Genetic Diversity and Population Genetic Structure in the South African Commercially Important Shark Species, the Common Smoothhound (*Mustelus mustelus*)

by

Simo Njabulo Maduna

Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in the Faculty of Natural Science at Stellenbosch University

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF

Supervisor: Dr A.E. Bester-van der Merwe
Co-supervisor: Prof R. Roodt-Wilding
Department of Genetics

December 2014

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

……

Date**: November 2014**

Abstract

Deciphering patterns of intraspecies population genetic structuring in commercially important shark species is essential for an integrated fisheries management approach to conservation of regional biodiversity. The common smoothhound shark, *Mustelus mustelus,* is an overexploited, commercially and recreationally important shark species in South Africa. Considering the vulnerable status of the common smoothhound shark and due to very limited available genetic information, this study aimed to develop molecular markers, assess patterns of genetic diversity and population connectivity along the South African coast using multilocus data generated from 12 microsatellite markers and the mitochondrial gene, *NADH dehydrogenase subunit 4* (*ND4*). The cross-species amplification of microsatellites proved useful for genetic diversity and population genetic analysis of the common smoothhound shark. These microsatellites could aid in the molecular characterisation of other endemic and cosmopolitan species and provide valuable tools for the conservation of potentially threatened or exploited shark species. For the microsatellite data, moderate levels of genetic diversity based on the heterozygosity, allelic richness and haplotype diversity were found in a total of 144 individuals sampled across eight study populations. Estimates for pairwise population differentiation, F-statistics, AMOVA and factorial correspondence analysis (FCA) indicated significant genetic structure within and between west- and east coast populations. Additionally, Bayesian clustering analyses detected two putative ancestral gene pools, supporting the presence of a biogeographic barrier at the Cape Agulhas region and therefore genetic discontinuity between the Indian and Atlantic Ocean samples. On the contrary, mitochondrial data indicated that common smoothhound shark is genetically homogenous with substantial interoceanic gene flow. Such conflicting signals found between nuclear and mitochondrial DNA (mitonuclear discordance) can be attributed to a number of factors and could simply be due to the inherent differences in marker properties or an indication of sex biased dispersal. Despite an indication of an expanding common smoothhound shark population based on both marker types, a contemporary genetic bottleneck may have gone undetected as genetic divergence was very low in some of the study populations. Nonetheless, contemporary restriction to gene flow and historical demographics such as range expansion are proposed as the most likely forces explaining genetic structure in present-day common smoothhound sharks in South Africa. For future sustainable exploitation

of common smoothhound shark, the possible existence of two genetically differentiated populations and observed asymmetric gene flow along the South African coast should be taken into consideration. It is also recommended that in the future further evaluations of fine-scale genetic structure and seasonal migration patterns in this commercially important species are conducted in order to allow integration of this knowledge into existing fisheries management practices.

Opsomming

Die ontsyfering van patrone van intraspesie populasie genetiese struktuur in kommersieel belangrike haai spesies is noodsaaklik vir 'n geïntegreerde bestuursbenadering tot visserue en bewaring van plaaslike biodiversiteit. Die hondhaai, *Mustelus mustelus*, is 'n oorbenutte, kommersiële en sporthengelary belangrike haai spesie in Suid-Afrika. Met inagneming van die kwesbare status van die hondhaai en as gevolg van baie beperkte beskikbare genetiese inligting, het hierdie studie gepoog om molekulêre merkers te ontwikkel, asook om die patrone van genetiese diversiteit en populasie struktuur te ondersoek langs die Suid-Afrikaanse kus deur middel van multilokus data gegenereer uit 12 mikrosatelliet merkers en die mitokondriale geen, *NADH dehidrogenase subeenheid 4* (*ND4*). Die kruis-spesie amplifisering van mikrosatelliete was nuttig vir genetiese diversiteit en populasie genetiese analise van die hondhaai. Hierdie mikrosatelliete kan moontlik help met die molekulêre karakterisering in ander inheemse en kosmopolitaanse spesies en kan as waardevolle hulpmiddels dien in die bewaring van potensieel bedreigde en oorbenutte haai spesies. Vir die mikrosatelliet data is matige vlakke van genetiese diversiteit gevind gebaseer op heterosigositeit, alleliese rykheid en haplotipe diversiteit gevind in 'n totaal van 144 individue getoets oor agt studie populasies. Skattings vir paarsgewyse populasie differensiasie, F-statistieke, AMOVA en faktoriale ooreenstemming analise het betekenisvolle genetiese struktuur aangedui binne en tussen wes- en ooskus populasies. Daarbenewens, het Bayesian groepering analise twee potensiele voorvaderlike geenpoele waargeneem, ter ondersteuning van die teenwoordigheid van 'n biogeografiese versperring by die Cape Agulhas gebied en dus genetiese diskontinuïteit tussen die Indiese en Atlantiese Oseaan monsters. In teenstelling het die mitokondriale data aangedui dat hierdie haai spesie geneties homogeen is met aansienlike interoseaniese geenvloei. Sulke teenstrydige tekens tussen kern en mitokondriale DNS (mitokern onenigheid) kan toegeskryf word aan 'n aantal faktore en kan eenvoudig wees as gevolg van die inherente verskille in merker eienskappe of 'n aanduiding van geslags sydigeverspreiding. Ten spyte van 'n aanduiding van 'n groeiende hondhaai populasie gebaseer op beide merker tipes, kon 'n hedendaagse genetiese bottelnek onopgemerk gegaan het aangesien genetiese divergensie baie laag was in sommige van die studie populasies. Nietemin, hedendaagse restriksie van geenvloei en historiese demografie soos verbreding van reeks voorkoming word voorgestel as die mees waarskynlike dryfkragte wat genetiese

struktuur in die hedendaagse hondhaaie in Suid-Afrika verduidelik. Vir toekomstige volhoubare benutting van die spesie, moet die moontlike bestaan van twee geneties verskillende populasies en waargenome asimmetriese geenvloei langs die Suid-Afrikaanse kus in ag geneem word. Vir die toekoms word dit ook aanbeveel dat verdere evaluerings van fyn-skaal genetiese struktuur en seisoenale migrasie patrone in hierdie kommersiël belangrike spesie uitgevoer word om die integrasie van hierdie kennis in die bestaande bestuur van visserye praktyke toe te laat.

Acknowledgements

I would like to extend gratitude (in alphabetical order) to the National Research Foundation of South Africa, the SASBi-SAGS Congress 2014 and Stellenbosch University for financial and travel support during my MSc studies. I would also like to thank the following persons and institutions for aiding in the acquisition of biological specimens (in alphabetical order): Adina Bosch, KwaZulu-Natal Sharks Board (Sheldon Dudley and Sabine Wintner), Michelle Soekoe, Oceans Research (Dylan Irion), South African Department of Agriculture, Forestry and Fisheries (DAFF; Charlene Da Silva and Melissa Goosen), South African Shark Conservancy (Katie Gledhill, Meaghen McCord and Tamzyn Zweig) and White Shark Africa (Gibbs Kuguru).

I take immense pleasure in thanking the Molecular Breeding and Bioversity (MBB) research group for lending their scientific wisdom during the course of my study. To my supervisor Dr Aletta Bester-van der Merwe, thank you for your advice and guidance, and providing me with an opportunity to conduct shark genetics research. To Prof Rouvay Roodt-Wilding, thank you for your valuable inputs and assistance with my project. Lastly, I would like to further extend my gratitude to my friends and family for their love and support but most importantly the laughter and adventures we had when I needed cheering up.

Preface

Scientific Contributions during Masters Candidature (2013-2014):

1. ***Published or submitted papers, to date, directly emanating from the work presented in this thesis:***

Maduna SN, Rossouw C, Roodt-Wilding R, Bester-van der Merwe AE (2014) Microsatellite cross-species amplification and utility in southern African elasmobranchs: A valuable resource for fisheries management and conservation. *BMC Research Notes* **7**:352.

Maduna SN, Roodt-Wilding R, Da Silva C, Wintner S, Bester-van der Merwe AE (in prep) Population genetic structure in common smoothhound shark (*Mustelus mustelus)* from the South-East Atlantic and South-West Indian Ocean: contrasting or concordant patterns in microsatellite and mtDNA sequence data? *Marine Ecology Progress Series*

2. ***Published or submitted papers with indirect relevance to the work presented in this thesis:***

Bitalo DN, Da Silva C, **Maduna SN**, Roodt-Wilding R and Bester-van der Merwe AE (in prep) Differential population structure of two commercially important shark species, tope (*Galeorhinus galeus*) and common smoothhound (*Mustelus mustelus*) along the south-west coast of South Africa. *Fisheries Research*

Contributions: Provided data and contributed to the preparation of the manuscript.

3. ***Local conference presentations:***

Maduna SN*, Roodt-Wilding R, Bester-van der Merwe AE. Oral presentation: Population genetic structure of the common smoothhound shark (*Mustelus mustelus*) in South

Africa. *Southern African Shark and Ray Symposium*. April 2013. Mossel Bay, South Africa: 6

Maduna SN*, Roodt-Wilding R, Bester-van der Merwe AE. Oral presentation: Evaluating genetic connectivity amongst *Mustelus mustelus* populations across the Indian/Atlantic boundary. *15th Southern African Marine Science Symposium*. July 2014. Stellenbosch, South Africa: 98

Maduna SN*, Roodt-Wilding R, Bester-van der Merwe AE. Oral presentation: Regional population genetic structure of a declining coastal shark species, *Mustelus mustelus*, in the South-East Atlantic and South-West Indian Ocean. *2nd Joint SASBi-SAGS Congress*. September 2014. Pretoria, South Africa: S4-5

Rossouw C*, **Maduna SN**, Roodt-Wilding R, Slabbert R, Bester-van der Merwe AE. Poster presentation: Microsatellite cross-species amplification and high throughput development of single nucleotide polymorphisms in commercially important sharks. *2nd Joint SASBi-SAGS Congress*. September 2014. Pretoria, South Africa: P4-6

[**Presenting author*]

4. International conference presentations

Maduna SN, Rossouw C*, Roodt-Wilding R and Bester-van der Merwe AE. Microsatellite cross-species amplification and utility in southern African elasmobranchs: A valuable resource for fisheries management and conservation. *Sharks International*. June 2014. Durban, South Africa: 126

Maduna SN*, Roodt-Wilding R, Bester-van der Merwe AE. Spatio-temporal assessment of genetic variation in the South African commercially important shark species, the common smoothhound (*Mustelus mustelus*). *Sharks International*. June 2014. Durban, South Africa: 156

[**Presenting author*]

Table of Contents

List of Figures

List of Tables

List of Abbreviations

%	Percentage
(Pty)	Ltd Property Limited
<	Less than
>	Greater than
®	Registered Trademark
µl	Microlitre
µM	Micromole
3’	Three prime
5’	Five prime
A	Adenine
A_E	Effective number of alleles
AFLPs	Amplified Fragment Length Polymorphisms
AMOVA	Analysis of Molecular Variance
A_N	Number of alleles
ANG	Angola Population
AO	Atlantic Ocean
A_R	Allelic Richness
BLAST	Basic Local Alignment Search Tool
bp	Basepair
C	Cytosine
CB	*Carcharhinus brachyurus*
CI	Confidence Interval
CL	*Carcharhinus limbatus*
CO	*Carcharhinus obscurus*
COI	*Cytochrome c Oxidase subunit I*

CP	*Carcharhinus plumbeus*
CSH	Common Smoothhound haplotype identifier
CTAB	Cetyltrimethylammonium Bromide [$((C_{16}H_{33})N(CH_3)_3Br]$
Cytb	*Cytochrome b*
D_A	Nei's (1983) genetic distance
DUR	Durban Population
DEA	Department of Environmental Affairs
dH_2O	Distilled Water
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EEZ	Economic Exclusive Zone
EST	Expressed Sequence Tag
ETGD	Exact Test *P*-values for pairwise genotypic differentiation
F	Forward Primer
FAM	Blue (R100); 5-carboyfluirescein (ABI-fluorescent label)
FB	False Bay Population
FCA	Factorial Correspondence Analysis
F_{CT}	Derivative of Wright's Fixation Index adapted for hierarchical AMOVA (group of populations relative to the total population)
F_{IS}	Wright's Fixation Index (individual relative to the sub-population, equal to the inbreeding coefficient - *f*)
Fr_{NULL}	Null allele frequency
F_{SC}	Derivative of Wright's Fixation Index adapted for hierarchical AMOVA (sub-population relative to the group of populations)
F_{ST}	Wright's Fixation Index (subpopulation relative to the total population)
g	Grams
G	Guanine
GenBank No.	GenBank Accession Number at www.ncbi.nih.nlm.gov

GG	*Galeorhinus galeus*
GN No.	Elasmobranch specimen identifier for the associated molecular data at Global Cestode Database: Elasmobranchs Specimens (http://elasmobranchs.tapewormdb.uconn.edu)
H_O	Observed Heterozygosity
H_E	Expected Heterozygosity
HEd	*Haploblepharus edwardsii*
HP	*Haploblepharus pictus*
I	Information Index
IAM	Infinite Allele Model
IBD	Isolation by Distance
IEF-PAGE	Isoelectric Focusing Polyacrylamide Gel Electrophoresis
IO	Indian Ocean
IUCN	International Union for Conservation of Nature
JB	Jeffreys Bay Population
K2P	Kimura 2-parameter model
KB	Kalk Bay Population
Km	Kilometre
LD	Linkage Disequilibrium
LL	Langebaan Lagoon Population
LMPA	Langebaan Lagoon Marine Protected Area
L_T	Total Length
M	Molar (Moles per Litre)
mg/ml	Milligram per Millilitre
MgCl2	Magnesium Chloride
min	Minutes
ml	Millilitre
MLRA	Marine Living Resource Act

mM	Millimole
MM	*Mustelus mustelus*
MP	*Mustelus palumbes*
MPA	Marine Protected Area
MPS	Multiplex Systems (Assays)
MYA	Million Years Ago
ND2	NADH Dehydrogenase subunit 2
ND4	NADH Dehydrogenase subunit 4
NED	Yellow (Tamra) (ABI-fluorescent label)
NJ	Neighbor-Joining
^{o}C	Degrees Celsius
P	Polymorphic
PA	*Poroderma africanum*
PCR	Polymerase Chain Reaction
PET	Red (ABI-fluorescent label)
$P_{E\text{-}W}$	Ewens-Watterson Probability
PIC	Polymorphic Information Content
PP	Percentage of Polymorphism
PPa	*Poroderma pantherinum*
P-value	Probability value (as a statistically significant limit)
R	Reverse Primer
RA	*Raja alba*
RI	Robben Island Population
RS	*Raja straeleni*
RFLPs	Restriction Fragment Length Polymorphisms
s	Seconds
SB	Struis Bay Population

SL	*Sphyrna lewini*
SMM	Stepwise Mutation Model
SNP	Single Nucleotide Polymorphism
spp.	Several Species
SQ	*Scylliogaleus quecketti*
SSR	Simple Sequence Repeat
STR	Short Tandem Repeat
SZ	*Sphyrna zygaena*
T	Thymine
T_A	Annealing Temperature
TAC	Total Allowable Catch
TAE	Total Allowable Effort
Taq	*Thermus aquaticus* DNA polymerase
™	Trademark
U	Units (enzyme)
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
VIC	Green (ABI-fluorescent label)
v/v	Volume per Volume
w/v	Weight per Volume
YBP	Year Before Present

"*…ignorance more frequently begets confidence than does knowledge: it is those who know little, not those who know much, who so positively assert that this or that problem will never be solved by science.*"

-Charles Darwin 1871-

(The Descent of Man, and Selection in Relation to Sex)

Chapter 1

Introduction: Literature Survey, Research Aims and Objectives

1.1 Species Biology: An introduction to *Mustelus mustelus*

1.1.1 Classification, Evolutionary History and Phylogeny of Common Smoothhound Shark

The class Chondrichthyes (cartilaginous fishes), which sharks belong to (Compagno *et al*. 2005), is divided into two subclasses: Elasmobranchii [all modern sharks and rays (elasmobranchs)] and Holocephali [modern chimaeroids (holocephalans)] (Maisey 2012). A close phylogenetic relationship between these groups is strongly supported by morphological and molecular data (Maisey 2012). Elasmobranchs are distinguished from the holocephalans by their gill architecture: elasmobranchs are characterised by multiple (five to seven) paired gill slits on the side of their heads (in sharks) or the ventral surface (in rays) whereas holocephalans are characterised by a soft gill cover with a single slit on both sides of the head (Compagno *et al*. 2005). According to the fossil record, the evolutionary history of chondrichthyans stretches back to the early Devonian (roughly 400 million years ago, MYA) (Corrigan and Beheregaray 2009; Maisey 2012) and possibly Silurian (roughly 416 MYA), where they radiated to become globally distributed; representing diverse morphological and ecological types (Grogan and Lund 2004; Corrigan and Beheregaray 2009).

Elasmobranchii is the largest subclass of Chondrichthyes (over 1000 species have been described) (Compagno *et al*. 2005) and elasmobranch fish are considered one of the most ancient existing vertebrate lineages (Corrigan and Beheregaray 2009). They have survived four mass extinction events (Raup and Sepkoski 1982) and most present day taxa are thought to be derived from Mesozoic forms (Maisey *et al*. 2004; Maisey 2012). Elasmobranchs' historically low economic value, sampling challenges and the paucity of studies that use molecular methods to study these fish may explain why they are a relatively under-researched group; particularly at the genetic and taxonomic level (Walker 1998; Corrigan and Beheregaray 2009). Corrigan and Beheregaray (2009) underwrite that the majority of molecular phylogenetic considerations of elasmobranchs are limited to higher taxonomic levels and mainly deal with the origin and placement of study taxa. Although relationships at

or below the family level have rarely been constructed (Eitner 1995; López *et al*. 2006; Cavalcanti 2007; Corrigan and Beheregaray 2009; Lim *et al*. 2010; Boomer *et al*. 2012), Naylor *et al*. (2012) described the first comprehensive phylogenetic relationships across families in elasmobranchs. Maisey (2012) recommended that the phylogeny of the order Carcharhiniformes required re-evaluation since morphological and molecular data from various studies have revealed that some families (Scyliorhinidae and Triakidae), as presently recognised, may be paraphyletic (Maisey 1984; Iglésias *et al*. 2005; Human *et al*. 2006), including some para- or polyphyletic genera *e.g. Mustelus* and *Triakis* (**Figure 1.1**) (López *et al*. 2006).

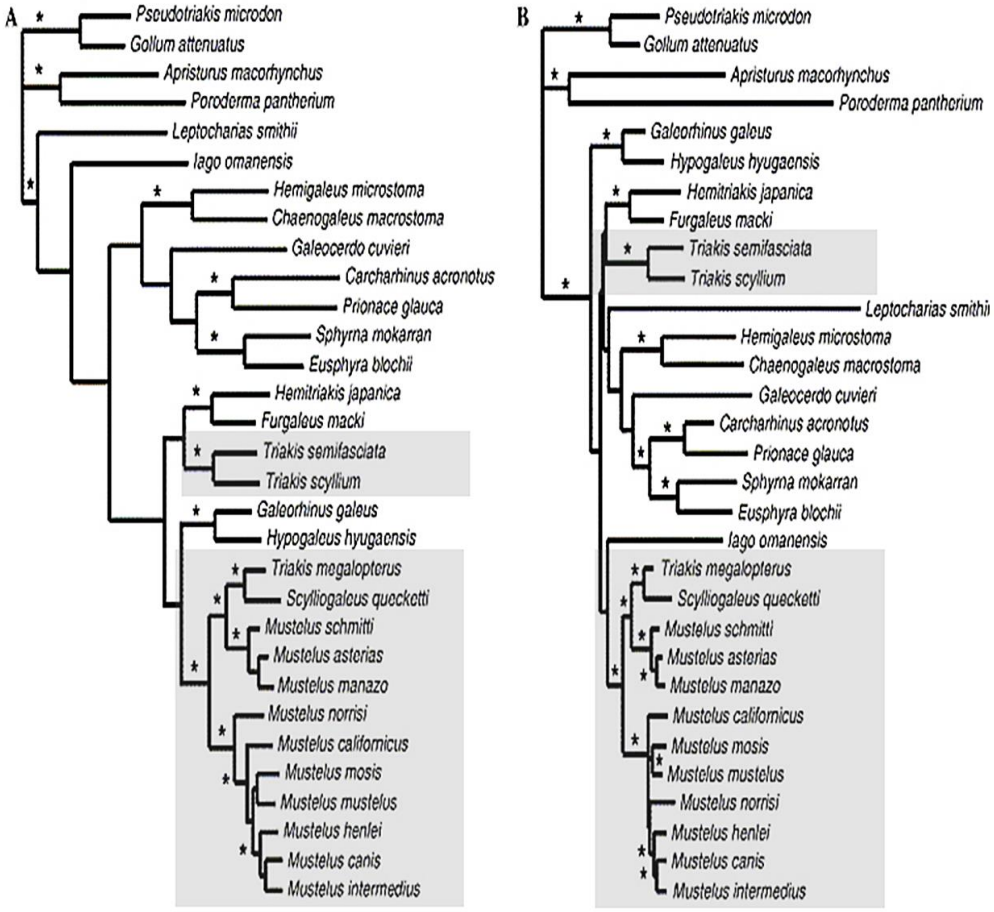

Figure 1.1 Mitochondrial DNA (*Cytb*, *ND4*, *ND2*) maximum parsimony (MP) (A) and maximum likelihood (ML) (B) bootstrap consensus topologies. Asterisks indicate clades that appear in >80% of the bootstrap pseudoreplicates. Para- or polyphyletic genera are in a grey background. Adapted and modified from López *et al*. (2006).

Mustelus Linck, 1760 (family Triakidae, order Carcharhiniformes) (**Table 1.1**) is a species-rich genus represented by at least 28 recognised species of small to medium-sized demersal sharks (Compagno *et al*. 2005) found globally in continental temperate and tropical waters (Smale and Compagno 1997). *Mustelus* species (spp.) are collectively termed smoothhounds,

houndsharks or gummy sharks (Smale and Compagno 1997), although the latter is the common name for the best known Australian species, *Mustelus antarcticus*. In the *Mustelus* genus there is a high degree of conserved interspecific morphology which in turn leads to confusion in unambiguously distinguishing *Mustelus* species (Rosa and Gadig, 2010). Consequently the genus has been deemed challenging systematically (Heemstra 1973; White and Last 2006, 2008; Boomer *et al*. 2012). Misidentification of *Mustelus* spp. is a widespread concern and a common occurrence *e.g*., in the Mediterranean and Black Sea (*M. asterias* and *M. Mustelus*; Farrell *et al*. 2009), Australia (*M. antarcticus*, *M. ravidus* and *M. stevensi*; Boomer *et al*. 2012), northern Gulf of California (*M. albipinnis*, *M. californicus*, *M. henlei* and *M. lunulatus*; Pérez-Jiménez *et al*. 2013) and in South Africa, where the genus is represented by three species, *M. mosis, M. mustelus* and *M. palumbes* (Smale and Compagno 1997; Da Silva and Bürgener 2007). These species together with the spotted gully shark, *Triakis megalopterus*, are readily confused in fisheries despite revision of the *Mustelus* genus by Heemstra (1973).

Table 1.1 Classification of the common smoothhound shark modified from Serena *et al*. (2009)

Kingdom	Animalia
Phylum	Chordata
Class	Chondrichthyes
Order	Carcharhiniformes
Family	Triakidae
Scientific Name:	*Mustelus mustelus*
Species Authority:	Linnaeus 1758
Common Name/s:	English – Common Smoothhound; Afrikaans – Hondhaai
Synonym/s:	*Squalus mustelus* Linnaeus (1758)
Taxonomic Notes:	The morphology of *Mustelus* spp. is highly conserved leading to misidentification of species since numerous early field observational research may refer to either one of the species in the Mediterranean and Black Sea (*M. asterias* and *M. mustelus*) and in South Africa (*M. mosis, M. mustelus* and *M. palumbes*).
Genetic Notes:	Molecular approaches have been adopted to discriminate *Mustelus mustelus* from *M. asterias* (Renon *et al*. 2001; Farrell *et al*. 2009; Barbuto *et al*. 2010) and from other *Mustelus* species (Naylor *et al*. 2012; Giresi *et al*. 2013).

Various molecular approaches have been adopted to discriminate between exploited *Mustelus* spp. and assist in species identification in the commercial trade. In Italy, during the late 90s it was noted that *M. asterias* and *M. mustelus* are commonly subjected to fraudulent substitution with lesser valued sharks (Weaver *et al*. 1999; Renon *et al*. 2001; Barbuto *et al*. 2010). Therefore, in 2001 Renon and co-workers introduced a biochemical identification method, isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE), to discriminate between these species and several other shark species of minor commercial value (Renon *et al*. 2001). Barbuto *et al*. (2010) extended the work of Renon *et al*. (2001) by employing a DNA barcoding approach to identify species substitutions using the *cytochrome c oxidase subunit I* (*COI*) barcode sequence (Hebert *et al*. 2003) and barcode reference databases [GenBank and Barcode of Life Database (BOLD)].

In addition, the *Mustelus* genus has also received some taxonomic attention in the last few years. Recent research efforts have been conducted in the western Atlantic to decipher the aforementioned taxonomic problems using anatomic, morphometric and meristic data (Rosa and Gadig 2010), in conjunction with molecular data (Giresi *et al*. 2013; Pérez-Jiménez *et al*. 2013). Molecular approaches combined with anatomic and meristic data have also been used in the central Indo-Pacific and Australasia to resolve these issues (Boomer *et al*. 2012). In the latter study, it was postulated that the troublesome systematics of the *Mustelus* genus may in part be attributed to a recent radiation following dispersal from a northern hemisphere ancestor. Additionally, Boomer *et al*. (2012) verified the "aplacental" and "placental" clades documented by López *et al*. (2006). The *Mustelus* spp. that lack white spots and were of placental reproductive mode, grouped separately from those with white spots exhibiting aplacental reproductive mode and, in general, the *Mustelus* spp. showed low levels of genetic divergence (particularly within the aplacental, white spot group) (**Figure 1.2**).

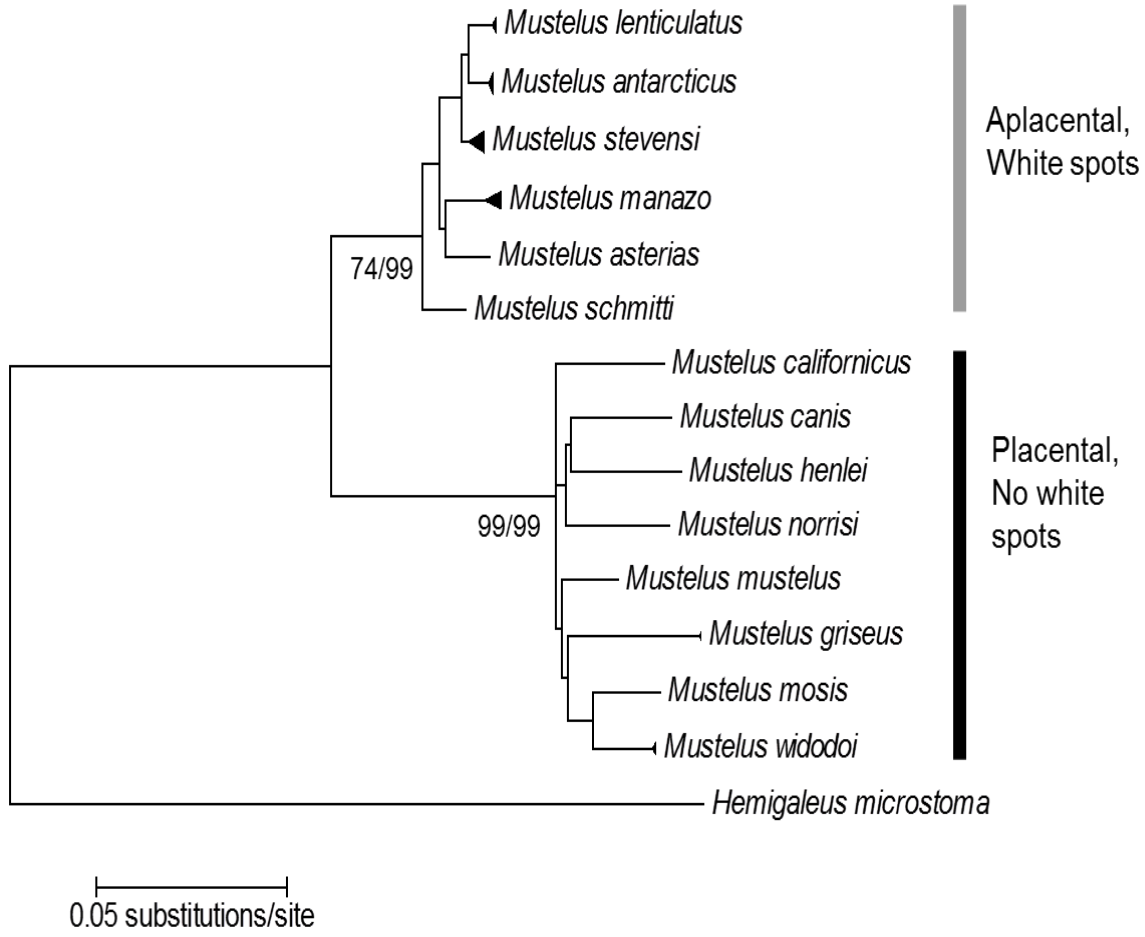

Figure 1.2 Phylogenetic hypothesis of 14 species of smoothhound sharks (*Mustelus*) based on 1055 bp of mtDNA (*ND4, ND2*). Bootstrap values are given for ML/MP analyses for white spotted/aplacental and non-spotted/placental clades (Boomer *et al*. 2012, their supplementary data 1).

1.1.2 Distribution, Ecology, Population Trends and Commercial Importance

The common smoothhound sharks, *Mustelus mustelus*, are active, strong-swimming epibenthic (living on or near the seafloor) sharks that are fairly slender with flattened ventral surfaces on the head and body (Smale and Compagno 1997). They are furthermore characterised by a grey to grey-brown body, mostly lacking spots, short head and round snout, a broad internarial space, large eyes, teeth with low bluntly rounded cusps arranged in multiserial rows and by the upper labial furrows being slightly longer than the lower (**Figure 1.3**) (Ebert and Stehmann 2013).

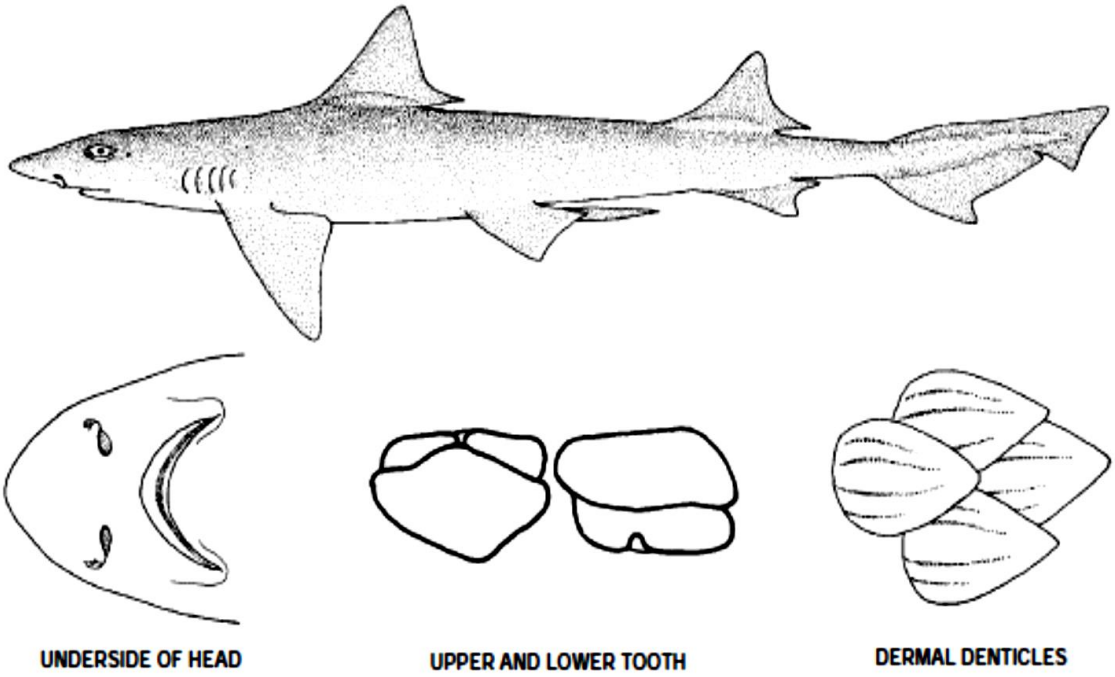

Figure 1.3 Anatomical features of common smoothhound shark *Mustelus mustelus* (Ebert and Stehmann 2013).

Common smoothhounds are cosmopolitan species, *i.e.* found across a wide distribution range from northern Europe to South Africa (eastern Atlantic and South-West Indian Ocean), including the Mediterranean Sea (**Figure 1.4**) (Whitehead *et al*. 1984; Compagno *et al*. 2005; Serena 2005). They inhabit continental shelves and uppermost slopes, from the intertidal region to at least 350 m in depth in temperate and tropical waters (Serena *et al*. 2009), where they may have a major impact on their prey populations (Smale and Compagno 1997). While some sharks are opportunistic apex (top) predators, others, such as *Mustelus* spp., are mesopredators (mid-level predators) (Belleggia *et al*. 2012). For example, with a trophic level of 3.8 (Cortés 1999) common smoothhound sharks are considered mesopredators in their niche. Mesopredators are at risk of predation from top predators and therefore play a vital role in marine ecosystems regulating prey populations. In doing so, they transmit effects of top predators to lower trophic levels (Heithaus *et al*. 2008). Common smoothhounds feed mainly on anchovy (fish), crustaceans and mollusks (Smale and Compagno 1997; Filiz 2009). Adaptive traits, such as the anatomy, dentition (**Figure 1.3**) and behaviour, of these animals render them well-adapted for this feeding mode (Smale and Compagno 1997).

Figure 1.4 Global distribution of common smoothhound shark *M. mustelus* (modified from http://www.zeeinzicht.nl; Compagno *et al*. 2005).

In the northern Atlantic, the common smoothhound is data deficient, deterring population trend estimates (Serena *et al*. 2009). Population declines in the Mediterranean Sea have been reported since 1997 (Aldebert 1997) and lately a similar trend has been observed in the eastern central Atlantic Ocean (Gascuel *et al*. 2007), eastern Atlantic and South-West Indian Ocean (Da Silva 2007). Consequently, global common smoothhound populations have been listed as decreasing and the species listed as vulnerable by the *IUCN Red List of Threatened Species* (Serena *et al*. 2009). This overall decline in populations is a combined response to the K-selected (low fecundity, late maturity and long gestation period) life history traits and fishing (artisanal, recreational and commercial) (Tillett *et al*. 2012*a*) and other anthropogenic pressures (Stevens *et al*. 2000). A drastic reduction in population size (population bottleneck) can result in loss of genetic diversity due to genetic drift, resulting in small populations experiencing accumulating effects of inbreeding (Nei *et al*. 1975). This in turn may result in severe declines in effective population size (N_E) (Wilson *et al*. 2012) and a relatively low population fitness (Ozerov *et al*. 2013), rendering a population vulnerable to extinction (Bouzat 2010).

In South Africa, common smoothhound shark is one of the topmost shark species harvested commercially (Da Silva and Bürgener 2007) and is also recreationally important (Department of Agriculture, Forestry and Fisheries 2013). Consumers generally prefer smoothhounds over other species as smoothhound fillets are greatly appreciated as a fish dish in Italy (Renon *et*

al. 2001), Asia and Australia (Da Silva and Bürgener 2007). In order to mitigate the recent global decrease in common smoothhound populations, more species-specific demographic and genetic knowledge are required (see later).

1.1.3 Life History and Reproduction

Sharks employ a K-selected life history strategy. Previous work on various shark species has shown that reproductive variables in elasmobranchs could be attributed to phenotypic plasticity (Yamaguchi *et al*. 2000; Saïdi *et al*. 2008) and are influenced by geographic variation, specifically latitude (Parsons 1993; Taniuchi *et al*. 1993; Yamaguchi *et al*. 2000; Saïdi *et al*. 2008). However, the patterns of variation in reproductive biology of common smoothhound sharks among regions are not coherent with latitudinal variation and Saïdi *et al*. (2008) suggested that further investigation is needed to confirm this. The reproductive variables of the common smoothhound in different regions are summarised in **Table 1.2**. It can be deduced that males reach sexual maturity sooner [matured at a smaller L_T (total length) than females, see **Table 1.2**] and reach a smaller maximum L_T, corroborating the sexual dimorphism in sharks (Taniuchi *et al*. 1993; Smale and Compagno 1997; Khallahi 2002, 2004; Capapé *et al*. 2006; Saïdi *et al*. 2008). Noteworthy, the reproductive variables in the Senegal population deviated from the expectation of latitudinal variation, *i.e.* increasing rather than decreasing (Saïdi *et al*. 2008). Little is known about the lifespan of ocean-dwelling common smoothhound sharks; however, those held in captivity live to an average age of 25 years and those in the wild are believed to live longer (Da Silva 2007).

Common smoothhound sharks are viviparous (live-bearing) with a yolk-sac placenta (Compagno 1984; Boomer *et al*. 2012); embryos develop a placental connection with the mother through the interaction of the yolk sac, egg envelope and uterine wall, and reproduce seasonally where each cycle may take one year or longer, depending on the resting periods between pregnancies (**Figure 1.5**) (Smale and Compagno 1997). Litter size has been positively correlated with maternal length and thus age (Smale and Compagno 1997; Saïdi *et al*. 2008).

Table 1.2 Summary of common smoothhound, *Mustelus mustelus*, reproductive variables observed from five different regions

		Smale & Compagno (1997)	Capapé *et al.* (2006)	Khallahi (2004)	Capapé (1974)	Saïdi *et al.* (2008)
Regions		South Africa	Senegal	Mauritania	Gulf of Tunis	Gulf of Gabès
Maximum total length (L_T; mm)	M	1450	1390	900	—	1445
	F	1650	1450	1100	1420	1650
L_T at maturity (mm) ($L_{T50\%}$)	M	950–1300	820–950	570–520 (670)	960	880–1120 (971)
	F	1250–1300	900–1040	590–930 (720)	1080–1170	1075–1230 (1173)
L_T at birth (mm) (mean)		368–410	360–450 (403)	240–320 (280)	390	340–420 (364)
Litter size (mean)		2–23 (11·54)	4–21 (9·20)	1–13 (4)	12–22	4–18 (11·8)
Gestation period (months)		9–11	12	7–10	12	10–11

M, males; F, females.

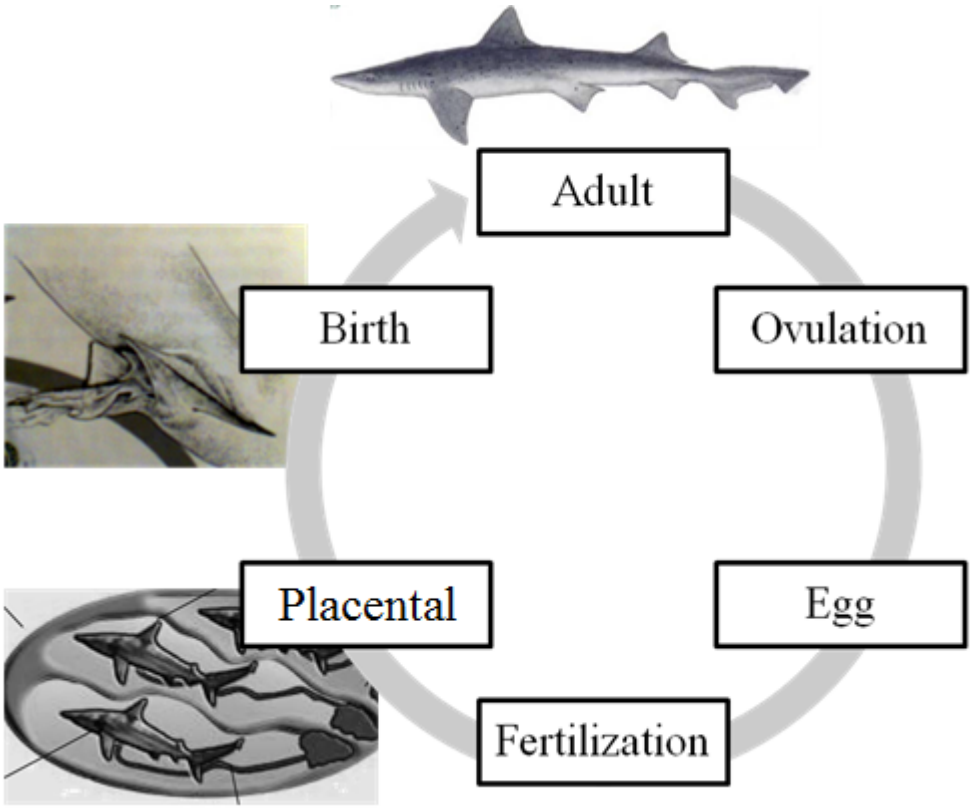

Figure 1.5 Viviparity in common smoothhound *Mustelus mustelus*.

Molecular genetics have also been employed to study the genetic mating systems of various shark species using microsatellite markers with the main objective of elucidating multiple paternity (a single brood of offspring sired by multiple males), polyandry (females mating with more than one male) (*e.g.*, Lage *et al*. 2008; Daly-Engel *et al*. 2010; Chapman *et al*. 2013; Boomer *et al*. 2013; Farrell *et al*. 2014) and reproductive philopatry (repeated use of specific nursery areas for parturition) (*e.g.*, Keeney and Heist 2006, Portnoy *et al*. 2010, Karl *et al*. 2011, Blower *et al*. 2012, Tillett *et al*. 2012*b*, Feldheim *et al.* 2014). Although multiple matings could benefit fitness in males, the indirect genetic benefits are still uncertain and

most likely negligible (di Battista *et al*. 2008). A study by Karl (2008) showed that multiple paternity could even result in lower genetic diversity due to an increased variance in male reproductive success. This in turn could reduce effective population size and limit population genetic diversity. An understanding of reproductive strategies is therefore also important for species-specific management and the conservation of commercially-important sharks.

1.2 The Demersal Shark Fishery, Management and Socio-Economic Issues in South Africa

1.2.1 Historical Development of the South African Demersal Shark Fishery

The South African shark fishery was initiated in the 1930s off the coast of Durban, Kwa-Zulu Natal (Kroese *et al*. 1995; Sauer *et al*. 2003) and was initially targeted at the tope shark *Galeorhinus galeus* (Van Zyl 1992; Da Silva and Bürgener 2007). A high demand for shark liver oil as a source of vitamin A during World War II resulted in elevated shark catches, roughly 1500 sharks per trip (Lees 1969). However, the advent of artificial synthesis of vitamin A during 1950 led to the collapse of the shark liver market (Lees 1969; Van Zyl 1992). Despite this, sharks were still caught incidentally and exported as various meat products to central Africa (dried and/or salted meat), Europe, the Far East and Australia (frozen carcasses) (Kroese *et al*. 1995). Shark exports from South Africa started to increase since 1995 (Stuttaford 1995; Da Silva 2007), owing to the collapse of the Australian tope shark industry (McGregor 1991), and South Africa is the only country in sub-equatorial Africa reporting substantial yields (> 1 000 tons in aggregate over 1985-2000) in terms of shark production and trade (Fowler 2005). Currently, a new directed shark fishery exists and has since expanded into the fin trade and, more recently, into the shark fillet industry, mainly for Australia (Da Silva 2007; Da Silva and Bürgener 2007).

1.2.2 Structure of the Fishery

South Africa's demersal shark trade primarily targets five shark species with commercial value. In order of commercial importance they are: common smoothhound (*Mustelus mustelus*), tope shark (*Galeorhinus galeus*), copper shark (*Carcharhinus brachyurus*), dusky shark (*Carcharhinus obscurus*), and whitespotted smoothhound (*Mustelus palumbes*) (Da Silva and Bürgener 2007). To a limited extent, hammerhead species (*Sphyrna* spp.), gully

sharks (*Triakis megalopterus*) and cow sharks (*Notorhynchus cepedianus*) may also form part of the trade (Da Silva 2007).

The recognised fisheries impacting sharks in South Africa comprise 16 sectors (including both commercial and non-commercial) that are divided into two principle components, directed and non-directed (bycatch) fisheries, in order to conform to global regulation of shark catches (McCord 2005). Directed fisheries denote fishing activities that target sharks, namely demersal shark longline-, traditional line-, and St. Joseph shark net-fisheries (McCord 2005; Da Silva 2007). Sharks are also caught as both bycatch and as targeted species in the large pelagic longline fishery and the recreational linefishery (Department of Agriculture, Forestry and Fisheries 2013; Sharks Biodiversity Management Plan 2014).

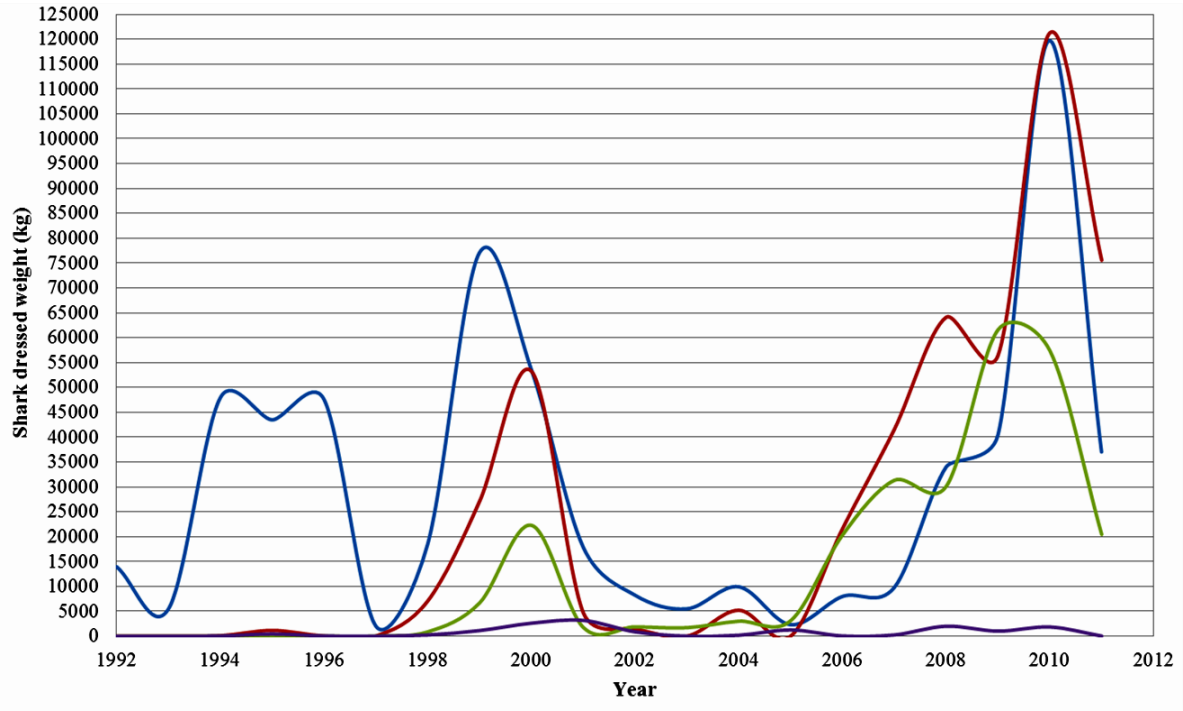

Figure 1.6 Catches (kg) of demersal sharks in the South African longline fishery, 1992-2011. These figures may reflect the weight of the shark after being headed and gutted. Blue line represents tope sharks; red, smoothhound sharks (*Mustelus* spp.); green, requiem sharks (*Carcharhinus* spp.) and purple, cowsharks (*Notorhynchus cepedianus*) (Da Silva and Bürgener 2007; Bosch 2012).

The total annual shark catches in South Africa are estimated at 6 562 tons (**Figure 1.6**) and South Africa is the second largest shark landing country in sub-equatorial Africa (Fowler 2005; Department of Agriculture, Forestry and Fisheries 2013), although not listed under the top 20 shark fishing countries in the world (Lack and Sant 2011). Since shark meat is of little importance in South Africa, the bulk of processed demersal shark meat is exported to

Australia (fish and chip trade) (Da Silva and Bürgener 2007) and Asian countries (shark fin trade) (Fowler 2005). Exports of frozen shark surpassing 100 tons per annum from the sub-equatorial African region by 2005 were only reported by South Africa and Angola, with the former engaging largely in shark fin exports to China (Fowler 2005). Imports of shark meat have also been reported but it is currently unclear what this meat is being used for (Warman 2004).

Shark population declines have been reported worldwide and this could in part be ascribed to legal and illegal fisheries to support the increased demand for shark meat. Notably, in South Africa shark catches have been fluctuating since 1992, with a sharp increase between 2008 and 2010, and a drastic decline in 2011 (**Figure 1.6**). These results corroborate the results of stock assessments of the tope (McCord 2005) and common smoothhound (Da Silva 2007) demersal sharks exploited in southern Africa. McCord (2005) and Da Silva (2007) found that these sharks are overexploited and threatened. In other commercially important marine organisms, a sudden and drastic decline in population size has been shown to impact the levels of genetic diversity (Teske *et al*. 2011; Dudgeon *et al*. 2012) and, therefore, the observed decline has raised concerns for the conservation and management of sharks occurring in South African waters.

1.2.3 Regulation and Management of the Fishery

South Africa has a coastline that spans some 3650 km and an Exclusive Economic Zone (EEZ) of just over 1 million km^2 that includes two oceans, the south-east Atlantic and south-west Indian Ocean, including all marine bio-zones (Griffiths *et al*. 2010). Sharks are managed and regulated under the *Marine Living Resources Act 18 of 1998* (MLRA) (Department of Agriculture, Forestry and Fisheries 2013; Sharks Biodiversity Management Plan 2014). Coastal Marine Protected Areas (MPAs), *e.g.* the Langebaan Lagoon Marine Protected Area (LMPA), have also been implemented to offer partial protection to various coastal shark species, such as ragged tooth sharks, cow sharks, smoothhounds, catsharks and juvenile requiem sharks (Griffiths *et al*. 2010). A recent study by Da Silva *et al*. (2013) on the degree of protection by MPAs to shark populations, using *M. mustelus* as a candidate species, found that no-take area protection may be a practical management option for common smoothhound since this species demonstrated high levels of site fidelity. This information may be applied to other species with similar life history traits. However, it is debated that many targeted species are too mobile to benefit from zone protection [*e.g.* blacktip reef sharks (Vignaud *et*

al. 2013)] and that MPAs are only suitable for resident species (Gell and Roberts 2003; Kerwath *et al*. 2013) and, therefore, various management tools are needed for the conservation and sustainable fishing of sharks (Department of Agriculture, Forestry and Fisheries 2013). These include special protection of some species under the MLRA, *e.g.* sawfishes (*Pristis* spp.) and the spotted gully shark (*Triakis megalopterus*) due to their compromised conservation status (Department of Agriculture, Forestry and Fisheries 2013). Fisheries management also monitors entry into any commercial fishery by a rights allocation process, which is based on scientific recommendations in limiting the number of vessels, crew and Total Allowable Catch (TAC) or Total Allowable Effort (TAE) for target species, in addition to precautionary catch limits for bycatch species (Department of Agriculture, Forestry and Fisheries 2013; Sharks Biodiversity Management Plan 2014).

South Africa has developed and implemented shark management actions since the launch of an International Plan of Action for Sharks in 1999 (IPOA-Sharks, which also includes skates, rays, and chimaeras) and adopted a Nation Plan of Action for Sharks in 2001 (NPOA-Sharks) (Department of Agriculture, Forestry and Fisheries 2013). The South African NPOA-Sharks aims to enhance the conservation and management of sharks and their sustainable use, while improving data collection and the monitoring and management of shark fisheries (Department of Agriculture, Forestry and Fisheries 2013). The South African NPOA-Sharks is implemented in conjunction with the national Sharks Biodiversity Management Plan (SBMP) with the goal to improve the status of sharks within South African waters. Specifically, the SBMP intends to achieve and maintain a favourable conservation status for resident and migratory sharks within South African waters, taking into account the socio-economic and other values of these species, based on the best available scientific information (Sharks Biodiversity Management Plan 2014).

1.2.4 Socio-Economic Aspects Governing the Fishery

Marine fisheries contribute to the global economy, but the general lack of data and uncertainty about the level of employment in marine fisheries may deter sound estimation of fishing effort, leading to overexploitation of marine resources. This in turn may result in inaccurate projections of economic and societal costs and benefits (Teh and Sumaila 2013). Coastal artisanal fisheries in developing countries may exacerbate illegal shark fishing countries by various coastal communities that depend primarily on shark meat as an important source of protein (Andrew *et al*. 2007). Teh and Sumaila (2013) estimated that 260

± 6 million people are involved in global marine fisheries, including both full-time and part-time jobs in the direct and indirect sectors. In South Africa, previous work established that 78% of fishermen depend on fishing for 100% of their income (Da Silva 2007) and, in that respect, fishermen may practice coastal artisanal fisheries to optimise their income.

Nature-based tourism (ecotourism) involving marine areas and species has expanded in the last two decades (Dobson 2006) and offers opportunities for economic, educational and environmental benefit (Techera and Klein 2013). Apart from their fishery importance, sharks also play a vital role in shark based ecotourism, an emerging conservation tool that, when managed appropriately, allows for recreational use of MPAs, provides means of alternative livelihoods for fishers, facilitates marine research and encourages public awareness of threatened shark species (Techera and Klein 2013). Globally, there are 376 established shark ecotourism operations across 29 different countries (Gallagher and Hammerschlag 2011). Shark-based ecotourism may have negative impact on the behaviour of some already-threatened shark species; for instance, a dependency on tourist food, fostering aggression towards humans, or through incidental disease or injury (Orams 2002). In South Africa, the effect of establishing ecotourism on the behaviour of white sharks (Carcharodon carcharias) was tested around a seal colony on a small island, Seal Island in False Bay (Laroche *et al*. 2007). The study found that moderate levels of ecotourism had a minor impact on the behaviour of white sharks, indicating no impact on behavioural effects at the ecosystem level. Because of an increase in shark ecotourism operations in South Africa in the last six years, further research is necessary to validate this observation.

