Molecular species identification and spatio-temporal assessment of genetic diversity in the smooth hammerhead shark *Sphyrna zygaena* in South Africa

by

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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

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March 2017

Declaration

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Abstract

The South African coast hosts a unique oceanographic regime with an enriched habitat able to support a diverse biota of chondrichthyans (sharks, skates, rays and chimaeras). Investigating these species and populations on a molecular level could aid in conserving this rich chondrichthyan biodiversity. As a precursor, a case study regarding the composition of species in three different South African fisheries was evaluated to determine the utility of the mitochondrial cytochrome oxidase subunit 1 (CO1) gene in delimiting species identity. Through this, a number of issues surrounding misidentification and cryptic speciation were recognized, and the efficacy of CO1 was tested and proved to be useful in identifying chondrichthyans affected in South African fisheries. One of these species, the smooth hammerhead shark (*Sphyrna zygaena*) displays a high degree of site fidelity to Mossel Bay as evidenced by the rise in the number of neonate and juvenile hammerheads during the summer season. This species is vulnerable as they are in danger of overfishing and the destruction of their natural habitat. With a low fecundity and a long generational time, recovery of near-depleted populations is prolonged. In this study mitochondrial sequence data and microsatellite markers were used to assess genetic diversity within and between *S. zygaena* sampling cohorts collected from Mossel Bay to the KwaZulu Natal coast. Additionally, kinship between the juvenile individuals was determined and parental genotypes were reconstructed from the neonate and juvenile smooth hammerhead sharks sampled in the Mossel Bay area. Significant population subdivision was evident between individuals sampled in the warm temperate south coast (Mosselbay) and the subtropical east coast (Algoa Bay and KwaZulu Natal), with asymmetric gene flow mainly from the south to the east coast. Highly significant population differentiation was seen between sampling years, indicative of differential temporal stocks utilizing Mossel Bay each year. Analysis of kinship revealed a high degree of sibling relationships within and between seasons, which is likely due to an overlap of some parental genotypes across seasons. The results obtained here can assist with decisions regarding the conservation of chondrichthyan biodiversity in South Africa while it is also recommended that genetic structure and temporal variation of *S. zygaena* populations be evaluated on a finer scale in the future.

Opsomming

Die Suid-Afrikaanse kus huisves 'n unieke oseanografiese gebied wat 'n diverse biota van ‘chondrichthyan’ spesies (haaie, rôe, pylsterte en chimaeras) ondersteun. Molekulêre ondersoeke van hierdie spesies en populasies kan bydrae tot die bewaring van hierdie ryk chondrichthyan biodiversiteit. ’n Gevallestudie is gedoen met betrekking tot die samestelling van spesies in drie verskillende Suid-Afrikaanse visserye om die bruikbaarheid van die mitokondriale sitochroom oksidase subeenheid 1 (SO1) geen te evalueer vir spesies identifisering. Dit het aanleiding gegee tot 'n aantal kwessies rondom die misidentifikasie en kriptiese spesiasie in visserye. Die doeltreffendheid van die SO1 geen is ook bevestig vir die identifisering van ‘chondrichthyans’ geaffekteer in Suid-Afrikaanse visserye. Een van hierdie spesies, die gladde hammerkop haai (*Sphyrna zygaena*) vertoon 'n hoë mate van affiniteit aan Mosselbaai soos blyk uit die toename in die aantal jong hammerkop haaie in die somer seisoen. Hierdie spesie is kwesbaar as gevolg van oorbenutting in visserye asook die vernietiging van hul natuurlike habitat. Met 'n lae vrugbaarheid en 'n lang generasie tyd, kan herstel van geaffekteerde populasies aansienlik verleng word. In hierdie studie is mitokondriale DNS volgorde data en mikrosatelliet merkers gebruik om genetiese diversiteit binne en tussen *S. zygaena* individue gekollekteer van Mosselbaai tot die KwaZulu Natal kus te evalueer. Daarbenewens is die verwantskap tussen die jong individue bereken asook die ouerlike genotipes gerekonstrueer vanaf die jong gladde hammerkop haaie vanaf Mosselbaai. Beduidende populasie struktuur is tussen individue waargeneem afkomstig vanaf die warm gematigde suidkus (Mosselbaai) en die subtropiese ooskus (Algoabaai en KwaZulu-Natal), met asimmetriese geenvloei hoofsaaklik van die suide tot die ooskus. Hoogs beduidende populasie verskille is waargeneem tussen die twee seisoene, wat ‘n aanduiding kan wees van twee verskillende populasies wat Mosselbaai elke jaar besoek. Verwantskap analise het 'n hoë mate van verwantskappe tussen sibbe gewys binne en tussen seisoene, en kan waarskynlik as gevolg van 'n oorvleueling van sommige ouer genotipes oor seisoene wees. Die resultate wat in hierdie studie verkry is, kan help met die verdere besluitneming rakende die bewaring van mariene biodiversiteit in Suid-Afrika. Dit word wel aanbeveel dat die genetiese struktuur en temporale variasie van *S.*

