, Katsares V *et al.* (2005) Mitochondrial DNA variation in the European lobster (*Homarus gammarus*) throughout the range. *Marine Biology* **146**, 223-235.

Turner TF, Richardson LR, Gold JR (1998) Polymorphic microsatellite DNA markers in red drum (*Sciaenops ocellatus*). *Molecular Ecology* **7**, 1771-1788.

Turpie JK, Beckley LE, Katua SM (2000) Biogeography and the selection of priority areas for conservation of South African coastal fishes. *Biological Conservation* **92**, 59-72.

Van der Elst RP (1988) *A guide to the common sea fishes of southern Africa* Struik, Cape Town.

Vorwerk PD, Whitfield A, Cowley PD, Paterson AW (2003) The influence of selected environmental variables on fish assemblage structure in a range of southeast African estuaries. *Environmental Biology of Fishes* **66**, 237-247.

Wake DB (1991) Homoplasy: the result of natural selection, or evidence of design limitations? *The American Naturalist* **138**, 543-567.

Wakeley J (2005) The limits of theoretical population genetics. *Genetics* **169**, 1-7.

Wallace JH (1975a) The estuarine fishes of the east coast of Southern Africa. Part I. Species composition and length distribution in the estuarine and marine environments. Part II. Seasonal abundance and migrations. *Investigational Report of the Oceanographic Research Institute* **40**, 1-72.

Wallace JH (1975b) The estuarine fishes of the east coast of Southern Africa. Part III. Reproduction. *Investigational Report of the Oceanographic Research Institute* **41**, 1-51.

Wallace JH, Kok HM, Beckley LE *et al.* (1984) South African estuaries and their importance to fishes. *South African Journal of Science* **80**, 203-207.

Wallace JH, van der Elst RP (1975) The estuarine fishes of the east coast of Southern Africa. Part IV. Occurrence of juveniles in estuaries. Part V. Ecology, estuarine dependence and status. *Investigational Report of the Oceanographic Research Institute* **42**, 1-62.

Waples RS (1998) Separating the wheat from the chaff: patterns of genetic differentiation in high gene flow species. *The Journal of Heredity* **89**, 438-450.

Ward RD (2000) Genetics in fisheries management. *Hydrobiologia* **420**, 191-201.

Ward RD, Woodwark M, Skibinski (1994) A comparison of genetic diversity levels in marine, freshwater, and anadromous fishes. *Journal of Fish Biology* **44**, 213-232.

Ward TM, Staunton-Smith J, Hoyle S, Halliday IA (2003) Spawning patterns of four species of predominantly temperate pelagic fishes in the sub-tropical waters of southern Queensland. *Estuarine, Coastal and Shelf Science* **56**, 1125-1140.

Webb GA (2002) *Biology and demography of the spotted grunter Pomadasys commersonnii (Haemulidae) in South African waters.* Masters dissertation, Rhodes University.

Weir BS, Cockerham CC (1984) Estimating *F*-statistics for the analysis of population structure. *Evolution* **38**, 1358-1370.

Whitfield AK (1988) The fish community of the Swartvlei estuary and the influence of food availability on resource utilization. *Estuaries* **11**, 160-170.

Whitfield AK (1989) Fish larval composition, abundance and seasonality in a southern African estuarine lake. *South African Journal of Zoology* **24**, 217-224.

Whitfield AK (1998) *Biology and ecology of fishes in Southern African estuaries* JLB Smith Institute of Ichthyology, Grahamstown.

Whitfield AK (1999) Ichthyofaunal assemblages in estuaries: a South African case study. *Reviews in Fish Biology and Fisheries* **9**(2), 151-186.

Whitfield AK, Beckley LE, Bennett BA *et al.* (1989) Composition, species richness and similarity of Ichtyofaunas in eelgrass *Zostera capensis* beds of Southern Africa. *South African Journal of Marine Science* **8**, 251-259.

Whitfield AK, Blaber SJM, Cyrus DP (1981). Salinity ranges of some southern African fish species occurring in estuaries. South African Journal of Zoology **16**, 151-155.