1.3 Applied Molecular Population Genetics for Fisheries Management and Conservation of Sharks

1.3.1 Molecular Genetic Markers

Molecular markers have been extensively applied in population genetics and ecology of many terrestrial, freshwater and marine animals (O'Connell and Wright 1997; Chenuil 2006; Portnoy and Heist 2012) to characterise and understand the apportioning of genetic variation at multiple levels, from intra-individual to interspecific using mitochondrial (matrilineal) and/or nuclear DNA (bi-parentally inherited) (Chenuil 2006). Early molecular work on elasmobranchs was based on nuclear allozymes (enzymes which possess allelic variation at a single locus), amplified fragment length polymorphisms (AFLPs) and restriction fragment

length polymorphisms (RFLPs) (Dudgeon *et al*. 2012; Portnoy and Heist 2012). Allozyme analysis is not ideal for delineating genetic divergence among chondrichthyan stocks because allozyme heterozygosity in these animals is low (Smith 1986; Heist and Gold 1999*a*; Heist 2004*a*). The disadvantage of AFLPs and RFLPs is that they are dominant markers and scoring and analysis of alleles can be difficult (Smith 1986; Heist and Gold 1999*a*; Heist 2004*a*). These markers are also not consistent and easily reproducible between laboratories (Chenuil 2006). Therefore molecular genetic studies on elasmobranchs have extended to typically employ mtDNA markers [*e.g. ND4*, *ND2*, CR (control region)] and, more recently, microsatellites due to their hyper-variability offering increased resolution (Dudgeon *et al*. 2012). These molecular markers are now widely deployed to discern population genetic structure and demographic history in sharks (*e.g*. Veríssimo *et al*. 2010; Karl *et al*. 2011).

1.3.1.1 Microsatellite Markers

Microsatellites are simple sequence repeats (SSRs) of one to six base pairs motifs that are tandemly arranged *e.g*., GA_n and $GACA_n$, (where n refers to the number of times the unit is repeated) (Tautz 1989; O'Connell and Wright 1997; Chenuil 2006; Liu 2007). They are characterised by multiple alleles per locus (*i.e.* are highly polymorphic), co-dominance (each allele can be scored) (Chenuil 2006) and random dispersal throughout genomes (Litt and Luty 1989; Tautz 1989; O'Connell and Wright 1997). These markers occur in genic (type I) and non-genic (type II) regions (Liu *et al*. 2007). Type II markers are typically used in molecular population genetics to elucidate demographic and historic processes since, in most cases, these markers are selectively neutral. However, type I markers are derived from known genic regions (*e.g.* Expressed Sequence Tags, ESTs) and are, therefore, gene-linked markers that may be subjected to selection (Guichoux *et al*. 2011). Type I markers have a higher probability of conferring phenotypic effects or being closely linked to a causal mutation and, therefore, delineating the adaptive potential of species (Liu and Cordes 2004; Guichoux *et al*. 2011).

The lack of molecular markers for many shark species has impeded population- and conservation genetic studies; for instance, microsatellites for most species need to be developed *de novo*, a process that is often costly and laborious (Hoffman and Nichols 2011). To save time and cost, cross-species amplification of microsatellites from closely related species are generally employed (see Chapter 2 for a detailed discussion). However, various novel approaches have been developed to speed up the process of generating polymorphic

microsatellite markers; the approach of Dawson *et al*. (2010) that results in a high cross-species utility and the Hoffman and Nichols (2011) approach for mining polymorphic microsatellite markers *in silico*.

However, microsatellites also have limitations that should be taken into account when interpreting microsatellite genetic data, particularly when the objective is to infer population genetic structure, demographic history, parentage analysis, etc. These markers have technical difficulties such as null alleles (failure of an allele to amplify due to primer binding site sequence variation) and stuttering (*in vitro* slippage of *Taq* polymerase causing multiple bandings of a single allele), leading to genotyping errors (Hoffman and Amos 2005; Girard and Angers 2008). Microsatellites also display size homoplasy (alleles of the same size but different lineages, Estoup *et al*. 2002), which reduces the true allelic diversity of populations (Blankenship *et al*. 2002; Epperson 2005; Selkoe and Toonen 2006). Despite these limitations, microsatellite markers have successfully been applied in elasmobranchs for genetic stock characterization (degree of genetic connectivity), individual identification, and for discerning genetic mating systems, kinship, relatedness, sex-biased dispersal and philopatry (Dudgeon *et al*. 2012; Portnoy and Heist 2012).

1.3.1.2 Mitochondrial DNA

Mitochondrial DNA is a minute portion of the DNA of eukaryotic cells that is non-nuclear and is devoid of introns, and is located within cytoplasmic organelles called mitochondria (Martin *et al*. 1992; Heist 2004*b*). It is maternally inherited, haploid and not subjected to recombination. Mitochondrial DNA is also a mosaic molecule with faster and slower evolving DNA regions permitting the design of conserved primers for addressing phylogenetic questions at various taxonomic levels (López *et al*. 2006; Cavalcanti 2007; Corrigan and Beheregaray 2009). Most of these characteristics contrast that of nuclear DNA markers (for example, microsatellites). The major drawback of mtDNA is maternal inheritance, which reduces the effective population size to a quarter (1/4) of that of nuclear DNA (Ovenden 1990). In the past, molecular mtDNA studies of sharks employed *NADH dehydrogenase subunits 2* and *4* (*ND2*, *ND4*) and *Cytochrome b* (*Cytb*) genes due to their high levels of polymorphism (high evolutionary rates) compared to the Control Region (CR), and their assumed neutrality (Tavares *et al*. 2013). These regions have been used to elucidate population genetic structure, phylogeography and evolutionary history within species (*e.g.* Chabot and Allen 2009; Karl *et al*. 2011; Benavides *et al*. 2011*a*, *b*; Vignaud *et al*. 2013) and,

more recently, to investigate the interaction of species biology and distribution with past climatic changes in multiple shark species (O'Brien *et al*. 2013).

1.3.2 Integrating Molecular Population Genetic Data into Fisheries Management

1.3.2.1 Population Genetic Structure in Sharks

Population genetic structure among natural populations is governed by the interaction between evolutionary forces (gene flow, mutation, genetic drift and natural selection), life history traits and environmental (seascape) features such as currents, upwelling oscillations, temperature and biogeographic barriers which limits gene flow amongst populations (Dudgeon *et al*. 2012; Ovenden 2013). Therefore, genetic connectivity among natural populations in such complex environments may be investigated by comparing the genetic composition of several spatially separated samples taken throughout the species distribution range (Waples *et al*. 2008; Lowe and Allendorf 2010; Dudgeon *et al*. 2012; Ovenden 2013). Various hypotheses of genetic population structure have been proposed. For example, isolation by distance (IBD), assuming the stepping stone model of gene flow, postulates that gene flow is more likely to occur among populations in close proximity than distant ones, such that remote populations are connected via a series of 'stepping stones' (Wright 1943). Then, abrupt genetic discontinuity describes a sudden change in genetic variation between two adjacent populations that results from limited gene flow across a biogeographic barrier (Ovenden 2013). Various traditionally-recognised biogeographic barriers (*e.g.* Isthmus of Panama Barrier, Eastern Pacific Barrier, Mid-Atlantic Barrier, Benguela Barrier, Sunda Shelf Barrier and Amazon Barrier) have been shown to affect population connectivity in marine species and, therefore, impact phylogeographic patterns (Keeney and Heist 2006; Schultz *et al*. 2008; Benavides *et al*. 2011*b*; Daly-Engel *et al*. 2012). South Africa has a long and diverse coastline that stretches from the mouth of the Orange River on the west coast to Kosi Bay on the east coast. South Africa is located at the transition zone between the Atlantic Ocean and Indian Ocean biomes constituting a total of nine marine bioregions across these oceans (Lombard 2004) making it of great interest from a biogeographic point of view (**Figure 1.7**). For a variety of marine fish, phylogeographic breaks that coincide with the biogeographic disjunction between cool temperate and warm-temperate biota have been reported near Cape Point and Cape Agulhas (Teske *et al*. 2011). South Africa has featured in a number of key phylogeographic studies of sharks with a global focus and it was found that

the Cape Agulhas Boundary, the Atlantic/Indian Ocean phylogeographical break, does not restrict gene flow of cosmopolitan species, such as copper shark (Benavides *et al*. 2011*a*) and tope shark (Chabot and Allen 2009). Lastly, clinal variation shows that other factors may also influence the pattern of allelic variation besides gene flow. Clinal variation stipulates that differences in allele frequencies are gradual along a geographical or other environmental gradient (*e.g.* ocean basins) (Manly 1985; Storz 2002) due to differential adaptation to conditions, *e.g.* temperature, pH, salinity or depth (Teske *et al*. 2011) and isolation by distance, resulting in population divergence and leading to the development of ecotypes (Lowry 2012; de Bruyn *et al*. 2013; Louis *et al*. 2014).

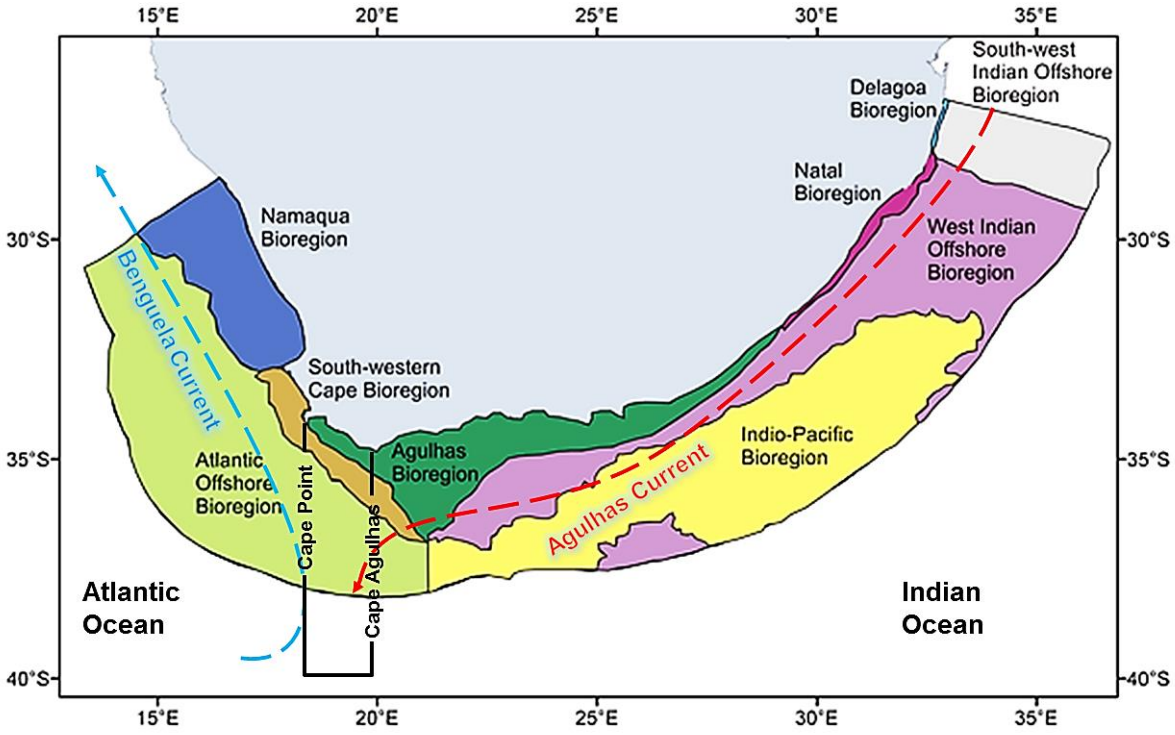

Figure 1.7 South Africa's nine marine bioregions, as defined by Lombard (2004), and the recognised coastal phylogeographic break, the Benguela Barrier (westernmost - Cape Point, easternmost - Cape Agulhas). Modified from Griffiths *et al*. (2010).

Elucidating historical and contemporary processes responsible for observed patterns of spatial and temporal genetic differentiation in elasmobranch populations (Dudgeon *et al*. 2012) is of vital importance for defining reproductively isolated stocks, characterising genetic variability and assessing the direction and strength of gene flow between populations (Ovenden 2013; Vignaud *et al*. 2013). Different levels of subdivision over large and smaller spatial scales have recently been reported for various shark species (Ovenden *et al*. 2009; Pereyra *et al*. 2010; Benavides *et al*. 2011*a*, *b*; Mourier *et al*. 2013; Vignaud *et al*. 2013).

Identifying regional stocks is a key component to achieve a sustainable fishery (Grant and Bowen 1998; Ovenden *et al*. 2013) since species distributions may extend across national and international boundaries where protection and management legislation may differ for any given species (Ovenden *et al*. 2013). Recent studies of detailed regional-scale population genetic structure have been reported for some species with important implications for fisheries management (Mendonça *et al*. 2013; Mourier *et al*. 2013; Vignaud *et al*. 2013). Deciphering temporal patterns of population genetic structure can additionally assist in evaluating the consequences of demographic and environmental changes on population stability and persistence (Ozerov *et al*. 2013). The use of temporally-replicated samples may also assist in the comparison of historical and contemporary patterns of changes in effective population size (N_E) over time, which is critical for setting conservation priorities and identifying management units (Waples *et al*. 2008; Wilson *et al*. 2012). Furthermore, a temporal approach may help to evaluate the genetic consequences of physical and biological environmental changes (Schwartz *et al*. 2007; Ozerov *et al*. 2013), particularly in MPAs where fishing is regulated, *e.g.* the Langebaan Lagoon Marine Protected Area (LMPA). To assess whether the genetic stability of commercial shark species affected by human activity, it is vital to evaluated the effect of fishing pressure on the N_E in these regions.

Reproductive philopatry is the return of adults to specific nurseries to either mate or give birth (Feldheim *et al*. 2004; Hueter *et al*. 2005). It is one of the most important behaviours (Speed *et al*. 2010) since the degree of segregation of these sites can directly affect the level of population subdivision and genetic divergence among geographic regions (Hueter *et al*. 2005). Specifically, if local extinctions of philopatric species occur, the chance of recovery is greatly reduced as the likelihood of an individual re-utilising an area is not random (Hueter *et al*. 2005). Given the variance in philopatric behaviour amongst species, knowledge of particular nursery areas for individual species (either sex-specific or not) has important implications for the spatial scale of management and conservation strategies (Karl *et al*. 2011; Tillett *et al*. 2012*b*).

1.3.2.2 Historical Demography of Sharks

Climate change is among the most significant factors affecting species distribution and demographic patterns (growth rate, reproduction and survival) in a variety of shark species leading to changes in abundance and disruptions to community structure (Dulvy *et al*. 2003; Perry *et al*. 2005; Planque *et al*. 2010; O'Brien *et al*. 2013). Given that these can also result

from complex interations between habitat, environmental conditions and species biology, it is vital to investigate how species responded to historic climate fluctuations (O'Brien *et al*. 2013). In doing so will allow for a better understanding into a species' response to current climate change. Inferring the demographic history of populations, such as historical migration rates and fluctuations in effective population sizes over time (N_E; contractions, expansions) (Excoffier 2004), will therefore allow for effective management of commercially important shark species (Planque *et al*. 2010; O'Brien *et al*. 2013). The fossil record for sharks in general is relatively poor and mutation rates are unknown, but these are thought to be about an order of magnitude slower than in mammals (Martin *et al*. 1992; Martin 1995). This presents a challenge in calculating the timing of expansions and effective population sizes as both are highly dependent on the chosen mutation rate (μ). A few studies on sharks have examined the neutral substitution rate in mitochondrial genes using divergence estimates between species and calibrating these using biogeographic events (*e.g*. closure of the Isthmus of Panama) (Martin *et al*. 1992; Duncan *et al*. 2006; Keeney and Heist 2006; Schultz *et al*. 2008).

These molecular clock estimates have also been applied to non-congeneric species (*e.g*. Murray *et al*. 2008; Pereyra *et al*. 2010; Verríssimo *et al*. 2010; Karl *et al*. 2011; Boomer *et al*. 2012), regardless of the accuracy of this approach being contested (Ho *et al*. 2011; Grant *et al*. 2012; Shapiro and Ho 2014). Studies reconstructing the demographic history of various sharks species, characterised by different life history traits and habitat preferences, demonstrated that climate changes during the Pleistocene epoch (approximately 2.6 million to 11 700 years before present, YBP) had a major impact in shaping the demographic history of sharks (Karl *et al*. 2011; Boomer *et al*. 2012; O'Brien *et al*. 2013). Particularly following the end of the last glacial maximum (LGM, approximately 20 000 YBP), population expansion events have been reported for the whale shark (*Rhincodon typus*; Vignaud *et al*. 2014) and *Mustelus* species (Pereyra *et al*. 2010; Boomer *et al*. 2012), whereas for the scalloped hammerhead shark (*Sphyrna lewini*) population contraction (*i.e*. bottleneck) was reported (Nance *et al*. 2011). The latter is unexpected considering that warming after the LGM possibly caused population expansions and, because the sea levels were elevated, increased suitable coastal habitats for various marine organisms (Peltier 1988; Miller *et al*. 1995; Nance *et al*. 2011; Teske *et al*. 2011; Mendonça *et al*. 2013). These studies highlight that, in addition to discerning stock structure, management decisions should also consider vulnerability of commercially important species to future changes in climate.

1.3.2.3 Population Inference Methods

Describing and quantifying spatial patterns of intraspecific variation is vital in population studies (Avise 2000; François and Durand 2010). Around the 1960s, inference about population genetic structure was improved by the debut of principal component analysis (PCA) and tree-based clustering algorithms (Cavalli-Sforza and Edwards 1965), which made no assumptions about the biological processes that generated the data (François and Durand 2010) Recently, inferring population genetic structure from these descriptive methods advanced to model-based parametric Bayesian methods (François and Durand 2010), catered for by various computer programs (Blair *et al*. 2012).

Summary Statistics: The most widely used statistical measures of population genetic structure are Wright's hierarchical F-statistics (Wright 1931; Evanno *et al*. 2005), particularly the fixation index F_{ST} or the mitochondrial DNA analogue ϕ_{ST}, which quantify the degree to which a polymorphic population is subdivided into subpopulations (Wright 1951; Balloux and Lugon-Moulin 2002; Meirmans and Hedrick 2011). F_{ST} is defined in terms of the expected heterozygosity of the overall population and the mean expected heterozygosity across the subpopulations (Nei 1977) and was extended by Cockerham and Weir (1987) to couple it to the probabilities of identities; therefore, F_{ST} varies from -1 to +1. F_{ST} is also a measure of the Wahlund effect (*i.e.* a heterozygote deficiency due to population subdivision) (Wahlund 1928). F_{ST} is based on the island model of migration (distance has no part in genetic differentiation between groups) and assumes an infinite alleles model (IAM) (Balloux and Lugon-Moulin 2002). Since microsatellites follow a stepwise mutation model (SMM) an F_{ST} equivalent, (rho) R_{ST}, was formulated by Slatkin (1995). R_{ST} is calculated from the variances of allele sizes, whereas F_{ST} will typically be derived from the variances of allele frequencies (Slatkin 1995; Balloux and Lugon-Moulin 2002). Nevertheless, these measures have some drawbacks, for instance, dependency on within-population diversity, and standardised measures have therefore recently been developed. These include F'_{ST} and D (Meirmans and Hedrick 2011). F'_{ST} is the normal F_{ST} standardised by the maximum value it can obtain, given the observed within-population diversity. D uses a multiplicative partitioning of diversity, based on the effective number of alleles rather than on the expected heterozygosity. F'_{ST} is recommended because it is most suited for inferences of the influence of demographic processes such as genetic drift and migration on genetic population structure.

Methods not assuming predefined structure include multivariate analysis such as factorial correspondance analysis (FCA; the genetic relationship among populations determined by

visualizing the ordination of populations along the factorial axes in a three-dimensional space) (Belkhir *et al*. 2004) and the Analysis of Molecular Variance (AMOVA) (Excoffier *et al*. 1992). The AMOVA is another widely used descriptive multivariate analysis to infer genetic structure using allele frequency variation to determine the apportioning of the total genetic variation within populations, among populations within groups and among groups (Excoffier and Lischer 2010). Clustering methods are also commonly used in population differentiation analyses; tree-based methods where genetic distance is estimated between individuals and trees are then constructed (UPGMA or NJ) to group them in clusters are also commonly used (François and Durand 2010).

Model-based methods: Model-based parametric methods involve the monitoring of the performance of a given model by a set of parameter values (François and Durand 2010). These methods presently provide a more realistic and accurate approach towards making inferences on the processes underlying population dynamics (François and Durand 2010). Model-based methods that have revolutionised the most important theoretical and computational advances in molecular population genetics is the Bayesian computation analysis, implemented in computer programs such as STRUCTURE (Pritchard *et al*. 2000), GENELAND (Guillot *et al*. 2005), TESS (Durand *et al*. 2009) and BAPS (Corander *et al*. 2008). Bayesian analysis permits the integration of variable parameter values as prior information into the model to estimate certain population parameters, given the properties of an observed dataset, and is computed by updating the prior distribution based on the data using a Markov chain Monte Carlo (MCMC) algorithm (François and Durand 2010).

1.3.2.4 Population Demography

Several indirect methods based on the analysis of neutral genetic variation have been developed to infer historical demographic processes such as migration rates, founder or bottleneck effects and population expansion using microsatellite and sequence data (Tajima 1989; Fu 1997; Cornuet and Luikart 1996; Beerli 1998; Reich *et al*. 1999). The various approaches widely used to infer demographic changes based on microsatellite data include those implemented in the programs BOTTLENECK (Cornuet and Luikart 1996; Piry *et al*. 1999), the *M*-ratio method (Garza and Williamson 2001), KGTESTS Excel (Bilgin 2007) and MSVAR (Beaumont 1999; Storz and Beaumont 2002). The first three are moment-based methods that rely on summary statistics, while the MSVAR method uses a full-likelihood Bayesian approach (Beaumont 1999; Storz and Beaumont 2002). Past population

demography is commonly investigated using sequence data with Tajima's *D* and Fu's F_s statistics, which detect departures from neutrality/population expansion (Fu 1997; Tajima 1989). Secondly, haplotype networks are constructed to qualitatively assess demographic history under the expectation that populations that had been stable over time would have a complex network of unique haplotypes, whereas populations that underwent a recent population expansion will have networks with one frequently occurring haplotype and several haplotypes differing by one or few mutation(s) (starlike phylogeny, Slatkin and Hudson 1991). Lastly, demographic history can be inferred using the mismatch distribution analysis, where pairwise differences between sequences and their expected frequency under a sudden demographic expansion model are calculated. Inferences about changes in population sizes are then made following the expectations that a multi-modal or ragged distribution suggests a stable population, whereas a smooth unimodal distribution suggests a rapid population expansion (Slatkin and Hudson 1991; Rogers and Harpending 1992).

1.4 Ethics Statement, Research Aims and Objectives

The collection of specimens from various shark species used in this study complied with the Convention on Biological Diversity (http://www.cbd.int/convention/) and the Convention on the Trade in Endangered Species of Wild Fauna and Flora (http://www.cites.org/). Muscle or fin clips were taken from dead shark specimens caught by local fishermen during fishing campaigns and from humanely killed sharks (as stated by the provider). Fin clips were also taken from live shark specimens and, to our knowledge, all efforts were made to minimise suffering. All permits to collect fin clips or muscle tissue for research purposes were granted by the Department of Agriculture, Forestry and Fisheries (Republic of South Africa) and Stellenbosch Universdity ethical committee.

The aim of the study in Chapter 2 was the development and optimisation of medium-throughput microsatellite multiplex panels for use in studies of regional and cosmopolitan elasmobranch species. The study describes the cross-species amplification approach to develop putative microsatellite markers for a wide range of applications, including genetic diversity assessment and species identification in *Mustelus mustelus*. In Chapter 3, two microsatellite multiplex assays, selected based on levels of polymorphism, are employed to investigate population genetic structure and demographic history of common smoothhound shark in South Africa, in order to identify putative management units for short-term conservation. This chapter applied the traditional approach of defining genetic structure

between populations and regions, using F_{ST}, AMOVA, FCA and the Bayesian clustering model-based method (STRUCTURE). In Chapter 4, the goal is to investigate and confirm the observed population structuring patterns of the microsatellite data and determine whether, historically, the common smoothhound shark is also divided into evolutionary significant units that coincide with the identified management units by analysing the mitochondrial *ND4* region. Overall, this information will contribute to the growing body of research on population genetic structure and demographic history of sharks. The findings of this study could be incorporated into existing fisheries management practices for commercially important species.

Chapter 2

Cross-Species Amplification of Microsatellites and Development of Multiplex Assays for Southern African Elasmobranchs

Abstract

This chapter describes the testing and optimisation of microsatellite markers in *Mustelus mustelus* and the design of four microsatellite multiplex assays for cross-species utility in southern African elasmobranchs. Thirty five microsatellite primer sets previously developed for five elasmobranch species were selected from literature for testing cross-species amplification in 16 elasmobranch species occurring in southern Africa. Cross-species amplification success rates ranged from 28.6%-71.4%. From the successfully amplified microsatellites, 22 loci were selected and evaluated for levels of polymorphism, and four multiplex assays comprising of the 22 microsatellites were successfully constructed, optimised and characterised in a panel of 87 *Mustelus mustelus* individuals. A total of 125 alleles were observed across all loci, with the number of alleles ranging from 3-12. Cross-species amplification of the four optimised multiplex assays was further tested in 11 commercially-important and/or endemic southern African elasmobranch species. Percentage polymorphism ranged from 31.8%-95.5% in these species with polymorphic information content decreasing exponentially with evolutionary distance from the source species. Cross-species amplification of the 35 microsatellites proved to be a time- and cost-effective approach to marker development in elasmobranchs and enabled the construction of four novel multiplex assays for characterising genetic diversity in a number of southern African elasmobranch species. This study successfully demonstrated the usefulness of these markers in down-stream applications, such as genetic diversity assessment and species identification, which could potentially aid a more integrative, multidisciplinary approach to management and conservation of commercially-important cosmopolitan and endemic elasmobranch species occurring in southern Africa.

The results of this chapter was published: Maduna et al. *(2014)* BMC Research Notes *7:353.*
The results of this chapter was presented: Sharks International Symposium. June 2014. Durban, South Africa (Oral presentation)

2.1 Introduction

The subclass Elasmobranchii (sharks, skates and rays) comprises a diverse group of over 1000 species, and is representative of one of the most ancient extant vertebrate lineages (Corrigan and Beheregaray 2009). Recently, pressures from direct and indirect fisheries have resulted in the depletion of elasmobranch populations globally (Worm *et al*. 2013). Decline in wild populations of elasmobranchs is further compounded by their life history traits that are more similar to those of mammals (*e.g*. low fecundity, late maturity and long gestation periods) than those of teleost fishes (Stevens *et al*. 2000; Shivji *et al*. 2002). In comparison, elasmobranchs may not respond well to high fishing pressures. This trend has been particularly pronounced for sharks due to unregulated harvesting to support an increase in demand for shark products (*e.g*. fins, meat, liver oil, skin and cartilage). A drastic reduction in population size (population bottleneck) can result in small populations experiencing the accumulating effects of inbreeding, leading to severe loss of genetic diversity (Nei *et al*. 1975; Glenn *et al*. 1999). These trends have previously been reported for species such as the basking shark (*Cetorhinus maximus*, Hoelzel *et al*. 2006) and the narrownose smoothhound shark (*Mustelus schmitti*, Pereyra *et al*. 2010). Assessing genetic diversity and population structure of these exploited species is, therefore, important for sustainable long-term management of the global shark fishery industry.

Misidentification of shark species in fisheries operations is a widespread concern (Myers and Worm 2003; Barker and Schluessel 2005; Petersen *et al*. 2008; Attwood *et al*. 2011) and molecular identification methods have been developed to alleviate this problem (Pank *et al*. 2001; Shivji *et al*. 2002; Abercrombie *et al*. 2005; Farrell *et al*. 2009; Naylor *et al*. 2012; Domingues *et al*. 2013; Giresi *et al*. 2013). Identification issues have previously been addressed using the nuclear ribosomal internal transcribed spacer 2 (ITS2) region (Pank *et al*. 2001; Shivji *et al*. 2002; Abercrombie *et al*. 2005) and the mitochondrial *NADH dehydrogenase subunit 2* (*ND2*) (Naylor *et al*. 2012; Giresi *et al*. 2013) and *ND4* (Tillett *et al*. 2012*c*; Boomer *et al*. 2013; Geraghty *et al*. 2013) genes. To integrate genetic knowledge with fisheries management, it is imperative for shark fisheries to report shark landings by species instead of lumping them into species- or family groups (*e.g.* houndsharks, carcharinids, hammerheads, etc.). This stems from the difficulties involved with unambiguously identifying species within and across families (Pank *et al*. 2001; Da Silva and Bürgener 2007) *e.g*., carcharinids (*Carcharhinus brachyurus*, *C. obscurus* and *C. plumbeus*) and houndsharks (*Mustelus mustelus*, *M. palumbes* and *Galeorhinus galeus*) due to a high degree of conserved interspecific morphology (Pank *et al*. 2001; Domingues *et al*. 2013). Neglecting to report

shark landings by species overlooks important differences in species susceptibility and population vulnerability to exploitation (Abercrombie *et al*. 2005) and that, in turn, has important implications for species-specific conservation, management and trade monitoring programmes (Clarke 2004).

The general lack of molecular genetic markers (*e.g*. microsatellites) for many elasmobranch species impedes population and conservation genetic studies as these markers can provide valuable information relating to population dynamics (spatial and temporal genetic variation) of individual species. Microsatellites are highly polymorphic due to their high mutation rate (between 10^{-3} and 10^{-4} mutations per gamete per generation), resulting in extensive length polymorphism (Tautz 1989; Weber and Wong 1993). This makes microsatellite markers one of the most powerful molecular genetic tools with a remarkable array of applications, ranging from genetic diversity (Edwards *et al*. 2012; Ozerov *et al*. 2013) and population structure inference (Urian *et al*. 2009; Mourier *et al*. 2013) to discerning genetic mating systems (Mobley *et al*. 2009; Boomer *et al*. 2013) and the identification of species (Martin *et al*. 2002; Sekino and Hara 2007; Costa *et al*. 2012).

The *de novo* development of microsatellites is challenging in general but even more so in elasmobranchs due to the notoriously low rates of polymorphism (Heist and Gold 1999*a*; Dudgeon *et al*. 2012). The development of microsatellite markers through cross-species amplification is the most effective alternative approach to *de novo* development of microsatellites and has recently also been reported in sharks (Boomer and Stow 2010; Chabot and Nigenda 2011). Microsatellite cross-species amplification relies on the presence of conserved microsatellite flanking sequences (Primmer *et al*. 2005; Barbara *et al*. 2007), which markedly demonstrate a high degree of conservation in some organisms following millions of years of divergent evolution [*e.g*., 250 million years in sharks (Martin *et al*. 2002) and 470 million years in fish (Rico *et al*. 1996)]. The success rate of microsatellite cross-species amplification has been correlated directly to the evolutionary distance between the source species and the target species (Primmer *et al*. 2005; Barbara *et al*. 2007).

The underrepresentation of endemic taxa in many cross-species amplification studies is unfortunate as endemics should be of great interest for conservation of biodiversity on a regional scale. Southern Africa has one of the most diverse chondrichthyan faunas in the world, consisting of some 181 species in 44 families, of which 34 species are endemic to southern Africa (Compagno 2000; Department of Agriculture, Forestry and Fisheries 2013). Growing concerns regarding the sustainability of the southern African shark fishery, stemming from local declines of cosmopolitan and endemic species, led to stricter regulations

being imposed to avert the collapse of natural populations (Department of Agriculture, Forestry and Fisheries 2013; Sharks Biodiversity Management Plan 2014).

Accordingly, this chapter reports the development and optimisation of microsatellite markers through cross-species amplification of species-specific primers from closely related species. This included the design and optimisation of four microsatellite multiplex assays and their cross-species utility in genetic diversity analysis of 11 southern African elasmobranch species.

2.2 Materials and Methods

2.3.1 Study Species and DNA Extraction

Sixteen elasmobranch species (belonging to five families within two orders) occurring in southern African waters were selected for cross-species amplification (**Figure 2.1**). Where possible, specimens were collected from at least two sampling locations to better capture allelic diversity present within populations of each of the respective species. However, due to opportunistic sampling for a majority of the study species, samples were obtained from only a single location (**Figure 2.1**). Muscle tissue or finclips were preserved in 99% ethanol and stored at room temperature until further use. Total genomic DNA was isolated using the standard cetyltrimethylammonium bromide (CTAB) method of Saghai-Maroof *et al*. (1984). The extracted DNA was quantified using a NanoDrop ND-1000 spectrophotometer v.3.0.1 (*NanoDrop*®). For testing cross-species amplification, each DNA sample was adjusted to a working concentration of 50 ng/µl and stored at -20 ºC prior to polymerase chain reaction (PCR) analysis.

2.3.2 Microsatellite Primer Transfer

A total of 35 microsatellite markers, previously developed for five elasmobranch species [*G. galeus* (Chabot and Nigenda 2011), *M. canis* (Giresi *et al*. 2012), *M. henlei* (Byrne and Avise 2012; Chabot 2012), *Raja pulchra* (Kang *et al*. 2012) and *Scyliorhinus canicula* (Griffiths *et al*. 2011)], were selected for testing cross-species amplification. Primer sequences and the annealing temperature (T_A) of each primer set, optimised for each of the respective source species, are shown in **Table 2.1**. Polymerase chain reaction conditions optimised for the the source species (*G. galeus*, *M. henlei*, *R. pulchra* and *S. canicula*) were applied except for the *M. canis* [Chabot (2012) PCR protocol] and *R. pulchra* [modified Byrne and Avise (2012) PCR protocol] primer sets. Polymerase chain reactions for all individuals were executed in a

Family	Species: vernacular	Distribution[a]	N	Sample Location
Order Carcharhiniformes				
Triakidae	*Mustelus mustelus*: Common smoothhound/ houndshark		8	Angola (2), False Bay (4) and Durban (4)
	Mustelus palumbes: Whitespotted smoothhound shark		8	Mossel Bay (3), Struisbaai (5)
	Galeorhinus galeus: Tope shark		8	Robben Island (2), Langebaan Lagoon (4) and False Bay (2)
	Scylliogaleus quecketti: Flapnose houndshark		1	Mossel Bay
Carcharhinidae	*Carcharhinus brachyurus*: Bronze whaler/ copper shark		5	Durban
	Carcharhinus limbatus: Blacktip shark		4	Mossel Bay
	Carcharhinus obscurus: Dusky shark		8	Durban
	Carcharhinus plumbeus: Sandbar shark		5	Durban
Scyliorhinidae	*Haploblepharus edwardsii*: Puffadder/ Happy eddie shyshark		4	Hermanus
	Haploblepharus pictus: Dark shyshark		8	Hermanus
	Poroderma africanum: Pyjama shark		1	Hermanus
	Poroderma pantherinum: Leopard catshark		5	Hermanus (2), Mossel Bay (3)
Sphyrnidae	*Sphyrna lewini*: Scalloped hammerhead		5	Mossel Bay
	Sphyrna zygaena: Smooth hammerhead		5	Mossel Bay
Order Rajiformes				
Rajidae	*Raja (Rostroraja) alba*: Spearnose skate		3	Port Elizabeth (1), Mossel Bay (2)
	Raja straeleni: Biscuit skate		1	West Coast

Figure 2.1 The 16 elasmobranch species from southern Africa selected for cross-species amplification, including family, species, distribution and sampling locations. [a] Compagno *et al*. (1989).

GeneAmp® PCR System 2700.

The PCR amplicons, together with negative controls and a Promega 100 bp molecular size ladder, were visualised on a 2% agarose gel stained with ethidium bromide for preliminary size determination. Success or failure of PCR amplification in cross-species trials was determined simply on the basis of whether band intensity was sufficient to score alleles. In most instances, less stringent PCR conditions were not employed in the cross-species assays so as to minimise the risk of amplification of non-orthologous loci in the target species. The number of markers that showed amplification success for all or a percentage of individuals in the target species ("+/++" in **Table 2.2**) were counted as an index to measure the cross-species microsatellite amplification performance.

Table 2.1 The 35 microsatellite markers developed from five closely related species for cross-species amplification in the study taxa, including the primers sequences, microsatellite repeat motif, annealing temperature (T_A) and GenBank accession numbers

Locus	Primer sequence (5'-3')	Repeat unit	T_A	Accession number	References
Mh1	F: GGAGGAGGGAAGCCTATGG R: TCTCTGGCTCCATTCAGGG	$(AG)_n$	59	N/A	Chabot (2012)
Mh2	F: ACTACACTGCATATAAACAGGC R: TTTTCAGAGGGCATAACTCAC	$(GA)_n$	56	N/A	Byrne and Avise (2012)
Mh6	F: CATGTCCACTTCCCATCGC R: GGAGAGATTAGAACAGGTGGC	$(CT)_n$	59	N/A	Chabot (2012)
Mh9	F: CAACCATCTTTACTACACTG R: GATGGACCTCACATTTAACAC	$(GA)_n$	56	N/A	Byrne and Avise (2012)
Mh25	F: TGCAATAACCGTTCTGCGTC R: TCACACCCGCAGTTAGATCC	$(CT)_n$	59	N/A	Chabot (2012)
Mca25	F: ACACACTTTCACGCACAAGC R: TCGCTCAAGTGAGACCAGAG	$(CA)_n(CT)_n$	59	JN129145	Giresi *et al*. (2012)
Mca31	F: GGCAGATCAGTTGAGGAAGG R: AATGGGGAGACTTCTCTTTGC	$(ATC)_n$	59	JN083992	Giresi *et al*. (2012)
McaB33	F: TCTCCTAATGGAACGTGTGC R: GGTATGCGTATGGGTGTCG	$(CA)_n$	59	JN084002	Giresi *et al*. (2012)
Mca44	F: TTTCCGCTGTATCACACATACAC R: GCATCTATATGTCTGCGTGTGTC	$(AC)_n$	59	JN083995	Giresi *et al*. (2012)
McaB35	F: AGTGCGTGCCAGTGTATGAG R: GTTCTGCATGGGACGTGAC	$(TG)_n$	59	JN084003	Giresi *et al*. (2012)
McaB5	F: TAATCGACACGCAGTCATCG R: AAGCTCCAATTCTCACTGTGC	$(GT)_n$	59	JN083996	Giresi *et al*. (2012)
McaB6	F: AGGATAAATACACGCACACAGG R: TTTTTGTTTTGCAATCTCACG	$(CA)_n$	59	JN083997	Giresi *et al*. (2012)
McaB22	F: TCCTCTCCAGGACAAACACAC R: TCCCACCTGCCATAGTAATTG	$(AC)_n$	59	JN083999	Giresi *et al*. (2012)
McaB27	F: ATCCAGTGGTTTTGAAATGC R: CCTCGTAGGTCTCGTC	$(GT)_n$	59	JN129154	Giresi *et al*. (2012)
Mca33	F: CATTTGAACCCCGACAGAAC R: TCCAAGTAAGGATGAGTGACACC	$(ATC)_n$	59	JN083993	Giresi *et al*. (2012)
McaB37	F: TCTGCCTCTGTGTCTCATCC R: TTTCCATTTCCGACATAGGG	$(GT)_n$	59	JN084005	Giresi *et al*. (2012)
McaB39	F: GGACAGGCAGCATCTGTGTA R: CCCAGGGGGATTAGGATATT	$(CA)_nGAT(AC)_n$	59	JN129156	Giresi *et al*. (2012)
Gg2	F: TGGCTCAGTCCAGAAACCC R: CCCTATTCGAGAGGCCCAG	$(TG)_n$	59	N/A	Chabot and Nigenda (2011)
Gg3	F: CCGTGACTGAAAGCAGCC R: CCCTCAACCATGGCAAGTG	$(GATT)_n$	59	N/A	Chabot and Nigenda (2011)
Gg7	F: CTGTGGAACCAAACTCCAGC R: AGCTGGTCGAGGTGAATGC	$(AG)_n$	59	N/A	Chabot and Nigenda (2011)
Gg11	F: AAGTTGCACGTTTCCCAGC R: TACTGCAGGACCGGTTTCC	$(TCCC)_n$	59	N/A	Chabot and Nigenda (2011)
Gg12	F: TGTCAAACACCATCGCAGG R: TGCTCTGAAGTCTACAAGAATGG	$(TA)_n$	59	N/A	Chabot and Nigenda (2011)

Gg15	F: GGCTGAATGGTTTCCCAGC R: GCCTCCAACTTAGCATAGCC	$(GA)_n$	59	N/A	Chabot and Nigenda (2011)
Gg17	F: CCTGCTTGTGACAGTTACCC R: ACAGGCATCACCTCTGTGC	$(AC)_n$	59	N/A	Chabot and Nigenda (2011)
Gg18	F: TCCACTTCAGGAAGGCCAG R: CAAAGCCAGGTGGTTCTCC	$(GA)_n$	59	N/A	Chabot and Nigenda (2011)
Gg22	F: TCCTGGGATGGCAACTTCG R: AGGCCACCCAACTATCCTG	$(GT)_n$	59	N/A	Chabot and Nigenda (2011)
Gg23	F: ACAGACCACAGGGCATGG R: TGCAGAGCAGGCTAGATGG	$(AC)_n$	59	N/A	Chabot and Nigenda (2011)
Rp16-nfrdi	F: AGGAAGGCTTCAGCACATAAT R: CTCATCTGGAAGAGCACACAC	$(TG)_{13}$	54	JQ433557	Kang *et al*. (2012)
Rp35-nfrdi	F: CTTACTGGTGAGGAATCTGAGC R: GCATACACTCCACACACCAC	$(TG)_9$	61	JQ433564	Kang *et al*. (2012)
Scan02	F: TGCAGCTTCGCTATCTTATGC R: AAATCTGCTGCTCGCTTCAT	$(TG)_9$	60	N/A	Griffiths *et al*. (2011)
Scan06	F: GGCAGTGATTGCATTCTTGA R: CAGAAACTGTGCAGAAATCACA	$(TG)_9$	60	N/A	Griffiths *et al*. (2011)
Scan12	F: GCCAGTGGCTATAACGGA AC R: TCC CAC ACA GTC CTG TTGAA	$(AG)_9$	60	N/A	Griffiths *et al*. (2011)
Scan14	F: AACCATCCTCCGCAAATAAA R: GAACAGTGCCCCAAGTTCAT	$(CA)_9$	60	N/A	Griffiths *et al*. (2011)
Scan15	F: TCATCATCATCACCACCAGAA R: GAGCTATGCTGGCAATTCGT	$(CA)_{15}$	60	N/A	Griffiths *et al*. (2011)
Scan16	F: CCGACTCCTTTGGATGTGTT R: GGACGCTCTCGTTCTTATGC	$(TG)_9$	60	N/A	Griffiths *et al*. (2011)

2.3.3 Multiplex Design and Optimisation

As the primary objective of this chapter was to develop molecular markers for the common smoothhound shark *Mustelus mustelus*, levels of polymorphism were initially assessed in a panel of eight individuals of *M. mustelus*. Amplicons were subjected to electrophoresis for two hours at 150 volts on a 12% polyacrylamide gel to detect size variants. Microsatellites were considered to be polymorphic when two bands were distinguishable in a single individual (*i.e.* heterozygote) and/or clear size differences were detected between different individuals.

Twenty-two polymorphic microsatellite loci were selected, and primers were fluorescently labelled and optimised in four multiplex assays (5-6 loci per MPS), using a strategy outlined by Guichoux *et al*. (2011), with one of the following dyes: FAM, VIC, PET, or NED. A two-dimensional multiplex plot was created to illustrate the spatial (in base pairs, *x* axis) and spectral (four fluorescent dye labels, *y* axis) design of the four multiplex systems (*e.g.* MPS1 and MPS2, **Figure 2.2**). The use of different dyes was to facilitate co-amplification of multiple microsatellite markers in a single reaction for cost- and time-efficient genotyping (Multiplex PCR).

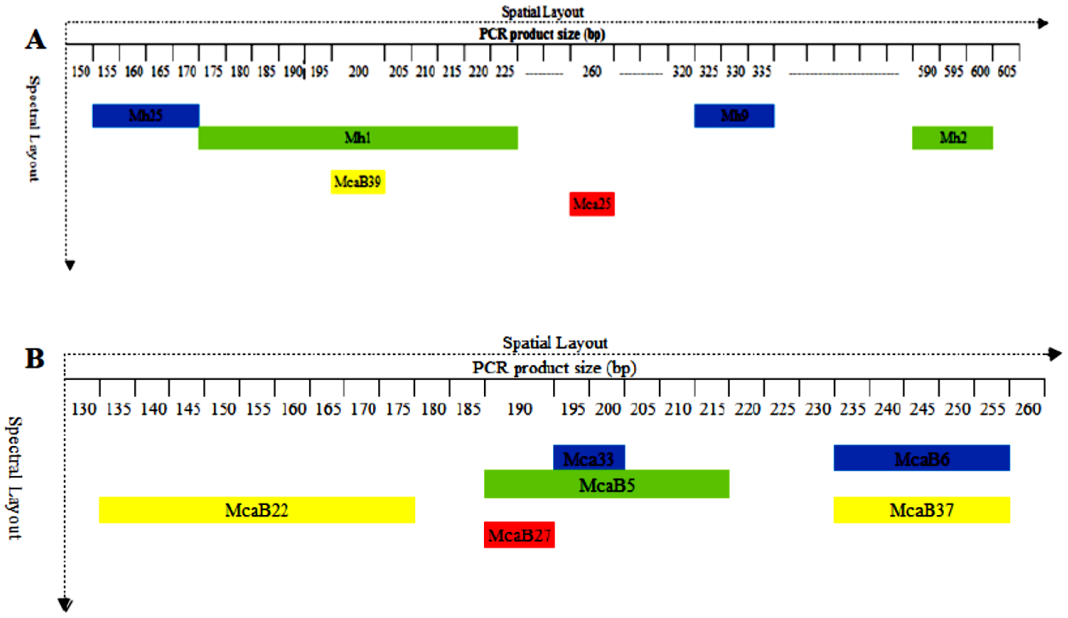

Figure 2.2. Two of the four microsatellite multiplex assays [A; multiplex assay 1 (MPS1) and B; multiplex assay 2 (MPS2)] design layout using spatial (PCR product size) and spectral (dye label colour) dimensions [FAM (blue), VIC (green), NED (yellow) and PET (red)] for *M. mustelus*.

After optimisation of the newly designed MPS (MPS1, MPS2, MPS3 and MPS4), a panel of 87 *M. mustelus* individuals from across the distribution range in southern Africa was genotyped for marker characterisation purposes. The multiplex assays were then tested on 11 additional species to show their overall application in genetic diversity and population structure analysis. Finally, a total of 12 microsatellite loci that were successfully genotyped across the study species (*Mh1*, *Mh25*, *Mca25*, *McaB39*, *McaB5*, *McaB22*, *Mca33*, *Gg15*, *Gg17*, *Gg18*, *Gg22* and *Gg23*) were selected to demonstrate the potential use of microsatellite loci in species identification.

For the multiplex reaction, the Qiagen Multiplex PCR kit was used and PCR conducted according to the manufacturer's instructions, except for varying T_A: 59 ºC for MPS1, MPS3 and MPS4; and 56 ºC for MPS2. For subsequent analysis on an ABI 3730XL DNA Analyser, PCR products were diluted in distilled water and fragment analysis performed together with the LIZ600 internal size standard. Individual genotypes were scored based on fragment size via PEAK SCANNER® v1.0 (*Life Technologies*). AUTOBIN v0.9 macro for Excel [http://www.bordeaux-aquitaine.inra.fr/biogeco/Ressources/Logiciels/Autobin; Guichoux *et al*. (2011)] was used to detect discrete size variants where allele binning of genotype data obtained from PEAK SCANNER was based on raw size.

2.3.4 Genetic Diversity Analysis

The percentage of polymorphism (*PP*) was calculated using the formula:

$$PP = \frac{N_P}{N_T} X 100$$

where N_P is the total number of polymorphic loci and N_T is the total number of loci multiplied by 100. MICROCHECKER v2.2.3 (Van Oosterhout *et al*. 2002) was used to evaluate the presence of genotypic errors caused by allele dropout, stuttering and null alleles. Null allele frequencies (Fr_{NULL}) were calculated using the Brookfield 1 estimator implemented in this program. Fixation indices (F_{IS}) for each locus and over all loci was estimated to measure departure from Hardy-Weinberg equilibrium using the exact probability test (20 batches, Dememorization; 10000 and 5000 iterations) using GENEPOP v4.0 (Rousset 2008). Linkage disequilibrium between all pairs of loci was calculated using an exact test, also implemented in GENEPOP. Slatkin's exact test (1000 permutations) for neutrality, based on Ewens-Watterson sampling theory (Slatkin 1994), was used to detect loci under selection as implemented in ARLEQUIN v3.5.1.2 (Excoffier and Lischer 2010). The abovementioned analyses were done for the multiplex characterisation of 87 *Mustelus mustelus* individuals). The number (A_N) of alleles at each microsatellite locus, as well as the effective number of alleles A_E:

$$A_E = 1 / \sum_{i=1}^{n} p_i^2$$

where p_i is the frequency of the i^{th} allele and n is the number of alleles, were calculated using the GENALEX v6.5 program (Peakall and Smouse 2012). The proportion of individual samples that were heterozygous [direct count heterozygosity (H_O) and expected under Hardy-Weinberg equilibrium (H_E)] was calculated using MSATTOOLS v1.0 (Park 2001). MSATTOOLS was also used to calculate the polymorphic information content (*PIC*) of each marker according to the following equation in Botstein *et al*. (1980):

$$PIC = 1 - \sum_{i=1}^{n} p_i^2 - 2\left[\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} p_i^2 p_j^2\right]$$

where p_i and p_j are the frequency of the i^{th} and j^{th} allele, respectively, and n is the number of alleles.

Due to different sample sizes, direct comparison of genetic diversity estimates (H_E, A_E and *PIC*) across the 11 species was not plausible and species were therefore grouped according to sample size: (1) *M. mustelus*, *M. palumbes, G. galeus*, *C. brachyurus* and *H.*

pictus (*n* = 8); (2) *P. pantherinum*, *S. lewini* and *S. zygaena* (*n* = 5), and (3) *C. obscurus*, *C. limbatus, C. plumbeus* and *H. edwardsii* (*n* = 4). The potential use of microsatellite data for species-assignment was assessed through principle coordinate analysis (PCoA) in GENALEX, using genetic distances between individuals. To evaluate cross-species amplification performance, DNA sequences derived from the mitochondrial *ND2* gene (1044 bp**)** of each species were downloaded from GenBank and Global Cestode Database: Elasmobranchs Specimens (http://elasmobranchs.tapewormdb.uconn.edu) (Appendix A: **Table S2.1**). Using these sequences, the genetic distances among the study taxa were estimated using the Kimura 2-parameter model (Kimura 1980) with the rate variation among sites modelled with a gamma distribution (shape parameter = 5) implemented in MEGA v5 (Tamura *et al*. 2011).

2.3 Results and Discussion

2.3.1 Cross-Species Amplification

Cross-species amplification proved useful in establishing genetic markers for use in several shark species of commercial importance in South Africa and those (typically endemics) that are indirectly affected by fisheries' operations. Amplification results of the 35 microsatellites in 50 individuals from 16 different elasmobranch species (1-4 individuals per species) are summarized in **Table 2.2**. Cross-species amplification success rates (percentage of microsatellites that amplified successfully) ranged from 60.00%-71.40% in the Triakidae and Carcharhinidae families, and from 28.57%-48.57% in the Scyliorhinidae, Sphyrnidae and Rajidae families (**Figure 2.3**). The higher success rates in the Triakidae and Carcharhinidae are expected as most microsatellites tested in this study were originally developed for species within the Triakidae family. Overall, the microsatellites showed less successful cross-species amplification to the taxa more divergent from the source species (see below). Notably, none of the individuals showed PCR amplification at any of the six *Scyliorhinus canicula* microsatellites. This may in part be attributed to *S. canicula* being more distantly related to the study species. The mean genetic distance between the all taxa and source taxon was 21.4±1.7% (mean±SD) (*G. galeus* as source species; **Figure 2.4; Table S2.2**) and 18.7±1.5% (*M. canis* as source species; **Figure 2.5; Table S2.3**). *Haploblepharus pictus* could not be represented in the distance plot due to the lack of genetic information available in GenBank and the Global Cestode Database: Elasmobranchs Specimens.

Table 2.2 Cross-species amplification of the 35 microsatellites among 16 elasmobranch species from southern Africa

Species / Loci	MM (*n* = 4)	MP (n = 3)	GG (*n* = 4)	SQ (*n* = 1)	CB (*n* = 4)	CL (n = 3)	CO (*n* = 4)	CP (*n* = 4)	HP (*n* = 4)	HEd (*n* = 4)	PPa (n = 4)	PA (*n* = 1)	SL (n = 3)	SZ (n = 3)	RS (*n* = 1)	RA (*n* = 3)
Mh1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	++
Mh2	+	+	+	+	+	+	+	+	-	-	-	-	+	+	-	-
Mh6	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-
Mh9	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-
Mh25	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	++
Mca25	+	-	-	-	+	+	++	++	+	-	+	+	-	-	+	+
Mca31	-	-	-	+	+	+	+	-	-	-	-	-	-	+	-	-
Mca33	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+	+
Mca44	++	-	+	-	-	-	-	-	+	+	+	+	-	-	+	++
McaB5	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
McaB6	+	-	+	+	+	+	+	+	+	+	-	-	+	-	-	-
McaB22	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	-
McaB27	+	+	+	+	+	-	-	+	-	+	-	-	-	+	-	-
McaB33	-	-	-	-	+	+	+	+	-	-	-	-	+	-	-	-
McaB35	+	+	-	+	-	-	-	-	+	-	+	+	-	-	-	++
McaB37	+	+	+	+	-	+	-	-	-	-	-	-	+	-	-	-
McaB39	+	-	+	-	+	+	+	+	+	+	+	+	-	-	+	-
Gg2	+	+	+	+	+	-	-	-	+	-	-	-	-	+	++	+
Gg3	+	+	+	+	+	+	+	+	+	+	-	-	+	-	++	+
Gg7	+	+	+	-	+	-	+	+	-	+	-	-	-	+	-	+
Gg11	+	+	+	++	++	+	++	++	+	+	-	-	-	+	-	-
Gg12	+	+	+	-	+	-	+	+	+	-	-	-	-	-	-	-
Gg15	+	+	+	+	+	+	++	++	+	+	+	+	+	+	+	-
Gg17	+	-	+	+	+	+	+	+	-	+	-	-	+	-	-	+
Gg18	+	+	+	+	+	+	++	+	+	-	-	-	-	++	+	+
Gg22	+	+	+	+	+	+	+	+	+	-	-	-	-	++	+	-
Gg23	+	+	+	+	+	+	+	+	-	-	-	-	+	++	+	+
Rp16-nfrdi	+	++	-	+	+	++	+	+	-	-	-	+	++	+	+	++
Rp35-nfrdi	-	+	-	++	-	+	-	-	-	-	-	+	+	++	+	++
Scan02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scan06	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scan12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scan14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scan15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scan16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

–, no visible band or faint bands with insufficient band intensity for scoring alleles were observed; +, solid bands with sufficient intensity for scoring alleles were detected; ++, solid bands with artifacts were produced but with at least one band of expected allele size. *Mustelus mustelus* (MM), *Mustelus palumbes* (MP), *Galeorhinus galeus* (GG), *Scylliogaleus quecketti* (SQ), *Carcharhinus brachyurus* (CB), *Carcharhinus limbatus* (CL), *Carcharhinus obscurus* (CO), *Carcharhinus plumbeus* (CP), *Haploblepharus pictus* (HP), *Haploblepharus edwardsii* (HEd), *Poroderma africanum* (PA), *Poroderma pantherinum* (PPa) *Sphyrna lewini* (SL), *Sphyrna zygaena* (SZ), *Raja straeleni* (RS) and *Raja alba* (RA).

