Population genetics of *Galeorhinus galeus*, *Carcharhinus brachyurus* and *Rhinobatos annulatus*- implications for regional fisheries and elasmobranch conservation

Thesis presented in fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of AgriScience at Stellenbosch University

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Declaration

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March 2016

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Abstract

Elasmobranchs (sharks, skates and rays) are highly exploited world-wide and more vulnerable than most teleosts due to their life history traits (e.g. late age at maturity, low fecundity and slow growth). Most elasmobranchs are either targeted by commercial fisheries or unintentionally taken as bycatch in mixed-species fisheries. Among these, the tope shark *Galeorhinus galeus*, the copper shark *Carcharhinus brachyurus* and the southern African endemic lesser sandshark *Rhinobatos annulatus*, are targeted globally and locally in demersal, pelagic and recreational fisheries. Across the Southern Hemisphere, the International Union for the Conservation of Nature (IUCN) categorizes both the tope and copper sharks as "vulnerable" while the lesser sandshark as "data deficient" within its region of endemism. Information is urgently needed on their regional genetic structure and diversity to help delineate management units (MUs) for better fisheries monitoring and conserving local biodiversity.

Regional and local population genetic structure of these species was assessed using previously optimised cross-species microsatellite panels and/or the mitochondrial *NADH2* and *NADH4* genes. Patterns of evolutionary and demographic history were inferred using coalescent and Bayesian statistical methods. For *G. galeus*, the data showed a lack of contemporary gene flow and deep historical divergence across the Southern Hemisphere. Two geographically distinct mitochondrial clades were recovered, one including the Atlantic and Indo-Pacific collections (ARG, SA and AUS) and one comprising the Pacific samples (NZ and CHI) as well as single divergent haplotype restricted to South Africa. Nuclear data also revealed large population subdivisions (F_{ST} = 0.050 to 0.333, $P < 0.05$) indicating very limited gene flow for tope sharks across ocean basins. On a local scale, F-statistics, multivariate and clustering analyses supported gene flow with substantial admixture along the South African coastline (F_{ST} = 0.016 to 0.048, $P > 0.05$), with some degree of genetic structure between the Atlantic and Indian Ocean samples. The east coast samples of Port Elizabeth were significantly differentiated from the rest (F_{ST} = 0.023 to 0.091, $P > 0.05$).

For *C. brachyurus*, estimates of pairwise population differentiation were significant (average F_{ST} = 0.031, $P = 0.000$) indicating some degree of gene flow between sampling sites while the sub-structuring observed at Strandfontein indicated the existence of a possible distinct, more admixed group of individuals. Neither AMOVA (F_{CT} = -0.011, $P = 1.000$) nor Bayesian clustering analyses indicated genetic discontinuity or significant population structure across

the Atlantic/Indian boundary. Although the *ND4* results also alluded to historical dispersal across this boundary, the population of Mossel Bay harboured four highly divergent haplotypes, indicating that this region might be a potential nursery site for *C. brachyurus*.

The genetic diversity and genetic connectivity of *R. annulatu*s was inferred using cross-amplified polymorphic microsatellite loci across the Agulhas bioregion that coincides with the warm temperate biogeographical province of South Africa. Significant genetic differentiation was observed over a small sampling range (F_{ST} = 0.016 to 0.094, *P* < 0.050) implying that the species might be highly structured throughout its entire geographical range. Overall effective population size for *R. annulatu*s was very low (N_e = 106) and not in accordance to the abundance proposed for the species. As this is the first regional assessment for all three of these species, the findings of this study could have immediate implications for the regional management and conservation of commercial and recreational sharks.

Acknowledgements

Preface

Scientific Contributions (2013-2015)

Poster presentation – 2nd Southern African Shark and Ray Symposium 2013

Population dynamics of the commercially important shark species *Galeorhinus galeus* and its implications for management and conservation.

Oral presentation – 2nd Sharks International 2014

Population structure of tope shark (*Galeorhinus galeus*) across the Southern Hemisphere inferred by microsatellite markers.

Oral presentation – Joint SASBi-SAGS Congress 2014

Regional genetic connectivity and phylogeography of the tope shark (*Galeorhinus galeus*) across the South Atlantic and Indian Oceans.

Oral presentation – 3rd Southern African Shark and Ray Symposium 2015

Genetic connectivity of the commercially important copper shark, *Carcharhinus brachyurus*, associated with South Africa's contrasting oceanic current system.

Article published – Fisheries Research

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Abbreviations

ATP	Adenosine triphosphate
ß-ME	ß-Mercaptoethanol
bp	Base pairs
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
cm	centimetres
CTAB	Cetyltrimethylammonium bromide
^{o}C	Degrees celsius
DAFF	Department of Agriculture Forestry and Fisheries
ddH$_2$O	Double distilled water
DNA	Deoxyribonucleic acid
DAPC	Discriminant Analysis of Principal Components
dNTPs	Deoxyribonucleotidetriphosphate
EtBr	Ethidium bromide
FCA	Factorial Correspondence Analysis
IUCN	International Union for Conservation of Nature
m	metres
mins	Minutes
NaCl	Sodium chloride
NPOA	National Plan of Action SA
PCA	Principal Component Analysis
PIC	Polymorphism Information Content
SA	South Africa
SASC	South African Shark Conservancy
sec	Seconds

TBE   Tris-Borate-Ethylenediaminetetraacetic acid

USA   United States of America

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Language and style used in this thesis are in accordance with the requirements of the Harvard referencing style. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetitions between chapters have, therefore, been unavoidable.

General Introduction

Elasmobranchs (sharks, skates and rays) are a group of marine organisms with a long evolutionary history (400 million years) and a range of ecological niches. These species are highly exploited and are in dire need of better global and regional management. There are currently over 1150 species known worldwide. Most of these species are extremely vulnerable to overexploitation given the life history traits they display such as slow growth, late sexual maturity, long gestation periods and low fecundity (Compagno *et al.* 2005; Naylor *et al.* 2012). Despite this, the majority of shark fisheries around the globe are unmonitored or poorly managed, leading to severe population declines as the demand for shark products, especially dried fins, has escalated (Musick *et al.* 2000; Clarke *et al.* 2006; Dudley & Simpfendorfer 2006; Best *et al.* 2013; Worm *et al.* 2013). The commercial exploitation of demersal shark species in South Africa alone is more than 80 years old and has led to declines in population numbers (da Silva & Bürgener 2007; da Silva *et al.* 2015). Across the South African coastline, there are a number of species that are of commercial importance including shortfin mako (*Isurus oxyrinchus*), blue shark (*Prionace gluaca*), tope shark (*Galeorhinus galeus*), common smoothhound (*Mustelus mustelus*) and copper shark (*Carcharhinus brachyurus*) (da Silva *et al.* 2015). The exploitation of these species in South Africa has gradually increased over the last two decades with shark fins and fillets mainly being exported to support a demand in the international market (da Silva & Bürgener 2007; DAFF 2013).

Elasmobranchs are currently regarded as one of the most vulnerable extant vertebrate groups and many of the species are threatened with extinction (Dulvy *et al.* 2014). For example, tope shark (*Galeorhinus galeus* Linnaeus 1758) was assessed by the International Union for Conservation of Nature (IUCN) and is listed as "vulnerable" globally (Walker *et al.* 2006) while the copper shark (*Carcharhinus brachyurus* Günther 1870) is listed as "near threatened" (Duffy & Gordon 2003). Both these species are distributed globally in temperate waters where they are commercially exploited on a large scale. They are both highly susceptible to the pressures of fishing due to the *K*-selected traits they exhibit (*e.g.* long generation time, low fecundity and late sexual maturity) (Musick *et al.* 2000; Compagno *et al.* 2005). The lesser sandshark (*Rhinobatos annulatus* Müller & Henle 1841) on the other hand, is one of the most abundant endemic species in southern Africa and is mainly caught as bycatch by commercial fisheries. The species is not as vulnerable to fishing pressures since it

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does not exhibit strictly *K*-selected traits but has a short generation time and early sexual maturity (Rossouw 1983; Compagno *et al.* 1991; Rossouw 2014). Although the species is listed on the IUCN red list as "least concern", population trends in southern Africa and the impact of fisheries on the species remain for the most part unknown (Burgess *et al.* 2006).

The assessment of populations (observable and inferred from molecular data) in southern Africa have thus far been hampered by a lack of fisheries independent data, species-specific assessments and limited understanding of transoceanic movement patterns (Department of Agriculture and Fisheries, DAFF-2013; da Silva *et al.* 2015). In order to implement regional management strategies for elasmobranchs, patterns of migration and resulting population structure needs to be elucidated for each species. Information on population genetic structure is needed to monitor the effect of fishing on different stocks and areas and ultimately to preserve species-specific genetic diversity (Ovenden *et al.* 2013). Ideally this could lead to a more integrated approach to fisheries management, where species showing different levels of population subdivision over similar spatial scales, are co-managed (Keeney *et al.* 2003; Ovenden *et al.* 2009; Pereyra *et al.* 2010).

In South Africa, the National Plan of Action (NPOA-sharks) has identified that *G. galeus* and *C. brachyurus* are mostly exploited across the south-west coastline (DAFF 2013) while *R. annulatus* has a more south to eastern coast exploitation (Burgess *et al.* 2006). The South African coastline encompasses the Atlantic and Indian Oceans with two oceanic currents (*i.e.* Benguela and Agulhas) previously shown to affect dispersal of various marine species (Teske *et al.* 2011; Teske *et al.* 2013; Henriques *et al.* 2014). A review by Cochrane *et al.* (2004) revealed that fisheries in South Africa have impacts beyond the target species and that an ecosystem approach to fisheries (EAF) is required for a long-term sustainability of the living marine resources. With the aid of molecular markers such as microsatellites and mitochondrial sequence data the genetic diversity and population connectivity of species such as *G. galeus, C. brachyurus* and *R. annulatus* can be evaluated to help understand the influence of contemporary and historical features on the distribution of populations seen today for a comprehensive EAF management. This study was therefore conducted under two major themes with the following questions in mind:

Research questions pertaining to commercially exploited sharks

(i)   What is the genetic connectivity and phylogeography of *Galeorhinus galeus* across the Southern Hemisphere and where does South African *Galeorhinus galeus* fit in from an evolutionary perspective?

(ii)   What is the local genetic connectivity and phylogeography of *Galeorhinus galeus* across the south Atlantic and Indian oceans?

(iii)   What is the regional population connectivity of *Carcharhinus brachyurus* across the Atlantic/Indian boundary and how does this compare to *Galeorhinus galeus*?

(iv)   Could this have implications for the sustainable exploitation of these two shark species in South Africa?

Research question pertaining to non-targeted endemic species

(i)   What is the level of genetic diversity in the endemic *Rhinobatos annulatus* in South Africa?

(ii)   Is there any indication of population structure across a limited sampling range in the Eastern Cape?

The overarching hypothesis to be tested is whether, for any of these species, the null hypothesis of panmixia can be rejected and if so, what the possible drivers of the observed structure may be. This study will be the first regional account of genetic diversity and patterns of gene flow (or absence thereof) for these species and aims to be of significance to sustainable shark fisheries and bycatch regulations in South Africa. Furthermore, the study will reflect on whether an ecosystem approach will be suitable in the management of the study species especially with regards to the Benguela and Agulhas ecosystems. This study will mainly focus on sharks collected from the west and south coasts of South Africa, the regions in which these species are most exploited or seen as most vulnerable. As such, sampling was concentrated across the cool and warm temperate regions of the South African coastline, coinciding with the area known to include the Atlantic/Indian oceans boundary at Cape Agulhas and where the cold Benguela and warm Agulhas currents define distinct oceanographic conditions. Additionally, samples of *Galeorhinus galeus* were obtained from Argentina, Australia, New Zealand and Chile to investigate gene flow and historical dispersal over a larger oceanic expanse across the Southern Hemisphere. A smaller sampling region was included for *Rhinobatos annulatus* as this species is endemic only to southern Africa and is mainly caught along the south and east coasts. Due to opportunistic sampling, attaining

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representative samples evenly throughout the respective distribution ranges proved to be difficult but where possible, this was compensated for by more rigorous statistical analysis for some datasets.

To better understand the flow of this study, some key terms are defined:

(i)   Sampling population- Samples sourced from a specific location. This does not necessarily represent the true population of the samples.

(ii)   Population- The true clustering of samples based on the microsatellite genotypes and mitochondrial haplotypes.

In this dissertation, 'patterns of gene flow' are described as either panmictic or genetically differentiated populations. Panmixia describes a highly admixed population as a result of a high degree of gene flow while genetic differentiation describes populations with very little or no gene flow, leading to isolated or discreet populations. In the fisheries sense, the latter are often referred to as 'stocks', referring to populations that are genetically and demographically distinct from other populations. These genetic 'stocks' can further be considered either as management units (MUs) or evolutionary significant/conservation units (ESUs). Management units are usually defined based on patterns of contemporary gene flow whereas conservation units are typically defined based on historical or long term restriction to gene flow (Moritz 1994; Waples 1998; Avise 2000). This dissertation is divided in to six chapters including four experimental research chapters. Chapter 1 is a critical review of literature on the biodiversity, management and conservation genetics of regional elasmobranchs. Chapter 2 entails the optimisation of microsatellite multiplex panels for the three species in question as well as their application in species composition of a commercial catch and evaluating gene flow patterns of the two shark species exploited most in the South African fisheries. Chapter 3 examines the contemporary and historical patterns of gene flow for *G. galeus* across the Atlantic/Indian Ocean transitional zone of South Africa and also include analysis of gene flow and phylogeography across the Southern Hemisphere. Chapter 4 examines the contemporary patterns of gene flow of *C. brachyurus* across southern Africa based on microsatellites and mitochondrial sequence data while Chapter 5 details the first evaluation of microsatellite genotypic variation of *R. annulatus,* endemic to southern Africa. Finally, chapter 6 will conclude with a summary of the findings and discuss implications and possible implementation thereof for future management and research.

Chapter 1:   Literature Review

1.1.   Global and regional elasmobranch biodiversity

Chondrichthyes is a taxonomic class of cartilaginous fish that is divided into two subclasses; the Elasmobranchii (sharks, skates and rays) and the Holocephalii (chimaeras). Elasmobranchs are further divided in to four suborders, Squalomorphii, Galeomorphii, Squatinomorphii and Batoidea (Compagno *et al.* 2005; Ebert & Compagno 2007). Up to 1144 species of elasmobranchs have been identified and these include 500 shark species and 650 batoids (Ebert & Compagno 2007; Kyne & Simpfendorfer 2007; Ebert & van Hees 2015). Sharks are grouped in to eight orders with the dominant order, the ground sharks (Carchariniformes) comprising 56% of all sharks. The other three major groups are the dogfish (Squaliformes), carpet sharks (Orectolobiformes) and mackerel sharks (Lamniformes). The smallest four orders are the frilled and cow sharks (Hexanchiformes), the angel sharks (Squatiniformes), the bullhead sharks (Heterodontiformes) and the saw sharks (Pristiophoriformes) (Compagno *et al.* 2005). Batoids are grouped in to four main orders including electric rays (Torpendiniformes), sawfishes (Pristiformes), skates, wedgefishes and guitarfishes (Rajiformes) and sting rays (Myliobatiformes) (McEachran & de Carvalho 2002; Compagno *et al.* 2005.)

Most sharks exhibit similar life history traits that confer a lower inherent rate of population increase (*K*-selected reproduction) in comparison to bony fish. Shark species have a simple skeleton made of cartilage; they have transverse jaws with rows of replicating teeth and dermal denticles. They are larger than bony fish, have later maturity stages (2-22 years), live longer (8-65 years), have long gestation periods (9-18 months) and low fecundity rates (Compagno *et al.* 2005). The slow life-history traits and low production rates of elasmobranchs make them more vulnerable to the pressures of fishing compared to bony fish (Dulvy *et al.* 2008; Ferretti *et al.* 2010). Most sharks inhabit marine water (about 95%) and a small percentage inhabits fresh water during all or part of their lives (*e.g.* bull shark *Carcharhinus leucas*). Marine dwelling species are divided based on habitation with most of the species inhabiting continental shelves (up to 55%) at depths of 200m, followed by continental slopes (up to 35%) which range from depths of 200 to 2000m. Only a small percentage (2%) is entirely oceanic (*e.g.* blue shark *Prionace glauca* and oceanic whitetip

shark *Carcharhinus longimanus*) and around 8% of species move between shelves, slopes and oceans (*e.g.* white shark *Carcharodon carcharias* and tiger shark, *Galeocerdo cuvier*) (Compagno 1990). The presence of fresh water elasmobranchs could be an indication of apical predation as is seen with the requiem sharks that spend a portion of their lives in temperate lakes and rivers (*e.g.* bull shark *Carcharhinus leucas*) (Compagno *et al.* 2005). Sharks have a very diverse ecology, morphology and behaviour, comprising 22 different ecomorphotypes. Ecomorphotypes show the discrete measures of morphological traits that are related to habitat and activity levels of species across taxonomic groups (defined by Compagno 1990). Sharks also have diverse reproductive modes that are classified based on foetal nutrition and where the embryo develops. The two forms of foetal nutrition are lecithotrophy where the developing embryo is only nourished by the yolk and matrotrophy where part of the foetal development is supplemented by a maternal input of nutrients. Embryo development is oviparous when it occurs externally or viviparous when it occurs internally (Wourms & Demski 1993). Oviparous modes of development are all lecithotrophic. Viviparity on the other hand is mostly matrotrophic with the exception of yolk-sac viviparity which is lecithotrophic (Compagno 1990; Musick & Ellis 2005).

Batoids are distinguished from sharks morphologically by their ventral gill slits and lack of anal fins. Sawfishes and guitarfishes are very shark-like in appearance although they are flat in structure. Guitarfishes are sometimes also referred to as shovelnose rays. Most batoids are small in size with sizes ranging from 20 cm to 1 m (total length, TL). However, sawfishes tend to be larger in size and can grow to a TL of 7 m. Batoids have a shallow coastal to continental shelf distribution and are mostly found close to the sea bed. Some batoids are known to inhabit fresh brackish estuaries and rivers (*e.g.* sawfishes) but only one family of rays, the Potamotrygonidae is confined to fresh waters located in South American rivers. Most skates have extensive latitude and depth ranges, with representatives at most latitudes and depths to about 2000 m, but are rare in tropical shallow waters and coral reef areas. Some electric rays (Torpedinidae) are also abundant in temperate latitudes while all other batoid families are restricted to tropical and warm-temperate areas, and show a preference for relatively shallow waters. Moreover, some of these families show a high propensity for endemism (*e.g.* guitarfishes). Batoids also undergo internal fertilization and have diverse forms of reproduction ranging from oviparity to aplacental viviparity (Musick & Ellis 2005).

The southern African region that includes Angola, Mozambique, Namibia and South Africa, boasts 18% of the world's total chondrichthyan fauna, with about 177 elasmobranch species found in South African waters alone. In South Africa, elasmobranchs inhabit three different areas that are broadly labelled as the continental shelves, the continental slopes and the oceanic zone. Most of the elasmobranchs in South Africa inhabit continental shelves and slopes, 6% inhabit the oceanic zone and about 1% inhabits a wider range of habitats including fresh water. Amongst some of the shelf species and some of the deep-slope species, the distribution can be further sub-divided into three zoogeographical regions. There is a west coast cool-temperate fauna found west of Cape Point, an east coast warm-temperate fauna from Cape Point to East London, and a subtropical-tropical fauna found east of East London. Cases of overlap in the zoogeographical regions are not uncommon and the diversity in these regions increases from the west to east (Compagno *et al.* 1991; Compagno 1999).

1.2. Status of exploitation and implications for ecosystems

The exploitation of elasmobranchs has been steadily increasing raising concerns over the sustainability of this marine resource and the impacts to the marine ecosystem globally (Worm *et al.* 2013). Most elasmobranchs (especially sharks) are vulnerable to fishing pressures due to the *K*-selected traits they exhibit such as low fecundity, late sexual maturity and a long lifespan with slow growth rates. Added to this is the limited amount of baseline data on species-specific landed catch since, historically, they were of low economic value and a lesser priority in terms of fisheries management. Since the 1980s, there has been a high demand for shark fins and meat, dramatically increasing the value of elasmobranchs and thus escalating the number of unreported catches (Clarke *et al.* 2006; Worm *et al.* 2013; Gallagher *et al.* 2014). From the year 2000, the landed catch reported globally ranged between 63 and 273 million sharks caught annually which exceeds the average rebound rate of many shark and ray species estimated from the life-history traits. Unfortunately, little is known of species-specific catches as these or unintentional catch are never reported (Molina & Cooke 2012; Worm *et al.* 2013; Gallagher *et al.* 2014). Despite the inadequacies in reporting catch data, the available reports show that one-quarter of the world's chondrichthyans are threatened by extinction (Dulvy *et al.* 2014) with 67 elasmobranch species classified as critically endangered or endangered by the International Union for the Conservation of Nature (IUCN) (Ward-Paige *et al.* 2012).

The over-fishing of elasmobranchs (especially sharks), which are at the top of the marine ecosystem, could affect the harvested species directly or indirectly through the trophic interactions within the ecosystem. Fishing directly affects the abundance and biomass, and indirectly the size structure, life-history parameters that are density-dependent, as well as species diversity. A decrease in the abundance of larger sized fishes is usually a feature of exploited fish populations. In earlier times, shark harvesting was driven by the market's demand for liver oil which is enriched in vitamin-A while the current market demands fins, meat and liver, driving a growth in the harvesting of specific species. Cited in this, is the demand for tope shark (*Galeorhinus galeus*) fin soup and meat which led to the stock collapse of the species in California and southern Australia (Stevens 2000).

Exploitation of marine ecosystems has been reported to make a shift in the size and age structure of some species (Stevens 2000; Brunel 2010). This could be due to the size-selective gear used during fishing, which ultimately makes fishing a selective force. Studies carried out in fished marine ecosystems around the world have shown a direct correlation between the change in the structure of fish assemblages and the intensity of fishing over time. Several studies have also reported significant changes in the size composition of fished communities using a variety of size-based indicators (Dulvy *et al.* 2004; Yemane *et al.* 2008; Brunel 2010; Atkinson *et al.* 2011). An overall decrease in target species size has been observed in fished communities, the cause of which has been attributed partially to the strong size discrimination of fishing activities. Yemane *et al.* (2008) investigated changes in size-based indicators of demersal fish assemblages from the south coast of South Africa from 1986 to 2003, and showed a decrease in mean length, mean maximum length and the proportion of large fish over time. Selective fishing could also have significant impacts on the reproduction output since fecundity increases with body size. Observations for gummy sharks (*Mustelus antarticus*) showed that the litter size increases with the maternal size (Walker *et al.* 1998).

Though there is little evidence to support an increase in fecundity or growth rate as compensatory mechanisms for the over-exploitation, the net recruitment rate and therefore juvenile survival rate is believed to play a key role. Stevens and West (1997) for example reported observations of apparent density-dependent changes; increments in the growth rate of tope shark juveniles in Australia following heavy fishing pressure. Heavy fishing may also affect the community structure of the ecosystem by impacting the predator/prey relationships

(trophic cascade) since most elasmobranchs are at the top or near the top of the food chain. Ecological effects such as increases in prey composition and dips in predator composition have been noted, as well as changes in habitat distribution (Myers *et al.* 2007; Ferretti *et al.* 2010). Myers *et al.* (2007) showed that declines in the populations of 11 large shark species larger than 2 m in the northwest Atlantic coastal ecosystems led to a trophic cascade effect. There was a reported increase of inferior elasmobranchs such as rays, skates and small-sized sharks, due to the over-exploitation of the higher ranking predators.

1.3.   Different fisheries and their impacts

Fishing for elasmobranchs incorporates the use of varying fishing gear, which is dependent on the species being targeted and the ecosystem it inhabits (Camhi *et al.* 2008). Contemporary sharks inhabit coastal, demersal and pelagic habitats in all oceans (Compagno, 1990). While most species are limited to the continental shelves, there is a small number of exclusively oceanic species (*e.g.* blue shark *Prionace glauca*, oceanic whitetip *Carcharhinus longimanus*, shortfin mako *Isurus oxyrinchus*), and some that migrate between coastal and oceanic waters (*e.g.* scalloped hammerhead *Sphyrna lewini*, tiger shark *Galeocerdo cuvier*, white shark *Carcharodon carcharias*, tope shark *Galeorhinus galeus*) (Ferretti *et al.* 2010). The largest percentage of elasmobranch species inhabits demersal ecosystems on continental shelves and slopes (Compagno, 1990) and these are targeted mainly by trawl fishing (Shepherd & Myers 2005).

Trawl fisheries exploits not just the target species but leaves a trail of incidental catch with devastating consequences for elasmobranchs in particular (Ferretti *et al.* 2010). In the Mediterranean for example, a century of trawl fishing led to the loss of 16 of 31 recorded elasmobranch species in the Tyrrhenian Sea and six of 33 species in the Adriatic Sea (Aldebert 1997). A study by Fennessy (1994) performed on bycatch species in the prawn fishing industry showed that during 1989 to 1992, six large coastal sharks and 21 small elasmobranchs were recorded as bycatch on just a small shallow bank in eastern South Africa. In a similar case in Australia, prawn trawl surveys during the period 1990 to 1998 recorded a catch of 10 large coastal sharks and 46 smaller sized elasmobranchs (Stobutzki *et al.* 2001). Since this type of incidental fishing of large sharks continues, the ecosystem becomes dominated by smaller elasmobranch meso-predators as was seen in the northeast Atlantic (Ellis *et al.* 2005). The pelagic ecosystem is most prone to longline fishing which accounts for the largest share of shark catches around the world (Camhi *et al.* 1998). Up to

100 kilometres of ground can be covered by a longline, which is attached to shorter branch lines connected to hooks (Gilman *et al.* 2007). These lines are hauled on to a boat baring the captured sharks. During the haul back, it's highly likely that the sharks are dead or seriously injured. In cases where the shark species caught aren't desired, they are released back in to the water albeit with fatal injuries (Gilman *et al.* 2007; Morgan *et al.* 2010).

1.3.1. Direct fisheries (with focus on the South African chondrichthyan fisheries)

The economic potential of chondrichthyan fisheries in South Africa was first discussed by von Bonde (1934) who noted that virtually the whole shark carcass was of multi-produce entity. This led to years of an irregular export of mainly shark products from South Africa to foreign markets in Asia, Europe and Australia (da Silva & Bürgener 2007) mainly depending on several factors, including:

(i)   relaxation of the mercury concerns due to effective quality control

(ii)   good political and trade relationships with countries such as Australia

(iii)   favourable exchange rates for exporting, and

(iv)   increased demand for shark meat and fins overseas

Today, the South African chondrichthyan fisheries include a fishing area within an exclusive economic zone (EEZ) encompassing the South Atlantic Ocean and the Indian Ocean (**Figure 1.1**). This fishing zone was only declared in 1977. Catch data for elasmobranchs within the South African EZZ is labelled under shark fisheries and is limited to commercial grounds which include three major trawling zones; the west coast from Cape Agulhas, the south/east coast including the Agulhas Bank, and the Natal coast along the east coast. These areas encompass data from both the direct fisheries and the bycatch fisheries where catches differ significantly due to the varying fishing methods used. Elasmobranchs in South African waters are affected by direct fisheries methods such as the demersal longline, the traditional linefishery, St. Joseph net-fishery, the bather protection program and shark fishing for aquarium trade (NPOA-sharks 2012). Interestingly, the large pelagic longline fishery and the recreational linefishery where elasmobranchs are caught as bycatch, are classified under the direct fisheries due to the very high catch numbers. Elasmobranchs are otherwise landed as bycatch in the hake-longline, the inshore and offshore trawl, the beach seine and the prawn trawl (NPOA-sharks 2012).
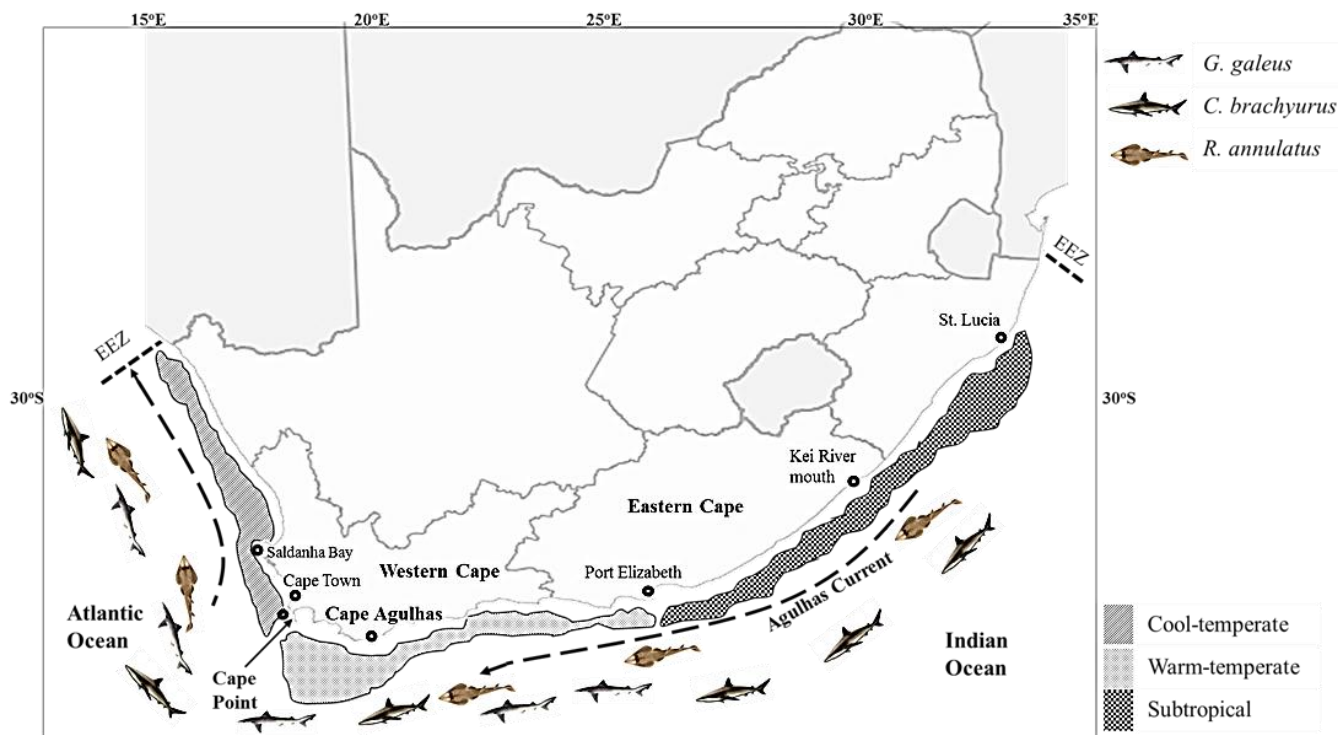
Figure 1.1. Map showing the exclusive economic zone (EZZ) of South Africa, the major biogeographic regions and the distribution ranges of the study species.

The demersal longline shark fishery operates in coastal waters from the west to south coast within the Atlantic Ocean and targets mainly demersal shark species of commercial importance as well as some skates. The fishery targets demersal shark species mainly at Port Elizabeth, Mossel Bay, Vleesbaai, Stilbaai, Struisbaai and Gansbaai (da Silva & Bürgener 2007). This fishery started off on a low note, with the number of permits issued reduced from 30 in 1990 to six in 2005 due to the lack of consumer market for shark products in South Africa. By 2006 however, there was a high demand for shark products mainly from Australia and this led to a boom in the targeted fishing of sharks. In a recent study by da Silva *et al.* (2015), estimated landings of 408 t, 175 t and 88 t of shark were reported in 2010, 2011 and 2012, respectively. This included mostly smoothhound sharks (*Mustelus* species), tope shark *G. galeus*, copper shark *C. brachyurus*, dusky shark *C. obscurus* and unidentified skates (NPOA-sharks 2012). Unfortunately, there is still a lack of species-specific data within this fishery due to the generic reporting of landed catch (NPOA-sharks 2012; 2013).The traditional linefishery is one of the oldest fisheries in South Africa, and targets elasmobranchs when insufficient number of line fish has been caught. The use of linefishery in targeting elasmobranchs dates back to the 1940s when *G. galeus* was the main target. This fishery

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overlaps with the demersal longline as it also targets pelagic and demersal shark species throughout the South African coastline and is mostly inshore. Just like the demersal longline, landings in the traditional linefishery are not properly monitored; despite the required monthly report from vessel logbooks, and species-level data is lacking. Annual landings of chondrichthyans were reported as 277 t, 175 t and 165 t between 2010 and 2012, with the main target species being *M. mustelus*, *G. galeus*, *C. brachyurus* and broadnose sevengill shark *Notorynchus cepedianus* (da Siva *et al.* 2015).

Despite being classified under direct fisheries, the large pelagic longline was established to target swordfish and tuna species. Up to 30 or 40% of the landed catch in this fishery is made up of pelagic sharks especially *Prionace glauca*, *Isurus oxyrinchus* and *Sphyrna lewini*. In 2010 alone, 66 t of *I. oxyrinchus* and 100 t of *P. glauca* were landed making the large pelagic longline fishery a large contributor to South African shark fisheries (NPOA-sharks 2012; 2013).

1.3.2. Indirect fisheries

Indirect fisheries or bycatch refers to the unintentional capture of a non-targeted species by means of non-selective fishing gear. The unintentional catch is discarded when found to be of low or no commercial value (Crowder & Murawski, 1998). Bycatch poses a threat to especially elasmobranchs due to their life history characteristics resulting in slow rates of population increase and growth (Dulvy *et al.* 1998). For instance, most of the elasmobranch species listed as "threatened" on IUCN red list are believed to be most threatened via bycatch (Molina & Cooke 2012). However, very little quantitative data is available on the number of sharks, rays and skates caught as bycatch in various commercial fishing industries as much of the discarded catch isn't documented (Attwood *et al.* 2011; Molina & Cooke 2012).

The interest in bycatch has been raised over the last decade as it poses one of the greatest threats in managing elasmobranch populations. Fortunately, research spanning over a decade has shown a reduction in the number of fish caught as bycatch reducing from 39.5 million tons to 6.8 million tons (Kelleher 2005). This decline could be due to the implementation of bycatch reduction methods (Kelleher 2005) or simply inaccurate numbers due to a lack of reporting and studies on bycatch. Also, the few studies that do focus specifically on bycatch of elasmobranchs (Herndon *et al.* 2010; Worm *et al.* 2013), concentrate only on a few commercial species or on a limited geographical area (Megalofonou *et al.* 2005; Godin &

Worm 2010). All these factors could bias the global bycatch numbers and impact whatever conservation policies have been put in place thus far (Molina & Cooke 2012).

Locally, elasmobranchs are also caught as bycatch in the inshore and offshore trawl fisheries, which operate in the Eastern and Western Cape respectively. The bycatch is mainly made up of *G. galeus*, *Mustelus* species, *R. annulatus* and *C. brachyurus*. Annual chondrichthyan landed bycatch has been recorded at 1 727 t, 1 625 t and 1 576 t for 2010, 2011and 2012, respectively and according to the national observer database, approximately 52 chondrichthyan species are caught in these fisheries (da Silva *et al.* 2015). On a per-weight basis, the inshore trawl fishery comprises of 42% bycatch making it a multi-species fishery in comparison to the offshore trawl fisheries (Attwood *et al.* 2011). To summarise, harvesting of elasmobranchs in South Africa increased from 3500 t landed in 2010 (NPOA-sharks 2012) to more than 6500 t landed during 2012 (NPOA-sharks 2013). Almost 50% of local chondrichthyan species are affected by nine different fisheries in South Africa. The species included in this study are affected by all nine these fisheries, highlighting the importance of assessing their population structure and patterns of gene flow.

Table 1.1. Fisheries impacting the study species in South African waters

Fishery	Area	Species	Target/bycatch
Demersal shark longline	West and south coast	*Mustelus* spp.*, G. galeus*	Target
Traditional linefish	Inshore to 200m	*Mustelus* spp., *G. galeus*	Target
Hake longline	West and south coast to 500m	*Mustelus* spp., *G. galeus*	Bycatch
Inshore trawl	South and east coast to 200m	*Mustelus* spp., *G. galeus, R. annulatus*	Bycatch
Offshore trawl	West coast (Agulhas bank to shelf edge at 600m depth)	*G. galeus*	Bycatch
Gillnet/beach seine	West and south coast	*Mustelus* spp., *G. galeus*	Target and bycatch
Bather protection program	East coast	*C. brachyurus*	Target
Recreational linefishery	Inshore to 200m	*C. brachyurus*	Target
Prawn trawl	Kwazulu Natal, east coast to 600m	*C. brachyurus*	Bycatch

1.4.   Current status of elasmobranch management in South Africa

Although there is concern over elasmobranch exploitation, stemming from consideration of their *K*-selected traits, and from historical exploitation patterns, protection of these species from the impacts of fisheries is not impossible (Worm *et al.* 2013). Ward-Paige *et al.* (2012) suggest that management measures that help restore elasmobranch populations require monitoring of population trends, proper assessment of diversity estimates, assessment of harvesting practices, protection of critical habitats and enforcement of fishing limits. Only through this improved knowledge, can there be effective management and protection of elasmobranchs and this is highlighted in case studies in the U.S.A where recovery was seen in populations of *Pristis pectinata*, *Galeocerdo cuvier* and *Sphyrna lewini* (Ward-Paige *et al.* 2012).

Two-thirds of the reported catch was bycatch. Regulations aimed at limiting chondrichthyan catches, coupled with species-specific permit conditions, currently exist in these fisheries: demersal shark longline, pelagic longline, recreational line, and beach-seine and gillnet. However, few management measures exist for many elasmobranchs in South Africa with the exception of *Carcharodon carcharias* and the *Pristidae* species, which are currently protected under Appendix II of CITES and *Carcharhinus taurus*, *Triakis megalopterus*, *T. megalopterus* and several *Poroderma* species, which are commercially de-listed (da Silva *et al.* 2015). Several factors are at play in hindering proper management measures in South Africa, including the multi-species nature of the fisheries, species misidentification, limited biological knowledge of commercially exploited elasmobranch species and a lack of data required for stock assessments (Silva & Bürgener 2007; Best *et al.* 2013; NPOA-sharks 2013). For instance, catch and effort data suitable for stock assessments exist for fewer than ten elasmobranch species including *Galeorhinus galeus*, *Mustelus mustelus*, *Carcharodon carcharias*, *Carcharias taurus* and *Triakis megalopterus* (da Silva *et al.* 2015).

However, these factors have not prevented the development of precautionary management recommendations especially for some commercially exploited elasmobranchs. For instance, McCord (2005) did a stock assessment of *Galeorhinus galeus* and suggested that catch limits be implemented, that further assessments be done in the inshore and offshore fisheries of the species and that genetic data be included for this species. In another case study, the commercially important shark species *Mustelus mustelus* is afforded protection in the no-take

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area Langebaan Lagoon Marine Protected Area (LLMPA) which has been shown to be an effective management measure in the Langebaan region. However, the LLMPA only provides about 35% protection in the entire Saldanha Bay area and even much less of the entire distribution range of this species (da Silva *et al.* 2013).

1.5. Historical and contemporary gene flow patterns in elasmobranchs

Both historical and contemporary barriers to gene flow can influence present-day patterns of genetic diversity and population connectivity within species. In the marine environment, contemporary oceanographic features such as currents, thermal fronts and upwelling systems can influence dispersal of adults and juveniles, in the case of elasmobranchs, resulting in population substructure. In the same way, historical processes such as climatic changes during the Pleistocene have been linked to significant changes in demographic history and driving population substructure in marine species. As it is not always easy to discern the exact role of contemporary and historical processes in shaping populations, various approaches have been used, including comparing patterns of differentiation of molecular markers with differing mutation rates, such as mitochondrial DNA (mtDNA) sequence data and nuclear microsatellite loci (Portnoy *et al.* 2010). The matrilineal transmitted, noncoding, mitochondrial control region (mtCR) is considered a selectively neutral marker that lacks recombination and mutates at a relatively high rate, making it effective for detecting historical divergence among populations (Avise 2004; Wang 2010). On the other hand, microsatellites provide a more contemporary glimpse into the gene flow of species due to their relatively high mutation rates and bi-parental mode of transmission (Avise 2004; Wang 2010).

1.5.1. Phylogeography

Phylogeography is a discipline in which the geographical distribution of the genetic variation of natural populations is studied in a historical context (Avise *et al.* 1987), rendering it very important to conservation biology (Avise 2000; Beheregaray 2008; Teske *et al.* 2011; Garca 2012; Teske *et al.* 2013). The geographic distribution of genealogical lineages within and amongst species gives an indication of the evolution of reproductively isolated populations, the processes underlying the origin and distribution of a species, as well as the level of biodiversity within a species using appropriate genes (mitochondrial or nuclear).

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The phylogeographic patterns observed for elasmobranchs are different from those of bony fish since dispersal is dependent mainly upon the movement ability (vagility) of adults and not larvae. Vagility tends to be highest for larger species (greater than 1.5 m), pelagic species and oceanic dwellers. These patterns of dispersal are not always straight forward since in the marine realm, elasmobranchs are often subjected to a number of oceanographic features. In addition, females and males may exhibit different patterns of dispersal, sometimes complicating interpretation of results (Portnoy *et al.* 2010; Portnoy & Heist 2012). Despite this, molecular data such as from mitochondrial and nuclear genes are currently used to discern phylogeography and biogeographic barriers for elasmobranchs on a global and regional scale (Dudgeon *et al.* 2012).

There are a number of globally recognised biogeographic barriers, such as the Isthmus of Panama barrier, Eastern Pacific barrier, mid-Atlantic barrier, Benguela barrier and the Agulhas barrier (Dudgeon *et al.* 2012). In relation to southern Africa, the Benguela barrier restricts the mixing of Atlantic and Indian Ocean populations due to the cold Benguela current on the western side that runs off the southern tip of Africa. This was for example shown in the globally distributed *Galeorhinus galeus* (Chabot & Allen 2009), *Mustelus mustelus* (Maduna *et al.* 2016) and *Carcharhinus falciformis* (Clarke *et al*. 2015a). However, the Benguela current was shown not to be a barrier to more vagile temperate shark species such as *Carcharhinus brachyurus* (Benavides *et al.* 2011) and *Carcharodon carcharias* (Andreotti *et al.* 2015). On the other hand, the warm Agulhas current that flows southward along the eastern side of the southern African coastline, has been shown to deflect away from the southern tip of South Africa and thus reducing its effect on the coastal biota the further south it flows (Briggs & Bowen 2012; Teske *et al.* 2013). Since most batoids are demersal, very little has been reported on their phylogeographic patterns and the few studies available are all on a regional scale (Plank *et al.* 2010). Most data has been reported for Northern Hemisphere endemics and none thus far for the endemic batoids of southern Africa such as the lesser sandshark. Regionally, the warm Agulhas current that flows southward along the east coast and collides with the Benguela current, causing upwelling also seem to have an effect on gene flow at the southern tip of South Africa.