zygaena populasies waargeneem in hierdie studie geëvalueer word op 'n fyner skaal in die toekoms.

Acknowledgements

I would like to thank everyone who has supported me in completing this thesis.

Firstly, I would like to thank my supervisor, Dr. Aletta van der Merwe for bestowing her wellspring of expertise and continued resolve to provide a genuine learning experience for me; this experience has changed my life. Thank you to Dr. Clint Rhode for always having a minute (even when there was only seconds to spare) to help patch up my golden thread and always taking my issues seriously. I want to give many thanks to Dr. Enrico Gennari for valuable input and guidance in pushing me to develop my skills as a scientist. I would like to thank Simo Maduna for always questioning my ideas and staying through to the end of every heated, scientific discourse.

To the White Shark Africa team: Christo Kruger, Craig Ferreira, and Elton Polly, huge thanks for providing me with a platform to work from. I would not be here without your support. Thanks to the PIs, field specialists, and interns who assisted in the sampling efforts; your contributions made hammertime, a great time.

I would like to extend gratitude to the National Research Foundation of South Africa and Stellenbosch University for financial and travel support during my MSc candidacy.

Lastly, I would like to thank my family and especially my parents, Peter and Annie, for always motivating me to be better and do better. You all have inspired me in many ways that I cannot begin to thank you for. This is a stock that I do not want to be differentiated from.

Preface

Scientific Contributions during Masters Candidature (2015-2016)

1. Publications in preparation based on the work presented in this thesis

Kuguru, G., Maduna, S.N., Bitalo, D.N., Gennari, E., Rhode, C., and Bester-van der Merwe, A.E., (in prep). DNA barcoding of chondrichthyans caught by fisheries in South Africa: a case study of molecular species identification. *Fisheries Research*.

2. Local Conference Presentations

Kuguru, G., Gennari, E., Rhode, C., and Bester-van der Merwe, A.E. Oral Presentation: Low genetic diversity and reduced population connectivity in a highly mobile coastal shark, the smooth hammerhead shark *Sphyrna zygaena*. *South African Genetics Society*. September 2016. Durban, South Africa.

Kuguru, G., Gennari, E., Rhode, C., and Bester-van der Merwe, A.E. Oral Presentation: Molecular Assessment of *Sphyrna zygaena* (smooth hammerhead shark): Genetic diversity and population connectivity in Mossel Bay. *Southern African Sharks and Rays Symposium*. September 2015. False Bay, South Africa. (Best oral presentation award)

3. International Conference Presentations

Kuguru, G., Gennari, E., Rhode, C., and Bester-van der Merwe, A.E. Oral Presentation: Genetic diversity of smooth hammerhead sharks *Sphyrna zygaena*: Reconstructing pedigrees and testing for population connectivity along the South African coastline using microsatellites and mitochondrial DNA. *Sharks International 2014*. June 2014. Durban, South Africa.