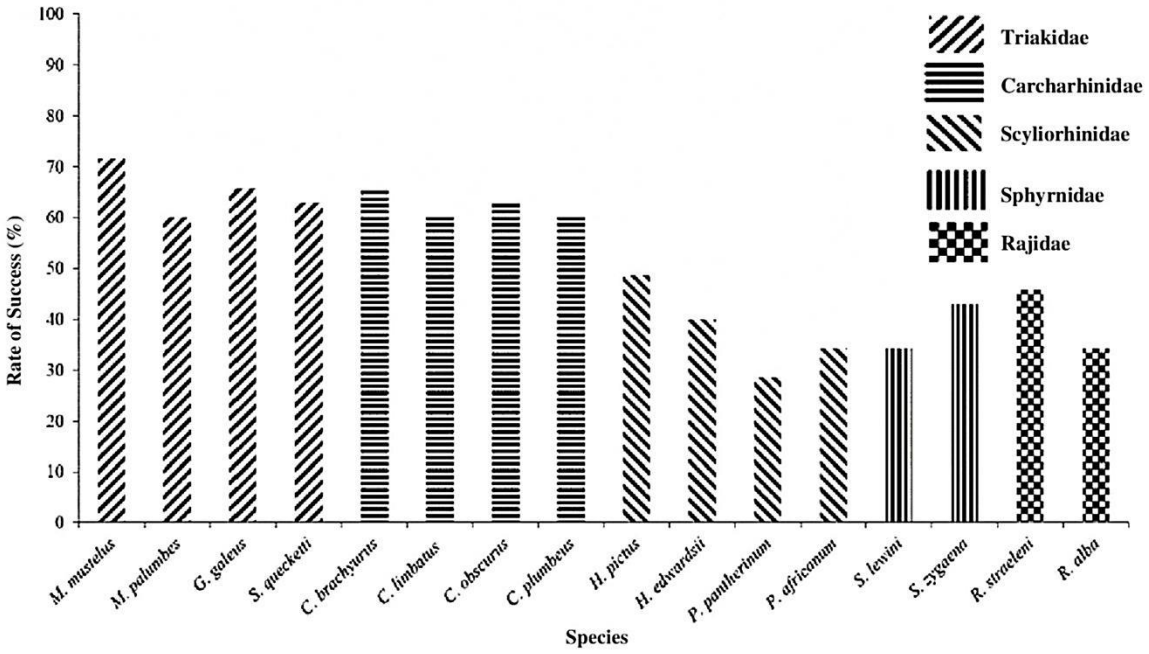

Figure 2.3 Amplification success rates of 35 microsatellite loci across 16 elasmobranch species (from five families) from southern Africa.

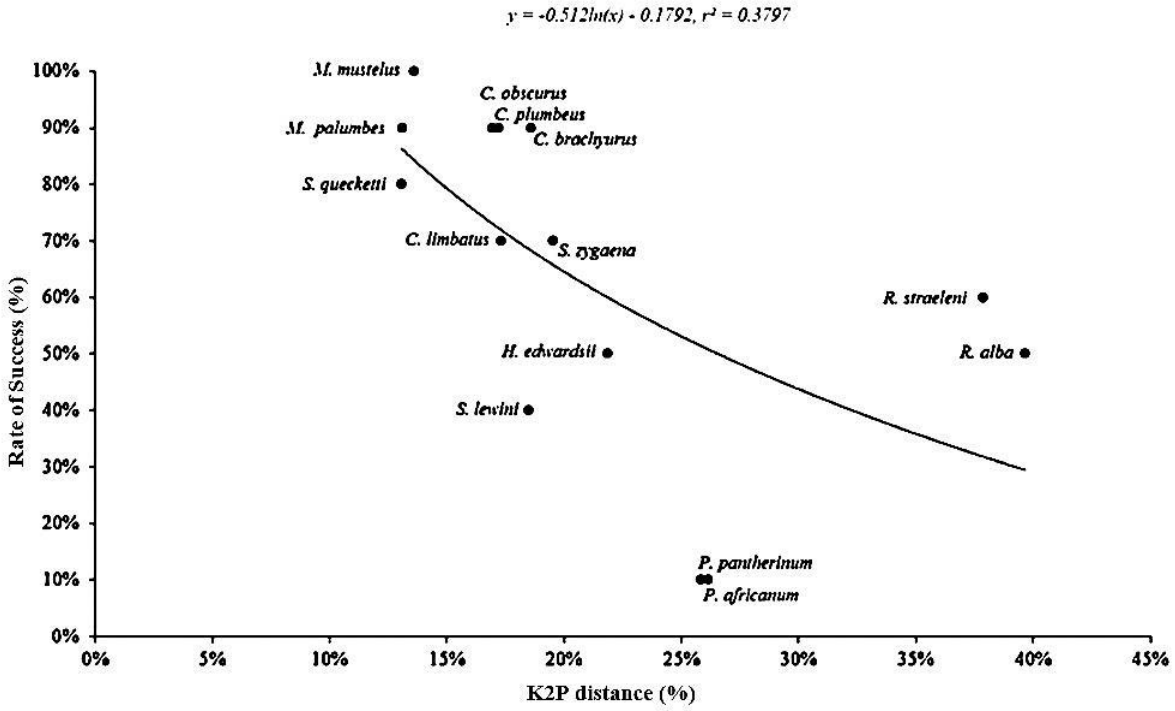

Figure 2.4 Cross-species amplification performance of *Galeorhinus galeus* microsatellites in 15 of the 16 elasmobranch species, and genetic divergence (K2P) between *G. galeus* and the target species based on *ND2* sequences.

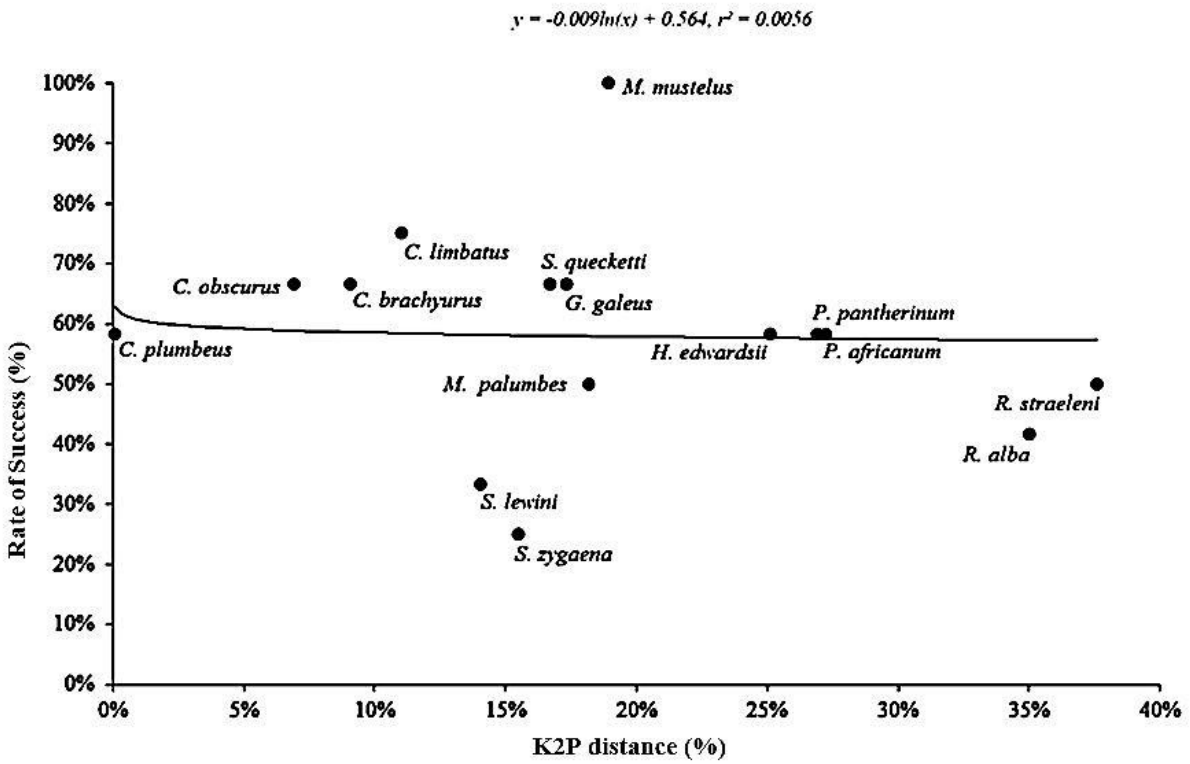

Figure 2.5 Cross-species amplification performance of *Mustelus canis* microsatellites in 15 of the 16 elasmobranch species, and genetic divergence (K2P) between *M. canis* and the target species based on *ND2* sequences.

Results of cross-species amplification performance of *G. galeus* microsatellites exhibited a logarithmic regression function (**Figure 2.4**), albeit non-significant (slope within the 95% CI for no difference from zero), that may explain the general trend of negative correlation between cross-species amplification performance and genetic divergence seen across taxa (Primmer *et al*. 2005; Barbara *et al*. 2007; Sekino and Hara 2007; Karaiskou *et al*. 2008; Kang *et al*. 2012). Cross-species amplification of microsatellite markers from source to target species is generally negatively correlated with evolutionary divergence (FitzSimmons *et al*. 1995; Primmer *et al*. 1996; Sekino and Hara 2007; Griffiths *et al*. 2011; Kang *et al*. 2012). Interestingly, this trend was not observed for the *M. canis* microsatellites (**Figure 2.5**). This can be due to different life history traits (*i.e*. mating system and generation time) and genome size (*C* value) between the source and target species, which have been previously found to have significant negative effects on cross-species amplification success (Barbara *et al*. 2007). However, apart from the source-target species evolutionary distance, other factors, such as mutations in microsatellite flanking sequences, may affect the success rate of cross-species amplification. Since microsatellites are usually found in non-coding regions, where the substitution rate is higher than in coding regions (Zane *et al*. 2002), these microsatellite

flanking sequences which serve as regions for PCR primer design and binding sites are prone to mutations (Primmer *et al*. 2005). Mutations (indels) in these regions may therefore result in null alleles and, in turn, affect the patterns of cross-species amplification as demonstrated in birds (Primmer and Ellegren 1998) and salmonids (Angers and Bernatchez 1997). Additionally, *M. canis* microsatellite loci were isolated from an enriched genomic library (Giresi *et al*. 2012), whereas the microsatellites for *G. galeus* were developed by a high-throughput sequencing approach (Roche 454 pyrosequencing) (Chabot and Nigenda 2011). Based on the observed data, it is hypothesised that these different approaches may have influenced the cross-species performance, possibly due to the different resolving power of each approach for capturing microsatellites distributed across different parts of the genome. Castoe *et al*. (2010) argue that enrichment-based approaches commonly use a few specific repeated motifs, which are largely selected without prior knowledge of their abundance in the genome and, therefore, could introduce potential bias in genome representativeness. In contrast, microsatellite identification from randomly sequenced genomic regions (*e.g*. Roche 454 NGS) allows for an unbiased assessment of all types of microsatellite loci present in a genome (Castoe *et al*. 2010).

Cross-species amplification of orthologous microsatellites, due to the presence of conserved microsatellite flanking sequences (Barbara *et al*. 2007), can persist over millions of years following divergent evolution, as previously shown in sharks (250 million years, Martin *et al*. 2002) and in fish (470 million years, Rico *et al*. 1996). This indicates lower mutation rates within microsatellite flanking regions in aquatic organisms (Rico *et al*. 1996; Sekino and Hara 2007). The reported cross-amplified microsatellite markers will thus contribute to establishing a molecular genetic marker repository for each of the southern African elasmobranch species included in this study. Future research efforts should be directed at generating microsatellite primers that have a high cross-species utility (*e.g*. Dawson *et al*. 2010) and at *in silico* mining of polymorphic microsatellite markers from expressed sequence tag data (*e.g*. Hoffman and Nichols 2011).

2.3.2 Multiplex Assay Characterisation

Twenty-two microsatellite loci that successfully cross-amplified across the study species and showed polymorphism in initial screening tests were used to develop four multiplex assays (MPS) comprised of at least five microsatellites each. These multiplex assays were characterised in a panel of 87 common smoothhound sharks (*Mustelus mustelus*) (**Table 2.3**).

All 22 microsatellite loci were polymorphic across the multiplexes. With the exception of one locus, *McaB22*, all the microsatellite loci were found to deviate significantly from Hardy-Weinberg equilibrium, most likely due to a Wahlund effect as samples were pooled from diverse geographical locations for analysis. MICROCHECKER detected no significant genotyping errors, but indicated that null alleles were present at two loci (*Mh9* and *Gg7*). Slatkin's exact test for neutrality indicated that two loci (*McaB22* and *Gg3*) were candidates for being subjected to selection.

Table 2.3 Characterisation of four multiplex systems for *Mustelus mustelus* based on 87 individuals from southern Africa

Locus	Microsatellite repeat motif	[P]	Dye	Size range (bp)	A_N	A_E	H_O	H_E	*PIC*	F_{IS}	Fr_{NULL}	$P_{E\text{-}W}$
Mh1	$(AG)_n$	0.2	VIC	191-211	7	2.2	0.885	0.544	0.443	−0.633**	−0.223	0.931
Mh2	$(GA)_9$	0.3	VIC	587-597	4	1.7	0.367	0.402	0.342	0.089**	0.023	0.688
Mh9	$(GA)_9$	0.4	FAM	312-326	5	1.7	0.337	0.429	0.373	0.214**	0.062[b]	0.723
Mh25	$(CT)_n$	0.2	FAM	122-148	8	1.6	0.356	0.404	0.385	0.118**	0.032	0.802
Mca25	$(CA)_n(CT)_n$	0.2	PET	232-240	3	1.9	0.563	0.463	0.382	−0.217**	−0.070	0.226
McaB39	$(CA)_{10}GAT(AC)_8$	0.2	NED	177-212	3	2.0	1.000	0.509	0.384	−0.977**	−0.328	0.501
MPS1 (mean)	-	-	-	-	**5**	**1.9**	**0.585**	**0.459**	**0.385**	**−0.234**	**−0.084**	**0.645**
McaB5	$(GT)_{11}$	0.2	VIC	189-210	10	3.5	0.826	0.716	0.674	−0.155*	−0.067	0.330
McaB6	$(CA)_{10}$	0.2	FAM	226-266	9	3.3	0.756	0.702	0.655	−0.077*	−0.034	0.498
McaB22	$(AC)_{18}$	0.2	NED	137-179	12	8.2	0.874	0.882	0.865	0.010	0.002	0.002
McaB27	$(GT)_6$	0.2	PET	138-199	4	2.1	0.965	0.536	0.424	−0.808**	−0.282	0.589
Mca33	$(ATC)_5$	0.2	FAM	189-199	6	3.0	0.872	0.674	0.609	−0.295**	−0.121	0.347
McaB37	$(GT)_5$	0.2	NED	219-251	11	1.9	0.483	0.486	0.431	0.007**	−0.016	0.997
MPS2 (mean)	-	-	-	-	**9**	**3.7**	**0.796**	**0.666**	**0.610**	**−0.220**	**−0.086**	**0.461**
Gg2	$(TG)_n$	0.2	NED	249-259	7	3.2	1.000	0.688	0.632	−0.458**	−0.188	0.324
Gg3	$(GATT)_n$	0.2	PET	257-265	2	2.0	1.000	0.503	0.375	−1.000**	−0.333	0.001
Gg7	$(AG)_n$	0.2	NED	296-312	4	1.6	0.310	0.393	0.343	0.212**	0.058[b]	0.584
Gg11	$(TCCC)_n$	0.2	NED	329-363	4	1.2	0.061	0.182	0.173	0.666**	0.000	0.792
Gg12	$(TA)_n$	0.2	FAM	276-296	4	1.8	0.610	0.454	0.361	−0.347**	−0.110	0.807
MPS3 (mean)	-	-	-	-	**4.2**	**2.0**	**0.596**	**0.444**	**0.377**	**−0.185**	**−0.115**	**0.495**
Gg15	$(GA)_n$	0.2	FAM	147-169	3	2.05	0.977	0.514	0.392	−0.910**	−0.308	0.370
Gg17	$(AC)_n$	0.2	PET	159-181	3	1.02	0.023	0.023	0.023	−0.003**	0.000	1.000
Gg18	$(GA)_n$	0.2	VIC	179-187	6	2.24	0.976	0.558	0.456	−0.759**	−0.272	0.776
Gg22	$(GT)_n$	0.2	FAM	237-247	4	2.25	0.964	0.559	0.455	−0.733**	−0.263	0.488
Gg23	$(AC)_n$	0.2	VIC	258-278	6	2.84	1.000	0.651	0.582	−0.540**	−0.214	0.562
MPS4 (mean)	-	-	-	-	**4.4**	**2.08**	**0.788**	**0.461**	**0.3816**	**−0.589**	**−0.211**	**0.615**
Overall (mean)	-	-	-	-	**5.7**	**2.4**	**0.691**	**0.512**	**0.444**	**0.010**	**−0.139**	**0.561**

Primer concentration in the final reaction as µM/primer ([P]); Number of alleles per locus (A_N); effective number of alleles (A_E); observed heterozygosity (H_O); expected heterozygosity (H_E); polymorphic information content (*PIC*); inbreeding coefficient (F_{IS}) with statistically significant deviations from Hardy-Weinberg expectations indicated by * ($P < 0.01$) and ** ($P < 0.001$); null allele frequency (Fr_{NULL}) with [b] indicating the presence of null alleles at statistical significance at the 5% nominal level and Ewens-Watterson probability ($P_{E\text{-}W}$). Mean values for each multiplex assay and overall are indicated in bold.

2.3.3 Multiplex Assay Cross-Species Amplification and Efficiency in Species Identification

Cross-species amplification of the four multiplex assays was tested for 11 other southern African shark species (**Table 2.4**). The number of alleles observed was highest in *G. galeus* and *M. palumbes*. Overall, A_N varied from 1 to 7, while the percentage polymorphism (*PP*) for each species over all the markers ranged from 31.8%-95.5%. The polymorphic information content (*PIC*) decreased exponentially with evolutionary distance from the source species (**Table 2.4**) and the four multiplex assays showed the highest *PIC* in *M. mustelus*, *M. palumbes* and *G. galeus*.

Table 2.4 Multiplex transferability of a total of 22 microsatellite loci, showing the number of alleles per locus for 11 elasmobranch species tested

Locus	**MP** *(n = 8)*	**GG** *(n = 8)*	**CB** *(n = 8)*	**CL** *(n = 4)*	**CO** *(n = 4)*	**CP** *(n = 4)*	**HP** *(n =8)*	**HEd** *(n = 4)*	**SL** *(n = 5)*	**SZ** *(n = 5)*	**PPa** *(n = 5)*
MPS1											
Mh1	4	3	1	2	1	1	1	1	4	2	3
Mh2	-	5	*	1	1	*	-	-	2	1	3
Mh9	4	4	*	-	*	*	-	-	**4**	5	**3**
Mh25	5	5	2	3	2	3	1	3	**4**	6	3
Mca25	3	1	1	3	2	1	2	1	**3**	**4**	3
McaB39	**3**	3	2	2	1	2	2	*	2	**3**	3
MPS2											
McaB5	3	1	2	4	2	3	1	1	**5**	**5**	7
McaB6	4	4	*	4	2	1	-	1	2	**3**	**5**
McaB22	2	1	1	4	2	4	2	4	**4**	7	7
McaB27	2	2	2	-	**1**	*	-	1	-	2	-
Mca33	4	2	2	4	2	2	2	3	**4**	**7**	6
McaB37	3	5	**1**	3	**1**	**1**	-	-	**4**	**6**	**7**
MPS3											
Gg2	5	4	1	-	**1**	**1**	2	-	-	2	-
Gg3	3	2	1	1	2	*	2	1	-	2	**2**
Gg7	4	1	1	-	1	1	**2**	-	-	1	1
Gg11	6	4	1	-	2	2	*	*	-	1	3
Gg12	4	5	-	-	1	*	1	**1**	2	2	**1**
MPS4											
Gg15	7	5	1	4	1	4	4	2	5	3	5
Gg17	4	4	1	4	1	2	**2**	2	2	3	2
Gg18	6	3	2	3	1	3	3	**3**	7	6	4
Gg22	6	5	2	3	2	2	2	*1*	3	2	3
Gg23	4	2	2	1	2	1	**3**	**2**	6	4	3
Total P loci	21	18	8	13	10	10	12	7	16	19	18
PP	95.5	81.8	36.4	59.1	45.5	45.5	54.5	31.8	72.7	86.3	81.8

n - number of individuals tested; *Failed to amplify but showed successful transferability initially (see **Table 2.2**); − No amplification; allele numbers in bold indicate loci that failed to cross-amplify according to **Table 2.2**; P – polymorphic and *PP* - percentage of polymorphism. For species abbreviations refer to **Table 2.2.**

The mean genetic diversity estimates for each species in terms of the number of alleles (A_N), effective number of alleles (A_E), observed heterozygosity (H_O), expected heterozygosity (H_E) and *PIC* are shown in **Figure 2.6**. In species group 1, the mean H_E, A_E and *PIC* varied from relatively low in *C. brachyurus* (mean H_E = 0.230; mean A_E = 1.4; mean *PIC* = 0.181) to relatively high in *M. palumbes* (mean H_E = 0.653; mean A_E = 3.3; mean *PIC* = 0.606). Species group 2 exhibited similar patterns of genetic diversity that varied from moderate in *S. zygaena* (mean H_E = 0.593; mean A_E = 3.2; mean *PIC* = 0.554) to relatively high in *P. pantherinum* (mean H_E = 0.662; mean A_E = 3.4; mean *PIC* = 0.603). For species group 3 (species with n = 4), the mean H_E, A_E and *PIC* ranged from relatively low in *C. plumbeus* (mean H_E = 0.249; mean A_E = 1.5; mean *PIC* = 0.193) to relatively high in *C. obscurus* (mean H_E = 0.429; mean A_E = 2.1; mean *PIC* = 0.367).

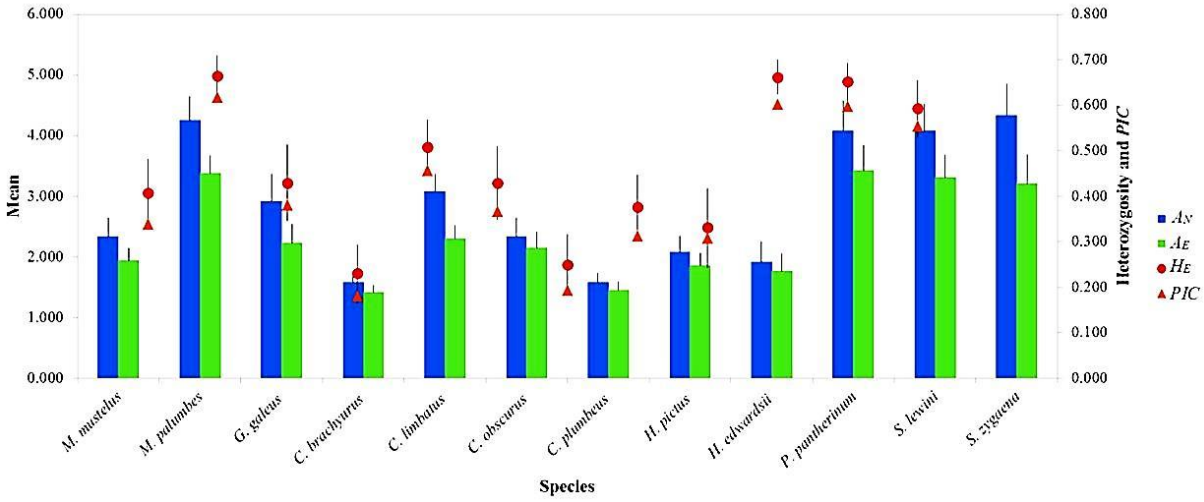

Figure 2.6 Mean genetic diversity estimates using 12 microsatellite loci shared between species: number of alleles (A_N), effective number of alleles (A_E), heterozygosity (H_E) and polymorphic information content (*PIC*). Error bars represent standard error.

The genotypic distribution of the study species is depicted in **Figure 2.7**. Most of the study species could be differentiated on PC1 and PC2 of the PCoA plot as can be seen with individuals of each respective species clustering together. However, individuals of the catshark species were dispersed across quadrant 3 and 4 (*H. edwardsii* and *H. pictus*), and quadrant 1 and 3 (*P. pantherinum*). The PCoA also revealed that one of the *M. mustelus* individuals was misidentified as *G. galeus*. The identity of this particular individual was subsequently confirmed using the genetic identification method developed specifically for smoothhound sharks (Farrell *et al*. 2009). Briefly, this method involves using four primers (one universal forward primer and three species-specific reverse primers) for the mitochondrial gene, *nicotinamide adenine dehydrogenase subunit 2* (*ND2*), in a multiplex PCR reaction. The reverse primers amplify a fragment of different length for each species (*G.*

galeus, 671 bp; *M. asterias*, 564 bp; *M. mustelus*, 392 bp) and can, therefore, be utilised for distinguishing species based on fragment size.

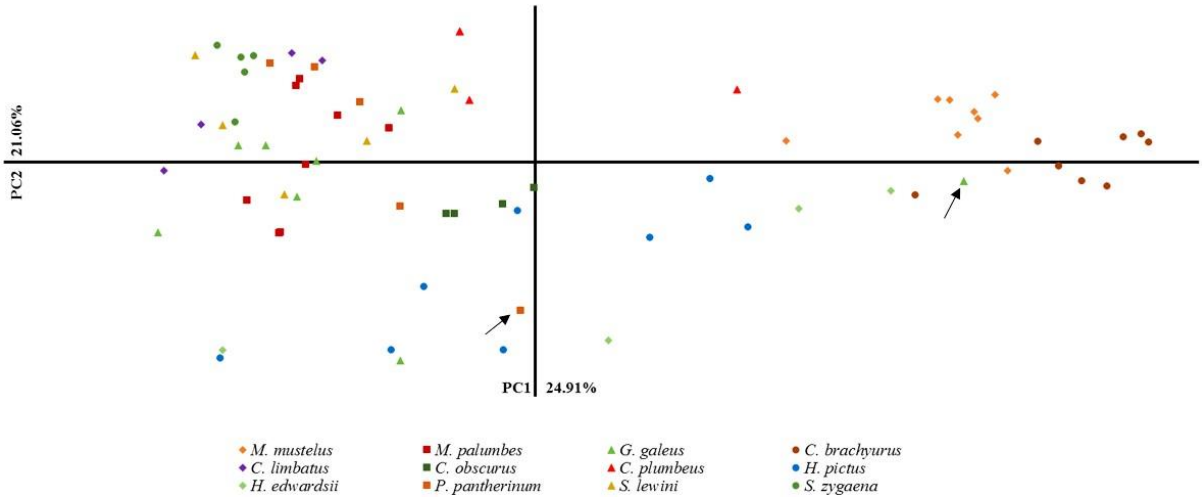

Figure 2.7 Principle coordinates analysis (PCoA) of study species based on 12 amplified microsatellite loci shared between species. Arrows depict misidentified/mislabelled individuals.

In this study, the potential use of microsatellites in species identification was successfully demonstrated for some of the species included in this study. The polymorphic information of these microsatellite loci was characterised by low genetic variation, as previously proposed for elasmobranchs (Dudgeon *et al*. 2012). The genotypic distribution of the study species could also be differentiated based on PCoA analysis. Markedly, the lack of differentiation between the catshark species (*H. edwardsii* and *H. pictus*) on the PCoA plot may be explained by the misidentification of the *Haploblepharus* species, which is a common occurrence in the catshark family (Human 2007). To further investigate whether the lack of differentiation detected with microsatellites was indeed due to misidentification, the *cytochrome b* (*Cytb*) and *ND2* genes were applied for species comparisons (Human 2007). For both genes, sequence analyses revealed individuals with cryptic identification (conflicting morphological and genetic identification), suggesting that, in the case of the catsharks, the microsatellites optimised in the current study were not successful in discriminating between the *Haploblepharus* species, but could in the future aid in the identification of cryptic speciation within the catshark family.

In South Africa, the aforementioned misidentification issue is prominent in fishing operations (particularly in longline and trawl fisheries) where there is a high rate of incidental capture of non-target shark species (Myers and Worm 2003; Barker and Schluessel 2005; Petersen *et al*. 2008; Attwood *et al*. 2011). This hinders the collection of reliable data on

shark catch and trade on a species-specific basis, making robust stock assessments and identification of overfished and potentially threatened species nearly impossible in most situations (Shivji *et al*. 2002). This was apparent in a study by Attwood *et al.* (2011), which assessed bycatch in South Africa's inshore trawl fishery based on observer records. In the aforementioned study, certain taxa were difficult to identify and, therefore, taxonomic groups were lumped in species groups (*e.g. Raja* spp., *Mustelus* spp. and Scyliorhinidae), even though every attempt was made to analyse data at the species level. Therefore, the molecular genetic makers developed by the current study may facilitate obtaining species-specific catch data for stock assessment, characterising genetic diversity and delineating population genetic structure. This could, in turn, contribute to the implementation of future conservation and management plans on a species-specific level in southern Africa.

2.4 Conclusions

Cross-species amplification of available microsatellite loci to target species is more time and cost-effective in comparison to *de novo* development and permitted the optimisation and use of 22 microsatellite markers across 12 elasmobranch species. Genotyping data of the four multiplex assays developed in the current study was successfully used for characterising genetic diversity and also highlighted the potential use of microsatellites in species identification in a number of commercially important and endemic species. The molecular genetic markers developed in this study and their usefulness in down-stream applications could, therefore, aid a more integrative, multidisciplinary approach to conservation management of elasmobranchs in southern Africa.

Chapter 3

Microsatellite Variation in *Mustelus mustelus*: Regional Population Genetic Structure and Demographics of a Declining Coastal Shark

Abstract

In marine organisms dispersal may affect genetic variation within and among populations through gene flow, thereby influencing fitness and spatial organization at both local- and meta-population levels. The patterns of dispersal may be affected by the oceanic surroundings, attributed to the influence of climate, hydrodynamics and biogeographic barriers. In South Africa, the three recognised biogeographic provinces are the cool-temperate west coast, the warm-temperate south coast and the subtropical east coast. Known biogeographic barriers between these provinces may impact and restrict genetic connectivity of regional shark populations, leading to locally adapted stocks. Deciphering these patterns of genetic connectivity in commercially important shark species is, therefore, becoming increasingly important for integrated fisheries management of threatened shark populations. The common smoothhound, *Mustelus mustelus,* is an overexploited, commercially- and recreationally-important shark species in South Africa. Considering the vulnerable status of the common smoothhound shark and very limited available genetic information, this study assessed patterns of gene flow along the South African coast, using multilocus data from 12 highly variable microsatellite markers. The present study found moderate levels of genetic diversity, as based on the heterozygosity and allelic richness. Estimates for pairwise population differentiation, F-statistics, AMOVA and Factorial Correspondence Analysis (FCA) detected significant genetic structure within and between the Atlantic and Indian Ocean populations. Additionally, Bayesian clustering analyses detected two ancestral gene pools in the study populations, further supporting the presence of at least one oceanographic feature driving structure of common smoothhound shark along the South African coastline. For future sustainable exploitation of common smoothhound shark it is recommended that fisheries take into account the possible existence of, and observed asymmetric gene flow among, two genetically-differentiated populations along the South African coast.

3.1 Introduction

Nearly 70 years of demersal shark fishing for fins and meat, peaking in 2008-2010, and unregulated exploitation of various elasmobranch species in South Africa (Department of Agriculture, Forestry and Fisheries 2013) have resulted in local population declines (Sauer *et al*. 2003; Da Silva and Bürgener 2007). This decline is accentuated by the generally slow growth rate, late maturity and low reproductive output of these animals (Musick *et al*. 2000; Stevens *et al*. 2000). Growing concerns regarding the sustainability of the South African shark fishery led the South African government to participate in the International Plan of Action for the Conservation and Management of Sharks (IPOA-sharks) through implementing a National Plan of Action for Sharks (NPOA-Sharks), imposing increasingly stricter regulations to avert a collapse of stocks (Department of Agriculture, Forestry and Fisheries 2013). It is well recognised that a drastic reduction in population size can result in small and isolated populations becoming increasingly susceptible to a loss of genetic diversity and consequently also the general survival of populations (Nei *et al*. 1975; Frankham 2005). Characterising genetic variability, assessing the direction and strength of gene flow between populations and defining reproductively isolated stocks in marine organisms is important for elucidating historical and contemporary processes responsible for observed patterns of spatial genetic differentiation (Veríssimo *et al*. 2010, 2011; Ovenden 2013; Vignaud *et al*. 2013).

In sharks, different levels of subdivision have been reported over large and smaller spatial scales (Feldheim *et al*. 2001; Duncan *et al*. 2006; Schultz *et al*. 2008; Chabot and Allen 2009; Pereyra *et al*. 2010; Benavides *et al*. 2011*a*, *b*; Mourier *et al*. 2013; Mendonça *et al*. 2013; Vignaud *et al*. 2013, 2014) based on molecular data. These studies highlighted the reality of existing barriers to gene flow, shaping genetic structure in a range of small-benthic to large-oceanic shark species. For example, hydrodynamic barriers (open oceanic waters; Feldheim *et al*. 2001; Duncan *et al*. 2006; Schultz *et al*. 2008), thermal barriers (water temperature; Chabot and Allen 2009; Veríssimo *et al*. 2010) and biogeographic barriers (disconnection along continental coastlines; Duncan *et al*. 2006; Daly-Engel *et al*. 2012; Veríssimo *et al*. 2012; Vignaud *et al*. 2013) have been shown to limit gene flow. Integrating genetic information into conservation management of shark populations could aid in identifying Management Units (MUs), defined as demographically-distinct populations that

should be managed separately to ensure the viability of the larger metapopulation (Funk *et al*. 2012).

The likelihood of species misidentification in shark fishery operations is also a general concern hampering the estimation of species-specific catch rates (Myers and Worm 2003; Attwood *et al*. 2011) and is attributed to the conserved interspecific morphology of various sharks species occurring sympatrically (Pank *et al*. 2001). Accordingly, it is vital for fisheries to correctly identify sharks to the species level, particularly demersal species, as they are usually landed having been headed and gutted at sea (Da Silva and Bürgener 2007). For instance, species misidentification of *Mustelus* species is a common occurrence in the Mediterranean and Black Sea (involving *M. asterias* and *M. mustelus*), Australia (*M. antarcticus*, *M. ravidus* and *M. stevensi*), northern Gulf of California (*M. albipinnis*, *M. californicus*, *M. henlei* and *M. lunulatus*) and in South Africa (*M. mosis*, *M. mustelus* and *M. palumbes*) (Heemstra 1973; Smale and Compagno 1997; Da Silva and Bürgener 2007; Farrell *et al*. 2009; Boomer *et al*. 2012; Pérez-Jiménez *et al*. 2013). Moreover, these species are readily confused with other houndsharks, such as the tope (*Galeorhinus galeus*) and spotted gully (*Triakis megalopterus*) sharks (Da Silva and Bürgener 2007; Farrell *et al*. 2009). Species-specific catch rate data complemented with genetic data could prove useful in assessing species composition of commercially important shark species in fishing operations.

The common smoothhound shark, *Mustelus mustelus* Linnaeus 1958, is considered a cosmopolitan species *i.e*., widespread in distribution from the Mediterranean Sea and eastern Atlantic Ocean to the South-West Indian Ocean; in southern Africa the species' range includes the west, south and east coasts from Angola to KwaZulu-Natal (Smale and Compagno 1997). Common smoothhound shark is an active and strong-swimming, medium-sized (< 1.6 m), epibenthic (living on or near the seafloor) member of the houndshark family Triakidae that is fairly slender with flattened ventral surfaces on the head and body (Smale and Compagno 1997). These sharks dwell along continental shelves and uppermost slopes, from intertidal regions, mostly less than 100m in depth, although residence at 350m was reported in the tropics (Smale and Compagno 1997; Serena *et al*. 2009). Common smoothhound shark is a mesopredator (mid-level predator) (Belleggia *et al*. 2012) and feeds mainly on anchovy (fish), crustaceans and mollusks (Smale and Compagno 1997; Filiz 2009). The species is characterised by placental viviparity (live-bearing) (Compagno, 1984; Boomer *et al*. 2012) and reproduces seasonally where each cycle may take one year or longer, depending on the resting periods between gravidities (Smale and Compagno 1997). Litter

size has been positively correlated with maternal length and, thus, age (Smale and Compagno 1997; Saïdi *et al*. 2008), ranging from four to 23 pups per litter (Da Silva and Bürgener 2007). Little is known about the lifespan of ocean dwelling common smoothhound sharks; however, those held in captivity live to an average age of 25 years and those in the wild are believed to live longer (Da Silva 2007). Common smoothhound sharks are harvested commercially and recreationally across the species range (Da Silva 2007; Serena *et al*. 2009). A decrease in population size in the Mediterranean Sea has previously been reported (Aldebert 1997) and lately a similar trend has been observed in the eastern central Atlantic (Gascuel *et al*. 2007), eastern Atlantic and South-West Indian Ocean (Da Silva 2007). Globally, common smoothhound shark population trends have been listed as decreasing and the species is listed as vulnerable by the IUCN Red List of Threatened Species (Serena *et al*. 2009).

Identifying regional stocks is one of the key components in sustainable fisheries (Grant and Bowen 1998; Ovenden 2013) since species distributions may extend across national and international management boundaries, where protection and management legislation may differ for any given species (Ovenden 2013). A stock assessment of common smoothhound shark in southern Africa by Da Silva (2007) found that the species is overexploited and threatened, underlining the need for appropriate conservation and management strategies for the species in South Africa. In light of this, a recent investigation by Da Silva *et al*. (2013) on the degree of protection afforded to common smoothhound sharks by the Langebaan Lagoon Marine Protected Area (MPA) found, using telemetry, that this species demonstrated a high degree of site fidelity with an average distance travelled by individuals of 16 km. It was also concluded that no-take area protection may be a practical management option for the common smoothhound shark since the species demonstrated such site fidelity, and that this information may be applied to other species with similar life history traits.

Considering the vulnerable status and potential site fidelity of this species, this study aims to investigate the patterns of gene flow among populations along the South African coast. Using microsatellite data, the alterenate hypotheses of panmixia or population subdivision is tested among sampled populations of *M. mustelus* collected along its regional range. More specifically, the effect of hydrodynamics and biogeographic barriers on gene flow and the impact on genetic structuring of populations are investigated. Assessment of population demography could also increase understanding of the influence of fisheries activity on the genetic diversity of *M. mustelus* and aid in establishing a more integrated conservation management programme for the species in southern Africa.

3.2 Materials and Methods

3.2.1. Sample Collection and DNA Extraction

Fin clips and muscle tissue of 158 sharks were obtained from seven different locations along the South African coast, as well as from the Dunes, a seascape situated between Tombua and Baia dos Tigres in southern Angola (reference population; **Figure 3.1**). In South Africa, samples were collected from the west (Langebaan Lagoon MPA, Robben Island, False Bay and Kalk Bay), south (Struis Bay and Jeffreys Bay) and east (Durban) coasts. The west coast populations constituted the South-East Atlantic Ocean populations, west of the proposed Cape Agulhas Boundary, while the south- and east coast populations constituted the South-West Indian Ocean populations, east of the Cape Agulhas Boundary. These samples were collected by licensed shark ecology researchers who had volunteered to participate in this study (see Acknowledgements) and one commercial fishing company.

Total genomic DNA was isolated using the standard cetyltrimethylammonium bromide (CTAB) method of Saghai-Maroof *et al*. (1984). The extracted DNA was quantified using a NanoDrop ND-1000 spectrophotometer v3.0.1 (*NanoDrop*®) and each DNA sample was adjusted to a working concentration of 50 ng/µl and stored at -20ºC prior to polymerase chain reaction (PCR) analysis.

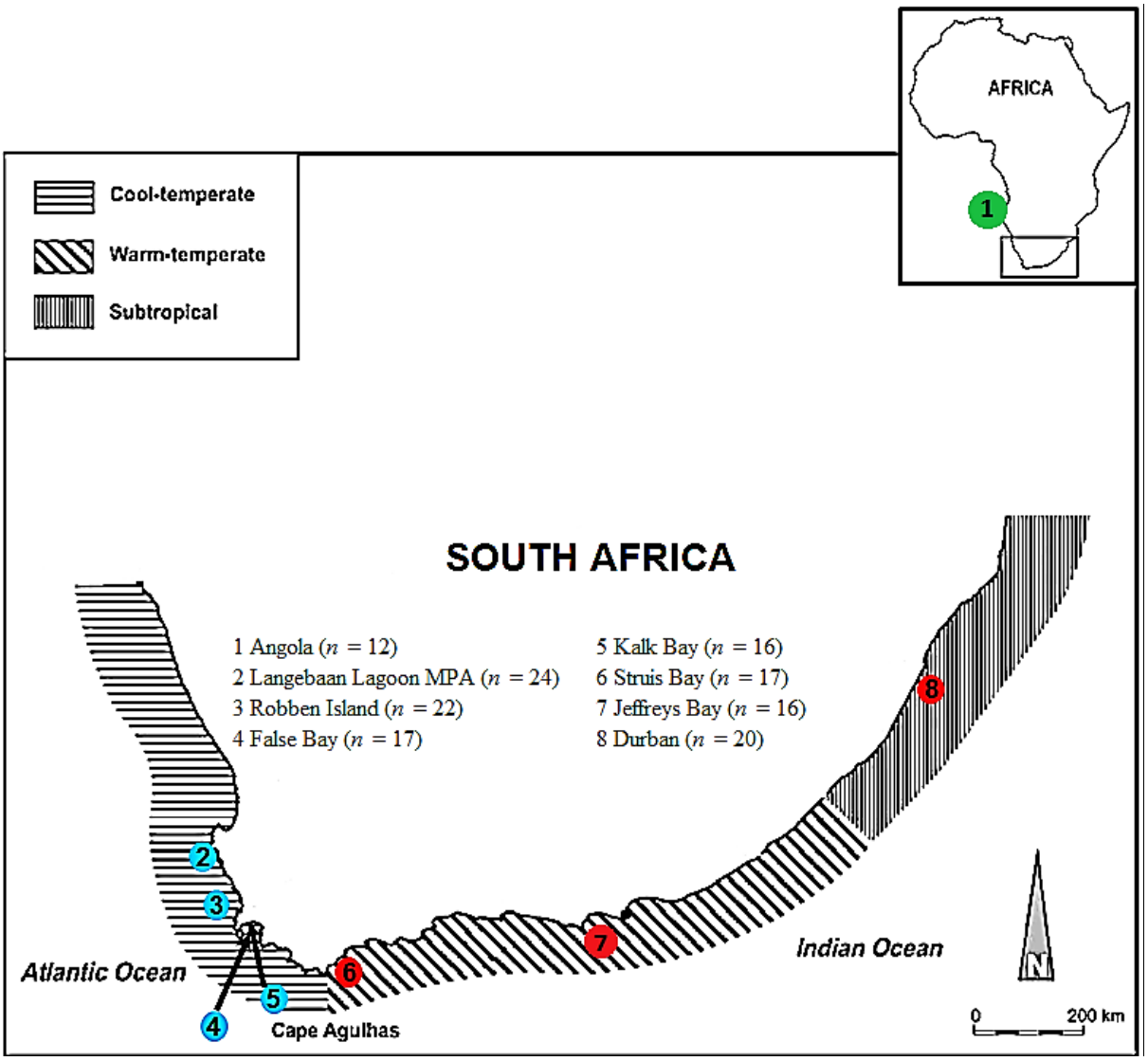

Figure 3.1 Sampling localities and sample sizes of *Mustelus mustelus* with the green circle representing Angola, and blue and red circles representing the South-East Atlantic and South-West Indian Ocean sampled populations, respectively. The three major coastal biogeographic regions are also shown. Map adapted with modification from Whitfield and Baliwe (2013).

3.2.2. Species Identification

Misidentification of houndsharks is a common occurrence as their morphology is highly conserved between species (Heemstra 1973; Smale and Compagno 1997; Farrell *et al*. 2009) and because of linguistic (ethnic) differences in understanding or interpreting common names (Burgess *et al*. 2005). Therefore, prior to genotyping, all available houndshark samples (158) were screened using the genetic identification method of Farrell *et al*. (2009) and/or barcoded with the mitochondrial *cytochrome oxidase I* (*COI*) gene, using the primers *FishF1* and *FishR1* according to the recommended PCR conditions outlined in Ward *et al*. (2005). Bi-directional sequencing was performed using standard Sanger sequencing chemistry

(BigDye® terminator v3.1 cycle sequencing kit, *Life Technologies*) and capillary electrophoresis conducted at the Central Analytical Facility of Stellenbosch University (DNA sequencing unit). Sequences were aligned using the CLUSTALW algorithm (Thompson *et al*. 1994) implemented in BIOEDIT v7.0.9 (Hall 1999). A local BLASTn analysis of these sequences was conducted for species identification using the Barcode of Life Database (BOLD).

3.2.3. Microsatellite Genotyping and Marker Validity

A set of 12 microsatellite markers, previously optimised in two multiplex reactions, multiplex assay 1 (MPS1; *Mh1*, *Mh2*, *Mh9*, *Mh25*, *Mca25* and *McaB39*) and multiplex assay 2 (MPS2; *McaB5*, *McaB6*, *McaB22*, *McaB27*, *Mca33* and *McaB37*), were selected and polymerase chain reaction (PCR) amplification performed as outlined in Chapter 2. For subsequent analysis, PCR products were diluted in distilled water and fragment analysis performed, together with the LIZ600 internal size standard, on an ABI 3730XL DNA Analyser. Allele scoring was done using GENEMAPPER v4.0 (*Life Technologies*).

The efficiency of the markers was tested by inspecting genotypic errors resulting from allele dropout, stuttering and null alleles, according to Brookfield (1996) using MICROCHECKER v2.2.3 (Van Oosterhout *et al*. 2002). Departure from Hardy-Weinberg equilibrium was tested using the exact probability test (500 batches, 10 000 iterations) in GENEPOP v4.0 (Rousset 2008). Linkage disequilibrium between all pairs of loci was calculated using an exact test, also implemented in GENEPOP. Slatkin's exact test (10 000 permutations) for neutrality, based on Ewens-Watterson sampling theory (Slatkin 1994), was used to detect loci subjected to selection as implemented in ARLEQUIN v3.5.1.2 (Excoffier and Lischer 2010). Additionally, the F_{ST}-outlier method as implemented in LOSITAN v1.44 (with 10 000 permutations, assuming the infinite alleles model) was used to test for neutrality (Antao *et al*. 2008).

3.2.4. Within-Population Patterns of Genetic Diversity

Genetic diversity was measured as the number of alleles (A_N) at each microsatellite locus, Information (Shannon-Weaver) Index (I), as calculated using the GENALEX v6.5 program (Peakall and Smouse 2012), observed heterozygosity (H_O), expected heterozygosity (H_E) and polymorphic information content (PIC), as calculated in MSATTOOLS v1.0 (Park 2001), and allelic richness standardized for sample sizes (A_R) as calculated in HP-RARE v1.0

(Kalinowski 2005). Mean relatedness was calculated for each population using the relatedness estimator, *r*, of Queller and Goodnight (1989), implemented in GENALEX. Significant within-population mean relatedness was tested using a permutation test (999 permutations, Peakall and Smouse 2006) and upper and lower 95% confidence intervals (CIs) for the expected range of *r* if reproduction were random across sampling sites were derived.

3.2.5. Among-Population Patterns of Genetic Diversity

To assess population differentiation, pairwise F_{ST}-values between populations [with Bonferonni correction at the 5% nominal level (Rice 1989)] were calculated in GENALEX v6.5 (Peakall and Smouse 2012), and an exact test for pairwise genotypic differentiation (ETGD) was implemented in GENEPOP v4.0 (Rousset 2008). A hierarchical locus by locus analysis of molecular variance (AMOVA), with 1 000 permutations to determine significance, was computed in ARLEQUIN v3.5.1.2; populations were grouped based on geographical origin (Angola, South-East Atlantic or South-West Indian Ocean) to test *a priori* hypothesised hydrodynamic barrier between South Africa and Angola; the Angola-Benguela Front. Additionally hierarchical AMOVA was used to examine *a priori* hypothesised biogeographic barrier across the South-East Atlantic (including Angola) and South-West Indian Ocean; the Cape Agulhas Boundary.To visualise population distinctness, a Factorial Correspondence Analysis (FCA) plot was drawn in GENETIX v4.03 (Belkhir *et al*. 1996–2004). Isolation by distance (IBD) was tested using a Mantel test (Mantel 1967), implemented via GENALEX (Peakall and Smouse 2012). Population genetic structure was further assessed through a Bayesian clustering, model-based method, implemented in STRUCTURE v2.3.4, in order to detect the number of genetic clusters (*K*) present in the southern African samples (Pritchard *et al*. 2000). The admixture model with correlated allele frequencies was applied for 10 replicates across $K = 1$ (panmaxia) to $K = 8$ (each sampling site distinct), with each run consisting of 2 000 000 MCMC iterations and an initial burn-in phase of 200 000 iterations, assuming no prior population information. To identify the most likely *K* value, the program STRUCTURE HARVESTER v0.3 was used to process the STRUCTURE result files (Earl and vonHoldt 2013). The results from the 10 replicates were averaged using the software CLUMPP v1.1.2 (Jakobsson and Rosenberg 2007) and the output was visualised using DISTRUCT v1.1 (Rosenberg 2004). Finally, between-population genetic distances D_A (Nei *et al*. 1983) were calculated and used to construct a phylogram according to the Neighbour-Joining (NJ) method (Saitou and Nei, 1987). The goodness of fit

(R^2) of the D_A NJ phylogram was tested using TREEFIT v1.2 (Kalinowski 2008). This program creates the tree from a genetic distance matrix, and then compares the observed genetic distance between populations with the genetic distance (fitted) in the tree and computes the R^2 value, the proportion of variation in the genetic distance matrix that is explained by the tree (Kalinowski 2008). Additionally, phylogram robustness was tested with 1 000 bootstraps among loci using the software POPULATIONS v1.2.3 (Langella 1999) and visualised using TREEVIEW v1.6.6 (Page 2001).

3.2.6. Demographical History Inference

Contemporary estimates of effective population size (N_E) were estimated with the linkage disequilibrium (LD) test (minimum allele frequency of 0.02) and heterozygosity excess method applied to all populations, as implemented in N_EESTIMATOR v2.01 (Do *et al*. 2014). The occurrence of recent bottlenecks and changes in N_E were evaluated using the Wilcoxon signed-rank test for significant deviation from heterozygosity excess and deficiency under all three mutation models [infinite alleles model (IAM), stepwise mutation model (SMM) and the two-phased model (TPM)], implemented in the program BOTTLENECK v1.2.02 (Piry *et al*. 1999). The TPM is thought to be the best fit for microsatellite data and recent bottleneck events (Piry *et al*. 1990; Williamson-Natesan 2005). Analysis in BOTTLENECK was performed using 1 000 replications at the 5% nominal level and a TPM composed of 70% SMM and 30% IAM and a variance of 30 (Piry *et al*. 1999). Features of past demographic events were further examined using the interlocus *g*-test (Reich *et al*. 1999), implemented in the KGTESTS Excel macro provided by Bilgin (2007). The interlocus *g*-test assumes that, in an expanding population, the variance in the widths of the allele length distribution across loci is usually lower than in a population of constant size; an unusually low value of the *g*-statistic may be interpreted as an indication of expansion. Significance was assessed by computer simulations that established cut-off values for a given number of samples and loci, as described in Reich *et al*. (1999). This test is appropriate for the low number of loci and small sample size considered in this study.

The study populations were grouped into three regional groups: Angola, South-East Atlantic Ocean (Langebaan, Robben Island, False Bay and Kalk Bay) and South-West Indian Ocean (Struis Bay, Jeffreys Bay and Durban), separated by two putative barriers: a hydrodynamic barrier (Angola-Benguela Front) and a biogeographic barrier (Cape Agulhas Boundary). Estimates of long-term effective migration rates among these regional groups and

their confidence intervals (CIs) were estimated by applying the coalescent-based approach, using maximum likelihood inference methods (Beerli 1998; Beerli and Felsenstein 1999, 2001). The simple electrophoretic ladder model and stepping-stone migration model with asymmetric rates were applied, as implemented in MIGRATE-N v3.6 (Beerli 2006; Beerli 2009; Beerli and Palczewski 2010). All possible combinations of migration that were either symmetrical or had no dispersal between populations were evaluated with the likelihood ratio test. Bidirectional M (mutation-scaled migration rate) among populations ($M=m/\mu$, where m is the immigration rate per generation) and the number of migrants per generation ($N_m = M\theta/4$) were also estimated. For the maximum likelihood runs of MIGRATE-N, one in every 20 reconstructed genealogies was sampled for each locus for ten short and three long chains. In the recorded 1 000 and 10 000 genealogies for short and long chains, respectively, the first 200 and 2 000 genealogies were discarded as burn-in.

3.3 Results

3.3.1. DNA Barcoding and Species Identification

For species identification prior to population genetic analysis, a total of 158 houndshark samples were screened and 144 (91.1%) were positively identified as *M. mustelus*. The remaining samples were identified as other houndshark species, such as *Galeorhinus galeus* and *M. palumbes*, based on the combination of *COI* barcoding and the identification method of Farrell *et al*. (2009). With the latter method, species are distinguished based on DNA fragment size, 392 bp for *M. mustelus* and 671 bp for *G. galeus*. The misidentified individuals showed amplification of a band at 671 bp or two fragments when species were assigned based on the fragment length visualized on the gel (**Figure 3.2**). The latter samples were barcoded using *COI* (Ward *et al*. 2005).