Both the Benguela and Agulhas currents play a vital role in defining the phylogeographic distribution of marine biota along the South African coastline; a region divided in to three major biogeographic provinces; cool temperate, warm temperate and subtropical (Teske *et al.*

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2011) (**Figure 1.1**). The cool temperate region is located on the west coast of South Africa, stretching from southern Angola in the north to Cape Point in the south, and is dominated by the northward flowing Benguela current which has been shown to facilitate strong unidirectional gene flow in some species (Duncan *et al.* 2006; Von der Heyden *et al.* 2008; Henriques *et al.* 2014). The warm temperate region stretches from the south to south-east coast of South Africa and has been shown to allow gene flow in either direction for some species such as *Caffrogobius caffer* (Neethling *et al.* 2008) and *Carcharodon carcharias* (Andreotti *et al.* 2015). Gene flow in this region is for the most part complex since it is an inter-oceanic transitional zone, however, the region between Cape Point and Cape Agulhas has been identified as the oceanographic transition zone between the cool temperate and warm temperate biota (Teske *et al.* 2007). On the east coast of South Africa is the subtropical region which is dominated by the southward flow of the Agulhas current (Teske *et al.* 2011). Oceanography and life-history characteristics are known to influence the genetic structure of marine species, however the relative role that these factors play in shaping phylogeographic patterns of elasmobranchs within southern Africa remains for the most part unknown (Teske *et al.* 2013). Four major phylogeographic breaks have been identified between the marine biogeographic provinces of South Africa and in most cases these break points coincide with the disjunctions between the provinces. These areas include a disjunction on the south-west coast between Cape Point and Cape Agulhas; a disjunction on the south-east coast between Algoa Bay and Wild Coast; and a disjunction between at the north-east coast found at St. Lucia (Teske *et al.* 2011).

1.5.2. Population connectivity and the stock concept

The definition of "stock" in the fisheries sense refers to a demographically and genetically distinct population or organism (Waples 1998). Stocks can be defined based on fisheries and biological data such as life history traits and migration patterns. The spatial and temporal distribution of individuals within a species could be dependent on biotic (*e.g.* predation, parasitism) and/ or abiotic (*e.g.* temperature, currents) variables (Lomolino *et al.* 2006). It is thus believed that almost all species are made up of discrete clusters of individuals that may or may not be reproductively isolated from each other and as a result could exhibit varying degrees of population connectivity (Avise 2000).

Gene flow is defined as the movement of alleles from one population to another (Slatkin 1985). The marine environment is a dynamic environment with currents and fluctuating

temperatures, which can act as drivers of specific dispersal patterns and hence population structure (Limborg *et al.* 2009). Most obvious is that some species have high dispersal abilities and exhibit relatively high levels of gene flow among distant locations, leading to reduced levels of population differentiation across large spatial scales *e.g.* scalloped hammerhead *Sphyrna lewini*, (Duncan *et al.* 2006; Nance *et al.* 2011) and whale shark *Rhincodon typus* (Vignaud *et al.* 2014). Other species have lower dispersal abilities and exhibit relatively low rates of gene flow *e.g.* spinner shark *Carcharhinus brevipinna* (Geraghty *et al.* 2013), banded guitarfish *Zapteryx exasperate* (Castillo-Páez *et al.* 2014) and brown smoothhound *Mustelus henlei* (Chabot *et al.* 2015). Despite high dispersal, several studies have shown different levels of population genetic subdivision over large and smaller spatial scales (*e.g.* Dudgeon *et al.* 2009, Ovenden *et al.* 2009; Geraghty *et al.* 2013; Hernández *et al.* 2015; Spaet *et al.* 2015). Also, certain life history traits such as natal philopatry of females to nursery areas, can lead to strong population differentiation even in highly migratory species *e.g.* blacktip shark *Carcharhinus limbatus* (Keeney *et al.* 2005) and lemon shark *Negaprion brevirostri* (Schultz *et al.* 2008; Ashe *et al.* 2015).

Actual migration can be measured using field techniques, whereby species' movements are physically tracked while gene flow is measured by indirect means through applying mathematical models to genetic data. Direct observation of movement however does not necessarily imply contribution of alleles via breeding and therefore actual gene flow. Direct observations only record the extent of movement over short periods of time and can't infer historical dispersal or population genetic structure (Souza *et al.* 2002). On the other hand, indirect measures of gene flow are based on genetic estimates and can elucidate population structure over varying scales and evolutionary time frames. These molecular based techniques can also discern possible barriers that might inhibit the dispersal of a species that are difficult to access with direct observation (Souza *et al.* 2002). Population genetic structure can be inferred by various types of molecular markers and can address different questions regarding the dynamics of populations. It does not only allow inferences regarding the distribution of genetic diversity among individuals and populations but it can also help describe the evolutionary factors (*e.g.* selection and migration) influencing these patterns of genetic variation (Hartl & Clark 1997).

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1.5.3. Mitochondrial DNA (mtDNA) sequence data

Mitochondrial DNA (mtDNA) are maternally inherited and most genes are fairly easy to sequence across species because of highly conserved regions (Nielsen *et al.* 2009). Mitochondrial evolution within elasmobranchs is said to be very slow with estimates being six to eight times lower than that of mammals (Dudgeon *et al.* 2012). However, this rate of evolution might not hold for all elasmobranch species or across all mtDNA genes. One of the most commonly used mtDNA regions used in assessing population structure is the mitochondrial control region (mtCR). It is non-coding with differing levels of variability across the region; with the central domain most conserved and the flanking regions more variable. Several studies have successfully discerned levels of divergence and population structure within elasmobranch species using the mtCR (Benavides *et al.* 2011; Castillo-Páez *et al.* 2014; Portnoy *et al.* 2014; Chabot *et al.* 2015; Hernández *et al.* 2015)

Other protein-coding genes such as cytochrome b (*cytb*), NADH-dehydrogenase subunit 2 (*ND2*) (Verssimo *et al.* 2012; Castillo-Páez *et al.* 2014) and NADH-dehydrogenase subunit 4 (*ND4*) (Boomer *et al.* 2012; Geraghty *et al.* 2013) have been used in discerning population structure and genetic variation. Some of these genes have also been used for species-identification and barcoding of elasmobranchs including the *ND2* gene (Farrell *et al.* 2009; Naylor *et al.* 2012) and the cytochrome oxidase 1 (*CO1*) gene (Geraghty *et al.* 2013). Despite the high variability of the control region, a number of studies have also found results to the contrary showing more variation in some of the protein-coding genes. For instance, *ND4* was reported to be more variable than the mtCR in the gummy shark *Mustelus antarcticus* (Boomer *et al.* 2012) while the *ND2* gene was also found to be more variable than the mtCR in the banded guitarfish *Zapteryx exasperate* (Castillo-Páez *et al.* 2014). For this reason, the utility of protein-coding mitochondrial genes in discerning intraspecific and interspecific relationships in elasmobranchs has steadily increased (Naylor *et al.* 2005, 2012; Farrell *et al.* 2009; Ashe *et al.* 2015; Maduna *et al.* 2016).

1.5.4. Microsatellite markers

Microsatellites are simple sequence repeats (SSRs) or short tandem repeats (STRs) consisting of one to six nucleotides repeats and remain the most popular marker type in population genetics and ecology studies. These repeat motifs are co-dominant, distributed all over the genome and the length of the repeat motifs varies among taxa and sometimes even among individuals (Guichoux *et al.* 2011). Microsatellites are believed to evolve by replication

slippage (Schlötterer & Tautz 1992) and a few mutational models have been proposed to describe the underlying mechanisms of microsatellite evolution. These include the infinite allele model (IAM), the stepwise mutational model (SMM), and the two-phase model (TPM) (Kimura & Crow 1964; Kimura & Ohta 1978; Di Rienzo *et al.* 1994).

The *de novo* development of microsatellite loci primers requires the availability of sequence data which is essentially searched for repeat motifs, and the sequences flanking the repeat motifs are used to design PCR primers. Microsatellites have been developed for several elasmobranch species (Keeney & Heist 2003; Chabot & Nigenda 2011; Fitzpatrick *et al.* 2011; Giresi *et al.* 2012; Chabot 2012). The traditional screening of genomic libraries to identify microsatellites is being replaced by the faster next-generation sequencing (NGS) technologies which generate enormous amounts of sequence data in a single run (Chabot & Nigenda 2011; Guichoux *et al.* 2011; Kang *et al.* 2012; Blower *et al.* 2015). However, most NGS platforms are still very expensive warranting the need for alternative means of microsatellite development. The use of cross-species microsatellite markers is the current most cost-effective alternative means of developing microsatellites for elasmobranch genetic studies (Barbará *et al.* 2007; Maduna *et al.* 2014). Since elasmobranchs are highly conserved species, there has been successful transfer of cross-species microsatellites across species of the same family (Chabot & Nigenda 2011) and even across genera (Keeney & Heist 2003; Fitzpatrick *et al.* 2011; Maduna *et al.* 2014; Pirog *et al.* 2014).

To detect the variation of the microsatellite allele length, the microsatellite-containing region is amplified by PCR and electrophoresis used to determine the size of the PCR amplicons. With the implementation of fluorescent dye labelling, several microsatellite loci can be pool-plexed and analysed in combination increasing the data output and lowering costs involved (Guichoux *et al.* 2011). Microsatellites markers have been widely applied from parentage analysis studies (Testerman *et al.* 2012; Chabot & Haggin 2014; Hernández *et al.* 2014), to assessing genetic diversity and population connectivity (Plank *et al.* 2010; Verssimo *et al.* 2012; Portnoy *et al.* 2014) and in the estimation of effective population size (Plank *et al.* 2010; Portnoy *et al.* 2014).

1.6. Study species (*Galeorhinus galeus* Linnaeus 1758)

The tope shark species (*Galeorhinus galeus* Linnaeus 1758) first became commercially valuable between the 1930's and 1940's due to its vitamin A rich liver and an estimated landing of 2000 tonnes was made in California alone (Ripley 1946). As a result of the heavy exploitation of this species, the California tope shark fisheries industry crashed (Stevens 2000). Recently, catches of *G. galeus* are on the increase in California and most of the Northern Hemisphere. However, the same cannot be said for the Southern Hemisphere where catches and landings are fast declining (Walker *et al.* 2006). Between 1994 and 2004, a total of 44000 tonnes of *G. galeus* shark were reported by FAO (FAO 2008) and the species is currently listed as vulnerable on the IUCN Red list (Walker *et al.* 2006).

Galeorhinus galeus exhibits *K*-selected traits found in most elasmobranchs making it vulnerable to fishing pressures (Walker *et al.* 2006). The species is long-lived (25 to 60 years), grows to a maximum length that varies for regions; ranging from 200 cm in the Mediterranean (Capape & Mellinger 1988) to a 155 cm in the southwest Atlantic (Peres & Vooren 1991). The species has a maximum length of 195 cm with males being on average 10 cm smaller than females. Similar differences are also noted for the size at sexual maturity with males at 107 to 117 cm and females at 118 to 128 cm (Peres & Vooren 1991; McCord 2005). The reproduction is aplacental viviparous which is characterised by a long ovarian and gestation period estimated to be three years (Peres & Vooren 1991; Walker 2005); with individual reports of every second year in Australia and every third year in Brazil (Capape & Mellinger 1988; Peres & Vooren 1991). Because pregnant females give birth approximately every three years, the tope shark populations are most vulnerable at three different reproductive stages:

(i) with one-third of the population having resting oviducts (post-parturition)

(ii) one-third of the population having large mature follicles (uteri ready for ovulation)

(iii) and the final one-third of the population having full-term embryos in the oviducts

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This has biological significance as only one-third of the mature females are able to support the annual recruitment of newborns (Peres & Vooren 1991; Punt *et al*. 2005). *Galeorhinus galeus* is a cosmopolitan hound shark that belongs to the family Triakidae. This species occurs in areas with anti-tropical temperatures and may be described as hemipelagic (occurring up to depths greater than 400 m) (Riede 2004). It inhabits the continental shelf from nearshore to 800 m in depth, and also occurs in the pelagic zone and offshore (Walker 1999; Riede 2004). Across the Southern Hemisphere, landings of *G. galeus* are reported for the Atlantic Ocean in Argentina, Brazil, Uruguay and off the coasts of South Africa and Namibia, parts of the southern Indian Ocean in Australia and the South Pacific Ocean in Chile, New Zealand and Peru. Commercial fisheries exist for most parts of the species' distribution range in the Southern Hemisphere with significant landings occurring in Australia, Argentina, Chile, New Zealand and South Africa (McCord 2005; Walker *et al.* 2006).

In Australia, *G. galeus* is commonly referred as school shark and is found in the temperate coastal waters of southern Australia including Moreton Bay, Queensland, Perth, offshore waters of Lord Howe Island and Tasmania. The inshore areas of Tasmania, parts of the south-east and Victoria have been pinpointed as hotspots for birthing and nursery sites making the species vulnerable to fishing pressures (Stevens 2005; Walker *et al.* 2006). The school shark fisheries in Australia began in the late1920's and at that time large and sexually-mature tope sharks were the main targets in the demersal longline fisheries. By the 1960's, there was an increase in the demand for tope shark leading to the implementation of gillnet fishing in this industry and ultimately the mortality of sexually-mature animals exponentially increased (Stevens 2005). As a result, the fishing impacts were detected in the drastic decline of adult-catch and were also reflected in the increased catch of juvenile animals. But it was not until concerns were raised over the high levels of mercury found in tope sharks that the fishing of large tope sharks was banned. This then led to a shift in fishing efforts towards the gummy shark (*Mustelus antarticus*) until the tope shark ban was lifted again in the 1980's. Despite having lifted the ban on tope shark fishing, this species is mainly landed as bycatch in the gummy shark fisheries in Australia (Stevens 2005; McLoughlin 2007).

Galeorhinus galeus is currently listed as 'conservation dependant" in Australia and stringent management plans have been put in place (*e.g.* reduction in total allowable catch, TAC, area closures, fishing gear restrictions, minimum legal-size limits) to help recover the population

to a given target biomass within a biologically reasonable timeframe but there has been little evidence of stock recovery (Francis 2010).

Galeorhinus galeus is also distributed in the SW Atlantic Ocean where the population is believed to be panmictic and migrating seasonally between wintering grounds off south Brazil and Uruguay, as well as summer grounds off Argentina where pupping and nursery areas (*e.g.*, Bahía Blanca and El Rincón) are believed to be situated (Chiaramonte 1998). Commercial fisheries for this species in Argentina operate inshore at depths of 18 to 57 m in the same time period when the sexually-mature males and females who are to copulate, and the gravid females (during the final phase of gestation), are migrating in to Argentina from south Brazil. This makes the species susceptible to recruitment overfishing especially in the known reproduction and nursery hotspots (Chiaramonte 1998; Elías *et al.* 2005). Drastic declines in catches of *G. galeus* have been noted in Argentina and this species is currently classified as "critically endangered" (Walker *et al.* 2006). Despite a drastic drop in catch per unit effort (CPUE) by 80%, there are no management measures in place for this species but some recommendations have been made regarding its fishery. These include enforcing artisanal and small-scale fishery, reducing the catch effort and the number and size of hooks-per-boat by permit, implementing an effective regional species conservation plan for the Southwest Atlantic population including fisheries authorities from Brazil, Uruguay and Argentina, and increasing research on species biology and fishing practises (Cuevas 2014; Elías *et al.* 2005).

Within South Africa, *G. galeus* is commonly referred to as soupfin and is distributed from the western to the southern coast of South Africa, across the South Atlantic Ocean and in some parts of the Eastern Cape across the Indian Ocean. The species is mostly found in the cool temperate and warm temperate bioregions of South Africa (**Figure 1.1**). Principal landing sites for this species have been reported at Cape Town, Hout Bay, Gansbaai and occasionally between Mossel Bay and East London. Soupfin is caught at depths exceeding 400 m, but is most frequently caught between 55 to 150 m (McCord 2005). It is thought that females give birth in lagoons and estuaries along the west and south coast of South Africa and although no specific nursery areas have been conclusively identified, shallow embayment areas such as in Struisbaai, St. Helena, Walker Bay, False Bay and Gansbaai are believed to be nursery hotspots (McCord 2005; Walker *et al.* 2006).

Galeorhinus galeus ranks as the second most commercially important shark species in South Africa with a landed dressed weight of 401-500 tonnes reported for the species in 2013; far outranking the common smoothhound (*Mustelus mustelus*) and copper shark (*Carcharhinus brachyurus*), respectively (NPOA-sharks 2013). The exploitation of tope shark in South Africa has been in existence since the 1930s (Kroese & Sauer 1998) and today this species is a principal target of direct fisheries as well as incidental bycatch in the hake long-line, demersal longline and trawling fisheries (NPOA-sharks 2012). Since there is a low demand for shark fillets and fins in South Africa, most of the harvested soupfin are exported to Australia where they are consumed in the fish and chips industry (McCord 2005; Silva & Bürgener 2007).

Soupfin in South Africa is not just threatened by over-exploitation; with recent stock assessments indicating that the species is in a depleted status (NPOA-sharks 2012; DAFF 2012; DEAT 2015), but this species also lacks species-specific data, non-cohesive fishing regulations and more importantly, regional population structure assessments. At present, there are no conservation regulations in place for South Africa's soupfin despite the IUCN Red list status of vulnerable but proposed policies by McCord (2005) suggest that multi-species permits be replaced with single-species permits to aid track the exploitation of the species. However, for single-species permits to be effective, proper guidelines are required to correctly identify the species.

1.7. Study species (*Carcharhinus brachyurus* Günther 1870)

The copper shark or bronze whaler (*Carcharhinus brachyurus* Günther, 1870) is a large apex predator that belongs to the family Carcharhinidae (requiem sharks) (Clarke *et al.* 2006). The copper shark has a cosmopolitan distribution using both coastal and offshore areas (Compagno *et al.* 2005) and has major population centres in the Southern Hemisphere. Distribution zones for *C. brachyurus* in the Southern Hemisphere include the Southwest

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Atlantic from southern Brazil to northern Argentina, the SE Atlantic from Namibia to the southwest coast of South Africa, the Indian Ocean from the southeast coast of South Africa to Australia, and the western Pacific from Australia to New Zealand (Garrick 1982). In contrast to other members of the genus *Carcharhinus*, *C. brachyurus* inhabits temperate waters and tends to give birth and have nursery areas at the highest latitudes of its distribution range (Lucifora *et al.* 2005).

Carcharhinus brachyurus is generally large in size and has been reported to reach a maximum TL of 3.5 m (Benavides *et al.* 2011). An assessment done by Walter & Ebert (1991) showed that *C. brachyurus* grows slower than other *Carcharhinus* species and that males grow larger (maximum TL 2.94 m) than females (TL 2.88 m), and live up to a maximum age of 30 and 25 years, respectively in South African waters. The species exhibits viviparous reproduction, has a low fecundity and is considered to be very unproductive (Lucifora *et al.* 2005), with sexual maturity attained at 13-19 years for males, and 19-20 years for females (Walter & Ebert 1991).

Carcharhinus brachyurus tends to dwell in temperate habitats and nursery grounds that are in very close proximity to human populations (*e.g.* Eastern cape in South Africa) making them easy targets for coastal fisheries and habitat degradation arising from coastal development (Smale 1991). Added to this are their life history traits which make them highly vulnerable to fishing pressures (Cortés 2000; Musick *et al.* 2000; Camhi 2009). In a global assessment of *C. brachyurus* for the IUCN, the species is listed as globally "near threatened", and "least concern" in Australia, New Zealand and southern Africa. Commercial catches in Australia appear to be stable, while New Zealand saw an almost four-fold increase in catches followed by a decrease in the last decade (Cavanagh *et al.* 2003). *Carcharhinus brachyurus* in South Africa is taken in commercial fisheries, recreational fisheries and protective beach meshing programs (Dudley & Simpfendorfer 2006) and as recently as 2011, the species was listed as near threatened (DEAT 2015). However population declines for many regions are likely to go unnoticed because catches are grouped under the Carcharhinid genus and not much species-specific data exists for this species (Duffy & Gordon 2003; NPOA-sharks 2012; 2013).

Carcharhinus brachyurus is the fifth most commercially important shark species in South Africa and is mainly targeted as direct catch in the recreational linefishery which is usually inshore to 200 m ranging from the Western Cape to Kwazulu-Natal (**Figure 1.1**). The species is also targeted in the bather protection nets in Kwazulu-Natal and as bycatch in the prawn

trawl on the east coast of South Africa. Harvests are mainly exported to Australia where the demand for shark fins and fillets is high (Silva & Bürgener 2007). An estimated dressed weight of 201-300 tonnes of *C. brachyurus* was harvested in 2010 in South Africa but it's likely that this number does not reflect the animals that were misidentified; as the reports for *Carcharhinus* species are usually combined (NPOA-shark 2012; 2013). There are currently no management regulations in place for *C. brachyurus* in South Africa as species-specific data is still lacking.

1.8.   Study species (*Rhinobatos annulatus* Müller & Henle 1841)
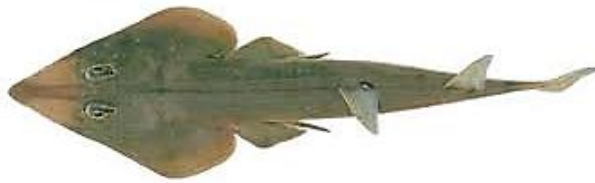
The lesser sandshark (*Rhinobatos annulatus* Müller & Henle 1841), also known as the lesser guitarfish belongs to the family Rhinobatidae. This is one of the most abundant endemic guitarfishes off the southern African coast and is listed as of "least concern" on the IUCN Red list. Despite this, the species lacks population trend data and is considered heavily data deficient (Burgess *et al.* 2006).

Rhinobatos annulatus is a southern African endemic batoid distributed from southern Angola, through Namibia to KwaZulu-Natal in South Africa. This species is found in inshore waters from the shoreline to 50 to 100 m depth, and also inhabits estuaries, the surf zone and enclosed bays. This species is relatively small in size with maximum sizes recorded at 140 cm total length. This species is termed as not purely *K*-selected because it reaches sexual maturity relatively quicker than most elasmobranchs (50% maturity at three years), has a short lifespan (maximum 7 years) and an annual fecundity giving birth to 2 to 10 young. This is of biological significance as this species is less vulnerable to fishing pressure (Rossouw 1987; Rossouw 2014).

Despite the paucity of data on *R. annulatus* in southern Africa, it is believed that there are two possible stocks in existence, one in the Western Cape area and another northward into southern Angolan waters (Compagno 1991). There have only been two observable population trends reported for this species and most of these reports were recorded for recreational angling in South Africa alone (Govender & Pradervand 2003; Vaughan & Chisholm 2010),

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with no records for Namibia and Angola. Reports from shore angling catch in the Eastern Cape showed that of a total 34 species caught, *R. annulatus* was the most commonly caught proving the likely threats faced by this species (Govender & Pradervand 2003).

1.9. Study Aims

Two previous studies that assessed the population structure of the commercially important shark species *G. galeus* and *C. brachyurus*, found that global samples of *G. galeus* showed distinct mtCR haplotypes that were largely restricted to their collecting locality (Chabot & Allen 2009) and three distinct populations (Africa, Oceania, Peru) were detected for samples of *C. brachyurus* in the Southern Hemisphere (Benavides *et al.* 2011). A later study by Hernández *et al.* (2015) assessed the population structure of *G. galeus* in the Southern Hemisphere using both nDNA and mtCR markers and this study found two populations including a large and genetically homogeneous population in Oceania (Australia and New Zealand) and Chile. In this case, not all the phylogeographic barriers in the Southern Hemisphere could be investigated. Therefore, the goal in this study is to investigate genetic variation across the entire Southern Hemisphere using the *ND2* gene and nuclear microsatellite markers. Samples from Argentina, Australia, New Zealand, Chile and from across the entire distribution range within South Africa were included. Secondly, the project aims to assess the genetic connectivity of *C. brachyurus* using the *ND4* gene and nuclear microsatellite markers with a further focus on the South African fisheries. Three levels of genetic structure will be tested for the commercial species:

(i) single panmictic population

(ii) isolation by distance (IBD) and

(iii) discrete populations

The first level involves genetic flow that is so extensive that the entire population could be classified as a single population or stock. The second level, IBD, involves genetic variation that is more or less continuous over distance. The gene flow among nearby individuals is more than between those separated by larger distances; resulting in genetic differentiation correlated to geographic distance. The last level is characterised by very little or no migration between populations. Two hypotheses are therefore tested:

(i) Null hypothesis: a single panmictic population

(ii) Alternative hypothesis: separate populations possibly due to IBD and/or barriers to dispersal

Lastly, the scarce literature on South Africa's endemic elasmobranchs prompts the need for genetic resources that can be incorporated in the regional fisheries management. As such, this research will embark on a pilot study aimed at assessing genetic diversity in the endemic *R. annulatus* across its distribution range in South Africa. The overall project aims are summarised in **Figure 1.2**.

Chapter 2
- Optimising microsatellite marker panels by cross-species amplification
- Application of marker panels in fisheries composition and gene flow assessment

Chapter 3
- Inferring levels of contemporary gene flow and phylogeography for *Galeorhinus galeus* across the Southern Hemisphere
- Assessing levels of population differentiation and demographic history of *Galeorhinus galeus* locally along the South African coastline

Chapter 4
- Evaluating genetic diversity and population genetic structure of *Carcharhinus brachyurus* along the southern and eastern coast of South Africa
- Inferring preliminary historical genetic signatures for *Carcharhinus brachyurus*

Chapter 5
- Assessing levels of genetic diversity and genetic differentiation of *Rhinobatos annulatus* across four sampling sites across the Agulhas bioregion of South Africa

Figure 1.2. A schematic summary of the study aims in the four experimental chapters.

Chapter 2:   **Optimizing microsatellite multiplex panels and their utility in South Africa's commercially exploited and endemic elasmobranchs**

Abstract

There is currently very limited population genetics data of elasmobranch species in South Africa. This is partly due to the lack of available genetic resources, species identification issues and challenging sampling. In this chapter, the optimisation of nuclear DNA markers through cross-species amplification of microsatellites previously developed for the Triakid species, *Galeorhinus galeus*, *Mustelus canis* and *Mustelus henlei*, are reported as an alternative approach to *de novo* marker development. Three microsatellite panels comprising of six primer pairs per panel were successfully optimised for cross-species utility in the commercially important and endemic elasmobranchs tope shark *G. galeus*, copper shark *C. brachyurus* and lesser sandshark *R. annulatus*, respectively. All 22 microsatellites were successfully amplified in *G. galeus* while only 77.2% of the cross-species microsatellites could be optimised for *C. brachyurus* and *R. annulatus*. Seventeen loci were polymorphic for *C. brachyurus* (N_A = 2 to 5, PIC = 50.3%) and for *R. annulatus* (N_A = 2 to 10, PIC = 66.3%). The microsatellite panels were then used in a catch composition case study where it proved to be useful in determining misidentification of fin clip samples obtained from hound sharks caught by commercial fishers. To further investigate the utility of these marker panels, a subset of 12 cross-species loci were used to assess the genetic diversity and compare population structuring of *M. mustelus* and *G. galeus* along the south-west coast of South Africa.

Keywords: cross-species amplification, gene flow, genetic diversity, microsatellites

2.1. Introduction

Microsatellites or simple sequence repeats (SSRs) are short repetitive DNA motifs of about 2 to 6 base pairs (bp) (Ashley & Dow 1994; O'Connell & Wright 1997) that exhibit a high mutation rate of copy number due to slipped-strand mispairing (Levinson & Gutman 1987). This high level of mutation rate allows detection of intraspecific variation, (O'Connell & Wright 1997, Guichoux *et al.* 2011) making these one of the most variable and popular markers of choice for studies related to intra- and interspecific population genetic analysis (Byrne & Avise 2012; Chabot & Haggin 2014; Hernández *et al.* 2015).

A major drawback is to identify and develop microsatellites specific to a species which can be expensive and time-consuming. In the past, microsatellite development was done either by hybridization selection (Armour *et al.* 1994), or by linker and ligation procedures (Hamilton *et al.* 1999). More recently, next-generation sequencing (NGS) technology has changed the approach to marker development. NGS has made the identification of microsatellite repeats much more affordable and faster since an entire genome can be searched for repeats. Primers can also be designed directly using various bioinformatics pipelines (Guichoux *et al.* 2011). Microsatellite development in elasmobranchs has been mainly focused on charismatic or commercially exploited species leaving a void for endemic and less important species. Contrary to other marine fish, microsatellites have only recently been developed for commercially important and other elasmobranch species such as dusky smoothhound *Mustelus canis* (Giresi *et al.* 2012), brown smoothhound *M. henlei* (Chabot 2012), tope shark *Galeorhinus galeus* (Chabot & Nigenda 2011), mottled skate *Raja pulchra* (Kang *et al.* 2012), bull shark *Carcharhinus leucas* (Pirog *et al.* 2014) and dusky shark *Carcharhinus obscurus* (Blower *et al.* 2015).

Another approach increasingly used by molecular ecologists, is cross-amplification or transfer of available microsatellites to study species providing a more cost-effective alternative to NGS. Such cross-amplified markers allow for comparisons among multiple co-existing species for patterns of genetic diversity and population structuring (Barbará *et al.* 2007). However, the success of the transferability of these loci hinges on the presence of conserved microsatellite flanking sequences, the evolutionary distance between source and recipient species and the genome size of the study species (Barbará *et al.* 2007). Since elasmobranchs are highly conserved species, there has been successful transfer of microsatellites across species of the same genera and even across families (Keeney & Heist

2003; Chabot & Nigenda 2011; Fitzpatrick *et al.* 2011; Kang *et al.* 2012; Maduna *et al.* 2014; Pirog *et al.* 2014).

Despite achieving successful cross-species amplification, utility of these markers for downstream applications comes with additional challenges relating to optimisations and error checks. Low levels of polymorphism, presence of null alleles and ascertainment bias have to be taken in to account when analysing data generated from cross-species markers. For instance, Kang *et al.* (2012) amplified 11 microsatellites developed for *Raja pulchra* across ten species from the four families Rajidae, Dasyatididae, Scyliorhinidae and Triakidae. Successful cross-species amplification was highest for the Rajid species and lowest for the Dasyatididae, Scyliorhinidae and Triakidae families. Even more so, the levels of polymorphism declined significantly for the families most divergent from the source family (Rajidae) with loci being mostly monomorphic in the evolutionary distant families. In another study by Maduna *et al.* (2014), a total of 35 microsatellite loci developed for *Mustelus canis*, *M. henlei*, *G. galeus*, *R. pulchra,* small-spotted catshark *S. canicula* were tested for cross-species amplification across five elasmobranch families. As expected, the amplification success rate and the level of polymorphism was highest for Triakid species and lowest for the Sphyrinidae and Rajidae families since most of the loci were developed for Triakidae species. This confirmed that the rate of success and level of polymorphism depends on the evolutionary distance between source and recipient species.

This chapter will report on the amplification and optimisation of cross-species microsatellites previously developed for the Triakid species *G. galeus*, *M. canis* and *M. henlei*, in three elasmobranch species occurring in South African waters: *G. galeus, C. brachyurus, M. mustelus*, and *R. annulatus*. Since the microsatellites to be tested are developed in the Triakidae family, the level of successful cross-species amplification within the study species in the Triakidae and Carcharhinidae families are expected to be high while cross-species amplification rate is expected to be lower for *R. annulatus* (Maduna *et al.* 2014). With that in mind, this study will also aim to test these loci for their utility in genetic diversity and population structure assessment and possibly for assisting in species identification in commercial catches.

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2.2. Materials and Methods

2.2.1   *Sampling and DNA extraction*

Fin clips were taken from *G. galeus, C. brachyurus, M. mustelus* and *R. annulatus* during opportunistic sampling efforts coordinated with recreational angling seasons and collected by observers and fisheries biologists at the Department of Fisheries and Forestry (DAFF) as well as through the South African Shark Conservancy (SASC). In total, 14 sampling locations between the south-west and eastern coast of South Africa were included: Langebaan, Robben Island, Kalk Bay, False Bay, Strandfontein, Gordon's Bay, Kleinmond, Agulhas Bank, Struisbaai, Die Plaat, De Mond, Mossel Bay, Jeffrey's Bay and Port Elizabeth (**Figure 2.1**). Sampling of the commercially important species in these regions was somewhat inadequate due to seasonal sightings; with data such as animal length and gender not provided for most of the samples. Also, *G. galeus* samples supplied by commercial fisheries were either inappropriately labelled or misidentified and provided the opportunity to examine the utility of the microsatellites for species identification. Additionally, *G. galeus* samples from Chile, Argentina, Australia and New Zealand were also included. Overall, a total of 185 *G. galeus*, 105 *M. mustelus*, 94 *C. brachyurus* and 83 *R. annulatus* samples were included for analyses in this chapter.
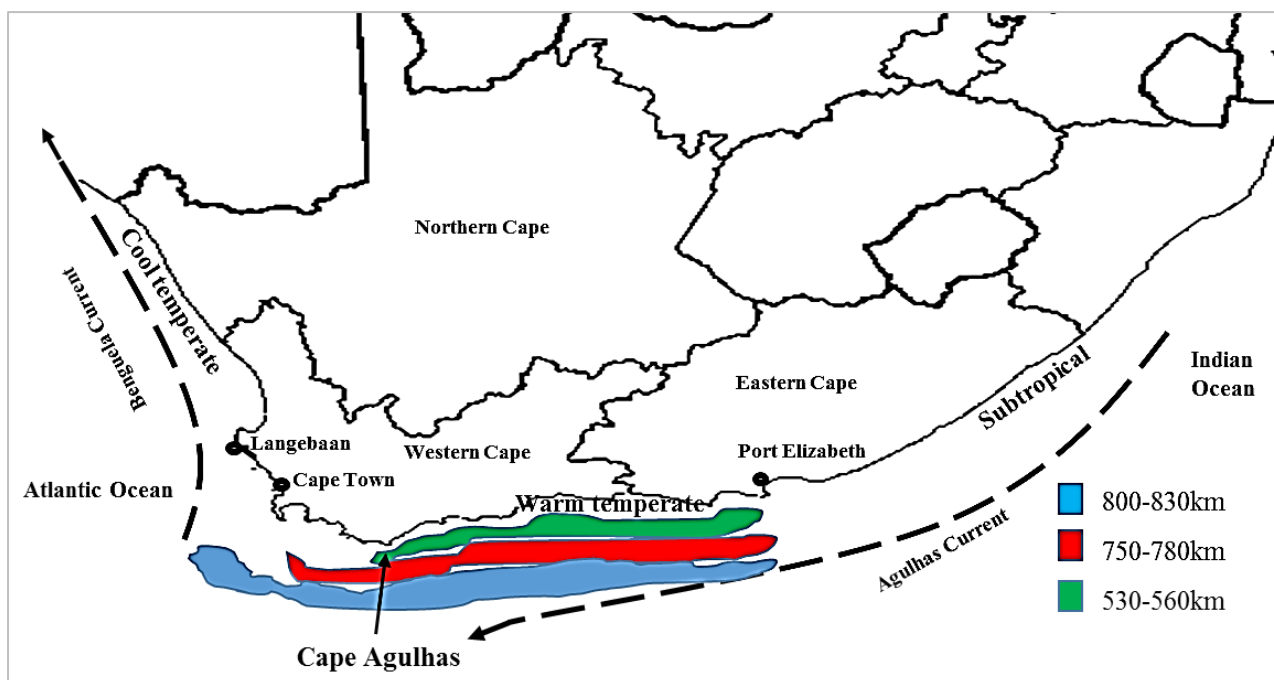
Figure 2.1. Map of South Africa indicating major biogeographic regions, oceanic currents, the Atlantic/Indian boundary at Cape Agulhas and the western and eastern most sampling sites, Langebaan and Port Elizabeth. The sampling ranges of *Galeorhinus galeus*, *Carcharhinus brachyurus* and *Rhinobatos annulatus* are shown in blue, red and green respectively

Genomic DNA was extracted from approximately 1 g of each fin clip using a modified protocol of the CTAB extraction method described by Saghai-Maroof *et al.* (1984). In brief, tissue was added to a 150 µl of extraction buffer [comprising of 0.2% (w/v) beta-mercaptoethanol (β-ME), 2% (v/v) CTAB (5M NaCl, 0.5M EDTA (pH 8) and 1M Tris-Cl (pH 8) buffer] and 200mg proteinase K were added. The solutions were incubated overnight in a water bath set at 37ºC. Subsequent to the incubation, 150 µl chloroform: isoamylic alcohol (C:I) (24:1) was added followed by centrifugation at top speed for 5 minutes. The supernatant was then transferred to a clean microcentrifuge tube to which 150 µl of C:I (24:1) was added followed by centrifugation at top speed for 5 minutes. The supernatant was again transferred to a clean tube and two volumes of ice cold 100% ethanol were added. All tubes were then incubated overnight at -20ºC for precipitation to occur. After incubation, the tubes were centrifuged at top speed for 20 minutes at 4ºC and the supernatant carefully discarded. DNA pellets were then washed with 200 µl of ice cold 70% ethanol and centrifuged at 4ºC for 10 minutes. A further discarding of the supernatant was done and the pellets were air dried and resuspended in 30-50 µl MiliQ water. DNA quantity and quality was determined

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using a NanoDrop® (ND-1000) Spectrophotometer. DNA samples were diluted to working stocks of 50ng/µl and stored at -20ºC or 4ºC until needed.

2.2.2 Microsatellite PCR cross-amplification

A total of 22 microsatellite loci sourced from literature were tested for cross-species amplification in three commercially exploited shark species *Galeorhinus galeus, Mustelus mustelus* and *Carcharhinus brachyurus*, as well as the endemic lesser sandshark *Rhinobatos annulatus*. Six of the microsatellite loci were sourced from *M. henlei* (Byrne & Avise 2012; Chabot 2012), 13 from *M. canis* (Giresi *et al.* 2012) and ten from *G. galeus* (Chabot & Nigenda 2011). Additionally, two microsatellite loci developed for *R. pulchra* (*Rp16* and *Rp35*) (Kang *et al.* 2012) were tested for cross-species amplification in *R. annulatus*. These microsatellite loci were selected based on their ability to detect four or more polymorphic alleles and their cross-species transferability within Carcharhiniformes and Rajiformes. Initially, a subset of individuals from the four elasmobranch species sampled at different locations was selected to test for cross-species amplification following PCR conditions described by Chabot & Nigenda (2011). A 10 µl touchdown single locus PCR amplification performed in an Applied Biosystems (ABI) (Life Technologies, California, USA) thermal cycler version 2.09 contained the following: 10 ng template DNA, 1X Go Taq buffer, 200 µM dNTPs, 0.2 µM of each primer, 1.5 mM $MgCl_2$, 0.1 U GoTaq DNA polymerase (Anatech, South Africa).

The PCR profile consisted of 15 minutes of initial denaturation at $95^{o}C$, followed by 25 cycles of denaturation ($94^{o}C$ for 30 seconds), annealing ($59^{o}C$ for 90 seconds), extension ($72^{o}C$ for 60 seconds) and another 20 cycles of denaturation ($94^{o}C$ for 30 seconds), annealing ($53^{o}C$ for 90 seconds), extension ($72^{o}C$ for 60 seconds), and a final extension of $60^{o}C$ for 30 minutes. The cycle was completed with a holding phase at $4^{o}C$. PCR amplicons were separated on a 2% agarose gel for 1 hour at 100V immersed in 1X Tris-Borate-Ethylenediaminetetraacetic acid (TBE) buffer and stained in ethidium bromide and visualised under UV-light using a Syngene 'Gene Genius' gel documentation system.

2.2.3 Multiplex optimisation and marker efficiency

Since none of the microsatellite loci from the Rajidae family showed cross-species amplification in *R. annulatus*, only the microsatellite loci from the Triakidae family were used in further analyses. A subset of loci were selected for each respective species and

grouped into multiplex panels containing a maximum of six loci. All multiplex panels were compiled using Multiplex Manager 1.2 (Holleley & Geerts 2011). The primers of each microsatellite locus were labelled with either of the fluorescent dyes PET, FAM, NED and VIC. This resulted in four multiplex panels (22 microsatellites in total) for *G. galeus* and 17 loci in three multiplex panels for both *C. brachyurus* and *R. annulatus*. These panels were tested for their utility and polymorphism content using the PCR conditions recommended for the Qiagen Multiplex kit-PCR conditions. The following were added to a 10µl reaction: 50 ng template DNA, 1X Qiagen Mastermix, 0.2 µM unlabelled primer and 0.2 µM of labelled primer (IDT). PCR amplifications were carried out following the cycle conditions as recommended in the Qiagen manual: Initial denaturation at 95°C for 15 minute, preceded by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C or 59°C for 90 seconds, extension at 72°C for 60 seconds and final extension at 60°C for 30 minutes. PCR amplification was confirmed with agarose gel electrophoresis and diluted in a 1:1 ratio with miliQ water and sent to the Central Analytical Facility (CAF) of Stellenbosch University for capillary electrophoresis on an ABI3730 Genetic Analyzer. Allele sizes were determined using the LIZ$^{®}$ 600 internal size standard and GeneMapper$^{®}$ 4.0 (Applied Biosystems).

Preliminary data analyses entailed inspecting the markers for the presence of null alleles, allele size shifts and scoring errors due to stuttering using MICRO-CHECKER 2.2.3 (van Oosterhout *et al.* 2004). Departures from Hardy Weinberg equilibrium (HWE) were examined across loci for each species and populations using a Fisher's exact test (500 batches, 10 000 iterations) implemented in GENEPOP 4.2 (Rousset 2008). Sequential Bonferroni corrections were performed to correct for multiple tests (Rice 1989). Tests for linkage disequilibrium (LD) between pairs of loci were carried out using FSTAT 2.9.3.2 (Goudet 2002). Genetic diversity estimates such as the number of alleles per locus (N_A), observed (H_O), and unbiased expected (uH_E) heterozygosities were calculated using GENALEX 6.5 (Peakall & Smouse 2012).

2.2.4   *Application of microsatellites for species identification in addition to barcoding*

Primers that were previously designed for the NADH dehydrogenase subunit 2 (*ND2*) gene in order to distinguish between *M. mustelus* and *G. galeus* from the Northeast Atlantic (Farrell *et al.* 2009), were used to amplify 57 samples obtained from a commercial catch. More specifically, DNA was amplified using a multiplex-PCR reaction containing one universal forward primer MUNDF 3'-TGTGAATAGGCCTCGAAATCA-5' and two target-species

reverse primers MMND2R 3'-AATGCCAAGGAATAGTAGGAGGT-5' (specific for *M. mustelus*) and GGND2R 3'-TCCTAAGGAAAGGAGAGTCAGTAA-5' (specific for *G. galeus*). Each of the target-specific primer sets amplified a fragment of specific size (671 bp for *G. galeus* and 392 bp for *M. mustelus*). To validate the specificity of these primers, four taxonomically identified *M. mustelus* and *G. galeus* individuals were used as positive controls. Multiplex-PCR amplification were as described by Farrell *et al.* (2009) and consisted of 100 ng template DNA, 1X GoTaq buffer, 200 µM dNTPs, 0.4 µM of each primer, 2 mM MgCl$_2$ and 0.5 U GoTaq polymerase. PCR cycling conditions comprised of an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 minute, 56°C for 1 minute, 72°C for 1 minute and final extension at 72°C for 7 minutes. Based on the amplification of the *ND2* gene and a size standard, 26 samples were identified as *G. galeus*, 20 as *M. mustelus*, four were cryptic (amplified both fragments 392 and 671 bp) and eight showed no amplification (**Figure 2.1**). The 50 samples that showed successful amplification with the *ND2* primers were subsequently genotyped using all four optimised multiplex panels to verify whether these markers were also able to distinguish between the hound shark species. The same PCR conditions were used as described above and also included four known *M. mustelus* and *G. galeus* individuals as positive controls. To visualise the relationships among individual genotypes, a principal component analysis (PCA) plot was generated in GENALEX 6.5 (Peakall & Smouse, 2012) and depicted in two dimensions.