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List of Abbreviations

°C	Degrees celcius
µL	Microliter
µM	Micromole
%	Percent
π	Nucleotide Diversity
3’	Three prime
5’	Five prime
A	Adenine
AB	Algoa Bay Population
Ae	Effective Number of Alleles
AMOVA	Analysis of Molecular Variance
BOLD	Barcode of Life Data System
bp	Base Pair
BSP	Bayesian Skyline Plot
C	Cytosine
CI	Confidence Interval
COI	Cytochrome Oxidase Subunit I
cm	Centimeter
CITES	The Convention on International Trade in Endangered Species of Wild Fauna and Flora
CMS	The Conservation of Migratory Species of Wild Animals
CTAB	Cetyltrimethylammonium Bromide
DAFF	The South African Department of Agriculture, Forestry and Fisheries
DAPC	Discriminant analysis of principal components
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EEZ	Exclusive Economic Zone
FAM	Blue (R100); 5-carboyfluirescein (ABI-fluorescent label)

F_{IS}	Inbreeding Coefficient (the mean reduction in H of an individual due to non-random mating within a subpopulation)
Fr_{NULL}	Null allele frequency
FS	Full sibling
F_{ST}	Fixation Index (the mean reduction in H of a subpopulation, relative to the total population, due to genetic drift among subpopulations)
G	Guanine
h	Haplotype Diversity
HE	Heterozygote Excess
He	Expected heterozygosity
Ho	Observed heterozygosity
HS	Half sibling
HWE	Hardy-Weinberg Equilibrium
ITS2	Internal Transcribed Spacer 2
IUCN	The International Union for Conservation of Nature
K2P	Kimura Two-Parameter
kya	A thousand years ago
KZN	KwaZulu Natal Population
km	Kilometer
LD	Linkage Disequilibrium
m	Meter
MB/MB1	Mossel Bay 1 Population (December 2013-February 2014)
MB2	Mossel Bay 2 Population (December 2014-February 2015)
MBO	Mossel Bay Out-of Season Population (August 2014)
MBP1	Mossel Bay Reconstructed Parental Generation 1
MBP2	Mossel Bay Reconstructed Parental Generation 2
MCMC	Markov Chain Monte Carlo
$MgCl_2$	Magnesium Chloride
min	Minutes
mtCR	Mitochondrial Control Region

mtDNA	Mitochondrial Deoxyribonucleic Acid
mya	A million years ago
Na	Number of alleles
Ne	Effective Population Size
ND2	NADH Dehydrogenase Subunit 2
ND4	NADH Dehydrogenase Subunit 4
NED	Yellow (Tamra) (ABI-fluorescent label)
NJ	Neighbor-Joining
PCA	Principle Components Analysis
PCR	Polymerase Chain Reaction
PI	Probability of Identity
PIC	Polymorphic information content
R	Related
R	Transition/Transversion Bias
R_{ST}	An analogue of F_{ST} that assumes a stepwise mutational model
S	Segregating Sites
sec	Seconds
SEM	Scanning Electron Micrograph
SMM	Stepwise Mutation Model
SNP	Single-Nucleotide Polymorphisms
spp.	Several Species
T	Thymine
Ta	Annealing Temperature
Taq	*Thermus aquaticus* DNA polymerase
U	Units (enzyme)
UR	Unrelated
US$	US Dollars
VIC	Green (ABI-fluorescent label)