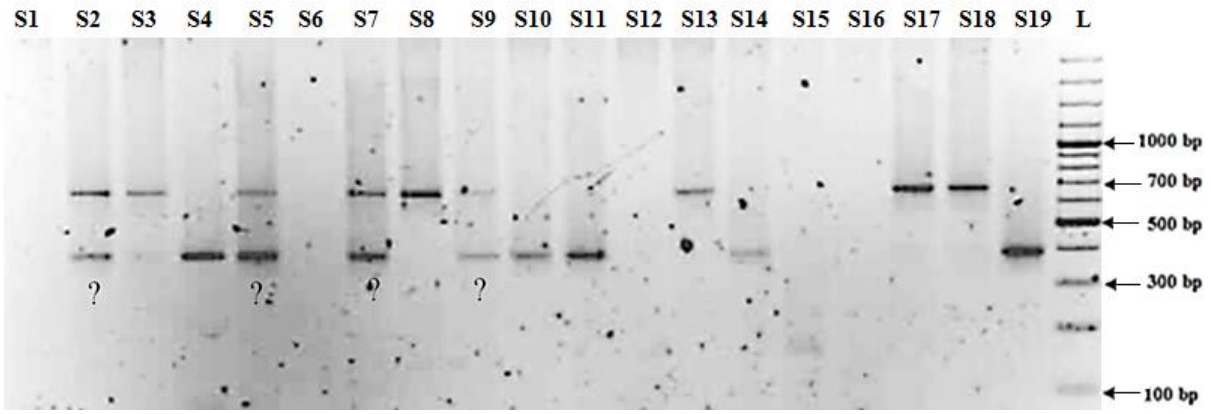

Figure 3.2 Results of the multiplex PCR amplification of the *ND2* gene for houndshark species identification on a 2% agarose gel. Lanes S1-19 are smoothhound samples and Lane L is the 100 bp molecular ladder. The ? symbol indicates individuals that amplified for both fragments.

3.3.2. Within-Population Genetic Diversity

A total 113 alleles were observed across all loci, with allele numbers ranging from 2-14 per locus. No significant genotyping errors, due to stuttering and allelic dropout, were identified. Fixation indices F_{IS} were highly significant in most populations, with values ranging from -1 to 0.835, mostly because of significant heterozygote excess or deficiencies at some loci (Appendix B: **Table S3.1**). Null alleles were present at some of the loci (*Mh2* and *Mh25*) at significantly high frequencies ($P < 0.05$) and most likely explain the significant deviations from Hardy-Weinberg expectation at these loci (**Table S3.1**). Slatkin's exact test for neutrality, based on Ewens-Watterson sampling theory, indicated that two loci (*McaB22* and *McaB39*) were candidates for being subjected to selection (**Table S3.1**), whereas the F_{ST}-outlier test showed that four loci, including *McaB37* and *Mh2* (under directional selection), did not conform to neutrality (**Figure 3.3**).

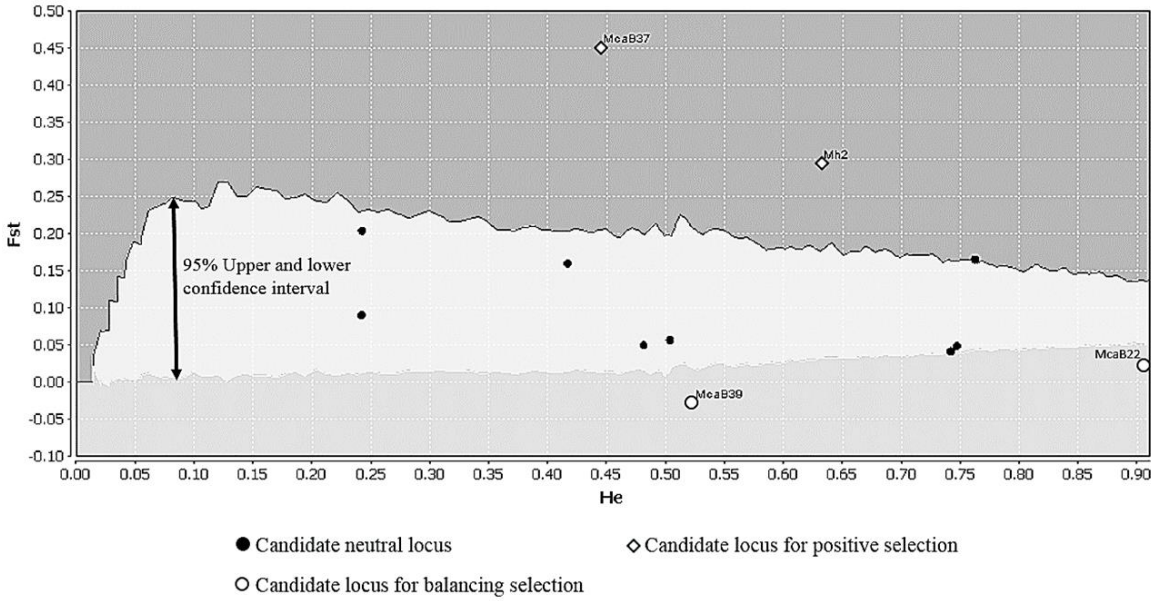

Figure 3.3 LOSITAN results indicating outlier loci as candidate loci under directional (white squares in dark grey area) and balancing selection (white circles in light grey area).

Genetic diversity, based on all measures, was moderate and comparable across the sampling populations (**Figure 3.4**; **Table S3.1**). Genetic diversity levels were relatively high for Jeffreys Bay (mean H_E = 0.597; mean A_R = 2.3; mean I = 0.988) and relatively low for Langebaan (mean H_E = 0.427; mean A_R = 1.6; mean I = 0.613). Markedly, the Robben Island and Jeffreys Bay populations had the highest mean number of private alleles (**Figure 3.4**).

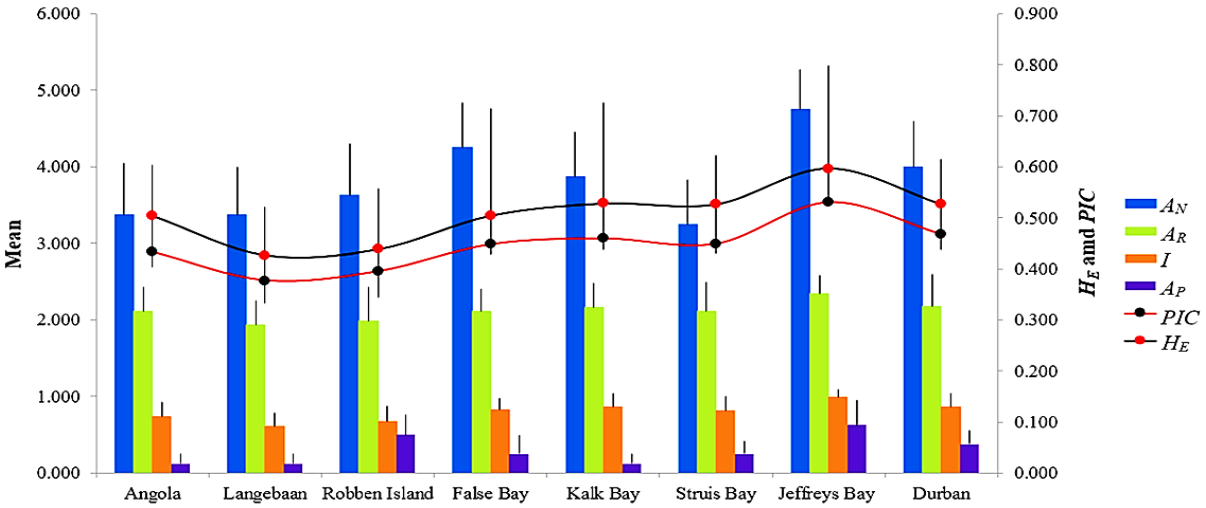

Figure 3.4 Mean genetic diversity estimates using 12 microsatellite loci; number of alleles (A_N), allelic richness (A_R), information (Shannon-Weaver) index (I), number of private alleles (A_P), polymorphic information content (PIC) and heterozygosity (H_E). Error bars represent standard error.

Three of the 66 pairs of loci were in linkage disequilibrium ($P < 0.01$), supporting the presence of allele frequency differentials between populations: *Mh9-Mca25*, *McaB5-McaB6*

and *McaB27-Mca33*. Four out of eight populations (Angola, Langebaan, Robben Island and Struis Bay) showed significantly higher degrees of relatedness (estimates beyond the 95% CI for no difference between populations) than expected from randomly sampled individuals (**Figure 3.5**). Although *r* estimates within most populations were statistically higher than expected, values among populations were not exceedingly high (ranging from -0.106 to 0.359; **Figure 3.5**).

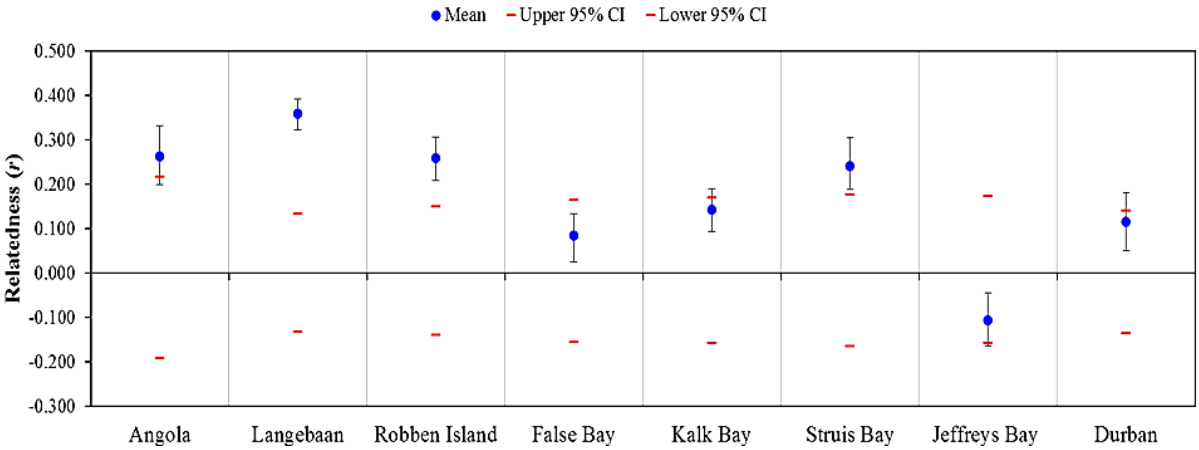

Figure 3.5 Mean within-population pairwise relatedness, *r*, for the study populations. Error bars represent standard error.

Estimates of population genetic structure and demographic history were computed using a subset of markers (eight microsatellites), excluding loci not conforming to Hardy-Weinberg equilibrium, neutrality, and/or exhibiting high null allele frequencies (*Mh2*, *Mh25*, *McaB22* and *McaB39*).

3.3.3. Among-Population Patterns of Genetic Diversity

Pairwise genotypic differentiation as estimated by the exact test indicated highly significant population differentiation amongst almost all populations, with the majority of the *P*-values reaching the 1% significance level (Appendix B: **Table S3.2**). Pairwise F_{ST} estimates, varying from 0.007 to 0.296, were congruent with the results obtained for the exact test for genotypic differentiation, indicating moderate-to-high genetic differentiation amongst most populations ($P < 0.05$, **Table 3.1**). Noticeably, F_{ST} estimates were large for almost all Atlantic *vs.* Indian Ocean comparisons, with F_{ST}-values reaching 0.296. Hierarchical AMOVA for the three geographic clusters (Angola *vs.* South-East Atlantic Ocean populations *vs.* South-West Indian Ocean populations) supported regional population genetic structure, with significant differentiation amongst regions ($F_{CT} = 0.084$, $P < 0.01$), within regions ($F_{SC} = 0.055$, $P < 0.01$) and over all regions and populations ($F_{ST} = 0.134$, $P < 0.01$) (**Figure 3.6a**).

Hierarchical AMOVA for the oceanic clusters (Angola+South-East Atlantic Ocean populations *vs.* South-West Indian Ocean populations) also indicated the separation of the oceans, with significant differentiation amongst oceans (F_{CT} = 0.097, P < 0.01), within oceans (F_{SC} = 0.071, P < 0.01) and over all oceans and populations (F_{ST} = 0.161, P < 0.01) (**Figure 3.6b**). Factorial Correspondence Analysis (FCA) as depicted in **Figure 3.7** clearly revealed the same interoceanic pattern of population genetic structure.

Table 3.1 Pairwise F_{ST}-values among populations with P-values shown above diagonal

	F_{ST}							
	A	**LL**	**RI**	**FB**	**KB**	**SB**	**JB**	**D**
Angola (A)		0.001**	0.001**	0.003**	0.001**	0.001**	0.001**	0.001**
Langebaan (LL)	0.072		0.191	0.005**	0.001**	0.001**	0.001**	0.001**
Robben Island (RI)	0.090	0.007		0.003**	0.001**	0.001**	0.001**	0.001**
False Bay (FB)	0.053	0.033	0.043		0.007**	0.001**	0.002**	0.001**
Kalk Bay (KB)	0.103	0.150	0.172	0.039		0.001**	0.001**	0.001**
Struis Bay (SB)	0.200	0.207	0.175	0.201	0.296		0.001**	0.001**
Jeffreys Bay (JB)	0.078	0.110	0.088	0.045	0.075	0.134		0.001**
Durban (D)	0.180	0.171	0.145	0.129	0.210	0.132	0.082	

*Statistical significance at the 5% nominal level and ** statistical significance at the 1% nominal level.

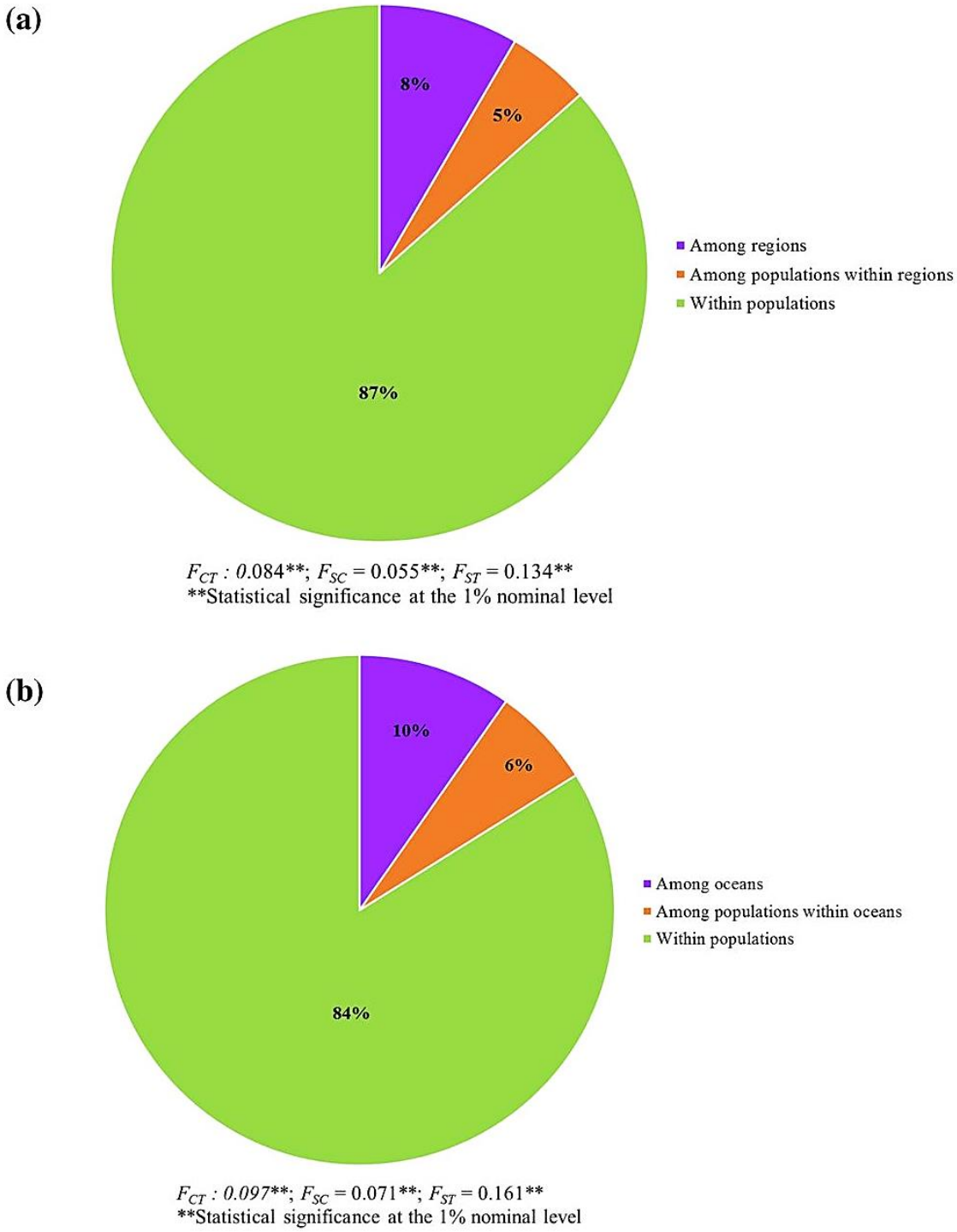

Figure 3.6 Locus by locus AMOVA results with populations clustered (a) in three geographic groups, Angola *vs*. Atlantic Ocean populations *vs.* Indian Ocean populations and (b) two oceainc groups, Angola+Atlantic Ocean populations *vs.* Indian Ocean populations (**significance at the 1% nominal level).

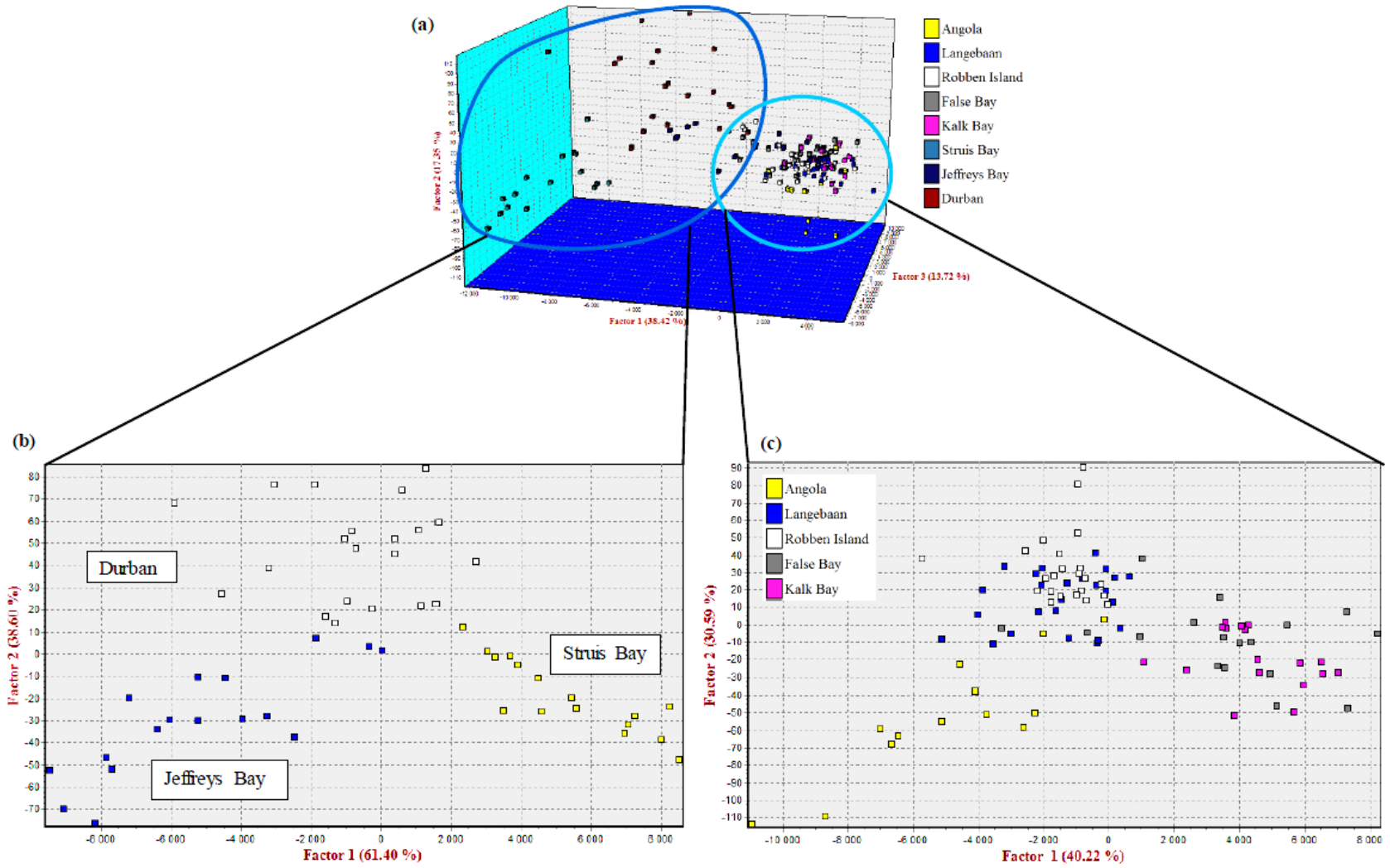

Figure 3.7 Factorial correspondence analysis plots. (a) Eight *Mustelus mustelus* populations grouped into Indian- and Atlantic Ocean. Heterogeneity within (b) Indian Ocean and (c) Atlantic Ocean along factor 1 and 2.

When considering the entire dataset, there was no significant correlation between the genetic- and geographic distances (isolation by distance; IBD) at microsatellite loci (r^2 = 0.0025; P = 0.584; **Figure 3.8a).** Excluding the peripheral population of Angola, according to groupings in the AMOVA, did not reveal a significant IBD relationship either **(**r^2 = 0.1152; P = 0.162; **Figure 3.8b**). Furthermore, excluding Angola and Indian Ocean populations did dot not reveal a significant IBD relationship within the South-East Atlantic either (not reported).

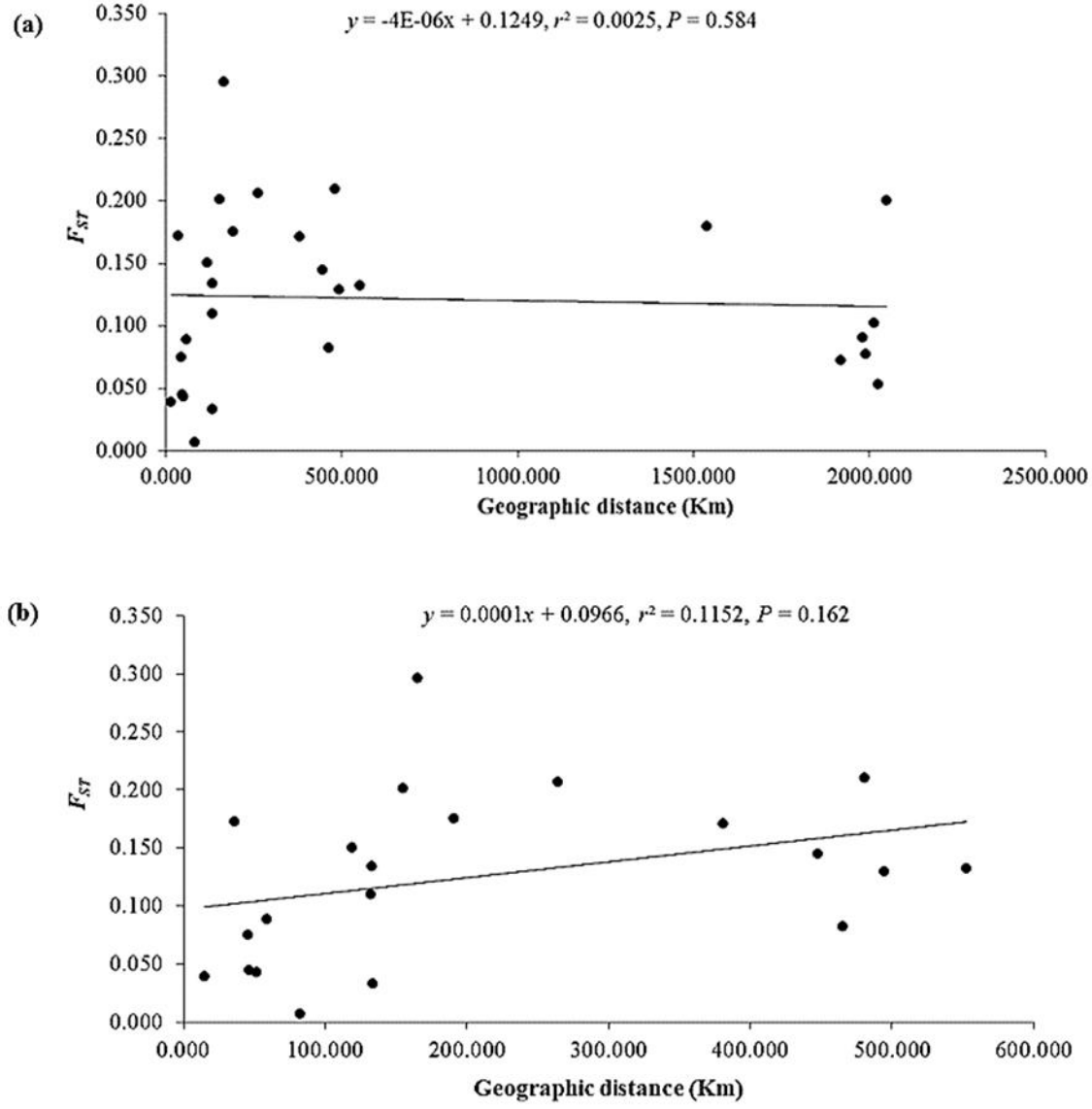

Figure 3.8 Isolation by distance scatterplots with (a) all sampling locations and (b) excluding samples from Angola.

Bayesian clustering analysis in STRUCTURE also indicated population subdivision and clustering of the South-West Indian Ocean populations separate from the rest; identifying the most likely number of populations as K = 2, based on the Evanno *et al*. (2005) method (ΔK statistic), implemented in STRUCTURE HARVESTER (**Figure 3.9a and b**). Additionally, K

= 3 is also shown (**Figure 3.9c and d**) to demonstrate clinal variation; gradual differences in allele frequencies along the southern African coastline.

Finally, limited gene flow between the Atlantic- and Indian Ocean is clearly illustrated in the phylogram based on the between-population genetic distances D_A (Nei *et al*. 1983; **Figure 3.10**), where the South-East Atlantic Ocean sampling sites grouped in two clades and the South-West Indian Ocean sampling sites in a separate clade. An R^2 value of 0.953 as estimated in TREEFIT indicates that this phylogram accurately represents the genetic distance reflected in the data set with a high degree of confidence.

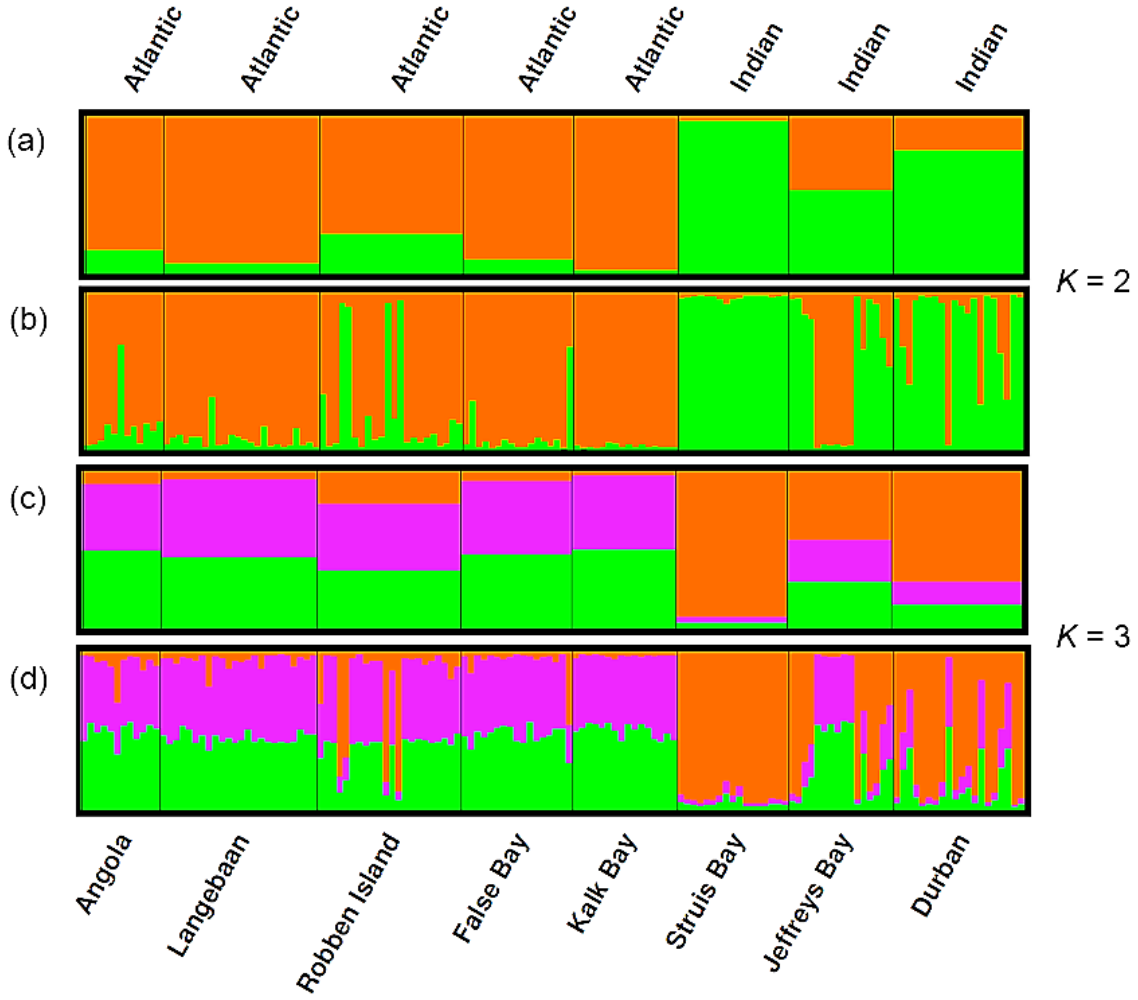

Figure 3.9 Genetic structure of *Mustelus mustelus* populations based on Bayesian clustering analyses (STRUCTURE). The number of populations (a) K = 2, population Q-matrix; (b) K = 2, individual Q-matrix; (c) K = 3, population Q-matrix and (d) K = 3, individual Q-matrix, are shown.

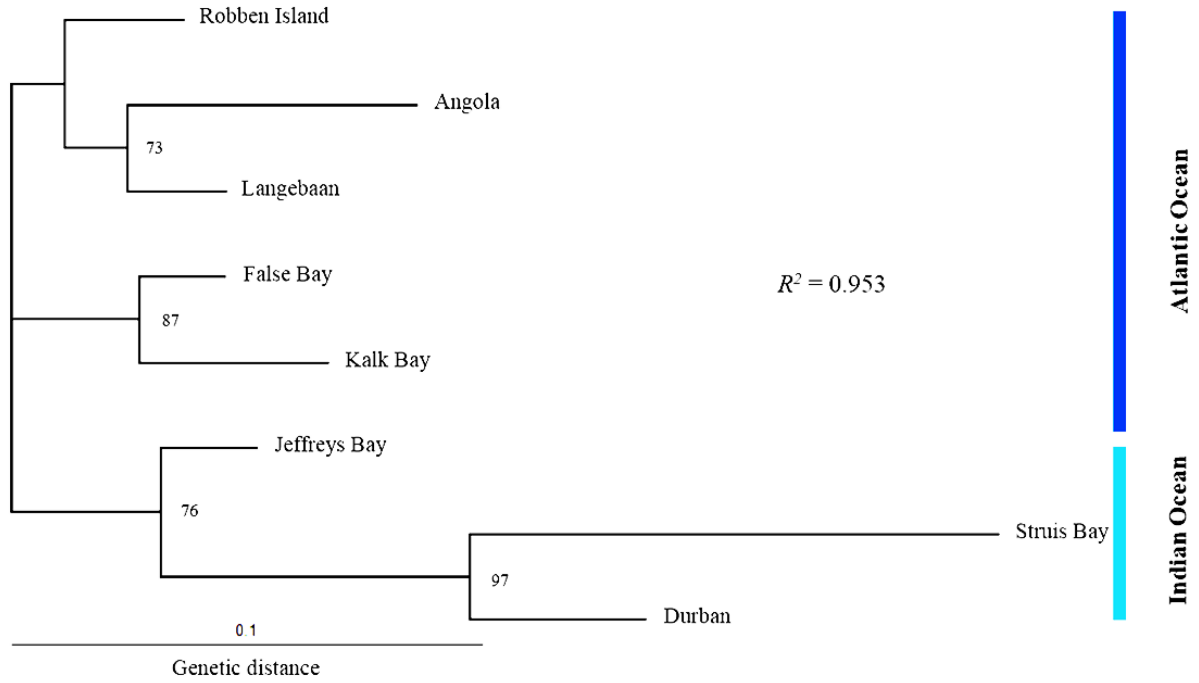

Figure 3.10 Neighbour-joining phylogram based on D_A genetic distances, demonstrating the genetic relationships between Atlantic- and Indian Ocean *Mustelus mustelus* populations in southern Africa. The numbers next to the nodes indicate the bootstrap values (percentage) obtained after 1000 replicates. Only values > 50% are shown.

3.3.4. Demographic History

Estimates of the contemporary N_E as estimated using the linkage LD method indicated very low effective population sizes in most populations (**Table 3.2**). Given that there was no significant IBD relationship and non-significant population differentiation between population pairs Langebaan-Robben Island and False Bay-Kalk Bay, a combined N_E was also estimated. The combined N_E for Langebaan and Robben Island was 17.2 [3.9-88.3], and for False Bay and Kalk Bay was 97.6 [17.8-∞]. There was no significant heterozygosity excess in any population under all three mutation models (TPM, SMM and IAM: Wilcoxon signed-rank test, $P > 0.05$). Across all the study populations (excluding Struis Bay), there was evidence for a recent demographic expansion event as these demonstrated a significant heterozygote deficiency (Wilcoxon signed-rank test, SMM and/or TPM: $P < 0.05$). However, values of the *g*-test ranged from 0.20 to 5.92 and none of these estimates indicated significant population expansion when compared tothe fifth percentile rejection values (≤ 0.19 and ≤ 0.16, respectively) appropriate for the number of loci and sample sizes (10 and 20, respectively) given in Reich *et al*. (1999; their Table 1).

The maximum likelihood estimates (MLE) showed that genetic diversity in southern African populations, as measured by mutation-scaled effective population size (θ), was highest in the South-West Indian Ocean (θ = 1.791) and lowest in the South-East Atlantic Ocean (θ = 0.178). The most probable estimates of migration rates (M) ranged from 13.025 to 40.762, with the highest migration observed from Angola to the South-East Atlantic Oceanic region (M = 40.762). In contrast, estimates of migration rates between the South-West Indian Ocean and the South-East Atlantic Ocean (M = 13.025) were lower, providing further evidence for interoceanic population genetic structure due to low gene flow. The migration between oceans was asymmetrical, as is shown by the number of migrants (N_m) in **Table 3.2** and **Figure 3.11**.

Table 3.2 N_E estimates amongst the study populations based on three methods, linkage disequilibrium, heterozygosity excess and the *g*-test. Combined N_E (LL and RI; FB and KB) in shaded area. *NS* = non-significant

	Method and estimates [95% CI]				*g*-test	
Population	Linkage Disequilibrium		Heterozygosity Excess		*g*-ratio	*P*-value
Angola (A)	∞ [8.6-∞]		∞ [6.7-∞]		2.94	*NS*
Langebaan (LL)	192.3 [13.1-∞]	17.2 [3.9-88.3]	∞ [6.8-∞]	∞ [15.8-∞]	5.92	*NS*
Robben Island (RI)	11.6 [2.9-84.4]		∞ [8.8-∞]		2.52	*NS*
False Bay (FB)	∞ [49.0-∞]	97.6 [17.8-∞]	∞ [7.7-∞]	∞ [5.2-∞]	3.60	*NS*
Kalk Bay (KB)	∞ [15.3-∞]		∞ [2.9-∞]		4.22	*NS*
Struis Bay (SB)	1.4 [0.9-2.3]		∞ [2.2-∞]		0.61	*NS*
Jeffreys Bay (JB)	24.2 [8.7-∞]		∞ [8.1-∞]		4.48	*NS*
Durban (D)	18.1 [5.4-∞]		∞ [∞-∞]		0.20	*NS*

Table 3.3 Mutation-scaled effective population size ($\theta = 4N_E\mu$) and migration rates (M) across Angola (A), the Atlantic- and Indian Ocean (AO and IO, respectively)

	Mean [95% CIs]
θ_A	2.287 [2.060-2.550]
θ_{AO}	0.178 [0.170-0.186]
θ_{IO}	1.791 [1.665-1.930]
$M_{AO > A}$	15.015 [14.236-15.820]
$M_{A > AO}$	40.762 [38.552-43.054]
$M_{IO > AO}$	23.295 [21.639-25.033]
$M_{AO > IO}$	13.025 [12.189-13.898]

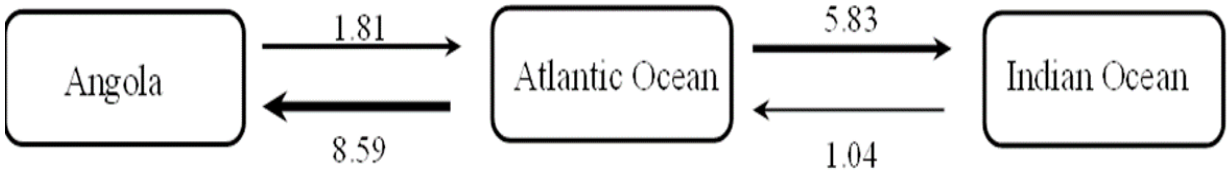

Figure 3.11 Number of migrants per generation ($N_m = M\theta/4$) between different oceanic regional *Mustelus mustelus* populations in southern Africa.

3.4 Discussion

3.4.1. Species Identification

Misidentification of shark species is a common phenomenon in fisheries operations, so much so that landings are reported at a species- or family group level (*e.g.* houndsharks, carcharinids, hammerheads *etc*.). This arises from the difficulties involved with unambiguously identifying species within and across families, *e.g.*, houndsharks (*Mustelus mustelus*, *M. palumbes* and *Galeorhinus galeus*), due to a high degree of conserved interspecific morphology (Heemstra 1973; Farrell *et al*. 2009; Smale and Compagno 1997; Da Silva and Bürgener 2007). Apart from conserved interspecific morphology, misidentification of these sharks is further compounded by linguistic (ethnic) differences in understanding or interpreting common names, which was also apparent in the current study.

In total, 14 sharks were misidentified (*i.e.* 91.1% observer accuracy) in this study, six of which were identified as tope shark and eight as the whitespotted smoothhound shark (*M. palumbes*). This occurred despite the species identification method based on morphological and anatomical traits developed by Da Silva (2007). This highlights the necessity for using

multidisciplinary approaches in confirming identification of shark species (*e.g*. Giresi *et al*. 2013; Pérez-Jiménez *et al*. 2013). Genetic identification methods using mitochondrial fragments *e.g*., *COI* (Ward *et al*. 2005, 2008; Caballero *et al*. 2012) and *ND2* (Farrell *et al*. 2009; Naylor *et al*. 2012) have been proposed and prove to be effective. Genetic identification based on allelic distributions of microsatellites has only been tested to a limited degree, but has proven to be successful in individual identification of great white sharks *Carcharodon carcharias* (Gubili *et al*. 2009) and species identification for some of the elasmobranch species included in Chapter 2.

3.4.2. Genetic Diversity

Levels of genetic variation in sharks, as represented by the number of alleles per locus, allelic richness and/or expected heterozygosity, hase previously been reported to be low compared to other fishes (Schmidt *et al*. 2009; Karl *et al*. 2011; Dudgeon *et al*. 2012; Portnoy and Heist 2012). For instance, low genetic diversity was detected in the sandbar shark (*Carcharhinus plumbeus*) and a large number of microsatellites had to be screened in order to obtain enough informative markers to test for population differentiation (Heist and Gold 1999*a*). Some studies have, however, reported moderate (mean $4 \leq A_N \leq 9$; mean $0.400 \leq H_E \leq 0.600$) to high (mean $10 \leq A_N \leq 20$; mean $0.650 \leq H_E \leq 0.900$) levels of genetic diversity in sharks *e.g*., the whale shark (*Rhincodon typus*; Schmidt *et al*. 2009; Vignaud *et al*. 2014), the spiny dogfish (*Squalus acanthias*, Veríssimo *et al*. 2010) and the bull shark (*Carcharhinus leucas*, Karl *et al*. 2011). In the present study, moderate levels of genetic diversity characterised all *M. mustelus* populations, similar to those described for other shark species (Ahonen *et al*. 2009; Ovenden *et al*. 2009; Schmidt *et al*. 2009; Vignaud *et al*. 2014). Moderate levels of genetic diversity may be explained by the moderate mean relatedness found across populations and significant in half the study populations, which was lower than that of expected full-sib relationship (r = 0.5; Queller and Goodnight 1989). Apart from relatedness, other factors, such as the life history strategy and reproductive mode of a species exert strong influences in shaping genetic diversity (Dudgeon *et al*. 2012; Portnoy and Heist 2012). The moderate level of relatedness is also exhibited by the FCA plot, where the genotypic distribution of individuals within each respective population with significant mean relatedness were tightly clustered, as well as by the AMOVA results, where a large percentage variation was within populations.

3.4.3. Interoceanic Population Genetic Structure

In combination, the pairwise F_{ST} estimates and the exact test for genotypic differentiation (ETGD) can provide valuable information about the genetic connectivity of populations. The exact test for genotypic differentiation is considered a more sensitive test for population differentiation if unique alleles persist in said populations (Balloux and Lugon-Moulin 2002; Holsinger and Weir 2009), as was the case for the current populations (particularly Robben Island and Jeffreys Bay, **Figure 3.4**). The present study suggests that regional common smoothhound sharks consist of two genetically differentiated populations, with low levels of gene flow between the South-East Atlantic and South-West Indian Ocean populations. This is also congruent with the known biogeographic boundaries *e.g*., the Cape Agulhas Boundary at the south-west coast, the Atlantic/Indian Ocean phylogeographical break in south-western Africa that coincides with the biogeographic disjunction between the cool temperate and warm-temperate biotas (westernmost – Cape Point, easternmost – Cape Agulhas; Teske *et al*. 2011) and obstructs gene flow and migration of many marine species (Duncan *et al*. 2006; Daly-Engel *et al*. 2012; Teske *et al*. 2014). The Angolan samples are most probably separated from the South African populations by the Angola-Benguela Front, resulting in a barrier to dispersal. The southward flow of the Angola Current along the narrow shelf of Angola is an extension of the south equatorial counter-current that forms the northern boundary of the Benguela Current (Hutching *et al*. 2009). The South-East Atlantic Ocean sampling sites are again separated from the South-West Indian Ocean by the Atlantic/Indian Ocean phylogeographical break, which has been suggested to impact the genetic connectivity of the scalloped hammerhead shark (*Sphyrna lewini*, Duncan *et al*. 2006; Daly-Engel *et al*. 2012). The retroflection of the Agulhas Current is proposed to act as a biogeographic barrier for the small coastal shark species *M. mustelus*, explaining the high degree of population genetic structure detected amongst the South-East Atlantic and South-West Indian Ocean sampling sites in this study. It has been demonstrated that strong population genetic structure in sharks may be linked to adaptation to ecological niches or other environmental conditions (e.g., salinity and temperature) (Schultz *et al*. 2008; Chabot and Allen 2009; Pereyra *et al*. 2010; Veríssimo *et al*. 2010, 2011; Benavides *et al*. 2011*a*, *b*; Mourier *et al*. 2013; Mendonça *et al*. 2013; Vignaud *et al*. 2013, 2014) and, therefore, such separated populations may represent distinct evolutionary units (Mendonça *et al*. 2013; Vignaud *et al*. 2014).

F_{ST} estimates and ETGD indicated significant population genetic differentiation amongst all population pairs, except for Langebaan-Robben Island and False Bay-Kalk Bay (**Table**

3.1), and this corroborates the two distinct clusters obtained with the FCA and STRUCTURE (**Figure 7a** and **Figure 9**, respectively) analyses. To better understand the patterns of gene flow within oceans, FCA plots were drawn for each individual ocean group since the high intra-ocean genetic variation of the Indian Ocean populations could bias results. Intraoceanic population genetic structure was also detected, indicating the presence of less prominent biogeographical barriers within the two areas (see **Figure 7b and c**).

Based on intraoceanic population genetic structure, the non-significant Mantel test of IBD and limited dispersal potential exhibited by the common smoothhound shark, the Cape Agulhas Boundary (thermal and/or biogeographic) is proposed as the barrier restricting gene flow amongst oceans. Further sampling along the east coast will assist in validating an additional barrier potentially located at Algoa Bay. In other shark species, similar barriers have been shown to limit gene flow, for instance, hydrodynamic barriers (open oceanic waters; Feldheim *et al*. 2001; Duncan *et al*. 2006; Schultz *et al*. 2008), thermal barriers (water temperature; Chabot and Allen 2009) and biogeographic barriers (disjunction in continental coastlines; Vignaud *et al*. 2013). Evidence for interoceanic and intraoceanic population genetic structure was further demonstrated by the AMOVA, with significant moderate differentiation on all hierarchical/grouping levels (amongst regions/oceans and over all regions/ oceans and populations). The Bayesian results strongly indicated restricted gene flow between the Atlantic and Indian Ocean populations, adding further support to the interoceanic structure hypothesised for *M. mustelus*. Using the D_A genetic distances, three clades congruent with the proposed biogeographic barriers between Atlantic and Indian Ocean were identified for *M. mustelus* populations. These barriers include the major Cape Agulhas Boundary.

Previous studies on sharks have shown that gene flow is largely male-mediated, with females displaying limited dispersal due to site fidelity (Pardini *et al*. 2001; Schrey and Heist 2003; Portnoy *et al*. 2010; Daly-Engel *et al*. 2012). Even though the sex ratio is slightly skewed towards females in the present study, the low yet significant levels of population differentiation within the Atlantic Ocean populations are indicative that for common smoothhound shark, male-mediated gene flow does occur, at least between the South-East Atlantic populations. Using mitochondrial DNA and an equal sex ratio of males to females will in future contribute to further understanding the dispersal patterns in the species.

Overall, the population genetic structure results suggest that the barrier at Cape Agulhas may impact to an extent genetic connectivity of common smoothhound shark populations since the Benguela Current restricts mixing of Atlantic and Indian Ocean populations along

western South Africa (Briggs 1995; Briggs and Bowen 2012). Some level of clinal variation was observed along the South African coast; however, future studies including more sampling sites could focus on achieving greater resolution in order to test the hypothesis that the observed clinal variation is a consequence of adaptation along an environmental gradient.

3.4.4. Demographic History

Effective population size N_E is a critical parameter in conservation genetics since it influences the rate of loss of genetic diversity in finite populations (Wright 1931; Saarinen *et al*. 2009). Contemporary estimates of N_E were estimated with the linkage disequilibrium (LD) test and heterozygosity excess method. The estimates of N_E based on the linkage disequilibrium method may be more reliable as this method corrects for bias associated with small sample size and heterozygosity excess due to a possible bottleneck event (Waples and Do 2010). Contemporary estimates of N_E based on the linkage disequilibrium method were mostly small (< 100) and below the theoretical critical minimum (> 1000) that is considered necessary to maintain genetic diversity and avoid the accumulation of deleterious allele (Frankham *et al*. 2003; Palstra and Ruzzante 2008; Waples and Do 2010). Franklin (1980) stated that inbreeding depression may occur if the N_E falls below 50, therefore, this raises concerns over the conservation status of common smoothhound shark in South Africa. Since Robben Island was not genetically differentiated from Langebaan, a combined N_e was estimated (N_E = 17) and was below the theoretical critical minimum. Furthermore, the Wilcoxon sign-rank test did not detect any genetic bottlenecks, but indicated a recent population expansion event (founder effect) for all study populations except Struis Bay. Recent bottlenecks are known to sometimes go undetected by demographic history inference as the loss of allelic diversity is not accompanied by an immediate decrease in heterozygosity in some instances, but by a transient heterozygosity excess (Cornuet and Luikart 1996; Luikart and Cornuet 1998). Further investigation of the detected population expansion using the interlocus *g*-tests did not provide support for population expansion and this may be due to an insufficient number of loci tested (usually n = 15 or 20). Additionally, Reich *et al*. (1999) also reported that, when using the interlocus *g*-tests, the use of more loci increased the power to reject size constancy. On the contrary, the Wilcoxon sign-rank test performs better than other methods when the number of loci is low (Luikart *et al*. 1998).

The recent population expansion signal detected with the Wilcoxon sign-rank test could likely coincide with the early Holocene (approximately 7 000-11 000 years ago) when sea

levels were elevated, increasing suitable coastal habitats for various marine organisms, including common smoothhound sharks (Miller *et al*. 1995; Ramsay 1995; Carr *et al*. 2010). An expansion in common smoothhound shark following the last glacial maximum (LGM; 18 000-23 000 years ago) is highly probable, considering that warming after the LGM caused population expansions in many marine and terrestrial organisms (Peltier 1988; Miller *et al*. 1995; Carr *et al*. 2010; Teske *et al*. 2011). This theorised timing of population expansion should in future be tested using mitochondrial DNA, despite the absence of a well-calibrated molecular clock for *M. mustelus*.

In shark species, migration rate estimates are rare and for the common smoothhound shark the migration rates estimated in this study were low and most likely facilitated by oceanic currents *e.g*., strong northward flow on the west coast of the Benguela Current (**Table 3.3**). The low levels of asymmetric gene flow occurring between oceanic regions coincide with the strong population genetic structure observed and the known influence of N_m on adaptive divergence among populations (*e.g*. Hendry *et al*. 2001). Likewise, non-random individual dispersal, such as sex-biased dispersal, can also impinge on gene flow patterns among populations by affecting rates of dispersal from source populations (Aars and Ims 2000; Portnoy *et al*. 2010; Daly-Engel *et al*. 2012). Interestingly, in rig smoothhound sharks (*M. lenticulatus*), a dispersal distance greater than 1 100 km was reported by Francis (1988), using a conventional tagging method. It is proposed that common smoothhound shark males may indeed be capable of dispersing over wider geographic areas than previously thought (*e.g*., South Africa to Angola ~1750 km, **Figure 3.11**). In future, migration rate estimates will prove useful when corroborated with tagging or telemetry data for the species. Effective population size estimates and founder effects are probably reflective, albeit speculative, of the intense fishing pressures experienced by the species and stress the need for appropriate conservation and management strategies for common smoothhound sharks in South Africa.

3.5 Conclusion

Microsatellite data indicate that modern populations of the common smoothhound shark (*Mustelus mustelus*) in South Africa have moderate levels of genetic diversity based on the observed number of alleles, allelic richness and expected heterozygosity. The results of the FCA, AMOVA and Bayesian clustering revealed pronounced interoceanic population differentiation. The discontinuity of the continental shelf at Cape Agulhas is regarded as a

substantial barrier to gene flow, limiting contact between common smoothhound shark populations from the Atlantic and Indian Oceans. Although finding an expanding common smoothhound shark population, a contemporary genetic bottleneck may have gone undetected as the effective population size was very low across populations. The use of mitochondrial DNA markers, in addition to microsatellites, may provide further insight into the mechanisms responsible for the observed population genetic structure of common smoothhound sharks, as well as elucidating the genetic divergence of the Indian Ocean populations. The observed significant decline in biomass of common smoothhound sharks in South Africa, together with the low effective population sizes, differential dispersal patterns and strong genetic partitioning amongst oceans, raises concerns about the conservation status of this species.

Chapter 4

Elucidating Genetic Divergence of *Mustelus mustelus* Across the Indian/Atlantic Boundary

Abstract

Sharks commonly have wide distribution ranges covering areas with different seascape features such as coastal, pelagic or benthic regions in tropical or temperate areas. As a consequence intraspecific populations may be present as divergent and even distinct genetic units. For long-term conservation purposes, it is therefore critical that evolutionary significant units (ESUs) are identified for exploited shark species. This is especially important in cases where species are restricted in distribution, have small population sizes and are subject to human induced mortality, which is the case for the common smoothhound shark *Mustelus mustelus*. In this chapter, the mitochondrial *ND4* region was analysed to investigate whether historically this species is also divided into evolutionary lineages that coincide with the recommended management units of Chapter 3. Low levels of divergence and less structure was observed than with the microsatellite data and this mitonuclear discordance can be explained by a number of different scenarios. These include the inherent differences in marker properties such as large effective population size of the nuclear DNA relative to mitochondrial DNA as well as selection, sex-biased dispersal and historical demographics such as range expansion. A significant and likely recent population expansion was detected and postulated to have occurred during the warm interglacial periods in the late Pleistocene to early Holocene. Despite the indication of an expanding common smoothhound shark population, a contemporary genetic bottleneck may have gone undetected as genetic divergence was very low in some of the study populations. Historical demographics such as range expansion and contemporary restriction to gene flow are proposed as the most likely forces explaining structure in the case of common smoothhound.

4.1 Introduction

Sharks are generally characterised by wide distribution ranges, often covering areas with different ecological features, such as coastal, pelagic or benthic regions in tropical or temperate areas (Dudgeon *et al*. 2012). As a consequence, intraspecific populations may be present as divergent and even distinct genetic units (Mendonça *et al*. 2013). In a marine environment, levels of gene flow are supposedly dictated by the dispersal ability of a species (Ovenden 2013), which may be affected by the oceanic surroundings through the influence of climate, hydrodynamics, and thermal and biogeographic barriers (Veríssimo *et al.* 2010). Typically, shark species that exhibit a high dispersal potential have little variation across populations, with shallow genetic structure over vast regions (Heist 2004*b*). For instance, low levels of population genetic structure among populations were reported for highly migratory species, with population genetic structure being detected only between ocean basins, *e.g*., in the basking shark (*Cetorhinus maximus*, Hoelzel *et al*. 2006) and whale shark (*Rhincodon typus*, Castro *et al*. 2007). In contrast, higher levels of population genetic structure across similar spatial scales have been reported for demersal or benthic shark species with low dispersal potential and disjunct geographic ranges *e.g*., zebra shark (*Stegostoma fasciatum*, Dudgeon *et al*. 2009) and Caribbean sharpnose shark (*Rhizoprionodon porosus*, Mendonça *et al*. 2013).

Barriers to gene flow, affecting patterns of dispersal, have been recognised for several shark species. The open oceanic waters appear to be a barrier to gene flow (hydrodynamic barrier) in species such as the scalloped hammerhead shark (*Sphyrna lewini*) and the lemon shark (*Negaprion brevirostris*) (Feldheim *et al*. 2001; Duncan *et al*. 2006; Schultz *et al*. 2008), whereas warm equatorial waters also function as a barrier to gene flow (thermal barrier) in temperate species such as the tope shark (*Galeorhinus galeus*) (Chabot and Allen 2009).