2.2.5   *Application in population genetic analysis of G. galeus and M. mustelus*

Since both *M. mustelus* and *G. galeus* are heavily exploited between the west and south coast of South Africa, a region that features two oceanic currents and the Atlantic/Indian Ocean boundary, it is important to investigate the possible differential effects of this barrier to gene flow and how this is relevant to the South African shark fisheries management. This section explores the use of two of the multiplex panels (panels 1 and 2) for population genetic analysis of *M. mustelus* (n = 105) and *G. galeus* (n = 124) samples collected from the south-west coast of South Africa from Langebaan to Struisbaai. A summary of genetic diversity estimates within populations of each species was computed in GENALEX 6.5 (Peakall and Smouse, 2012). Pairwise F-statistics (F_{ST}) (Weir & Cockerham 1984) were calculated in ARLEQUIN 3.5 (Excoffier *et al.* 2010) to determine levels of genetic differentiation. Type I and type II errors were reduced by performing sequential Bonferroni corrections (Rice 1989) and controlling the false discovery rate (FDR) using the Benjamini and Hochberg (B-H)

method (Benjamini & Hochberg, 1995). Experiment-wise critical values (α_{EW}) and (α_{B-H}) were determined for both tests and used to determine statistical significance among all pairwise tests. A hierarchical analysis of molecular variance (AMOVA) with 1,000 permutations was conducted in ARLEQUIN to compare the allocation of genetic variation on three different levels; 1) among groups (F_{CT}), 2) amongst populations (five sampling sites for each species) (F_{SC}) and 3) within populations (F_{ST}). The AMOVA analysis was performed firstly for all populations (G1; all five populations per species) and then to test for *a priori* oceanic grouping (G2; Atlantic- vs Indian Ocean). To visualise the relationships among individual genotypes of *M. mustelus* and *G. galeus*, factorial correspondence analysis (FCA) plots were generated in GENETIX 4.05 (Belkhir *et al.*, 2004) and depicted in three dimensions. Finally, a Bayesian clustering analysis was employed using STRUCTURE 2.3.4 (Pritchard *et al.* 2000) to detect the most likely number of ancestral genetic clusters (*K*) present in each species. Ten runs were performed for each assumed *K* (1-5). Markov chain Monte Carlo (MCMC) simulation runs of 10^6 iterations were made with 10^5 burn-in periods using the admixture model (Falush *et al.* 2003). The web-based STRUCTURE HARVESTER 0.6.93 (Earl and vonHoldt, 2012) was used to visualise STRUCTURE output data and determine the number of *K* using the ΔK method (Evanno *et al.* 2005). The STRUCTURE plots were compared for data based on all 12 loci as well as data excluding loci that deviated from HWE.

2.3. Results

The PCR cross-species amplification results for all microsatellite loci tested were tabulated to indicate successful amplifications (+) and failed reactions (-). All 12 of the microsatellite loci developed for the *Mustelus* species showed amplification in *G. galeus*. However, only 17 of the 22 microsatellites tested (77.27%) showed successful amplification in *C. brachyurus* and *R. annulatus*. None of the loci developed for *R. pulchra* showed successful amplification in *R. annulatus*.

2.3.1. *Characterisation of microsatellite loci for Galeorhinus galeus*

A total of 12 cross-species and ten species-specific microsatellite loci were characterised for 185 *G. galeus* individuals sampled across Chile, Argentina, Australia, New Zealand and six sampling populations in South Africa (Robben Island, False Bay, Kleinmond, Agulhas Bank, Struisbaai and Port Elizabeth). A summary of the characteristics of the microsatellite loci

considered for this study species are shown in **Table 2.1**. None of the loci showed evidence of scoring errors due to stuttering and large allele dropouts while null alleles were detected for three of the loci (*Mh2*; *Mh9*, *McaB6*). All 22 loci conformed to HWE with the exception of locus *Mca33* ($\alpha \leq 0.00042$); despite the presence of null alleles at some of the loci. Analyses for LD showed that only 17.1% of the loci pairwise comparisons were significant ($\alpha = 0.00029$) at the 5% nominal level.

All 22 loci were polymorphic in *G. galeus* exhibiting an average of seven alleles ranging from $N_A = 4$ for *Mca33* to $N_A = 10$ for *Gg2* and *Gg23*. High levels of polymorphism were detected for all loci with an average PIC of 68%. Notably, the species-specific loci (panels 3 and 4) were only slightly more polymorphic (PIC=70.2%) than the cross-species loci (panels 1 and 2) (PIC= 66.2%). Furthermore, PIC levels were above 50% for all loci except for locus *McaB22*. An overall low to moderate level of genetic diversity in terms of heterozygosity was detected across the loci ($H_O = 54.3\%$) ranging from 20% for *MaB27* to 93.2% for *McaB39*.

2.3.2. *Characterisation of cross-species loci for Carcharhinus brachyurus and Rhinobatos annulatus*

A total of 94 samples of *C. brachyurus* from five sampling populations (False Bay, Strandfontein, Gordon's Bay, Struisbaai, Mossel Bay and Jeffrey's Bay) were genotyped using 17 cross-species microsatellite loci (**Table 2.2**). Null alleles were detected at four of these loci (*McaB27, Mca33, Gg12* and *Gg22*) while none of the 17 loci characterised for *C. brachyurus* deviated from HW expectations except locus *Gg3* ($\alpha \leq 0.00042$). Analyses for LD showed that only 5% of the loci pairwise comparisons between were significant at an adjusted α of 0.000064 for 5% nominal level. All the loci were polymorphic ($N_A = 2$ to 5) with an average PIC of 50.3%. Multiplex panel 4 showed the highest level of polymorphism (PIC = 60%). On average, the heterozygosity levels were moderate (59.7%) but ranged from as low as 2.9% for *Gg3* to 87% for *McaB22*.

For *R. annulatus*, 83 samples were successfully genotyped using the same 17 microsatellite loci as for *C. brachyurus* (**Table 2.3**). Null alleles were again detected at four of the loci (*McaB27, Mca33, Gg12* and *Gg22*) while only genotypes at locus *McaB37* showed significant deviation from HWE ($\alpha \leq 0.00074$). Analyses for LD showed that none of the pairwise comparisons between loci were significant at an adjusted α of 0.00048 for 5% nominal level. All 17 of the cross-species loci characterised for *R. annulatus* were

polymorphic (N_A = 2 to 10) with an average of six alleles per locus. Polymorphism levels were at an average level of 61.8% with a significant difference in the levels of polymorphism detected for *Mustelus* loci as opposed to the *G. galeus* loci. Three *G. galeus* loci (*Gg3*, *Gg11* and *Gg12*) exhibited a PIC level lower than 50%. On average, the genetic diversity levels were relatively high at 71.3% ranging from 13% for *McaB27* to 88% for *McaB5*.

Table 2.1. Polymorphism and genetic diversity estimates for 22 microsatellite loci characterised in *Galeorhinus galeus*. Estimates shown are number of alleles N_A, effective number of alleles N_E, allelic richness A_R, observed H_O an unbiased heterozygosity uH_E, polymorphism information content PIC, inbreeding coefficient F_{IS} and probability of Hardy-Weinberg equilibrium P_{HWE}

Statistic	Panel 1								Panel 2					Panel 3				
	Mh1	*Mh2**	*Mh9**	*Mh25*	*Mca25*	*McaB39*	*McaB5*	*McaB6**	*McaB22*	*McaB27*	*McaB37*	*Mca33*	*Gg2*	*Gg3*	*Gg7*	*Gg11*	*Gg12*
N_A	6.400	5.000	7.000	7.600	8.600	5.200	8.000	8.000	8.800	4.600	7.600	4.000	7.800	4.600	8.000	5.200	6.000
N_E	3.980	2.000	2.000	4.279	4.862	3.633	3.622	3.000	3.901	2.681	3.469	2.178	3.887	3.168	4.313	3.003	2.964
A_R	1.371			1.561	1.471	1.233	1.530		1.273	0.936	1.338	0.858	1.344	1.172	1.547	1.109	1.242
H_O	0.674	0.297	0.233	0.719	0.514	0.932	0.573	0.679	0.676	0.200	0.507	0.557	0.417	0.358	0.642	0.297	0.423
uH_E	0.665	0.543	0.336	0.738	0.640	0.669	0.721	0.652	0.567	0.518	0.649	0.595	0.623	0.614	0.722	0.572	0.631
PIC	0.753	0.593	0.602	0.760	0.692	0.588	0.688	0.749	0.674	0.461	0.767	0.626	0.582	0.647	0.593	0.634	0.760
F_{IS}	-0.043	0.436	0.303	-0.005	0.172	-0.438	0.179	-0.070	-0.225	0.590	0.196	-0.198	0.308	0.399	0.080	0.458	0.290
P_{HWE}	1.000	1.000	N/D	0.0014	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000^	1.000	1.000	1.000	1.000	1.000

*only 121 individuals were genotyped

^loci deviating from HWE after Bonferroni correction ($\alpha \leq 0.00042$)

Table 2.2. Polymorphism and genetic diversity estimates for 17 microsatellite loci characterised in *Carcharhinus brachyurus*. Estimates shown are number of alleles N_A, effective number of alleles N_E, allelic richness A_R, observed H_O an unbiased heterozygosity uH_E, polymorphism information content PIC, inbreeding coefficient F_{IS} and probability of Hardy-Weinberg equilibrium P_{HWE}

Statistic	Panel 1						Panel 2							Panel 3			
	McaB5	*McaB6*	*McaB22*	*McaB27*	*McaB37*	*Mca33*	*Gg2*	*Gg3*	*Gg7*	*Gg11*	*Gg12*	*Mh1*	*Gg15*	*Gg17*	*Gg18*	*Gg22*	*Gg23*
N_A	3.000	4.571	2.571	2.000	3.429	3.143	4.286	1.571	2.857	3.571	4.143	3.714	4.571	3.000	4.429	3.429	4.286
N_E	2.342	3.050	1.172	1.318	2.286	2.090	3.484	1.034	1.838	1.790	2.645	2.084	2.767	1.703	2.990	2.072	2.715
A_R	0.908	1.186	0.240	0.330	0.932	0.843	1.271	0.067	0.673	0.715	0.937	0.865	1.035	0.630	1.175	0.848	1.080
H_O	0.900	0.764	0.087	0.171	0.595	0.659	0.900	0.029	0.623	0.462	0.550	0.721	0.899	0.387	0.952	0.674	0.964
uH_E	0.622	0.703	0.109	0.199	0.662	0.569	0.801	0.028	0.447	0.445	0.519	0.630	0.661	0.404	0.713	0.523	0.745
PIC	0.579	0.632	0.100	0.289	0.453	0.524	0.791	0.000	0.462	0.438	0.737	0.544	0.637	0.380	0.739	0.557	0.686
F_{IS}	-0.606	-0.200	0.181	0.083	-0.094	-0.304	-0.314	-0.070	-0.535	-0.128	-0.158	-0.423	-0.532	-0.043	-0.476	-0.418	-0.564
P_{HWE}	0.000	0.000	0.000	0.989^	0.458	0.660^	0.003	N/D	0.003	0.092	1.000^	1.000	1.000	0.639	0.001	0.009^	1.000

^loci deviating from HWE after Bonferroni correction ($\alpha \leq 0.00042$)

Table 2.3. Polymorphism and genetic diversity estimates for 22 microsatellite loci characterised in *Rhinobatos annulatus*. Estimates shown are number of alleles N_A, effective number of alleles N_E, allelic richness A_R, observed H_O an unbiased heterozygosity uH_E, polymorphism information content PIC, inbreeding coefficient F_{IS} and probability of Hardy-Weinberg equilibrium P_{HWE}

Statistic	Panel 1						Panel 2							Panel 3			
	Mh1	*Mh25*	*Mca25*	*McaB39*	*McaB5*	*McaB6*	*McaB22*	*McaB27*	*McaB37*	*Mca33*	*Gg2*	*Gg3*	*Gg7*	*Gg11*	*Gg12*	*Gg15*	*Gg18*
N_A	6.250	8.000	5.500	6.500	5.750	6.000	8.250	4.500	4.500	5.000	3.500	2.000	5.000	3.750	3.000	9.750	7.250
N_E	4.442	4.438	3.565	4.112	3.398	3.089	4.823	2.932	3.000	3.279	2.453	1.815	3.329	2.721	2.461	6.635	3.961
A_R	1.615	1.657	1.367	1.558	1.397	1.347	1.741	1.202	1.136	1.303	0.984	0.637	1.317	1.010	0.864	2.009	1.585
H_O	0.652	0.739	0.831	0.733	0.876	0.386	0.663	0.131	0.361	0.383	0.875	0.259	0.287	0.442	0.208	0.704	0.421
uH_E	0.821	0.776	0.738	0.780	0.736	0.698	0.809	0.688	0.591	0.739	0.618	0.475	0.719	0.731	0.539	0.879	0.779
PIC	0.737	0.716	0.654	0.702	0.657	0.619	0.746	0.588	0.532	0.628	0.502	0.345	0.625	0.496	0.437	0.816	0.705
F_{IS}	0.154	0.010	-0.177	0.013	-0.245	0.417	0.148	0.798	0.358	0.434	-0.483	0.418	0.574	0.225	0.566	0.158	0.431
P_{HWE}	0.069	0.176	0.132	0.131	0.075	0.113	0.198	0.113	0.312^	0.114	0.060	0.026	0.185	0.267	0.313	0.183	0.119

^loci deviating from HWE after Bonferroni correction ($\alpha \leq 0.00074$)

2.3.3. Application in resolving species identification

Based on the amplification of the *ND2* gene, 26 samples of the commercial catch were identified as *G. galeus*, 20 as *M. mustelus*, four were cryptic (amplified both fragments of 392- and 671 bp) and eight were not successfully amplified (**Figure 2.2**). Subsequently, 49 samples were then genotyped using the four multiplexes previously optimised. The generated PCA plot depicted three genotypic clusters; one comprising *G. galeus* samples (as identified with the *ND2* gene), another containing only *M. mustelus* samples and a third cluster containing the four cryptic samples (those showing amplification of both *ND2* fragments) (**Figure 2.2**). Interestingly, two samples that showed amplification of the 392 bp fragment of the *ND2* gene and therefore identified as *M. mustelus* also clustered with the *G. galeus* group while one clustered with the cryptic samples (red arrows in **Figure 2.2**). Additionally, one of the samples identified as cryptic by *ND2* amplification also clustered with *G. galeus*. This discrepancy was investigated by barcoding all the outlier samples using the FishF1 and FishR1 primers designed to amplify the *cytochrome oxidase 1* (*CO1*) gene (Ward *et al.* 2005). Sequences were subsequently compared to reference sequences on the Barcode of Life Data System (BOLD). BLAST searches confirmed misidentification/mislabelling and that the four cryptic samples were either *M. palumbes* or *C. brachyurus*. The microsatellite genotypes grouped most of the samples into either *M. mustelus* or *G. galeus* while also enabling the identification of the cryptic samples and thereby showing their potential application in assisting with species identification. In total, 28 samples of *G. galeus*, 17 samples of *M. mustelus* and 1 sample of *C. brachyurus* could be incorporated in further analyses.
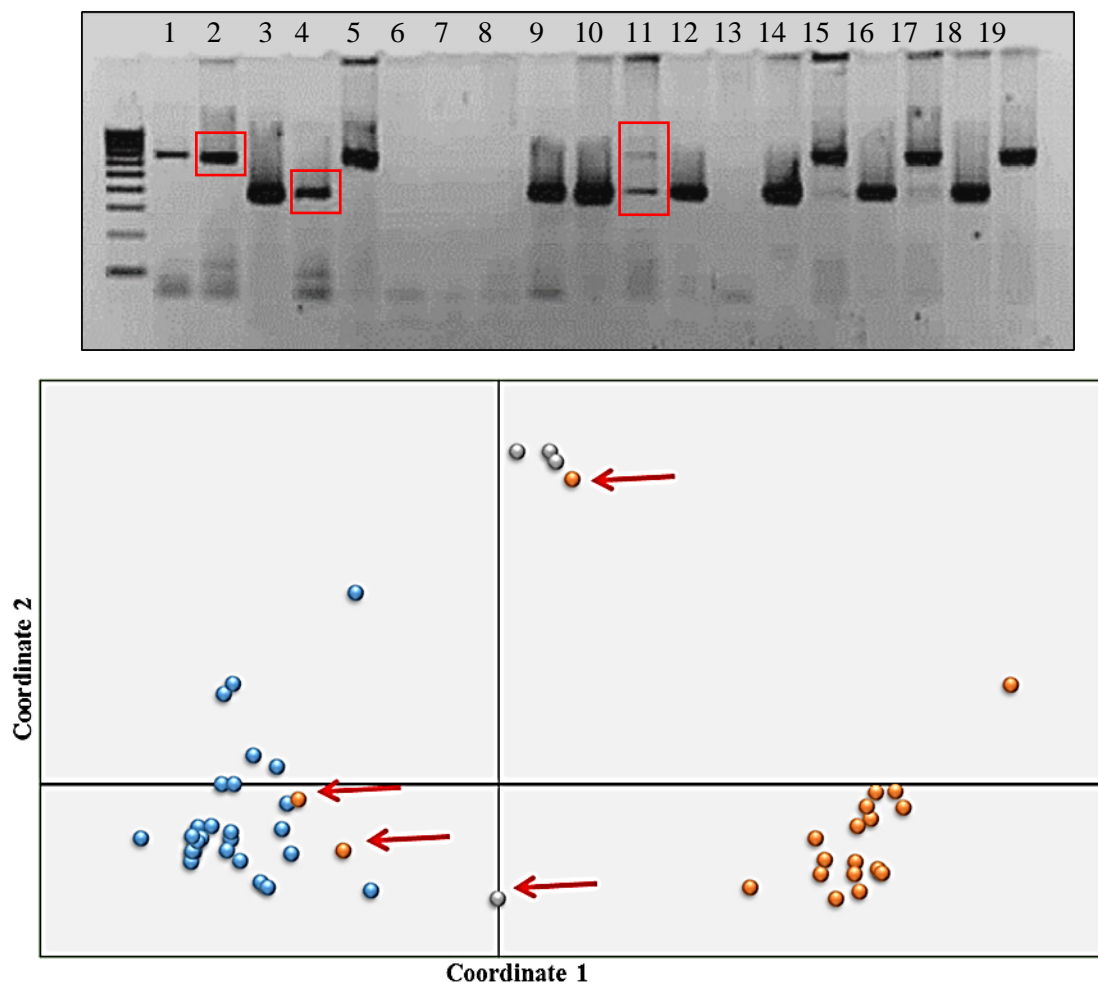
Figure 2.2. A 2% agarose gel showing PCR amplification of the *ND2* gene for *G. galeus* (lane 2), *M. mustelus* (lane 4), and one of the cryptic samples (lane 11) at the top and a principal component analysis (PCA) plot showing species clustering based on 22 microsatellite loci (bottom).

2.3.4. *Differential gene flow patterns of Mustelus mustelus and Galeorhinus galeus*

When two of the multiplex panels were tested on *M. mustelus* and *G. galeus,* the allele-scoring ranges differed between the source species, *M. canis* and *M. henlei* and the study species, *M. mustelus* and *G. galeus*, and a similar trend was detected for the N_a. For instance, loci that were monomorphic in the source species *M. canis* (*Mca25*, *McaB39*, *McaB27*) (Giresi *et al.* 2011) were polymorphic when characterised for the study species and this was similarly noted by Maduna *et al.* (2014). Cross-species utility tests carried out by Maduna *et al.* (2014) confirmed that when microsatellite loci isolated in *G. galeus*, *M. canis* and *M.*

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henlei were characterised for *M. mustelus*, there was a shift in the allele-scoring range and a drop in the N_A. None of the microsatellites showed evidence of scoring errors due to stuttering and large allele dropouts in MICRO-CHECKER. None of loci characterised for *M. mustelus* and *G. galeus* deviated from HWE except for the locus *McaB39* characterised for *M. mustelus* whose *P*-value ($P = 0.000$) was below the Bonferroni-corrected significance value ($\alpha = 0.00083$). Analyses for LD showed that only 8% of the pairwise comparisons between loci characterised for *M. mustelus* were significantly linked while 24% of pairwise comparisons for *G. galeus* were linked according to an adjusted *P*-value of 0.000757 at 5% nominal level.

Moderate levels of genetic diversity were detected for the five sampling sites of *M. mustelus* (H_e = 0.51 to 0.55) while fairly higher and more varying levels were detected across the sampling sites of *G. galeus* (H_e = 0.58 to 0.72, N_a = 4-7). The fact that the samples of *G. galeus* showed similar levels of genetic diversity to samples of *G. galeus* from Chile, Australia and New Zealand, (H_e = 0.61, N_a = 4-6) based on species-specific markers (Hernández Muñoz, 2013), supported the usefulness and reliability of the cross-species microsatellite markers in genetic diversity estimates of these two species.

The statistical significance of pairwise F_{ST} values was confirmed after controlling for the FDR at a corrected critical value ($\alpha_{B-H} \leq 0.009$) and Bonferroni critical value ($\alpha_{EW} \leq 0.005$). Results indicated a significantly moderate to great inter-oceanic genetic differentiation ($F_{ST} = 0.118$ to 0.202, $P \leq 0.005$) exists between sampling populations of *M. mustelus*. Pairwise F_{ST} values between sampling sites of *G. galeus* indicated no inter-oceanic and no intra-oceanic differentiation ($F_{ST} = -0.006$ to 0.037, $P \geq 0.005$). Factorial correspondence analysis (FCA) of the individual genotypes confirmed inter-oceanic differentiation between sampling populations of *M. mustelus* sampled from the Atlantic Ocean and the Indian Ocean while *G. galeus* showed a higher degree of overlap between sampling sites with only the False Bay individuals clustering separately from the rest. Results for the AMOVA hierarchical analysis for all sampling populations (G1) showed that both species exhibit very little but significant variation amongst sampling populations (*M. mustelus*: $F_{ST} = 0.072$, $P = 0.000$; *G. galeus*: $F_{ST} = 0.023$, $P = 0.024$) with the highest level of variation seen within sampling populations. When looking at the oceanic variation (G2), a moderate level of inter-oceanic variation was indicated for *M. mustelus* (14.27%, $F_{CT} = 0.143$, $P = 0.000$) but very little variation was detected among the sampling populations (1.12%, $F_{SC} = 0.013$, $P = 0.000$). AMOVA showed

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no inter-oceanic variation for *G. galeus* (1.15%, $F_{CT} = 0.012$, *P* = 0.189) confirming the pairwise F_{ST} results. The cluster assignment patterns in STRUCTURE differed markedly for the two species (**Figure 2.3**) with strong inter-oceanic structure (Atlantic vs Indian sampling populations) detected for *M. mustelus* as opposed to varying levels of admixture observed for *G. galeus*. Interestingly, individuals of *G. galeus* sampled west of the proposed Agulhas barrier showed a more significant level of admixture than those sampled east of the barrier and this could explain why, in contrast to the pairwise F_{ST} and AMOVA analyses, Bayesian analysis failed to reject the null hypothesis of panmixia for this species.
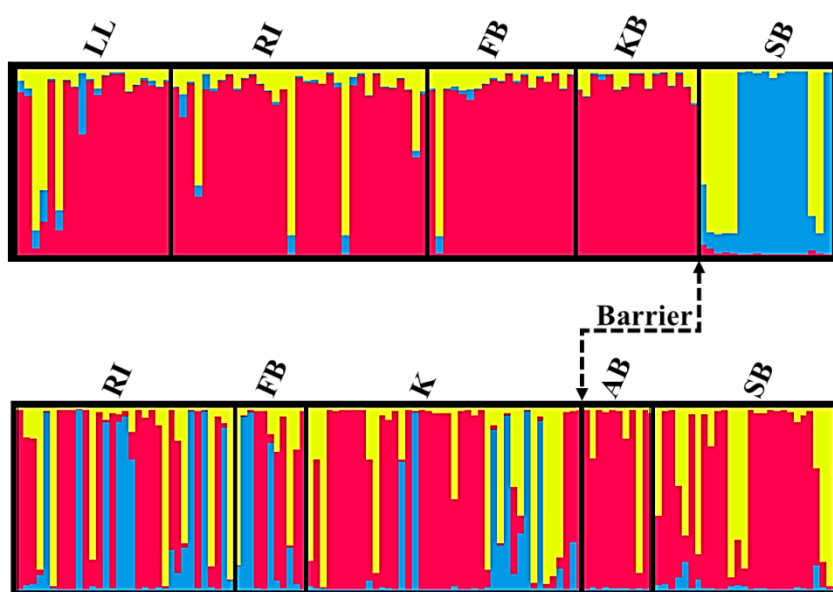
Figure 2.3. STRUCTURE plots showing individual assignments for *Mustelus mustelus* (top) and *Galeorhinus galeus* (bottom). The proposed Atlantic/Indian barrier in the vicinity of Cape Agulhas is indicated for both species.

2.4. Discussion

Cross-species amplification of 22 microsatellite markers in *G. galeus*, *C. brachyurus* and *R. annulatus* showed a decrease in success of amplification with increasing phylogenetic distance between source and recipient species (**Figure 2.4).** However, this was not reflected when comparing levels of polymorphism; whereby polymorphism levels were seen to be higher for cross-species characterisation of loci within *R. annulatus* than for *C. brachyurus*. This was unexpected since *R. annulatus* is the most divergent from all the rest of the study species. This study also showed similar cross-species amplification success and level of polymorphism as reported in Maduna *et al.* (2014). The latter study also detected size

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homoplasy for some of the loci characterised in the target/recipient species. Size homoplasy such as shifts in size regions scored and number of loci detected were mostly observed within lesser sandshark *R. annulatus*, which also happens to be the most divergent of the target species. A study by Yue *et al.* (2010) showed an existence of non-homologous products when transferring microsatellite markers between catfish species from different families and postulated that size homoplasy in cross-species amplification of microsatellites is locus dependent and does not neccessarily reflect phylogenetic relationships. However, size homoplasy at microsatellite loci does not represent a significant limitation for many population genetics statistics, as the extensive polymorphism exhibited at those loci often compensates for their homoplasious evolution (Estoup *et al.* 2002).
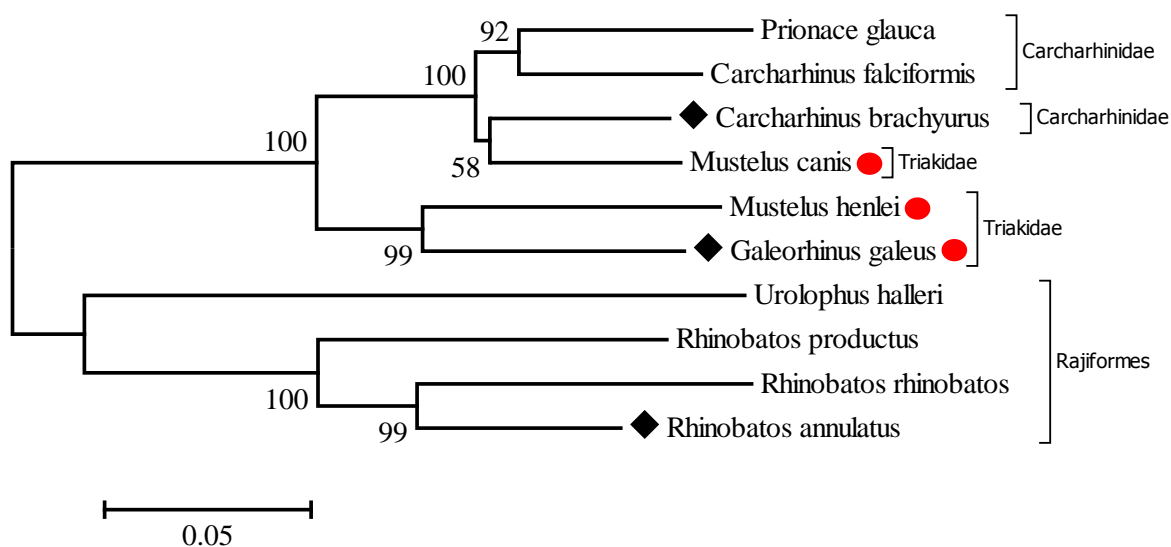
Figure 2.4. A neighbour-joining topology based on mitochondrial *ND2* sequences sourced from Naylor *et al.* (2012) showing the relationships between the study species (indicated with black diamonds) and the source species (indicated with red circles) classified under two shark families Carcharhinidae, Triakidae and one batoid order Rajiformes.

It is common knowledge that fisheries have a history of both unintentional and deliberate misidentification and taxonomic lumping of elasmobranch and other marine species. *Carcharhinus brachyurus* and *M. mustelus* are often misidentified as *G. galeus*, under the name 'vaalhaai' by fisheries in South Africa (Silva & Bürgener 2007; Best *et al.* 2013). *Mustelus palumbes* and *M. mustelus* are readily confused with one another, because of their morphological similarity (Farrell *et al.* 2009; Best *et al.* 2013) and despite the recent de-

commercialisation of *T. megalopterus* in South Africa, this species is being passed off as *M. mustelus* or *G. galeus* to obtain a better price (Best *et al.* 2013). Also, *C. obscurus* and *C. brevipinna* are often confused and lumped with *C. brachyurus* (Cliff & Dudley 1992). All these identification issues could lead to discrepancies in population trends of elasmobranch species. Despite the limitations noted with using only one set of markers in species identification, the usefulness and ease with which the four microsatellite panels distinguished between samples of *M. mustelus*, *G. galeus*, *C. brachyurus* and *M. palumbes*; together with the ND2 and *CO1* validation, makes them an important tool for elasmobranch research and fisheries management in South Africa. Moreover, the high rate of misidentification observed in the 57 samples from Struisbaai believed to be *G. galeus*, highlights the existential rate of confusion and demonstrates a need for large-scale application of genetic identification methods.

Since a minimum of 17 polymorphic loci were generated for each study species; far surpassing the minimum number of 10 polymorphic loci required for inferences of standard population genetic statistics, a subset of 12 loci in two multiplexes were used to investigate gene flow patterns for the commercially important *M. mustelus* and *G. galeus* in South Africa. The microsatellite multiplexes proved not only to discern genetic diversity within both species but were also successful in identifying different patterns of gene flow across one of the major barriers (Indian/Atlantic barrier in the vicinity of Cape Agulhas) recognised along the South African coast. The results also indicate that different management strategies might apply for these species. With regards to the smaller of the two species, *M. mustelus*, two populations are most likely present with hindrance of gene flow between the cool temperate and warm temperate biogeographic regions, in the vicinity of Cape Agulhas. On the other hand, no strong genetic structure was detected for *G. galeus* and gene flow for the species seems substantial. However, varying levels of admixture were detected along the studied area indicating that gene flow for this species may not necessarily be even throughout the entire distribution range.

Both *M. mustelus* and *G. galeus* are currently over-exploited in South Africa. This is worsened by the lack of spatio-temporal behaviour studies for both species in this area, with the exception of da Silva *et al.* (2013). Added to that are the relaxed regulations currently at play; with no seasonal or area restriction permits dispensed despite the existence of several no-take Marine Protected Areas (MPAs) in the region (NPOA-sharks 2012). Thus it is

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suggested that these differential gene flow patterns are taken into consideration in regulations relating to directed shark fisheries. Management policies should ideally be based on a combination of actual movement and genetic data in order to preserve the underlying contemporary population structure of species across the areas of exploitation in South Africa.

2.5. Conclusion

In conclusion, microsatellite multiplex panels were successfully optimised for species-specific and cross-species loci and showed to be reliable for use in the three study species. In combination with mitochondrial DNA barcoding, these panels showed some potential in resolving issues of species identification. Furthermore, a subset of the cross-species microsatellites were successful in detecting varying levels of gene flow amongst two of South Africa's most exploited shark species, *M. mustelus* and *G. galeus*. The contemporary gene flow across the Atlantic/Indian Ocean boundary is important information that could be incorporated for defining management units for the respective species in the future; most likely two inter-oceanic populations to be considered for *M. mustelus* and one panmictic population with high levels of admixture for *G. galeus*. This project nonetheless highlights the importance of incorporating molecular genetic tools in achieving an ecosystems approach to shark fisheries management in South Africa since the microsatellite panels developed in this chapter could be used to describe the genetic diversity and genetic connectivity of several elasmobranch species on a regional scale.

Chapter 3: Population connectivity and phylogeography of tope shark (*Galeorhinus galeus*) on a local and wider regional scale

Abstract

The tope shark (*Galeorhinus galeus* Linnaeus, 1758) is a temperate, coastal hound shark with an anti-tropical distribution in the Atlantic and Indo-Pacific oceans. Despite having been assessed on a global level, the population structure of this species is still undetermined across South Africa's coastline and the entire Southern Hemisphere. In this study, an analysis of the local and wider regional population structure of *G. galeus* was conducted on a total of 185 samples using 19 nuclear microsatellite markers and a 671 bp fragment of the NADH dehydrogenase subunit 2 (*ND2*) gene. Across the Southern Hemisphere, two geographically distinct clades were recovered, one including the Atlantic and Indo-Pacific collections (Argentina, South Africa and Australia) and one comprising the Pacific samples (New Zealand and Chile) as well as single divergent haplotype restricted to South Africa. Nuclear data also revealed significant population subdivisions (F_{ST} = 0.192 to 0.376, p<0.05) indicating very limited gene flow for tope sharks across ocean basins. On a local scale, F-statistics and multivariate analysis supported high gene flow along the South African coast (F_{ST} = 0.035 to 0.044, p<0.05), with exception of the East coast samples (Port Elizabeth) that was significantly differentiated from the rest. Bayesian clustering analysis implemented in STRUCTURE also showed substantial admixture in all sampling populations, decreasing from west to east. Mitochondrial sequence data recovered 11 haplotypes (h = 0.714, π = 0.0068) for South Africa, with one haplotype unique to Port Elizabeth separated by 12 mutational steps. As with many other coastal shark species with cosmopolitan distribution, this study confirms the lack of both historical dispersal as well as inter-oceanic gene flow but also suggests that factors such as oceanic currents and thermal fronts may play a role in driving genetic structuring of *G. galeus* locally.

Keywords: demographic history, fisheries, gene flow, mitochondrial *ND2*, South Africa, Southern Hemisphere

3.1. Introduction

The tope shark (*Galeorhinus galeus* Linnaeus 1758) is a commercially important shark species distributed in temperate waters around the world. This species has been harvested for centuries and is most commonly used for liver oil and making a delicacy fin soup. Across the Southern Hemisphere, the species takes second to third priority target in the demersal shark fisheries and is listed as vulnerable by the International Union for the Conservation of Nature (IUCN) (Walker *et al.* 2006). In Chilean waters for example, there is very limited data on the landings of *G. galeus*. It is usually lumped with landings of *Mustelus mento*, *M. whitney* and *Squalus acanthias* under the local name "tollo" (Sebastian *et al.* 2008). Also, reports of bycatch were noted in the artisanal fisheries targeting *Genypterus* species (Lamilla *et al.* 2005). Catches of *G. galeus* are typically sold in local Chilean markets while the dried fins are exported to Asia (Sebastian *et al.* 2008).

In the south-western Atlantic (SWA), *G. galeus* is ranked as critically endangered and is exploited by coastal fisheries from Brazil to Argentina at different stages during its seasonal migrations (Chiaramonte 1998; Elías *et al.* 2005). It is believed that *G. galeus* comprises only one population extending across Brazil, Uruguay and Argentina, with migrations occuring during spring (Lucifora *et al.* 2004). Large aggregations of sharks have been seen moving in to closed bays of northern Argentina during this time and these areas are considered to be the primary parturation grounds for the species (Lucifora *et al.* 2004; Cuevas 2014).

Galeorhinus galeus also has an Indo-South Pacific distribution in the Southern Hemisphere wherein it occupies the temperate waters of Australia and New Zealand (Walker *et al.* 2006). In Australia, this species is mainly landed in the southern waters including Tasmania and is very rarely caught in the western waters off Perth (Punt *et al.* 2000; Punt *et al.* 2005; Walker *et al.* 2006). The species is ranked as vulnerable and a decline in catch numbers has been reported over the last 20 years, due in part to it being landed as bycatch in the *Mustelus antarticus* fisheries (McLoughlin 2007). *Galeorhinus galeus* occurs throughout New Zealand's entire exclusive economic zone (EZZ) and is considered a sustainable fishery. The New Zealand fisheries mandated numerous restrictions on the commercial harvesting of *G. galeus* and as of 1986, implemented eight quota management areas (QMAs), despite the presence of only one stock. Tag data also showed that sharks move as far as 5000 km outside of these QMAs with a significant number of sharks travelling into Australian waters. Despite

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this and genetic evidence for one panmictic population (Hernández *et al.* 2015), *G. galeus* in Australia and New Zealand are currently managed as separate stocks (Francis 2010).

Galeorhinus galeus is a principal target species in South Africa's shark fisheries where it is locally referred to as "soupfin" (DAFF 2012). The commercial fishery for *G. galeus* has existed in South Africa since the 1930s with major landing sites situated off the south-west coast at Saldanha Bay, Cape Town, Hout Bay, Gans Bay and Struisbaai. Occasional landings also occur between Mossel Bay and East London on the East coast (McCord 2005). Frozen fillets from the landed catch are exported to Australia whereas the dried fins are mostly traded on the Asian market. The landed value for *G. galeus* ranges from ZAR4 to ZAR20 per kg, and sharks larger than 10kg are generally not exported due to stringent mercury tests applied to larger animals in the international market (McCord 2005; da Silva & Bürgener 2007).

Galeorhinus galeus is ranked as vulnerable in South Africa and is threatened by over-exploitation, lack of species-specific catch data and non-cohesive fishing regulations (McCord 2005; da Silva & Bürgener 2007; Best *et al.* 2013). With the exception of catch-trend analysis (McCord 2005) and preliminary population genetic data (Bitalo *et al.* 2015), very little information exists for the migratory patterns of this species in South African waters. However, fisheries-dependant data shows that catches for *G. galeus* are highest during autumn (March to May) and spring (September to November), while very small catches are reported for the rest of the year. A high proportion of gravid females caught by fishers in areas such as Walker Bay, False Bay, Gans Bay, and Struisbaai during spring suggest that females move inshore to pup (McCord 2005).

Although sharks have previously been considered an under-exploited resource, the potential for growth within the chondrichthyan fisheries in South Africa and across the Southern Hemisphere, has been noted for the last few decades (da Silva *et al.* 2015). This warrants a transition from short-term to long-term management allocations, presenting an ideal opportunity for fisheries scientists and managers to develop species-specific management plans for commercially exploited shark species. Also given its inherit susceptibility to overexploitation due to *K*-selected traits; which essentially confound its path to a quick population recovery (Walker *et al.* 2006), knowledge of population genetic structure is essential for attaining effective management of the species locally (Ward-Paige *et al.* 2012).

Population genetic studies aim to infer contemporary and historical processes responsible for observed patterns of spatial genetic structure, in an effort to identify genetic management and conservation units (Ward-Paige *et al.* 2012; Worm *et al.* 2013). Biogeographic barriers such as oceanic currents are known to have an impact on population connectivity while the gene flow patterns defined across these barriers vary between species (Dudgeon *et al.* 2012). There are a number of traditionally recognised biogeographic barriers across the Southern Hemisphere most notably the Benguela Barrier (BB) and the Eastern South Pacific Barrier (EPB) (Briggs 1999). The EPB encompasses over 7000km of oceanic expanse and has been noted to result in complete isolation between populations of coastal species associated with continental shelves (Schultz *et al.* 2008; Chabot & Allen 2009; Benavides 2011; Daly-Engel *et al.* 2012; Hernández *et al.* 2015). Conversely, the EPB barrier shows no effect in pelagic species that are highly vagile (Schrey & Heist 2003; Keeney & Heist 2006; Duncan *et al.* 2006; Veríssimo *et al.* 2010; Taguchi *et al.* 2015). The Benguela Barrier (BB), the cold-water upwelling of the Benguela Current in southern Africa, is characterised for bringing in a cold current across the southern tip of Africa. This barrier has also been shown to restrict gene flow between southern Atlantic and south Indian Ocean populations of tropical and subtropical sharks such as *Sphyrna lewini* (Duncan *et al.* 2006) and *M. mustelus* (Maduna *et al.* 2016).

Added to these known biogeographic barriers, are the thermal barriers created by contrasting oceanic currents. For example, there is a sharp transition zone along the SWA where the warm Brazil current from the north meets the cold Malvina current from the south. These two currents have distinct characteristics and constitute a subtropical shelf front. This transition zone is known to create a diverse estuarine environment with varying stratification on a thermal and salinity level (Piola *et al.* 2000; Garca 2012), and these physical processes have been shown to drive migratory movements associated with seasonal reproductive behaviour as is seen for *G. galeus* (Elías *et al.* 2005) and *M. schmitti* (Pereyra *et al.* 2010). Locally, along the South African coastline, the cold Benguela current from the Atlantic Ocean meets the warm Agulhas current from the south Indian Ocean. This transition zone (Atlantic/Indian boundary) influences the distribution patterns of marine populations of the west and east coast of southern Africa due to the seasonal intensities of the currents (Teske *et al.* 2011). For instance, contemporary gene flow for *Mustelus mustelus* was shown to be restricted across this transition zone (Bitalo *et al.* 2015; Maduna *et al.* 2016), while it does not seem to have

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an effect on the temperate *C. brachyurus* which exhibits anti-tropical distribution (Benavides *et al.* 2011).

Studies using microsatellite and mitochondrial control region (mtCR) data have supported distinct continental populations of *G. galeus*, which are structured along a latitudinal gradient. These studies suggested *G. galeus* to have a strong affinity for cool temperate waters limiting its ability to cross warm temperate waters (Chabot & Allen 2009; Hernández *et al.* 2015). However, none of these studies resolved the genetic connectivity of *G. galeus* across all the know barriers and transition zones of the Southern Hemisphere. Considering the wide geographic distribution and potential for long distance dispersal of *G. galeus*, it is postulated here that restricted gene flow along the species' distribution range is associated with regions of unsuitable environmental conditions, such as warm waters at low latitudes and/or upwelling resulting from contrasting oceanic currents rather than with geographical distance per se. More specifically, the following hypothesises are tested: (1) genetic discontinuity across the south Pacific, south Atlantic and south Indian Oceans and (2) genetic discontinuity across Benguela/Agulhas current system of South Africa. A dual-marker approach is applied where variation in the mitochondrial *ND2* gene and 19 microsatellite markers are used to assess levels of genetic diversity and patterns of gene flow across the Southern Hemisphere and more locally around South Africa. Although recommendations can be made for stock management across the Southern Hemisphere oceans, these assessments are mainly for management purposes relevant to the South African commercial shark fisheries.

3.2. Materials and Methods

3.2.1. *Sample acquisition and DNA extraction*

A total of 185 fin clip or muscle tissue samples were taken from fisheries specimens collected across three ocean basins: the temperate south Pacific including samples from Santiago, Chile and Solander Island, New Zealand; the warm temperate south Indian Ocean comprising samples from the East coast of South Africa (Port Elizabeth) and the west coast of Australia; and the cool temperate southern Atlantic including samples from Bahía San Blas, Argentina and the south-west coast of South Africa **Figure 3.1**. These collection sites span the biogeographic features of interest including the East South Pacific Barrier (EPB) which is about 7000 km of oceanic expanse between Chile and New Zealand, the Great Australian

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Bight (GAB) that stretches across the Indo-South Pacific region between southern Australia and New Zealand, the Mid-Atlantic Barrier (MAB) between South America and South Africa, and the Benguela Barrier (BB) in combination with the Agulhas return current potentially acting as a barrier between South Africa and Australia (as reviewed in Dudgeon *et al.* 2012). Note that exactly the same samples from Chile and New Zealand were previously used for a regional study spanning the EPB and the Great Australian Bight (Hernández *et al.* 2015).