Whitfield AK, Kok HM (1992) Recruitment of juvenile marine fishes into permanently open and seasonally open estuarine systems on the southern coast of South Africa. *Ichthyological Bulletin of the J.L.B. Smith Institute of Ichthyology* **57**, 1-39.

Whitfield AK, Taylor RH, Grange N *et al.* (2000) Functional ecosystems: estuaries. In: *Summary marine biodiversity status report for South Africa* (eds. Durham BD, Pauw JC), pp. 6-9. National Research Foundation, Pretoria.

Wilson AC, Cann RL, Carr S *et al.* (1985) Mitochondrial DNA and two perspectives on evolutionary genetics. *Biological Journal of the Linnean Society* **26**, 401-405.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Appendix I: Microsatellite loci tested for amplification in *Rhabdosargus holubi.*

Locus name	Family	Reference	Locus name	Family	Reference
Pma1	Sparidae	Adcock *et al.* 2000	Soc 060	Sciaenidae	Turner *et al.* 1998
Pma2	Sparidae	Adcock *et al.* 2000	Soc 077	Sciaenidae	Turner *et al.* 1998
Pma3	Sparidae	Adcock *et al.* 2000	Soc 083	Sciaenidae	Turner *et al.* 1998
Pma5	Sparidae	Adcock *et al.* 2000	Soc 085	Sciaenidae	Turner *et al.* 1998
GA1A	Sparidae	Adcock *et al.* 2000	Soc 086	Sciaenidae	Turner *et al.* 1998
GA1B	Sparidae	Adcock *et al.* 2000	Soc 099	Sciaenidae	Turner *et al.* 1998
GA2A	Sparidae	Adcock *et al.* 2000	Soc 105	Sciaenidae	Turner *et al.* 1998
GA2B	Sparidae	Adcock *et al.* 2000	Soc 125	Sciaenidae	Turner *et al.* 1998
GT2	Sparidae	Adcock *et al.* 2000	Soc 133	Sciaenidae	Turner *et al.* 1998
GT3	Sparidae	Adcock *et al.* 2000	Soc 137	Sciaenidae	Turner *et al.* 1998
GT4	Sparidae	Adcock *et al.* 2000	Soc 138	Sciaenidae	Turner *et al.* 1998
GT6	Sparidae	Adcock *et al.* 2000	Soc 140	Sciaenidae	Turner *et al.* 1998
			Soc 156	Sciaenidae	Turner *et al.* 1998
SaGT 01	Sparidae	Batargias *et al.* 1999	Soc 177	Sciaenidae	Turner *et al.* 1998
SaGT 25	Sparidae	Batargias *et al.* 1999	Soc 201	Sciaenidae	Turner *et al.* 1998
SaGT 31	Sparidae	Batargias *et al.* 1999	Soc 204	Sciaenidae	Turner *et al.* 1998
SaGT 35	Sparidae	Batargias *et al.* 1999	Soc 206	Sciaenidae	Turner *et al.* 1998
SaGT 37	Sparidae	Batargias *et al.* 1999	Soc 232	Sciaenidae	Turner *et al.* 1998
SaGT 59	Sparidae	Batargias *et al.* 1999	Soc 243	Sciaenidae	Turner *et al.* 1998
			Soc 247	Sciaenidae	Turner *et al.* 1998
Hpl 018	Haemulidae	Chapman pers.com.	Soc 400	Sciaenidae	O'Malley *et al.* 2003
Hpl 030	Haemulidae	Chapman pers.com.	Soc 401	Sciaenidae	O'Malley *et al.* 2003
Hpl 034	Haemulidae	Chapman pers.com.	Soc 402	Sciaenidae	O'Malley *et al.* 2003
Hpl 042	Haemulidae	Chapman pers.com.	Soc 403	Sciaenidae	O'Malley *et al.* 2003