A shift in geographical distribution for various species is facilitated by the influence of climate change on oceanic currents, which, in turn, affects the patterns of spatial genetic diversity through genetic bottleneck events (O'Brien *et al*. 2013). Genetic bottleneck effects occur when a population experiences a severe reduction in effective population size (Avise 1994). During a genetic bottleneck event, genetic drift reduces genetic variation within, and increases genetic divergence among populations (François and Durand 2010). A founder effect is a bottleneck event associated with the founding of a new population, possibly followed by population expansion (François *et al*. 2010). Population expansion has previously been described in other *Mustelus* species with similar distribution patterns. In *M.*

antarcticus, *M. lenticulatus* and *M. schmitti*, for example, it is proposed that expansions occurred during the Pleistocene epoch (approximately 2.6 million to 11 700 years ago) in the central Indo-Pacific and off Australia, and in the South-West Atlantic Ocean, based on molecular data (Pereyra *et al*. 2010; Boomer *et al*. 2012). Understanding historical demographical processes and resultant patterns of population genetic structure is important for attaining information critical to the identification of Management Units (MUs), defined as demographically-distinct populations that should be managed separately to ensure viability of the larger meta-population (Funk *et al*. 2012). From an evolutionary perspective, it is also critical to further identify relevant biological units to be conserved, such as a group of historically-isolated populations with unique genealogical and adaptive legacies *i.e*., evolutionary significant units (ESUs) (Funk *et al*. 2012). This is especially important in cases where populations are restricted in distribution, have small population sizes and are subject to human induced mortality, which is the case for the common smoothhound shark, *Mustelus mustelus*.

The common smoothhound shark is of considerable socio-economic importance in southern Africa, even though the species has relatively low reproductive capacity (Da Silva and Bürgener 2007; Department of Agriculture, Forestry and Fisheries 2013). This is a small, coastal shark species, which has limited dispersal ability (Smale and Compagno 1997; Da Silva *et al*. 2013), probably due to their complex interactions with coastal environments, such as the use of nursery areas, which in itself lends to a theoretically complex evolutionary history of the species. In Chapter 3, microsatellite markers detected shallow population genetic structure within the South-East Atlantic and South-West Indian Ocean, and strong interoceanic population genetic structure (Chapter 3). Additionally, significant evidence of population expansion was found based on the Wilcoxon sign-rank test. Accordingly, it is postulated that the common smoothhound shark is genetically structured into at least two populations which have undergone a recent demographic and geographic population event. The goal of this chapter was to confirm the observed patterns of the microsatellite data and determine whether, historically, this species is also divided into evolutionary significant units that coincide with the recommended management units of Chapter 3, using sequence data from on the mitochondrial *ND4* region.

4.2 Materials and Methods

4.2.1 Sample collection, DNA Sequencing and Alignment

A total of 78 individuals were randomly selected across the eight populations used in the microsatellite analysis of the previous chapter. A portion (approximately 800 bp) of the mitochondrial gene *nicotinamide adenine dehydrogenase subunit 4* (*ND4*) was targeted for population genetic structure inference, and polymerase chain reaction (PCR) was conducted using primers *MaND4F* (5′-ACC MAA AGC YCA CGT WGA AGC-3′) and *MaND4R* (5′-TCT TGC TTG GAG TTG CAC CA-3′) according to the recommended PCR conditions outlined in Boomer *et al*. (2010). The PCR amplicons were bi-directionally sequenced using standard Sanger sequencing chemistry (BigDye® terminator v3.1 cycle sequencing kit, *Life Technologies*) and sent to the Stellenbosch University Central Analytical Facility (DNA sequencing unit) for capillary electrophoresis. Sequences were aligned using the CLUSTALW algorithm (Thompson *et al*. 1994), implemented in MEGA v5.2 (Tamura *et al*. 2011), manually corrected and trimmed to equal lengths. A local BLASTn search of these sequences was first conducted to confirm species identification using GENBANK and unique haplotypes were subsequently identified in ARLEQUIN v3.5 (Excoffier and Lischer 2010).

4.2.2 ND4 Sequence Analysis

To display the evolutionary relationship among haplotypes, maximum-parsimony haplotype networks (Polzin and Daneshmand 2003) were constructed using the median joining algorithm (Bandelt *et al*. 1999) with default parameters in the software NETWORK v4.6.1.2 (http://www.fluxus-engineering.com). The Hasegawa-Kishino-Yano (HKY) (Hasegawa *et al*. 1985) nucleotide substitution model fitted the current data best, as determined by the Maximum Likelihood test implemented in JMODELTEST SERVER 2 (accessed at http://jmodeltest.org/user), with models ranked using Akaike and Bayesian Information Criteria (AIC and BIC) with correction for small sample size (Darriba *et al*. 2012). Phylogenetic relationships among haplotypes were inferred using the maximum likelihood method, based on the HKY model (Hasegawa *et al*. 1985), and phylograms generated in MEGA with 1 000 bootstrap replicates. Furthermore, to assess the ability of the *ND4* region to differentiate between different hounds sharks, the phylogram was rooted with five morphologically-similar species as outgroups (*Galeorhinus galeus*, *M. asterias*, *M. mosis*, *M. schmitti* and *Triakis megalopterus*).

4.2.3 Molecular Diversity and Spatial Patterns of Genetic Differentiation

Using DNASP v5.10.1 (Librado and Rozas 2009), the following genetic diversity parameters were estimated for each population: number of polymorphic sites (S), haplotype diversity (h), nucleotide diversity (π) and number of pairwise nucleotide differences amongst individuals. The degree of genetic differentiation among populations was estimated using pairwise ϕ_{ST} values (with significance determined using 1 000 bootstrapped replicates), calculated in ARLEQUIN. Additionally, using ARLEQUIN, the average regional population pairwise differences were estimated with three calculations: (1) average number of pairwise differences between populations (π_{XY}); (2) average number of pairwise differences within population (π_X and π_Y) and (3) corrected average pairwise difference [π_{XY} - (π_X + π_Y)/2]. Hierarchical population structure was evaluated through an analysis of molecular variance (AMOVA), as implemented in ARLEQUIN; populations were grouped based on geographical origin (Angola, South-East Atlantic or South-West Indian Ocean) to test *a priori* hypothesised hydrodynamic barrier between South Africa and Angola; the Angola-Benguela Front. Additionally hierarchical AMOVA was used to examine *a priori* hypothesised biogeographic barrier across the South-East Atlantic (including Angola) and South-West Indian Ocean; the Cape Agulhas Boundary. The best available model in ARLEQUIN for these analyses was the Tamura-Nei model (Tamura and Nei 1993), as determined by JMODELTEST SERVER. Isolation by distance (IBD) was tested for all sampling locations using a Mantel test (Mantel 1967) implemented via the software GENALEX v6.5 (Peakall and Smouse 2012).

4.2.4 Population Demographics

Demographic analyses were performed in ARLEQUIN using the mtDNA sequence data. Deviations from selective neutrality (or population expansion) were also tested with Tajima's D (Tajima 1989) and Fu's F_s (Fu 1997) (20 000 permutations; α = 0.05 and α = 0.02, respectively), based on an infinite-site model without recombination. These tests assume no selective advantage among haplotypes and that significant mutational unbalance is associated with a recent population expansion event (possibly following a genetic bottleneck). Mismatch distributions (*i.e.* the observed number of differences between pairs of haplotypes) will be multi-modal or ragged for populations at demographic equilibrium (*i.e.* stationary), where the

generation of new mutations is offset by random drift, and uni-modal for expanding populations, where new mutations accumulate faster than their loss due to drift (Harpending 1994). Harpending's raggedness index (H_{RI}) was estimated for each population unit (20 000 permutations) to infer changes in population size based on the frequency of pairwise differences among haplotypes (Schneider and Excoffier 1999). Divergence from an ancestral population size θ_0 at T-generations in the past was estimated over all populations. The value of T, scaled by the mutation rate μ, *i.e.* $\tau = 2\mu T$, was estimated assuming 1) isolation after divergence and 2) constant but unequal populations sizes (Schneider and Excoffier 1999). Calculating the timing of expansions and effective population sizes is highly dependent on the chosen mutation rate (μ). The fossil record for *Mustelus* and sharks in general is relatively poor and mutation rates are unknown for smoothhound sharks. Mutation rates from a variety of shark species (Martin *et al*. 1992; Duncan *et al*. 2006; Keeney and Heist 2006; Schultz *et al*. 2008) have been applied to non-congeneric species to estimate T and effective population size ($\theta = 2N_{ef}\mu$, where N_{ef} is the effective female population size) (*e.g*. Murray *et al*. 2008; Pereyra *et al*. 2010; Veríssimo *et al*. 2010; Karl *et al*. 2011; Boomer *et al*. 2012). For the present study, attempts at dating a population expansion event and estimating N_{ef} were unfortunately not possible in the absence of mutation-rate estimates for *ND4* in elasmobranchs.

4.2.5 Coalescent Estimation of Migration between Oceanic Regions

The magnitude and direction of contemporary gene flow among groupings of the eight populations were estimated applying the coalescent-based approach, using the maximum likelihood inference methods implemented in MIGRATE-N v3.6 (Beerli 1998; Beerli and Felsenstein 1999, 2001; Beerli 2009). MIGRATE-N uses coalescent simulations of genetic data to infer effective population sizes and past migration rates among n populations. It assumes that the migration matrix model is asymmetric and population sizes need not be equal. The study populations were grouped into three regional groups: Angola, South-East Atlantic Ocean (Langebaan, Robben Island, False Bay and Kalk Bay) and South-West Indian Ocean (Struis Bay, Jeffreys Bay and Durban), separated by two putative barriers: a hydrodynamic barrier (Angola-Benguela Front) and a biogeographic barrier (Cape Agulhas Boundary). The stepping-stone migration model with asymmetric rates was applied (Kimura and Weiss 1964). Long-term migration rates (M), $4N_e$ generations in the past, were estimated and all possible combinations of migration that were either asymmetrical or had no dispersal

between populations were evaluated with the likelihood ratio test. Bidirectional migration rates ($M = m/\mu$, where m is the immigration rate per generation) were also estimated. Maximum likelihood analysis was performed with 20 short chains followed by five long chains with a sample increment of 100 for both runs, and the first 15 000 generations were discarded as burn-in at the beginning of each chain. An adaptive heating scheme with four chains and a swapping interval of one was applied. Maximum likelihood estimates were verified with three replicate Markov Chain Monte Carlo (MCMC) simulation runs to ensure the convergence of similar values for θ.

4.3 Results

4.3.1 Haplotype Networks

A 793 bp fragment of the mitochondrial *ND4* locus was successfully amplified and sequenced for 78 common smoothhound sharks and was composed of base frequencies of 29.8% adenine, 30.8% thymine, 26.1% cytosine and 13.4% guanine over all samples. Fifteen haplotypes were identified across the study area. The haplotype network incorporating the eight putative populations was shaped in a distinct starlike pattern, characterised by one central haplotype (CSH2) surrounded by an array of low frequency variants (CSH1; CSH3-CSH15) (**Figure 4.1**). A high degree of haplotype sharing was observed with the most common haplotype (CSH2) shared by 54 individuals across all the study area; two lower frequency haplotypes (CSH4 and CSH9) were shared between two or more locations (**Figure 4.1a**). The haplotype network indicated that Angola samples are characterised by 2 unique haplotypes, CSH1 and CSH3, the latter shared by three individuals and providing evidence for the divergence of this population from South African samples. A phylogenetic tree placed all haplotypes into a single, shallow clade (**Figure 4.2**). The *ND4* region was highly variable among houndshark species and was able to differentiate among the species (**Figure 4.2**).

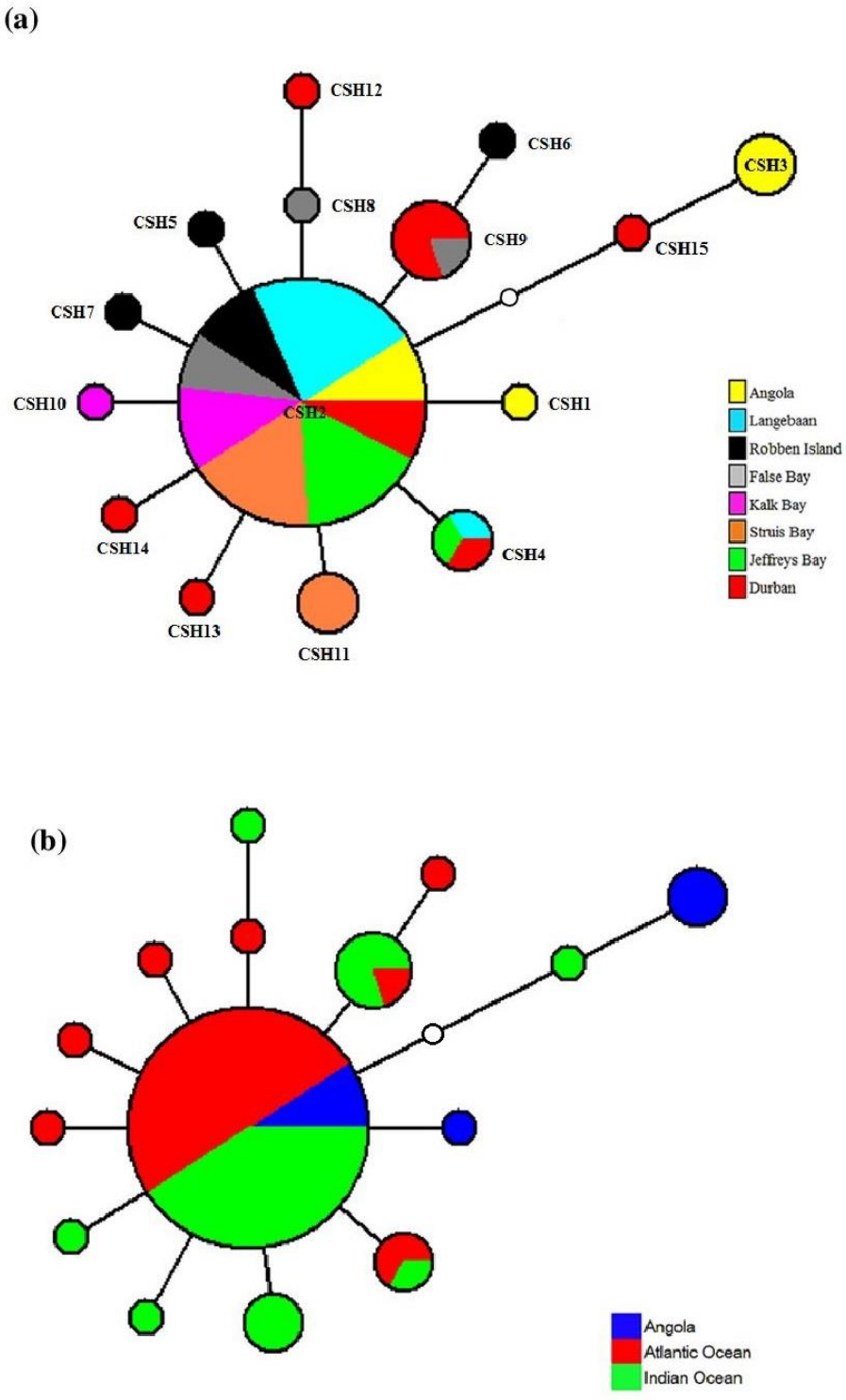

Figure 4.1 Median-joining network of *Mustelus mustelus* mtDNA *ND4* haplotypes (a) shown by sampling site and (b) region/ocean. All haplotypes are separated by one mutation and the *white circle* represents a hypothetical haplotype not sampled in the study. The sizes of the circles are proportional to the frequency of the haplotypes.

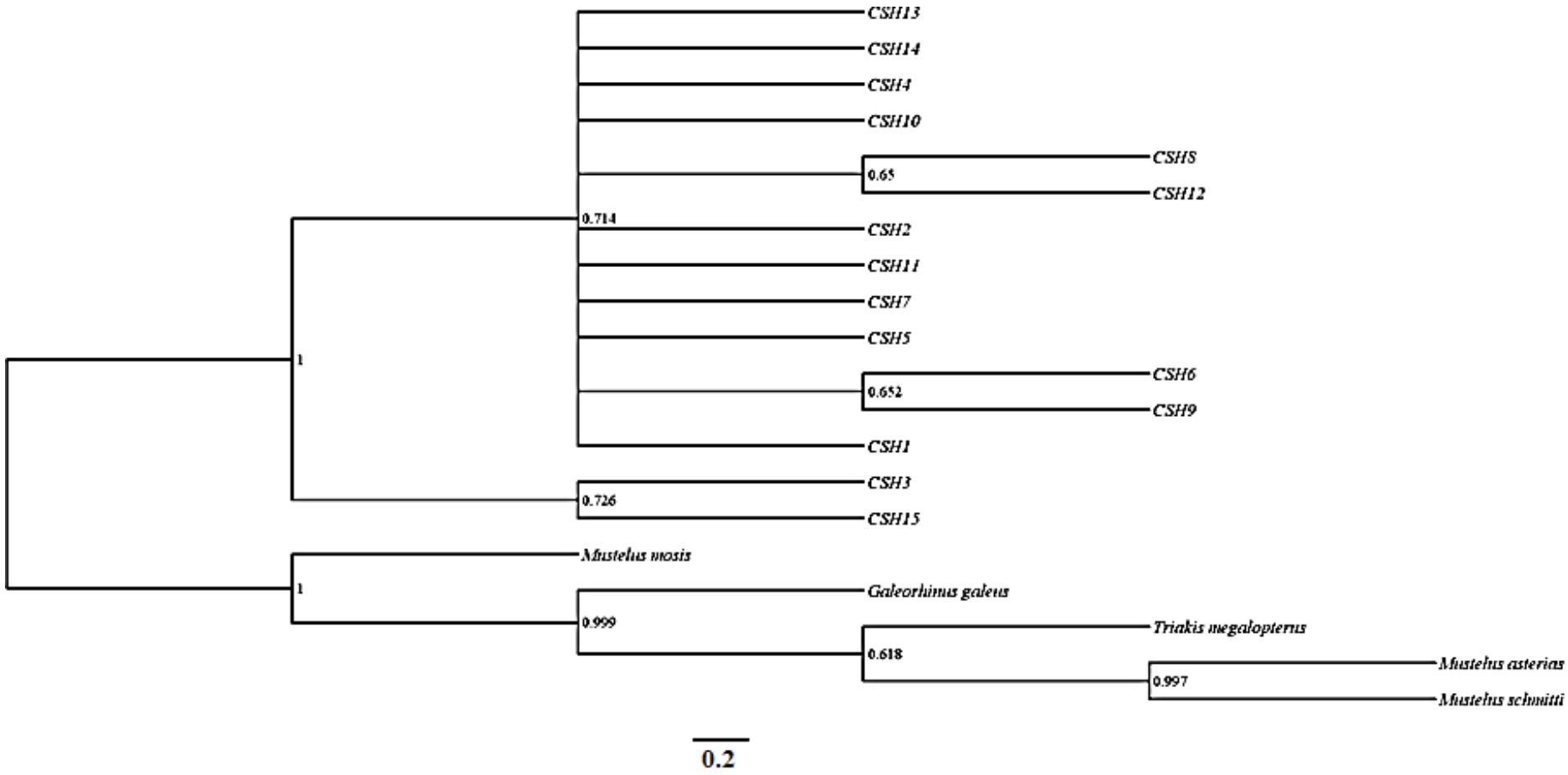

Figure 4.2 Maximum likelihood phylogenetic tree depicting relationships among *Mustelus mustelus* haplotypes. Bootstrap support is displayed where ≥ 60%. The scale represents the proportion of polymorphic sites between haplotypes.

4.3.2 Within-Population Patterns of Genetic Diversity

A total of 15 polymorphic sites (*S*), of which seven were parsimony informative and eight were singleton mutations, characterised 15 haplotypes (**Table 4.1, 4.2, 4.3**). The absolute number of pairwise differences between distinct haplotypes ranged from one to 10 (**Figure 4.3**). Over all the populations, the haplotype diversity (*h*) and nucleotide diversity (π) were 0.517 ± 0.069 and 0.00104 ± 0.00386, respectively (**Table 4.3**). Genetic diversity was low to moderate across populations and comparable in most cases. Genetic diversity levels were relatively high for Durban (h = 0.846, π = 0.00194) and relatively low for Langebaan (h = 0.154, π = 0.00019).

Table 4.1 Polymorphic nucleotide positions for *Mustelus mustelus* mtDNA *ND4* haplotypes. A dot indicates that the base in that position is the same as the base in Haplotype 1

Haplotype number	Nucleotide positions														
	0	0	0	0	0	2	3	3	3	3	4	4	6	7	7
	0	1	1	2	4	1	3	6	7	9	3	9	2	2	7
	6	4	5	2	5	8	5	9	1	5	3	1	8	6	7
CSH1	C	G	C	A	T	A	T	G	T	C	C	C	T	A	T
CSH2	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CSH3	T	.	.	.	.	.	.	A	.	T	.	T	.	.	.
CSH4	T	.	.	.	.	.	.	.	.	.	.	.	.	T	.
CSH5	T	.	.	.	.	G	.	.	.	.	.	.	.	.	.
CSH6	T	.	T	.	.	.	.	.	C	.	.	.	.	.	.
CSH7	T	A	.	.	.	.	.	.	.	.	.	.	.	.	.
CSH8	T	.	.	.	.	.	.	.	.	.	T	.	.	.	.
CSH9	T	.	T	.	.	.	.	.	.	.	.	.	.	.	.
CSH10	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.
CSH11	T	.	.	G	.	.	.	.	.	.	.	.	.	.	.
CSH12	T	.	.	.	C	.	.	.	.	.	T	.	.	.	.
CSH13	T	.	.	.	.	.	C	.	.	.	.	.	.	.	.
CSH14	T	.	.	.	.	.	.	.	.	.	.	.	.	.	C
CSH15	T	.	.	.	.	.	.	.	.	T	.	T	.	.	.

Table 4.2 Geographic distributions of *Mustelus mustelus* haplotypes and the number of individuals in each sampling region

Location	*n*	Haplotype (CSH) number														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Angola	9	1	5	3	0	0	0	0	0	0	0	0	0	0	0	0
Langebaan	13	0	12	0	1	0	0	0	0	0	0	0	0	0	0	0
Robben Island	8	0	5	0	0	1	1	1	0	0	0	0	0	0	0	0
False Bay	6	0	4	0	0	0	0	0	1	1	0	0	0	0	0	0
Kalk Bay	7	0	6	0	0	0	0	0	0	0	1	0	0	0	0	0
Struis Bay	12	0	9	0	0	0	0	0	0	0	0	3	0	0	0	0
Jeffreys Bay	10	0	9	0	1	0	0	0	0	0	0	0	0	0	0	0
Durban	13	0	4	0	1	0	0	0	0	4	0	0	1	1	1	1

Table 4.3 Summary of population diversity statistics for *Mustelus mustelus* integrated over all mtDNA ND4 haplotypes from each sampling location. *n*, number of samples; N_H, number of haplotypes (unique haplotypes); *h*, haplotype diversity; π, nucleotide diversity

Group	**Population**	***n***	N_H	***h***	π
Atlantic Ocean	Angola	9	3 (2)	0.639	0.0022
	Langebaan	13	2 (0)	0.154	0.0002
	Robben Island	8	4 (3)	0.643	0.0013
	False Bay	6	3 (1)	0.600	0.0008
Indian Ocean	Kalk Bay	7	2 (1)	0.286	0.0004
	Struis Bay	12	2 (1)	0.409	0.0005
	Jeffreys Bay	10	2 (0)	0.200	0.0003
	Durban	13	7 (4)	0.846	0.0019

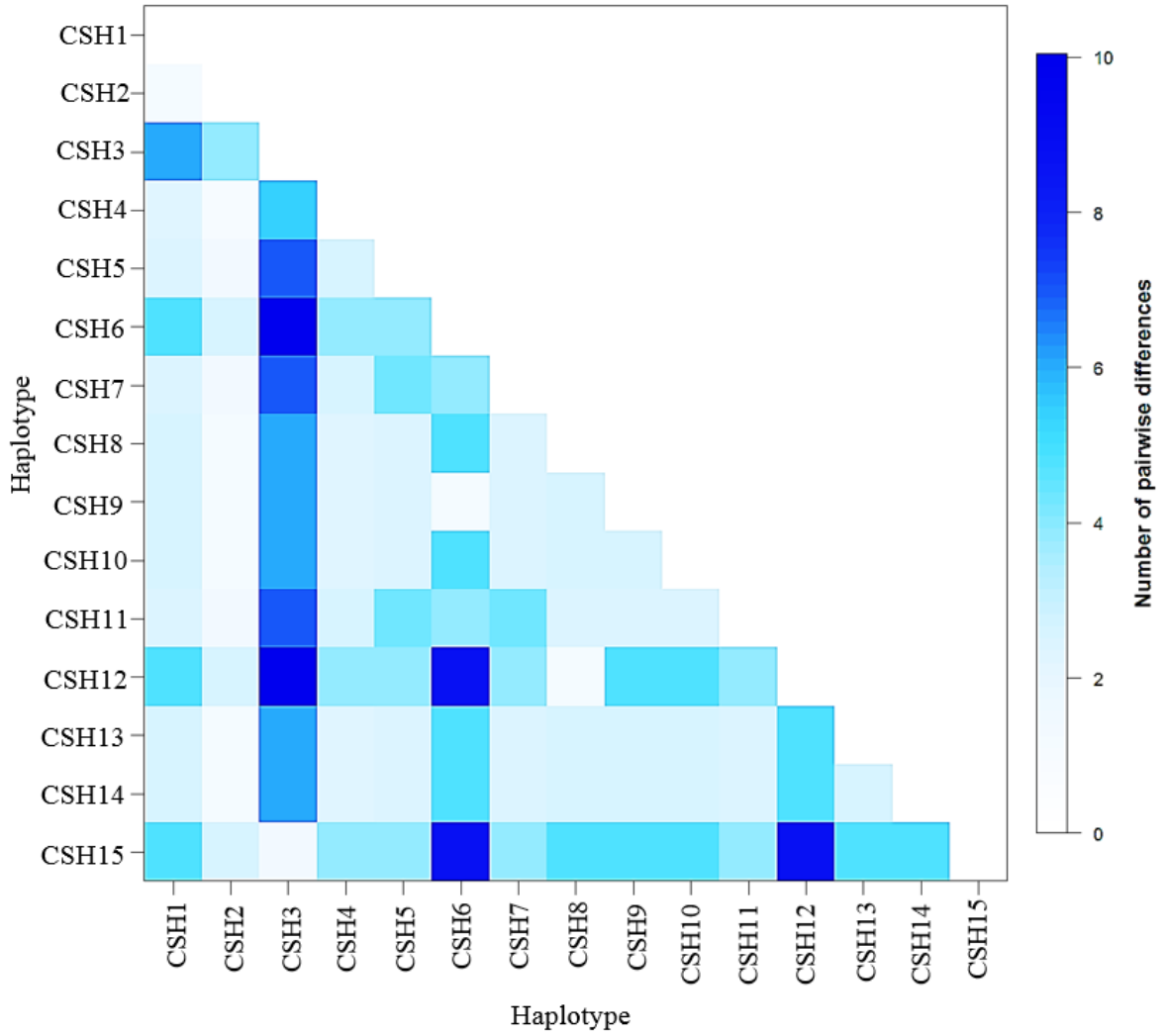

Figure 4.3 Haplotype distance matrix showing the number of molecular differences between 15 haplotypes across eight locations of *Mustelus mustelus* in southern Africa.

4.3.3 Spatial Patterns of Genetic Diversity

Genetic differentiation based on pairwise ϕ_{ST} estimates among populations was low to high, ranging from zero to 0.288 (**Figure 4.4**), and were non-significant for almost all population comparisons. Exceptions were significant ϕ_{ST}-values between Angola and three of the South African populations (Robben Island, Struis Bay and Durban), and between Robben Island and Struis Bay. When populations were grouped by oceans (South Atlantic or South-West Indian Ocean), pairwise ϕ_{ST} estimates were low and non-significant. Significant average numbers of pairwise differences between populations were detected between Angola, Durban and almost all the study populations (**Figure 4.5**). Notably, the Angola and Durban populations harboured comparably large average numbers of pairwise differences within populations (π_X; 2.280 and 2.078, respectively); Langebaan and Jeffreys Bay harboured low π_X (0.164 and

0.213, respectively). Large corrected average pairwise differences between the populations were detected between Angola and the rest of the study populations and moderate pairwise differences between Struis Bay and all the other study populations, indicating the divergence of these two populations from the remainder (**Figure 4.5**).

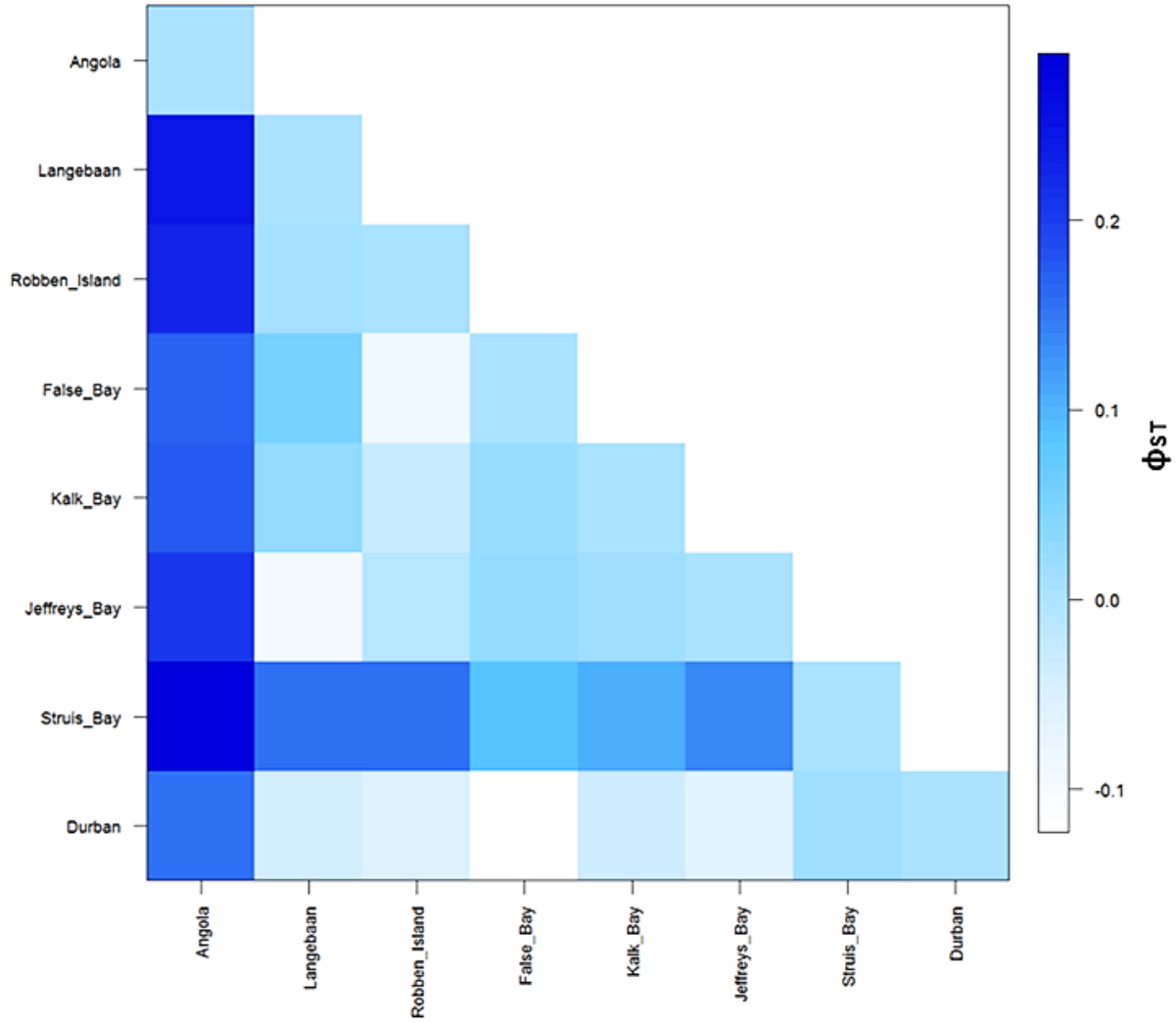

Figure 4.4 Genetic divergence as described by ϕ_{ST} computed between pairs of populations.

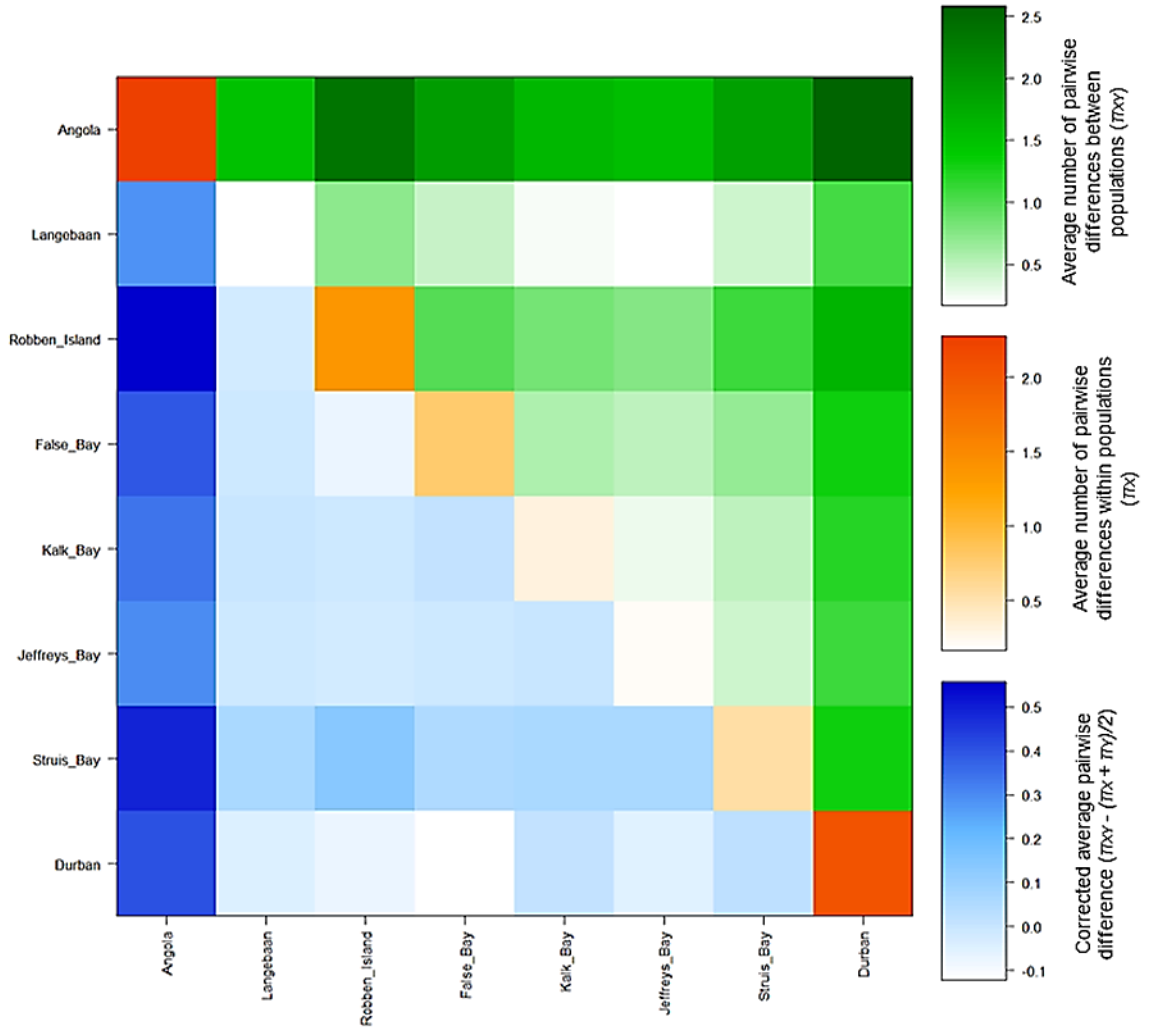

Figure 4.5 This graphic depicts the average number of pairwise differences between each population in the upper half of the matrix (green), the average number of pairwise differences within each population is shown in the diagonal (orange) and the lower half of the matrix (blue) shows the corrected average pairwise difference between the populations.

Results of the AMOVA indicated strong genetic divergence between the Angolan, South-East Atlantic and South-West Indian Ocean, with significant differentiation amongst regions (ϕ_{CT} = 0.151, P = 0.043) and over all regions and populations (ϕ_{ST} = 0.128, P = 0.007) (**Table 4.4**). However, there was a lack of within-region genetic variation (ϕ_{SC} = -0.027, P = 0.538), highlighting the genetic connectivity of populations within regions. Addtionally there was no interoceanic population genetic structure between the South-East Atlantic (including Angola and South-West Indian Ocean (**Table 4.4**). The haplotype network revealed no relationship between haplotype genealogy and geographic location, but supported the existence of a genetic connectivity between populations (**Figure 4.1**). Particularly, haplotypes CSH2 and CSH4 were shared by the Atlantic and Indian Ocean populations, indicating past connectivity.

Table 4.4 Analysis of Molecular Variance of *Mustelus mustelus* populations clustered in regional and oceanic groups: Angola *vs*. Atlantic Ocean populations *vs*. Indian Ocean populations and Angola+Atlantic Ocean populations *vs*. Indian Ocean populations, respectively

Population grouping	Source of variation	Sum of squares	Variance components	% of Variation
Angola *vs*. Atlantic Ocean populations *vs*. Indian Ocean populations	Among regions	4.641	0.084	15.10
	Within regions	1.830	-0.013	-2.27
	Within populations	34.091	0.487	87.17
	Total	**40.562**	**0.559**	
	ϕ_{CT}: 0.151	*P*: 0.043*		
	ϕ_{SC}: -0.027	*P*: 0.538		
	ϕ_{ST}: 0.128	*P*: 0.007**		
Angola+Atlantic Ocean populations *vs*. Indian Ocean populations	Among oceans	0.913	-0.00178	-0.33
	Within oceans	5.559	0.04633	8.72
	Within populations	34.091	0.48701	91.62
	Total	**40.562**	**0.53156**	
	ϕ_{CT}: -0.003	*P*: 0.521		
	ϕ_{SC}: 0.087	*P*: 0.002*		
	ϕ_{ST}: 0.084	*P*: 0.005*		

*Statistical significance at the 5% and ** 1% nominal level

Mantel tests revealed a positive and significant correlation between ϕ_{ST} and geographic distance, indicating a pattern of isolation by distance when all populations were compared (r^2 = 0.642, *P* = 0.017; **Figure 4.6a**). However, when considering only populations along the South African coastline, no correlation between ϕ_{ST} and geographic distance was detected (r^2 = 0.0405, *P* = 0.185; **Figure 4.6b**). These results are congruent with the patterns of genetic diversity and population genetic structure described above.

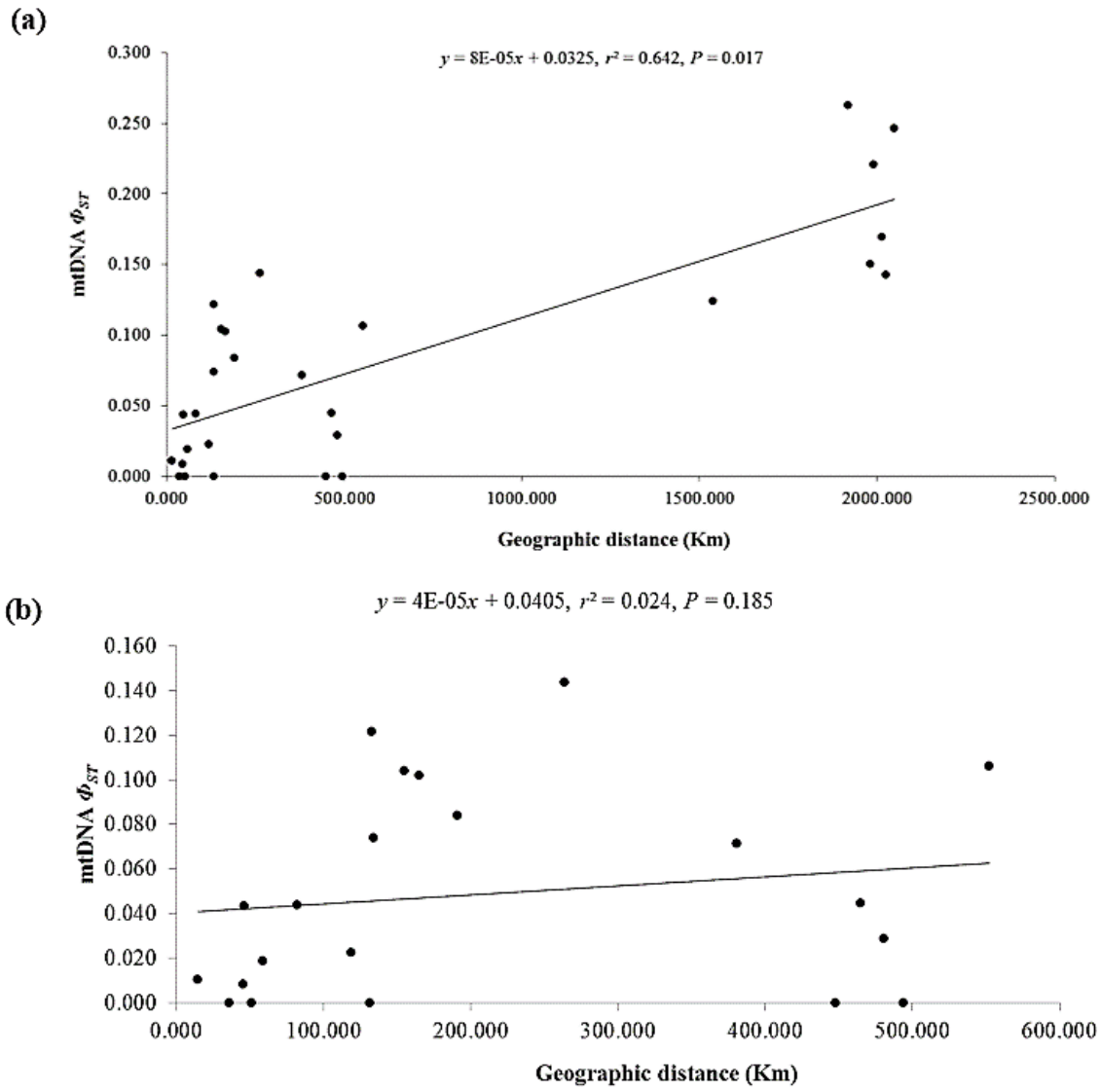

Figure 4.6 Isolation by distance with (a) all sampling locations and (b) excluding Angolan samples.

4.3.4 Demographical History

Overall Tajima's *D* and Fu's *F* statistics for neutrality were negative and non-significant (*D* = -0.783, *P* > 0.02 and *Fs* = -0.505, *P* = 0.042, respectively) indicating no excess of alleles that would be expected following a population expansion event (Fu 1997). Tajima's *D* showed significant values only for Robben Island and Durban (*D* = -1.535, *P* = 0.038 and *D* = -1.562, *P* = 0.042, respectively; **Table 4.5**), whereas Fu's *F* statistics showed significant values only for Durban (*Fs* = -3.156, *P* = 0.011) indicating population expansion (**Table 4.5**). All mismatch distributions were consistent with the sudden population expansion model, except the Angolan samples which exhibited a bi-modal distribution (**Table 4.5**, **Figure 4.7**). Harpending's raggedness index (H_{RI}) was non-significant and ranged from 0.080-0.360. Overall, the model of population expansion could not be rejected by any of the tests

performed (sum of squares distances [SSD]): $P_{(\text{Sim. SSD} \geq \text{Obs. SSSD})} = 0.677$); Harpending's raggedness index: $P_{(\text{Sim. Rag.} \geq \text{Obs. Rag.})} = 0.805$ (**Figure 4.7**). Overall the sampling sites, the mismatch analysis estimated an ancestral population size (θ_0) of 0.3 and actual population size (θ_1) of 5.1, with a τ value of 0.5. The large difference between θ_0 and θ_1 resulted in a large θ ratio ($\theta_1/\theta_0 = 17$), which is also indicative of population expansion.

Table 4.5 Demographic history estimates for *Mustelus mustelus* in southern Africa

Oceans	**Population**	***D***	***P***	F_S	P_{FS}	τ	θ_0	θ_1	H_{RI}	P_{HRI}
Atlantic	Angola	0.692	0.756	1.450	0.793	0.5	0.00	2.902	0.163	0.628
	Langebaan	-1.149	0.184	-0.537	0.022*					
	Robben Island	-1.535	0.038*	-1.236	0.061					
	False Bay	-1.132	0.145	-0.858	0.068					
	Kalk Bay	-1.006	0.220	-0.095	0.226					
	Struis Bay	0.541	0.806	0.735	0.475					
Indian	Jeffreys Bay	-1.112	0.177	-0.339	0.172	0.9	0.00	99999.000	0.080	0.535
	Durban	-1.562	0.042*	-3.156	0.011*					

D = Tajima's test, *P* = the *P*-value of Tajima's test, F_S = Fu's F_S test, P_{FS} = the *P*-value of F_S test, $\boldsymbol{\tau}$ = expansion time in scaled coalescent units, θ_0 = the ancestral population size, θ_1 = the ancestral population size, H_{RI} = Harpending's raggedness index and P_{HRI} = *P*-value of Harpending's raggedness index

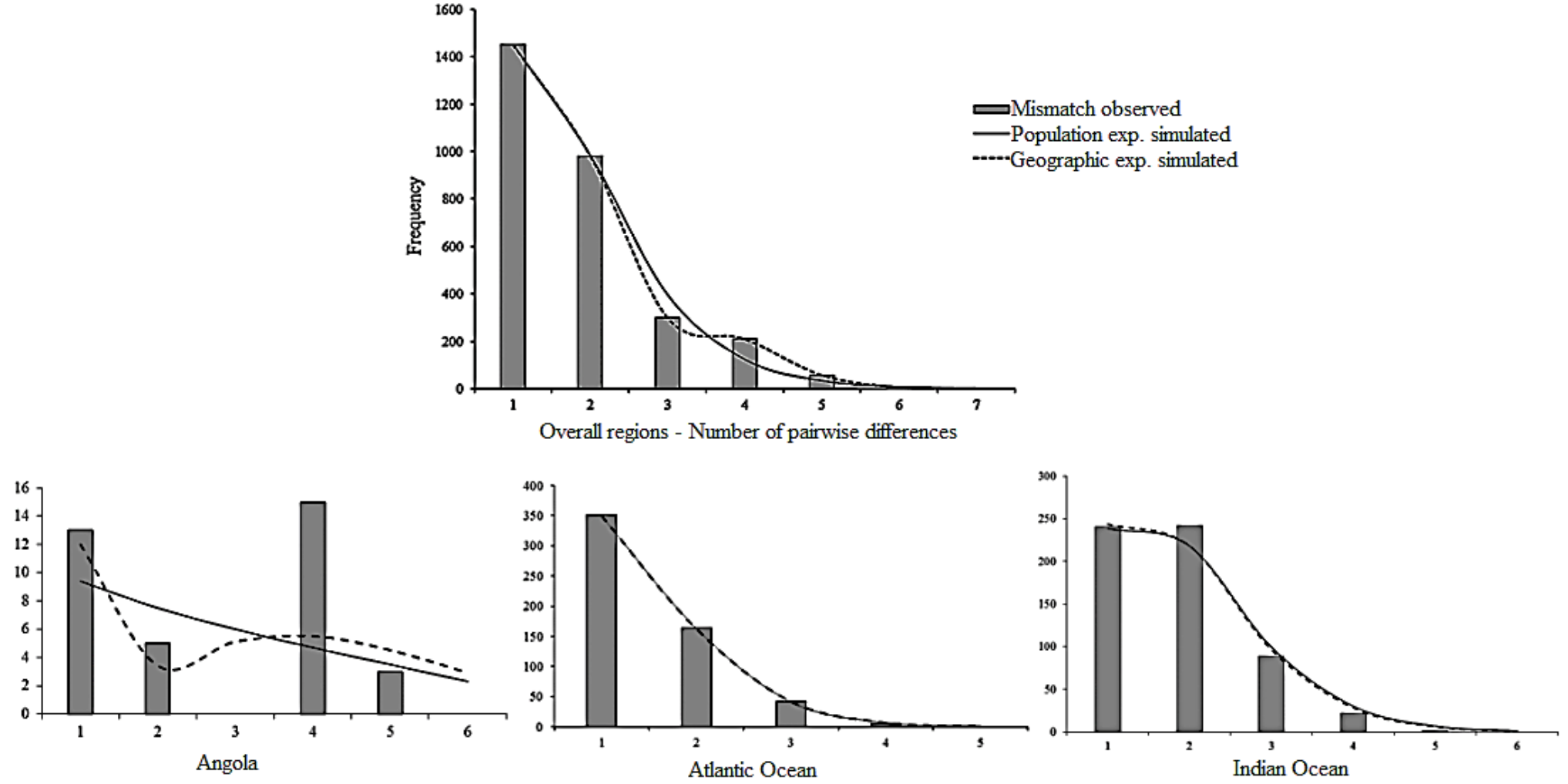

Figure 4.7 Pairwise mismatch distribution and the hypothesis of population expansion and geographic expansion of *Mustelus mustelus* in southern Africa.

4.3.5 Migration Rates between Oceans

Coalescent-based maximum likelihood estimates (MLE) by MIGRATE-N indicated asymmetric migration among oceans. The MLE showed that mutation-scaled effective population size (θ) was highest in the South-East Atlantic Ocean (θ = 0.0143) and lowest in the Angola population (θ = 0.0010). The most probable estimates of migration rates (M) ranged from zero to 27 800, with the highest migration observed from the South-East Atlantic to South-West Indian Oceanic region (M = 27 800). Interstingly, there is a lack of migration from the South-West Indian Oceanic to South-East Atlantic region (M = 0). The migration between oceans was asymmetrical, as is shown by the number of migrants (N_m) in **Table 4.6** and **Figure 4.8**.

Table 4.6 Mutation-scaled effective population size ($\theta = 4N_e\mu$) and migration rates (M) across Angola (A), the Atlantic and Indian Ocean (AO and IO, respectively)

	Mean [95% CIs]
θ_A	0.0010 [0.0005 - 0.0013]
θ_{AO}	0.0143 [0.0121 - 0.0239]
θ_{IO}	0.0067 [0.0010 - 0.0208]
$M_{AO > A}$	2880 [2060 - 4650]
$M_{A > AO}$	9670 [6600 - 11600]
$M_{IO > AO}$	0
$M_{AO > IO}$	27800 [19100-31300]

Figure 4.8 Number of migrants per generation ($N_m = M\theta/2$) between different oceanic regional *Mustelus mustelus* populations in southern Africa.

4.4 Discussion

Mustelus mustelus is one of the top five species harvested commercially in South Africa (Da Silva & Bürgener 2007). Stock assessment of the species revealed it was overexploited with populations gradually declining (Da Silva 2007; Serena *et al*. 2009). As a consequence, it may require several decades for the species to recover. Despite its commercial importance locally and globally, no population genetics analysis, evaluating the impact of fisheries and

recreational activities on levels of genetic diversity and population structuring, have been conducted on common smoothhound shark. To our knowledge, this is the first study aimed at characterising genetic diversity and population differentiation of *M. mustelus* in the southern African region using sequence data (based on the mitochondrial *ND4* region). This will provide information for population monitoring efforts and long-term management goals by defining evolutionary significant units.

4.4.1 Genetic Diversity

The mtDNA sequence data analysis revealed low to moderate nucleotide (π) and haplotype (h) diversity across populations of this threatened coastal shark (mean π = 0.00104± 0.00386; mean h = 0.517±0.069). The overall levels of nucleotide and haplotype diversity for *M. mustelus* were similar to those reported for other *Mustelus* species: *M. antarcticus* (π = 0.0008±0.0008, h = 0.456±0.05) and *M. lenticulatus* (π = 0.0009±0.0008, h = 0.531±0.05) (Boomer *et al*. 2012), but lower than that reported for other elasmobranchs (*e.g*. Veríssimo *et al*. 2010; Geraghty *et al*. 2013). Interestingly, low levels of genetic diversity in the Langebaan population agreed with the low genetic diversity estimates revealed by the microsatellite analysis (Chapter 3) and this might indicate that the population at Langebaan has undergone a recent genetic bottleneck not detected by the demographic analysis. In contrast, the low levels of genetic diversity found in the Jeffreys Bay population, suggesting either a founder or recent bottleneck event, were not in agreement with the high intra-population genetic diversity detected using microsatellites (Chapter 3). Loss of genetic diversity can occur actively through selection (Amos and Harwood 1998; Bazin *et al*. 2006). Although the present data did not indicate departure from neutrality for Jeffreys Bay, the mitochondrial genome of this population may have been subjected to a recent selective sweep or strong background selection. Alternatively, the nuclear genome may have been subjected to balancing selection. Lower levels of genetic variation in mtDNA than nuclear DNA have also been reported in other shark species *e.g*., *Negaprion brevirostris*, *N. acutidens* and *Carcharhinus leucas* (Schultz *et al*. 2008; Karl *et al* 2011); however, this pattern is not common to all sharks. For example, high levels of mtDNA genetic diversity on a background of lower nuclear DNA diversity have previously been reported for *Prionace glauca* (Ovenden *et al*. 2009) and *Rhincodon typus* (Ramírez-Macías *et al*. 2007). Overall, the intermediate levels of genetic diversity found in this study and Chapter 3 for the common smoothhound

shark conform to the patterns described for most shark species and raises further concerns over the conservation status of this shark.

4.4.2 Spatial Patterns of Genetic Diversity

The mitochondrial haplotype data indicated genetic homogeneity within and between the South-East Atlantic and South-West Indian Ocean and did not coincide with the interoceanic population genetic structure found with the microsatellite analyses. The weak but significant genetic divergence [ϕ_{ST} and F_{ST} (Chapter 3)] between the Angolan and the three South African populations (Robben Island, Struis Bay and Durban) is probably indicative that Angola is genetically divergent from common smoothhound shark populations in South Africa. Including more samples could in the future assist in resolving the genetic divergence between Atlantic and Indian Ocean samples from South Africa. The haplotype network did not indicate geographic structure and supported by the AMOVA which did not detect tnteroceanic genetic divergence (Angola+Atlantic Ocean populations *vs*. Indian Ocean populations; ϕ_{CT} = -0.003, $P > 0.05$). The haplotype network indicated the presence of shared ancestral polymorphisms (due to recent population divergence) between the oceans but indicated divergence of the Angolan samples (**Figure 4.1** and **4.2**). Additionally, the lack of significant isolation by distance further supports substantial levels of historical interoceanic gene flow across the Cape Agulhas Boundary (Benguela Barrier). The latter coincides with the hypothesis that the leakage of the Agulhas Current during the warm interglacial periods throughout the Pleistocene epoch enabled gene flow between Atlantic and Indian Ocean populations (Peeters *et al*. 2004; Dudgeon *et al*. 2012) (**Figure 4.6b**). The AMOVA (Angola *vs*. Atlantic Ocean populations *vs*. Indian Ocean populations; ϕ_{CT} = 0.151, $P < 0.05$) and the significant isolation by distance further supported the divergence of the Angolan samples from the Atlantic and Indian Ocean samples. This indicates that the hydrodynamic barrier, the Angola-Benguela Front is a potential barrier that may restrict gene flow between South African and Angolan populations.