Locally, sampling was performed at six locations off the coast of South Africa according to protocols and permits approved by the Department of Agriculture, Fisheries and Forestry (DAFF) (Republic of South Africa). Sampling covered most of the distribution and exploitation range of the species traversing the Benguela/Agulhas transition zone across the cool and warm temperate Indo-Atlantic **Figure 3.2**. Samples from the cool temperate Atlantic region were obtained from Robben Island (RI), False Bay (FB), Kleinmond (KL) and the western end of the Agulhas Bank (AB), while those from the warm temperate Indian Ocean included samples from Struisbaai (SB) and Port Elizabeth (PE). Samples were collected between 2008 and 2015 with sampling carried out by DAFF or commercial fishers. All samples from Robben Island to Agulhas Bank as well as Port Elizabeth originated from fishery observer programs and research captures operated by DAFF. Genomic DNA was extracted from fin clips or tissue samples as described in chapter 2 using a modified CTAB extraction method described by Saghai-Maroof *et al.* (1984) with minor modifications.
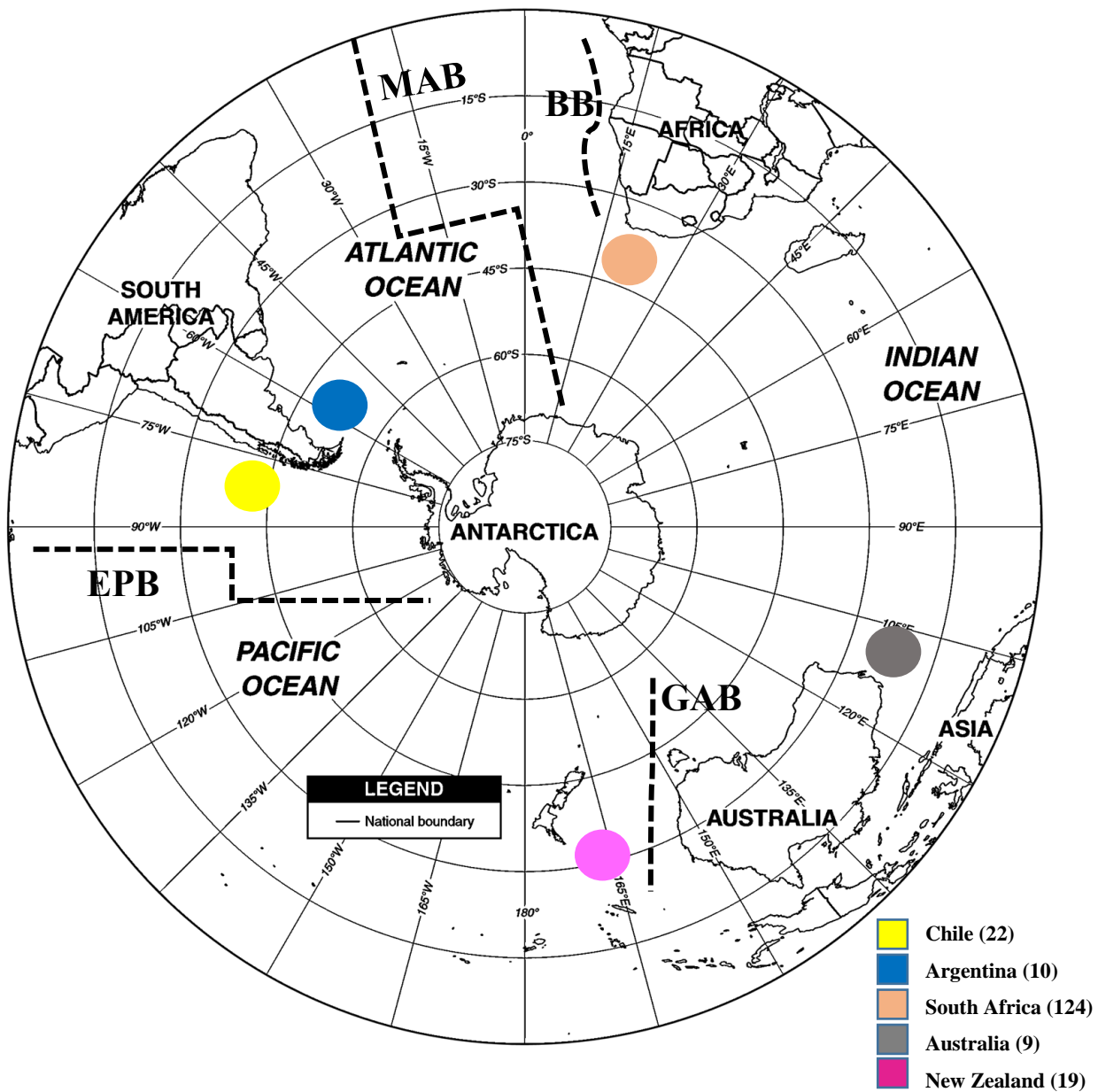
Figure 3.1. Map showing the major biogeographic barriers across the Southern Hemisphere. Geographic sampling sites of *G. galeus* include Chile (CHI), Argentina (ARG), South Africa (SA), Western Australia (AUS) and New Zealand (NZ). Sample numbers collected are shown in parenthesis. The biogeographic barriers are the Benguela Barrier (BB); the Eastern South Pacific Barrier (EPB); the Great Australian Bight (GAB) and the Mid-Atlantic Barrier (MAB).
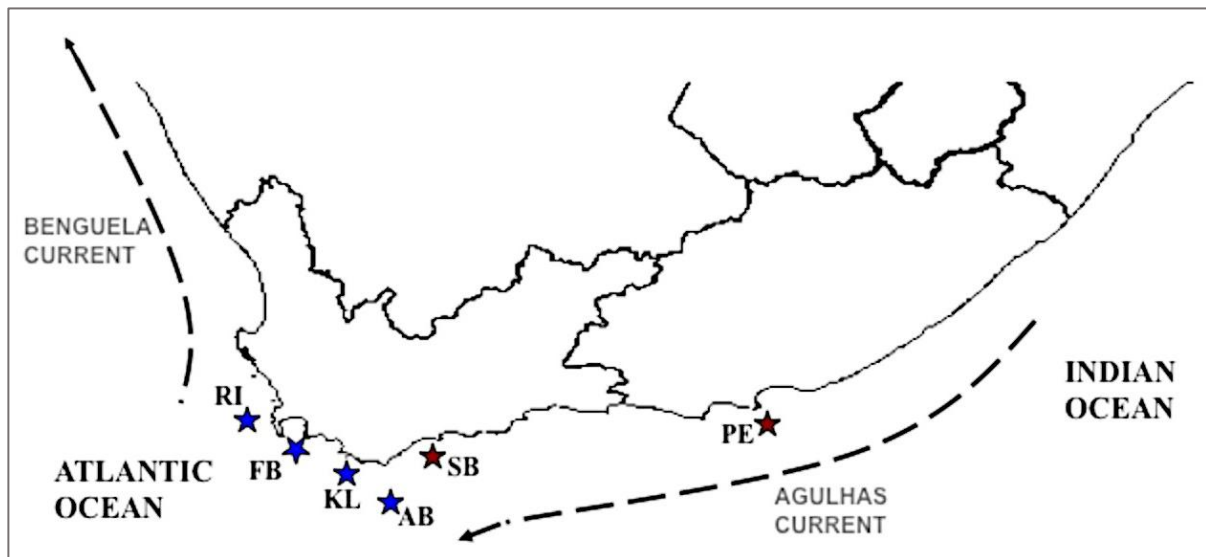
Figure 3.2. Map of South Africa showing regional sampling sites of *Galeorhinus galeus* traversing the Atlantic/South Indian Ocean transition zone. Samples from the Atlantic Ocean (blue stars) include Robben Island (RI), False Bay (FB), Kleinmond (K) and Agulhas Bank (AB). Samples from the South Indian Ocean (red stars) include Struisbaai (SB) and Port Elizabeth (PE).

3.2.2. *Mitochondrial DNA sequencing*

The DNA sequences of the mitochondrial gene NADH dehydrogenase subunit 2 (*ND2*) were analysed for a total of 96 samples of *G. galeus* using the species-specific primers sourced from Farrell *et al.* (2009). The primers MUND2F1 5'-TGTGAATAGGCCTCGAAATCA-3' and GGND2R 5'-TCCTAAGGAAAGGAGAGTCAGTAA-3 were used to confirm species identity of the commercial catch. Southern Hemisphere (SH) samples included Chile (11), Argentina (9), South Africa (57), Australia (9) and New Zealand (9) while South African (SA) samples included Robben Island (10), False Bay (7), Kleinmond (12), Agulhas Bank (4), Struisbaai (15) and Port Elizabeth (7). Mitochondrial DNA (mtDNA) sequence amplification was performed in a 20µl total volume reaction containing 100 ng template DNA, 1X GoTaq buffer (Anatech, South Africa), 200 µM dNTPs, 0.4 µM of each primer (Integrated DNA Technologies, IDT, South Africa), 2 mM $MgCl_2$ (Promega, Wisconsin, USA) and 0.5 U GoTaq DNA polymerase (Anatech, South Africa). PCR amplifications were performed in an Applied Biosystems (ABI) (Life Technologies, California USA) thermal cycler version 2.09 using cycling conditions as described by Farrell *et al.* (2009). Mitochondrial *ND2* amplicons were subjected to bi-directional sequencing using the

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BigDye[®] Terminator 3.1 Cycle Sequencing Kit (Life Technologies, California USA) and a ABI 3730xl Genetic Analyser. All mtDNA sequences were manually edited and aligned using the MUSCLE alignment algorithm available in MEGA 6 (Tamura *et al.* 2013). Aligned sequences were trimmed to 590 bp and exported to DNASP 5.10.01 (Librado & Rozas 2009) for further analysis.

3.2.3. *Microsatellite genotyping*

A total of 185 samples of *G. galeus* were genotyped using ten species-specific microsatellites developed by Chabot & Nigenda (2011) and nine cross-species markers previously developed for *Mustelus henlei* and *M. canis* (Giresi *et al.* 2012). Southern Hemisphere samples included Chile (22), Argentina (10), South Africa (124), Australia (9) and New Zealand (19). South African samples included Robben Island (26), False Bay (11), Kleinmond (39), Agulhas Bank (10), Struisbaai (28) and Port Elizabeth (10). Multiplex PCRs were conducted in three assays based on primer pair combinations and multiplex panels described in Chapter 2. The PCR cycling profile recommended in the Qiagen Multiplex kit user's manual was used. Subsequent to capillary electrophoresis, microsatellite allele sizes were scored manually using the LIZ[®] 600 internal size standard and GeneMapper[®] 4.0 software (ABI, Life Technologies, California, USA). Particular care was taken with allele scoring and control samples were added with each independent capillary electrophoresis run.

3.3. Genetic data analyses

3.3.1. *Mitochondrial analyses*

The software DNASP and ARLEQUIN 3.5 (Excoffier *et al.* 2005) were used to calculate molecular diversity indices such as the number of segregating sites (K), number of haplotypes (H), haplotype diversity (h) and nucleotide diversity (π). Genetic structure across sampling sites was investigated using two different approaches. Firstly, pairwise genetic differences (Φ_{ST}) based on haplotype frequencies were estimated across the Atlantic, South Indian and South Pacific oceans. Pairwise Φ_{ST} values were computed in ARLEQUIN using 20,000 permutations for two separate datasets; Southern Hemisphere (regional) and South Africa (local). Sequential false discovery rate (FDR) corrections of the significant values were performed in order to minimise type I error (probability of incorrectly rejecting a true null hypothesis) (Narum 2006). Secondly, an analysis of molecular variance (AMOVA)

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(Excoffier *et al.* 1992) was conducted in ARLEQUIN to determine the variance components and fixation indices (Φ-statistics) at three levels of hierarchical subdivision: among groups (Φ_{CT}), among populations (Φ_{SC}), and within populations (Φ_{SC}). On a wider regional scale, spatial AMOVA was undertaken to test the hypothesis of genetic subdivision between collections from Chile, Argentina, South Africa, Australia and New Zealand across the three Southern Hemisphere oceans. The AMOVA for the SA samples alone included testing for 1) the hypothesis of panmixia through pooling all six sampling sites, 2) the hypothesis of substructure across the Cape Agulhas boundary by comparing pooled samples from the Atlantic Ocean (Robben Island, False Bay, Kleinmond, Agulhas Bank), against pooled samples from the South Indian Ocean (Struisbaai, Port Elizabeth). The reconstruction of genealogies was performed using phylogenetic algorithms in order to estimate the relationship between haplotypes without ambiguities or unresolved connection (Salzburger *et al.* 2011). A phylogenetic tree of the mtDNA sequences was estimated using a maximum likelihood (ML) approach in PHYML 3.0 (Guindon *et al.* 2010) based on the Kimura-2 (K2) model selected according to the Bayesian Information Criterion (BIC). For tree searching and level of branch support, default settings were used. The ML tree was imported into HAPLOVIEWER (Salzburger *et al.*, 2011) to illustrate the evolutionary relationships among haplotypes.

To assess the demographic history of the populations, past demographic and population expansions were evaluated using two methods. Firstly, using the neutrality test, computation of Tajima's *D* (Tajima 1989) and Fu's F_S (Fu 1997) statistics and significance values were tested by 20,000 coalescent simulations (significance at $\alpha \leq 0.05$) under the infinite-sites model in ARLEQUIN. The values of these indices should be close to zero to show that a population does not deviate significantly from the neutral model of evolution. Positive values indicate reduced polymorphism and could suggest a deviation caused by a bottleneck, balancing selection or population subdivision. Negative values indicate high levels of polymorphism, possibly as a result of population expansion (an excess of novel haplotypes) (Tajima 1989; Fu 1997). Secondly, nucleotide mismatch distributions of the pairwise differences were assessed between haplotypes. Mismatch distributions were obtained for each sampling population (20,000 permutations) to infer changes in population size based on the frequency of pairwise differences among haplotypes. Parametric bootstrap indices were applied to corroborate the significance between observed and expected mismatch distribution

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patterns. In addition, corresponding Harpending's raggedness (H_R) and sum of squared deviations (SSD) indices (Harpending 1994) were calculated in ARLEQUIN to determine whether any observed mismatch distributions were drawn from an expanded population (small values) or a stationary one (large values). The parameters of demographic expansion (mutational timescale) Θ_0 and Θ_1 were obtained in ARLEQUIN to determine mutational parameters before and after demographic expansion (Harpending 1994). Population expansion times were determined from time (τ), which is the age of expansion expressed as a unit of mutation derived from mismatch distributions calculated in ARLEQUIN. The mutational timescale τ were estimated by the equation $\tau = 2\mu t$ (with t = the number of generations and μ = the mutation rate) (Rogers & Harpending 1992). With the lack of species-specific mutation rate (μ) for *G. galeus*, $\mu = 2.15 \times 10^{-9}$ substitutions/sites/year was used as suggested by Dudgeon *et al.* (2012). Expansion time, T, was estimated by multiplying t by the average generation time of *G. galeus* (17.7 years) (Cortes 2002).

3.3.2. *Microsatellite analyses*

Microsatellite scoring errors due to stuttering, large allele dropouts and null alleles were assessed in MICROCHECKER 2.2.3 (van Oosterhout *et al.* 2004). Tests for departures from Hardy Weinberg equilibrium (HWE) were performed using a Fisher's exact test implemented in GENEPOP 4.2 (Rousset, 2008) while pairwise linkage disequilibrium (LD) was determined using FSTAT 2.9.3 (Goudet 2002). Indices of genetic diversity such as mean number of alleles (N_A), number of effective alleles (N_E), number of private alleles (N_P), observed and unbiased expected heterozygosity (H_O and uH_E) and inbreeding coefficient (F_{IS}), were estimated for each sampling population in GENALEX 6.5 (Peakall & Smouse 2012).

To determine levels of genetic differentiation, pairwise F-statistics (F_{ST}) (Weir & Cockerham 1984) were calculated in ARLEQUIN. A false discovery rate was determined for multiple tests and applied to minimise type I errors. Additionally, calculations of pairwise G''_{ST} were performed in GENALEX since this F_{ST} analogue provides a standardized allele frequency based estimator of population differentiation (Meirmans & Hedrick 2011). This measure was also compared to the pairwise F_{ST} values for a more robust scrutiny of statistical significance. For population structure assessment, levels of genetic variation within and among populations and ocean basins (defined as Atlantic- and South Indian Ocean) were calculated using the standard AMOVA implemented in ARLEQUIN (Excoffier & Lischer 2010). For both

regional and local analysis, the variance components and fixation indices were partitioned at three levels of hierarchical subdivision: among ocean basins, within populations, and the interaction of both (among + within). On a regional scale across the Southern Hemisphere, AMOVA were conducted to test for genetic subdivision across the three ocean basins. The AMOVA performed on the local sampling populations tested for; 1) panmixia, 2) *a priori* defined grouping of Atlantic- (RI, FB, KL, AB) versus South Indian Ocean sampling populations (SB, PE). The genetic distance matrix for AMOVA was estimated by pairwise differences and the significance levels of the variance components and F-statistics values were tested by 20,000 nonparametric permutations. Tests for isolation-by-distance (IBD) were performed as described for mitochondrial data. Tests for isolation-by-distance (IBD) were performed in GENALEX 6.5 (Peakall & Smouse 2012) by plotting linearized F_{ST} values against geographic distance for both regional and local datasets. Geographic distances were estimated by converting GPS coordinates in to UTM coordinates using the online GPS Coordinate Converter and Maps application (http://netvicious.com/gps/). The distance calculations consisted of the shortest path, via sea, between any two sampling locations. Significance was evaluated for each location using a Mantel test implemented in GENALEX using 10,000 permutations.

For a visual representation of genotypic partitioning, a discriminant analysis of principal components (DAPC) (Jombart *et al*. 2010) was carried out using ADEGENET (Jombart 2008) in R 3.0.2 (R Development Core Team 2013). Finally, a Bayesian clustering analysis was performed in STRUCTURE 2.3.4 (Pritchard *et al.* 2000) to detect the most likely number of ancestral genetic clusters (K). Ten runs were performed for each *K* ranging from 2 to 5 and 2 to 6 for the regional and local datasets respectively. Markov chain Monte Carlo (MCMC) simulation runs of 10^6 iterations were made with 10^5 burn-in periods using the admixture model (Falush *et al.* 2003). The web-based STRUCTURE HARVESTER 0.6.93 (Earl & vonHoldt 2012) was used to visualise STRUCTURE output data and determine the number of *K* using the Δ*K* method (Evanno *et al.* 2005). Individuals were considered as being correctly assigned to a population with a *q* value (*i.e.* its posterior probability of belonging to original population) of at least 80% (Pritchard *et al.* 2000). The STRUCTURE HARVESTER output files were processed in CLUMPP v1.1.2 (Jackobsson & Rosenberg 2007) and structure display plots were visualised in DISTRUCT v.1.1 (Rosenberg 2004). The

STRUCTURE plots were compared for data based on all 19 loci and data based on only the ten species-specific loci.

Demographic parameters were estimated for both the regional and local datasets. The contemporary effective population size (N_e) was estimated for each sampling site using the linkage-disequilibrium (LD) method implemented in NE ESTIMATOR 2.0 (Do *et al.* 2014). Subsequently, two approaches were used to investigate whether there was evidence of a recent population size contraction. BOTTLENECK 1.2.02 (Cornuet & Luikart 1996; Piry *et al.* 1999) was used to determine if sampling populations experienced significant recent reductions in size. The Wilcoxon signed-rank test implemented in BOTTLENECK, was used to examine whether each population exhibited an excess of observed heterozygotes relative to that predicted for a population at mutation-drift equilibrium. Heterozygote excess and allele frequencies were tested with 10,000 simulations under the infinite alleles model (IAM), two-phase model (TPM), and the step-wise model (SMM) (Cornuet & Luikart 1996). Emphasis was placed on the TPM of mutation as it is recommended for microsatellite loci due to a better fit with observed allele frequency data than the IAM and SMM (Di Rienzo *et al.* 1994; Piry *et al.* 1999). The TPM option was set at 95% single-step mutations and 5% multiple-step mutations (Cornuet & Luikart 1996; Piry *et al.* 1999). The average expected equilibrium heterozygosity (H_{eq}) for each locus was compared to Hardy-Weinberg heterozygosity (H_e) and used to assess whether there was a heterozygote excess or deficit. The second approach also used BOTTLENECK but tested for a mode shift of the allele frequency distribution from an L-shaped distribution that develops when the population is at mutation-drift equilibrium (Piry *et al.* 1999).

3.4. Results

3.4.1. *Regional mitochondrial and nuclear descriptive statistics*

Since a few recent studies have indicated the potential for other mtDNA regions to be more informative than the control region for detection of population structure in elasmobranchs (Naylor *et al.* 2012; Tillet *et al.* 2012; Feutry *et al.* 2014), a 671 bp fragment of the *ND2* gene was sequenced and analysed for a total of 96 *G. galeus* samples. Across the Southern Hemisphere, this resulted in a total of 22 haplotypes ranging from one (NZ) to ten (SA) per geographical location. The overall haplotype diversity (*h*) was 0.820 ± 0.039 with a

nucleotide diversity (π) of 0.013 ± 0.010 **Table 3.1**. Only one haplotype was shared between Chile and New Zealand across the Indo-South Pacific region, while all other haplotypes were unique to their geographical locations. No haplotypes were shared between Argentina and South Africa and therefore across the Atlantic Ocean. Atlantic Ocean collections (ARG) showed the highest haplotype diversity ($h = 0.822 ± 0.097$) while collections from the eastern South Pacific (NZ) exhibited no diversity at all. The haplotype network indicated that haplotypes were almost exclusively associated with either of two distinct *ND2* lineages, one including all Atlantic- and Indian Ocean samples and one including mostly south Pacific samples and one divergent haplotype of SA origin **Figure 3.3**.

Table 3.1. Genetic diversity estimates for mtDNA *ND2* sequences of the Southern Hemisphere sampling populations of *Galeorhinus galeus*. Genetic diversity estimates include number of haplotypes (*H*), private haplotypes (H$_P$), polymorphic sites (K), haplotype- (*h*) and nucleotide diversity (π).

Site	N	*H*	H$_P$	K	*h*	Π
CHI	6	4	3	8	0.800 ± 0.172	0.007 ± 0.108
ARG	10	5	5	6	0.822 ± 0.097	0.004 ± 0.002
SA	52	10	10	22	0.599 ± 0.072	0.007 ± 0.021
AUS	9	3	3	4	0.417 ± 0.191	0.002 ± 0.001
NZ	3	1	0	0	0.000 ± 0.000	0.000 ± 0.000
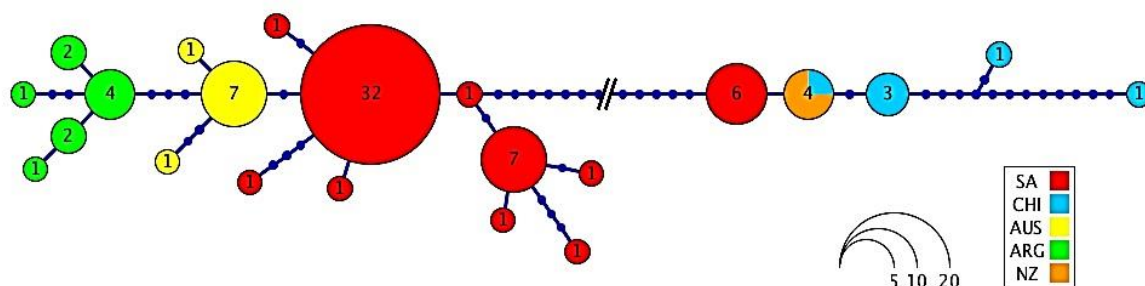
Figure 3.3. Global haplotype genealogy of *Galeorhinus galeus* based on a maximum likelihood tree of *ND2*. Circles represent the haplotypes with area being equivalent to

frequency. Each line indicates one mutational step between haplotypes and small dark blue circles indicate hypothetical missing haplotypes.

For the microsatellites, all diversity estimates including number of alleles (N_A), number of effective alleles (N_E), observed heterozygosity (H_O), unbiased expected heterozygosity (uH_E), and inbreeding coefficient (F_{IS}) are given for each geographic region in **Table 3.2**. The total number of alleles (N_A) ranged from 3 to 11 per sampling site. Unbiased expected heterozygosity (uH_E) shows nuclear genetic diversity to be higher in the Pacific Ocean (NZ, CHI) relative to the rest of the geographic locations. This is also corroborated by the much higher number of effective alleles (N_E) seen for the two Pacific Ocean sampling sites.

Table 3.2. Genetic diversity and demographic estimates of geographical sampling populations of *Galeorhinus galeus* based on 19 microsatellite loci. Diversity estimates include number of alleles (N_A), number of effective alleles (N_E), observed heterozygosity (H_O), unbiased expected heterozygosity (uH_E), and inbreeding coefficient (F_{IS}).

Site	N	N_A	N_E	H_O	uH_E	F_{IS}
CHI	22	10	6.040	0.682	0.807	0.166
ARG	10	3	1.958	0.352	0.373	0.040
SA	124	11	3.333	0.668	0.681	0.028
AUS	9	4	2.545	0.626	0.506	-0.190
NZ	19	8	5.518	0.500	0.763	0.349

3.4.2. *Local mitochondrial and nuclear descriptive statistics*

A total of 52 mtDNA *ND2* sequences from six sampling sites across the coastline of South Africa were analysed. The genetic diversity estimates are summarised in **Table 3.3**. The sequences generated a total of ten haplotypes, with relatively moderate levels of haplotype- ($h = 0.599 \pm 0.072$) and relatively low levels of nucleotide ($\pi = 0.007 \pm 0.021$) diversity. Two common haplotypes were shared amongst 73% of individuals and a single divergent haplotype restricted to Port Elizabeth is separated from the rest by 12 mutational steps. Except for False Bay and Agulhas Bank samples, all other sampling sites exhibited unique

haplotypes with Robben Island and Kleinmond containing three private haplotypes each **Figure 3.4**. Interestingly, haplotype diversity seems to be lowest for sampling populations at and adjacent to the transition zone (*i.e.* SB).

Table 3.3. Genetic diversity and demographic estimates for mtDNA *ND2* sequences of South Africa's regional sampling populations of *G. galeus*. Diversity estimates include number of haplotypes (*H*), private haplotypes (H_P), polymorphic sites (K), haplotype- (*h*) and nucleotide diversity (π). (n.d. Not determined due to lack of polymorphism)

Site	N	*H*	H_P	K	*h*	π
RI	10	5	3	12	0.905 ± 0.103	0.010 ± 0.007
FB	7	1	0	0	n.d.	n.d.
KL	12	5	3	6	0.593 ± 0.144	0.067 ± 0.048
AB	4	2	0	3	0.667 ± 0.314	0.102 ± 0.094
SB	15	3	1	5	0.362 ± 0.145	0.068 ± 0.049
PE	7	1	1	0	n.d.	n.d.

n.d. Not determined due to lack of polymorphism
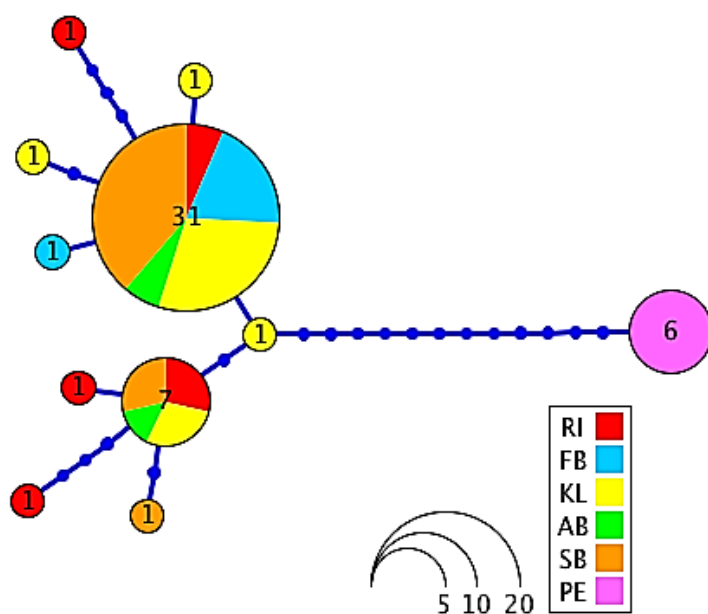
Figure 3.4. Local haplotype genealogy of *Galeorhinus galeus* based on a maximum likelihood tree of *ND2*. Circles represent the haplotypes with area being equivalent to

frequency. Each line indicates one mutational step between haplotypes and small blue circles indicate hypothetical missing haplotypes.

For the microsatellites, MICROCHECKER indicated no scoring errors due to stuttering or the presence of null alleles. All regional sampling populations were in HWE except for Agulhas Bank that showed significant departure from HWE ($P < 0.05$). No linkage disequilibrium was present for any of the loci pairwise comparisons. Nuclear genotypic diversity such as unbiased expected heterozygosity and allelic richness were comparable for *G. galeus* across the Atlantic and Indian Ocean. The overall number of alleles ranged from $N_A = 5$ to 6 in the Indian Ocean, and from $N_A = 4$ to 8 in the Atlantic Ocean. Expected heterozygosity was highest for Robben Island ($uH_E = 0.707$) and lowest for Struisbaai ($uH_E = 0.600$) **Table 3.4**.

Table 3.4. Genetic diversity and demographic estimates of South Africa's regional sampling populations of *Galeorhinus galeus* based on 19 microsatellite loci. Diversity estimates include number of alleles (N_A), number of effective alleles (N_E), observed heterozygosity (H_O), unbiased expected heterozygosity (uH_E), and inbreeding coefficient (F_{IS}). Demographic estimates include coancestry coefficient (Θ_H) and effective population size (N_e).

Site	N	N_A	N_E	H_O	uH_E	F_{IS}	θ_H	N_e
SA (RI)	26	8	3.686	0.615	0.707	0.141	2.591	7826
SA (FB)	11	5	3.050	0.630	0.641	0.021	2.047	3048
SA (KL)	39	7	3.232	0.692	0.679	-0.025	2.114	6455
SA (AB)	10	4	2.851	0.705	0.615	-0.134	1.836	Infinite
SA (SB)	28	6	2.726	0.648	0.600	-0.045	1.575	3402
SA (PE)	10	5	3.003	0.786	0.646	-0.210	2.181	2192

3.4.3. *Regional population connectivity*

Based on the *ND2* gene, genetic differentiation was evident among geographic sampling populations spanning the three oceans (global $\Phi_{ST} = 0.153$, $P = 0.000$). All of the geographic

pairwise comparisons of Φ_{ST} values showed statistically significant differentiation after correcting for multiple tests ($\Phi_{ST} = 0.355$, $P < 0.05$ between Chile and New Zealand, to $\Phi_{ST} = 0.933$, $P = 0.000$ between New Zealand and Australia) **Table 3.5**. This indicated strong inter-oceanic structure among ocean basins. Interestingly, significant genetic differentiation was found between samples from South Africa and Western Australia ($\Phi_{ST} = 0.416$, $P = 0.000$), indicating population isolation even within the Indian Ocean. Apart from overwater distance, the Mid-Atlantic Barrier, the East Pacific Barrier and the Great Australian Bight are biogeographical features proposed to hinder gene flow on an intra-oceanic level.

Table 3.5. Mitochondrial DNA *ND2* sequence pairwise Φ_{ST} values (below diagonal) and *P*-values (above diagonal) compared across the Southern Hemisphere. Statistically significant values are shown indicated with an asterisk.

	CHI	ARG	SA	AUS	NZ
CHI		0.000	0.000	0.000	0.047
ARG	0.853**		0.000	0.000	0.003
SA	0.737**	0.669**		0.000	0.000
AUS	0.874**	0.760**	0.416**		0.004
NZ	0.355*	0.912**	0.695**	0.933**	

**Statistically significant after a false discovery rate ($P \leq 0.027$).

*Statistically significant at $P < 0.05$

For all *a priori* biogeographic scenarios tested using AMOVA, a high percentage of variation was explained by the within-population level of subdivision while also noting a significant level of variation amongst the geographic populations ($\Phi_{ST} = 0.691$, $P = 0.000$). Further grouping hypothesis to test for structuring between ocean basins were not significant ($\Phi_{CT} = 0.145$ to 0.559, $P > 0.05$), irrespective of SA being grouped with the Atlantic- or Indian

Ocean **Table 3.6.** Genetic differentiation across the Southern Hemisphere was further investigated using microsatellite nuclear data. Pairwise F_{ST} values indicated high levels of genetic differentiation on an inter-oceanic and intra-oceanic level across the Southern Hemisphere. Pairwise F_{ST} values ranged from 0.050 to 0.330, $P = 0.000$; with the lowest genetic differentiation found between NZ and CHI on opposite sides of the South Pacific Ocean **Table 3.7**.

Table 3.6. An AMOVA across the Southern Hemisphere of *Galeorhinus galeus* based on mtDNA *ND2* sequence data. Significant fixation indices indicated with an asterisk for *P* < 0.05.

Hypothesis tested	Source of variation	% variation	Fixation indices	*P*-value
Panmixia	Among populations	69.14	$\Phi_{ST} = 0.691^*$	0.000
	Within populations	30.86		
Pacific vs. Atlantic vs. Indian (SA with Atlantic)	Among groups	14.51	$\Phi_{CT} = 0.145$	0.263
	Among populations	56.01	$\Phi_{SC} = 0.655^*$	0.000
	Within populations	29.47	$\Phi_{ST} = 0.705^*$	0.000
Pacific vs. Atlantic vs. Indian (SA with Indian)	Among groups	55.89	$\Phi_{CT} = 0.559$	0.066
	Among populations	18.08	$\Phi_{SC} = 0.410^*$	0.000
	Within populations	26.03	$\Phi_{ST} = 0.740^*$	0.000

Table 3.7. Microsatellite pairwise F_{ST} values (below diagonal) and *P*-values (above diagonal) compared across the Southern Hemisphere. Statistically significant values are shown indicated with an asterisk for $P \leq 0.009$ after a false discovery rate.

	CHI	ARG	SA	AUS	NZ
CHI		0.000	0.000	0.000	0.000
ARG	0.236*		0.000	0.000	0.000
SA	0.136*	0.138*		0.000	0.000
AUS	0.171*	0.330*	0.097*		0.000
NZ	0.050*	0.287*	0.131*	0.163*	

Similarly, AMOVA showed high molecular variation amongst sampling populations (F_{CT} = 0.137, P = 0.000) while none of the *a priori* grouping hypotheses tested was significant **Table 3.8**. The genetic differentiation and molecular variation exhibited here was also corroborated by significant isolation-by-distance (R^2 = 0.063, P = 0.010) **Figure 3.5**.

Table 3.8. An AMOVA across sampling sites of *Galeorhinus galeus* across the Southern Hemisphere based on 19 microsatellite loci. Significant fixation indices indicated with an asterisk for $P < 0.05$.

Hypothesis tested	Source of variation	% variation	Fixation indices	*P*-value
Panmixia	Among populations	13.65	F_{ST} = 0.137	0.000*
	Within populations	86.35		
Pacific vs. Atlantic vs. Indian (SA with Atlantic)	Among groups	5.70	F_{CT} = 0.057	0.135
	Among populations	8.88	F_{SC} = 0.094	0.000*
	Within populations	85.42	F_{ST} = 0.146	0.000*
Pacific vs. Atlantic vs. Indian (SA with Indian)	Among groups	8.060	F_{CT} = 0.081	0.067
	Among populations	6.810	F_{SC} = 0.074	0.000*
	Within populations	85.130	F_{ST} = 0.149	0.000*
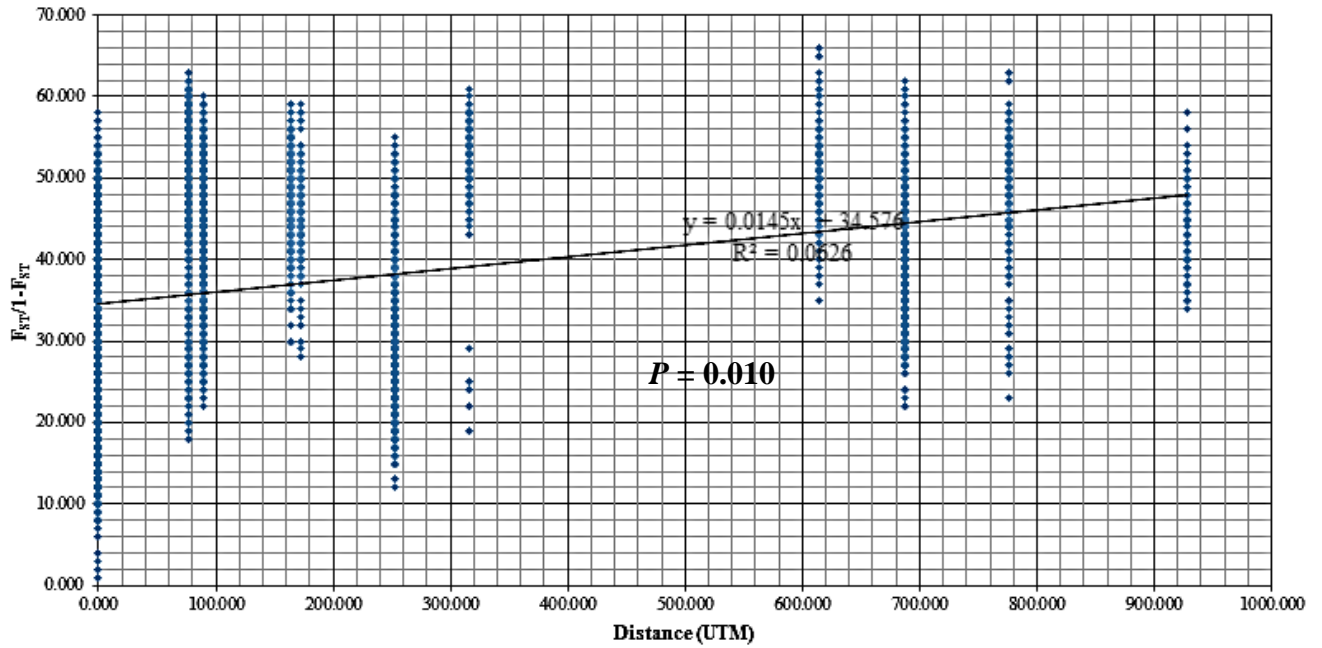
Figure 3.5. A mantel test investigating isolation-by-distance (IBD) between regional sampling sites of *G. galeus* based on microsatellite data.

Population structuring was further investigated by ascertaining the relationship between individual genotypes through DAPC. The DAPC plot confirmed strong separation between the five geographically distinct populations of *G. galeus* with South Africa and Australia clustering the closest. Across the first axis explaining 44% of the variation, the geographic populations could be separated into the South Pacific and the remaining sampling sites
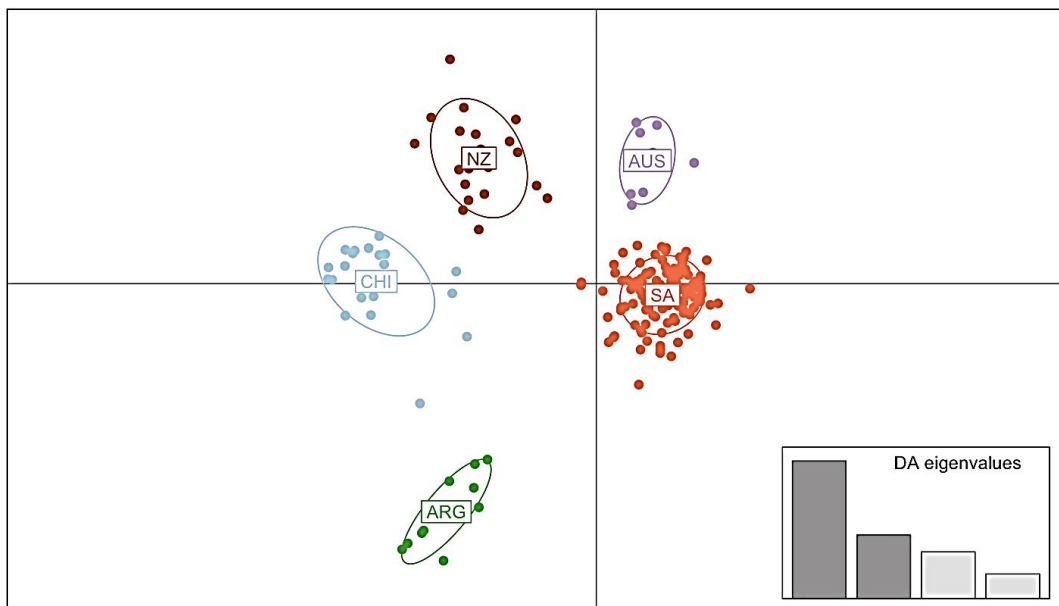
. On a

three-dimensional level of analysis, three main clusters were evident including two clusters

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across the Pacific Ocean (NZ and CHI) and one Atlantic/Indian Ocean cluster (SA, AUS, ARG). The latter only appeared to be separated into a South Western Atlantic (ARG) and an Indian Ocean cluster (SA and AUS) according to the second and third axis.
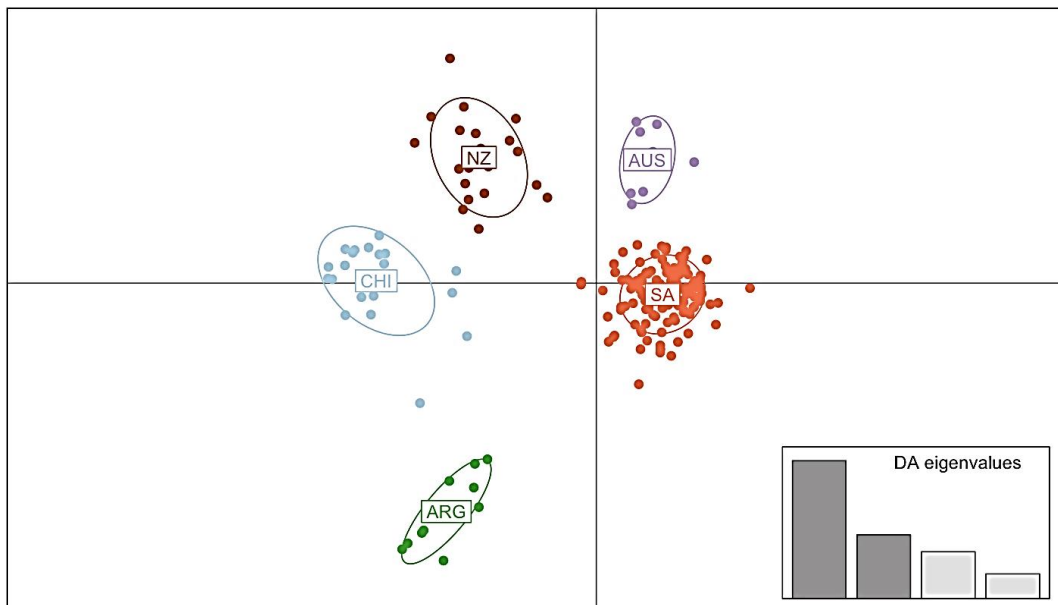
Figure 3.6. A discriminant analysis of principal components (DAPC) plot obtained with the ADEGENET package (Jombart 2008) of five *G. galeus* populations across the three major oceans of the Southern Hemisphere.

Finally, the true number of populations (K) was investigated using Bayesian clustering analysis. Prior to the application of the Evanno method, the normal distribution of L(K) Two clusters (K = 2) were identified based on the delta *K* method and all 19 microsatellites, most likely detecting the lowest level of hierarchical structure (see **Figure 3.** in supplementary material). The assignment plots were investigated for K up to 5 or until genetic subdivision was no longer evident. For both *K* = 4 and *K* = 5 tested, the assignment plots implied a maximum of four populations. Therefore, to rule out possible bias of cross-species microsatellite markers for Bayesian analysis, STRUCTURE were performed based on only the ten species-specific loci and assignment plots were drawn for *K* = 4 and *K* = 5. Both plots displayed a similar pattern of structure of four populations albeit with minor variations in individual assignment. Quite surprisingly, the samples of Chile and NZ exhibited exactly the same pattern of admixture, while samples from Argentina, South Africa and Australia seemed to denote distinct populations **Figure 3.7.** These results are also more consistent with the F-statistics than the multi-variate analysis depicted by the DAPC plots.