CHAPTER 1

Introduction: Literature Review

1.1 An introduction to *Sphyrna zygaena*

1.1.1 Classification

The chondrichthyan class is comprised of two main superorders of cartilaginous fish: selachimorpha (sharks) and batoidea (rays, skates, and sawfish). Within this grouping, there are about 1,200 species that are functionally specialized across diverse ecosystems (Naylor *et al.* 2012). Able to consume a variety of prey, sharks play a dynamic role in marine ecosystems (Fowler, 2005). New species are continually being discovered and characterized attesting the need for more targeted taxonomic as well as molecular assessment of regional chondrichthyan biodiversity (Fowler, 2005; Ebert & van Hees, 2015). Within the order Carcharhiniformes, the *Sphyrna* genus is one of many genera that have experienced changes to its classification as well as reconfigurations to its evolutionary placement based on molecular phylogenetics (Cavalcanti, 2007). Prior to the application of molecular techniques, phylogenetic inferences were based purely on morphometric analyses. This approach based only on morphology proved itself challenging when attempting to distinguish the three large-bodied hammerheads: *Sphyrna zygaena*, *Sphyrna lewini*, and *Sphyrna mokarran* (Figure 1.1) (Linnaeus, 1758; Compagno, 1988). Their laterally expanded, prebranchial head, known as the cephalofoil, easily identifies hammerheads. While identification of the hammerhead sharks at the genus level is simple, species level identifications are considerably more complex (Compagno, 1984). Though similar in appearance to *S. lewini* and *S. mokarran*, *S. zygaena* lacks a median indentation centered on its cephalofoil, which readily distinguishes it from the other two species (Bass *et al.* 1975; Stevens, 1984). Data from multiple nuclear and mitochondrial genes suggest a common ancestor to all hammerhead sharks, resulting in a new divergent lineage 10 million years ago (Lim *et al.* 2010). In addition to this, a new species of hammerhead, *Sphyrna gilberti*, was recently discovered.

Having long been confused for *S. lewini*, it was only distinguishable through a vertebral count (Quattro *et al.* 2013).

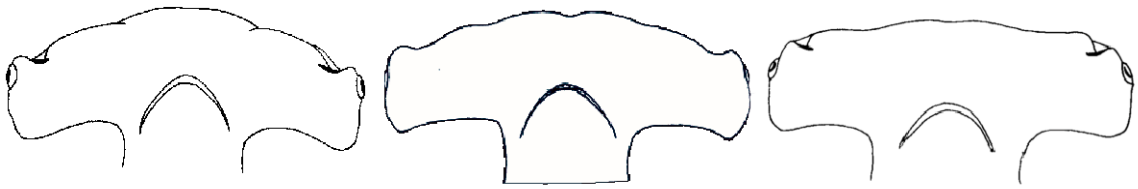

Figure 1.1 Comparison of the cephalofoil shapes between the three large-bodied hammerheads. Difficulty in identifying these species is owed to similarity in the shape of the cephalofoil and a lack of unobscured distinguishable features.

Since the 18th century, *S. zygaena* carried several names including *Squalus zygaena*, *Cestracion zygaena*, and *Zygaena malleus* (Hussakof, 1916; Latham, 1917; Herre, 1930; Springer, 1940). The British ichthyologist, A. Fraser-Brunner, listed the hammer shaped head of the shark (known as the cephalophoil) as the main difference in the Sphyrnidae family, differentiating it from other Carcharhinid sharks (Gudger, 1907; Gudger, 1947; Fraser-Brunner, 1950). Though hammerhead sharks have the most distinguishable features amongst shark species, they maintain the very basic characteristics shared amongst most members of the Carcharhiniformes order. These characteristics include two dorsal fins, the presence of an internal nictitating eyelid, bladelike teeth with a single cusp, and viviparity (Hayes, 2007; Froese & Pauly, 2010).

1.1.2 Distribution and Habitat

As a cosmopolitan species, *S. zygaena* lives in almost all coastal and offshore waters, primarily encompassing the continental and insular shelves in up to 20m depths (Ebert, 2003). They prefer temperate and tropical water conditions and occur in high concentrations during the summer along the South African coast ranging from the Cape coast all the way up to KwaZulu Natal (Compagno, 1988). The majority of inshore encounters are dominated by neonates/juveniles (<150cm) that are generally found swimming in non-uniform shoals near the surface, with rare sightings of adult females (Bass *et al.* 1975). Adults have been found over deep (>100m) reefs at the edge of the

continental shelf, but information on their movement patterns remains for the most part unclear (Smale, 1991; Diemer *et al.* 2011).