MIGRATE-N estimates of migration rates showed high migration events, albeit highly asymmetrical, across the Cape Agulhas Boundary, further supporting interoceanic gene flow. Furthermore, gene flow asymmetry may also be indicative of range expansion (Excoffier *et al*. 2009), which frequently leads to the formation of secondary contact zones between differentiated populations of a species (Fedorka *et al*. 2012).

The presented results are congruent to the pattern described for other coastal shark species *e.g*., narrownose smoothhound shark (*Mustelus schmitti*) and sandbar shark (*Carcharhinus plumbeus*), where interoceanic movements and reproductive mixing is female-mediated (Pereyra *et al*. 2010; Portnoy *et al*. 2010). Incongruence of genetic structure found based on mitochondrial and nuclear DNA data sets (mitonuclear discordance) can be attributed to different factors: (1) mode of inheritance of the molecular marker type, (2) recent selective sweeps in the mitochondrial genome or balancing selection in the nuclear, (3) population size changes, (4) sampling bias (unequal gender ratios), and/or (5) sex-specific reproductive behaviour, specifically female fidelity to mating and nursery areas (Karl *et al*. 2011). These, along with asymmetric migration rates, are discussed in greater detail in Chapter 5.

4.4.3 Demographic History

Both mitochondrial and microsatellite (Chapter 3) analyses of demographic history of the southern African common smoothhound shark populations detected a founder event. Population expansion was supported by the starlike phylogeny of haplotypes, mismatch distribution analyses and the overall significant negative values of D and F_S. The profiles of the mismatch analyses were very similar for the demographic and geographic expansion hypotheses tested in the present study, indicating concurrent occurrence of these expansion events. The higher support for the constant population size for the Angolan sample indicates that this population represents a more stable population and has not undergone demographic expansion (**Figure 4.7**). Various authors have suggested that the Pleistocene glaciations had a major demographic impact on shaping the phylogeography of mtDNA patterns and population genetic structure in fish species (Pereyra *et al*. 2010; Teske *et al*. 2011; Boomer *et al*. 2012; Mendonça *et al*. 2013; O'Brien *et al*. 2013). The absence of a well-calibrated molecular clock for *M. mustelus* presents a challenge in dating the event of population expansion and determining the effective population sizes associated with the ancestral and actual θ values, since all these estimates require a gene-specific mutation rate. Estimates of mutation rates in elasmobranch taxa are only available for the noncoding mitochondrial DNA control region (CR) (~10^{-5} mutations per generation) [scalloped hammerheads *Sphyrna lewini* (Duncan *et al*. 2006); blacktip shark *Carcharhinus limbatus* (Keeney and Heist 2006); lemon shark *Negaprion brevirostris* (Schultz *et al*. 2008)] and the mitochondrial cytochrome *b* gene (per-site divergence rate of 0.0414/million years) [bonnethead sharks *Sphyrna tiburo* (Martin *et al*. 1992); sleeper sharks subgenus *Somniosus* (Murray *et al*. 2008)]. These mutation rates

have been applied to distantly related species, which were not congeneric, making the accuracy of this approach questionable (Ho *et al*. 2011; Grant *et al*. 2012; Shapiro and Ho 2014). For instance, the CR mutation rates were applied to the analysis of the coding regions of the mitochondrial genome, *ND2* (Veríssimo *et al*. 2010) and *ND4* (Boomer *et al*. 2012). For *Mustelus* species, Boomer *et al*. (2012) (*M. antarcticus* and *M. lenticulatus*) and Pereyra *et al*. (2010) (*M. schmitti*) used averaged mutation rates from the scalloped hammerhead shark and lemon shark, and bonnethead sharks, respectively, which are very distant relatives of smoothhound sharks. In most cases the authors of the original studies that reported these rates claimed to be uncertain as to the reliability of these rate estimates (Duncan *et al*. 2006; Keeney and Heist 2006; Murray *et al*. 2008). The equivalent mutation rates for smoothhound sharks are considerably lower due to these animals' potentially lower metabolic rates (Smale and Compagno 1997; Bosch *et al*. 2013). For the reasons outlined above, no mutation rate was selected and applied in the present study. Nevertheless, the overall demographic inferences suggested that the population expansion, followed by the possible reduction in population size, that occurred in common smoothhound shark is very likely to be recent given the small τ value of 0.5 and the large θ ratio (θ_1/θ_0 = 17). It is hypothesised this recent population expansion occurred during the warm interglacial periods in the late Pleistocene (approximately 50 000 years ago) to early Holocene (approximately 7 000-11 000 years ago).

In the future, estimates of a locus-specific mutation rate for the *ND4* gene in *M. mustelus* could assist in supporting the hypothesis of population expansion. Interestingly, some selective events (*e.g*. positive selection) acting on mtDNA can also mimic demographic population expansion due to the resulting excess of low-frequency haplotypes, making it difficult to unambiguously discern between evidence for natural selection and demographic population expansion (Babbucci *et al*. 2010; Pereyra *et al*. 2010; Geraghty *et al*. 2013). A population expansion event in common smoothhound shark should therefore be confirmed using several unlinked loci in the genome, such as CR and/or sequences of nuclear genes (*e.g. recombination-activating gene 1*, *RAG1*).

4.5 Conclusions

In summary, this study found that based on mitochondrial sequence data, *Mustelus mustelus* populations in South Africa most likely constitute a single panmictic population and indicated substantial historical gene flow between the oceans. Based on the AMOVA, ϕ_{ST} and haplotype network it is proposed that the Angolan samples are divergent

from the South African populations. Generally, mtDNA is only indicative of long-term female dispersal history with no genetic differentiation expected between populations of species in which females disperse widely. The distinct and opposing patterns of genetic population structure detected with mitochondrial DNA and microsatellite markers can arise from various factors, such as a founder event, range expansion in the past or even male philopatry; hypotheses supported by the mitochondrial sequence data. In addition, discordance could be due to locus-specific artefacts such as selection and differences in substitution rates. Further sampling, tagging studies and acoustic tracking could in the future assist in a more robust evaluation of the latter findings. A significant and likely recent population expansion is postulated to have occurred during the warm interglacial periods in the late Pleistocene to early Holocene. Nonetheless, despite finding an expanding common smoothhound shark population, a contemporary genetic bottleneck may have gone undetected as genetic diversity was very low in some of the study populations. This chapter, in conjunction with Chapter 3, highlights the importance of using multilocus genetic data before conclusions are drawn about conservation management of commercially important shark populations.

Chapter 5

Concluding Remarks and Future Perspectives

5.1 Overview of Research Findings

The socio-economic importance of the common smoothhound shark *Mustelus mustelus* has had a significant negative impact on global population trends of this species (Aldebert 1997; Da Silva 2007; Gascuel *et al*. 2007; Serena *et al*. 2009; Da Silva *et al*. 2013). The species has recently started to receive some ecological attention (Smale and Compagno 1997; Da Silva 2007; Da Silva *et al*. 2013), but there is still very little genetic information available for this shark. In this study, the objectives were to quantify genetic diversity and assess population genetic structure of common smoothhound shark along the South African coast using multilocus data generated from 12 microsatellite markers and the mitochondrial gene *NADH dehydrogenase subunit 4* (*ND4*).

With regard to effective management strategies, it is important to understand the population dynamics and structuring of species in a marine environment and how fishing effort and mortality are distributed (Begg and Waldman 1999). In fisheries science, stock structure is generally defined based on geographic area and meristic and morphometric measurements (Ovenden 1990; Begg and Waldman 1999). The definition of modern stock structure integrates genetic knowledge to account for the number and geographic limits of non-interbreeding, self-recruiting populations within an exploited species, *i.e*. population genetic structure (Ovenden 1990, 2013). Such an understanding is vital for recognising management and evolutionary significant units for short and long-term conservation. Delimiting population genetic structure for various elasmobranch species is largely incomplete due to the general lack of molecular genetic markers, *e.g*. microsatellites, single nucleotide polymorphism (SNPs) and, to a lesser extent, mitochondrial DNA. These can provide valuable information relating to the population dynamics (spatial and temporal genetic variation, and demographics) of individual species.

In Chapter 2, the first larger scale (in terms of species used and number of loci) development and optimisation of medium-throughput microsatellite multiplex assays for regional and cosmopolitan elasmobranch species were presented. In short, a microsatellite

repository for the common smoothhound shark and other elasmobranch species was successfully established through cross-species amplification, a less costly and time-efficient proxy approach to *de novo* development of microsatellite markers. The cross-species amplification success rate and percentage of polymorphism in common smoothhound were 71% and 100%, respectively, with four multiplex assays consisting of a total of 22 microsatellite loci successfully optimised and characterised in the species. Cross-species amplification of these assays to 11 other elasmobranch species highlighted the usefulness of microsatellites for characterising genetic diversity and potentially also species identification in houndsharks.

In Chapter 3, two microsatellite multiplex assays (MPS1 and MPS2) were selected based on levels of polymorphism to investigate the extent of population genetic structure and historical demographics of common smoothhound shark in southern Africa. Genetic diversity summary statistics, analysis of molecular variance (AMOVA), factorial correspondence analysis (FCA) and Bayesian clustering indicated moderate levels of genetic diversity across nearly all sampling populations and that regional common smoothhound potentially consisted of two genetically-differentiated populations with low levels of gene flow between the South-East Atlantic and South-West Indian Ocean. The contemporary migration rates among oceans were considerably low and could in part explain the observed patterns of population genetic structure. The Cape Agulhas Boundary (westernmost extent – Cape Point, easternmost extent – Cape Agulhas; Teske *et al*. 2011) that coincides with the biogeographic disjunction between the cool-temperate and warm-temperate biotas was identified as the most probable biogeographic barrier between Atlantic and Indian Ocean common smoothhound sharks. In addition, no population bottlenecks were detected across the study populations, despite the considerably low contemporary estimates of effective population size (N_E). Two putative management units were, therefore, identified for the common smoothhound shark based on the AMOVA, FCA and Bayesian clustering analysis of microsatellite data.

In Chapter 4, to confirm the observed population structure patterns of the microsatellite data and determine whether, historically, this species is also divided into evolutionary significant units that coincide with the recommended management units, the mitochondrial *ND4* region was analysed. Mitochondrial sequence data was for the most part not in concordance with the microsatellite analysis results, indicating that, historically, common smoothhound shark populations in South African most likely constitute a single population with higher levels of interoceanic gene flow for this species. A similar trend has also been observed for copper or bronze whaler shark (*Carcharhinus brachyurus*) populations from southern Africa, where no

population genetic structure was detected between the South-East Atlantic (Namibia) and South-West Indian Ocean (South Africa) (ϕ_{ST} = -0.002; Benavides *et al*. 2011*a*). In other coastal shark species, females generally exhibit philopatry to nursery areas. For instance, high levels of population genetic structure among populations were reported for the scalloped hammerhead shark (*Sphyrna lewini*) from the South-East Atlantic (West Africa) and South-West Indian Ocean (South Africa) (ϕ_{ST} = 0.566, Daly-Engel *et al*. 2012). The findings of a recent population and geographic expansion event, nonetheless, validated those obtained with the microsatellites. Population expansion was proposed to have occurred during the warm interglacial periods in the late Pleistocene to early Holocene.

Overall, the work presented in this thesis constitutes one of the first regional population genetic studies for any elasmobranch species in southern Africa. Contemporary restriction to gene flow and historical demographics, such as a founder event followed by range expansion, are proposed as the most likely forces explaining genetic structure observed in present-day common smoothhound.

5.2 Significance of the Biological Findings

5.2.1 Molecular Genetic Markers and Outlier Loci

The greatest challenge in elasmobranch population and conservation genetics is the scarcity of molecular genetic resources currently available. In general, this impedes research efforts aiming to characterise and understand the apportioning of genetic variation at multiple levels, from intra-individual to interspecific (Dudgeon *et al*. 2012; Portnoy and Heist 2012). To circumvent this issue, early molecular work on elasmobranchs was based on genetic markers that did not require prior knowledge of a species' genome *e.g*., nuclear allozymes, amplified fragment length polymorphisms (AFLPs) and restriction fragment length polymorphisms (RFLPs) (Dudgeon *et al*. 2012; Portnoy and Heist 2012). Currently, microsatellite markers and mitochondrial DNA are the most widely used molecular markers in elasmobranchs due to their hyper-variability, and ease in scoring and down-stream analysis (Chabot and Allen, 2009; Portnoy *et al*. 2010; Veríssimo *et al*. 2010; Karl *et al*. 2011; Benavides *et al*. 2011*a, b*; Geraghty *et al*. 2013; Mendonça *et al*. 2013; Mourier *et al*. 2013; Vignaud *et al*. 2013). In this study, 12 microsatellite markers and a 793 bp fragment of the mitochondrial *ND4* gene were employed to delineate population genetic structure and reconstruct the demographic history of the common smoothhound shark in southern Africa.

Generally, outlier loci are known to distort population genetic structure and demographic history estimates and, in most instances, are attributed to heterogeneous genomic divergence due to selection (Nosil *et al*. 2009; Bierne *et al*. 2010). This occurs when the effective population size is small, as is the case for the common smoothhound shark, which increases population divergence through background selection against deleterious mutations and, thus, inflates allelic variance at loci with differential recombination rates (Bierne *et al*. 2010). Furthermore, selection may occasionally occur in neutral loci to facilitate ecological adaptation in fluctuating environmental conditions (Nielsen *et al*. 2009). Using various outlier detection methods, four of the microsatellites tested in the current study were identified as outlier loci; two loci were under directional selection and two under balancing selection. The *ND4* region also exhibited deviations from neutrality. This mitochondrial region did not detect population differentiation. Generally, selection can significantly reduce genetic variation; therefore, it is possible that the lower variation in the *ND4* region constrained the resolution of this locus for detecting weak population subdivision.

5.2.2 Species Identification in Sharks

Misidentification of shark species is a common phenomenon; so much so that that these animals are reported at a species- or family group level (*e.g*. houndsharks, carcharinids, hammerheads *etc*.). In South Africa, this occurs despite the species identification method based on morphological and anatomical traits developed by Da Silva (2007) and this highlights the necessity for using multidisciplinary approaches in confirming identification of shark species, for instance, using molecular data to corroborate morphological data (*e.g*. Giresi *et al*. 2013; Pérez-Jiménez *et al*. 2013). Genetic identification methods using mitochondrial fragments *e*.g., *COI* (Ward *et al*. 2005, 2008; Caballero *et al*. 2012) and *ND2* (Farrell *et al*. 2009; Naylor *et al*. 2012) have proven to be effective for species identification in the past. Microsatellite markers have also been proven to be successful in individual identification of great white sharks *Carcharodon carcharias* (Gubili *et al*. 2009). The present study tested and successfully demonstrated the potential for shark genetic identification based on microsatellite markers using multi-variant analysis. In future these markers can be integrated to existing morphology identification keys for a more multidisciplinary approach.

5.2.3 Observer Accuracy in the South African Demersal Shark Fishery

The generally conserved interspecific morphology of sharks confounds identification keys used to discriminate between species and is a long-standing concern in fisheries operations

(Da Silva and Bürgener 2007; Department of Agriculture, Forestry and Fisheries 2013). Another aspect compounding shark misidentification is linguistic (ethnic) differences in understanding or interpreting common names, which was also apparent in this study. Observer accuracy in this study was fairly high (91.1%) in the identification of common smoothhound shark, but relatively low compared to other shark species with conserved interspecific morphology *e.g.*, *Carcharhinus* species (Geraghty *et al*. 2013; Tillett *et al*. 2012*c*). Geraghty *et al*. (2013) reported that observer accuracy was high in delineating between *Carcharhinus* species (98.4% for *C*. *brevipinna*, 96.6% for *C*. *obscurus* and 99.4% for *C*. *plumbeus*) in the New South Wales Ocean Trap and Line Fishery (NSW OTLF). Interestingly, observer accuracy for *C*. *brevipinna* in the Northern Territory Offshore Net and Line Fishery (NT ONLF) was estimated only at 87.2% (Tillett *et al*. 2012*c*), suggesting the officials in certain fisheries may lack the species identification skills necessary to correctly identify sharks to the species level. In the South African demersal shark fishery, fishery-observer data remain contested due to the practice of landing demersal sharks having been headed and gutted at sea, making species identification nearly impossible (Da Silva and Bürgener 2007; Department of Agriculture, Forestry and Fisheries 2013).

5.2.4 Population Dynamics of Common Smoothhound Shark

South Africa has a long and diverse coastline that stretches from the mouth of the Orange River on the west coast to Kosi Bay on the east coast and is located at the transition zone between the Atlantic Ocean and Indian Ocean biomes (**Figure 1.6**). Although the exact locations of the boundaries remain contested, the coastline is divided into nine marine bioregions (Lombard 2004; Griffiths *et al*. 2010), as shown in **Figure 1.6**. Strong environmental gradients (*e.g*. temperature, salinity), currents and upwelling cells across these bioregions have been shown to hinder along-shore dispersal of various marine species, leading to restricted gene flow and eventually to genetic divergence (Teske *et al*. 2011). Interestingly, many organisms with ranges spanning multiple biogeographical regions exhibit genetic structure across the transition zones between these regions [*e.g*. invertebrates (Bester-van der Merwe *et al*. 2011; Teske *et al*. 2014) and teleosts (Klopper 2005; Oosthuizen 2007)], while others appear to be genetically homogenous (Teske *et al*. 2014).

Whether there are also genetic boundaries that relate to the ecologically-determined, coastal biogeographical boundaries in South African shark populations remains poorly understood. This study is the first to provide some insight into the impact of biogeographical boundaries on the degree of population structuring of a coastal shark species with a fairly wide

distribution along the South African coast. Assessment of genetic structure in the common smoothhound shark revealed distinct and opposing patterns of population genetic structure using microsatellite markers and mitochondrial DNA. Microsatellite markers indicated significant interoceanic population genetic structure, while mitochondrial DNA detected moderate to high levels of interoceanic gene flow. The most appropriate boundary that coincided with the break obtained with the microsatellite data was identified as the Cape Agulhas Boundary. Even though the sample size was small, this study found that the Angolan samples are divergent from South-East Atlantic and South-West Indian Ocean, and the Angola-Benguela Front was proposed as a semi-permeable barrier to gene flow. In light of mitonuclear discordance, the observed patterns of genetic structure between South African and Angolan samples may indicate that these regions are connected via a series of stepping stone populations, such that Namibia links South Africa with Angola.

5.2.5 Mitonuclear Discordance

Generally, the conflicting patterns of population structure found with the nuclear and mitochondrial genomes (mitonuclear discordance) can arise from various factors such a differences in effective population size, recombination, ploidy and rates of nucleotide substitution (de Biasse *et al*. 2014). In this study, mitonuclear discordance could have resulted from locus-specific artefacts at the *ND4* gene region, such as retention of an ancestral polymorphism and incomplete lineage sorting between populations (Teske *et al.* 2014). These were also evident from the starlike phylogeny obtained in this study which showed genetic homogeneity across oceans. In addition, discrepancies between microsatellites and mtDNA may result from differential effects of genetic drift, differential mutation rates and migration or selection on the particular marker class, or may also result from sex-biased dispersal. Indeed, sexes often differ in their degree of dispersal and, hence, in their contribution to spatial genetic structure both within and among populations (Francis 1988; Pereyra *et al*. 2010; Karl *et al*. 2011; Benavides *et al*. 2011*b*; Daly-Engel *et al*. 2012). Asymmetric migration rates among sexes, but also spatio-temporal variation in sex ratio, will then facilitate differential genetic signals between nuclear and mitochondrial markers, as was found in this study. In most other coastal and pelagic shark species studied thus far, contrasting maternally- and bi-parentally inherited genetic markers indicated dispersive males and philopatric females (Pardini *et al*. 2001; Schrey and Heist 2003; Portnoy *et al*. 2010; Daly-Engel *et al*. 2012). Conventional and acoustic tagging studies have been conducted to better understand the movement patterns of common smoothhound shark in South Africa

(Mann and Bullen 2009; Da Silva *et al*. 2013). Both these studies reported that common smoothhound shark demonstrate site fidelity since these animals were generally recaptured close to their release site, regardless of time at liberty (Mann and Bullen 2009; Da Silva *et al*. 2013). The aforementioned studies did, however, not report any sex-bias in dispersal, while tagging studies of the related species *M. antarticus* and *M. lenticulatus* found that females had a higher dispersal capacity than males (Francis 1988; Pereyra *et al*. 2010). Therefore, the discordance found between microsatellite markers and mitochondrial DNA data of this study could be an indication of female-mediated dispersal in common smoothhound shark.

Additionally, the findings suggest a recent population expansion event that potentially coincides with the early Holocene and the rise in sea levels, and warming (Miller *et al*. 1995; Ramsay 1995; Carr *et al*. 2010; Dudgeon *et al*. 2012; Portnoy and Heist 2012) that increased suitable coastal habitats for common smoothhound sharks. An expansion following the last glacial maximum (LGM; 18 000-23 000 years ago) in common smoothhound shark is highly probable, considering that warming after the LGM caused population expansions in many marine and terrestrial organisms (Peltier 1988; Miller *et al*. 1995; Carr *et al*. 2010; Dudgeon *et al*. 2012; Portnoy and Heist 2012). This study detected no signal of a recent population bottleneck, which is not entirely unexpected since recent bottlenecks are transient and could go undetected by demographic history inferences due to variability in sampling and sampling period (Luikart and Cornuet 1998; Heller *et al*. 2013; Vignaud *et al*. 2014). Rates of gene flow (*i.e*., the number of migrants per generation) were asymmetrical between oceans, indicating the possible effects of temperature gradients and ocean currents. Interestingly, both the microsatellite and mitochondrial DNA exhibited similar patterns (in direction) of gene flow, indicating that a large proportion of migrants move from the Atlantic to the Indian Ocean. This may be indicative of these animals moving with the Agulhas Current when it retroflects back into the South-West Indian Ocean and that westward movement is influenced by the Benguela Current.

5.3 Smoothly-Hounding for Conservation Management

The high fishing pressure currently experienced by the common smoothhound shark in southern Africa may be unsustainable given that this shark has decreased in biomass across its distribution range (Aldebert 1997; Gascuel *et al*. 2007; Da Silva, 2006; Serena *et al*. 2009; Da Silva *et al*. 2013). Although this study reports a significant and probably recent population expansion, a recent bottleneck (subsequent to expansion) might have gone undetected. These

findings, together with the low resilience to exploitation of this species, raise concerns about the future conservation of common smoothhound shark. Da Silva *et al*. (2013) proposed that, since common smoothhound shark exhibit site-fidelity within a small area, these sharks might therefore benefit from spatial fishery closures in the absence of species-specific management for this species. This shark is heavily exploited on the south-west coast of South Africa (South-East Atlantic Ocean) and if this stock becomes extinct it will most likely not be replenished by the South-East Indian Ocean stock, given the asymmetrical migration rates detected in this study. It is therefore vital that stricter regulations should be put in place, such as limiting the number of vessels, crew and Total Allowable Catch (TAC) or Total Allowable Effort (TAE) for this species. In South Africa, this has proved valuable for specific protection of some species under the *Marine Living Resources Act 18* of 1998 (MLRA), *e.g.* the spotted gully shark (*Triakis megalopterus*) and sawfish (*Pristis* spp.), due to their compromised conservation status (Department of Agriculture, Forestry and Fisheries 2013; Sharks Biodiversity Management Plan 2014).

5.4 Project Limitations and Future Perspectives

This study was subject to a range of limitations requiring careful consideration. Firstly, sample size bias was a major limitation as this study relied on opportunistic sampling, making targeting particular sampling sites and obtaining representative samples difficult. The sample sizes were also weighted towards the South-East Atlantic Ocean. In future, additional sampling from, for example, East London (a sampling site between Jeffreys Bay and Durban) and Namibia (linking South Africa with Angola) could assist with more robust allocations of management units and, hence, the sustainable exploitation of this target species. Secondly, the lack of gene-specific mutation rate estimates for the *ND4* region in elasmobranchs deterred estimating the time of expansions and effective population sizes. Lastly, due to financial constraints, only a subset of individuals were sequenced for the *ND4* region and in future screening a larger sample size may provide further information on the retrospective historical demographic events reported in this study. In light of these limitations, it is recommended that this work be considered as a starting point for further evaluations of genetic structure in this commercially important species, and incorporating these into existing fisheries management practices, rather than results upon which definitive management decisions are made. Additionally, since tagging methods (*e.g.*, acoustic tracking) only measure animal movement and do not detect whether the movement has resulted in

reproduction in the adopted population *i.e.*, interbreeding (Ovenden 2013), the value of using acoustic tracking and genetic data in conjunction to better understand the movement patterns is highlighted.

References

Aars J, Ims RA (2000) Population dynamic and genetic consequences of spatial density-dependent dispersed in patchy populations. *American Naturalist* **155**: 252-265.

Abercrombie DL, Clarke SC, Shivji MS (2005) Global-scale genetic identification of hammerhead sharks: Application to assessment of the international fin trade and law enforcement. *Conservation Genetics* **6**: 775-788.

Ahonen H, Harcourt RG, Stow AJ (2009) Nuclear and mitochondrial DNA reveals isolation of imperiled grey nurse shark populations (*Carcharias taurus*). *Molecular Ecology* **18**: 4409-4421.

Aldebert Y (1997) Demersal resources of the Gulf of Lions (NW Mediterranean). Impact of exploitation on fish diversity. *Vie et Millieu*. **47**: 275-284.

Amos W, Harwood J (1998) Factors affecting levels of genetic diversity in natural populations. *Philosophical Transactions of the Royal Society B* **353**: 1-12.

Andrew NL, Béné C, Hall SJ, Allison EH, Heck S, Ratner BD (2007) Diagnosis and management of small-scale fisheries in developing countries. *Fish and Fisheries* **8**: 227-240.

Angers B, Bernatchez L (1997) Complex evolution of a salmonid microsatellite locus and its consequences in inferring allelic divergence from size information. *Molecular Biology and Evolution* **14**: 230-238.

Antao T, Lopes A, Lopes RJ, Beja-Pereira A, Luikart G (2008) LOSITAN: a workbench to detect molecular adaptation based on a F_{ST}-outlier method. *BMC Bioinformatics* **9**: 323.

Attwood CG, Peterson SL, Kerwath SE (2011) Bycatch in South Africa's inshore trawl fishery as determined from observer records. *ICES Journal of Marine Science* **68**: 2163-2174.

Avise JC (1994) *Molecular Markers, Natural History, and Evolution*. Chapman and Hall: New York. 51 pp.

Avise JC (2000) *Phylogeography: The History and Formation of Species*. Harvard University Press: Cambridge. 447 pp.

Babbucci M, Buccoli S, Cau A, Cannas R, Goñi R, Díaz D, Marcato S, Zane L, Patarnello T (2010) Population structure, demographic history, and selective processes: contrasting evidences from mitochondrial and nuclear markers in the European spiny lobster *Palinurus elephas* (Fabricius, 1787). *Molecular Phylogenetics and Evolution* **56**: 1040-1050.

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

Bandelt HJ, Forster P, Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16**: 37-48.

Barbara T, Palma-Silva C, Paggi GM, Bered F, Fay MF, Lexer C (2007) Cross-species transfer of nuclear microsatellite markers: potential and limitations. *Molecular Ecology* **16:** 3759-3767.

Barbuto M, Galimberti A, Ferri E, Labra M, Malandra R, Galli P, Casiraghi M (2010) DNA barcoding reveals fraudulent substitutions in shark seafood products: the Italian case of "palombo" (*Mustelus* spp.). *Food Research International* **43**: 376-381.

Barker MJ, Schluessel V (2005) Managing global shark fisheries: suggestions for prioritising management strategies. *Aquatic Conservation: Marine and Freshwater Ecosystems* **15**: 325-347.

Bazin E, Glemin S, Galtier N (2006) Population size does not influence mitochondrial genetic diversity in animals. *Science* **312**: 570-572.

Beaumont MA (1999) Detecting population expansion and decline using microsatellites. *Genetics* **153**: 2013-2029.

Beerli P (1998) Estimation of migration rates and population sizes in geographically structured populations. In: *Advances in Molecular Ecology* (Carvalho G, Ed.). NATO-ASI workshop series. IOS Press: Amsterdam. 39-53 pp.

Beerli P, Felsenstein J (1999) Maximum-likelihood estimation of migration rates and effective population numbers in two populations using a coalescent approach. *Genetics* **152**: 763-773.

Beerli P, Felsenstein J (2001) Maximum likelihood estimation of a migration matrix and effective population sizes in n subpopulations by using a coalescent approach. *Proceedings of the National Academy of Sciences of the USA* **98**: 4563-4568.

Beerli P (2006) Comparison of Bayesian and maximum likelihood inference of population genetic parameters. *Bioinformatics* **22**: 341-345.

Beerli P (2009) How to use migrate or why are markov chain Monte Carlo programs difficult to use? In *Population Genetics for Animal Conservation* (Bertorelle G, Bruford MW, Hauffe HC, Rizzoli A, Vernesi C, Eds.), Cambridge UK: Cambridge University Press. Volume 17: 42-79 pp.

Beerli P, Palczewski M (2010) Unified framework to evaluate panmixia and migration direction among multiple sampling locations. *Genetics* **185**: 313-326

Begg GA, Waldman JR (1999) An holistic approach to fish stock identification. *Fisheries Research* **43**: 35-44.

Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F (2004) GENETIX 4.05, *logiciel sous Windows TM pour la génétique des populations*. Montpellier, France: Université de Montpellier II.

Belleggia M, Figueroa DE, Sánchez F, Bremec C (2012) The feeding ecology of *Mustelus schmitti* in the southwestern Atlantic: geographic variations and dietary shifts. *Environmental Biology of Fishes* **95**: 99-114.

Benavides MT, Feldheim KA, Duffy CA, Wintner S, Braccini JM, Boomer J, Huveneers C, Rogers P, Mangel JC, Alfaro-Shigueto J, Cartamil DP, Chapman DD (2011*a*) Phylogeography of the copper shark (*Carcharhinus brachyurus*) in the southern hemisphere: implications for the conservation of a coastal apex predator. *Marine and Freshwater Research* **62**: 861-869.

Benavides MT, Horn RL, Feldheim KA, Shivji MS, Clarke SC, Wintner S, Natanson L, Braccini M, Boomer JJ, Gulak SJB, Chapman DD (2011*b*) Global phylogeography of the dusky shark *Carcharhinus obscurus*: implications for fisheries management and monitoring the shark fin trade. *Endangered Species Research* **14**: 13-22.

Bester-van der Merwe AE, Roodt-Wilding R, Volckaert FAM, D'Amato ME (2011) Historical isolation and hydrodynamically constrained gene flow in declining populations of the South-African abalone, *Haliotis midae*. *Conservation Genetics* **12**: 543-555.

Bierne N, Roze D, Welch JJ (2013) Pervasive selection or is it…? Why are F_{ST} outliers sometimes so frequent?. *Molecular ecology* **22**: 2061-2064.

Bilgin R (2007) KGTESTS: a simple excel macro program to detect signatures of population expansion using microsatellites. *Molecular Ecology Notes* **7**: 416-417.

Blair C, Weigel DE, Balazik M, Keeley ATH, Walker FM, Landguth E, Cushman S, Murphy M, Waits L, Balkenhol N (2012) A simulation-based evaluation of methods for inferring linear barriers to gene flow. *Molecular Ecology Resources* **12**: 822-833.

Blankenship SM, May B, Hedgecock D (2002) Evolution of a perfect simple sequence repeat locus in the context of its flanking sequence. *Molecular Biology and Evolution* **19**: 1943-1951.

Blower D, Pandolfi J, Bruce B, Gomez-Cabrera M, Ovenden J (2012) Population genetics of Australian white sharks reveals fine-scale spatial structure, transoceanic dispersal events and low effective population sizes. *Marine Ecology Progress Series* **455**: 229-244.

Boomer JJ, Harcourt RG, Francis MP, Stow AJ (2012) Genetic divergence, speciation and biogeography of *Mustelus* (sharks) in the central Indo-Pacific and Australasia. *Molecular Phylogenetics and Evolution* **64**: 697-703.

Boomer JJ, Harcourt RG, Francis MP, Walker TI, Braccini JM, Stow AJ (2013) Frequency of multiple paternity in gummy shark, *Mustelus antarcticus*, and rig, *Mustelus lenticulatus*, and the implications of mate encounter rate, postcopulatory influences, and reproductive mode. *Journal of Heredity* **104**: 371-379.

Boomer JJ, Peddemors V, Stow AJ (2010) Genetic data show that *Carcharhinus tilstoni* is not confined to the tropics, highlighting the importance of a multifaceted approach to species identification. *Journal of Fish Biology* **77**: 1165-1172.

Boomer JJ, Stow AJ (2010) Rapid isolation of the first set of polymorphic microsatellite loci from the Australian gummy shark, *Mustelus antarticus* and their utility across divergent shark taxa. *Conservation Genetics Resources* **2**: 393-395.

Bosch AC (2012) Investigation of the chemical composition and nutritional value of smoothhound shark (*Mustelus mustelus*) meat. Unpublished M.Sc. thesis. Department of Food Science. Stellenbosch University: South Africa.

Bosch AC, Sigge GO, Kerwath SE, Cawthorn D.-M, Hoffman LC (2013) The effects of gender, size and life-cycle stage on the chemical composition of smoothhound shark (*Mustelus mustelus*) meat. *Journal of the Science of Food and Agriculture* **93**: 2384-2392.

Botstein D, White RL, Skolnick M, Davis RW (1980) Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics* **32**: 314-331.

Bouzat JL (2010) Conservation genetics of population bottlenecks: the role of chance, selection, and history. *Conservation Genetics* **11**: 463-478.

Briggs JC (1995) *Global Biogeography*. Elsevier Science: Amsterdam. 207-284 pp.

Briggs JC, Bowen BW (2012) A realignment of marine biogeographic provinces with particular reference to fish distributions. *Journal of Biogeography* **39**: 12-30.

Brookfield JFY (1996) A simple new method for estimating null allele frequency from heterozygote deficiency. *Molecular Ecology* **5**: 453-455.

Burgess GH, Beerkircher LR, Cailliet GM, Carlson JK, Cortés E, Goldman KJ, Grubbs RD, Musick JA, Musyl MK, Simpfendorfer CA (2005) Is the collapse of shark populations in the Northwest Atlantic Ocean and Gulf of Mexico real? *Fisheries* **30**: 19-26.

Byrne RJ, Avise JC (2012) Genetic mating system of the brown smoothhound shark (*Mustelus henlei*), including a literature review of multiple paternity in other elasmobranch species. *Marine Biology* **159**: 749-756.

Caballero S, Cardenosa D, Soler G, Hyde J (2012) Application of multiplex PCR approaches for shark molecular identification: feasibility and applications for fisheries management and conservation in the Eastern Tropical Pacific. *Molecular Ecology Resources* **12**: 233-237.

Capapé C, Diatta Y, Diop M, Vergne Y, Guélorget O (2006) Reproductive biology of the smoothhound, *Mustelus mustelus* (Chondrichthyes: Triakidae) from the coast of Senegal (eastern tropical Atlantic). *Cybium* **30**: 273-282.

Capapé, C (1974) Observations sur la sexualité, la reproduction et la fécondité de 8 sélaciens pleurotrêmes vivipares placentaires des côtes tunisiennes. *Archives de l'Institut Pasteur de Tunis* **51**: 329-344 (As cited by Saïdi *et al*. 2008).

Carr AS, Bateman MD, Roberts DL, Murray-Wallace CV, Jacobs Z, Holmes PJ (2010) The last interglacial sea-level high stand on the southern Cape coastline of South Africa. *Quaternary Research* **73**: 351-363.

Castoe TA, Poole AW, Gu W, Jason De Koning AP, Daza JM, Smith EN, Pollock DD (2010) Rapid identification of thousands of copperhead snake (*Agkistrodon contortrix*) microsatellite loci

from modest amounts of 454 shotgun genome sequence. *Molecular Ecology Resources* **10**: 341-347.

Castro ALF, Stewart BS, Wilson SG, Hueter RE, Meekan MG, Motta PJ, Bowen BW, Karl SA (2007) Population genetic structure of Earths largest fish, the whale shark (*Rhincodon typus*). *Molecular Ecology* **16**: 5183-5192.

Cavalcanti M (2007) A phylogenetic supertree of the hammerhead sharks (Carcharhiniformes: Sphyrnidae). *Zoological Studies* **46**: 6-11.

Cavalli-Sforza LL, Edwards AWF (1965) Analysis of human evolution. In: *Genetics Today*. Proceedings of the XI International Congress of Genetics, The Hague, The Netherlands, September, 1963 (Geerts SJ, Ed.), Pergamon Press: Oxford. **3**: 923-933 pp.

Chabot CL (2012) Characterization of 11 microsatellite loci for the brown smoothhound shark, *Mustelus henlei*, discovered with next-generation sequencing. *Conservation Genetics Resources* **4**: 23-25.

Chabot CL, Allen LG (2009) Global population structure of the tope (*Galeorhinus galeus*) inferred by mitochondrial control region sequence data. *Molecular Ecology* **18**: 545-552.

Chabot CL, Nigenda S (2011) Characterization of 13 microsatellite loci for the tope shark, *Galeorhinus galeus*, discovered with next generation sequencing and their utility for eastern Pacific smoothhound sharks (*Mustelus*). *Conservation Genetics Resources* **3**: 553-555.

Chapman DD, Wintner SP, Abercrombie DL, Ashe J, Bernard AM, Shivji MS, Feldheim KA (2013) The behavioral and genetic mating system of the sand tiger shark, *Carcharias taurus*, an intrauterine cannibal. *Biology Letters* **9**: 3.

Chenuil A (2006) Choosing the right molecular genetic markers for studying biodiversity: from molecular evolution to practical aspects. *Genetica* **127**: 101-120.

Clarke SC (2004) Understanding pressures on fishery resources through trade statistics: A pilot study of four products in the Chinese dried seafood market. *Fish and Fisheries* **5**: 53-74.

Cockerham CC, Weir BS (1987) Correlations, descent measures: Drift with migration and mutation. *Proceedings of the National Academy of Sciences of the USA* **84**: 8512-8514.

Compagno LJV (1984) An annotated and illustrated catalogue of shark species known to date. Carcharhiniformes. *FAO Fisheries Synopsis*. **4**: 250-655.

Compagno LJV (2000) An overview of chondrichthyans systematics and biodiversity in Southern Africa. *Transactions of the Royal Society of South Africa* **54**: 75-120.

Compagno L, Dando M, Fowler S (2005) *Sharks of the World*. Princeton: Princeton University Press. 480 pp.

Compagno LJV, Ebert DA, Smale MJ (1989) *Guide to the sharks and rays of southern Africa*. Cape Town: Struik Publishers. 158 pp.

Corander J, Siren J, Arjas E (2008) Bayesian spatial modeling of genetic population structure. *Computational Statistics* **23**: 111-129.

Cornuet JM, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data.*Genetics* **144**: 2001-2014.

Corrigan S, Beheregaray LB (2009) A recent shark radiation: molecular phylogeny, biogeography and speciation of wobbegong sharks (family: Orectolobidae). *Molecular Phylogenetics and Evolution* **52**: 205-216.

Cortés E (1999) Standardized diet compositions and trophic levels of sharks. *ICES Journal of Marine Science: Journal du Conseil* **56**: 707-717.

Costa M, Fernandes C, Rodrigues M, Santos-Reis M, Bruford MW (2012) A panel of microsatellite markers for genetic studies of European polecats (*Mustela putorius*) and ferrets (*Mustela furo*). *European Journal of Wildlife Research* **58**: 629-633.

Darriba D, Taboada GL, Doallo R, Posada D (2012) JMODELTEST 2: more models, new heuristics and parallel computing. *Nature Methods* **9**: 772.

Da Silva C (2007) The smoothhound shark (*M. mustelus*) fishery: status and prognosis. Unpublished M.Sc. thesis. Department of Ichthyology and Fisheries Science, Rhodes University: South Africa.

Da Silva C, Bürgener M (2007) South Africa's demersal shark meat harvest. *Traffic Bulletin* **21**: 55-65.

Da Silva C, Kerwath SE, Attwood CG, Thorstad EB, Cowley PD, Økland F, Wilke CG, Næsje TF (2013) Quantifying the degree of protection afforded by a no-take marine reserve on an exploited shark. *African Journal of Marine Science* **35**: 57-66.

Daly-Engel TS, Grubbs RD, Feldheim KA, Bowen BW, Toonen RJ (2010) Is multiple mating beneficial or unavoidable? Low multiple paternity and genetic diversity in the shortspine spurdog *Squalus mitsukurii*. *Marine Ecology Progress Series* **403**: 255-267.

Daly-Engel TS, Seraphin KD, Holland KN , Coffey JP, Nance HA, Toonen RT, Bowen BW (2012) Global phylogeography with mixed marker analysis reveals male-mediated dispersal in the endangered scalloped hammerhead shark (*Sphyrna lewini*). *PLoS ONE* **7**: e29986.

Dawson DA, Horsburgh GJ, Küpper C, Stewart IR, Ball AD, Durrant KL, Burke T (2010) New methods to identify conserved microsatellite loci and develop primer sets of high cross-species utility–as demonstrated for birds. *Molecular Ecology Resources* **10**: 475-494.

de Biasse MB, Nelson BJ, Hellberg ME (2014) Evaluating summary statistics used to test for incomplete lineage sorting: mito-nuclear discordance in the reef sponge *Callyspongia vaginalis*. *Molecular Ecology* **23**: 225-238.

de Bruyn PJN, Tosh CA, Terauds A (2013) Killer whale ecotypes: is there a global model? *Biological Reviews* **88**: 62-80.

Department of Agriculture, Forestry and Fisheries (DAFF) (2013) South Africa's National Plan of Action for the Conservation and Management of Sharks.

http://www.iotc.org/sites/default/files/documents/2014/02/IOTC-2013-SC16-INF15_-_South_Africa_NPOA-sharks.pdf. [Accessed on 12 January 2014]

di Battista, JD, Feldheim KA, Gruber SH, Hendry AP (2008) Are indirect genetic benefits associated with polyandry? Testing predictions in a natural population of lemon sharks. *Molecular Ecology* **17**: 783-795.

Do C, Waples RS, Peel D, Macbeth GM, Tillett BJ, Ovenden JR (2014) N_EESTIMATOR version 2: re-implementation of software for the estimation of contemporary effective population size (N_E) from genetic data. *Molecular Ecology Resources* **14**: 9-214.

Dobson J (2006) Sharks, wildlife tourism, and state regulation. Tourism in Marine Environment **3**:15-23.

Domingues RR, de Amorim AF, Hilsdorf AWS (2013) Genetic identification of *Carcharhinus* sharks from the southwest Atlantic Ocean (Chondrichthyes: Carcharhiniformes). *Journal of Applied Ichthyology* **29**: 738-742.

Dudgeon CL, Blower DC, Broderick D, Giles JL, Holmes BJ, Kashiwagi T, Krück NC, Morgan JAT, Tillett BJ, Ovenden JR (2012) A review of the application of molecular genetics for fisheries management and conservation of sharks and rays. *Journal of Fish Biology* **80**: 1789-1843.

Dulvy NK, Sadovy Y, Reynolds JD (2003) Extinction vulnerability in marine populations. *Fish and Fisheries* **4**: 25-64.

Duncan KM, Martin AP, Bowen BW, de Couet GH (2006) Global phylogeography of the scalloped hammerhead shark (*Sphyrna lewini*). *Molecular Ecology* **15**: 2239-2251.

Durand E, Jay F, Gaggiotti OE, François O (2009) Spatial inference of admixture proportions and secondary contact zones. *Molecular Biology and Evolution* **26**: 1963-1973.

Earl DA, vonHoldt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources* **4**: 359-361.

Ebert DA, Stehmann MFW (2013) *Sharks, batoids, and chimaeras of the North Atlantic*. FAO Species Catalogue for Fishery Purposes. FAO: Rome. **7**: 523 pp.

Edwards CJ, Soulsbury CD, Statham MJ, Ho SYW, Wall D, Dolf D, Iossa G, Baker PJ, Harris S, Sacks BN, Bradley DG (2012) Temporal genetic variation of the red fox, *Vulpes vulpes*, across western Europe and the British Isles. *Quaternary Science Reviews* **57**: 95-104.

Eitner BJ (1995) Systematics of the genus *Alopias* (Laminiformes: Alopiidae) with evidence for the existence of an unrecognised species. *Copeia* **1**: 562-571.

Epperson BK (2005) Mutation at high rates reduces spatial structure within populations. *Molecular Ecology* **14**: 703-710.

Estoup A, Jarne P, Cornuet JM (2002) Homoplasy and mutation model at microsatellite loci and their consequences for population genetics analysis. *Molecular Ecology* **11**: 1591-1604.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* **14**: 2611-2620.

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

Excoffier L (2004) Patterns of DNA sequence diversity and genetic structure after a range expansion: lessons from the infinite-island model. *Molecular Ecology* **13**: 853-864.

Excoffier L, Hofer T, Foll M (2009) Detecting loci under selection in a hierarchically structured population. *Heredity* **103**: 285-298.

Excoffier L, Laval G, Schneider S (2005) ARLEQUIN version 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1**: 47-50.

Excoffier L, Lischer HEL (2010) ARLEQUIN suite version 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* **10**: 564-567.

Farrell ED, Clarke MW, Mariani S (2009) A simple genetic identification method for northeast Atlantic smoothhound sharks (*Mustelus* spp.). *ICES Journal of Marine Science* **66**: 561-565.

Farrell ED, O'Sullivan N, Sacchi C, Mariani S (2014) Multiple paternity in the starry smoothhound shark *Mustelus asterias* (Carcharhiniformes: Triakidae). *Biological Journal of the Linnean Society* **111**: 119-125.

Fedorka KM, Winterhalter WE, Shaw KL, Brogan WR, Mousseau TA (2012) The role of gene flow asymmetry along an environmental gradient in constraining local adaptation and range expansion. *Journal of Evolutionary Biology* **25**: 1676-1685.

Feldheim KA, Gruber SH, Ashley MV (2001) Population genetic structure of the lemon shark (*Negaprion brevirostris*) in the western Atlantic: DNA microsatellite variation. *Molecular Ecology* **10**: 295-303.

Feldheim KA, Gruber SH, Ashley MV (2004) Reconstruction of parental microsatellite genotypes reveals female polyandry and philopatry in the lemon shark, *Negaprion brevirostris*. *Evolution* **58**: 2332-2342.

Feldheim KA, Gruber SH, DiBattista JD, Babcock EA, Kessel SA, Hendry AP, Pikitch EK, Ashley MV, Chapman DD (2014) Two decades of genetic profiling yields first evidence of natal philopatry and long-term fidelity to parturition sites in sharks. *Molecular Ecology* **23**: 110-117.

Filiz H (2009) Diet composition of smooth-hound, *Mustelus mustelus* (Linnaeus, 1758), in Aegean Sea, Turkey. *Belgian Journal of Zoology* **139**: 81-84.

FitzSimmons NN, Moritz C, Moore S (1995) Conservation and dynamics of microsatellite loci over 300 million years of marine turtle evolution. *Molecular Biology and Evolution* **12**: 432-440.

Fowler SL (2005) *Sharks, rays and chimaeras: the status of the Chondrichthyan fishes: status survey*. Gland, Switzerland and Cambridge, UK: IUCN. 461 pp.

Francis MP (1988) Movement patterns of rig (*Mustelus lenticulatus*) tagged in southern New Zealand. *New Zealand Journal of Marine and Freshwater Research* **22**: 259-272.

François O, Durand E (2010) The state of the art - Spatially explicit Bayesian clustering models in population genetics. *Molecular Ecology Resources* **10**: 773-784.

Frankham R (2005) Genetics and extinction. *Biological Conservation* **126**: 131-140.

Frankham R, Ballou JD, Briscoe DA (2003) *Introduction to conservation genetics*. Cambridge: Cambridge University Press UK. 617 pp.

Franklin IR (1980) Evolutionary change in small populations. In *Conservation Biology: An Evolutionary–Ecological Perspective* (Soule ME, Wilcox BA, Eds.), Sunderland, MA: Sinauer Associates. 135-150 pp.

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

Funk WC, McKay JK, Hohenlohe PA, Allendorf FW (2012) Harnessing genomics for delineating conservation units. *Trends in Ecology and Evolution* **27**: 489-496.

Gallagher AJ, Hammerschlag N (2011) Global shark currency: the distribution, frequency, and economic value of shark ecotourism. *Current Issues in Tourism* **8**: 797-812.

Geraghty PT, Williamson JE, Macbeth WG, Wintner SP, Harry AV, Ovenden JR, Gillings MR (2013) Population Expansion and Genetic Structure in *Carcharhinus brevipinna* in the Southern Indo-Pacific. *PLoS ONE* **8**: e75169.

Garza JC, Williamson EG (2001) Detection of reduction in population size using data from microsatellite loci. *Molecular Ecology* **10**: 305-318.

Gascuel D, Labrosse P, Meissa B, Taleb-Sidi MO, Guénette S (2007) Decline of demersal resources in North-West Africa: an analysis of Mauritanian trawl-survey data over the past 25 years. *African Journal of Marine Science* **29**: 331-345.

Gell GR, Roberts CM (2003) Benefits beyond boundaries: the fishery effects of marine reserves. *Trends in Ecology and Evolution* **18**: 448-455.

Girard P, Angers B (2008) Assessment of power and accuracy of methods developed for detection and frequency-estimation of null alleles. *Genetica* **134**: 187-197.

Giresi, MM, Grubbs RD, Portnoy DS, Gold JR (2013) A morphological key to distinguish among smoothhound sharks (genus *Mustelus*) in the Gulf of Mexico. *Gulf and Caribbean Fisheries Institute* **65**: 143-146.

Giresi M, Renshaw MA, Portnoy DS, Gold JR (2012) Isolation and characterization of microsatellite markers for the dusky smoothhound shark, *Mustelus canis*. *Conservation Genetics Resources* **4**: 101-104.

Glenn TC, Stephan W, Braun MJ (1999) Effects of a population bottleneck on whooping crane mitochondrial DNA variation. *Conservation Biology* **13**: 1097-1107.

Grant W, Bowen B (1998) Shallow population histories in deep evolutionary lineages of marine fishes: insights from sardines and anchovies and lessons for conservation. *Journal of Heredity* **89**: 415-426.

Grant WS, Liu M, Gao T, Yanagimoto T (2012) Limits of Bayesian skyline plot analysis of mtDNA sequences to infer historical demographies in Pacific herring (and other species). *Molecular Phylogenetics and Evolution* **65**: 203-212.

Griffiths AM, Casane D, McHugh M, Wearmouth VJ, Sims DW, Genner MJ (2011) Characterisation of polymorphic microsatellite loci in the small-spotted catshark (*Scyliorhinus canicula* L.). *Conservation Genetics Resources* **3**: 705-709.

Griffiths CL, Robinson TB, Lange L, Mead A (2010) Marine biodiversity in South Africa: an evaluation of current states of knowledge. *PLoS One* **5**: e12008.

Grogan E, Lund R (2004) The origin and relationships of early chondrichthyes. In *Biology of Sharks and their Relatives* (Carrier J, Musick J, Heithaus M, Eds.), Washington D.C: CRC Press. 3-31 pp.

Gubili C, Johnson R, Gennari E, Oosthuizen WH, Kotze D, Meÿer M, Sims DW, Jones CS, Noble LR (2009) Concordance of genetic and fin photo identification in the great white shark, *Carcharodon carcharias*, off Mossel Bay, South Africa. *Marine Biology* **156**: 2199-2207.

Guichoux E, Lagache L, Wagner S, Chaumeil P, Léger P, Lepais O, Lepoittevin C, Malaus T, Revardel E, Salin F, Petit RJ (2011) Current trends in microsatellite genotyping. *Molecular Ecology Resources* **11**: 591-611.

Guillot G, Estoup A, Mortier F, Cosson JF (2005) A spatial statistical model for landscape genetics. *Genetics* **170**: 1261-1280.

Hall A (1999) BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95-98.

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

Hasegawa M, Kishino H, Yano T (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA**.** *Journal of Molecular Evolution* **22**: 160-174.

Heemstra PC (1973) *A Revision of the Shark Genus Mustelus (Squaliformes: Carcharhinidae)*. University of Miami: Coral Gables, Florida. 187 pp.

Heist EJ (2004*a*). Genetics of sharks, skates and rays. In *Biology of Sharks and Their Relatives* (Carrier JC, Musick JA, Heithaus MR, Eds.). Boca Raton, FL: CRC Press. 471-485 pp.

Heist EJ (2004*b*) Genetics: stock identification. In *Elasmobranch Fisheries Management Techniques* (Musick JA, Bonfil R, Eds.). Singapore: Asia-Pacific Economic Cooperation. 79-96 pp.

Heist EJ, Gold JR (1999*a*) Microsatellite DNA variation in sandbar sharks (*Carcharhinus plumbeus*) from the Gulf of Mexico and Mid-Atlantic Bight. *Copeia* **1999**: 182-186.

Heist EJ, Gold JR (1999*b*) Genetic identification of sharks in the US Atlantic large coastal shark fishery. *Fishery Bulletin* **97**: 53-61.

Heithaus MR, Frid A, Wirsing AJ, Worm B (2008) Predicting ecological consequences of marine top predator declines. *Trends in Ecology and Evolution* **23**: 202-210.

Heller R, Chikhi L, Siegismund HR (2013) The confounding effect of population structure on Bayesian skyline plot inferences of demographic history. *PLoS One* **8**: e62992.

Hendry AP, Taylor EB, McPhail JD (2002) Adaptive divergence and the balance between selection and gene flow: lake and stream stickleback in the Misty system. *Evolution* **56**: 1199-1216.

Ho SYW, Lanfear R, Bromham L, Phillips MJ, Soubrier J, Rodrigo AG, Cooper A (2011) Time-dependent rates of molecular evolution. *Molecular Ecology* **20**: 3087-3101.

Hoelzel AR, Shiviyi MS, Magnussen J, Francis MP (2006) Low worldwide genetic diversity in the basking shark. *Biology Letters* **2**: 630-642.

Hoffman JI, Amos W (2005) Microsatellite genotyping errors: detection, approaches, common sources and consequences for paternal exclusion. *Molecular Ecology* **14**: 599-612.

Hoffman JI, Nichols HJ (2011) A novel approach for mining polymorphic microsatellite markers *in silico*. *PLoS One* **6**: e23283.

Holsinger KE, Weir BS (2009) Genetics in geographically structured populations: Defining, estimating and interpreting F_{ST}. *Nature Reviews Genetics* **10**: 639-650.

Hueter RE, Heupel MR, Heist EJ, Keeney DB (2005) The implications of philopatry in sharks for the management of shark fisheries. *Journal of Northwest Atlantic Fishery Science* **35**: 239-247.

Human BA (2007) A taxonomic revision of the catshark genus *Haploblepharus* Garman 1913 (Chondrichthyes: Carcharhiniformes: Scyliorhinidae). *Zootaxa* **1451**: 1-40.