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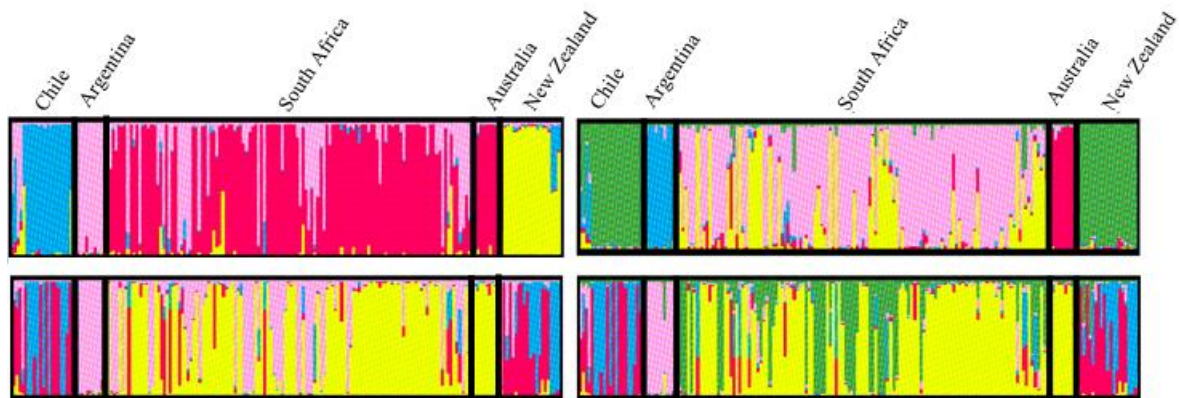
Figure 3.7. STRUCTURE plots showing individual assignments across the Southern Hemisphere. Top plots show structure based on 19 loci and bottom plots are based on 10 species-specific loci. Left is $K = 4$ and right is $K = 5$.

3.4.4. *Local population connectivity*

Analysis of the *ND2* sequence variation across six local populations resulted in ten haplotypes, with one common haplotype shared among all the sampling populations except Port Elizabeth. The pairwise Φ_{ST} values shown in **Table 3.9** corroborated this haplotype's genealogy; reflecting high connectivity across the south-west coast populations ($P > 0.05$). Interestingly, the Port Elizabeth samples showed stronger genetic divergence from all the other samples ($\Phi_{ST} = 0.799$ to 1.000, $P \leq 0.009$). Although significant molecular variation was noted among the populations ($\Phi_{ST} = 0.619$, $P = 0.000$), most of the variation was attributed to amongst individuals within populations. There was also no significant variation between Indian and Atlantic Ocean samples ($\Phi_{CT} = -0.167$, $P = 0.401$) **Table 3.10**.

Table 3.9. Mitochondrial DNA *ND2* sequence pairwise Φ_{ST} values (below diagonal) and *P*-values (above diagonal) for six South African *G. galeus* sampling populations. Statistically significant values are shown indicated with an asterisk for $P \leq 0.009$ after a false discovery rate.

	RI	FB	KL	AB	SB	PE
RI		0.071	0.111	0.481	0.143	0.001
FB	0.286		0.233	0.304	0.320	0.001
KL	0.164	0.006		0.949	0.985	0.000
AB	-0.167	0.300	-0.149		1.000	0.011
SB	0.147	0.036	-0.048	-0.183		0.000
PE	0.779**	1.00**	0.899**	0.937*	0.900**	

For the nuclear data, low to moderate levels of genetic differentiation was exhibited by *G. galeus* across the Atlantic/Indian Ocean transition zone. Pairwise F_{ST} values ranged from 0.003 to 0.091, $P \leq 0.0363$ with the highest pairwise value between the two Indian Ocean populations (PE and SB) **Table 3.11**. The G''_{ST} values which ranged from 0.071 to 0.288, $P \leq 0.0363$ corroborated this **Table 3.12.** Interestingly, there was low but significant differentiation between both Struisbaai and Agulhas Bank and the rest of the populations. No molecular variation was detected amongst the two oceanic groups tested for *a priori* (F_{CT} = -0.000, P = 0.273) while low but significant molecular variation was detected amongst the sampling populations (F_{ST} = 0.025, P = 0.000) **Table 3.13**. The genetic differentiation and molecular variation detected within oceanic groups are most likely a consequence of isolation-by-distance (R^2 = 0.012, P = 0.010) **Figure 3.8**.

Table 3.10. AMOVA across among South Africa's regional sampling sites of *Galeorhinus galeus* based on mtDNA *ND2* sequence data. Significant fixation indices indicated with an asterisk for $P < 0.05$.

Hypothesis tested	Source of variation	% variation	Fixation indices	*P*-value
Regional Panmixia	Among populations	61.92	$\Phi_{ST} = 0.619*$	0.000
	Within populations	33.08		
Oceanic (Atlantic vs. Indian)	Among groups	-16.72	$\Phi_{CT} = -0.167$	0.401
	Among populations	76.15	$\Phi_{SC} = 0.652*$	0.000
	Within populations	40.57	$\Phi_{ST} = 0.594*$	0.000

Table 3.11. Microsatellite pairwise F_{ST} values (below diagonal) and *P*-values (above diagonal) compared across the south-west coast of South Africa. Statistically significant values are indicated with an asterisk for $P \leq 0.0363$ after a false discovery rate.

	RI	**FB**	**KL**	**AB**	**SB**	**PE**
RI		0.064	0.446	0.036	0.000	0.000
FB	0.011		0.027	0.009	0.000	0.036
KL	-0.000	0.017*		0.118	0.000	0.000
AB	0.016*	0.048*	0.003		0.005	0.000
SB	0.030*	0.045*	0.028*	0.020*		
PE	0.030*	0.023*	0.040*	0.073*	0.091*	

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Table 3.12. Microsatellite pairwise G"$_{ST}$ values (below diagonal) and *P*-values (above diagonal) compared across the south-west coast of South Africa. Statistically significant values are shown indicated with an asterisk for *P* ≤ 0.0363 after a false discovery rate.

	RI	FB	KL	AB	SB	PE
RI		0.318	0.487	0.090	0.001	0.004
FB	0.011		0.052	0.005	0.001	0.009
KL	-0.001	0.045		0.060	0.001	0.001
AB	0.052	0.130*	0.037		0.002	0.001
SB	0.084*	0.110*	0.081*	0.071*		0.001
PE	0.139*	0.108*	0.170*	0.249*	0.288*	

Table 3.13. An AMOVA across six South African sampling populations of *Galeorhinus galeus* using 19 microsatellite loci. Significant fixation indices indicated with an asterisk for *P* < 0.05.

Hypothesis tested	Source of variation	% variation	Fixation indices	*P*-value
Panmixia	Among populations	2.48	$F_{ST} = 0.025$	0.000*
	Within populations	97.52		
	Among groups	-0.01	$F_{CT} = -0.000$	0.273
Oceanic (Atlantic vs. Indian)	Among populations	2.48	$F_{SC} = 0.025$	0.000*
	Within populations	97.53	$F_{ST} = 0.0247$	0.000*

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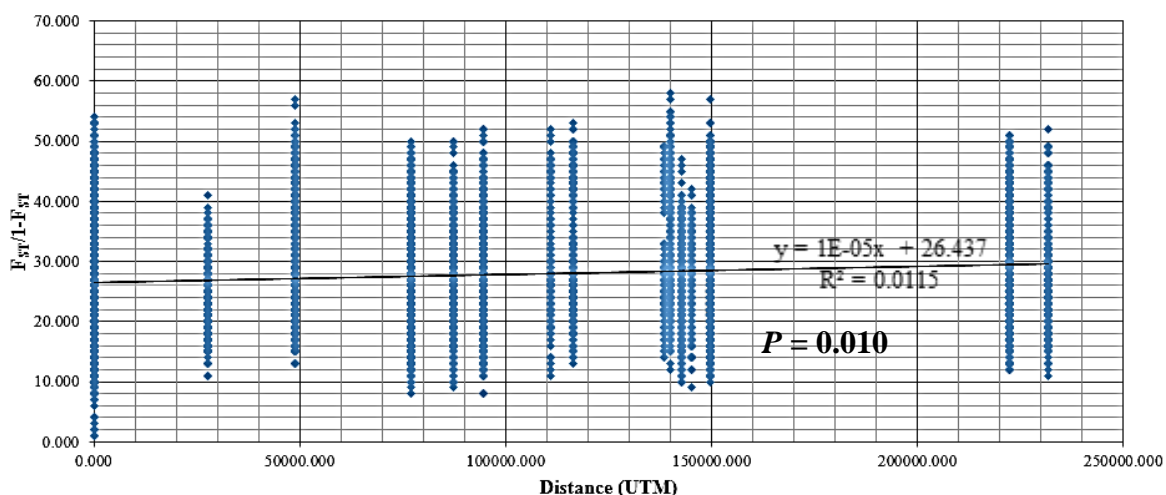
Figure 3.8. Results of a mantel test for isolation-by-distance (IBD) between local sampling sites of *G. galeus*.

With the DAPC, the sampling population of Port Elizabeth clustered separately while the Struisbaai population also showed less overlap with the rest of the sampling populations **Figure 3.9**. Bayesian clustering analysis in STRUCTURE determined $K = 2$ for the local populations. When tested at higher hierarchical levels, the assignment plots again depicted up to three distinct populations but with considerable admixture across the Atlantic Ocean samples **Figure 3.10**. The Indian Ocean samples showed the least level of admixture and a clear distinction between Port Elizabeth and Struisbaai for all the assignment plots **Figure 3.11**.
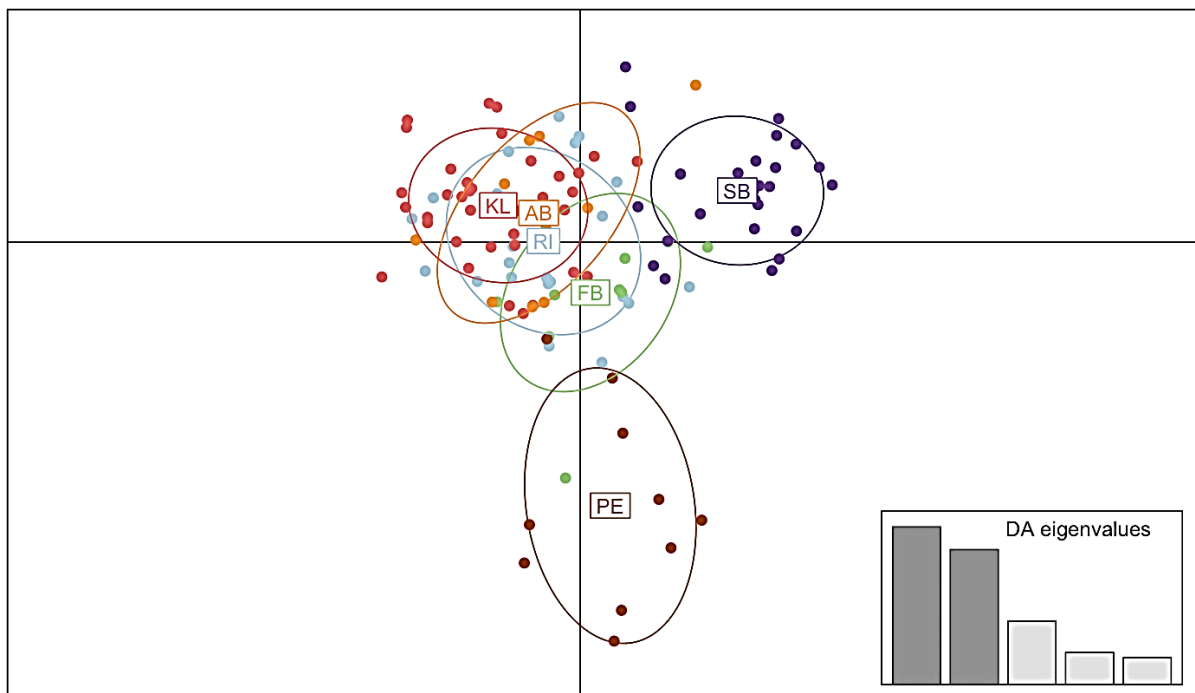
Figure 3.9. A discriminant analysis of principal components (DAPC) plot depicting the relationships of *G. galeus* populations across the South African coastline.
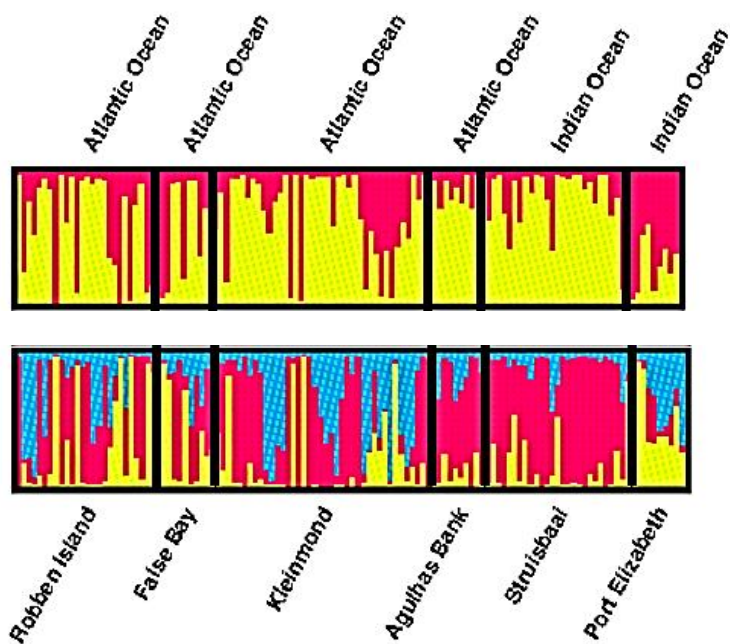
Figure 3.10. STRUCTURE plots showing individual assignments across South Africa showing top $K = 2$ and bottom $K = 3$.
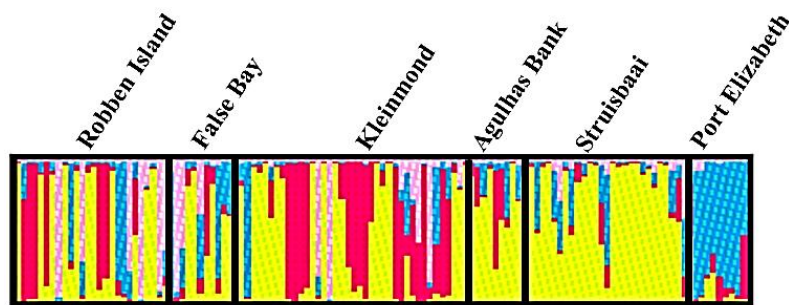
Figure 3.11. STRUCTURE plots showing individual assignments across South Africa at *K* = 4 based on 19 microsatellite loci.

3.4.5. *Demographic history of Galeorhinus galeus*

The parameters of neutrality and population demographic history for *G. galeus* presented by sampling location were not indicative of population expansions or bottlenecks within the Southern Hemisphere or South Africa. Across the Southern Hemisphere, the distribution of the expected and observed pairwise differences for mtDNA *ND2* sequences from all ocean basins (the south Pacific, the Atlantic and Indian Ocean) deviated from the sudden expansion model. These patterns were corroborated by results of goodness-of-fit tests **Table 3.14** for the observed mismatch distributions, which were non-significant for four of the geographic sampling populations (ARG, CHI, NZ, AUS). These findings were further supported by non-significant Tajima's D values ($P > 0.05$) **Table 3.14**.

On a local scale, collections from Port Elizabeth and Struisbaai showed signatures of past population expansion that was corroborated by the significant Tajima's D value (D = -2.478, $P = 0.000$) reflecting an excess of rare polymorphisms and population expansion in the past **Table 3.15**. However, since the haplotype genealogies depict a deep separation of two clades across the Southern Hemisphere **Figure 3.3** and locally in South Africa **Figure 3.4**, the analysis of demographic history was thus presented by clade rather than by sampling location. Two clades were investigated regionally; the Indo-Atlantic clade and the Indo-Pacific clade. Tests for neutrality indicated a departure from mutation-drift equilibrium for both clades with statistically significant negative Tajma's D values **Table 3.14**. A process of expansion is suggested by the unimodal curve detected for the Indo-Atlantic clade **Figure 3.12**, but was not statistically supported ($F_S = -0.493$, $P = 0.490$) and (SSD = 0.050, $P = 0.192$). However, the sharp drop from the crest in mismatch distributions for the Indo-Atlantic clade indicates a rapid loss of genetic diversity. Athough the expansion model was

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rejected, the start of this decline is roughly estimated around 1.8 million years ago. A display of a multimodal curve for the mismatch distributions within the Indo-Pacific clade indicates a deviation from the population expansion model **Figure 3.12** and this was corroborated by a non-significant Fu's F_S value ($F_S = 4.028$, $P = 0.990$).

On a local scale, the unimodal curve of mismatch distributions suggested a period of population expansion for the Atlantic clade (including Struisbaai) **Figure 3.13** although this was not statistically supported by the Fu's Fs value **Table 3.15**. There was a deviation from the expansion model for the Indian Ocean clade (Port Elizabeth) as signified by the multimodal curve of mismatch distributions, although deviation from mutation-drift equilibrium ($D = -1.108$, $P = 0.000$) was evident.
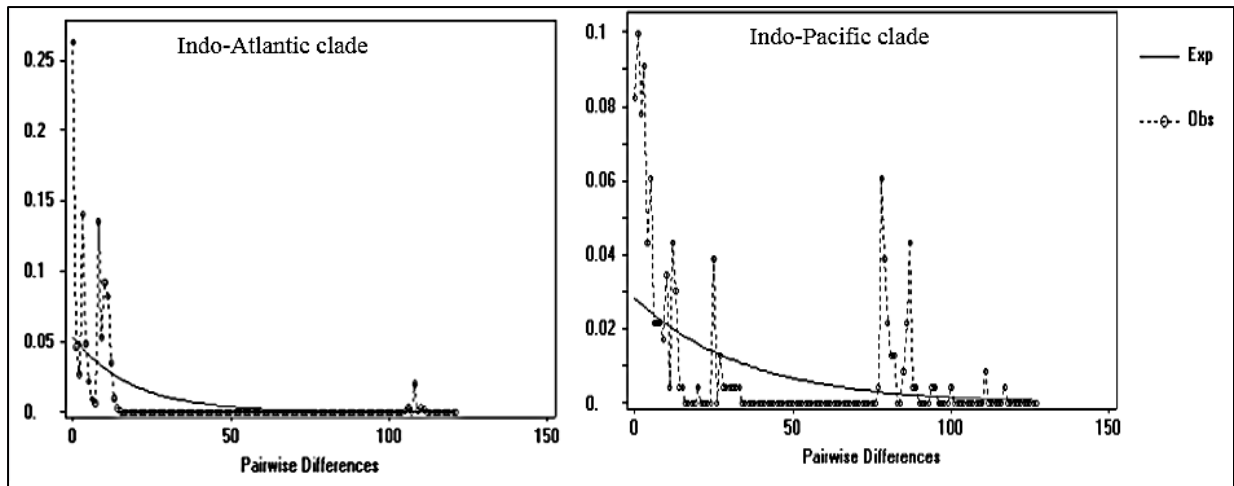
Figure 3.12. Distribution of the expected (Exp) and observed (Obs) pairwise differences for mtDNA *ND2* sequences across the Indo-Atlantic clade; Argentina (ARG), South Africa (SA), Australia (AUS) and the Indo-Pacific clade; Chile (CHI), Port Elizabeth, SA (PE), New Zealand (NZ).
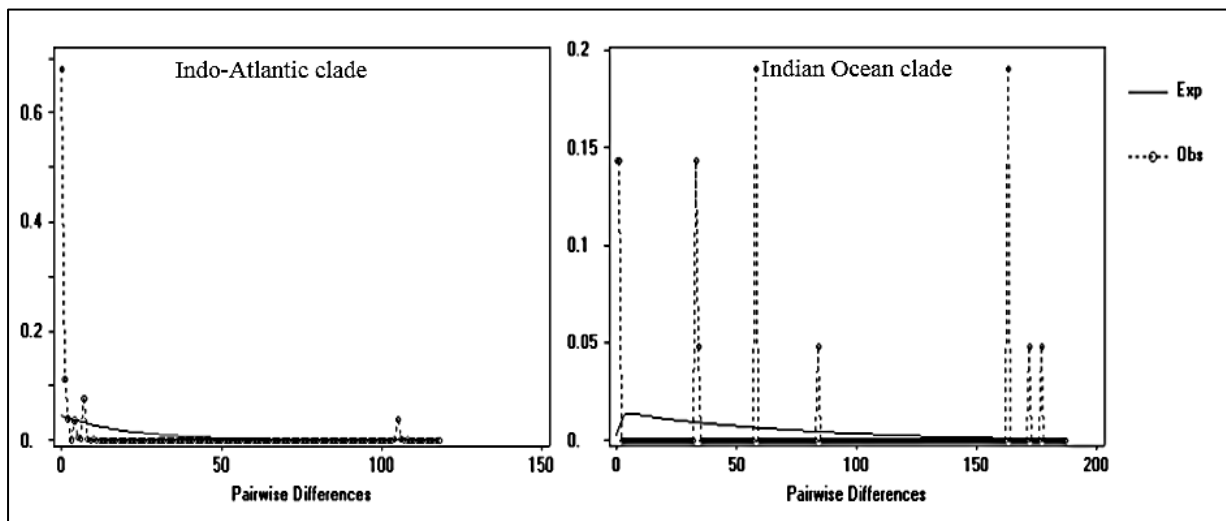
Figure 3.13. Distribution of the expected (Exp) and observed (Obs) pairwise differences for mtDNA *ND2* sequences in the local sampling clades of South Africa. Indo-Atlantic clade; Robben Island (RI), Kleinmond (K), Struisbaai (SB) and Indian Ocean clade; Port Elizabeth (PE).

Table 3.14. Demographic analysis parameters for mtDNA *ND2* sequences of the Southern Hemisphere sampling populations and two major clades of *Galeorhinus galeus* including neutrality test estimates Tajima's test (D) and Fu's test (F_S), sum of squared distribution (SSD), Harpending's raggedness index (H_R), age of population mutational time (τ), population size before (Θ_0) and after expansion (Θ_1), time since population expansion occurred for mutational rate 2.15 X 10^{-9} (T) and the coancestry coefficient (Θ_S).

Site/clade	Neutrality tests		Demographic estimates						
	D	F_S	SSD	H_R	τ	Θ_0	Θ_1	T	Θ_S
CHI	-1.072 ($P = 0.186$)	0.153 ($P = 0.477$)	0.030 ($P = 0.677$)	0.0933 ($P = 0.812$)	4.076	0.000	9.536	1.678E^5	3.504
ARG	-0.973 ($P = 0.208$)	-1.084 ($P = 0.153$)	0.331 ($P = 0.080$)	0.400 ($P = 0.250$)	2.929	0.900	3.600	1.206E^5	0.353
SA	-2.656 ($P = 0.000$)	7.209 ($P = 0.980$)	0.009 ($P = 0.390$)	0.253 ($P = 0.630$)	3.000	0.000	0.452	1.210E^5	26.239
AUS	-1.088 ($P = 0.230$)	-0.264 ($P = 0.130$)	0.307 ($P = 0.100$)	0.358 ($P = 0.300$)	2.929	0.900	3.600	1.206E^5	0.368
NZ	no polymorphism	no polymorphism	n.d.	n.d.	0.000	0.000	0.000	0.000	0.000
Indo-Atlantic	-2.308 ($P = 0.000$)	-0.493 ($P = 0.490$)	0.050 ($P = 0.192$)	0.098 ($P = 0.084$)	10.721	0.002	6.026	1.858E^6	24.429
Indo-Pacific	-1.713 ($P = 0.000$)	4.028 ($P = 0.990$)	0.016 ($P = 0.940$)	0.019 ($P = 0.910$)	81.656	0.004	7.556	1.415E^7	43.650

n.d. Not determined due to lack of polymorphism.

Table 3.15. Demographic estimates for regional mtDNA *ND2* sequences of *Galeorhinus galeus*. Demographic analysis parameters include Tajima's test (D) and Fu's test (F_S), sum of squared distribution (SSD), Harpending's raggedness index (H_R), age of population mutational time (τ), population size before (Θ_0) and after expansion (Θ_1), time since population expansion occurred for mutational rate 2.15 X 10^{-9} (T) and coancestry coefficient (Θ_S) .

Site/clade	Neutrality tests		Demographic analysis						
	D	F_S	SSD	H_R	τ	Θ_0	Θ_1	T	Θ_S
RI	-0.279 (P = 0.430)	1.955 (P = 0.860)	0.384 (P = 0.00)	0.388 (P = 0.950)	0.000	0.000	99999	0.000	2.121
FB	no polymorphism	no polymorphism	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
KL	no polymorphism	no polymorphism	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
AB	no polymorphism	no polymorphism	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
SB	-2.478 (P = 0.000)	16.083 (P = 1.000)	0.026 (P = 0.090)	0.787 (P = 0.740)	0.300	0.000	0.079	$1.235E^4$	27.679
PE	-1.108 (P = 0.000)	5.744 (P = 0.980)	0.132 (P = 0.070)	0.211 (P = 0.080)	136.014	0.000	31.865	$5.600E^6$	67.755
Indo-Atlantic	-2.186 (P = 0.000)	3.880 (P = 0.880)	0.018 (P = 0.26)	0.345 (P = 0.580)	3.000	0.000	0.380	$5.199E^5$	25.256

n.d. Not determined due to lack of polymorphism.

For effective population size estimates, emphasis was placed on the South African samples of *G. galeus* with higher sample numbers. Comparisons of the contemporary and the historical effective population sizes were therefore only made for local samples. Higher levels of contemporary effective population sizes (N_e) were detected for samples from the Atlantic Ocean (N_e = 3048 to 7826) versus the Indian Ocean (N_e = 2192 to 3402). The highest effective population size was detected for Kleinmond (infinite). There was only evidence of a bottleneck with the Wilcoxon test under the two-phase model (TPM) for samples from Agulhas Bank and Port Elizabeth (**Table 3.16**).

Table 3.16. Test for mutation-drift equilibrium in BOTTLENECK under the IAM, TPM and SMM. Significant *P*-values for Wilcoxon's test are indicated with an asterisk.

Site	IAM	TPM	SMM	Mode shift
RI	0.002*	0.768	0.079	L-mode
FB	0.001*	0.275	0.374	L-mode
KL	0.000*	0.859	0.002*	L-mode
AB	0.000*	0.005*	0.374	Shifted
SB	0.000*	0.332	0.079	L-mode
PE	0.000*	0.026*	0.798	L-mode

3.5. Discussion

3.5.1. *Genetic diversity of Galeorhinus galeus with focus on South Africa*

As this is the first time that *G. galeus* are being assessed along the South African coastline, more focus was placed on comparing genetic diversity estimates with other elasmobranch species exhibiting similar life history patterns. The overall *ND2* haplotypic diversity of *G. galeus* in South Africa (*h* = 0.599) is slightly lower than those reported for other commercially exploited shark species (Benavides *et al.* 2011; Karl *et al.* 2012; Chabot *et al.* 2015; Clarke *et al.* 2015; Hernández *et al.* 2015). This was also slightly lower than what was reported for the South African common smoothhound shark, *Mustelus mustelus*, the only

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other commercially exploited species assessed for this region (Maduna 2014). The latter study reported overall *ND4* haplotype (*h*) and nucleotide diversity (*π*) of 0.517 ± 0.069 and 0.00104 ± 0.00386, respectively for pooled samples spanning a similar coastal distance. It is quite possible that this low diversity reflects the inherent properties of the mitochondrial gene used or even the low sample numbers investigated for some populations. However, Naylor *et al.* (2012) analysed 4,283 *ND2* sequences from 595 different elasmobranch species and found substantial intraspecific divergences among populations. Also, a recent study using whole mitogenomes of 93 critically endangered speartooth shark, *Glyphis glyphis,* demonstrated that among the single gene sub-datasets, *ND5* was the most diverse followed by *ND2* while the control region was not able to detect the existing substructure at all (Feutry *et al.* 2014).

In general, higher haplotype diversities have been reported for pelagic shark species with wider distribution ranges (*e.g. Prionace glauca* and *Carcharhinus falciformis*) than for coastal sharks with smaller distribution ranges (Karl *et al.* 2011; Clarke *et al.* 2015). In South Africa, although not commercially exploited and a protected species, the white shark *Carcharodon carcharias* exhibits a remarkably low haplotypic diversity based on >800 bp of the control region and 238 samples (Andreotti *et al.* 2015). Nonetheless, the low mtDNA *ND2* haplotype diversity levels reported here could well be a reflection of the substantial exploitation the species has encountered in the past and continues to encounter along the South African coastline (McCord 2005; Silva & Bürgener 2007; DAFF 2012). Reductions in genetic diversity related to overexploitation have been well documented in a number of shark species (Ferretti *et al.* 2010; Worm *et al.* 2013). Based on 19 microsatellite loci characterized for *G. galeus*, a moderate level of genetic diversity was exhibited across all six sampling sites ($H_O = 0.679$). The levels of genetic diversity were relatively similar across the coastline with the exception of Port Elizabeth with high levels of heterozygosity ($H_O = 0.786$). Taking into account the directed fishing efforts for *G. galeus* that are most lucrative in the Western Cape and very rarely occur in the Eastern Cape, it is likely that the higher levels of genetic diversity in the Eastern Cape region could be as a result of lower fishing pressure. Alternatively, the PE population constitutes historically admixed sharks (as supported by the divergent haplotype and close affinity with New Zealand and Chile) and harbours a mixture of all the regional stocks thus reflecting a higher diversity. Interestingly, Port Elizabeth bay is characterized by shallow pockets of cool water (McCord 2005; Griffiths *et al.* 2010) which are believed to harbour the species during parturition (McCord 2005).

3.5.2. *Patterns of historical and contemporary dispersal across the Southern Hemisphere*

This study provides new insight into historical and contemporary connectivity of *G. galeus* across the Southern Hemisphere, with findings including the entire distribution range of the species in this region. This study shows that, based on mtDNA *ND2* haplotypes, historical dispersal does exist for *G. galeus* across the South Pacific with NZ and CHI sharing a single haplotype. This was supported by pairwise Φ_{ST} values and haplotype genealogy. These findings and the lack of haplotype connectivity between ARG and CHI are in contrast to previous findings suggesting that there is no connectivity across the South Pacific and that gene flow does in fact exist between the Atlantic and the Pacific oceans (Chabot & Allen 2009). The latter study also postulated that South America had only one historical population but placed uncertainty on the interpretation of results due to low sample sizes used *e.g.* one sample from Argentina pooled together with 11 samples from Peru. Additionally, results for the current study show that *G. galeus* from Western Australia and New Zealand exhibit a high degree of genetic differentiation, while *G. galeus* from South Africa and Western Australia show high levels of historical gene flow. The haplotype genealogy also confirmed that Port Elizabeth is more connected to haplotypes from the Indo-Pacific region than with the rest of South Africa.

The deep separation of clades suggests that the lineage split is much older than the current distribution of genetic diversity. This historical range devide seen in *G. galeus* is most likely explained by either vicariance or lineage sorting (Moritz *et al*. 2004; Cunha *et al*. 2012). Since it is difficult to confidently ascertain lineage sorting, two models of vicariance were considered; the closure of the Tethyan corridor (12 to 20 million years ago) (Briggs 1974) and the emergence of the Isthmus of Panama (3.5 million years ago) (Coates *et al*. 2004). The closure of the Tethyan corridor occured at a time when the African and Eurasian plate converge and the warm coastal Tethyan corridor is eliminated between the Atlantic and Indian oceans (Musick *et al*. 2004). The network genealogies revealed two clades that more or less corresponded to an Atlantic and a Pacific clade indicating a closure of the subtropical seaway connecting the Atlantic and Pacific oceans. This could suggest vicariance as a result of the emergence of the Isthmus of Panama also previously explained for the copper shark, a similar cosmopolitan coastal shark (Benavides *et al*. 2011). Dating the separation of the two clades is complicated by the lack of information on the evolutionary rate of the *ND2* gene in sharks but a rough comparison of the two clades point to a genetic partition older than 3.5

million years ago. Nonetheless, the haplotypes of *G. galeus* from the Atlantic/Indian clade (SA, ARG, AUS) are separated by a significant number of mutations (> 25 mutations) from the Pacific clade (NZ, CHI) supporting deep historical vicariance and it is proposed that both the rise of the Isthmus of Panama and the Tethyan closure played a role in shaping the present day distribution of the cosmopolitan tope shark. In addition, the mixed ancestry seen for the South African populations of *G. galeus* suggest that South African waters were a site of secondary contact following colonisation from at least two refugia.

Based on the microsatellites, contemporary gene flow was not evident between all geographic sampling sites implying that known biogeographic barriers in the Southern Hemisphere can hinder gene flow for *G. galeus* over smaller and larger spatial scales. For instance, the presence of the Benguela Barrier in the Atlantic Ocean combined with the presence of gyres and straits, most likely restricts gene flow between SA and ARG while the Great Australian Bight seems to be a barrier between Western Australia and NZ. It should be noted that the panmictic population of *G. galeus* previously found between Australia and New Zealand (Hernández *et al.* 2015), is due to the fact that the latter study included samples from Southern Australia on the same side of the GAB barrier.

Previous tagging efforts across the Southern Hemisphere have shown that *G. galeus* does exhibit extensive migratory patterns within two of the major ocean basins (McCord 2005; Francis 2010; Cuevas *et al*. 2014). On a local scale, McCord 2005 showed that *G. galeus* aggregates to South Africa during autumn (March to May) and spring (September to November) when water temperatures are slightly cooler. Across the Tasman Sea, Francis 2010 showed that *G. galeus* migrates between New Zealand and Southern Australia and that these migrations occur more often over time. Within the South West Atlantic, Cuevas *et al*. 2014 studied the movement patterns of this species and showed that, similar to aggregation patterns noted in South Africa, *G. galeus* tends to aggregate in to embayments and shallow coastal areas during spring and late summer. This study also further showed that the species prefers cool temperate waters ranging from temperatures between 17°C and 19°C and exhibits a yo-yo oscillatary movement within the ocean. The seasonal migratory patterns revealed by the aforementioned studies seem to indicate that *G. galeus* uses coastal bay areas as nursery grounds and seasonally aggregates towards these sites. These migratory patterns could also be very important in making more informed regulatory policies when it comes to protecting these nursery grounds.

3.5.3. *Genetic connectivity on a local scale*

The regional sampling populations across the Atlantic/Indian Ocean transition zone of South Africa did not show substantial inter-oceanic *ND2* divergence, except for the Indian Ocean population of Port Elizabeth. Two main genealogical clades were detected; an Indo-Atlantic clade in which Struisbaai almost exclusively shared haplotypes with the East Atlantic Ocean samples indicating historical admixture between the two oceans, and an exclusive clade containing only Port Elizabeth samples. The presence of a highly divergent Port Elizabeth cohort indicates a possibility of a localized haplotype in that area resulting either from philopatry or historical isolation. The presence of this very distinct haplotype at Port Elizabeth suggests that perhaps the thermal front in this particular area also plays a role in defining the phylogeography of local *G. galeus*. In an attempt to ascertain the origin of the private haplotypes found at Port Elizabeth, a different dataset was compiled in which mtDNA *ND2* sequences from Port Elizabeth were compared to the samples from the Indo-Pacific. This analysis confirmed that Port Elizabeth does indeed harbour different ancestral haplotypes and most likely share a historical link with the South-Pacific. It is possible that the remnant historical genetic link across a large expanse of Indo-Pacific overlay could be associated with an intensified Agulhas return current that occurred during the last warm inter-glacial period (Pleistocene) and has persisted for 17,000 years since the Pleistocene. The Agulhas return current has been shown to also aid gene flow among shark species that have wide distribution ranges such as *Squalus acanthias* (Veríssimo *et al.* 2010), *Sphyrna lewini* (Daly-Engel *et al.* 2012) and *Carcharhinus brevipinna* (Geraghty *et al.* 2013).

Furthermore, the F-statistics based on the microsatellite dataset confirmed low levels of genetic differentiation amongst local populations. Intra-oceanic differentiation was however evident amongst samples of the Indian Ocean illustrating some level of contemporary restriction to gene flow. Both these estimates were used to account for the bias generated when using cross-species markers that show high levels of heterozygosity and to account for the differences in the number of samples genotyped. The STRUCTURE analysis showed a clinal variation at $K = 4$, with higher levels of admixed assignment at Atlantic Ocean samples, lesser admixture for Agulhas Bank and Struisbaai, and an almost 100% assignment of Port Elizabeth to a distinct cluster of Indian Ocean origin. This finding supports the hypothesis that the barrier between the Atlantic/Indian Ocean transition zone occurs at Agulhas Bank and this fragmentation is as a result of isolation-by-distance and could also be

driven by the cold water pockets found at the thermal front found at Port Elizabeth (Teske *et al*. 2011).

Overall, the hypothesis that *G. galeus* exhibits genetic discontinuity across the Atlantic/ Indian Ocean transition zone in South Africa cannot be rejected as clear restrictions of historical and contemporary gene flow were detected. Rather an additional boundary was detected and genetic differentiation is not just as a result of the Cape Agulhas boundary but rather driven by a combination of habitat preference, thermal fronts that generate cold water pockets and upwelling which could perhaps alter the vertical oscillations of the species as was shown across the South West Atlantic (Cuevas *et al*. 2014). In a previous study including only one collection of Indian Ocean samples, varying levels of genetic admixture for South African *G. galeus* were predicted to occur as a result of habitat preference (Bitalo *et al*. 2015). Furthermore, the phylogeographic and contemporary gene flow patterns of the species are linked to the bioregions found at the south-west coastline and are indicative of both historical admixture and isolation by distance. The heterogeneity found with the nuclear data supports limited dispersal of both sexes while the mitochondrial heterogeneity seen between Port Elizabeth and the remaining samples could indicate possible reproductive philopatry for females. The latter however warrants further investigation constituting a bigger sampling effort.

3.5.4. *Demographic history*

For this data set, recent population expansions were identified for all of the Indo-Atlantic collections (AUS, SA, ARG) to occur approximately during the same time frame. This demographic event is characterised by a long historical period of population expansion that began before the last glacial Pleistocene (18,000 years before present). However, there was no statistical support for this trend and could be due to the changes caused by the ice age which could have disrupted migration patterns more than what have been established during the interglacial periods over 50,000 to 70,000 years ago (Duncan *et al.* 2006). Interestingly enough, more recent expansions were detected for the Indo-Atlantic clade in comparison to the Pacific clade. It is likely that after the rise of the Isthmus of Panama and the subsequent warmer interglacial period over the last 25,000 years new habitats opened up and promoted population expansion in the Southern Hemisphere counries within Atlantic waters. A similar pattern was seen in other shark species such as *Sphyrna lewini* (Duncan *et al.* 2006), *Carcharhinus limbatus* (Keeney & Heist 2006), *Carcharhinus brachyurus* (Benavides *et al.*

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2011) and *Carcharhinus leucas* (Tillett *et al.* 2012) which showed dramatic population expansion trends during the Pleistocene.

Despite the evidence for population expansion in many species, the time of such expansions may differ among species and populations since they depend on for example the mutation rate, life history traits and population structuring (Avise 2000; Dudgeon *et al.* 2012). Coordinated expansion events across populations are not necessarily expected to be observed unless shared environmental and historical factors obscured evidence of lineage specific adaptation, as was seen in elasmobranchs inhabiting a similar environment across a small spatial scale [e.g. for *G. galeus* in southern Australia and New Zealand (Hernández *et al.* 2015) and *P. glauca* in the Pacific Ocean (Taguchi *et al.* 2015)]. This synchrony in population expansions supports the argument that current genetic variation may be the result of a major regional event over all populations. Currently, this study does not have the necessary information or samples from the appropriate temporal scales to determine the environmental changes associated with the historical events that influenced population dynamics of *G. galeus* across the Southern Hemisphere. However, statistical analyses have been used to propose an estimation of the timeframe in which these demographic events occurred and hypothesize on the environmental causes behind these observations; especially in regards to the implications for sustainable fisheries of *G. galeus* within South Africa. Thus, if the expansion event occurred across the southern African region approximately 25,000 years ago, it can be suggested that this population expansion reflects the much more recent colonisation of *G. galeus* in this region as opposed to New Zealand and Chile for example.

3.6. Management implications

Genetic data analysis plays an integral role in making recommendations for fisheries management policies. When genetic markers show that fishery populations are genetically subdivided, the best explanation is that the differences have arisen because of restricted gene flow. In this study, the genetic data suggests that there are two differentially admixed populations of *G. galeus* within the Atlantic Ocean and Indian Ocean, plus one distinct population in Port Elizabeth. The data also suggests that the Atlantic/Indian Ocean transition zone restricts both contemporary and historical gene flow with the barrier found at Agulhas Bank. However, this transitional zone may not be the only driver behind these gene flow patterns but rather a combination of habitat preference by the species, thermal fronts and bathymetric properties are also at play. Furthermore, the distinct mtDNA *ND2* haplotype

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detected at Port Elizabeth is believed to represent females that are sheltered for reproduction purposes and this area should be investigated as a potential nursery for future conservation efforts. On a geographical scale, all sampled populations should ideally be managed as separate stocks. This implies that any form of replenishment in the Pacific, Atlantic and Indian oceans will have to be done locally and there should not be any input from geographically distant populations.