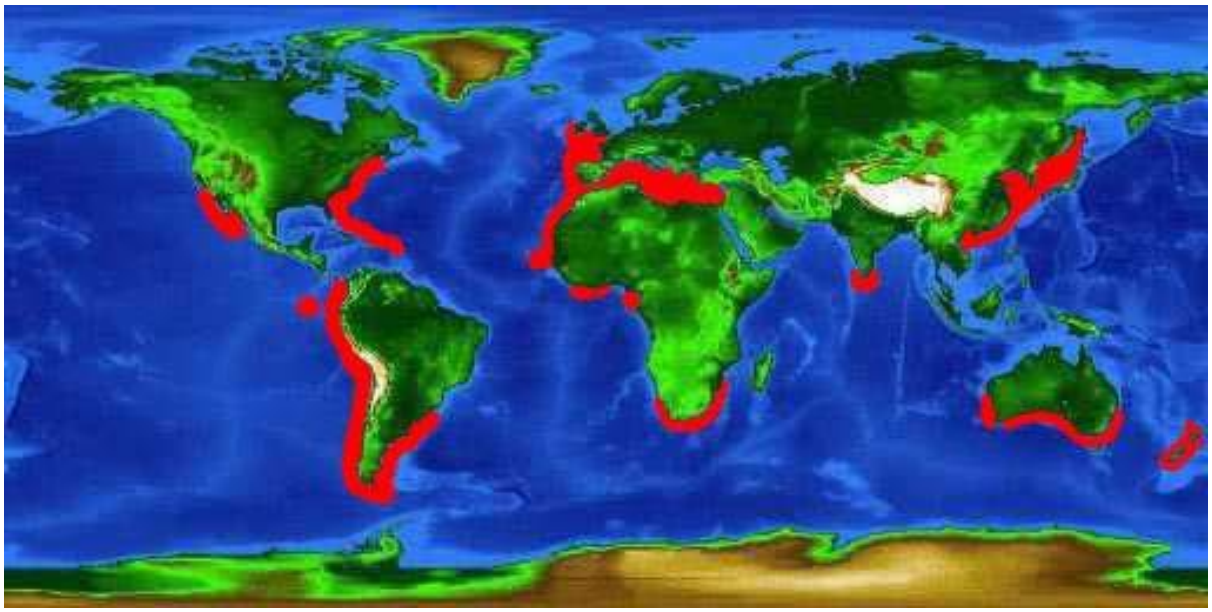

Figure 1.2. Global distribution of *S. zygaena*; their ranges extend beyond national and regional boundaries. (https://www.flmnh.ufl.edu/fish/discover/species-profiles/sphyrna-zygaena)

Some shark species utilize specific areas as nurseries because of resource availability, as well as reduced inter or intra-specific risk of predation. Some evidence exists that certain areas may be used as nurseries, but mainly on the grounds that neonate and juvenile individuals are present in those areas (Beck *et al.* 2001). This becomes too broad of a definition due to the high frequency of sites containing young-of-the-year sharks, with extended home ranges for some species. The suggested interpretation for a nursery ground requires that newborn sharks spend extended periods of time in an area (more so than others), and that these sites are used across multiple years (Heupel *et al.* 2007). Taking this information into account, a more robust definition for a nursery should be attained, and applied to fisheries management.