Human BA, Owen EP, Compagno LJV, Harley EH (2006) Testing morphologically based phylogenetic theories within the cartilaginous fishes with molecular data, with special reference to the catshark family (Chondrichthyes; Scyliorhinidae) and the interrelationships within them. *Molecular Phylogenetics and Evolution* **39**: 384-391.

Hutchings L, van der Lingen CD, Shannon LJ, Crawford RJM, Verheye HMS, Bartholomae CH, van der Plas AK, Louw D, Kreiner A, Ostrowski M, Fidel Q, Barlow RG, Lamont T, Coetzee J, Shillington F, Veitch J, Currie JC, Monteiro PMS (2009) The Benguela Current: An ecosystem of four components. *Progress in Oceanography* 83: 15-32.

Iglésias SP, Lecointre G, Sellos DY (2005) Extensive paraphylies within sharks of the order carcharhiniformes inferred from nuclear and mitochondrial genes. *Molecular Phylogenetics and Evolution* **34**: 569-583.

Jakobsson M, Rosenberg NA (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* **23**: 1801-1806.

Kalinowski ST (2005) HP-RARE 1.0: a computer program for performing rarefaction on measures of allelic richness. *Molecular Ecology Notes* **5**: 187-189.

Kalinowski ST (2008) TREEFIT. A computer program for evaluating how well evolutionary trees fit genetic distance data. http://www.montana.edu/kalinowski/Software/TreeFit.htm.

Kalinowski ST (2009) How well do evolutionary trees describe genetic relationships between populations? *Heredity* **102**: 506-513.

Kang J, Park J, Jo H (2012) Rapid development of microsatellite markers with 454 pyrosequencing in a vulnerable fish, the mottled skate, *Raja pulchra*. *International Journal of Molecular Science* **13**: 7199-7211.

Karaiskou N, Buggiotti L, Leder E, Primmer CR (2008) High degree of transferability of 86 newly developed zebra finch EST-linked microsatellite markers in 8 bird species. *Journal of Heredity* **99**: 688-693.

Karl SA (2008) The effect of multiple paternity on the genetically effective size of a population. *Molecular Ecology* **17**:3973-3977.

Karl SA, Castro ALF, Lopez JA, Charvet P, Burgess GH (2011) Phylogeography and conservation of the bull shark (*Carcharhinus leucas*) inferred from mitochondrial and microsatellite DNA. *Conservation Genetics* **12**: 371-382.

Keeney DB, Heist EJ (2006) Worldwide phylogeography of the blacktip shark (*Carcharhinus limbatus*) inferred from mitochondrial DNA reveals isolation of western Atlantic populations coupled with recent Pacific dispersal. *Molecular Ecology* **15**: 3669-3679.

Kerwath SE, Thorstad EB, Næsje TF, Cowley PD, Økland F, Wilke C, Attwood CG (2009) Crossing invisible boundaries: the effectiveness of the Langebaan lagoon marine protected area as a harvest refuge for a migratory fish species in South Africa. *Conservation Biology* **23**: 653-661.

Kerwath SE, Winker H, Gotz A, Attwood CG (2013) Marine protected area improves yield without disadvantaging fishers. *Nature Communications* **4**: 2347.

Khallahi B (2004). Ecologie et biologie de l'émissole lisse *Mustelus mustelus* (Linné, 1758) sur les côtes de Mauritanie. PhD Thesis, Université de Bretagne Occidentale, Brest, France (As cited by Saïdi *et al*. 2008).

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**: 111-120.

Kimura M, Weiss GH (1964) Stepping stone model of population structure + decrease of genetic correlation with distance. *Genetics* **49**: 561-576.

Klopper AW (2005) Intraspecific genetic variation in the percoid teleosts *Argyrosomus japonicus* (Temminck and Schlegel, 1843) and *Pomadasys commersonnii* (Lacepède, 1801) as inferred from the mitochondrial control region. Unpublished M.Sc. thesis. University of Pretoria: South Africa.

Kroese M, Sauer W, Penney A (1995) An overview of shark catches and bycatches in South African fisheries. *Collective Volume of Scientific Papers-International Commission for the Conservation of Atlantic Tunas* **45**: 318-328.

Lack M, Sant G (2011). *The future of sharks: A review of action and inaction*. Cambridge and Washington: TRAFFIC International and the Pew Environment Group.

Lage CR, Petersen CW, Forest D, Barnes D, Kornfield I, Wray C (2008) Evidence of multiple paternity in spiny dogfish (*Squalus acanthias*) broods based on microsatellite analysis. *Journal of Fish Biology* **73**: 2068-2074.

Langella O (1999) POPULATIONS 1.2.28 (12/5/2002): a population genetic software.Montpellier: Laboratoire Populations, génétique et evolution; CNRS UPR9034. Available at http://bioinformatics.org/~tryphon/populations/.

Laroche RK, Kock AA, Dill LM, Oosthuizen WH (2007) Effects of provisioning ecotourism activity on the behaviour of white sharks *Carcharodon carcharias*. *Marine Ecology Progress Series* **338**: 199-209.

Lees R (1969) *Fishing for Fortunes: The Story of the Fishing Industry in Southern Africa—and the Men who Made It*. Cape Town and London: Purnell.

Librado P, Rozas J (2009) DnaSP version 5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**:1451-1452.

Lim DD, Motta P, Mara K, Martin AP (2010) Phylogeny of hammerhead sharks (Family Sphyrnidae) inferred from mitochondrial and nuclear genes. *Molecular Phylogenetics and Evolution* **55**: 572-579.

Linck HF (1790) Versuch einter Eintheilung der Fische nach den Zähnen. *Magazin für das Neueste aus der Physik und Naturgeschichte* **6**: 28-38.

Linnaeus C (1758) *Systema Naturae per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis*. Editio decima, reformata. Laurentius Salvius: Holmiae **2**: 824 pp.

Litt M, Luty JA (1989) A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. *American Journal of Human Genetics* **44**: 397-401.

Liu Z (2007) Chapter 5 Microsatellite Markers and Assessment of Marker Utility. In *Aquaculture Genome Technologies* (Liu Z, Ed.). Blackwell Publishing Ltd, Oxford, UK. 43-58 pp.

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

Lombard AT (2004) Marine component of the National Spatial Biodiversity Assessment for the development of South Africa's National Biodiversity Strategic and Action Plan. National Botanical Institute. 101 pp.

López JA, Ryburn JA, Fedrigo O, Naylor GJP (2006) Phylogeny of sharks of the family Triakidae (Carcharhiniformes) and its implications for the evolution of Carcharhiniform placental viviparity. *Molecular Phylogenetics and Evolution* **40**: 50-60.

Louis M, Viricel A, Lucas T, Peltier H, Alfonsi E, Berrow S, Brownlow A, Covelo P, Dabin W, Deaville R, de Stephanis R, Gally F, Gauffier P, Penrose R, Silva MA, Guinet C, Simon-Bouhet B (2014) Habitat-driven population structure of bottlenose dolphins, *Tursiops truncatus*, in the North-East Atlantic. *Molecular Ecology* **23**: 857-874.

Lowe WH, Allendorf FW (2010) What can genetics tell us about population connectivity? *Molecular Ecology* **19**: 3038-3051.

Lowry DB (2012) Ecotypes and the controversy over stages in the formation of new species. *Biological Journal of the Linnean Society* **106**: 241-257.

Luikart G, Allendorf FW, Cournuet JM, Sherwin WB (1998) Distortion of allele frequency distributions provides a test for recent population bottlenecks. *Journal of Heredity* **89**: 238-247.

Luikart G, Cornuet JM (1998) Empirical evaluation of a test for identifying recently bottlenecked populations from allele frequency data. *Conservation Biology* **12**: 228-237.

Maisey JG (1984) Higher elasmobranch phylogeny and biostratigraphy. *Zoological Journal of the Linnaean Society* **82**: 33-54.

Maisey JG (2012) What is an 'elasmobranch'? The impact of palaeontology in understanding elasmobranch phylogeny and evolution. *Journal of Fish Biology* **80**: 918-951.

Maisey JG, Naylor GJP, Ward DJ (2004) Mesozoic elasmobranchs, neoselachian phylogeny and the rise of modern elasmobranch diversity. In *Mesozoic Fishes 3* (Arratia G, Tintori A, Eds.), Munich: Verlag F. Pfeil. 17-56 pp.

Manly BFJ (1985) The statistics of natural selection. In *Spurious Test Results Due to Isolation By Distance*. Chapman and Hall: London. 186-95 pp.

Mann BQ, Bullen EM (2009) *ORI/WWF-SA Tagging Project: summary of tag and recapture data for smooth houndsharks (Mustelus mustelus) caught along the southern African coast from 1984-2008*. Durban: Oceanographic Research Institute.

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

Martin AP (1995) Mitochondrial DNA sequence evolution in sharks: rates, patterns and phylogenetic inferences. *Molecular Biology and Evolution* **12**: 1114-1123.

Martin AP, Naylor GJP, Palumbi SR (1992) Rates of mitochondrial DNA are slow in sharks compared to mammals. *Nature* **357**: 153-155.

Martin AP, Pardini AT, Noble LR, Jones CS (2002) Conservation of a dinucleotide simple sequence repeat locus in sharks. *Molecular Phylogenetics and Evolution* **23**: 205-213.

McCord ME (2005) Aspects of ecology and management of the soupfin shark (*Galeorhinus galeus*) in South Africa. Unpublished MSc thesis. Department of Ichthyology and Fisheries Science. Rhodes University: South Africa.

McGregor C (1991) Sharks in Crisis. Australian Fisheries. 10-16 pp.

Meirmans PG, Hedrick PW (2011) Assessing population structure: F_{ST} and related measures. *Molecular Ecology Resources* **11**: 5-18.

Mendonça FF, Oliveira C, Gadig OBF, Foresti F (2013) Diversity and genetic population structure of the Brazilian sharpnose shark *Rhizoprionodon lalandii*. *Aquatic Conservation: Marine and Freshwater Ecosystems* **23**: 850-857.

Miller DE, Yates RJ, Jeradino A, Parkington JE (1995) Late Holocene coastal change in the southwestern Cape, South Africa. *Quaternary International* **29/30**: 3-10.

Mobley KB, Amundsen T, Forsgren E, Svensson PA, Jones AG (2009) Multiple mating and low incidence of cuckoldry for nest-holding males in the two-spotted goby, *Gobiusculus flavescens*. *BMC Evolutionary Biology* **9**: 6.

Mourier J, Mills SC, Planes S (2013) Population structure, spatial distribution and life-history traits of blacktip reef sharks *Carcharhinus melanopterus*. *Journal of Fish Biology* **82**: 979-993.

Murray BW, Wang JY, Yang SC, Stevens JD, Fisk A, Svavarsson J (2008) Mitochondrial cytochrome *b* variation in sleeper sharks (Squaliformes: Somniosidae). *Marine Biology* **153**: 1015-1022.

Musick JA, Burgess GH, Camhi M, Cailliet G, Fordham S (2000) Management of sharks and their relatives (Elasmobranchii). *Fisheries* **25**: 9-13.

Myers RA, Worm B (2003) Rapid worldwide depletion of predatory fish communities. *Nature* **423**: 280-283.

Namibia's National Plan of Action to prevent, deter and eliminate illegal, unreported and unregulated fishing.http://209.88.21.36/opencms/export/sites/default/grnnet/MFMR/downloads/docs/Namibia_NPOA_IUU_Final.pdf. [Accessed 7 March 2014]

Nance H, Klimley P, Galvan-Magana F, Martinez-Ortiz J, Marko PB (2011) Demographic processes underlying subtle patterns of population structure in the scalloped hammerhead shark, *Sphyrna lewini*. *PLoS One* **6**: e21459.

Navigating Global Shark Conservation: Current Measures and Gaps. http://www.pewenvironment.org/uploadedFiles/PEG/Publications/Report/Navigating%20Global%20Shark%20Conservation_Current%20Measures%20and%20Gaps%207%206%2012.pdf [Accessed on 7 March 2014]

Naylor GJP, Caira JN, Jensen K, Rosana KAM, White WT, Last PR (2012) A DNA sequence based approach to the identification of shark and ray species and its implications for global elasmobranch diversity and parasitology. *Bulletin of the American Museum of Natural History* **367**: 262 pp.

Nei M (1977) *F*-statistics and analysis of gene diversity in subdivided populations. *Annals of Human Genetics* **41**: 225-233.

Nei M, Maruyama T, Chakraborty R (1975) The bottleneck effect and genetic variability in populations. *Evolution* **29**: 1-10.

Nei M, Tajima F, Tateno Y (1983) Accuracy of estimated phylogenetic trees from molecular data. *Journal of Molecular Evolution* **19**: 153-170.

Nielsen EE, Hemmer-Hansen J, Larsen PF, Bekkevold D (2009) Population genomics of marine fishes: identifying adaptive variation in space and time. *Molecular Ecology* **18**: 3128-3150.

Nosil P, Funk DJ, Ortiz-Marrientos D (2009) Divergent selection and heterogeneous genomic divergence. *Molecular Ecology* **18**: 375-402.

O'Brien SM, Gallucci VF, Hauser L (2013) Effects of species biology on the historical demography of sharks and their implications for likely consequences of contemporary climate change. *Conservation Genenetics* **14**:125-144.

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

Oosthuizen CJ (2007) Genetic variation within the Cape Stumpnose, *Rhabdosargus holubi* Steindachner (Teleostei: Sparidae). MSc thesis. University of Pretoria: South Africa.

Orams MB (2002) Feeding wildlife as a tourism attraction: a review of issues and impacts. *Tourism Management* **23**: 281-293.

Ovenden JR (1990) Mitochondrial DNA and marine stock assessment: A review. *Austrailian Journal of Marine and Freshwater Research* **41**: 835-853.

Ovenden JR (2013) Crinkles in connectivity: combining genetics and other types of data to estimate movement and interbreeding between populations. *Marine and Freshwater Research* **64**: 201-207.

Ovenden JR, Berry O, Welch DJ, Buckworth RC, Dichmont CM (2013) Ocean's eleven: a critical evaluation of the role of population, evolutionary and molecular genetics in the management of wild fisheries. *Fish and Fisheries* **15**: 1-35.

Ovenden JR, Kashiwagi T, Broderick D, Giles J, Salini J (2009) The extent of population genetic subdivision differs among four co-distributed shark species in the Indo-Australian archipelago. *BMC Evolutionary Biology* **9**: 40.

Ozerov MY, Veselov AE, Lumme J, Primmer CR (2013) Temporal variation of genetic composition in Atlantic salmon populations from the Western White Sea Basin: influence of anthropogenic factors? *BMC Genetics* **14:** 88.

Page RD (2002) Visualizing phylogenetic trees using TreeView. *Current Protocols in Bioinformatics* **6**: 1-15.

Palstra FP, Ruzzante DE (2008) Genetic estimates of contemporary effective population size: what can they tell us about the importance of genetic stochasticity for wild population persistence? *Molecular Ecology* **17**: 3428-3447.

Pank M, Shivji MS, Stanhope M, Natanson L, Kohler N (2001) Rapid and simultaneous identification of body parts from the morphologically similar sharks *Carcharhinus obscurus* and *Carcharhinus plumbeus* (Carcharhinidae) using multiplex PCR. *Marine Biotechnology* **3**: 231-240.

Pardini AT, Jones CS, Noble LR, Kreiser B, Malcolm H, Bruce BD, Stevens JD, Cliff G, Scholl MC, Francis M, Duffy CAJ, Martin AP (2001) Sex-biased dispersal of great white sharks – in some respects, these sharks behave more like whales and dolphins than other fish. *Nature* **412**: 139-140.

Park S (2001) The Excel microsatellite toolkit. http://animalgenomics.ucd.ie/sdepark/ms-toolkit [Downloaded on 27 February 2012]

Parsons GR (1993) Geographic variation in reproduction between two populations of the bonnethead shark, *Sphyrna tiburo*. *Environmental Biology of Fishes* **38**: 25-35.

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6**: 288-295.

Peakall R, Smouse PE (2012) GENALEX 6.5: genetic analysis in Excel: population genetic software for teaching and research – an update. *Bioinformatics* **28**: 2537-2539.

Peeters FJC, Acheson R, Brummer GJA, de Ruijter WPM, Schneider RR, Ganssen GM, Ufkes E, Kroon D (2004) Vigorous exchange between the Indian and Atlantic oceans at the end of the past five glacial periods. *Nature* **430**: 661-665.

Peltier WR (1988) Lithospheric thickness, Antarctic deglaciation history, and ocean basin discretization effects in the global model of post-glacial sea-level change: A summary of some sources of nonuniqueness. *Quaternary Research* 29: 93-112.

Pereyra S, Garcia G, Miller P, Oviedo S, Domingo A (2010). Low genetic diversity and population structure of the narrownose shark (*Mustelus schmitti*). *Fisheries Research* **106**: 468-473.

Pérez-Jiménez JC, Rocha-Olivares A, Sosa-Nishizaki O (2013) Morphological and molecular differentiation of smoothhound sharks (Genus *Mustelus*, Family Triakidae) from the Gulf of California. *Journal of Applied Ichthyology* **29**: 268-270.

Perry AL, Low PJ, Ellis JR, Reynolds JD (2005) Climate change and distribution shifts in marine fishes. *Science* **308**: 1912.

Petersen SL, Honig MB, Ryan PG, Underhill LG, Compagno LJV (2008) Pelagic shark bycatch in the pelagic longline fishery off southern Africa. In *Understanding and Mitigating Vulnerable Bycatch in Southern African Trawl and Longline Fisheries*. (Petersen SL, Nel DC, Ryan PG, Underhill LG, Eds). WWF South Africa Report Series - 2008/Marine/002. 59-79 pp.

Piry S, Luikart G, Cornuet JM (1999) BOTTLENECK: a computer program for detecting recent reductions in the effective population size using allele frequency data. *Journal of Heredity* **90**: 502-503.

Planque B, Fromentin J-M, Cury P, Drinkwater KF, Jennings S, Perry RI, Kifani S (2010) How does fishing alter marine populations and ecosystems sensitivity to climate? *Journal of Marine Systems* **79**: 403-417.

Polzin T, Daneshmand SV (2003) On Steiner trees and minimum spanning trees in hypergraphs. *Operations Research Letters* **31**: 12-20.

Portnoy DS, Heist EJ (2012) Molecular markers: progress and prospects for understanding reproductive ecology in elasmobranchs.*Journal of Fish Biology* **80**: 1120-1140.

Portnoy DS, McDowell JR, Heist EJ, Musick JA, Graves JE (2010) World phylogeography and male-mediated gene flow in the sandbar shark, *Carcharhinus plumbeus*. *Molecular Ecology* **19**: 1994-2010.

Primmer CR, Ellegren H (1998) Patterns of molecular evolution in avian microsatellites. *Molecular Biology and Evolution* **15**: 997-1008.

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

Primmer CR, Painter JN, Koskinen MT, Palo JU, Merilä J (2005) Factors affecting avian cross-species microsatellite amplification. Journal of Avian Biology **36**: 348-360.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics* **155**: 945-959.

Queller DC, Goodnight KF (1989) Estimating relatedness using genetic markers. *Evolution* **43**: 258-275.

Ramírez-Macías D, Vázquez-Juárez R, Galván-Magaña F, Munguía-Vega A (2007) Variations of the mitochondrial control region sequence in whale sharks (*Rhincodon typus*) from the Gulf of California, Mexico. Fisheries Research **84**: 87-95.

Ramsay PJ (1995) 9000 years of sea-level change along the southern African coastline. *Quaternary International* **31**: 71-75.

Raup D, Sepkoski J (1982) Mass extinctions in the marine fossil record. *Science* 215: 1501-1503.

Reich DE, Feldman MW, Goldstein DB (1999) Statistical properties of two tests that use multilocus data sets to detect population expansions. *Molecular Biology and Evolution* **16**: 453-466.

Renon P, Colombo MM, Colombo F, Malandra R, Biondi PA (2001) Computer-assisted evaluation of isoelectric focusing patterns in electrophoretic gels: identification of smoothhounds (*Mustelus mustelus*, *Mustelus asterias*) and comparison with lower value shark species. *Electrophoresis* **22**: 1534-1538.

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

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

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

Rosa MR, Gadig OB (2010) Taxonomic comments and an identification key to species for the Smooth-hound sharks genus *Mustelus* Link, 1790 (Chondrichthyes: Triakidae) from the Western South Atlantic. *Pan-American Journal of Aquatic Sciences* **5**: 401-413.

Rosenberg NA (2004) DISTRUCT: a program for the graphical display of population structure. *Molecular Ecology Notes* **4**: 137-138.

Rousset F (2008) GENEPOP'007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Resources* **8**: 103-106.

Saghai-Maroof MA, Solima KM, Jorgenson RA, Allard RW (1984) Ribosomal DNA spacerlength polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proceedings of the National Academy of Sciences of the USA* **81**: 8014-8018.

Saïdi B, Bradaï MN, Bouaïn A (2008) Reproductive biology of the smoothhound shark *Mustelus mustelus* (L.) in the Gulf of Gabès (south-central Mediterranean Sea). *Journal of Fish Biology* **72**: 1343-1354.

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**: 406-425.

Sauer WJJ, Hecht T, Brits PJ, Mather D (2003) *The Shark Longline Fishery. An Economic and Sectoral Study of the South African Fishing Industry.* Volume 2: Fishery profiles. Report prepared for Marine and Coastal Management by Rhodes University. 293 pp.

Schmidt JV, Schmidt CL, Ozer F, Ernst RE, Feldheim KA, Ashley MV, Levine M (2009) Low genetic differentation across three major ocean populations of the whale shark, *Rhincodon typus*. *PLoS ONE* **4**: e4988.

Schneider S, Excoffier L (1999) Estimation of demographic parameters from the distribution of pairwise differences when the mutation rates vary among sites: Application to human mitochondrial DNA. *Genetics* **152**: 1079-1089.

Schrey AW, Heist EJ (2003) Microsatellite analysis of population structure in the shortfin mako (*Isurus oxyrinchus*). *Canadian Journal of Fisheries and Aquatic Sciences* **60**: 670-675.

Schultz JK, Feldheim KA, Gruber SH, Ashley MV, McGovern TM, Bowen BW (2008) Global phylogeography and seascape genetics of the lemon sharks (genus *Negaprion*). *Molecular Ecology* **17**: 5336-5348.

Schwartz MK, Luikart G, Waples RS (2007) Genetic monitoring as a promising tool for conservation and management. *Trends in Ecology and Evolution* **22**: 25-33.

Sekino M, Hara M (2007) Individual assignment tests proved genetic boundaries in a species complex of Pacific abalone (genus *Haliotis*). *Conservation Genetics* **8**: 823-841.

Selkoe KA, Toonen RJ (2006) Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters* **9**: 615-629.

Serena F (2005) *Field identification guide to the sharks and rays of the Mediterranean and Black Sea*. FAO Species Identification Guide for Fishery Purposes. FAO: Rome. 97 pp.

Serena F, Mancusi C, Clò S, Ellis J, Valenti SV (2009) *Mustelus mustelus*. In *IUCN 2012*. IUCN Red List of Threatened Species. Version 2014.2. http://www.iucnredlist.org [Accessed on 07 June 2014]

Shapiro B, Ho SYW (2014) Ancient hyaenas highlight the old problem of estimating evolutionary rates. *Molecular Ecology* **23**: 499-501.

Sharks Biodiversity Management Plan 2014 Draft (Gazette 37586 General Notice 331). http://www.polity.org.za/article/national-environmental-management-biodiversity-act-biodiversity-management-plan-for-shark-draft-gazette-37586- [Accessed on 12 May 2014]

Shivji M, Clarke S, Pank M, Natanson L, Kohler N, Stanhope M (2002) Genetic identification of pelagic shark body parts for conservation and trade monitoring. *Conservation Biology* **16**: 1036-1047.

Slatkin M (1994) An exact test for neutrality based on the Ewens sampling distribution. *Genetics Research* **64**: 71-74.

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

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

Smale MJ, Compagno LJV (1997) Life history and diet of two southern African smooth-hound sharks, *Mustelus mustelus* (Linnaeus, 1758) and *Mustelus palumbes* Smith, 1957 (Pisces: Triakidae). *South African Journal of Marine Science* **18**: 229-248.

Smith PJ (1986) Low genetic variation in sharks (Chondricthyes). *Copeia* **1986**: 202-207.

Speed CW, Field IC, Meekan MG, Bradshaw CJA (2010) Complexities of coastal shark movements and their implications for management. *Marine Ecology Progress Series* **408:** 275-293.

Stevens JD, Bonfil R, Dulvy NK, Walker PA (2000) The effects of fishing on sharks, rays, and chimaeras (chondrichthyans), and the implications for marine ecosystems. *ICES Journal of Marine Science* **57**: 476-494.

Storz JF (2002) Contrasting patterns of divergence in quantitative traits and neutral DNA markers: analysis of clinal variation. *Molecular Ecology* **11**: 2537-2551.

Storz JF, Beaumont MA (2002) Testing for genetic evidence of population expansion and contraction: an empirical analysis of microsatellite DNA variation using a hierarchical Bayesian model. *Evolution* **56**: 154-166.

Stuttaford M (1995) *Fishing Industry Handbook. South Africa, Namibia and Mozambique*. Stellenbosch: Marine Information cc. 433 pp.

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

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10**: 512-526.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**: 2731-2739.

Taniuchi T, Tachikawa H, Shimizu M, Nose Y (1993) Geographical variations in reproductive parameters of shortspine spurdog in the North Pacific. *Nippon Suisan Gakkaishi* **59**: 45-51.

Tautz D (1989) Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Research* **17**: 6463-6471.

Tavares W, Da Silva-Rodrigues-Filho LF, Sodré D, Souza RFC, Schneider H, Sampaio I, Vallinoto M (2013) Multiple substitutions and reduced genetic variability in sharks. *Biochemical Systematics and Ecology* **49**: 21-29.

Techera EJ, Klein N (2013) The role of law in shark-based eco-tourism: lessons from Australia. *Marine Policy* **39**: 21-28.

Teh LCL, Sumaila UR (2013) Contribution of marine fisheries to worldwide employment. *Fish and Fisheries* **14**:77-88.

Teske PR, Papadopoulos I, Barker NP, McQuaid CD, Beheregaray LB (2014) Mitonuclear discordance in genetic structure across the Atlantic/Indian Ocean biogeographical transition zone. *Journal of Biogeography* **41**: 392-401.

Teske PR, von der Heyden S, McQuaid CD, Barker NP (2011) A review of marine phylogeography in southern Africa. *South African Journal of Science* **107**: 43-53.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673-4680.

Tillett BJ, Field IC, Bradshaw CJA, Johnson G, Buckworth RC, Meekan MG, Ovenden RG (2012*c*) Accuracy of species identification by fisheries observers in a north Australian shark fishery. *Fisheries Research* **127**: 109-115.

Tillett BJ, Meekan MG, Broderick D, Field IC, Cliff G, Ovenden JR (2012*a*) Pleistocene isolation, secondary introgression and restricted contemporary gene flow in the pigeye shark, *Carcharhinus amboinensis* across northern Australia. *Conservation Genetics* **13**: 99-115.

Tillett BJ, Meekan MG, Field IC, Thorburn DC, Ovenden JR (2012*b*) Evidence for reproductive philopatry in the bull shark *Carcharhinus leucas*. *Journal of Fish Biology* **80**: 2140-2158.

Urian KW, Hofmann S, Wells RS, Read AJ (2009) Fine-scale population structure of bottlenose dolphins (*Tursiops truncatus*) in Tampa Bay, Florida. *Marine Mammal Science* **25**: 619-638.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICROCHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* **4**: 535-538.

Van Zyl K (1992) Marine Products: Die eerste 50 jaar. Goodwood, Cape Town: Nasionale Boekdrukkery (As cited by Kroese and Sauer 1998). 279 pp.

Veríssimo A, McDowell JR, Graves JE (2010) Global population structure of the spiny dogfish *Squalus acanthias*, a temperate shark with an antitropical distribution. *Molecular Ecology* **19**: 1651-1662.

Veríssimo A, McDowell JR, Graves JE (2011). Population structure of a deep-water squaloid shark, the Portuguese dogfish (*Centroscymnus coelolepis*). *ICES Journal of Marine Science* **68**: 555-563.

Veríssimo A, McDowell JR, Graves JE (2012). Genetic population structure and connectivity in a commercially exploited and wide-ranging deepwater shark, the leafscale gulper (*Centrophorus squamosus*). *Marine and Freshwater Research* **63**: 505-512.

Vignaud T, Clua E, Mourier J, Maynard J, Planes S (2013) Microsatellite analyses of blacktip reef sharks (*Carcharhinus melanopterus*) in a fragmented environment show structured clusters. *PLoS One* **8**: e61067.

Vignaud TM, Maynard JA, Leblois R, Meekan MG, Vázquez-Juárez R, Ramírez-Macías D, Pierce S J, Rowat D, Berumen ML, Beeravolu C, Baksay S, Planes S (2014) Genetic structure of populations of whale sharks among ocean basins and evidence for their historic rise and recent decline. *Molecular Ecology* **23**: 2590-2601.

Wahlund S (1928) Zusammensetzung von Populationen und Korrelationsers-chinungen von Standpunkt der Vererbungslehre aus betrachtet. *Hereditas* **11**: 65-108. English translation: In *Demographic genetics*. (Weiss KM, Ballanoff PA, Eds.), Dowden, Hutchinson and Ross Inc, Stroudsburg: Pennsylvania, USA. 224-263 pp.

Walker TI (1998) Can shark resources be harvested sustainably? A question revisited with a review of shark fisheries. *Marine and Freshwater Research* **49**: 553-572.

Waples RS, Do C (2010) Linkage disequilibrium estimates of contemporary N_E using highly variable genetic markers: a largely untapped resource for applied conservation and evolution. *Evolutionary Applications* **3**: 244-262.

Waples RS, Punt AE, Cope JM (2008) Integrating genetic data into management of marine resources: how can we do it better? *Fish and Fisheries* **9**: 423-449.

Ward RD, Holmes BH, White WT, Last PR. (2008) DNA barcoding Australian chondrichthyans: results and potential uses in conservation. *Marine and Freshwater Research* **59**: 57-71.

Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PDN (2005) DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society B* **360**: 1847-1857.

Warman G (2004). *Fishing Industry Handbook. South Africa, Namibia, Mozambique*. Stellenbosch: George Warman Publications. 505 pp.

Weaver LA, Lundstrom RC, Colbert AA (1999) Identification of shark species by polyacrylamide gel isoelectric focusing of sarcoplasmic proteins. *Journal of AOAC International* **82**: 1163-1169.

Weber JL, Wong C (1993) Mutation of human short tandem repeats. *Human Molecular Genetics* **2**: 1123-1128.

White WT, Last PR (2006) Description of two new species of smoothhounds, *Mustelus widodoi* and *M. ravidus* (Carcharhiniformes: Triakidae) from the western central Pacific. *Cybium* **30**: 235-246.

White WT, Last PR (2008) Description of two new species of gummy sharks, genus *Mustelus* (Carcharhiniformes: Triakidae), from Australian waters. In *Descriptions of New Australian Chondrichthyans (*Last PR, White WT, Pogonoski JJ, Eds.), CSIRO: Hobart. 189-202 pp.

Whitehead PJP, Bauchot ML, Hureau JC, Nielsen J, Tortonese E (Eds.) (1984) *Fishes of the North-eastern Atlantic and the Mediterranean*. UNESCO: Paris. **1**: 1473 pp.

Whitfield AK, Baliwe NG (2013) A numerical assessment of research outputs on South African estuaries. *South African Journal of Science* **109**: 01-04.

Williamson-Natesan EG (2005) Comparison of methods for detecting bottlenecks from microsatellite loci. *Conservation Genetics* **6**: 551-562.

Wilson AS, Marra PP, Fleischer RC (2012) Temporal patterns of genetic diversity in Kirtland's warblers (*Dendroica kirtlandii*), the rarest songbird in North America. *BMC Ecology* **12**: 8.

Worm B, Davis B, Kettemer L, Ward-Paige CA, Chapman D, Heithaus MR, Kessel ST, Gruber SH (2013) Global catches, exploitation rates, and rebuilding options for sharks. *Marine Policy* **40**: 194-204.

Wright S (1931) Evolution in Mendelian populations. *Genetics* **16**: 97-159.

Wright S (1943) Isolation by distance. *Genetics* **28**: 139-156.

Wright S (1951) The genetical structure of populations. *Annals of Eugenics* **15**: 323-354.

Yamaguchi A, Taniuchi T, Shimizu M (2000) Geographic variations in reproductive parameters of the starspotted dogfish, *Mustelus manazo*, from five localities in Japan and in Taiwan. *Environmental Biology of Fishes* **57**: 221-233.

Zane L, Bargelloni L, Patarnello T (2002) Strategies for microsatellite isolation: a review. *Molecular Ecology* **11**: 1-16.

Appendix A

Supplementary Information for Chapter 2

Table S2.1 The *ND2* sequence information of the study taxa used to estimate the genetic distance to evaluate cross-species performance, including ID verified, availability of images (yes or no), specimen identifier (GN No.) which are available in the on-line host specimen database (http://elasmobranchs.tapewormdb.uconn.edu) and GenBank accession numbers

Genus	Species	ID Verified	No. Images	GN No.	GenBank No.	Unique ID
Mustelus	*mustelus*	Yes	5	7218	JQ518709.1	5963
Mustelus	*palumbes*	Yes	5	7322	JQ518710.1	6067
Mustelus	*canis*	?	?	917	JQ518711.1	?
Galeorhinus	*galeus*	Yes	5	7236	JQ518695.1	5981
Scylliogaleus	*quecketti*	?	?	-	DQ422121.1	-
Carcharhinus	*plumbeus*	?	?	903	JQ518632.1	?
Carcharhinus	*brachyurus*	?	?	3	JQ518611.1	?
Carcharhinus	*obscurus*	Yes	5	3213	JQ518612.1	4679
Carcharhinus	*limbatus*	?	?	1303	JN082204.1	?
Haploblepharus	*edwardsii*	Yes	5	7237	JQ518679.1	5982
Poroderma	*africanum*	?	?	1772	JQ518682.1	?
Poroderma	*pantherinum*	Yes	5	7325	JQ518683.1	6070
Sphyrna	*lewini*	?	?	5663	JQ518691.1	?
Sphyrna	*zygaena*	?	?	1097	JQ519079.1	?
Raja	*straeleni*	Yes	5	7192	JQ518894.1	5937
Rostroraja	*alba*	Yes	5	7302	JQ518900.1	6047

Table S2.2 Estimates of evolutionary divergence between *ND2* sequences of source species *Galeorhinus galeus* and target species using Kimura-two-parameter distances (K2P: Kimura 1980)

Target species	K2P distance	Std. Error	No. of SSRs transferred
Mustelus mustelus GN7218	0.136	0.013	10
Mustelus palumbes GN7322	0.131	0.013	9
Scylliogaleus quecketti	0.131	0.013	8
Carcharhinus brachyurus GN3	0.186	0.016	9
Carcharhinus limbatus GN1303	0.173	0.015	7
Carcharhinus obscurus GN3213	0.169	0.015	9
Carcharhinus plumbeus GN903	0.172	0.015	9
Haploblepharus edwardsii GN7237	0.219	0.018	5
Poroderma africanum GN1772	0.261	0.020	1
Poroderma pantherinum GN7325	0.258	0.019	1
Sphyrna lewini GN5663	0.185	0.016	4
Sphyrna zygaena GN1097	0.195	0.017	7
Raja straeleni GN7192	0.379	0.025	6
Rostroraja alba GN7302	0.397	0.025	5
Mean:	21.4%	1.7%	6.4

Table S2.3 Estimates of evolutionary divergence between *ND2* sequences of source species *Mustelus canis* and target species using Kimura-two-parameter distances (K2P: Kimura 1980)

Target species	K2P distance	Std. Error	No. of SSRs transferred
Mustelus mustelus GN7218	0.190	0.016	12
Mustelus palumbes GN7322	0.182	0.016	6
Galeorhinus galeus GN7236	0.173	0.015	8
Scylliogaleus quecketti	0.167	0.014	8
Carcharhinus brachyurus GN3	0.091	0.010	8
Carcharhinus limbatus GN1303	0.110	0.011	9
Carcharhinus obscurus GN3213	0.069	0.008	8
Carcharhinus plumbeus GN903	0.001	0.001	7
Haploblepharus edwardsii GN7237	0.251	0.019	7
Poroderma africanum GN1772	0.273	0.021	7
Poroderma pantherinum GN7325	0.269	0.021	7
Sphyrna lewini GN5663	0.140	0.013	4
Sphyrna zygaena GN1097	0.155	0.014	3
Raja straeleni GN7192	0.376	0.026	6
Rostroraja alba GN7302	0.350	0.024	5
Mean:	18.7%	1.7%	7.0

Appendix B

Supplementary Information for Chapter 3

Table S3.1 Summary genetic diversity estimates at 12 microsatellite loci in eight *Mustelus mustelus* sampling sites in southern Africa

Population	Locus	A_N	A_R	H_O	H_E	*PIC*	F_{IS}	Fr_{NULL}	$P_{E\text{-}W}$
Angola	*Mh1*	3	1.5	0.273	0.255	0.228	-0.071	-0.023	0.903
	Mh2	3	2.2	0.3	0.611	0.492	0.522	0.177	0.211
	Mh9	5	2.6	0.556	0.712	0.617	0.231	0.07	0.604
	Mh25	6	2.8	0.273	0.749	0.669	0.647**	0.258[b]	0.544
	Mca25	4	2	0.455	0.463	0.411	0.02	-0.009	0.732
	McaB39	2	1.9	1	0.524	0.375	-1.000**	-0.333	0.025*
	McaB5	6	2.7	0.583	0.717	0.641	0.194	0.062	0.63
	McaB6	5	2.5	0.75	0.674	0.59	-0.119	-0.063	0.606
	McaB22	10	3.3	0.75	0.877	0.823	0.150*	0.049	0.514
	McaB27	1	1	0	0	0	0	0	N/A
	Mca33	2	1.8	0.5	0.464	0.346	-0.082	-0.039	0.207
	McaB37	1	1	0	0	0	0	0	N/A
	Average	4	2.1	0.453	0.504	0.433	0.041	0.012	0.498
Langebaan	*Mh1*	1	1	0	0	0	0	0	N/A
	Mh2	3	1.9	0.391	0.474	0.39	0.178	0.05	0.374
	Mh9	3	1.9	0.391	0.474	0.39	0.178	0.05	0.376
	Mh25	5	2.5	0.174	0.657	0.594	0.740**	0.285[b]	0.201
	Mca25	4	1.8	0.348	0.378	0.347	0.081	0.016	0.567
	McaB39	2	1.9	1	0.511	0.375	-1.000**	-0.333	0.011*
	McaB5	7	2.7	0.913	0.733	0.671	-0.252	-0.114	0.622
	McaB6	4	2.4	0.455	0.644	0.571	0.299	0.107	0.157
	McaB22	10	3.3	0.857	0.875	0.838	0.02	-0.002	0.036**
	McaB27	2	1.1	0.043	0.043	0.042	0	-0.001	1
	Mca33	3	1.3	0.174	0.165	0.154	-0.054	-0.011	0.881
	McaB37	3	1.3	0.174	0.165	0.154	-0.054	-0.011	0.881
	Average	3.9	1.9	0.41	0.427	0.377	0.011	0.003	0.464
Robben Island	*Mh1*	1	1	0	0	0	0	0	N/A
	Mh2	2	1.5	0.111	0.286	0.239	0.618*	0.13	0.405
	Mh9	3	1.6	0.19	0.324	0.279	0.418	0.096	0.711
	Mh25	6	2.9	0.316	0.78	0.721	0.602**	0.252[b]	0.164
	Mca25	3	1.5	0.318	0.28	0.247	-0.14	-0.035	0.756
	McaB39	3	2	1	0.534	0.407	-0.913**	-0.314	0.456
	McaB5	7	2.8	0.773	0.761	0.71	-0.016	-0.017	0.248
	McaB6	5	2.8	0.773	0.768	0.71	-0.006	-0.012	0.021*

	McaB22	10	3.4	0.955	0.885	0.851	-0.081	-0.048	0.009**
	McaB27	5	1.4	0.182	0.175	0.168	-0.037	-0.009	1
	Mca33	3	1.4	0.182	0.173	0.163	-0.05	-0.011	0.803
	McaB37	2	1.6	0.05	0.296	0.247	0.835**	0.185[b]	0.381
	Average	4.2	2	0.404	0.439	0.395	0.103	0.018	0.45
False Bay	*Mh1*	2	1.1	0.063	0.063	0.059	0	-0.002	1
	Mh2	4	2	0.188	0.466	0.417	0.605**	0.182[b]	0.512
	Mh9	2	1.5	0.167	0.29	0.239	0.436	0.087	0.468
	Mh25	7	2.6	0.385	0.683	0.625	0.447**	0.164[b]	0.821
	Mca25	5	2.1	0.438	0.51	0.462	0.146	0.038	0.71
	McaB39	2	1.9	1	0.516	0.375	-1.000**	-0.333	0.016*
	McaB5	6	2.7	0.647	0.725	0.66	0.111	0.034	0.443
	McaB6	5	2.5	0.647	0.679	0.61	0.049	0.007	0.37
	McaB22	14	3.4	0.882	0.889	0.853	0.008	-0.01	0.629
	McaB27	6	1.9	0.471	0.414	0.385	-0.143	-0.049	0.968
	Mca33	3	2	0.824	0.542	0.436	-0.545*	-0.195	0.318
	McaB37	5	1.6	0.176	0.275	0.258	0.364	0.071	0.993
	Average	5.1	2.1	0.49	0.504	0.448	0.04	-0.001	0.604
Kalk Bay	*Mh1*	2	1.1	0.063	0.063	0.059	0	-0.002	1
	Mh2	4	1.8	0.385	0.403	0.363	0.048	0.002	0.79
	Mh9	3	2.3	0.25	0.607	0.468	0.625	0.184	0.744
	Mh25	5	2.5	0.214	0.656	0.584	0.682**	0.256[b]	0.438
	Mca25	3	1.9	0.467	0.503	0.396	0.076	0.014	0.558
	McaB39	2	1.9	1	0.516	0.375	-1.000**	-0.333	0.017*
	McaB5	6	2.8	0.688	0.758	0.696	0.096	0.027	0.231
	McaB6	5	2.5	0.875	0.657	0.588	-0.346	-0.146	0.385
	McaB22	10	3.4	1	0.901	0.86	-0.114	-0.068	0.010*
	McaB27	6	2.4	0.813	0.615	0.558	-0.336	-0.136	0.814
	Mca33	4	2.2	0.875	0.597	0.51	-0.489	-0.188	0.376
	McaB37	2	1.1	0.063	0.063	0.059	0	-0.002	1
	Average	4.3	2.2	0.558	0.528	0.46	-0.063	-0.033	0.53
Struis Bay	*Mh1*	3	2.3	0.294	0.633	0.532	0.543*	0.198[b]	0.083
	Mh2	3	2	0.556	0.503	0.404	-0.111	-0.054	0.59
	Mh9	4	2.3	0.588	0.62	0.522	0.053	0.009	0.356
	Mh25	5	2.5	0.941	0.679	0.603	-0.403**	-0.17	0.272
	Mca25	2	1.8	0.706	0.471	0.352	-0.524**	-0.171	0.172
	McaB39	2	1.9	1	0.515	0.375	-1	-0.333	0.015*
	McaB5	6	2.8	1	0.759	0.695	-0.330**	-0.151	0.239
	McaB6	5	2.8	1	0.75	0.684	-0.347**	-0.157	0.076
	McaB22	9	3.1	1	0.822	0.772	-0.225**	-0.113	0.438
	McaB27	1	1	0	0	0	0	0	N/A
	Mca33	3	1.2	0.118	0.116	0.109	-0.016	-0.005	1
	McaB37	2	1.8	0.647	0.451	0.342	-0.455	-0.146	0.2

	Average	3.8	2.1	0.654	0.527	0.449	-0.234	-0.091	0.313
Jeffreys Bay	*Mh1*	5	1.6	0.313	0.29	0.271	-0.079	-0.024	0.993
	Mh2	3	2.4	0.636	0.671	0.567	0.054*	0.003	0.041*
	Mh9	2	1.8	0.625	0.458	0.337	-0.4	-0.137	0.277
	Mh25	5	2.6	0.333	0.683	0.626	0.521**	0.197[b]	0.111
	Mca25	5	2.6	0.563	0.7	0.638	0.201**	0.069	0.122
	McaB39	5	2.5	1	0.677	0.593	-0.500**	-0.208	0.429
	McaB5	6	2.5	0.813	0.653	0.599	-0.254	-0.11	0.583
	McaB6	6	2.6	0.5	0.69	0.616	0.281	0.101	0.634
	McaB22	11	3.5	0.813	0.921	0.883	0.122**	0.042	0.002**
	McaB27	6	1.8	0.438	0.391	0.366	-0.123	-0.043	0.981
	Mca33	3	1.8	0.5	0.417	0.367	-0.206	-0.068	0.359
	McaB37	5	2.2	0.467	0.611	0.507	0.243	0.078	0.853
	Average	5.2	2.3	0.583	0.597	0.531	-0.012	-0.008	0.449
Durban	*Mh1*	5	1.9	0.278	0.43	0.396	0.361**	0.099	0.811
	Mh2	4	2.3	0.444	0.601	0.522	0.273**	0.079	0.525
	Mh9	4	1.9	0.412	0.437	0.385	0.059	0.009	0.707
	Mh25	5	2.3	0.5	0.595	0.542	0.164**	0.05	0.434
	Mca25	3	1.6	0.368	0.317	0.275	-0.167	-0.046	0.732
	McaB39	2	1.9	1	0.514	0.375	-1.000**	-0.333	0.011*
	McaB5	6	2.5	0.563	0.681	0.612	0.179**	0.059	0.734
	McaB6	6	3	0.429	0.812	0.75	0.482**	0.199[b]	0.071
	McaB22	12	3.5	0.875	0.913	0.874	0.043	0.005	0.055
	McaB27	1	1	0	0	0	0	0	N/A
	Mca33	4	2.5	0.467	0.674	0.587	0.315*	0.112	0.279
	McaB37	3	1.7	0.267	0.343	0.294	0.228	0.048	0.737
	Average	4.6	2.2	0.467	0.526	0.468	0.078	0.023	0.463
Overall Populations	*Mh1*	2.8	1.4	0.161	0.217	0.193	-0.025	0.007	0.798
	Mh2	3.3	2.0	0.376	0.502	0.424	0.159	0.055	0.487
	Mh9	3.3	2.0	0.397	0.490	0.405	0.200	0.046	0.530
	Mh25	5.5	2.6	0.392	0.685	0.621	0.425	-0.060	0.373
	Mca25	3.6	1.9	0.458	0.453	0.391	0.003	-0.016	0.544
	McaB39	2.5	2.0	1.000	0.538	0.406	-1.000	-0.315	0.443
	McaB5	6.3	2.7	0.748	0.723	0.661	-0.020	-0.026	0.466
	McaB6	5.1	2.6	0.679	0.709	0.640	0.026	-0.023	0.328
	McaB22	10.8	3.4	0.892	0.885	0.844	-0.025	-0.018	0.409
	McaB27	3.5	1.5	0.243	0.205	0.190	-0.080	-0.030	0.953
	Mca33	3.1	1.8	0.455	0.394	0.334	-0.150	-0.051	0.528
	McaB37	2.9	1.5	0.231	0.276	0.233	0.047	0.005	0.721
	Average	4.4	2.1	0.502	0.507	0.445	-0.005	-0.010	0.471

Number of alleles per locus (A_N); allelic richness (A_R); observed heterozygosity (H_O); expected heterozygosity (H_E); polymorphic information content (*PIC*); inbreeding coefficient (F_{IS}) with statistically significant deviations from Hardy-Weinberg expectations indicated by * ($P < 0.01$) and ** ($P < 0.001$); null allele frequency (Fr_{NULL}) with [b] indicating the presence of null alleles at statistical significance at the 5% nominal level and Ewens-Watterson probability ($P_{E\text{-}W}$).

Table S3.2 Exact test *P*-values for pairwise genotypic differentiation for eight *Mustelus mustelus* sampling sites in southern Africa using 12 microsatellite markers. $P > 0.01$ are underlined

	ETGD						
	A	**LL**	**RI**	**FB**	**KB**	**SB**	**JB**
Angola (A)	-						
Langebaan (LL)	0.000						
Robben Island (RI)	0.000	0.042					
False Bay (FB)	0.000	0.000	0.001				
Kalk Bay (KB)	0.000	0.000	0.000	0.981			
Struis Bay (SB)	0.000	0.000	0.000	0.000	0.000		
Jeffreys Bay (JB)	0.001	0.000	0.000	0.085	0.000	0.000	
Durban (D)	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Appendix C

Published Papers

Maduna SN, Rossouw C, Roodt-Wilding R, Bester-van der Merwe AE (2014) Microsatellite cross-species amplification and utility in southern African elasmobranchs: A valuable resource for fisheries management and conservation. *BMC Research Notes* **7**:352.

BMC
Research Notes

Microsatellite cross-species amplification and utility in southern African elasmobranchs: A valuable resource for fisheries management and conservation

Maduna *et al.*

BioMed Central

Maduna *et al. BMC Research Notes* 2014, **7**:352
http://www.biomedcentral.com/1756-0500/7/352

Maduna et al. BMC Research Notes 2014, 7:352
http://www.biomedcentral.com/1756-0500/7/352

BMC Research Notes

RESEARCH ARTICLE Open Access

Microsatellite cross-species amplification and utility in southern African elasmobranchs: A valuable resource for fisheries management and conservation

Simo N Maduna, Charné Rossouw, Rouvay Roodt-Wilding and Aletta E Bester-van der Merwe*

Abstract

Background: Similarly to the rest of the world, southern Africa's diverse chondrichthyan fauna is currently experiencing high fishing pressures from direct and non-direct fisheries to satisfy market demands for shark products such as fins and meat. In this study, the development of microsatellite markers through cross-species amplification of primer sets previously developed for closely related species is reported as an alternative approach to *de novo* marker development. This included the design of four microsatellite multiplex assays and their cross-species utility in genetic diversity analysis of southern African elasmobranchs. As this study forms part of a larger project on the development of genetic resources for commercially important and endemic southern African species, *Mustelus mustelus* was used as a candidate species for testing these multiplex assays in down-stream applications.

Results: Thirty five microsatellite primer sets previously developed for five elasmobranch species were selected from literature for testing cross-species amplification in 16 elasmobranch species occurring in southern Africa. Cross-species amplification success rates ranged from 28.6%-71.4%. From the successfully amplified microsatellites, 22 loci were selected and evaluated for levels of polymorphism, and four multiplex assays comprising of the 22 microsatellites were successfully constructed, optimised and characterised in a panel of 87 *Mustelus mustelus* individuals. A total of 125 alleles were observed across all loci, with the number of alleles ranging from 3–12 alleles. Cross-species amplification of the four optimised multiplex assays was further tested on 11 commercially important and endemic southern African elasmobranch species. Percentage of polymorphism ranged from 31.8%-95.5% in these species with polymorphic information content decreasing exponentially with evolutionary distance from the source species.

Conclusions: Cross-species amplification of the 35 microsatellites proved to be a time- and cost-effective approach to marker development in elasmobranchs and enabled the construction of four novel multiplex assays for characterising genetic diversity in a number of southern African elasmobranch species. This study successfully demonstrated the usefulness of these markers in down-stream applications such as genetic diversity assessment and species identification which could potentially aid in a more integrative, multidisciplinary approach to management and conservation of commercially important cosmopolitan and endemic elasmobranch species occurring in southern Africa.

Keywords: Cross-species amplification, Microsatellites, Multiplex assays, Genetic diversity, Species identification, Conservation management

* Correspondence: aeb@sun.ac.za
Department of Genetics, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa

Background

The subclass Elasmobranchii (sharks, skates and rays) comprises a diverse group of over 1000 species, and is representative of one of the most ancient extant vertebrate lineages [1]. Recently, pressures from direct and non-direct fisheries have resulted in the depletion of elasmobranch populations globally [2]. Decline in wild populations of elasmobranchs is further compounded by their life history traits that are more similar to those of mammals (*e.g.* low fecundity, late maturity and long gestation periods) than those of teleost fishes [3,4]. In comparison, elasmobranchs may not respond well to the high fishing pressures. This trend has been particularly pronounced for sharks due to unregulated harvesting to support an increase in demand for shark products (*e.g.* fins, meat, liver oil, skin and cartilage). A drastic reduction in population size (population bottleneck) can result in small populations experiencing the accumulating effects of inbreeding leading to severe loss of genetic diversity [5,6]. These trends have previously been reported for species such as the basking shark (*Cetorhinus maximus*) [7] and the narrownose smoothhound shark (*Mustelus schmitti*) [8]. Assessing genetic diversity and population structure of wild populations is therefore important for sustainable long-term management of the global shark fishery industry.

Misidentification of shark species in fisheries operations is also a widespread concern [9-12], and molecular individual identification methods have been developed to alleviate this problem [4,13-17]. To integrate genetic knowledge with fisheries management, it is imperative for shark fisheries to report shark landings by species instead of lumping them into species- or family groups (*e.g.* houndsharks, carcharinids, hammerheads *etc.*). This stems from the difficulties involved with unambiguously identifying species within and across families [13,18] *e.g.*, carcharinids (*Carcharhinus brachyurus*, *C. obscurus* and *C. plumbeus*) and houndsharks (*Mustelus mustelus*, *M. palumbes* and *Galeorhinus galeus*) due to a high degree of conserved interspecific morphology [14]. Neglecting to report shark landings by species overlooks important differences in species susceptibility and population vulnerability to exploitation [15], and that in turn has important implications for species-specific conservation, management and trade monitoring programmes [19].

The general lack of molecular genetic markers (*e.g.* microsatellites) for many elasmobranch species impedes population and conservation genetic studies in that these markers can provide valuable information relating to population dynamics (spatial and temporal genetic variation) of individual species. Microsatellites are highly polymorphic due to their high mutation rate (between 10^{-3} and 10^{-4} mutations per gamete per generation) resulting in extensive length polymorphism [20,21]. This makes microsatellite markers one of the most powerful molecular genetic tools with a remarkable array of applications ranging from genetic diversity [22,23] and population structure inference [24,25] to discerning genetic mating systems [26,27] and the identification of species [28-30].

Because the *de novo* development of microsatellites is challenging due to notoriously low rates of polymorphism in elasmobranchs [31], the development of microsatellite markers through cross-species amplification is the most effective alternative approach to *de novo* development of microsatellites and has recently also been reported in sharks [32]. Microsatellite cross-species amplification relies on the presence of conserved microsatellite flanking sequences [33], which in some organisms markedly demonstrate a high degree of conservation following millions of years of divergent evolution (*e.g.*, 250 million years in sharks [28] and 470 million years in fish [34]). The success rate of microsatellite cross-species amplification has directly been correlated to the evolutionary distance between the source species and the target species [33,35].