Supplementary data

Table 3.17. Regional and local sampling sites, sample numbers (N) and sampling dates

Group	Site	N	Coordinates	Sampling date
Pacific Ocean	Chile (CHI)	22	52.55"33°SE 07.14"17°SE	2006
	New Zealand (NZ)	19	47.4"33°SE 24.16"18°SE	2010
Atlantic Ocean	Argentina (ARG)	10	48.8"34°SE 58.17"18°SE	2010
Atlantic/Indian	South Africa (SA)	124	54.09"34°SE 17.57"18°SE	2008-2015
Indian Ocean	Australia (AUS)	9	37.68"34°SE 12.12"20°SE	2013
Atlantic Ocean	Robben Island (RI)	33	47.4"33°SE 24.16"18°SE	2009-2012
	False Bay (FB)	11	54.09"34°SE 17.57"18°SE	2012
	Kleinmond (K)	37	45"34°SE 32.77"19°SE	2012
Transition zone	Agulhas Bank (AB)	10	50"33°SE 24"18°SE	2012
Indian Ocean	Struisbaai (SB)	26	37.68"34°SE 12.12"20°SE	2013
	Port Elizabeth	10		2015
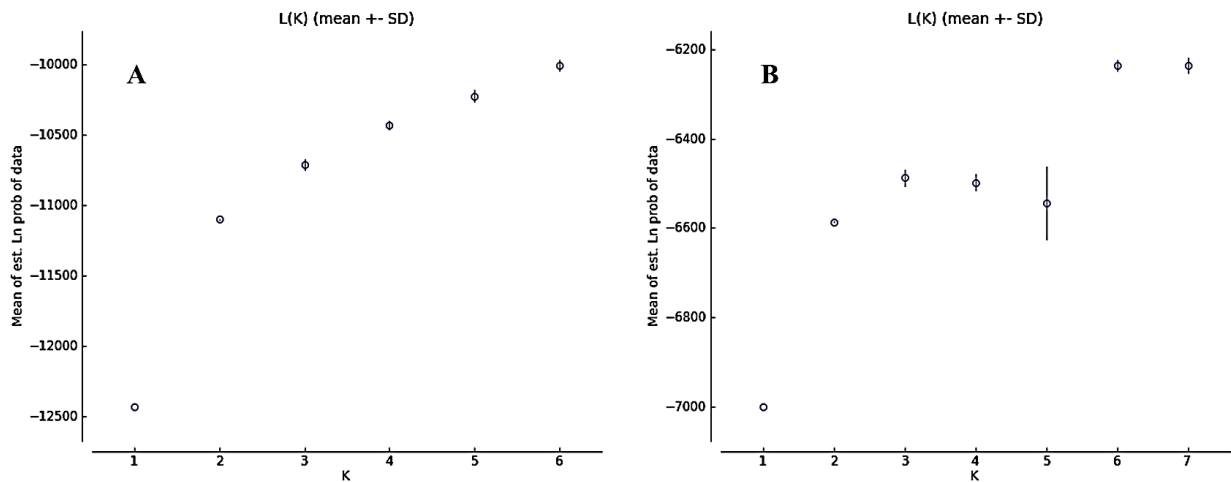
Figure 3.14. L(K) distributions using the "log probability of data" (Mean of LnP±1) approach prior to application of Evanno method. **A**. across the Southern Hemisphere, **B**. on a regional level across the south-west coast of South Africa.
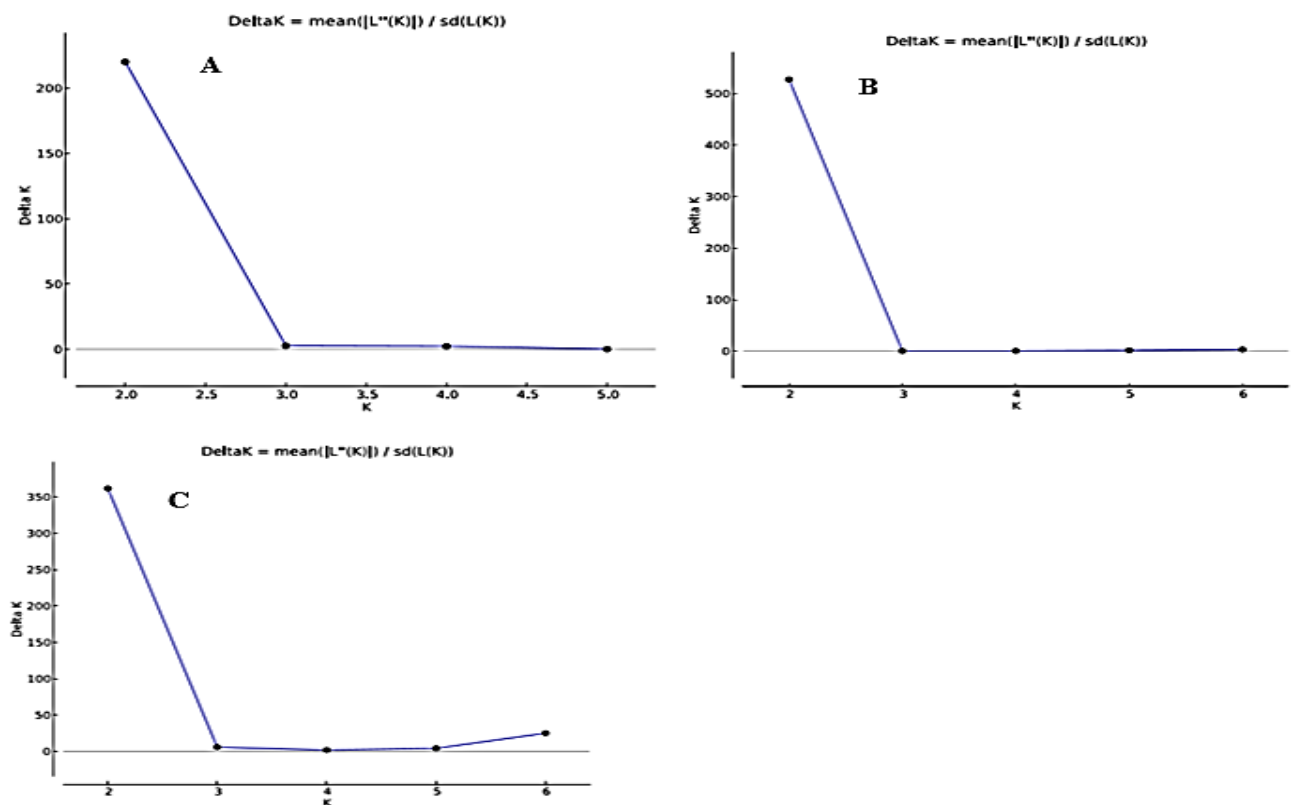
Figure 3.15. Delta *K* analysis of the true number of clusters for *Galeorhinus galeus* **A.** across the Southern Hemisphere based on 19 microsatellite loci, **B**. across the Southern Hemisphere

based on 10 species-specific microsatellite loci and **C**. on a regional level along the south-west coast of South Africa based on 19 microsatellite loci.

Chapter 4:   **Genetic diversity, population structure and demographic history of *Carcharhinus brachyurus* in South Africa**

Abstract

The copper shark *Carcharhinus brachyurus* is a cosmopolitan Carcharinid that is commonly found in the temperate, subtropical, and coastal shelves of southern Africa. In South Africa, this species is targeted and taken as bycatch by commercial and recreational fishers. Due to the *K*-selected traits of the species coupled with anthropogenic effects, there has been a severe decline in local populations. Regional genetic diversity and population connectivity of *C. brachyurus* was investigated using 13 cross-species microsatellite loci and a 713 bp fragment of the NADH dehydrogenase subunit 4 (*ND4*) gene. Levels of genetic diversity and population differentiation were assessed for four major sampling areas (False Bay, Strandfontein, Struisbaai and Mossel Bay) across the Atlantic/Indian boundary. Overall, nuclear diversity for *C. brachyurus* was low to moderate, while the moderate haplotype diversity ($h = 0.527 \pm 0.100$) and low nucleotide diversity ($\pi = 0.001 \pm 0.000$) were slightly lower estimates indicated in other regional studies of elasmobranchs. Estimates of pairwise population differentiation were low to moderate (average $F_{ST} = 0.031$, $P = 0.000$) indicating some degree of gene flow between sampling sites while the Strandfontein population was the most distinct from the rest of the populations. Neither AMOVA ($F_{CT} = -0.011$, $P = 1.000$) nor Bayesian clustering analyses, indicated genetic discontinuity or significant population structure across the Atlantic/Indian boundary. Although the *ND4* results also alluded to historical dispersal across the boundary, the population of Mossel Bay harboured four distinct haplotypes of which two were divergent from the common haplotype shared by 68% of the individuals. Despite an indication that *C. brachyurus* could well be managed as a single stock in South Africa, this study provides a narrow-range stock assessment and future work should include more sampling to confirm possible site-fidelity in this species.

Keywords: Atlantic/Indian boundary, copper shark, genetic connectivity, site-specificity

4.1. Introduction

The copper shark (*Carcharhinus brachyurus*, Günther 1870), also locally known as the bronze whaler, belongs to the Carcharhinidae family. This species inhabits antitropical, temperate coastal areas, but has also been reported in freshwater and the lower parts of rivers and bays (Duffy & Gordon 2003; Compagno *et al.* 2005). *Carcharhinus brachyurus* tends to dwell in temperate habitats and nursery grounds (e.g. Mossel Bay, Port Elizabeth, Algoa Bay), making it an easy target for coastal fisheries and habitat degradation arising from coastal development (Smale 1991). Added to this is their *K*-selected life history strategy which make them highly vulnerable to fishing pressures (Cortés 2000; Musick *et al.* 2000; Camhi 2009). This species exhibits viviparity, produces small litters and is considered a very unproductive species, with the minimum population doubling time exceeding 14 years (Duffy & Gordon 2003) and is currently listed as "*near threatened*" on the IUCN Red List of Threatened Species (Duffy & Gordon 2003).

Generating estimates of genetic diversity and metapopulation structure for highly vagile marine species such as those of the family Carcharhinidae, presents with several challenges; in most part due to the cryptic barriers to gene flow, the lack of information on movement patterns and the generally low number of samples available for analysis. Various members of the Carcharhinidae represent large, cosmopolitan shark species occupying predominantly continental-shelf waters (Compagno 1984). These species, including *Carcharhinus brachyurus*, *C. obscurus*, *C. plumbeus*, *C. leucas*, *C. limbatus* and *C. brevipinna*, are highly vagile, and are suspected to undertake long-range migrations (Duffy & Gordon 2003; Burgess 2009; Musick *et al.* 2009; Simpfendorfer & Burgess 2009). Furthermore, these species have been shown to rely on shallow coastal habitats for birthing and offspring development (Hussey *et al.* 2009; Taylor & Bennett 2013), with mounting evidence of seasonally stratified philopatric behaviour in juveniles and gravid females (Karl *et al.* 2011; Brooks *et al.* 2012; Tillet *et al.* 2012). This trait suggests that, for some carcharhinid sharks, genetic connectivity may be lower than what otherwise would have been predicted based on vagility and existing patterns of movement. The contrast between long-range dispersal ability and the potential for gender-specific site specificity provides a complex context within which to decipher genetic structure. Given the implications for management and conservation, this highlights the need to understand spatial genetic subdivision in shark species by taking in to account aspects such as oceanic expanses, oceanic currents and biogeographical regions; and

how these can shape the within species' genetic connectivity. Studies on the global phylogeography of carcharhinids have shown that large oceanic expanses are robust barriers to gene flow and in some cases, genetic structure has been shown over finer regional scales with philopatric behaviour and historic events such as genetic bottlenecks pinpointed as the bases for subdivision (Portnoy *et al.* 2010; Benavides *et al.* 2011; Karl *et al.* 2011; Tillet *et al.* 2012; Geraghty *et al.* 2013; Spaet *et al.* 2015). In the case of *C. brachyurus,* coastal populations are believed to be demographically independent, resulting in highly structured populations (Duffy & Gordon 2003). This was demonstrated by Benavides *et al.* (2011), which showed based on the mitochondrial control region (mtCR), restricted gene flow between three coastal regions separated by large oceanic expanses: South Africa-Namibia, Australia-New Zealand and Perú. This is to date the only study that investigated gene flow patterns in *C. brachyurus*.

From a regional perspective, the South African coastline has two contrasting currents, the warm Agulhas Current and the cold Benguela Current that meet between Cape Agulhas and the Cape Peninsula. Oceanographic conditions and ecological factors determined three major biogeographical regions along the coast of South Africa: the cool-temperate West Coast, the warm-temperate South Coast and the subtropical East Coast (Heydorn *et al.* 1978; Neethling *et al.* 2008). The three main areas previously described as oceanic barriers to gene flow are Cape Point, Cape Agulhas and Algoa Bay. The upwelling of the Benguela Current causes large temperature fluctuations in the region between Cape Point and Cape Agulhas, whereas the Algoa Bay region has a persistent thermal front (Von der Heyden *et al*. 2011). It is thought that these currents and thermal fluctuations play a major part in defining genetic connectivity and phylogeographic patterns of marine species but that the extent of this depends on the vagility and preferred environmental conditions of a given species (Neethling *et al.* 2008; Teske *et al.* 2011). Various studies have shown that the permeability levels of these barriers vary greatly for different marine species; with some species showing genetic discontinuities and others not (Von der Heyden *et al.* 2008; Bester-van der Merwe *et al.* 2011; Bitalo *et al.* 2015; Maduna *et al.* 2016).

In southern Africa, *Carcharhinus brachyurus* has great commercial importance and is mostly landed in Namibia, Angola and South Africa through recreational angling and targeted and non-targeted fisheries. In southern Africa, it is believed that there may be two discrete populations of *Carcharhinus brachyurus;* one which inhabits the temperate waters of South

Africa and is believed to follow the sardine run eastwards to the tropical waters of KwaZulu Natal, and another off the coast of Namibia from just south of Walvis Bay stretching northwards (Walter & Ebert 1991; Cliff & Dudley 1992; Dudley & Simpfendorfer 2006). However, most of this information is based on observed differences in breeding seasonality, which may be compromised due to species misidentification among carcharhinids species, and likely leading to misrepresentations of actual movement patterns.

In South African waters, considerable numbers of *C. brachyurus* are landed along the coastlines of the Western and Eastern Cape where they are harvested inshore to 200m using recreational handlines (da Silva *et al.* 2015). Off the KwaZulu Natal coastline on the east coast, this species is targeted by the bather protection gillnets and is landed as bycatch in the prawn trawl (Duffy & Gordon 2003; NPOA-shark 2012, 2013). However, population declines for many regions are likely to go unnoticed since catches of *C. brachyurus* in South Africa are grouped under carcharhinids and very little species-specific data exists, despite its high commercial value (NPOA-shark 2012, 2013; da Silva *et al.* 2015). A study by Domingues *et al*. (2013) found situations where more than one species of the Carcharhinidae family was being traded off under the same commercial designation, including those *Carcharhinus* species that are listed as vulnerable in federal legislation. This misreporting directly affects the long-term sustainability of *C. brachyurus* (Domingues *et al.* 2013). Recreational catch rates are also considered to be substantial. However, as is the case for most shark species, they remain unquantified (Worm *et al.* 2013).

For South African *C. brachyurus*, it is critical to generate more genetic resources in order to allow proper species identification. In addition, it is important to investigate whether the biogeographic patterns described for the South African coastline are reflected in the genetic connectivity of the species traversing an inter-oceanic transition zone. This study aims to investigate the genetic diversity and population structure of *C. brachyurus* in South Africa using a combination of microsatellites and NADH dehydrogenase subunit 4 (*ND4*) sequence data. The hypothesis of genetic discontinuity across the different biogeographic regions along the South African coastline will be tested.

4.2. Materials and methods

4.2.1 Sampling and DNA extraction

A total of 94 fin clips of *Carcharhinus brachyurus* were collected by recreational landing efforts from the Western and Eastern Cape of South Africa (**Figure 4.1**). Sampling populations were from False Bay (FB), Strandfontein (SF), Gordon's Bay (GB), Struisbaai (SB), Mossel Bay (MB) and Jeffrey's Bay (JB) **Figure 4.1**. Sampling occurred during the spring and summer months (October to March) of 2011, 2012, 2013 and 2014 **Table 4.9**. To avoid replicate sampling, all animals were tagged by the South African Shark Conservancy (SASC) or obtained from Mossel Bay as part of a tagging initiative by the Oceanographic Research Institute (ORI). All animals were released after tagging. Genomic DNA was extracted from fin clips as described in chapter 2 using a modified CTAB extraction method (Saghai-Maroof *et al.* 1984).
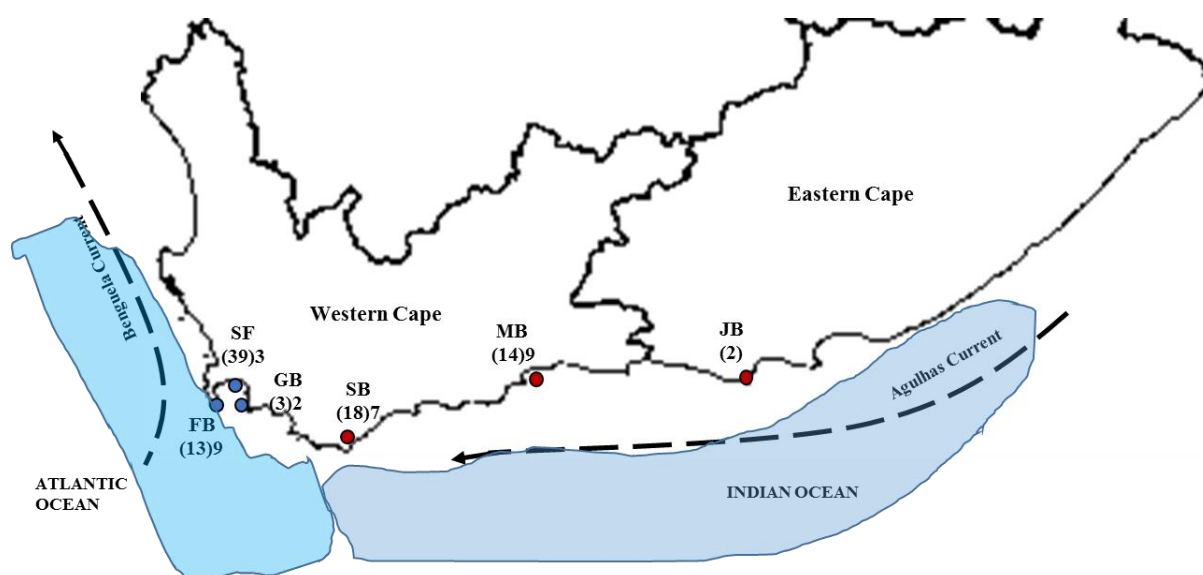
Figure 4.1. Map of the Western- and Eastern Cape showing the sampling sites of *C. brachyurus* across the Atlantic- and Indian Ocean. Number of samples genotyped per population is indicated in parentheses and those sequenced for *ND4* without parentheses. Sampling sites in the Atlantic Ocean (blue dots) are False Bay (FB), Strandfontein (SF) and Gordon's Bay (GB). Sampling sites in the Indian Ocean (red dots) are Struisbaai (SB), Mossel Bay (MB) and Jeffrey's Bay (JB).

4.2.2   Microsatellite Genotyping

Initially, all 94 individuals were genotyped using 17 cross-species microsatellite markers that were previously optimised for utility in *C. brachyurus* (described in Chapter 2:). However, five individuals and four microsatellite loci including Mca33, McaB27, Gg12 and Gg22, were excluded from the final data set due to inconsistent PCR amplification and missing genotypes (see Chapter 2:). A total of 13 microsatellites were therefore amplified by multiplex PCR using the recommended conditions for the Qiagen Multiplex kit and an ABI GeneAmp 2700 thermal cycler (Life Technologies, California USA). Allele sizes were determined using the LIZ$^®$ 600 internal size standard and the GeneMapper$^®$ 4.0 software from ABI (Life Technologies, California USA). To ensure reliable genotype calling, all microsatellite profiles were independently scored by two researchers.

4.2.3   Mitochondrial DNA Sequencing

A 737 bp fragment of the mtDNA gene NADH dehydrogenase subunit 4 (*ND4*) was amplified for 31 samples of *C. brachyurus* using primer sets sourced from Boomer *et al.* (2010). The low sample number was due to inconsistent amplification of this gene region in *C. brachyurus*; most likely due to using primers that were initially developed for amplification of this gene region in *Carcharhinus tilstoni*. Nonetheless, mitochondrial gene amplification was done in a 20µl total volume reaction containing 100ng template DNA, 1X Go*Taq* buffer, 200µM dNTPs, 0.4 µM of each primer, 2 mM MgCl$_2$ and 0.5U Go*Taq* DNA polymerase (Anatech, South Africa). PCR cycling conditions as described by Boomer *et al.* (2010) were performed in an Applied Biosystems (ABI, Life Technologies, California, USA) thermal cycler and amplification was confirmed on a 1.5% agarose gel. Mitochondrial *ND4* amplicons underwent bi-directional sequencing using the BigDye® Terminator 3.1 Cycle Sequencing Kit (ABI, Life Technologies, California USA) and the ABI 3730xl Genetic Analyzer at the Central Analytical Facility (CAF) of Stellenbosch University. All mtDNA sequences were manually edited using MEGA 6 and aligned using MUSCLE (Tamura *et al.* 2013).

4.3 Microsatellite Data Analysis

4.3.1 *Descriptive statistics*

Indices of microsatellite genetic diversity such as mean number of alleles (N_A), number of effective alleles (N_E), number of private alleles (N_P), observed and unbiased expected heterozygosities (H_O and uH_E) and inbreeding coefficient (F_{IS}), were estimated for each sampling population in GENALEX 6.5 (Peakall & Smouse 2012). For all loci, the presence of scoring errors and null alleles were determined using MICRO-CHECKER 2.2.3 (Van Oosterhout *et al.* 2004) while pairs of loci were tested for linkage disequilibrium (LD) in FSTAT 2.9.3 (Goudet 2002). Finally, loci were tested for neutrality in LOSITAN (Antao *et al.* 2008) by comparing F_{ST} values and expected heterozygosities.

4.3.2 *Genetic differentiation and population structure*

To determine genetic differentiation and signatures of population structure, pairwise F-statistics (F_{ST}) (Weir & Cockerham 1984) were determined in ARLEQUIN 3.5 (Excoffier & Lischer, 2010). Type I and type II errors were reduced by performing sequential Bonferroni corrections (Rice 1989) and controlling the false discovery rate (FDR) following the Benjamini and Hochberg (B–H) method (Benjamini & Hochberg 1995). Experiment-wise critical values (EW) and B–H were determined for multiple tests. Based on the pair-wise F_{ST} analyses, samples from Gordon's Bay (n=3) were pooled with Strandfontein (F_{ST} = -0.023; *P* = 0.336), its geographically closest sampling populations, due to low sample sizes and geographic proximity. The samples of Jeffrey's Bay (n=2) were excluded for further analysis due to low sample number. This small sample number was not combined with samples from Mossel Bay due to the big geographic distance between Jeffery's Bay and Mossel Bay. Therefore, all further statistical analyses were therefore performed on the four sampling areas of False Bay (FB), Strandfontein (SF), Struisbaai (SB) and Mossel Bay (MB).

To determine population structure, levels of genetic variation within and among populations and ocean basins (defined as Atlantic- and Indian Ocean) were calculated using the standard analysis of molecular variance (AMOVA) implemented in ARLEQUIN 3.5 (Excoffier & Lischer 2010). Analysis was undertaken to test the hypothesis of panmixia versus substructure among sampling sites. The variance components and fixation indices were partitioned at three levels of hierarchical subdivision: among ocean basins, within populations, and the interaction of both (among + within). The *a priori* hypothetical groups

included sampling populations from the Atlantic Ocean (FB, SF) versus those from the Indian Ocean (SB, MB). The genetic distance matrix for AMOVA was estimated by pairwise differences and the significance levels of the variance components and F-statistics values were tested by 20,000 permutations.

The genetic relationships among sampling populations across the two oceans basins were displayed by DAPC plots. Furthermore, a Bayesian approach was used to test for the existence of distinct and structured populations using the software STRUCTURE 2.3.4 (Pritchard *et al.* 2000; Falush *et al.* 2003). Ten runs were performed for each *K* ranging from 2 to 4. Markov chain Monte Carlo (MCMC) simulation runs of 10^6 iterations were made with 10^5 burn-in periods using the admixture model (Falush *et al.* 2003). The STRUCTURE HARVESTER output files were processed in CLUMPP v1.1.2 (Jackobsson & Rosenberg 2007) and structure display plots were visualised in DISTRUCT v.1.1 (Rosenberg 2004).

4.3.3   *Mutation-drift equilibrium*

The effective population size (N_e) reflects the number of breeding adults and the potential for inbreeding and genetic drift in the species. Effective population size was estimated based on the theta (θ) value in ARLEQUIN, which is estimated from expected homozygosity (Hom_E). Assuming that the populations are in mutation-drift equilibrium, $\theta = (1 - Hom_E)/ Hom_E$), where $Hom_E = 1 - H_E$, and H_E is the expected heterozygosity. Therefore, using the equation $\theta = 4N_e\mu$, where μ is the mutation rate, effective population sizes were determined for each sampling site. A mutation rate (μ) of 1 X 10^{-3} mutations/generation/locus was used (DeWoody & Avise 2000).

Two approaches were used to investigate whether there was evidence of a recent population size contraction. First, BOTTLENECK 1.2.02 (Cornuet & Luikart 1996; Piry *et al.* 1999) was used to determine if sampling populations might have undergone significant reductions in size (*i.e.* population bottleneck) in the recent past. The method is based on the assumption that large declines in effective population size (N_e) decrease allelic diversity at a greater rate than overall heterozygosity. Therefore, if a population exhibits an excess of heterozygotes relative to what would be expected on the basis of observed allelic diversity, then the population may have experienced a bottleneck. The Wilcoxon signed-rank test implemented in BOTTLENECK, was used to examine whether each population exhibited an excess of observed heterozygotes relative to that predicted for a population at mutation-drift

equilibrium. Because this method is sensitive to the mutational model under which the null range of alleles is simulated, heterozygote excess and allele frequencies were tested with 10,000 simulations under the infinite alleles model (IAM), two-phase model (TPM), and the step-wise model (SMM) (Cornuet & Luikart 1996). The interpretation of results focused on the TPM of mutation as it is recommended for microsatellite loci due to a better fit with observed allele frequency data than the IAM and SMM (Di Rienzo *et al.* 1994; Piry *et al.* 1999). The TPM option was set at 95% single-step mutations and 5% multiple-step mutations (Cornuet & Luikart 1996; Piry *et al.* 1999). The average expected equilibrium heterozygosity (H_{eq}) for each locus was compared to Hardy-Weinberg heterozygosity (H_e) and used to assess whether there was a heterozygote excess or deficit. The second approach used BOTTLENECK to test for a mode shift of the allele frequency distribution from an L-shaped distribution that develops when the population is at mutation-drift equilibrium.

4.3.4   *Mitochondrial ND4 Analysis*

Due to amplification difficulties, analysis could only be performed on a small but representative dataset including 31 *ND4* sequences of *C. brachyurus*. Genetic diversity estimates such as the number of segregating sites (K), number of haplotypes (H), haplotype diversity (h) and nucleotide diversity (π) were calculated using ARLEQUIN and DNASP 5.10 (Librado & Rozas 2009). Due to the small number of samples analysed, the best-fit substitution model was determined based on the corrected Akaike Information Criterion (AIC) scores determined in MEGA (Tamura *et al.* 2009), and the Jukes Cantor (JC) was selected as the most suitable substitution model for *C. brachyurus*. The reconstruction of genealogies was performed using phylogenetic algorithms in order to estimate the relationship between haplotypes without ambiguities or unresolved connection (Salzburger *et al.* 2011). A phylogenetic tree of the mtDNA sequences was estimated using a maximum likelihood (ML) approach in PHYML 3.0 (Guindon *et al.* 2010) based on the JC model. For tree searching and level of branch support, default settings were used. The ML tree was imported into HAPLOVIEWER (Salzburger *et al.*, 2011) to visualise the evolutionary relationship according to geographical sampling areas.

Patterns of gene flow were estimated based on pairwise genetic differences (Φ_{ST}) which were also computed in ARLEQUIN using 20,000 permutations. Sequential FDR correction of the significant values was estimated for pairwise differences. A hierarchical AMOVA with 20,000 permutations was conducted in ARLEQUIN to compare the allocation of genetic

variation on a spatial scale 1) among groups (Φ_{CT}), 2) within populations among groups (Φ_{SC}) and 3) within populations (Φ_{ST}). The same two oceanic groups were defined as for the microsatellite analysis; 1) sampling populations from the Atlantic Ocean (FB, SF), and 2) sampling populations from the Indian Ocean (SB, MB).

Past demographic and spatial population expansions of the mtDNA *ND4* sequences were evaluated using two methods. Using the neutrality test, computation of Tajima's *D* (Tajima 1989) and Fu's F_S (Fu 1997) statistics and their significance values were tested by 20,000 coalescent simulations under the infinite-sites model in ARLEQUIN. The values of these indices should be close to zero to show that the population does not deviate significantly from the neutral model of evolution for this locus. Positive values indicate reduced polymorphism and could suggest a deviation caused by a bottleneck, balancing selection or population subdivision. Negative values indicate high levels of polymorphism, possibly as a result of population expansion (Tajima 1989; Fu 1997).

Secondly, nucleotide mismatch distributions of the pairwise differences were assessed between haplotypes. Rogers & Harpending (1992) defined demographic expansion as a sudden increase in population size or sustained exponential growth, whereas a spatial expansion was defined as a range expansion in a subdivided population under the infinite-island model (Excoffier 2004). For both models, the mismatch analysis assumes that any population growth or decline will reveal distinct genetic signatures that are different from what is expected with a constant population size. For instance, a multimodal distribution of pairwise differences indicates demographic equilibrium, with a highly stochastic shape of gene trees; whereas a unimodal distribution indicates recent demographic expansion or spatial expansion with migration between neighbouring demes (Rogers & Harpending 1992). Parametric bootstrap indices were applied to corroborate the significance between observed and expected mismatch distribution patterns.

Corresponding Harpending's raggedness (H_R) and sum of squared deviations (SSD) indices (Harpending 1994) were also calculated in ARLEQUIN to determine whether any observed mismatch distributions were drawn from an expanded population (small values) or a stationary population (large values). The parameters of demographic expansion (mutational timescale) Θ_0 and Θ_1 were obtained in ARLEQUIN to determine mutational parameters before and after demographic expansion (Harpending 1994). Population expansion times were estimated from tau (τ) values derived from mismatch distributions calculated in

ARLEQUIN. Finally, estimates of effective female population size (N_{ef}) were determined by calculating θ_S in ARLEQUIN. With the lack of species-specific mutation rate (μ) for *C. brachyurus*, $\mu = 2.15 \times 10^{-9}$ substitutions/sites/year was used as suggested by Dudgeon *et al.* (2012).

4.4 Results

4.4.1 Genetic diversity and descriptive statistics

Due to the extremely small sample sizes, preliminary genetic differentiation tests and geographical proximity, samples from Gordon's Bay were pooled with the geographically closest sampling population of Strandfontein. The two samples from Jeffrey's Bay were excluded from all further analyses due to the large geographic distance from the closest population, Mossel Bay. Microsatellite indices of genetic diversity, such as expected heterozygosity (H_E), observed (H_O), and unbiased (uH_E) heterozygosities, for each sampling region are provided in **Table 4.1**. Samples from both the Atlantic Ocean and the Indian Ocean showed relatively similar levels of moderate genetic diversity ($uH_E = 0.615$ to 0.670) while a sharply high level of diversity was detected for Strandfontein samples ($N_A = 8$, $uH_E = 0.707$). However, none of the sampling populations showed signs of inbreeding ($F_{IS} = -0.564$ to -0.028).

Table 4.1. Descriptive statistics and genetic diversity estimates for each sampling population of *C. brachyurus* based on 13 microsatellite loci. Number of individuals genotyped N, number of alleles N_A, number of effective alleles N_E, number of private alleles N_P, expected heterozygosity H_O, unbiased expected heterozygosity uH_E, inbreeding coefficient F_{IS}, effective population size N_e.

Site	N	N_A	N_E	N_P	H_O	uH_E	F_{IS}	θ_H	N_e
FB	13	3	3.686	0.231	0.615	0.707	-0.564	2.923	244
SF	41	8	3.05	4.154	0.63	0.641	-0.028	8.231	686
SB	18	4	3.232	0.385	0.692	0.670	-0.215	3.538	295
MB	16	4	2.851	0.538	0.705	0.615	-0.300	4.077	340

Molecular diversity estimates based on mtDNA *ND4* sequences exhibited a total of seven haplotypes, a moderate haplotype diversity ($h = 0.527 \pm 0.100$) and low nucleotide diversity

(π = 0.001) **Table 4.2**. There was no polymorphism for samples collected from Struisbaai. One haplotype was found in all four sampling sites and was present in 68% of the samples sequenced, whilst some private haplotypes were detected at False Bay, Strandfontein and Mossel Bay. Interestingly, Mossel Bay had the highest number of private haplotypes, two of which were highly divergent and separated by eight mutational steps from the rest of the sampling populations **Figure 4.2**. Samples at Mossel Bay also exhibited the highest haplotype diversity ($h = 0.756 \pm 0.130$).

Table 4.2. Molecular diversity estimates for *C. brachyurus* based on mtDNA *ND4* sequences. Genetic diversity estimates include number of haplotypes (*H*), private haplotypes (H$_P$), polymorphic sites (K), haplotype- (*h*) and nucleotide diversity (π).

Site	N	*H*	H$_P$	K	*h*	π
FB	10	2	1	1	0.222 ± 0.166	0.003 ± 0.000
SF	5	1	1	1	0.400 ± 0.237	0.001 ± 0.000
SB	7	1	0	0	n.d.	n.d.
MB	10	5	4	6	0.756 ± 0.130	0.002 ± 0.000
Overall	31	15		7	0.527 ± 0.100	0.001 ± 0.000

n.d. not determined due to a lack of polymorphism.
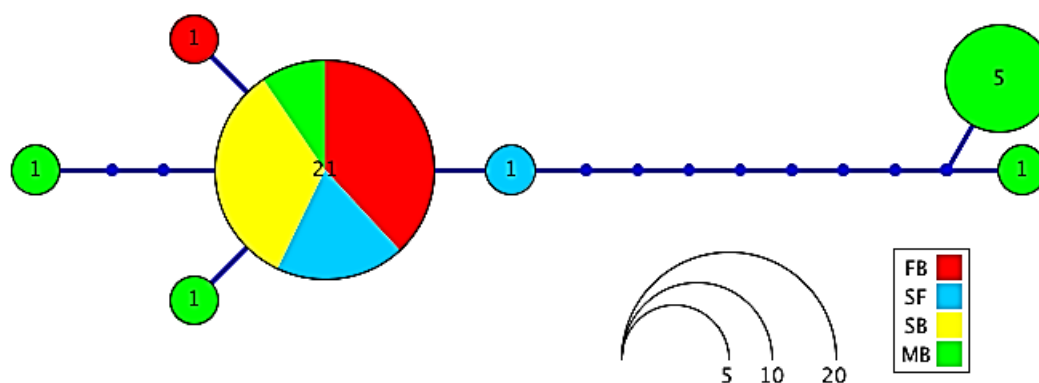
Figure 4.2. Haplotype genealogy of *Carcharhinus brachyurus* based on a maximum likelihood tree of *ND4*. Circles represent the haplotypes with area being equivalent to frequency. Each line indicates one mutational step between haplotypes and small blue circles indicate hypothetical missing haplotypes.

4.4.2   Genetic differentiation and population connectivity

The statistical significance of microsatellite pairwise F_{ST} values was confirmed after controlling for the FDR at a corrected critical value (B–H $\leq$ 0.025). Very low but statistically significant values were noted for all pairwise comparisons between the Atlantic- and Indian Ocean sampling populations except between False Bay and Struisbaai (F_{ST} = 0.027, *P* = 0.057) **Table 4.3**.

Table 4.3. Pairwise F_{ST} values below diagonal, *P* values above diagonal for four *C. brachyurus* sampling populations. Asterisks indicate statistical significance after Bonferroni corrections (*P* < 0.05).

Site	FB	SF	SB	MB
FB		0.000	0.057	0.002
SF	0.055*		0.010	0.001
SB	0.027	0.018		0.002
MB	0.081*	0.054*	0.068*	

Discriminant analysis of principal components (DAPC) of the individual microsatellite genotypes showed no inter-oceanic differentiation between sampling populations of *C. brachyurus* but a substantial overlap of populations. Noteworthy is the Strandfontein population clustering somewhat separately with a high percentage of distinct genotypes **Figure 4.3**.

Analysis of molecular variance indicated limited genetic variation among sites **Table 4.4**. When all sampling populations were grouped to test for global panmixia, very low but statistically significant variation was noted amongst the sampling populations (3.09%; F_{ST} = 0.031, *P* = 0.000) and most of the variation was within the sampling populations. In assessing molecular variation amongst the oceanic grouping of sampling populations, there was no inter-oceanic variation for *C. brachyurus* across the Atlantic- and Indian Ocean (-1.07%; F_{CT} = -0.011, *P* = 1.000).
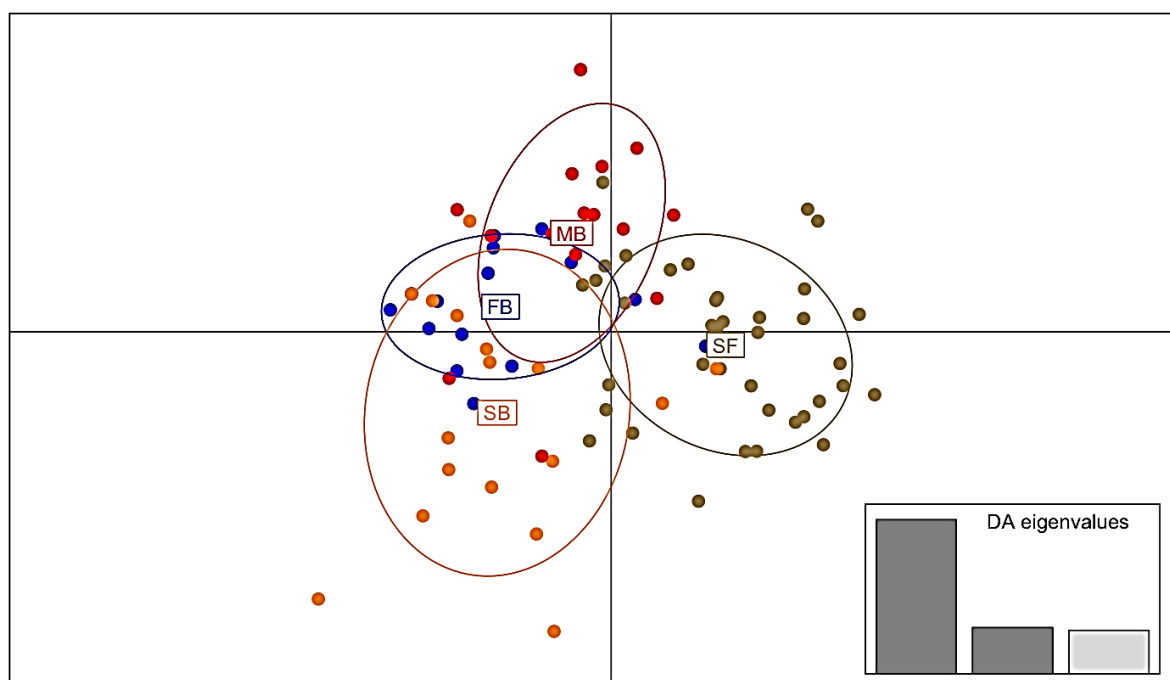
Figure 4.3. A discriminant analysis of principal components (DAPC) plot showing relationships of *C. brachyurus* genotypes among four local sampling populations, False Bay (FB), Strandfontein (SF), Struisbaai (SB) and Mossel Bay (MB).

Table 4.4. Molecular variance estimates among samples of *C. brachyurus* based on 13 microsatellite loci. Asterisks indicate statistical significance (*P* < 0.05).

Hypothesis tested	Source of variation	% variation	Fixation indices	*P*-value
Panmixia	Among populations	3.09	$F_{ST} = 0.031$*	0.000
	Within populations	96.91		
Oceanic (Atlantic vs. Indian)	Among groups	-1.07	$F_{CT} = -0.011$	1.000
	Among populations	3.59	$F_{SC} = 0.036$*	0.000
	Within populations	97.47	$F_{ST} = 0.025$*	0.000

Bayesian analysis obtained from all STRUCTURE runs identified three clusters (*K* = 3), but without indication of strong differentiation among sampling populations **Figure 4.4**. The cluster assignment patterns showed an overlap across populations with varying levels of admixture and no inter-oceanic structure. Two sub-groups were noted for Strandfontein; both showing samples with high genotype assignment to two different clusters. A second STRUCTURE analysis was performed excluding those individuals from Strandfontein that were the most divergent to see if these individuals biased clustering results. Even without these individuals, the Strandfontein population seemed to be the most distinct while Struisbaai, the area adjacent to the Atlantic/Indian boundary, was more admixed than the other populations **Figure 4.5**. In fact, the assignment plot now indicated clinal variation along the region sampled more in line with a stepping-stone model of migration than an abrupt break at Cape Agulhas.

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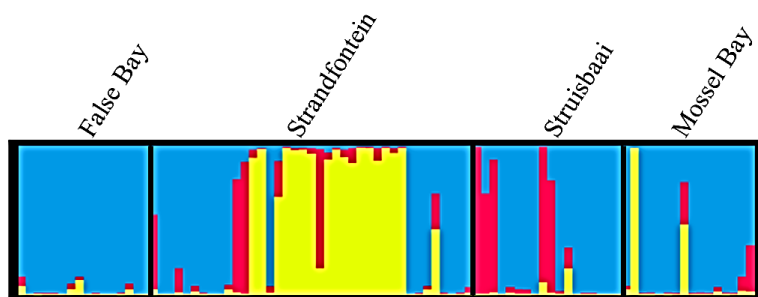
Figure 4.4. Individual STRUCTURE assignment plot showing genotype membership to $K = 3$ clusters for *C. brachyurus*.
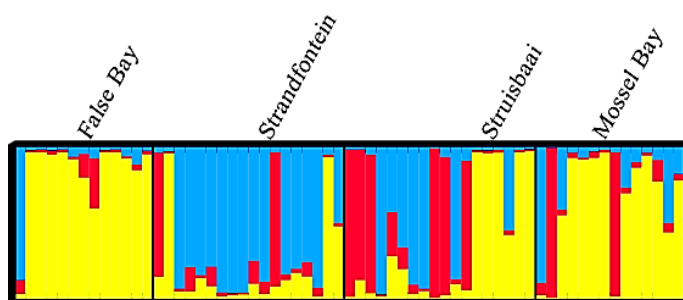
Figure 4.5. Individual STRUCTURE assignment plot showing genotype membership to $K = 3$ excluding divergent individuals from Strandfontein.

Statistical pairwise comparisons between mtDNA *ND4* haplotypes showed low to moderate levels of inter- and intra-oceanic genetic differentiation with non-significant pairwise comparisons between most of the sampling populations (Φ_{ST} = -0.031 to 0.164, *P* > 0.005). However, inter-oceanic genetic subdivision was detected between Mossel Bay and False Bay (Φ_{ST} = 0.262, *P* = 0.010); and intra-oceanic genetic subdivision between Struisbaai and Mossel Bay (Φ_{ST} = 0.245, *P* = 0.006) **Table 4.5**. AMOVA analysis showed global variation amongst the sampling populations (Φ_{ST} = 0.217, *P* = 0.002) but again an inter-oceanic mtDNA variation was not supported (Φ_{SC} = 0.018, *P* = 0.671) **Table 4.6**.

Table 4.5. Pairwise Φ_{ST} values below diagonal, *P* values above diagonal for four *C. brachyurus* sampling populations. Asterisks indicate statistical significance after Bonferroni corrections (*P* < 0.05).

Site	FB	SF	SB	MB
FB		1.000	1.000	0.010
SF	0.030		1.000	0.058
SB	-0.031	0.073		0.006
MB	0.262*	0.164	0.245*	

Table 4.6. Molecular variance estimates among samples of *C. brachyurus* based on *ND4* data. Asterisks indicate statistical significance (*P* < 0.05).

Hypothesis tested	Source of variation	% variation	Fixation indices	*P*-value
Panmixia	Among populations	21.68	$\Phi_{ST} = 0.217$	0.002*
	Within populations	78.32		
	Among groups	1.75	$\Phi_{CT} = 0.018$	0.671
Oceanic (Atlantic vs. Indian)	Among populations	20.37	$\Phi_{SC} = 0.207$	0.032*
	Within populations	77.87	$\Phi_{ST} = 0.221^*$	0.002

4.4.3 *Mutation-drift equilibrium and demographic dynamics*

Tests for mutation-drift equilibrium were performed based on all 13 microsatellite loci. All sampling populations were in mutation-drift equilibrium and showed a normal L-shaped distribution of allele frequencies. A significant *P*-value and thus deviation from equilibrium was seen in the Strandfontein population under the SMM model (*P* = 0.002). There was an excess of heterozygosity across all sampling populations except for Strandfontein. Effective population sizes ranged from $N_e = 686$ to 244 and were highest for Strandfontein and lowest for False Bay **Table 4.1**. Caution should however be taken in interpreting these results, as similar to the diversity estimates, these values correspond to sample sizes and could most likely therefore be an artifact of sampling.