Hpl 044	Haemulidae	Chapman pers.com.	Soc 404	Sciaenidae	O'Malley *et al.* 2003
Hpl 462	Haemulidae	Chapman pers.com.	Soc 405	Sciaenidae	O'Malley *et al.* 2003
			Soc 406	Sciaenidae	O'Malley *et al.* 2003
Hau 021	Haemulidae	Chapman pers.com.	Soc 407	Sciaenidae	O'Malley *et al.* 2003
Hau 034	Haemulidae	Chapman pers.com.	Soc 409	Sciaenidae	O'Malley *et al.* 2003
Hau 035	Haemulidae	Chapman pers.com.	Soc 410	Sciaenidae	O'Malley *et al.* 2003
Hau 045	Haemulidae	Chapman pers.com.	Soc 411	Sciaenidae	O'Malley *et al.* 2003
Hau 075	Haemulidae	Chapman pers.com.	Soc 412	Sciaenidae	O'Malley *et al.* 2003
Hau 090	Haemulidae	Chapman pers.com.	Soc 415	Sciaenidae	O'Malley *et al.* 2003
Hau 109	Haemulidae	Chapman pers.com.	Soc 416	Sciaenidae	O'Malley *et al.* 2003
Hau 117b	Haemulidae	Chapman pers.com.	Soc 417	Sciaenidae	O'Malley *et al.* 2003
Hau 151	Haemulidae	Chapman pers.com.	Soc 418	Sciaenidae	O'Malley *et al.* 2003
Hau 170	Haemulidae	Chapman pers.com.	Soc 419	Sciaenidae	O'Malley *et al.* 2003
Hau 183	Haemulidae	Chapman pers.com.	Soc 421	Sciaenidae	O'Malley *et al.* 2003
Hau 196	Haemulidae	Chapman pers.com.	Soc 422	Sciaenidae	O'Malley *et al.* 2003
Hau 206	Haemulidae	Chapman pers.com.	Soc 423	Sciaenidae	O'Malley *et al.* 2003
Hau 220	Haemulidae	Chapman pers.com.	Soc 424	Sciaenidae	O'Malley *et al.* 2003
Hau 232	Haemulidae	Chapman pers.com.	Soc 425	Sciaenidae	O'Malley *et al.* 2003
Hau 236	Haemulidae	Chapman pers.com.	Soc 426	Sciaenidae	O'Malley *et al.* 2003
Hau 239	Haemulidae	Chapman pers.com.	Soc 428	Sciaenidae	O'Malley *et al.* 2003
Hau 315	Haemulidae	Chapman pers.com.	Soc 429	Sciaenidae	O'Malley *et al.* 2003
Hau 330	Haemulidae	Chapman pers.com.	Soc 430	Sciaenidae	O'Malley *et al.* 2003
			Soc 431	Sciaenidae	O'Malley *et al.* 2003
Soc 009	Sciaenidae	Turner *et al.* 1998	Soc 432	Sciaenidae	O'Malley *et al.* 2003
Soc 011	Sciaenidae	Turner *et al.* 1998	Soc 433	Sciaenidae	O'Malley *et al.* 2003
Soc 012	Sciaenidae	Turner *et al.* 1998	Soc 434	Sciaenidae	O'Malley *et al.* 2003
Soc 014	Sciaenidae	Turner *et al.* 1998	Soc 435	Sciaenidae	O'Malley *et al.* 2003
Soc 017	Sciaenidae	Turner *et al.* 1998	Soc 437	Sciaenidae	O'Malley *et al.* 2003
Soc 019	Sciaenidae	Turner *et al.* 1998	Soc 438	Sciaenidae	O'Malley *et al.* 2003
Soc 029	Sciaenidae	Turner *et al.* 1998	Soc 439	Sciaenidae	O'Malley *et al.* 2003
Soc 034	Sciaenidae	Turner *et al.* 1998	Soc 442	Sciaenidae	O'Malley *et al.* 2003
Soc 035	Sciaenidae	Turner *et al.* 1998	Soc 443	Sciaenidae	O'Malley *et al.* 2003
Soc 044	Sciaenidae	Turner *et al.* 1998	Soc 444	Sciaenidae	O'Malley *et al.* 2003
Soc 049	Sciaenidae	Turner *et al.* 1998	Soc 445	Sciaenidae	O'Malley *et al.* 2003
Soc 050	Sciaenidae	Turner *et al.* 1998			