The underrepresentation of endemic taxa in many cross-species amplification studies is unfortunate as endemics should be of great interest for conservation of biodiversity on a regional scale. Southern Africa has one of the most diverse chondrichthyan faunas in the world, consisting of some 181 species in 44 families of which 34 species are endemic to southern Africa [36,37]. Growing concerns regarding the sustainability of the southern African shark fishery, stemming from the local declines of cosmopolitan and endemic species, lead to stricter regulations being imposed so as to avert the collapse of natural populations [38-40]. Accordingly, we report here the development of microsatellite markers through cross-species amplification of species-specific primers from closely related species. This included the design and optimisation of four microsatellite multiplex assays and their cross-species utility in genetic diversity analysis of 11 southern African elasmobranch species.

Results and discussion

Cross-species amplification

Development of microsatellite loci through cross-species amplification proved useful in establishing genetic markers for shark species that are commercially important and those (typically endemics) that are indirectly affected by fisheries' operations. Amplification of the 35 microsatellites in 50 individuals from 16 different elasmobranch species (1–4 individuals per species) proved to be effective (Table 1). Cross-species amplification success rates or the percentage of microsatellites that amplified successfully ranged from 60.00%-71.40% in the Triakidae and Carcharhinidae families and from 28.57%-48.57% in the Scyliorhinidae, Sphyrnidae and Rajidae families (Figure 1). The higher success rates in the Triakidae and Carcharhinidae is

Table 1 Cross-species amplification of the 35 microsatellites among 16 elasmobranch species of southern Africa

Species	References	MM	MP	GG	SQ	CB	CL	CO	CP	HP	HE	PP	PA	SL	SZ	RS	RA
Loci		(*n* = 4)	(n = 3)	(*n* = 4)	(*n* = 1)	(*n* = 4)	(n = 3)	(*n* = 4)	(*n* = 4)	(*n* = 4)	(*n* = 4)	(n = 4)	(*n* = 1)	(n = 3)	(n = 3)	(*n* = 1)	(*n* = 3)
Mh1	[56]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	++
Mh2	[57]	+	+	+	+	+	+	+	+	-	-	-	-	+	+	-	-
Mh6	[56]	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-
Mh9	[57]	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-
Mh25	[56]	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	++
Mca25	[49]	+	-	-	-	+	+	++	++	+	-	+	+	-	-	+	+
Mca31	[49]	-	-	-	+	+	+	+	-	-	-	-	-	-	+	-	-
Mca33	[49]	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+	+
Mca44	[49]	++	-	+	-	-	-	-	-	+	+	+	+	-	-	+	++
McaB5	[49]	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
McaB6	[49]	+	-	+	+	+	+	+	+	+	+	-	-	+	-	-	-
McaB22	[49]	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	-
McaB27	[49]	+	+	+	+	+	-	-	+	-	+	-	-	-	+	-	-
McaB33	[49]	-	-	-	-	+	+	+	+	-	-	-	-	+	-	-	-
McaB35	[49]	+	+	-	+	-	-	-	-	+	-	+	+	-	-	-	++
McaB37	[49]	+	+	+	+	-	+	-	-	-	-	-	-	+	-	-	-
McaB39	[49]	+	-	+	-	+	+	+	+	+	+	+	+	-	-	+	-
Gg2	[50]	+	+	+	+	+	-	-	-	+	-	-	-	-	+	++	+
Gg3	[50]	+	+	+	+	+	+	+	+	+	+	-	-	+	-	++	+
Gg7	[50]	+	+	+	-	+	-	+	+	-	+	-	-	-	+	-	+
Gg11	[50]	+	+	+	++	++	+	++	++	+	+	-	-	-	+	-	-
Gg12	[50]	+	+	+	-	+	-	+	+	+	-	-	-	-	-	-	-
Gg15	[50]	+	+	+	+	+	+	++	++	+	+	+	+	+	+	+	-
Gg17	[50]	+	-	+	+	+	+	+	+	-	+	-	-	+	-	-	+
Gg18	[50]	+	+	+	+	+	+	++	+	+	-	-	-	-	++	+	+
Gg22	[50]	+	+	+	+	+	+	+	+	+	-	-	-	-	++	+	-
Gg23	[50]	+	+	+	+	+	+	+	+	-	-	-	-	+	++	+	+
Rp16-nfrdi	[44]	+	++	-	+	+	++	+	+	-	-	-	+	++	+	+	++
Rp35-nfrdi	[44]	-	+	-	++	-	+	-	-	-	-	-	+	+	++	+	++
Scan02	[45]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scan06	[45]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scan12	[45]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scan14	[45]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scan15	[45]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scan16	[45]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

–, no visible band or faint bands with insufficient band intensity for scoring alleles were observed; +, solid bands with sufficient intensity for scoring alleles were detected; ++, solid bands with artefacts were produced but with at least one band of expected allele size. *Mustelus mustelus* (MM), *Mustelus palumbes* (MP), *Galeorhinus galeus* (GG), *Scyliogaleus quecketti* (SQ), *Carcharhinus brachyurus* (CB), *Carcharhinus limbatus* (CL), *Carcharhinus obscurus* (CO), *Carcharhinus plumbeus* (CP), *Haploblepharus pictus* (HP), *Haploblepharus edwardsii* (HE), *Poroderma africanum* (PA), *Poroderma pantherinum* (PP) *Sphyrna lewini* (SL), *Sphyrna zygaena* (SZ), *Raja straeleni* (RS) and *Raja alba* (RA).

expected as most microsatellites tested in this study were originally developed for species within the Triakidae family. Overall, the microsatellites showed less successful cross-species amplification to the taxa more divergent from the source species. Notably none of the individuals showed PCR amplification at any of the six *Scyliorhinus canacula* microsatellites. This may in part be attributed to *S. canacula* being more distantly related to the study species. The mean genetic distance between the taxa was 21.4 ± 1.7% (mean ± SD) (*G. galeus* as source species;

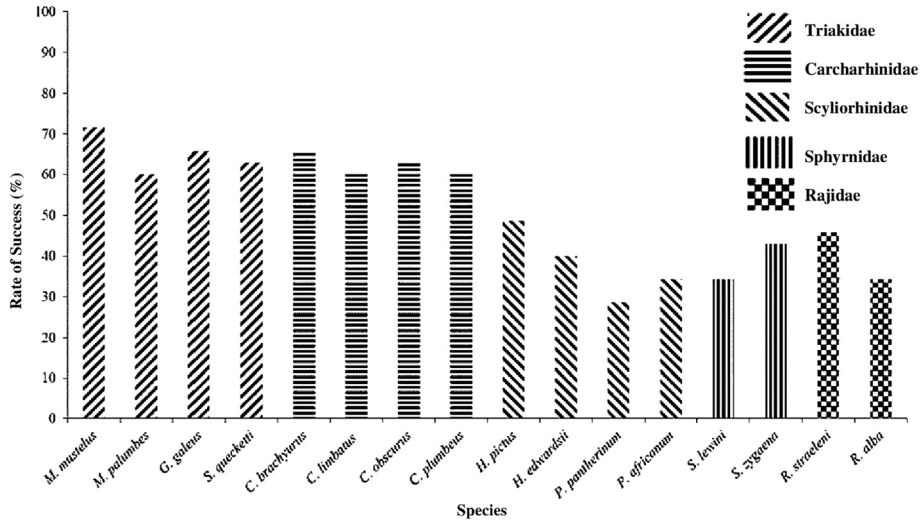

Figure 1 Success rates of 35 microsatellite loci across five families of southern African elasmobranch species.

Figure 2) and 18.7 ± 1.5% (*M. canis* as source species; Figure 3). *Haploblepharus pictus* could not be represented in the distance plot due to the lack of genetic information available in GenBank and Global Cestode Database: Elasmobranchs Specimens.

Results of cross-species amplification performance of *G. galeus* microsatellites exhibited a logarithmic regression function (Figure 2), albeit non-significant (slope within the 95% CI for no difference from zero), that may explain the general trend of negative correlation between cross-species amplification performance and genetic divergence seen across taxa [30,33,35,41]. Cross-species amplification of microsatellite markers from source to target species is generally negatively correlated with evolutionary divergence [30,42-45]. A similar trend was not observed for the *M. canis* microsatellites (Figure 3). This can be due to different life history traits (*i.e.* mating system and generation time) and genome size (*C* value) between the source and target species, which have been previously found to have significant negative effects on cross-species amplification success [33]. However, apart from the source-target species evolutionary distance other factors, such as mutations in microsatellite flanking sequences, may affect the success rate of cross-species amplification. Since microsatellites are usually found in non-coding regions where the substitution rate is higher than in coding regions [46], these microsatellite flanking sequences which serve as regions for PCR primer design and binding sites are prone to mutations [35]. Mutations (indels) in these regions may therefore result in null alleles and in turn affect the patterns of cross-species amplification as demonstrated in birds [47] and salmonids [48].

Additionally, *M. canis* microsatellite loci were isolated from an enriched genomic library [49] whereas for *G. galeus* the microsatellites were developed by a high-throughput sequencing approach (Roche 454 pyrosequencing) [50]. Based on the observed data it is hypothesised that these different approaches may have influenced the cross-species performance possibly due to the different resolving power of each approach for capturing microsatellites distributed across different parts of the genome. Castoe *et al.* [51] argues that enrichment-based approaches commonly use a few specific repeated motifs, which are largely selected without prior knowledge of their abundance in the genome and therefore could introduce potential bias in genome representativeness. In contrast, microsatellite identification from randomly sequenced genomic regions (*e.g.* Roche 454 NGS) allows for an unbiased assessment of all types of microsatellite loci present in a genome [51].

Cross-species amplification of orthologous microsatellites, due to the presence of conserved microsatellite flanking sequences [33], can persist over millions of years following divergent evolution as previously shown in sharks (250 million years [28]) and in fish (470 million years [34]). This indicates lower mutation rates within microsatellite flanking regions in aquatic organisms [30,34]. The reported cross-amplified microsatellite markers will thus contribute to establishing a molecular genetic marker repository for each of the Southern Africa elasmobranchs species included in this study. Future research efforts may be dedicated to generating microsatellite primers that have a high cross-species utility (*e.g.* [52]) as well as *in silico* mining of polymorphic microsatellite markers from expressed sequence tag data [53].

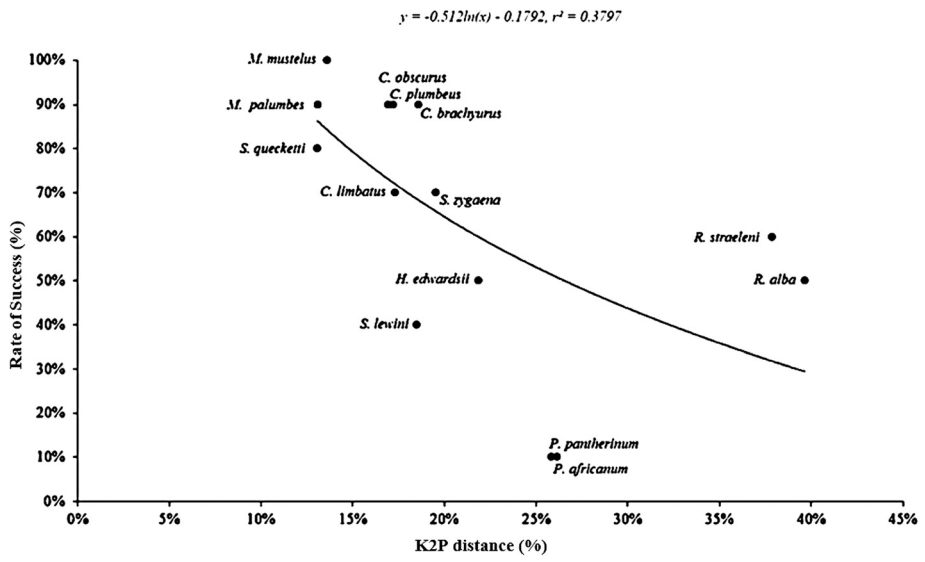

Figure 2 Cross-species amplification performance of *Galeorhinus galeus* microsatellites in 15 of the 16 elasmobranch species, and genetic divergence between *G. galeus* and the target species based on *ND2* sequences.

Multiplex assay characterisation

Twenty-two microsatellite loci that successfully cross-amplified across the study species and showed polymorphism in initial screening tests were used to develop four multiplex assays (MPS) comprised of at least five microsatellites each. These multiplex assays were characterised in a panel of 87 common smoothhound sharks (*Mustelus mustelus*) (Table 2). All 22 microsatellite loci were polymorphic across the multiplexes. With the exception of one locus, *McaB22*, all the microsatellite loci were found to deviate significantly from Hardy-Weinberg equilibrium most likely due to Wahlund effect as samples were pooled from diverse geographical locations for analysis. MicroChecker detected no significant genotyping errors but indicated that null alleles were present at two loci (*Mh9* and *Gg7*). Slatkin's exact test for neutrality indicated that two loci (*McaB22* and *Gg3*) were candidates for being subjected to selection.

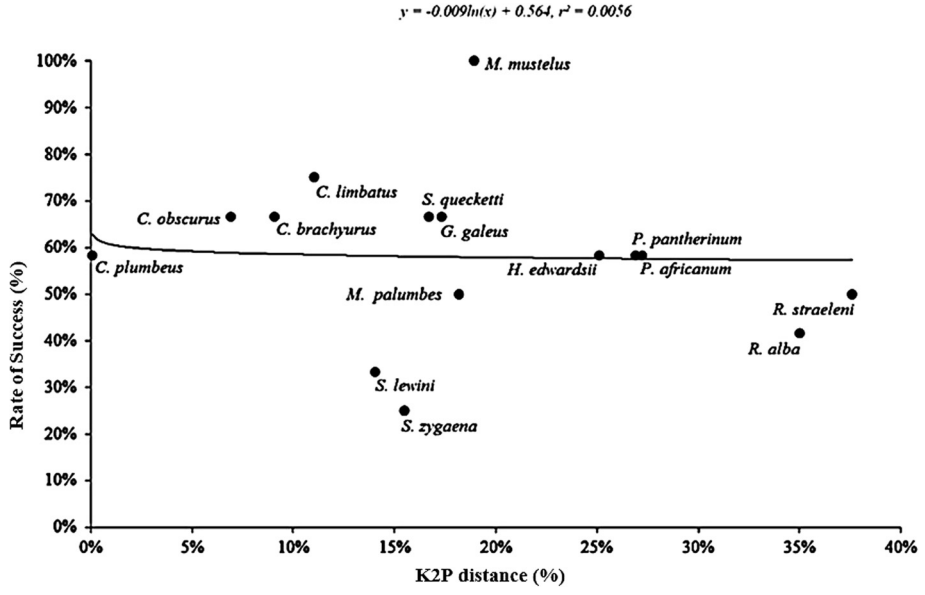

Figure 3 Cross-species amplification performance of *Mustelus canis* microsatellites in 15 of the 16 elasmobranch species, and genetic divergence between *M. canis* and the target species based on *ND2* sequences.

Table 2 Characterisation of four multiplex assays for *Mustelus mustelus* based on 87 individuals from southern Africa

Locus	Microsatellite repeat motif	[P]	Dye	Size range (bp)	A_N	A_E	H_O	H_E	*PIC*	F_{IS}	Fr_{NULL}	$P_{E\text{-}W}$
Mh1	$(AG)_n$	0.2	VIC	191-211	7	2.2	0.885	0.544	0.443	−0.633**	−0.223	0.931
Mh2	$(GA)_9$	0.3	VIC	587-597	4	1.7	0.367	0.402	0.342	0.089**	0.023	0.688
Mh9	$(GA)_9$	0.4	FAM	312-326	5	1.7	0.337	0.429	0.373	0.214**	0.062 [b]	0.723
Mh25	$(CT)_n$	0.2	FAM	122-148	8	1.6	0.356	0.404	0.385	0.118**	0.032	0.802
Mca25	$(CA)_n(CT)_n$	0.2	PET	232-240	3	1.9	0.563	0.463	0.382	−0.217**	−0.070	0.226
McaB39	$(CA)_{10}GAT(AC)_8$	0.2	NED	177-212	3	2.0	1.000	0.509	0.384	−0.977**	−0.328	0.501
MPS1 (mean)	**-**	**-**	**-**	**-**	**5**	**1.9**	**0.585**	**0.459**	**0.385**	**−0.234**	**−0.084**	**0.645**
McaB5	$(GT)_{11}$	0.2	VIC	189-210	10	3.5	0.826	0.716	0.674	−0.155*	−0.067	0.330
McaB6	$(CA)_{10}$	0.2	FAM	226-266	9	3.3	0.756	0.702	0.655	−0.077*	−0.034	0.498
McaB22	$(AC)_{18}$	0.2	NED	137-179	12	8.2	0.874	0.882	0.865	0.010	0.002	0.002
McaB27	$(GT)_6$	0.2	PET	138-199	4	2.1	0.965	0.536	0.424	−0.808**	−0.282	0.589
Mca33	$(ATC)_5$	0.2	FAM	189-199	6	3.0	0.872	0.674	0.609	−0.295**	−0.121	0.347
McaB37	$(GT)_5$	0.2	NED	219-251	11	1.9	0.483	0.486	0.431	0.007**	−0.016	0.997
MPS2 (mean)	**-**	**-**	**-**	**-**	**9**	**3.7**	**0.796**	**0.666**	**0.610**	**−0.220**	**−0.086**	**0.461**
Gg2	$(TG)_n$	0.2	NED	249-259	7	3.2	1.000	0.688	0.632	−0.458**	−0.188	0.324
Gg3	$(GATT)_n$	0.2	PET	257-265	2	2.0	1.000	0.503	0.375	−1.000**	−0.333	0.001
Gg7	$(AG)_n$	0.2	NED	296-312	4	1.6	0.310	0.393	0.343	0.212**	0.058[b]	0.584
Gg11	$(TCCC)_n$	0.2	NED	329-363	4	1.2	0.061	0.182	0.173	0.666**	0.000	0.792
Gg12	$(TA)_n$	0.2	FAM	276-296	4	1.8	0.610	0.454	0.361	−0.347**	−0.110	0.807
MPS3 (mean)	-	-	-	-	**4.2**	**2.0**	**0.596**	**0.444**	**0.377**	**−0.185**	**−0.115**	**0.495**
Gg15	$(GA)_n$	0.2	FAM	147-169	3	2.05	0.977	0.514	0.392	−0.910**	−0.308	0.370
Gg17	$(AC)_n$	0.2	PET	159-181	3	1.02	0.023	0.023	0.023	−0.003**	0.000	1.000
Gg18	$(GA)_n$	0.2	VIC	179-187	6	2.24	0.976	0.558	0.456	−0.759**	−0.272	0.776
Gg22	$(GT)_n$	0.2	FAM	237-247	4	2.25	0.964	0.559	0.455	−0.733**	−0.263	0.488
Gg23	$(AC)_n$	0.2	VIC	258-278	6	2.84	1.000	0.651	0.582	−0.540**	−0.214	0.562
MPS4 (mean)	-	-	-	-	**4.4**	**2.08**	**0.788**	**0.461**	**0.3816**	**−0.589**	**−0.211**	**0.615**
Overall (mean)	**-**	**-**	**-**	**-**	**5.7**	**2.4**	**0.691**	**0.512**	**0.444**	**0.010**	**−0.139**	**0.561**

Primer concentration in the final reaction as μM/primer ([P]); Number of alleles per locus (A_N); effective number of alleles (A_E); observed heterozygosity (H_O); expected heterozygosity (H_E); polymorphic information content (*PIC*); inbreeding coefficient (F_{IS}) with statistically significant deviations from Hardy-Weinberg expectations indicated by *($P < 0.01$) and **($P < 0.001$); null allele frequency (Fr_{NULL}) with [b]indicating the presence of null alleles at statistical significance at the 5% nominal level and Ewans-Watterson probability ($P_{E\text{-}W}$). Mean values for each multiplex assay and overall are indicated in bold.

Multiplex assay cross-species amplification and efficiency in species identification

Cross-species amplification of the four multiplex assays was tested for 11 other southern African shark species (Table 3). The number of alleles observed was highest in *G. galeus* and *M. palumbes,* varying from 1 to 7, while the percentage of polymorphism (*PP*) for each marker ranged from 31.8%-95.5%. The polymorphic information content (*PIC*) decreased exponentially with evolutionary distance from the source species (Table 3) and the four multiplex assays showed the highest *PIC* in *M. mustelus*, *M. palumbes* and *G. galeus*.

The mean genetic diversity estimates for each species in terms of number of alleles (A_N), effective number of alleles (A_E), observed heterozygosity (H_O), expected heterozygosity (H_E) and *PIC* are shown in Figure 4. In group 1, the mean H_E, A_E and *PIC* varied from relatively low in *C. brachyurus* (mean $H_E = 0.230$; mean $A_E = 1.4$; mean $PIC = 0.181$) to relatively high in *M. palumbes* (mean $H_E = 0.653$; mean $A_E = 3.3$; mean $PIC = 0.606$). Group 2 exhibited similar patterns of genetic diversity that varied from moderate in *S. zygaena* (mean $H_E = 0.593$; mean $A_E = 3.2$; mean $PIC = 0.554$) to relatively high in *P. pantherinum* (mean $H_E = 0.662$; mean $A_E = 3.4$; mean $PIC = 0.603$). For group 3 with $n = 4$, the mean H_E, A_E and *PIC* ranged from relatively low in *C. plumbeus* (mean $H_E = 0.249$; mean $A_E = 1.5$; mean $PIC = 0.193$) to relatively high in *C. obscurus* (mean $H_E = 0.429$; mean $A_E = 2.1$; mean $PIC = 0.367$).

The genotypic distribution of the study species is depicted in Figure 5. Most of the study species could be differentiated on PC1 and PC2 of the PCoA plot as can

Table 3 Multiplex transferability results of a total of 22 microsatellite loci showing the number of alleles per locus for the 11 elasmobranch species tested

Locus	MP	GG	CB	CL	CO	CP	HP	HE	SL	SZ	PP
	(n = 8)	*(n = 8)*	*(n = 8)*	*(n = 4)*	*(n = 4)*	*(n = 4)*	*(n =8)*	*(n = 4)*	*(n = 5)*	*(n = 5)*	*(n = 5)*
MPS1											
Mh1	4	3	1	2	1	1	1	1	4	2	3
Mh2	-	5	*	1	1	*	-	-	2	1	3
Mh9	4	4	*	-	*	*	-	-	**4**	5	**3**
Mh25	5	5	2	3	2	3	1	3	**4**	6	3
Mca25	3	1	1	3	2	1	2	1	**3**	**4**	3
McaB39	**3**	3	2	2	1	2	2	*	2	**3**	3
MPS2											
McaB5	3	1	2	4	2	3	1	1	**5**	**5**	7
McaB6	4	4	*	4	2	1	-	1	2	**3**	**5**
McaB22	2	1	1	4	2	4	2	4	**4**	7	7
McaB27	2	2	2	-	**1**	*	-	1	-	2	-
Mca33	4	2	2	4	2	2	2	3	**4**	**7**	6
McaB37	3	5	**1**	3	**1**	**1**	-	-	**4**	**6**	**7**
MPS3											
Gg2	5	4	1	-	**1**	**1**	2	-	-	2	-
Gg3	3	2	1	1	2	*	2	1	-	2	**2**
Gg7	4	1	1	-	1	1	**2**	-	-	1	1
Gg11	6	4	1	-	2	2	*	*	-	1	3
Gg12	4	5	-	-	1	*	1	**1**	2	2	**1**
MPS4											
Gg15	7	5	1	4	1	4	4	2	5	3	5
Gg17	4	4	1	4	1	2	**2**	2	2	3	2
Gg18	6	3	2	3	1	3	3	**3**	7	6	4
Gg22	6	5	2	3	2	2	2	*1*	3	2	3
Gg23	4	2	2	1	2	1	**3**	**2**	6	4	3
Total P loci	21	18	8	13	10	10	12	7	16	19	18
PP	95.5	81.8	36.4	59.1	45.5	45.5	54.5	31.8	72.7	86.3	81.8

n - number of individuals tested; *Failed to amplify but showed successful transferability initially (see Table 1); – No amplification; allele numbers in bold indicate loci that failed to cross-amplify according to Table 1; P – polymorphic and *PP* - percentage of polymorphism. For species abbreviations refer to Table 1.

be seen from individuals of each respective species clustering together. Individuals of the catshark species (*H. edwardsii*, *P. pantherinum* and *H. pictus*) however, were dispersed across quadrant 3 and 4. The PCoA also revealed that one of the *M. mustelus* individuals was misidentified as *G. galeus*. The identity of this particular individual was subsequently confirmed using the genetic identification method developed specifically for smoothhound sharks [16]. Briefly, this method involves using four primers (1 universal forward primer and 3 species-specific reverse primers) for the mitochondrial gene, *nicotinamide adenine dehydrogenase subunit 2* (*ND2*), in a multiplex PCR reaction. The reverse primers amplify a fragment of different length for each species (*M. asterias*, 564 bp; *M. mustelus*, 392 bp; *G. galeus*, 671 bp) and can therefore be utilised for distinguishing species based on fragment size.

In this study, the potential use of microsatellite loci in species identification was successfully demonstrated using shared microsatellite loci between species. The polymorphic information of these microsatellite loci was characterised by low genetic variation as previously proposed for elasmobranchs [31]. The genotypic distribution of the study species could also be differentiated based on PCoA analysis. Markedly, the lack of differentiation between the catshark species (*H. edwardsii* and *H. pictus*) on the PCoA plot may be explained by the misidentification of the *Haploblepharus* species that is a

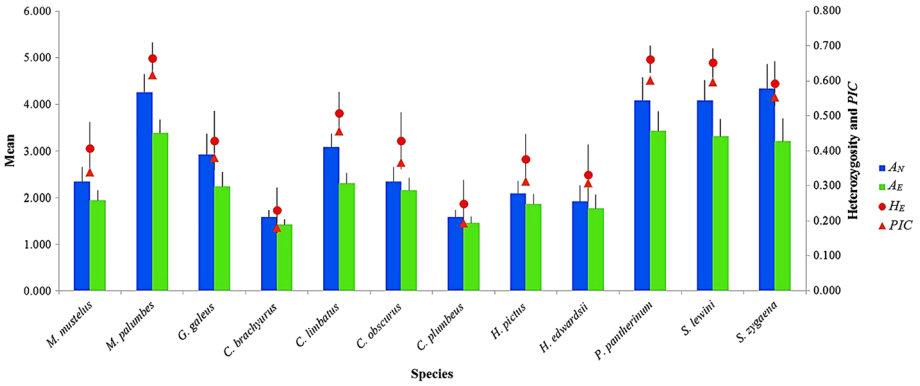

Figure 4 Mean genetic diversity estimates using 12 microsatellite loci shared between species: number of alleles (A_N), effective number of alleles (A_E), heterozygosity (H_E) and polymorphic information content (*PIC*). Error bars represent standard error.

common occurrence in the catshark family [54]. To further investigate whether the lack of differentiation detected with microsatellites was indeed due to misidentification, the *cytochrome b* (*Cyt b*) and *ND2* genes were applied for species comparisons [54]. For both genes, sequence analyses revealed individuals with cryptic identification, suggesting that in the case of the catsharks, the microsatellites optimised in the current study were not successful in discriminating between the *Haploblepharus* species but could in the future aid in the identification of cryptic speciation within the catshark family.

In South Africa, the aforementioned misidentification issue is prominent in fishing operations (particularly in longline and trawl fisheries) where there is a high rate of incidental capture of non-target shark species [9-12]. This hinders the collection of reliable data on shark catch and trade on a species-specific basis making robust stock assessments and identification of overfished and potentially threatened species nearly impossible in most situations [4]. This was apparent in a study by Attwood *et al.* [12], which assessed bycatch in South Africa's inshore trawl fishery based on observer records. In the aforementioned study, certain taxa were difficult to identify, and therefore taxonomic groups were lumped in species groups (*e.g. Raja* spp., *Mustelus* spp. and Scyliorhinidae), even though every attempt was made to analyse data at species level. Therefore, the molecular genetic makers developed by the current study may facilitate in obtaining species-specific catch data for stock assessment, characterising genetic diversity and delineating population genetic structure. This in turn will contribute to the implementation of future conservation and management plans on a species-specific level in southern Africa.

Conclusions

Cross-species amplification of available microsatellite loci to target species has proven to be more time- and cost-effective in comparison to the *de novo* development approach and permitted the cross-amplification of 22 markers across 12 elasmobranch species. Cross-species amplification of the four multiplex assays developed in the current study highlighted the usefulness of microsatellites for characterising genetic diversity and potentially also species identification of a number of commercially

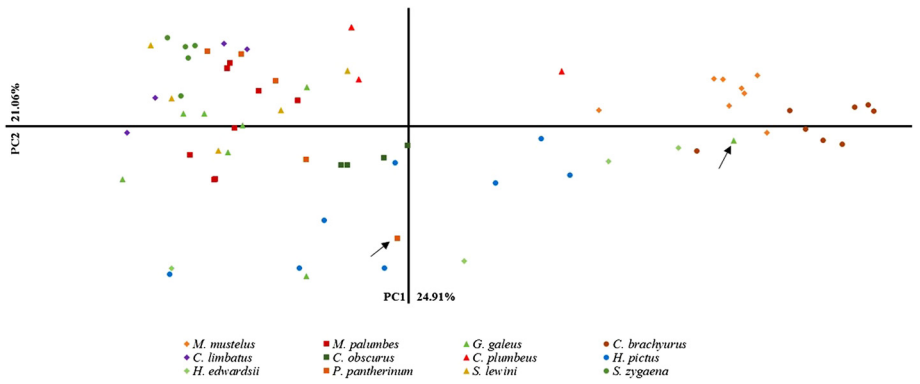

Figure 5 Principle coordinates analysis (PCoA) of study species based on 12 shared amplified microsatellite loci between species. Arrows depict misidentified/mislabelled individuals.

important and endemic elasmobranch species. The molecular genetic markers developed in this study and their usefulness in down-stream applications could therefore aid in a more integrative, multidisciplinary approach to conservation management of elasmobranchs in southern Africa.

Methods

Ethics statement

The collection of specimens from various shark species used in this study complied with the Convention on Biological Diversity (http://www.cbd.int/convention/) and the Convention on the Trade in Endangered Species of Wild Fauna and Flora (http://www.cites.org/). All permits to collect finclip or muscle tissue for research purposes were granted by the Department of Agriculture, Forestry and Fisheries (Republic of South Africa).

Study species and DNA extraction

Sixteen elasmobranch species occurring in southern African waters belonging to five families within two orders were selected for cross-species amplification (Additional file 1). Where possible, specimens were collected from at least two sampling locations to better capture allelic diversity present within populations of each respective species. However, due to opportunistic sampling for a majority of the study species, samples were obtained from only a single location (Additional file 1). Muscle tissue or finclips were preserved in 99% ethanol and stored at room temperature until further use. Total genomic DNA was isolated using the standard cetyltrimethylammonium bromide (CTAB) method of Saghai-Maroof *et al.* [55]. The extracted DNA was quantified using a NanoDrop ND-1000 spectrophotometer v.3.0.1 (*NanoDrop®*). For testing cross-species amplification, each DNA sample was adjusted to a working concentration of 50 ng/μl and stored at -20°C prior to polymerase chain reaction (PCR) analysis.

Microsatellite primer transfer

A total of 35 microsatellite markers previously developed in five elasmobranch species (*Raja pulchra* [44], *Scyliorhinus canacula* [45], *M. canis* [49], *G. galeus* [50] and *M. henlei* [56,57]) were selected for testing cross-species amplification. Primer sequences and annealing temperature (T_A) of each primer set optimised for each respective source species are shown in Table S2 (Additional file 2). Polymerase chain reaction conditions optimised for the majority of the source species [*G. galeus*, *M. henlei*, *R. pulchra* and *S. canacula*] were applied for cross-species microsatellite examinations except for the *M. canis* ([56] PCR protocol) and some *R. pulchra* (modified [57] PCR protocol) primer sets. Polymerase chain reactions for all individuals were executed in a GeneAmp® PCR System 2700.

The PCR amplicons were visualised on a 2% agarose gel stained with ethidium bromide together with negative controls and Promega 100 bp molecular size ladder for preliminary size determination. Success or failure of PCR amplification in cross-species trials was determined simply on the basis of whether band intensity was sufficient to score alleles. In most instances, less stringent PCR conditions were not employed in the cross-species assays so as to minimise the risk of amplification of non-orthologous loci in the target species. The number of markers that showed amplification success at all or a percentage of individuals in the target species ("+/++" in Table 1) were counted as an index to measure the cross-species microsatellite amplification performance.

Multiplex design and optimisation

As this study forms part of a larger project on the development of genetic resources for commercially important and endemic species of southern Africa, *Mustelus mustelus* was used as a candidate species for testing of the four novel multiplex assays. Levels of polymorphism were initially assessed at all the successfully cross-amplified microsatellite loci in a panel of eight individuals of *M. mustelus*. Amplicons were subjected to electrophoresis for two hours at 150 volts on a 12% polyacrylamide gel to detect size variants. Microsatellites were considered to be polymorphic when two bands were distinguishable in a single individual (*i.e.* heterozygote) and/or clear size differences were detected between different individuals.

Twenty-two polymorphic microsatellite loci were selected, and primers fluorescently labelled and optimised in four multiplex assays (5–6 loci per MPS) using a strategy outlined by Guichoux *et al.* [58] with one of the following dyes: FAM, VIC, PET, or NED. The use of different dyes was to facilitate co-amplification of multiple microsatellite markers in a single reaction for cost- and time-efficient genotyping (Multiplex PCR).

After optimisation of the newly designed MPS (MPS1, MPS2, MPS3 and MPS4), a panel of 87 *M. mustelus* individuals from across the distribution range in southern Africa was genotyped for marker characterisation purposes. The multiplex assays were then tested on 11 additional species to show their overall application in genetic diversity and population structure analysis. Finally, a total of 12 microsatellite loci that were successfully genotyped across the study species (*Mh1*, *Mh25*, *Mca25*, *McaB39*, *McaB5*, *McaB22*, *Mca33*, *Gg15*, *Gg17*, *Gg18*, *Gg22* and *Gg23*) were selected to demonstrate the potential use of microsatellite loci in species identification.

The percentage of polymorphism (*PP*) was calculated using the formula:

$$PP = \frac{N_P}{N_T} X\ 100$$

where N_P is the total number of polymorphic loci and N_T is the total number of loci multiplied by 100.

For the multiplex reaction, the Qiagen Multiplex PCR kit was used and PCR conducted according to the manufacturer's instructions except for varying T_A, 59°C for MPS1, MPS3 and MPS4; and 56°C for MPS2. For subsequent analysis on an ABI 3730XL DNA Analyzer, PCR products were diluted in distilled water and fragment analysis performed together with the LIZ600 internal size standard. Individual genotypes were scored based on fragment size via Peak Scanner® software v.1 (Life Technologies). Auto Bin v.0.9 macro for Excel (http://www.bordeaux-aquitaine.inra.fr/biogeco/Ressources/Logiciels/Autobin; see [58]) was used to detect discreet size variants where allele binning of genotype data obtained from Peak Scanner® software v.1 was based on raw size.

Genetic diversity analysis

MicroChecker v.2.2.3 [59] was used to evaluate the presence of genotypic errors caused by allele dropout, stuttering and null alleles. Null allele frequencies (Fr_{NULL}) were calculated using the Brookfield 1 estimator implemented in this program. Locus-specific fixation index (F_{IS}) and over all loci was estimated to measure departure from Hardy-Weinberg equilibrium using the exact probability test (20 batches, Dememorization; 10000 and 5000 iterations) using Genepop v.4.0 [60]. Linkage disequilibrium between all pairs of loci was calculated using an exact test implemented also in Genepop. Slatkin's exact test (1000 permutations) for neutrality, based on Ewens-Watterson sampling theory [61] was used to detect loci under selection as implemented in Arlequin v.3.5.1.2 [62]. The number (A_N) of alleles at each microsatellite locus, as well as the effective number of alleles A_E:

$$A_E = 1/\sum_{i=1}^{n} p_i^2$$

where p_i is the frequency of the i^{th} allele and n is the number of alleles was calculated using the GenAlEx v.6.5 program [63]. The proportion of individual samples that were heterozygous [direct count heterozygosity (H_O) and expected under Hardy-Weinberg equilibrium (H_E)] was calculated using MsatTools [64]. MsatTools was also used to calculate the polymorphic information content (*PIC*) of each marker according to the following equation in [65]:

$$PIC = 1 - \sum_{i=1}^{n} p_i^2 - 2\left[\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} p_i^2 p_j^2\right]$$ where p_i and p_j are the frequency of the i^{th} and j^{th} allele respectively and n is the number of alleles.

Direct comparison of genetic diversity estimates (H_E, A_E and *PIC*) across the 11 species was not plausible due to the different sample sizes that were used. Species were therefore grouped into three groups according to sample size: (1) *M. mustelus*, *M. palumbes*, *G. galeus*, *C. brachyurus* and *H. pictus* ($n = 8$); (2) *P. pantherinum*, *S. lewini* and *S. zygaena* ($n = 5$) and (3) *C. obscurus*, *C. limbatus*, *C. plumbeus* and *H. edwardsii* ($n = 4$). The potential use of microsatellite data for species-assignment was assessed through principle coordinate analysis (PCoA) in GenAlEx v.6.5 using genetic distances between individuals.

To evaluate cross-species amplification performance, DNA sequences derived from the mitochondrial *ND2* gene (1044 bp) of each species were downloaded from GenBank and Global Cestode Database: Elasmobranchs Specimens (http://elasmobranchs.tapewormdb.uconn.edu) (Additional file 3). The genetic distance of the study taxa was estimated using the Kimura 2-parameter model with the rate variation among sites modelled with a gamma distribution (shape parameter = 5) implemented in MEGA v.5 [66].

Additional files

Additional file 1: The 16 elasmobranch species of southern Africa selected for cross-species amplification, including family, species, distribution and sampling locations.

Additional file 2: The 35 putative microsatellite markers developed from five closely related species for cross-species amplification in the study taxa, including the primers sequence, microsatellite repeat motif, annealing temperature (T_A) and GenBank accession numbers.

Additional file 3: The *ND2* sequence information of the study taxa used to estimate the genetic distance to evaluate cross-species performance, including ID Verified, availability of images (yes or no) which are available in the on-line host specimen database (http://elasmobranchs.tapewormdb.uconn.edu) and GenBank accession numbers.

Abbreviations

CI: Confidence interval; *PP*: Percentage of polymorphism; *P*: Polymorphic; *PIC*: Polymorphic information content; A_N: Number of alleles; A_E: Effective number of alleles; H_O: Observed heterozygosity; H_E: Expected heterozygosity; F_{IS}: Inbreeding coefficient; Fr_{NULL}: Null allele frequency; $P_{E\text{-}W}$: Ewans-Watterson probability; K2P: Kimura 2-parameter model; *ND2*: *Nicotinamide adenine dehydrogenase subunit 2*; MM: *Mustelus mustelus*; MP: *Mustelus palumbes*; GG: *Galeorhinus galeus*; SQ: *Scylliogaleus quecketti*; CB: *Carcharhinus brachyurus*; CL: *Carcharhinus limbatus*; CO: *Carcharhinus obscurus*; CP: *Carcharhinus plumbeus*; HP: *Haploblepharus pictus*; HE: *Haploblepharus edwardsii*; PA: *Poroderma africanum*; PP: *Poroderma pantherinum*; SL: *Sphyrna lewini*; SZ: *Sphyrna zygaena*; RS: *Raja straeleni*; RA: *Raja alba*.

Competing interests

The authors have no competing interests to declare.

Authors' contributions

SNM performed cross-species amplification, microsatellite genotyping, genetic data analyses, organised the samples and drafted the manuscript. CR participated in cross-species amplification and microsatellite genotyping. RR-W participated in experimental design and coordination and contributed to manuscript preparation. AEB-vdM conceived the study, provided funds, participated in its design and coordination and contributed to manuscript preparation. All authors read and approved the final manuscript.

Acknowledgements
The authors gratefully acknowledge and thank the KwaZulu-Natal Sharks Board, Oceans Research, South African Department of Agriculture, Forestry and Fisheries (DAFF), South African Shark Conservancy and White Shark Africa for providing valuable samples. The authors also thank Michelle-Angelique Hallendorff for editorial revision and Clint Rhode and Luca Mirimin for valuable discussions. This work was funded by the National Research Foundation, South Africa.

Received: 7 February 2014 Accepted: 2 June 2014
Published: 10 June 2014

References

1. Corrigan S, Beheregaray LB: **A recent shark radiation: molecular phylogeny, biogeography and speciation of wobbegong sharks (family: Orectolobidae).** *Mol Phyl Evol* 2009, **52:**205–216.
2. Worm B, Davis B, Kettemer L, Ward-Paige CA, Chapman D, Heithaus MR, Kessel ST, Gruber SH: **Global catches, exploitation rates, and rebuilding options for sharks.** *Mar Policy* 2013, **40:**194–204.
3. Stevens JD, Bonfil R, Dulvy NK, Walker PA: **The effects of fishing on sharks, rays, and chimaeras (chondrichthyans), and the implications for marine ecosystems.** *ICES J Mar Sci* 2000, **57:**476–494.
4. Shivji M, Clarke S, Pank M, Natanson L, Kohler N, Stanhope M: **Genetic identification of pelagic shark body parts for conservation and trade monitoring.** *Conserv Biol* 2002, **16:**1036–1047.
5. Nei M, Maruyama T, Chakraborty R: **Bottleneck effect and genetic variability in populations.** *Evolution* 1975, **29:**1–10.
6. Glenn TC, Stephan W, Braun MJ: **Effects of a population bottleneck on whooping crane mitochondrial DNA variation.** *Conserv Biol* 1999, **13:**1097–1107.
7. Hoelzel AR, Shivyi MS, Magnussen J, Francis MP: **Low worldwide genetic diversity in the basking shark.** *Biol Lett* 2006, **2:**630–642.
8. Pereyra S, García G, Miller P, Oviedo S, Domingo A: **Low genetic diversity and population structure of the narrownose shark (*Mustelus schmitti*).** *Fish Res* 2010, **106:**468–473.
9. Myers RA, Worm B: **Rapid worldwide depletion of predatory fish communities.** *Nature* 2003, **423:**280–283.
10. Barker MJ, Schluessel V: **Managing global shark fisheries: suggestions for prioritising management strategies.** *Aquat Conserv Mar and Freshwat Ecosyst* 2005, **15:**325–347.
11. Petersen SL, Honig MB, Ryan PG, Underhill LG, Compagno LJV: **Pelagic shark bycatch in the pelagic longline fishery off Southern Africa.** In *Understanding and Mitigating Vulnerable Bycatch in Southern African Trawl and Longline Fisheries.* Edited by Petersen SL, Nel DC, Ryan PG, Underhill LG; 2008. WWF South Africa Report Series - 2008/Marine/002.
12. Attwood CG, Peterson SL, Kerwath SE: **Bycatch in South Africa's inshore trawl fishery as determined from observer records.** *ICES J Mar Sci* 2011, **68:**2163–2174.
13. Pank M, Shivji MS, Stanhope M, Natanson L, Kohler N: **Rapid and simultaneous identification of body parts from the morphologically similar sharks *Carcharhinus obscurus* and *Carcharhinus plumbeus* (Carcharhinidae) using multiplex PCR.** *Mar Biotechnol* 2001, **3:**231–240.
14. Domingues RR, de Amorim AF, Hilsdorf AWS: **Genetic identification of Carcharhinus sharks from the southwest Atlantic Ocean (Chondrichthyes: Carcharhiniformes).** *J Appl Ichthyol* 2013, **29:**738–742.
15. Abercrombie DL, Clarke SC, Shivji MS: **Global-scale genetic identification of hammerhead sharks: Application to assessment of the international fin trade and law enforcement.** *Conserv Genet* 2005, **6:**775–788.
16. Farrell ED, Clarke MW, Mariani S: **A simple genetic identification method for northeast Atlantic smoothhound sharks (*Mustelus* spp.).** *ICES J Mar Sci* 2009, **66:**561–565.
17. Naylor GJP, Caira JN, Jensen K, Rosana KAM, White WT, Last PR: **A DNA sequence based approach to the identification of shark and ray species and its implications for global elasmobranch diversity and parasitology.** *Bull Am Mus of Nat Hist* 2012, **367:**1–262.
18. Da Silva C, Bürgener M: **South Africa's demersal shark meat harvest.** *Traffic Bulletin* 2007, **21:**55–65.
19. Clarke SC: **Understanding pressures on fishery resources through trade statistics: A pilot study of four products in the Chinese dried seafood market.** *Fish Fish* 2004, **5:**53–74.
20. Tautz D: **Hypervariability of simple sequences as a general source for polymorphic DNA markers.** *Nucleic Acids Res* 1989, **17:**6463–6471.
21. Weber JL, Wong C: **Mutation of human short tandem repeats.** *Hum Mol Genet* 1993, **2:**1123–1128.
22. Edwards CJ, Soulsbury CD, Statham MJ, Ho SYW, Wall D, Dolf D, Iossa G, Baker PJ, Harris S, Sacks BN, Bradley DG: **Temporal genetic variation of the red fox, *Vulpes vulpes*, across western Europe and the British Isles.** *Quat Sci Rev* 2012, **57:**95–104.
23. Ozerov MY, Veselov AE, Lumme J, Primmer CR: **Temporal variation of genetic composition in Atlantic salmon populations from the Western White Sea Basin: influence of anthropogenic factors?** *BMC Genet* 2013, **14:**88.
24. Urian KW, Hofmann S, Wells RS, Read AJ: **Fine-scale population structure of bottlenose dolphins (*Tursiops truncatus*) in Tampa Bay, Florida.** *Mar Mamm Sci* 2009, **25:**619–638.
25. Mourier J, Mills SC, Planes S: **Population structure, spatial distribution and life-history traits of blacktip reef sharks *Carcharhinus melanopterus*.** *J Fish Biol* 2013, **82:**979–993.
26. Mobley KB, Amundsen T, Forsgren E, Svensson PA, Jones AG: **Multiple mating and low incidence of cuckoldry for nest-holding males in the two-spotted goby, *Gobiusculus flavescens*.** *BMC Evol Biol* 2009, **9:**6.
27. Boomer JJ, Harcourt RG, Francis MP, Walker TI, Braccini JM, Stow AJ: **Frequency of multiple paternity in gummy shark, *Mustelus antarcticus*, and rig, *Mustelus lenticulatus*, and the implications of mate encounter rate, postcopulatory influences, and reproductive mode.** *J Hered* 2013, **104:**371–379.
28. Martin AP, Pardini AT, Noble LR, Jones CS: **Conservation of a dinucleotide simple sequence repeat locus in sharks.** *Mol Phyl Evol* 2002, **23:**205–213.
29. Costa M, Fernandes C, Rodrigues M, Santos-Reis M, Bruford MW: **A panel of microsatellite markers for genetic studies of European polecats (*Mustela putorius*) and ferrets (*Mustela furo*).** *Eur J Wildl Res* 2012, **58:**629–633.
30. Sekino M, Hara M: **Individual assignment tests proved genetic boundaries in a species complex of Pacific abalone (genus *Haliotis*).** *Conserv genet* 2007, **8:**823–841.
31. Dudgeon CL, Blower DC, Broderick D, Giles JL, Holmes BJ, Kashiwagi T, Krück NC, Morgan JAT, Tillett BJ, Ovenden JR: **A review of the application of molecular genetics for fisheries management and conservation of sharks and rays.** *J Fish Biol* 2012, **80:**1789–1843.
32. Boomer JJ, Stow AJ: **Rapid isolation of the first set of polymorphic microsatellite loci from the Australian gummy shark, *Mustelus antarticus* and their utility across divergent shark taxa.** *Conserv Genet Resour* 2010, **2:**393–395.
33. Barbara T, Palma-Silva C, Paggi GM, Bered F, Fay MF, Lexer C: **Cross-species transfer of nuclear microsatellite markers: potential and limitations.** *Mol Ecol* 2007, **16:**3759–3767.
34. Rico C, Rico I, Hewitt G: **470 million years of conservation of microsatellite loci among fish species.** *Proc R Soc Lond B* 1996, **263:**549–557.
35. Primmer CR, Painter JN, Koskinen MT, Palo JU, Merilä J: **Factors affecting avian cross-species microsatellite amplification.** *J Avian Biol* 2005, **36:**348–360.
36. Compagno LJV: **An overview of chondrichthyans systematics and biodiversity in Southern Africa.** *Trans R Soc S Afr* 2000, **54:**75–120.
37. **Department of Agriculture, Forestry and Fisheries (DAFF): South Africa's National Plan of Action for the Conservation and Management of Sharks.** [http://www.daff.gov.za/doaDev/topMenu/DRAFT_NPOA_SHARKS.pdf]
38. **Navigating Global Shark Conservation: Current Measures and Gaps.** [http://www.pewenvironment.org/uploadedFiles/PEG/Publications/Report/Navigating%20Global%20Shark%20Conservation_Current%20Measures%20and%20Gaps%207%206%2012.pdf]
39. **Namibia's National Plan of Action to prevent, deter and eliminate illegal, unreported and unregulated fishing.** [http://209.88.21.36/opencms/export/sites/default/grnnet/MFMR/downloads/docs/Namibia_NPOA_IUU_Final.pdf]
40. Pierce SJ, Trerup M, Williams C, Tilley A, Marshall AD, Raba N: *Shark fishing in Mozambique: A preliminary assessment of artisanal fisheries.* Maputo: Eyes on the Horizon; 2008:1–28.
41. Karaiskou N, Buggiotti L, Leder E, Primmer CR: **High degree of transferability of 86 newly developed zebra finch EST-linked microsatellite markers in 8 bird species.** *J Hered* 2008, **99:**688–693.
42. FitzSimmons NN, Moritz C, Moore S: **Conservation and dynamics of microsatellite loci over 300 million years of marine turtle evolution.** *Mol Biol Evol* 1995, **12:**432–440.
43. Primmer CR, Møller AP, Ellegren H: **A wide-range survey of cross-species microsatellite amplification in birds.** *Mol Ecol* 1996, **5:**365–378.
44. Kang J, Park J, Jo H: **Rapid development of microsatellite markers with 454 pyrosequencing in a vulnerable fish, the mottled skate, *Raja pulchra*.** *Int J Mol Sci* 2012, **13:**7199–7211.

45. Griffiths AM, Casane D, McHugh M, Wearmouth VJ, Sims DW, Genner MJ: **Characterisation of polymorphic microsatellite loci in the small-spotted catshark (*Scyliorhinus canicula* L.).** *Conserv Genet Resour* 2011, **3**:705–709.
46. Zane L, Bargelloni L, Patarnello T: **Strategies for microsatellite isolation: a review.** *Mol Ecol* 2002, **11**:1–16.
47. Primmer CR, Ellegren H: **Patterns of molecular evolution in avian microsatellites.** *Mol Biol Evol* 1998, **15**:997–1008.
48. Angers B, Bernatchez L: **Complex evolution of a salmonid microsatellite locus and its consequences in inferring allelic divergence from size information.** *Mol Biol Evol* 1997, **14**:230–238.
49. Giresi M, Renshaw MA, Portnoy DS, Gold JR: **Isolation and characterization of microsatellite markers for the dusky smoothhound shark, *Mustelus canis*.** *Conserv Genet Resour* 2012, **4**:101–104.
50. Chabot C, Nigenda S: **Characterization of 13 microsatellite loci for the tope shark, *Galeorhinus galeus*, discovered with next generation sequencing and their utility for eastern Pacific smooth-hound sharks (*Mustelus*).** *Conserv Genet Resour* 2011, **3**:553–555.
51. Castoe TA, Poole AW, Gu W, Jason De Koning AP, Daza JM, Smith EN, Pollock DD: **Rapid identification of thousands of copperhead snake (*Agkistrodon contortrix*) microsatellite loci from modest amounts of 454 shotgun genome sequence.** *Mol Ecol Res* 2010, **10**:341–347.
52. Dawson DA, Horsburgh GJ, Küpper C, Stewart IR, Ball AD, Durrant KL, Burke T: **New methods to identify conserved microsatellite loci and develop primer sets of high cross-species utility–as demonstrated for birds.** *Mol Ecol Res* 2010, **10**:475–494.
53. Hoffman JI, Nichols HJ: **A novel approach for mining polymorphic microsatellite markers *in silico*.** *PLoS One* 2011, **6**:e23283.
54. Human BA: **A taxonomic revision of the catshark genus *Haploblepharus* Garman 1913 (Chondrichthyes: Carcharhiniformes: Scyliorhinidae).** *Zootaxa* 2007, **1451**:1–40.
55. Saghai-Maroof MA, Solima KM, Jorgenson RA, Allard RW: **Ribosomal DNA spacerlength polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics.** *Proc Natl Acad Sci U S A* 1984, **81**:8014–8018.
56. Chabot CL: **Characterization of 11 microsatellite loci for the brown smooth-hound shark, *Mustelus henlei*, discovered with next-generation sequencing.** *Conserv Genet Resour* 2012, **4**:23–25.
57. Byrne RJ, Avis JC: **Genetic mating system of the brown smoothhound shark (*Mustelus henlei*), including a literature review of multiple paternity in other elasmobranch species.** *Mar Biol* 2012, **159**:749–756.
58. Guichoux E, Lagache L, Wagner S, Chaumeil P, Léger P, Lepais O, Lepoittevin C, Malaus T, Revardel E, Salin F, Petit RJ: **Current trends in microsatellite genotyping.** *Mol Ecol Resour* 2011, **11**:591–611.
59. Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P: **MICROCHECKER: software for identifying and correcting genotyping errors in microsatellite data.** *Mol Ecol Notes* 2004, **4**:535–538.
60. Rousset F: **GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux.** *Mol Ecol Resour* 2008, **8**:103–106.
61. Slatkin M: **An exact test for neutrality based on the Ewens sampling distribution.** *Genet Res* 1994, **64**:71–74.
62. Excoffier L, Laval G, Schneider S: **Arlequin ver. 3.0: an integrated software package for population genetics data analysis.** *Evol Bioinform Online* 2005, **1**:47–50.
63. Peakall R, Smouse PE: **GenAlEx 6.5: genetic analysis in Excel: population genetic software for teaching and research – an update.** *Bioinformatics* 2012, **28**:2537–2539.
64. Park S: **The Excel microsatellite toolkit.** [http://animalgenomics.ucd.ie/sdepark/ms-toolkit]
65. Botstein D, White RL, Skolnick M, Davis RW: **Construction of a genetic linkage map in man using restriction fragment length polymorphisms.** *Am J Hum Genet* 1980, **32**:314–331.
66. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S: **MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods.** *Mol Biol Evol* 2011, **28**:2731–2739.

doi:10.1186/1756-0500-7-352
Cite this article as: Maduna *et al.*: Microsatellite cross-species amplification and utility in southern African elasmobranchs: A valuable resource for fisheries management and conservation. *BMC Research Notes* 2014 **7**:352.