To determine the robustness of the sampled populations, demographic history parameters were estimated to assess the changes, if any, in effective population sizes over time, by investigating the distribution of nucleotide frequencies over time as well as neutral evolution across the *ND4* locus. Since a limited number of samples were sequenced in each population, a mismatch distribution was illustrated for the *ND4* sequences pooled from all sampling sites **Figure 4.6**. The observed distribution of mismatch differences did not deviate from that expected for populations undergoing a demographic expansion. This was indicated by the unimodal distribution displayed by *ND4* sequences of *C. brachyurus.* However, this was not statistically supported by the non-significant H_R and SSD index values (H_R = 0.418, *P* = 0.420; SSD = 0.168, P = 0.338) **Table 4.8**.

Table 4.7. Test for mutation-drift equilibrium analysis for 13 microsatellite loci. Significant *P* values for Wilcoxon's test are indicated with an asterisk. Allele frequency distribution AFD, E/D number of loci showing excess or deficit when comparing observed and expected heterozygosity. SMM step-wise mutational model, IAM infinite allele model, TPM two-phase model.

Site	AFD	E/D	SMM	IAM	TPM
FB	Normal L-shaped	4/0	0.953	0.999	0.999
SF	Normal L-shaped	0/0	0.002*	0.999	0.916
SB	Normal L-shaped	5/0	0.847	1.000	0.997
MB	Normal L-shaped	2/0	0.878	1.000	0.999
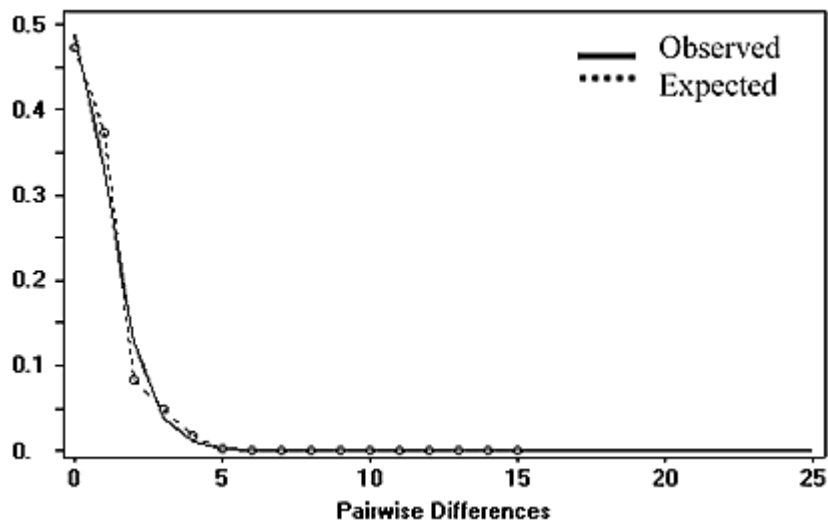
Figure 4.6. Comparison between observed and expected mismatch distributions of pairwise sequence differences for *C. brachyurus* under a growth-decline population model performed on a collection of four sampling sites. Solid lines represent the observed pairwise differences and dashed lines the expected distribution.

Table 4.8. Demographic analysis parameters for mtDNA *ND4* sequences of *C. brachyurus* including neutrality test estimates Tajima's test (D) and Fu's test (F_S), Harpending's raggedness index (H_R), sum of squared distribution (SSD), the coancestry coefficient (Θ_S) and female effective population size (N_{ef}).

Site	Neutrality tests		Demographic estimates			
	D	F_S	SSD	H_R	Θ_S	N_{ef}
FB	-1.088 ($P > 0.100$)	-0.263 ($P > 0.100$)	0.030 ($P = 0.677$)	0.0933 ($P = 0.812$)	4.076	275209
SF	-0.817 ($P > 0.100$)	0.090 ($P > 0.100$)	0.331 ($P = 0.080$)	0.400 ($P = 0.250$)	2.929	2298644
SB	no polymorphism	n.d.	n.d.	n.d.	n.d.	n.d.
MB	-1.146 ($P > 0.100$)	-1.215 ($P > 0.100$)	0.097 ($P = 0.089$)	0.039 ($P = 0.800$)	17.055	5114632

4.5 Discussion

This study is the first to assess the regional population structure of the large commercially important shark species, *C. brachyurus*, across the Atlantic/Indian Ocean transition zone of South Africa. Despite being based on a small sample cohort, the analyses included samples that spanned a distance of approximately 800km of the region most exploited by the demersal shark fisheries in South Africa. A previous phylogeographic study of the coastal samples of *C. brachyurus* across the Southern Hemisphere, suggested that this species warrants regional population assessment for management since its expansion across oceans traversed over evolutionary and most likely also ecological time scales (Benavides *et al.* 2011).

4.5.1 *Genetic diversity & population connectivity*

The regional genetic structure of *C. brachyurus* within and between four sampling populations was assessed using cross-species amplified microsatellites and a portion of the *ND4* mitochondrial gene. As previously described in Chapter 2, 17 microsatellite markers developed for *Mustelus henlei*, *M. canis* and *Galeorhinus galeus* were successfully amplified in *C. brachyurus*. Particular care was taken to select the loci that were not influenced by null alleles or selection. Genetic diversity estimates including number of alleles per locus, allelic richness, and observed- and expected heterozygosities for *C. brachyurus* were comparable to other regional studies of large coastally oriented shark species such as the scalloped hammerhead shark, *Sphyrna lewini*, the blacktip shark, *C. limbatus* (Spaet *et al.* 2015), and the lemon shark, *Negaprion brevirostris* (Ashe *et al.* 2015). Although a bias towards genetic diversity and population estimates cannot be ruled out due to data being generated from cross-amplified microsatellites, the overall lower genetic diversity found compared to other teleost fishes is not uncommon (Schmidt *et al.* 2009; Karl *et al.* 2011; Dudgeon *et al.* 2012; Portnoy & Heist 2012). Generally, lower genetic diversity levels are found in sharks and this is more likely a consequence of life history strategy and reproductive mode (Dudgeon *et al.* 2012, Portnoy & Heist 2012).

Observed *ND4* nucleotide diversity estimates were much lower than those generated by the mitochondrial control region (mtCR) diversity estimates seen in the study of Benavides *et al.*

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(2011) (0.1% versus 1.6%) on the global phylogeography of *C. brachyurus* and a similar pattern was noted for haplotype diversity estimates. Comparing these estimates with that of *G. galeus* (chapter 3) or published data from Hernández *et al.* (2015) for example, it shows that *C. brachyurus* exhibits lower levels of overall genetic diversity as opposed to the smaller, coastal sharks. The latter could be due to a combination of differenrences in life history strategies and vulnerability to overfishing of smaller and larger coastal sharks.

Given the contiguous shelf habitat around the south-west coastline of South Africa and the high vagility of the study species, the lack of overall genetic structuring and admixed individuals observed for *C. brachyurus*, was as expected. The study in its entirety failed to reject the hypothesis of panmixia while the detection of possible discreet subpopulations and varying degrees of admixture within sampling populations suggest that gene flow appears to be limited in some areas. A few of the pairwise population comparisons yielded significant levels of genetic differentiation based on both microsatellite and *ND4* sequence data sets. Sub-structuring within the Strandfontein population was corroborated by multi-variate and Bayesian clustering analysis, while a number of private mtDNA haplotypes were detected at Mossel Bay. The structuring of shark species over smaller spatial scales has been attributed to a number of contemporary and historical factors (Dudgeon *et al.* 2012). Furthermore, ecological and life-history differences amongst species appear to significantly influence the patterns of population structure. With regards to physical barriers, both the Benguela- and Agulhas Current systems have previously been shown to play a significant role in limiting gene flow along the southern African coastline. Global studies for *Sphyrna lewini* and *Carcharhinus limbatus* revealed that movement was hindered by the Benguela upwelling along the south-west coast of Africa (Duncan *et al.* 2006; Keeney & Heist 2006; Chapman *et al.* 2009) while locally, significant population genetic structure across the Atlantic/Indian transition zone was reported for *Mustelus mustelus* (Bitalo *et al.* 2015; Maduna *et al.* 2016). Based on the current nuclear results, there is less evidence that the ocean currents play a significant role in obstructing gene flow for *C. brachyurus* along the region investigated. However, the population substructure that was noted for Strandfontein could suggest that habitat discontinuity persists in this region and that the coastal populations of *C. brachyurus* are separated from a more offshore dwelling, more migratory population. In addition, the divergent haplotypes discovered for the Mossel Bay population could imply that perhaps the

species goes to very particular areas to breed and as suggested by previous studies, these breeding or nursery areas are most likely in embayment areas such as Mossel Bay, Algoa Bay and Storms River (Bester-van der Merwe & Gledhill 2015). Walter and Ebert (1991) also postulated that *C. brachyurus* has seasonal movement patterns pertaining exclusively to breeding while the global study by Benavides *et al.* (2011) suggested that their sampling populations most likely contained admixed individuals between Namibia and South Africa that might otherwise segregate to breed. With regards to life history traits, reproductive philopatry, or the fidelity of gravid females to nursery areas, is typically invoked to explain fine-scale genetic structuring (based on maternally-inherited mtDNA) in the absence of barriers to dispersal for highly-vagile sharks (Keeney *et al*. 2005; Hueter *et al*. 2005). Female philopatric behaviour can constitute a reasonable explanation for mtDNA variation observed on a spatial level such as reported for many other shark species including bonnethead shark, *Sphyrna tiburo* (Escatel-Luna *et al.* 2015) and porbeagle sharks *Lamna nasus* (Francis *et al.* 2015). Since deep genetic divergence with geographic correspondence **Table 4.6** is observed for *C. brachyurus*, and both nuclear and mtDNA data reflects genetic structure across sampling regions, it is postulated that the movement patterns of *C. brachyurus* are defined by a combination of factors including oceanic currents, seasonal changes correlated to breeding patterns and sex-biased behavioural traits. Confidently discerning sex-biased behavioural traits, however, is complex and relies on a robust experimental design involving the exclusive sampling of neonates, or adult females at time of parturition rather than during dispersal, from spatially discrete areas. Moreover, the collection of tissues in the present study was generally reliant on opportunistic sampling, rather than according to a dedicated experimental design. For instance, sampling was mostly done in the spring and summer months when *C. brachyurus* has been noted to move to the cool temperate regions of the Western Cape from KwaZulu Natal. Sampling was therefore during the parturition and some of the tissue from Mossel Bay was exclusively sampled from small juveniles and sexually mature females **Table 4.9**. While it is possible that the fine-scale genetic structuring observed in this study reflects female philopatry, the only meaningful test of this hypothesis would be at Mossel Bay for which the data detected significant mtDNA variation.

4.5.2 Mutation-drift equilibrium and demographic history

Population declines were investigated for all four sampling populations of *C. brachyurus* and based on the BOTTLENECK results; there was no indication of a significant recent population size decline. However, the presence of a heterozygosity excess in three of the four sampling populations indicates that perhaps these populations may be undergoing a decline that is still too recent to be detected by the molecular markers used. These subtle genetic signatures of population decline noted for *C. brachyurus* in False Bay, Struisbaai and Mossel Bay could be as a result of heavy exploitation in the region over the past decade. Coupled with the misidentification of the species in what is otherwise a mixed-species fishery, it is quite possible that this species has undergone severe declines not evident from the genetic assessment of population size. It is common knowledge among fisheries scientists that fishermen unintentionally misidentify taxa and that for instance, *C. obscurus* and *C. brevipinna* are often confused and lumped with *C. brachyurus* (Cliff & Dudley 1992; da Silva & Bürgener 2007; Attwood *et al.* 2011; Best *et al.* 2013). Preliminary tests for demographic history based on the distribution of mismatch pairwise differences and statistical support, show that if *C. brachyurus* had undergone a demographic expansion in the past, the very recent samples used in this study do not reflect such signatures yet. However, based on the small sample size and recent sampling, the results generated for demographic indices are not enough to make concrete extrapolations about the population status and health of *C. brachyurus*.

4.6 Management implications

The exploitation of *Carcharhinus brachyurus* in South Africa has been in existence for decades with the highest levels of exploitation resulting from recreational angling and commercial exports to Australia; and in some cases Asia (da Silva & Bürgener 2007). This and other factors have resulted in the species currently being classified as "vulnerable" in South Africa (NPOA-2014). Despite this decline in numbers, it is challenging to create sustainably lucrative management measures since the species lacks spatio-temporal data. This is mainly as a consequence of species misidentification of elasmobranchs which is quite common in the fisheries sector (da Silva & Bürgener 2007). A major challenge to the inclusion of genetic data in implementing fisheries management strategies is how to deal with

the temporary mixture of distinct populations of migratory fish species at regional fishing grounds. A key issue is the assessment of the level of variation required in populations to justify separate management approaches (Waples *et al.* 2008). Such evaluations could rely upon biological characteristics and the contemporary and historical population dynamics of the species, in conjunction with the underlying management and conservation objective (Galván-tirado *et al.* 2013; Bester-van der Merwe & Gledhill 2015). Statistically significant genetic structure was found between some sampling populations in this study, but the levels of genetic differentiation do not necessarily warrant classification of separate management units in the fisheries sense. Based on the observed genetic variation for *C. brachyurus* sampled across the Agulhas Bioregion of South Africa, failure to reject the hypothesis of a single panmictic population could compromise certain more vulnerable 'stocks' than others such as the subpopulation of Strandfontein or the divergent population found at Mossel Bay. These results should therefore be taken as a basis on which to combine genetic data with tagging data or even ecological data (*e.g.* contemporary climate change) to help ascertain the population dynamics of this commercially important species. Since this is the first available evidence for genetic connectivity of *C. brachyurus* in a region where it is most exploited, the resulting evidence should be considered in a fine scale context before making final management decisions for the species in South Africa. While this data did not provide a clear signal of genetic structure for *C. brachyurus*, it seems more prudent for future management purposes to assess sampling populations from the Atlantic- (including Namibia and Angola) and Indian Ocean separately; both likely being havens for gravid females and offspring based on the private alleles and haplotypes observed with this relatively small dataset.

Supplementary Material

Table 4.9. Sampling sites, sample details and sampling dates

Site	Gender	TL (cm)	Sampling date
False Bay (FB)	3M, 10F	82-279	2014
Strandfontein (SF)	12M, 19F	137-302	2012
Gordon's Bay (GB)	3F	141-235	2012
Struisbaai (SB)	8M, 10F	98-105	2012
Jeffrey's Bay (JB)	1M, 1F	159-168	2011
Mossel Bay (MB)	6M, 3F, 5U	82-279	2013-2014
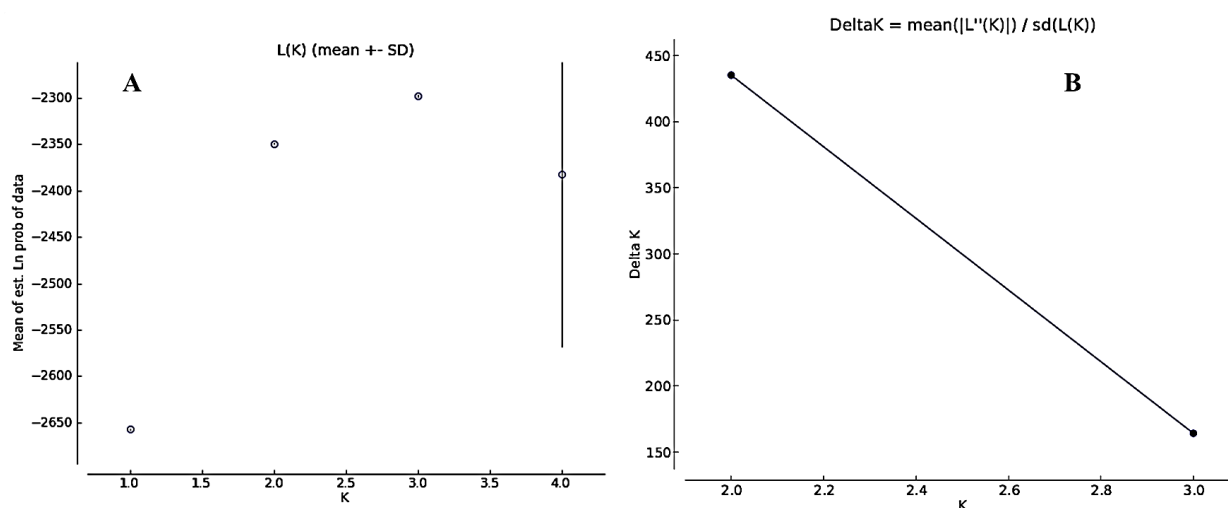
Figure 4.7. Identification of the number of genetic clusters (K) of best fit using the **A**. "log probability of data" (Mean of LnP±1) approach and **B**. the Evanno method to identify the highest "delta K" (ΔK).

Chapter 5:   **Genetic diversity and population structure of the endemic lesser**

sandshark (*Rhinobatos annulatus*) over a small regional scale

Abstract

The lesser sandshark *Rhinobatos annulatus* (Muller & Henle 1841) is an inshore guitarfish endemic to the southern African region that inhabits inshore, benthic habitats of this region. *Rhinobatos annulatus* is one of the most abundant endemic guitarfishes off the southern African coast, but little is known of its movement patterns or population structure. The population genetic structure of this species was inferred with 15 cross-amplified polymorphic microsatellite loci in samples collected at four sites across the Agulhas bioregion that also coincides with the warm temperate biogeographical region of the South African shoreline. F_{ST} pairwise estimates ranging from 0.016 to 0.094 indicated low but significant population differentiation with the highest observed between the western and eastern most sampling sites. Irrespective of which loci were included in different datasets, the Port Elizabeth population was revealed as the most distinct based on multivariate and Bayesian clustering analysis. This population also had the lowest allele richness and it is likely that this population is isolated from the rest by the thermal front caused by upwelling in this region. However, since Die Plaat was also significantly divergent (large $F_{ST} > 0.069$) from the eastern range samples but not from De Mond, suggest that the observed structure might simply be due to isolation-by-distance as confirmed by a mantel test. Nonetheless, the differentiation seen amongst these limited number of sample sites implies that the species might be highly structured throughout its entire geographical range which could have serious implications for the future preservation of genetic diversity within this species.

Keywords: Agulhas bioregion, lesser sandshark, microsatellites, genetic connectivity

5.1. Introduction

The southern African coastline is an ecologically and evolutionarily dynamic region featuring a rich assemblage of marine biodiversity and endemism that has evolved around a scenario of climatic oscillations encompassed by transitional frontiers between temperate and subtropical bioregions (Griffiths *et al.* 2010). Despite their ecological and evolutionary significance, the genetic structure of batoids remains the least attended to in comparison to that of other elasmobranchs. The very few investigations thus far do however show that there is intraspecific genetic heterogeneity in a number of batoid species. This has been attributed mainly to factors such as phylogeographic breaks between distinct maternal lineages as seen in the shovelnose guitarfish *Rhinobatos productus* (Sandoval-Castillo *et al.* 2004), thermal physiological barriers as reported in the common skate *Dipturus batis* (Griffiths *et al.* 2010) and reproductive isolation observed such as with the banded guitarfish *Zapteryx exasperata* (Castillo-Páez *et al.* 2014). Although these studies were performed over different geographical scales, these findings raise the question of how much an influence the habitat of different batoid species has on population genetic structure.

The lesser sandshark *Rhinobatos annulatus* (Muller & Henle 1841) is an inshore guitarfish endemic to the southern African region and a benthic dweller of the sandy shores of this region. *Rhinobatos annulatus* is one of the most abundant endemic guitarfishes off the southern African coast, yet it lacks population trend data and is seen as data deficient (Burgess *et al.* 2006). This species is found in inshore waters from the shoreline to depths of 50 - 100 m, and also inhabits estuaries, the surf zone and enclosed bay areas. This species is relatively small in size with maximum sizes recorded at 140 cm total length (TL). The species is termed *K*-selected and reaches sexual maturity later than most elasmobranchs (50% maturity at three years), has a short lifespan (maximum 7 years) and an annual fecundity giving birth to 2 to 10 young. A study by Rossouw (1984) showed that four years of reproductive activity in females yielded a mean number of only 24 embryos. This emphasises how vulnerable *R. annulatus* is to fishing pressures and how this could have detrimental consequences for the survival of this endemic species (Rossouw 1987; Rossouw 2014). Unfortunately the species is still listed as of least concern on the IUCN Redlist (Burgess *et al.* 2006). Globally, guitarfishes are considered as one of the most threatened elasmobranch families (Dulvy *et al.* 2014).

In South Africa, *R. annulatus* is a predominant bycatch within the commercial gillnet, beach seine and trawl fisheries where it is considered to be of very little to no value (Hutchings & Lambeth 2002; Walmsley *et al.* 2007; Attwood *et al.* 2011). Reports have shown that some commercial vessels return bycatch in to the water alive (Burgess *et al.* 2006). However, this is not based on recent information and might also be questionable since shark-like rays, especially sawfishes, wedgefishes and guitarfishes have been reported to have some of the most valuable fins (Dulvy *et al.* 2014). Also *R. annulatus* is often confused with *R. blochii* where catches for both species are reported under the generic name guitarfish, leading to unreliable species-specific catch data for this species. *Rhinobatos annulatus* is also caught during the shore angling competitions especially in the Eastern Cape of South Africa (Govender & Pradervand 2003).

There have been only two population trend assessments reported for this species and most of these reports were recorded for recreational angling in South Africa (Govender & Pradervand 2003; Vaughan & Chisholm 2010), with no records for Namibia and Angola. Reports from shore angling catch in the Eastern Cape showed that, of a total of 34 species reported, *R. annulatus* was the most commonly caught species (Govender & Pradervand 2003). Other information on *R. annulatus* describes the distribution patterns and feeding behaviour (Rossouw 1983; Harris *et al.* 1988), the biology of the species (Rossouw 1984; Rossouw 1987), parasite infestation (Vaughan & Chisholm 2010) and the seasonal reproductive activity of males (Rossouw & van Wyk 2014). *Rhinobatos annulatus* is a bottom feeder, occurring mainly in sandy beaches and lagoons where its distribution is correlated with prey biomass (Rossouw & van Wyk 2014). Studies conducted in Langebaan lagoon and Algoa Bay indicate that *R. annulatus* shows temporal and spatial changes in intensity of predation and distribution patterns with the biomass increasing during summer (Rossouw 1983; Harris *et al.* 1988). The ratio of males to females in Langebaan changed from 1:3.2 in April to 1:0.8 in August. Juveniles were also present in high numbers during April and absent in August (Harris *et al.* 1988). It was confirmed by Rossouw & van Wyk (2014) that male individuals of *R. annulatus* exhibit a seasonal reproductive activity; shown to be heightened around November and then lowered during late summer in May, when the females are giving birth. Interestingly, it has been shown that *R. annulatus* do not need its liver for buoyancy but rather uses the liver lipids as fuel for muscle movement especially during migration after parturition (Rossouw 1987).

Despite its low economic value, the species' low reproductive efficiency together with a general lack of catch and population trend data, highlights the importance of monitoring *R. annulatus* more closely. Inferring the genetic diversity and population structure of an endemic species could assist not only in conserving a species but also in maintaining the biodiversity and ecosystem of a particular region. It is speculated that there are possibly two separate stocks, one in the Western Cape area, South Africa and northward into southern Angolan waters, and the other along the southeastern African coast. However, these stocks have not been further characterised or confirmed. Since for this chapter, the study area was restricted to opportunistic sampling from a recreational fishing project, it was not possible to test for the existence of these proposed stocks. Rather, this chapter aimed to investigate, for the first time, the genetic diversity of any batoid species along the warm-temperate bioregion of South Africa. Since there are no species-specific markers available for *R. annulatus*, and very few mitochondrial primers were optimized for *Rhinobatos* at the time, using cross-species microsatellite markers, the study focused on determining contemporary dispersal patterns and effective population size of this endemic species across the Agulhas bioregion.

5.2. Materials and Methods

5.2.1 Sampling

Fin clip and muscle tissue was acquired from 83 individuals of both sexes across the warm temperate Agulhas bioregion of the South African coastline (**Figure 5.1**). Samples were from the western side of the warm temperate region including Die Plaat (DP) and De Mond (DM) and from the eastern side at Jeffrey's Bay (JB) and Port Elizabeth (PE). Animals were mostly caught during recreational fishing expeditions and were tagged by the South African Shark Conservancy (SASC) for an ongoing population assessment study. A summary of the total length, gender and catch date is shown in **Table 5.6** in the supplementary data.
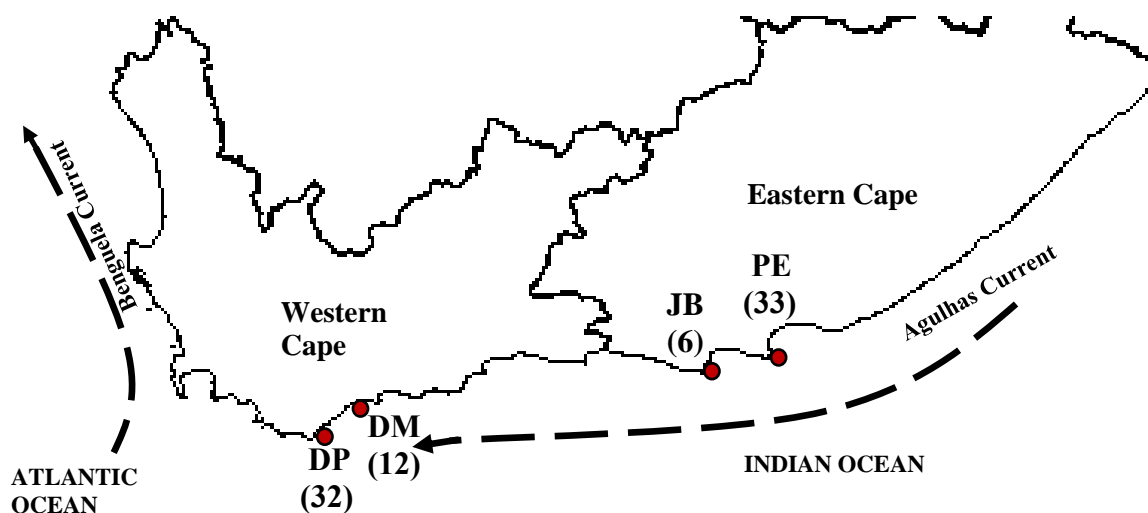
Figure 5.1. Map showing four sampling sites of *Rhinobatos annulatus* at the western side of the warm temperate region; Die Plaat (DP) and De Mond (DM), and the eastern side of the bioregion; Jeffery's Bay (JB) and Port Elizabeth (PE). Sample sizes are shown in parenthesis.

5.2.2   Microsatellite genotyping and Data analysis

A total of 83 individuals were genotyped using 17 cross-species microsatellite markers that were previously optimised for utility in *R. annulatus* (described in chapter 2). However, two of the loci (*McaB37* and *Gg22*) were excluded from the final data set due to inconsistent PCR amplification and missing genotypes. A total of 15 microsatellites were therefore amplified by multiplex PCR using the recommended conditions of the Qiagen Multiplex kit in an ABI GeneAmp 2700 thermal cycler (Life Technologies, California USA). Allele sizes were determined using the LIZ$^{®}$ 600 internal size standard and the GeneMapper$^{®}$ 4.0 software from ABI (Life Technologies, California USA). Genotype scoring was performed by two researchers.

Microsatellite loci were characterized for all four sampling populations of *R. annulatus* in terms of total number of alleles present (N_A), effective number of alleles (N_E), observed (H_O) and unbiased expected heterozygosity (uH_E), polymorphism information content (PIC) and the inbreeding coefficient (F_{IS}). A summary of these descriptive statistics was generated in GENALEX and the excel add-in MICROSATELLITE TOOLKIT 3.1.1 (Park 2001). Subsequently, sampling populations were tested for Hardy Weinberg equilibrium (HWE) and significance was calculated using the exact test (Guo & Thompson 1992) with a Markov chain of 10^4 steps and dememorization of 10^4 steps in GENEPOP 4.0 (Raymond & Rousset

1995). Allelic richness for each sampling site was estimated using FSTAT 2.9.3 (Goudet 2002).

F-statistics (pairwise F_{ST} values) (Weir & Cockerham 1984) were determined in ARLEQUIN 3.5 (Excoffier *et al.* 2010) to test for genetic differentiation between sampling populations. A false discovery rate (FDR) was determined for multiple tests and applied to obtain true significance. An AMOVA analysis was undertaken to test for the hypothesis of panmixia by pooling samples from all sampling sites. Since all sampling sites are within the Indian Ocean and same marine bioregion, the hypothesis of panmixia was tested against structure between sample pools from the western (DP and DM) and eastern most side of the bioregion (JB and PE). Tests for isolation-by-distance (IBD) were performed in GENALEX 6.5 (Peakall & Smouse 2012) for the four sampling sites. Geographic distances were estimated using the online GPS Coordinate Converter and Maps used in previous chapters. The mantel test was run with linearized F_{ST} values and geographic distances (km) between sampling sites.

Bearing in mind the cross-species microsatellite loci used and the study species *R. annulatus* being highly divergent from *G. galeus* and the *Mustelus* spp. (source species), data analysis was based on different datasets to investigate the level of connectivity for the study species. Firstly, simulations to evaluate statistical power of the microsatellites to differentiate populations were conducted in POWSIM 4.1 (Ryman 2011). This analysis simulated multiple populations that have diverged to predefined 'true' levels of divergence, and determined the power of a dataset with sample sizes, number of loci, and allele frequencies equal to the present study to differentiate populations. Simulations included true levels of divergence (F_{ST}) that ranged from 0.005 to 0.010, and significance for both Fisher's exact test and chi-squared tests from 1000 replicates. Three simulations with varying effective population size (N_e = 100, 200, 300 and 500) were performed. Secondly, the software WHICHLOCI 1.0 (Banks *et al.* 2003) was applied to the microsatellite dataset to select a subset of the most diagnostic loci in determining genetic structure of *R. annulatus*. The dataset was used to create four subpopulations of 100 simulated individuals to infer, under 100 iterations, which combinations of microsatellites allow correct assignment of the simulated individuals with a minimum and maximum inaccuracy of 95% and 5%, respectively. Locus scores were generated using the critical population method to ascertain the accuracy of the loci in assigning individuals to each population (Park 2001). A second dataset was also tested

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excluding those loci that exhibited null alleles and those that exhibited a PIC value lower than 50%.

A discriminant analysis of principal components (DAPC) plot was used to illustrate the relationship amongst individuals from the four different sampling sites based on 1) all 15 loci and 2) a subset of the loci ranked highest with WHICHLOCI and 3) a dataset containing the most robust loci taking the above criteria (PIC, POWSIM, WHICHLOCI) into account. Subsequently, Bayesian clustering analysis as implemented in STRUCTURE 2.3.4 (Pritchard *et al.* 2000; Falush *et al.* 2003) was used to infer the most likely number of clusters and to create assignment plots using the same run conditions as described in the previous chapters and testing for K = 1 - 4. Eventually, structure display plots were visualised in DISTRUCT 1.1 (Rosenberg 2004). Clustering and assignment results obtained for both datasets were compared to establish the robustness of the analysis with the least number of microsatellite loci.

BOTTLENECK 1.2.02 (Cornuet & Luikart 1996) was used to detect the presence of recent genetic bottlenecks by measuring heterozygosity excess. The Wilcoxon's test was used to test for significant deviations from population mutation-drift equilibrium, following the two-phase model (TPM) with 95% step mutations and a variance among multiple steps of 12. Lastly, an overall effective population size was estimated for *R. annulatus* using the linkage disequilibrium method implemented in NE ESTIMATOR 2.0 (Do *et al.* 2014).

5.3. Results

5.3.1   Genetic diversity

Three of the microsatellite loci showed evidence for null alleles (*McaB5*, *Gg2*, *Gg12*) with a significant level of heterozygote deficiency. Pairwise comparisons between loci revealed no linkage disequilibrium (P > 0.005). After correction for multiple tests, allelic frequencies conformed to expectations under HWE with the exception of samples from Die Plaat, which showed significant departures from HWE (P < 0.00083) at six of the microsatellite loci tested (*Mca25, McaB39, McaB22, McaB27, Gg2, Gg11*). Ten microsatellite loci were polymorphic in all four the sampling populations and only three loci (*Gg3, Gg11* and *Gg12*) exhibited a PIC value less than 50%. The majority of alleles occurred in multiple sampling sites (87%) and, as expected, the highest number of alleles occurred in the areas with the largest sample sizes (Die Plaat and Port Elizabeth). A small percentage (13%) of private alleles was

identified in De Mond and Port Elizabeth (Error! Reference source not found.). Interestingly, a higher number of effective alleles occurred in De Mond than in Port Elizabeth and a similar pattern was noted with the expected heterozygosity. Overall a moderate level of genetic diversity was observed for *R. annulatus* (H_O = 0.499-0.645) with the lowest expected heterozygosity exhibited at Port Elizabeth and the highest at Die Plaat. A previous study on the ray species *Urobatis halleri* exhibited much higher overall levels of genetic diversity (H_E = 0.85-0.89) (Plank *et al.* 2010) compared to *R. annulatus* but might in part be related to much larger sample sizes and the use of species-specific markers.

Table 5.1. Genetic diversity estimates for *R. annulatus* based on 15 cross-species microsatellite loci: sample number (N), number of alleles (N_A), effective number of alleles (N_E), number of private alleles (N_P), observed heterozygosity (H_O) and unbiased expected heterozygosity (uH_E), coefficient of inbreeding (F_{IS}), polymorphism information content (PIC). P_{HWE} with asterisk denote significant deviation from HWE.

Site	N	N_A	N_E	N_P	H_O	uH_E	F_{IS}	PIC	P_{HWE}
DP	32	7	4.188	0	0.499	0.750	0.346	0.679	0.030*
DM	12	5	3.697	1	0.645	0.739	0.137	0.645	0.305
JB	6	4	2.999	0	0.511	0.747	0.384	0.581	0.264
PE	33	7	3.612	2	0.532	0.650	0.194	0.590	0.057

5.3.2. Population differentiation

Prior to correction for multiple tests, significant genetic differentiation was evident between sampling sites (F_{ST} = 0.032, *P* = 0.049) **Table 5.2**. However, after FDR only pairwise comparisons between Die Plaat and the samples from the eastern region at Jeffrey's Bay and Port Elizabeth were significant with estimates ranging from 0.069 to 0.094 (*P* < 0.029). Overall, significant differentiation was exhibited between the far western and eastern *R. annulatus* of the Agulhas bioregion with very little differentiation detected between sampling sites within close proximity. The latter was consistent with the AMOVA results, which also depicted a low but significant level of molecular variation amongst the sampling populations (F_{ST} = 0.052, *P* = 0.000), with the highest level of variation evident within sampling populations. The testing of structure between *a priori* western and eastern grouped sampling

sites was not supported by the AMOVA ($F_{CT} = 0.046$, $P = 0.329$). Most of the genetic variation (93.71%) was accounted for by intra-individual variation (**Table 5.3**). The mantel test indicated that genetic differentiation is significantly correlated with geographical distance and that the molecular variance observed might be as a result of isolation-by-distance ($R^2 = 0.036$, $P = 0.010$) (**Figure 5.2**).

Table 5.2. Pairwise F_{ST} values based on 15 microsatellite loci (below diagonal) and *P*-values (above diagonal) compared across the sampling sites of *Rhinobatos annulatus* in the Agulhas bioregion.

	DP	DM	JB	PE
		0.285	0.028**	0.000**
DM	0.016		0.069	0.049*
JB	0.094**	0.061		0.149
PE	0.069**	0.032*	0.022	

*Significance at nominal level *P* < 0.05 before correction for multiple tests.

**Significance at nominal *P* < 0.029 after a false discovery rate was applied.

Table 5.3. AMOVA analysis based on 15 microsatellite loci. *Significance at nominal level P < 0.05.

Hypothesis tested	Source of variation	% variation	Fixation indices	*P*-value
Panmixia	Among populations	5.19	$F_{ST} = 0.052$	0.000*
	Within populations	94.81		
West vs East	Among groups	4.57	$F_{CT} = 0.046$	0.329
	Among populations	1.72	$F_{SC} = 0.018$	0.172
	Within populations	93.71	$F_{ST} = 0.063$	0.000*
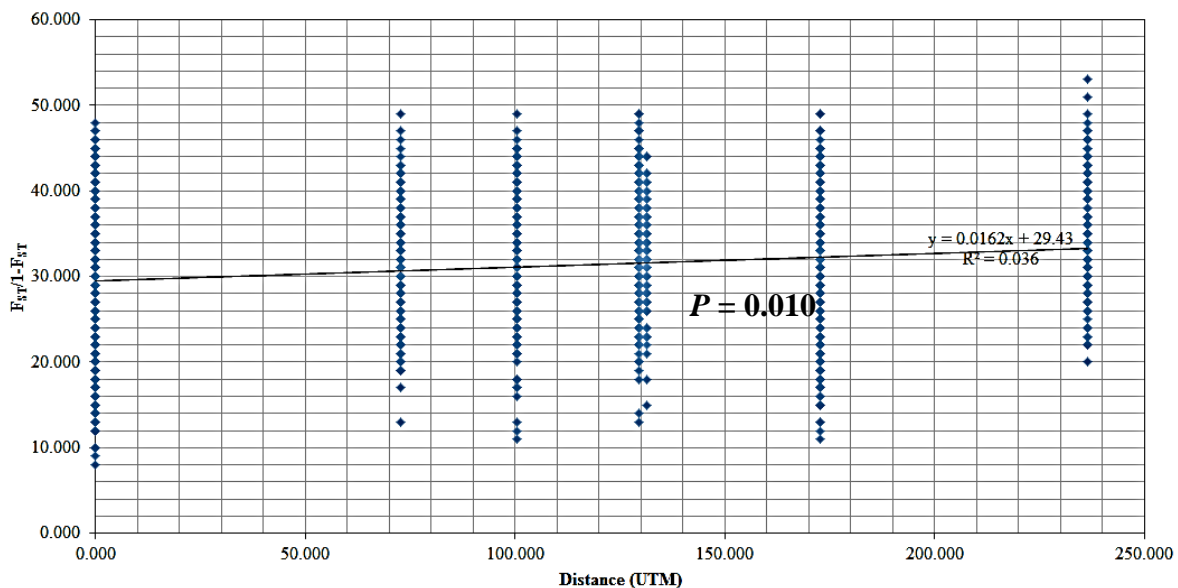
Figure 5.2. A mantel test investigating isolation-by-distance (IBD) between sampling sites of *Rhinobatos annulatus* based on microsatellite data.

5.3.3. Microsatellite marker evaluation

Four criteria were used to select the most reliable loci in detecting population structure; the absence of null alleles, the level of PIC, the statistical power of the loci as estimated in POWSIM and the assignment score as estimated using WHICHLOCI. Based on the first two criteria, five loci were excluded from the dataset; for presence of null alleles (McaB5, Gg2, Gg12) and PIC values lower than 50% (Gg3, Gg11, Gg12). Simulations of statistical power using POWSIM indicated that pairwise differentiation based on all 15 microsatellites at a level of $F_{ST} = 0.005$, was only significant in 62% of the 1,000 replicates (chi-squared = 68.2% and Fisher's exact test = 66.4%) while for levels of $F_{ST} > 0.01$ tested more than 90% of the replicates were significant. Individual locus tests showed that six of the 15 loci (Mh1, Mca25, McaB5, Mca33, Gg2 and Gg18) did not have sufficient statistical power to identify genetic differentiation levels below 0.005. These results indicated that the samples sizes and nine loci were large enough to detect genetic differentiation levels > 0.005. Interestingly, there was no direct correlation between PIC values and statistical power for each locus.

According to WHICHLOCI, all 15 microsatellites managed to correctly assign individuals to populations with an accuracy of 60.24%. The WHICHLOCI scores for the ten loci conforming to the first two criteria only managed to correctly assign individuals to populations by 37.35%. And of these ten, only a subset of four microsatellites (*Mh1, Gg18, Mh25, McaB22*) had a substantial locus score (> 90%). Based on this, different datasets were

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compared in their ability to assess population structure of *R. annulatus*; the first included all 15 loci (based on POWSIM results for levels > 0.01), the second data set included the ten loci with the highest PIC values ranked according to WHICHLOCI (**Table 5.4)** and the third dataset included six loci (Mh25, McaB22, McaB39, McaB27, Gg7, Gg15) taking into account all criteria and rankings.

Table 5.4. Polymorphism information content (PIC) for 15 microsatellite loci and their ranking in assigning individuals to the respective sampling populations: Die Plaat (DP), De Mond (DM), Jeffrey's Bay (JB) and Port Elizabeth (PE).

	WHICHLOCI locus rankings				
Locus	PIC	DP	DM	JB	PE
Mh1	0.737	1	1	5	4
Mh25	0.716	2	2	4	2
Mca25	0.654	3	3	6	5
McaB39	0.702	4	4	7	6
McaB5[A]	0.657	5	5	8	7
McaB22	0.746	6	6	2	8
McaB27	0.588	7	7	9	9
Mca33	0.628	8	8	10	10
Gg2[A]	0.502	9	9	11	11
Gg3[B]	0.346	10	10	12	12
Gg7	0.625	11	11	13	13
Gg11[B]	0.496	12	12	1	14
Gg12 [AB]	0.437	13	13	14	1
Gg15	0.817	14	14	3	15
Gg18	0.705	15	15	15	3

[A] Loci showing a presence of null alleles

[B] Loci with a PIC value less than 50%

5.3.4. Population connectivity of *Rhinobatos annulatus*

Discriminant analysis of principal components (DAPC) plots was generated based on the three datasets previously defined. All three DAPC plots based on 15, 10 and six loci, respectively, showed a similar pattern of genotypic distribution with varying degrees of clustering. Separation of Jeffrey's Bay and Port Elizabeth were evident with De Mond and Die Plaat overlapping the most **Figure 5.3**.

The STRUCTURE analysis assignment plots depicted a similar but less obvious pattern of three clusters with both the 15 and 10 loci datasets **Figure 5.4** using the L (K) approach for the admixture model (the highest for $K = 3$). Based on the 15 loci dataset, samples at Port Elizabeth showed the most homogeneity across the sampled region, while Die Plaat, De Mond and Jeffrey's Bay showed high levels of admixture and heterogeneity. Based on the 10 loci dataset, Port Elizabeth and Die Plaat showed a similar degree but distinct patterns of admixture, while the rest of the sampling sites were intermediate.
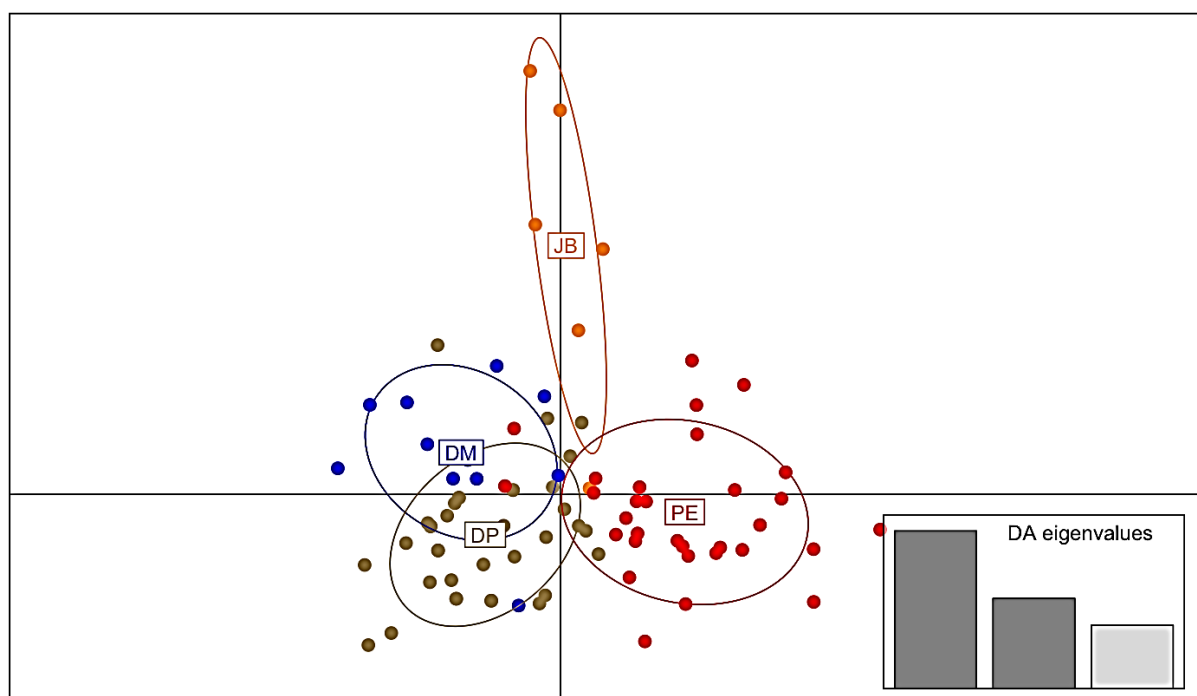
Figure 5.3. A discriminant analysis of principal components (DAPC) plot showing relationship of four *Rhinobatos annulatus* sampling populations based on 15 cross-species loci.
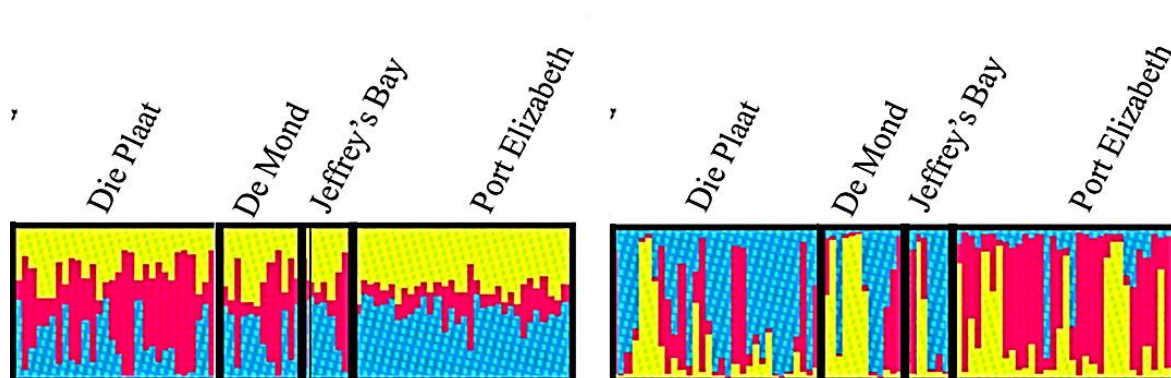
Figure 5.4. STRUCTURE analysis showing assignment of individuals at *K* = 3 based on ten loci (left) and 15 loci (right).

5.3.5. Mutation-drift equilibrium and effective population size

Very low levels of allelic richness were exhibited by *R. annulatus* across the Agulhas bioregion of South Africa. Allelic richness was highest for samples at Die Plaat and lowest for samples at Port Elizabeth. Samples at Jeffrey's Bay and De Mond also exhibited a higher allelic richness than at Port Elizabeth despite the large variance in sample sizes. Based on the BOTTLENECK analysis, *R. annulatus* showed no presence of a bottleneck at any of the four sampling sites. Non-significant probabilities of heterozygote excess were exhibited ($P > 0.05$) as well as a normal L-shaped mode of allele frequency distribution. An overall effective population size of $N_e = 106$ was detected for *R. annulatus* across the entire study region. Population-specific estimates of effective population were highest at De Mond and Jeffery's Bay and lowest at Port Elizabeth. Caution should however be taken in interpreting the results at Jeffery's Bay as this could most likely be an artifact of the very low sample size.

Table 5.5. Test for allelic richness (A_R) and mutation-drift equilibrium analysis based on 15 microsatellite loci showing the allele frequency distribution (AFD) and the *P* value under the two-phase model (TPM). Estimates for effective population size N_e.

Site	A_R	AFD	TPM	N_e
DM	1.739	Normal L-shaped	0.386	555
DP	1.749	Normal L-shaped	0.999	96
JB	1.747	Normal L-shaped	0.076	infinite
PE	1.651	Normal L-shaped	0.997	21

5.4. Discussion

5.4.1 Genetic diversity

An important aspect of biodiversity conservation is addressing the preservation of genetic diversity especially since human activity pushes many species closer to extinction (Greenbaum *et al.* 2014). This study provides the first genetic diversity assessment of *R. annulatus* across the warm temperate Agulhas bioregion of South Africa. This study was based on opportunistic sampling done during angling competitions in the Eastern Cape, therefore, providing only a glimpse in to what the status of *R. annulatus* might be across its entire distribution range. With only a very small area investigated, the moderate levels of genetic diversity noted in this study can not necessarily be used to draw conclusions on the current status of this species as a whole. Either way, it should be noted that the genetic diversity estimates observed for *R. annulatus* are well within the range of moderate genetic diversity reported for most other elasmobranchs ($H_E = 0.5$-0.7) (Dudgeon *et al.* 2012).

Of interest is the presence of private alleles at De Mond and Port Elizabeth and it is likely these private alleles indicate the presence of isolated individuals at these sites. Sampling on the eastern side of the bioregion occurred during December, while sampling on the western side occurred during February and March. Samples also mostly included females larger than 58 cm TL, implying that they were all sexually mature. It is likely that the females sampled

during March at De Mond were there to give birth since birthing is known to occur during late summer (Rossouw 1983). The presence of private alleles at Port Elizabeth during December; when the biomass of the species is at its lowest in the Eastern Cape (Rossouw 1983), could indicate site specificity of distinct individuals, similar to what was seen for *G. galeus* in Chapter 3. With frequencies ranging from 0.014 to 0.083, the contribution of private alleles was however not considerable enough to indicate that gene pools evolved independently from one another (Slatkin 1985).

5.4.2   Marker evaluation and genetic connectivity

Since the *de novo* development of microsatellites is challenging in elasmobranchs due to notoriously low rates of polymorphism and the costs involved, the development of microsatellite markers through cross-species amplification provides a promising alternative to *de novo* development of microsatellites. The success rate of microsatellite cross-species amplification has been directly correlated with the evolutionary distance between source and target species (Barbará *et al.* 2007; Dudgeon *et al.* 2012; Maduna *et al.* 2014). Thus far, no attempts have been made to develop species-specific markers for any of southern Africa's endemic rays and skates. Therefore, the use of cross-species microsatellite loci from highly divergent taxa in this study raised considerable concern with regards to bias. The 15 microsatellite loci developed in the *Mustelus* species and *G. galeus* detected significant levels of genetic differentiation amongst the sampling populations with a probability over 60% for both chi-squared and Fisher's exact test. This result was the same for the different effective population size simulations. Furthermore, pairwise F_{ST} estimates and fixation indices for AMOVA analyses were comparable amongst datasets, irrespective of which loci were included. Regardless of whether all 15 loci, only the ten with high PIC values, or the six of the most reliable and highly ranked loci by POWSIM and WHICHLOCI were included, the cross-amplified markers were reliable in detecting pairwise F_{ST} values as low as 0.005 (**Table 5.6**).

Many of southern Africa's endemic marine species are underrepresented as far as genetic studies are concerned with only but a few reporting on the phylogeographic barriers of historical gene flow (Teske *et al.* 2006; Neethling *et al.* 2008; von der Heyden *et al.* 2008; McLachlan 2011) and contemporary gene flow patterns (Bester-van der Merwe *et al.* 2010). This is unfortunate as the southern African coastline is a hotspot of biodiversity and endemic species should be a focal point for the conservation of this region's rich biodiversity. Overall,

the genetic structure observed for *R. annulatus* was consistent with low to moderate levels of genetic differentiation reported for other benthic elasmobranchs investigated over relatively small spatial scales. For example, the thornback ray *Raja clavata* showed low but significant structure in British waters while the round stingray *Urobatis halleri* revealed low contemporary structure in the Gulf of California (Plank *et al.* 2010). *Rhinobatos annulatu*s showed moderate levels of genetic differentiation across the continuous coastal stretch of the Agulhas bioregion with no significant differentiation between samples in close proximity to each other, *i.e.* between De Mond and Die Plaat, and between Jeffrey's Bay and Port Elizabeth. This was corroborated by multi-variate and Bayesian clustering analyses. However, the hypothesis that the western and eastern sample pools across the Agulhas bioregion are distinct could not be rejected. This was supported by the existence of distinct levels of admixture on either side of the warm temperate bioregion. Furthermore, a low but significant level of correlation between linearized genetic differentiation estimates and geographic distance was detected by the mantel test. This indicated further that there is some degree of isolation by distance being exhibited by this species.

The percentage of accurately assigning individuals to the respective populations was determined for all three datasets, and as expected the dataset based on all 15 loci showed the highest overall assignment success. However, only the Jeffrey's Bay individuals were correctly assigned irrespective of the number of loci used. This could most likely be due to the bias in sample size used (N = 6) for which the software cannot avoid inadvertent genetic drift for sample sizes below 500 (Banks *et al.* 2003).

Despite low fecundity, *Rhinobatos annulatus* does not exhibit strictly *K*-selected traits but are comparatively smaller-bodied, shorter-lived, and earlier-maturing. This implies that the species might not be as vulnerable to exploitation compared to the two shark species investigated in this study. Nonetheless, since it is predominantly caught as bycatch and as a recreational target across the area of study, this could have long term effects on the overall population status of this species with consequence to local biodiversity.

The Port Elizabeth population appeared to be the most distinct from all other sampling sites, which could indicate the benthic habits and unique breeding patterns noted by Rossouw (1983; 1984) in the Eastern Cape. In addition, the results indicated the highest levels of genetic differentiation between Die Plaat and Port Elizabeth, suggesting that geographical distance might be correlated with genetic distance as was corroborated by the test for IBD.

However, it should be noted that the test for IBD was based on only a few populations and does not cover the entire distribution range of the species. The high levels of genetic differentiation seen between the western and eastern Agulhas bioregion is more likely explained by the fidelity of these animals to specific bay areas, resulting in isolated or "pocket populations".

The relatively low effective population size found at Port Elizabeth and Die Plaat is concerning and below the approximately 500 to 1000 breeding individuals needed for an idealized population to retain enough genetic variability and ensure evolutionary potential (Franklin & Frankham 1998). It should also be noted that the differences between levels of genetic diversity may reflect differences in average effective population size (Frankham *et al*. 2002), and that the lower genetic diversity seen at Port Elizabeth and Die Plaat in fact reflects the smaller effective population sizes for those areas. This may indicate a greater susceptibility to exploitation at these bay areas. However, discrepancies in effective population size do not necessarily reflect parallel discrepancies in census size (Frankham 1995). The parallel comparison of effective population size and census size are complicated by evolutionary effects (e.g. genetic drift) on the nuclear markers used (Nielsen *et al*. 2009), the sex ratios of the sampling cohort and whether or not a population has experienced a fluctuation in size. The census number of *R. annulatus* in its region of endemism l*et al*one South Africa is for the most part unknown. However, the data presented here suggests an overall effective population size (N_e = 106). Given the highly skewed sex ratio in this particular study, this is a rough and biased estimation at best but. Even so, taking into account the suggested abundance of the species, this value is surprisingly low. In a meta-analysis, Frankham (1995) identified a median ratio of effective population size (N_e) to census population size (N_c) of 10%, though the range was very broad. Subsequently, later studies on elasmobranch species have identified that historical bottlenecks can depress the N_e/N_c ratio (Daly-Engel *et al*. 2012; Tillett *et al*. 2012). Therefore, it is imperative that future sampling across South Africa's bioregions should be done including equal sex ratios, to gauge information about reproductive capacity and effective population sizes in a crucial effort to examine whether exploitation of these animals in bay areas is sustainable.

5.4.3   Mutation-drift and equilibrium

Allelic richness is a strong indicator for the evolutionary potential of a population, and it has been suggested that this measure is of key importance in population conservation and

management. Allelic richness measures are also commonly presented in population genetic summaries but in practice conclusions pertaining to these measures are often merely comparative between populations and not quantitative (Caballero & García-Dorado 2013). The low allelic richness at Port Elizabeth is probably not an artefact of sample size, since De Mond and Jeffrey's Bay had fewer samples. Instead, low allelic richness could suggest a founder event, resulting in a reduction in the number of alleles introduced. Results from the BOTTLENECK, conversely, did not indicate significant deviations from heterozygosity expected at mutation-drift equilibrium that is characteristic of a genetic bottleneck. However, without any population trends recorded for this species, it is difficult to make any speculations on declines in effective population size.

5.5 Management implications

There have only been a few reported instances of genetic isolation amongst endemic fishes of the South African bioregions and those have mostly referred to species with larval dispersal. Population connectivity among endemic species deserves more attention. This study assessed the population structure of an elasmobranch whose exploitation levels remain unknown, using cross-species microsatellite markers and, although over a small spatial scale, revealed some degree of substructure across the Agulhas bioregion. In addition, this study showed surprising success using cross-species markers for population structure inference in *R. annulatus*. Based on the current results, it is clear that *R. annulatus* shows genetic differentiation over a small geographic distance and this implies that the species might be highly structured throughout its entire geographical range. The latter could have serious ramifications for the future sustainability of genetic diversity within this endemic and other ray species with a similar pattern of distribution *e.g. R. blochii*. The fact that the moderate levels of genetic diversity seen in this study are similar to those exhibited by most elasmobranchs, should not be justification for the continued listing of this species as of "least concern." Rather, more vigorous assessments should be carried out with strong emphasis on curbing incidents of bycatch and rationing recreational fishing effort.

Supplementary Material

Table 5.6. Sampling sites, sample details and sampling dates

Site	Gender	TL (cm)	Coordinates	Sampling date
Die Plaat (DP)	36F	78-99.3	47.4"33°SE 24.16"18°SE	2012
De Mond (DM)	4M, 8F	70.8-98	52.55"33°SE 07.14"17°SE	2012-2014
Jeffrey's Bay (JB)	1M, 5F	79-97	48.8"34°SE 58.17"18°SE	2011
Port Elizabeth (PE)	32F	69-110	54.09"34°SE 17.57"18°SE	2011

Table 5.7. Genetic diversity estimates based on 15 loci

Site	N	N_A	N_E	H_O	uH_E	F_{IS}	PIC	P_{HWE}
DP	32	7	4.188	0.499	0.750	0.346	0.679	1.000
DM	12	5	3.697	0.645	0.739	0.137	0.645	0.000
JB	6	4	2.999	0.511	0.747	0.384	0.581	0.000
PE	33	7	3.612	0.532	0.650	0.194	0.590	1.000

Table 5.8. Percentage of assignment of samples to populations for three different datasets. Dataset 1 included all 15 loci; Dataset 2 included ten loci selected based on PIC values and WHICHLOCI; and Dataset 3 included a subset of six loci selected based on PIC values, POWSIM and WHICHLOCI.

Site	DM	DP	JB	PE	Total
15 loci	75	15.63	100	100[C]	60.24
10 loci	66.67	21.88	100	30.30	37.35
6 loci	66.67	15.63	100	12.12	27.71

[C] required one locus (*Gg12*) to reach accuracy of assignment

Table 5.9. POWSIM simulations for statistical power of 15 microsatellites to differentiate sampling populations of *Rhinobatos annulatus* at a true differentiation level (F_{ST} = 0.005). Results are provided for both chi-squared and Fisher's exact tests for the proportion of simulations out of 1,000 that were significant with a critical value of 0.05.

True F_{ST}	Chi-squared	Fisher
0.005	0.682	0.664
Locus		
Mh1	0.010	0.083
Mh25	0.740	0.731
Mca25	0.077	0.022
McaB39	0.763	0.699
McaB5[A]	0.035	0.071
McaB22	0.138	0.103
McaB27	0.658	0.549
Mca33	0.006	0.049
Gg2[A]	0.001	0.004
Gg3[B]	0.791	0.799
Gg7	0.255	0.195
Gg11[B]	0.117	0.162
Gg12[A B]	0.143	0.229
Gg15	0.143	0.235
Gg18	0.033	0.042

[A] Loci showing a presence of null alleles

[B] Loci with a PIC value less than 50%

Table 5.10. Locus ranking performed by WHICHLOCI using ten microsatellite loci

Rank	Locus	Score	% Score
1	Mh1	0.1687	63.6364
2	Gg18	0.0361	13.6364
3	Mh25	0.0241	9.0909
4	McaB22	0.0241	9.0909
5	Mca25	0.0120	4.5455
6	McaB39	0.0000	0.0000
7	McaB27	0.0000	0.0000
8	Mca33	0.0000	0.0000
9	Gg7	0.0000	0.0000
10	Gg15	0.0000	0.0000
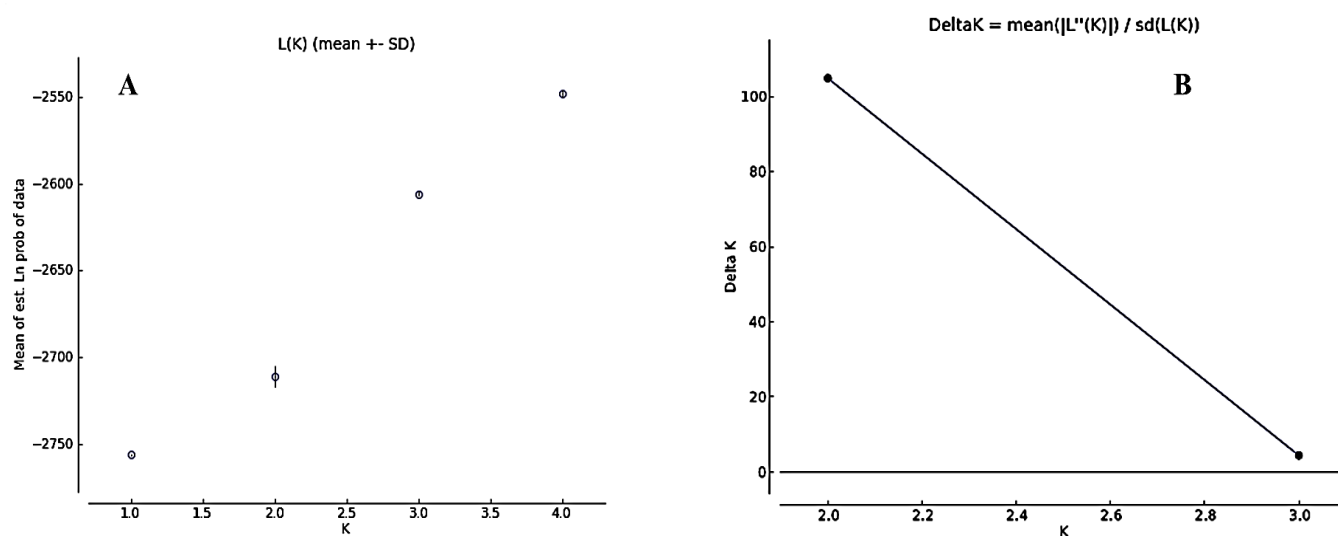
Figure 5.5. Identification of the number of genetic clusters (*K*) of best fit using the **A**. "log probability of data" (Mean of LnP±1) approach and **B**. the Evanno method to identify the highest "delta *K*" (Δ*K*).

Chapter 6: **General discussion of range-wide genetic diversity and connectivity: implications for regional shark fisheries and elasmobranch conservation**

6.1. Synopsis of regional genetic diversity

Similar to most other cosmopolitan and endemic elasmobranchs (Benavides *et al.* 2011; Geraghty *et al.* 2013; Castillo-Paez *et al.* 2014; Hernández *et al.* 2015), the regional genetic diversity of the three elasmobranch species *Galeorhinus galeus*, *Carcharhinus brachyurus* and *Rhinobatos annulatus* was low to moderate compared to teleost fishes ($H_O = 0.45 - 0.75$). For all three species, varying levels of genetic diversity were observed across the study areas investigated but most notably within the Atlantic Ocean with *G. galeus* showing a slightly higher level of heterogeneity than *C. brachyurus*. Interestingly, *Rhinobatos annulatus*, the least exploited of the species, showed the lowest level of genetic diversity consistently across all sampling populations. With *R. annulatus* being data-deficient for most of southern Africa, the few reports show that it is mostly threatened by anthropogenic factors such as settlements, angling and bycatch. Added to this is the fact that this species, in comparison to the two commercial sharks, inhabits very shallow waters close to sandy beaches further exposing it to habitat degradation and fishing effects. Unlike the other two species, *R. annulatus* is not listed as vulnerable and this can mostly be attributed to a lack of data likely due to its low commercial value. With regards to data collection and stock assessment, more emphasis is placed on species such as *G. galeus* and *C. brachyurus* that are both affected by targeted and non-targeted fisheries. It is also then important to note that for both these species, levels of diversity were considerably higher for Indian Ocean samples than for Atlantic Ocean samples coinciding with the latter area being most exploited.

Moderate levels of mitochondrial diversity were detected for the two commercial species *G. galeus* and *C. brachyurus* and were found to be marginally lower than those seen for other commercial shark species (Benavides *et al.* 2011; Karl *et al.* 2012; Chabot *et al.* 2015; Clarke *et al.* 2015; Hernández *et al.* 2015). The latter studies all used the mitochondrial control region (mtCR) and not the mitochondrial-coding genes used in this study (*ND2* and *ND4*). Studies conducted to compared the robustness of the mitochondrial coding genes relative to mtCR in detecting substructure, showed that mitochondrial-coding genes were informative enough to detect structure and in some cases even more so than the mtCR (Castillo-Paez *et*

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al. 2014; Feutry *et al.* 2014). Therefore, the low levels of mitochondrial diversity seen within samples of *G. galeus* and *C. brachyurus* in this study are not simply as a result of the inherent properties of the mitochondrial genes used but could in part reflect the severe exploitation these species are experiencing over the last few decades.

6.2. Synopsis of population connectivity of *G. galeus* across the Southern Hemisphere

Patterns of contemporary and historical gene flow were determined for *G. galeus* across the South Pacific, South Atlantic and the Indian Ocean. Both nuclear and mitochondrial data indicated that the species is highly divergent across the three ocean basins and the hypothesis of panmixia was strongly rejected based on statistical support. The haplotype genealogy showed two distinct clades that most likely emerged as a result of a Tethyan divergence followed by closure of the Isthmus of Panama. Furthermore, there was strong evidence to support the hypothesis of phylogeographic barriers to gene flow such as the Great Australian Bight (GAB) that stretches across the Indo-South Pacific region, the Mid-Atlantic Barrier (MAB) between South America and South Africa, and the Benguela Barrier (BB) in combination with the Agulhas return current. The presence of the BB in the Atlantic Ocean combined with the presence of gyres and straits, most likely restricts gene flow between South Africa and Argentina while the GAB seems to be a barrier between Western Australia and New Zealand. It should be noted that the panmictic population of *G. galeus* previously found between Australia and New Zealand (Hernández *et al.* 2015), is due to the fact that the latter study included samples from Southern Australia on the same side of the GAB barrier. What is interesting to note, is the subdivision of the South African collection and the fact that the Port Elizabeth samples are more connected to haplotypes from the Indo-Pacific region than the rest of South Africa. These findings coincide with the hypothesis of a secondary contact zone reported for many elasmobranch and other marine species along the southern tip of Africa (Benavides *et al*. 2011; Dudgeon *et al*. 2012).

6.3. Synopsis of genetic structure and population connectivity across South Africa

Based on the microsatellite data analysed in Chapters 3, 4 and 5, evidence for subtle to strong contemporary genetic differentiation was found within all three species. Higher levels of differentiation were evident among *G. galeus* and *R. annulatus* sampling populations than for *C. brachyurus*, coinciding with the relatively bigger body size and dispesal ability of the copper shark. Results depicted for all three elasmobranchs showed that the microsatellite multiplex assays optimised for each species were informative enough in detecting patterns of contemporary gene flow based on inference methods ranging from F- statistics to Bayesian clustering analyses. Varying levels of support were obtained with different analysis methods, but in all three species, it was reasonable to reject the hypothesis of panmixia based on statistical support. Furthermore, it was noted that the most prominent physical feature responsible for limiting gene flow appeared to be the barriers originating from the ocean currents, the Benguela and the Agulhas, and most importantly, the upwelling associated with the interchange between these currents **Figure 6.1**. Although differing patterns in structuring were displayed for *G. galeus* and *C. brachyurus*, a similar pattern is seen across the warm temperate region with the point of disjunction in the vicinity of Cape Agulhas and the surrounding region of Struisbaai corresponding to a zone of admixture **Figure 6.1**. Similarly for *R. annulatus*, the admixture zone seems to be in the region just east of Cape Agulhas with higher admixture exhibited for Die Plaat and De Mond than for Port Elizabeth. It is well documented that the complex interactions between contemporary and historical factors are responsible for the observed structure within species. In this study, the different patterns of gene flow across the three study species are therefore ascribed to a combination of factors such as differences in life history strategies, behaviours such as seasonal distribution, mating patterns, sex-biased behavioural traits and biological characteristics such as size and mobility.
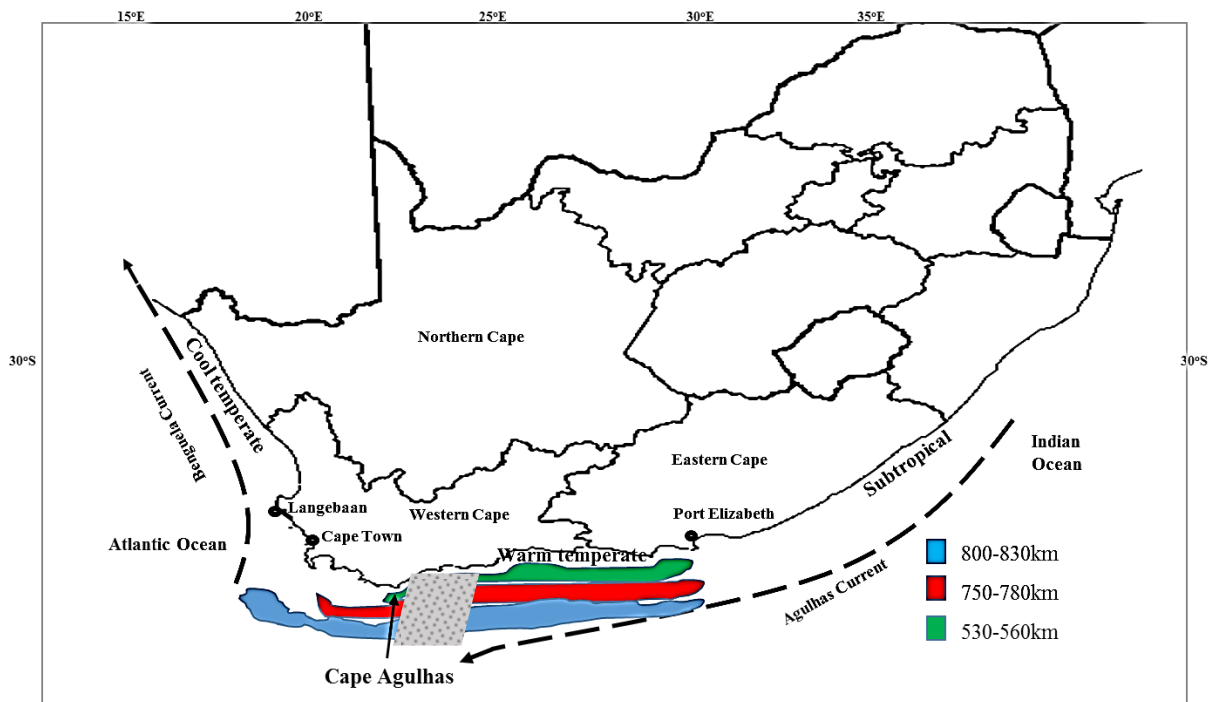
Figure 6.1. Map of South Africa indicating the oceanic currents, the point of disjunction (Cape Agulhas) and the proposed zone of admixture (shaded grey area). The sampling ranges of *Galeorhinus galeus*, *Carcharhinus brachyurus* and *Rhinobatos annulatus* for this study are shown in blue, red and green, respectively

The stock status of only the commercial species studied here is known; with *G. galeus* been assessed between 2001 and 2003 and shown to be fully exploited with a need to maintain the then current spawner biomass and recruitment levels (McCord 2005). Based on the current results, *G. galeus* exhibits shallow but significant genetic differentiation with the existence of two, possibly three genetic groups in South Africa. This implies that migrations across the distribution range of the species are not necessarily enough to maintain a sustainable recruitment level. And if this is indeed the case, critical spawner biomass has not been maintained since the last stock assessment of this species. Only fishery-dependent and fishery-independent stock data exists for *C. brachyurus* while no population trend data exists for *R. annulatus* in South Africa. Both these species exhibited some degree of structure over a fairly small geographical range with that observed for *R. annulatus* more pronounced than for *C. brachyurus*. Since most of the sampling for *C. brachyurus* occurred during the summer months (November to March), it is likely that a large number of the individuals contained an admixture of migrants from the western and eastern coastal regions, similar to what was noticed for the South African and Namibian samples in the study by Benavides *et al.* (2011);

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explaining the lack of inter-oceanic structure. Concurrently, high population connectivity could be explained by the comparatively larger size of *C. brachyurus* and the fact that the species is known to aggregate in schools during the summer months (Smale 1991), which could facilitate gene flow even more. Additionally, female philopatric behaviour can account for the observed distribution of genetic diversity in *C. brachyurus* especially with regards to samples at Mossel Bay. Despite also having been sampled during the summer months (December to March), *R. annulatus* showed a higher level of structure over a small sampling region in what is known to be its mating period. Since there is no evidence of schooling for this species, the genetic structure of *R. annulatus* was consistent with that of a typical benthic species that has restricted geographic distributions and it is likely that during mating in the inshore sandy beaches, some individuals of *R. annulatus* are anchored to particular regional sites for reproduction purposes. Overall, it is apparent that the elasmobranchs studies here are not only exhibiting gene flow patterns driven by physical barriers such as oceanic currents but by a combination of biogeographical features, seasonal distribution patterns, biological aspects *e.g.* size, mobility and habitat preference.

Across South Africa, historical gene flow patterns for the commercial species *G. galeus* and *C. brachyurus*, showed that a major phylogeographic discontinuity exists across the Atlantic and Indian Ocean. The phylogeographic patterns observed here were connected to phylogeographic barriers such as the Indian/Atlantic barrier, historical events such as vicariance, oceanographic and climate change patterns, as well sex-biased traits. The presence of private and very divergent haplotypes at Port Elizabeth and Mossel Bay for *G. galeus* and *C. brachyurus*, respectively, suggests that perhaps these haplotypes represent females that are anchoring in this region for reproduction purposes. These private haplotypes also raise concern that perhaps some individuals (most likely gravid females) are being exploited heavily in these areas leading to a long-term reduction in spawner biomass for the species. Alternatively, the persistent thermal front (also as a result of upwelling) in this particular area at the eastern most end of the Agulhas Bank (Lutjeharms *et al.* 2000) might also play a role in defining contemporary distribution of genetic diversity for these sharks.

6.4. Management implications for South African fisheries

The findings of this study could have immediate application to the regional management of the commercial sharks, which are exploited in many locations around the world for their fins and meat. Regional samples of *G. galeus* inhabiting different marine bioregions across two oceans, have been shown to comprise of distinct genetic groups and therefore also management units in fisheries terms. Furthermore, differences exhibited in mitochondrial haplotypes and microsatellite genotypes between these and other populations included from the Southern Hemisphere, might be able to facilitate future trade-monitoring efforts for internationally traded products such as fins and meat which are known to be exported from South Africa to Australia. Results of this study also confirmed that barriers to gene flow and historical demography contributed to the contemporary distribution of genetic diversity in this migratory species that inhabit an otherwise continuous marine habitat. Currently, local and global efforts on the regulation of cosmopolitan species are done under the auspices of each respective country involved. Regionally, *G. galeus* should therefore be managed not just on an ecosystems-based approach in line with the marine bioregions of South Africa, but it should be taken into account that since most of the fishery efforts are centered around the southwestern coast, one of these putative stocks (of Atlantic origin) might be more vulnerable that the others. However, we stop short of classifying all three genetic groups as fully differentiated 'stocks' in the classic fisheries sense and should rather focus on the existence of a highly admixed stock along the south-west coast and possibly two more discrete stocks on the eastern side of the Agulhas barrier.

As to whether the results from the regional assessment of *C. brachyurus* have immediate applications, could be arguable since only a small geographical range was included. Nonetheless, the fact that the area of sampling happens to be the most highly exploited for this species, and that some differentiation was indeed revealed, it warrants immediate further investigation in to a finer-scale structure of the species. Coupled to this are the highly divergent haplotypes observed at Mossel Bay, indicating that phylopatric behavior of females might also be at play. Alternatively, it can be argued that perhaps, due to the species ability for travelling long distances, this species is for the most part admixed along its southern African distribution range as reported by Benavides *et al.* (2011) where historical mitochondrial haplotypes were shared between samples from KwaZulu Natal and Namibia. *Carcharhinus brachyurus* in this study exhibited highly admixed microsatellite genotypes

with private alleles and ancestral haplotypes found at both False Bay and Mossel Bay. Thus, regional populations most likely rely on the slow processes of local reproduction and recruitment for replenishment and should therefore be carefully monitored and managed as populations associated with specific continental-shelf regions. This also only applies to the study range and cannot necessarily be applied directly to other areas across South Africa.

Based on the genetic diversity and connectivity of *R. annulatus* investigated across the Agulhas bioregion in this study, only speculations on how this species is performing within this region is justified, and these cannot be used to recommend management strategies for the species overall. However, the fact that *R. annulatus* exhibits genetic differentiation over the small coastal distance investigated here could mean that the species is in fact highly structured across the entirety of its distribution range. This could have serious ramifications for maintaining genetic diversity within this species with these results showing that the species does not rely on recruitment for replenishment. As mostly females were sampled in this study, it might also imply that females are harbouring in specific areas and do not even move over small regional scales. As mentioned before, the structure could also be related to the seasonal reproduction patterns and benthic nature of the species noted in the Eastern Cape by Rossouw (1984). All and all, the species is highly data deficient and being an endemic species, these results once again warrants further assessment for conservation of local biodiversity.

6.4.1. *Marine bioregional spatial scales*

Based on the molecular assessments, the movement patterns of these elasmobranchs seems to be driven mainly by oceanography specific to the biogeographical regions of South Africa as well as seasonal mating behaviour. However, management interests for South Africa's fisheries, are partitioned based on marine bioregions which when viewed, overlap with biogeographical regions **Figure 6.2**. Hence an integrated ecosystems-based approach in which the bioregions can be linked up to genetic discontinuities found across biogeographical regions is the most logical way forward to conserve as much of the biodiversity as possible.

For the species investigated here, the area between the cool and warm temperate biogeographical regions is shown as the region of admixture with the populations east or more offshore to the admixture zone as being more distinct. This implies that despite exhibiting varying levels of gene flow or having different distribution ranges and biology, the

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impact of the ecosystem on these species determines their bioregional spatial scales. Therefore, an ecosystems-based approach is warranted, the aim of which is to maintain the integrity of the ecosystem while enabling the sustainable management and conservation of these marine resources (Sardà *et al.* 2014). There is, however, a need for harmonized definitions of bioregions and standardized approaches in order to implement an ecosystems-based approach. According to Griffiths *et al.* (2010) the nine marine bioregions described for South Africa have to be revised as they were based on minimal biological sampling. Furthermore, these marine bioregions overlap with the coastal bioregions in which case, even with the limited sampling range covered in this study, a total of five marine bioregions could be covered. Interestingly, the defined coastal areas within these marine bioregions happen to be based on existing biota, while the offshore bioregions are based purely on physical criteria such as temperature, depth and substratum. With that information alone, it is apparent that these could raise some discrepancies in an ecosystems-management approach to sustainably maintain and conserve the elasmobranchs studied here. It should therefore not necessarily be used to implement policies but rather used as guidelines to protect individual stocks while taking into account the larger/broader ecosystem involved.
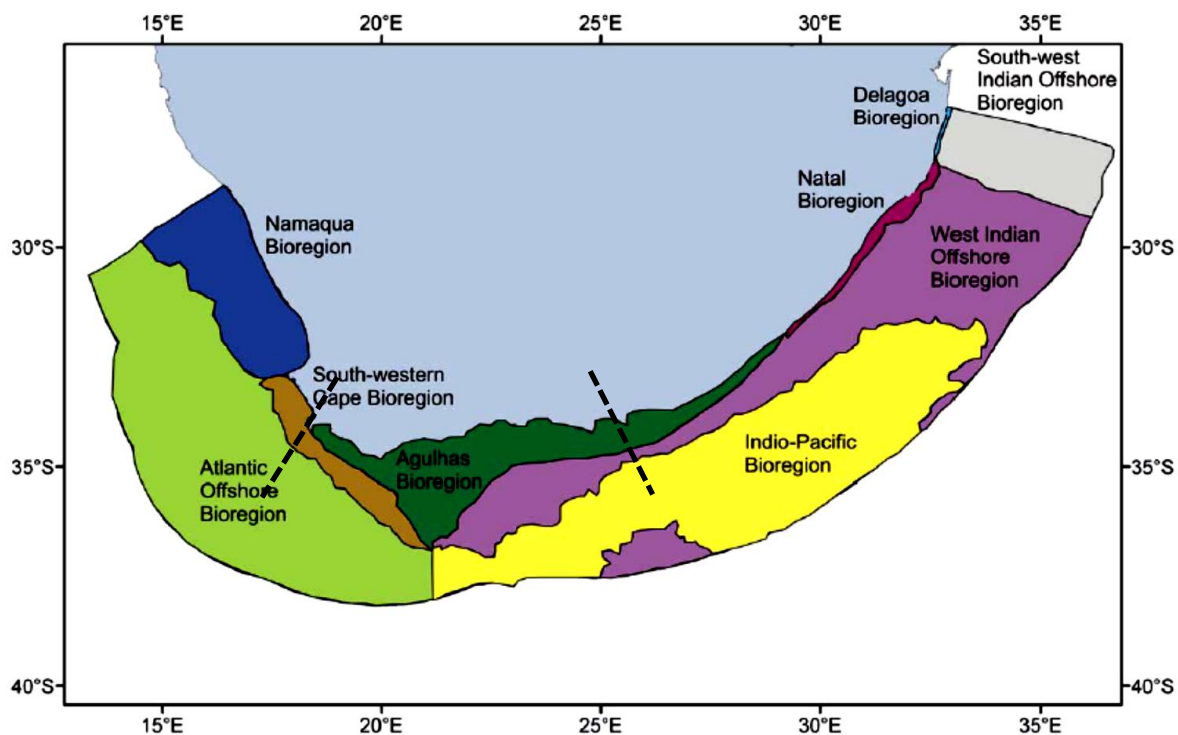
Figure 6.2. Map of South Africa showing the nine major bioregions as shown in Griffiths *et al.* (2010), and the separations between the three biogeographical regions defined as cool temperate, warm temperate and subtropical (indicated by dashed lines).

6.5. Future recommendations for South African fisheries

6.5.1. *Galeorhinus galeus*

This is the first regional assessment of contemporary and historical gene flow of *G. galeus* over its entire distribution range, with the last stock assessment conducted between 2001 and 2003. As such, the following are recommended as measures to be implemented over a time frame that is practically possible. The first measure recommended is site restrictive licensing and size restrictive licensing. This approach should be implemented as a precaution to prevent against the depletion of genetic variability and the spawner biomass. However, based on the results, this management approach should immediately be applied to those regions that have individuals showing the highest level of genetic uniqueness, especially at Port Elizabeth which is believed to be and could well be the source for genetic variability of South African *G. galeus*. The management approach can be applied by reducing the number of fishing permits currently issued for Port Elizabeth and by implementing size limitations (*i.e.*

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juveniles and gravid females must not be harvested) that come with incentives such as awarding of an additional permit to a reputable commercial fishing company that reports accurately on its catch data for a period of time.

In the long term , marine protected areas (MPAs) already implemented for the commercial shark species *M. mustelus* (da Silva *et al.* 2013) whose life history happens to be almost similar to that of *G. galeus*, should be revised to also include *G. galeus* to help maintain its current diversity levels under a marine bioregional jurisdiction and as an alternative to species-specific management. Furthermore, seasonal fishing restrictions should be applied in the future, prioritizing areas such as Port Elizabeth to help safeguard what is believed to be nursery areas for the species based on the heterogeneity in both marker types which supports either limited dispersal or reproductive philopatry by both sexes at this area. Further investigation constituting a bigger sampling effort is warranted at Port Elizabeth.

6.5.2. *Carcharhinus brachyurus*

This is the first assessment of population connectivity in *C. brachyurus* using a dual-marker approach. However, due to opportunistic and limited sampling a more extensive assessment should first be conducted before recommendations are made for regional management. For the moment it is suggested that *C. brachyurus* be monitored carefully due to local recruitment and also the possible existence of an admixed population at Strandfontein, most likely a vital source of genetic diversity for this species. In case this population is considered to be more offshore or migratory, according to Griffiths *et al.* (2010), it falls within a separate bioregion warranting separate management. In monitoring this species, permit licenses should be restricted for commercial vessels and especially for recreational anglers. This should then be followed up by a long term assessment of *C. brachyurus* in the south-west region to assess the purpose of permit limitations in maintaining the current levels of genetic diversity. Importantly, the possible existence of putative preferential areas for homing behaviour (such as embayments at Mossel Bay) should be further investigated to examine the possibility of sex-biased behaviour within *C. brachyurus* and stricter regulations for those areas.

6.5.3. *Rhinobatos annulatus*

Similar recommendations based on the current genetic assessment for commercial species cannot be enforced for the endemic ray *R. annulatus* since this species is completely data deficient and exhibits a very different life history. However, from a biodiversity perspective,

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R. annulatus should probably be seen as the most vulnerable species in this case and should take top priority in future assessments. Although regarded amongst anglers as abundant, this species falls under the seven most threatened families of elasmobranchs and without a concerted effort towards stock assessment, the impact of ongoing recreational fisheries or climate change on regional diversity cannot be predicted. Crucial to the sustainable management of this species is a robust stock assessment which includes information on reproductive capacity and effective population size assessments, allowing further insight into the role of mating behavior in maintaining the observed population structure. In addition, future fisheries management policies should not only be based on neutral genetic variability since neutral markers fail to recognize locally adapted populations, but rather on a mixed-marker approach. Future studies employing more than one class of marker are especially important for species with an unknown population size and potential for sex-biased dispersal. Focus should be placed on generating and assembling data for data deficient species such as *R. annulatus* that also happens to be one of the most vulnerable endemic species of this region.

6.5.4. Research directions

This study, although not first in its kind, highlights the value of (a) sampling the entire distribution range of a species including proximate sites and (b) using multiple marker types to separate historical dispersal (maternal lineage sorting) from contemporary gene flow patterns and (c) using several statistical analysis methods to infer population structure and demographic history and not to rely only on a single approach. Therefore, it is recommended that future population genetic studies on regional elasmobranchs should at the very least try to emulate such an approach in order to better define local population structure and delineate distributions and the key barriers driving differentiation between populations. In particular, larger sampling ranges should be covered for *C. brachyurus* and *R. annulatus*, while species-specific loci can be developed using new high throughput sequencing technologies. This will not only provide higher resolution for population genetic studies, but it will also elucidate the importance of local adaptive divergence in shaping the geographic distribution of different populations. In addition, mitogenome sequencing of neonates could resolve population structure on an even finer spatial scale (*i.e.,* identify nursery areas), thus providing even more compelling evidence of site specificity or natal philopatry hinted at for *C. brachyurus* and *R. annulatus*.

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