1.1.3 Biology & Ecology

1.1.3.1 Anatomy and Physiology

The size at birth for the smooth hammerhead shark is 40 – 60 cm while reaching a maximum length of 400cm (Compagno, 1988). The size at maturity is not known yet, but it has been proposed to be between 200 and 300cm (Bass *et al.* 1975). Individuals appear with a light grey to dark brown coloration, with varying black shading on fins. The dorsal fins are tall and narrow from the leading to the trailing edge, with a short free rear tip. A scanning electron micrograph (SEM) reveals that the *S. zygaena* dermal denticles are densely distributed similar to those of *S. lewini* and *S. mokarran*, but differ in the shape and the number of crests (Tanaka *et al.* 2002; Abercrombie *et al.* 2013). Hammerheads have accrued sensory adaptations that likely provide them with an increased propensity for foraging and navigation. In all sphyrnid sharks, the functional morphology of the optical, electrosensorial, and olfactory organs are enhanced and complemented by a larger brain relative to body mass (Kajiura *et al.* 2003; McComb *et al.* 2009; Mello, 2009; Rygg *et al.* 2013). This extension of their neurophysiology has given rise to a more complex cognitive function as evidenced by social behaviour, elaborate migrations, and prey-capture capabilities (Mara, 2010).

1.1.3.2 Diet

Smooth hammerheads feed primarily on fish, cephalopods, and crustaceans (Cortés, 1999). The majority of their diet is comprised of teleosts, but also prey on other elasmobranch species placing them as tertiary consumers based on diet composition and stable isotope analysis (Davenport *et al.* 2002; Houston & Haedrich, 1986).

1.1.3.3 Reproduction

A gestational period of 10 – 11 months normally yields a litter containing 20 – 50 pups that are released during summer to inshore areas. Females will sustain a year long, post-parturition recovery period, allowing them to only reproduce biennially (Bass *et al.* 1975; Smale, 1991). Like many other Carcharhiniformes, the mode of reproduction for *S. zygaena* is viviparous where a yolk-sac placenta nourishes embryos. Maximum age at

maturity is yet to be determined, but these sharks are thought to have a minimum lifespan of 20 years. Slow maturation and long generational times (birth to reaching sexual maturity), combined with a small number of offspring makes these sharks very susceptible to overfishing (Fowler, 2005).

An interesting phenomenon, polyandry, has been described in sharks and is thought to play a role in reducing genetic incompatibilities between mother and embryo (Feldheim *et al.* 2004). This activity is paired with an ability to store sperm from multiple sires until ovulation and fertilization, increasing the capacity of the species to produce viable offspring (Manire *et al.* 1995). On the contrary, evidence for genetic monogamy has been observed in some hammerhead sharks (*Sphyrna tiburo*), caused by an inability for female sharks to successfully store sperm from multiple sires and possibly resulting in broods that are sired by more than one male (Chapman *et al.* 2004). This can decrease the potential of the species to respond to evolutionary pressures since multiple mating efforts do not provide an increase in the amount of genetic diversity (Pearse & Avise, 2001). Assessments of reproductive biology are not currently available for *S. zygaena*.

Evidence of sharks utilizing inshore areas during certain developmental stages has been demonstrated in many studies and is an indicator for philopatry (Hueter *et al.* 2005) Philopatry is derived from the Latin of “home-loving” and can be described as a spatio-temporal pattern undertaken for reproductive purposes. The places these species return to are often sites in which the individuals were born, and return to for reproductive purposes (Feldheim *et al.* 2014). Unlike some species, which only have limited home ranges, philopatric sharks have a period with high site fidelity contrasted to a dispersal phase, which challenges the assumption that sharks remain in a persistent roving state (Tillet *et al.* 2012b). Molecular genetics has been useful in elucidating reproductive behaviour with respect to how sharks use particular areas through measuring variance in allelic frequencies among reproductive groups. Nuclear and mitochondrial genes are usually used in combination because they differ in pattern of inheritance, where the mitochondrial genome is maternally inherited and the nuclear genome is bi-parentally inherited. In general, a higher degree of population genetic structure seen with the

mitochondrial DNA than with nuclear DNA, showing little or no differentiation, could be indicative of philopatric behaviour (Feldheim *et al.* 2014).

1.2 Conservation of Shark Biodiversity

1.2.1 Threats

A decline of large, predatory sharks reduces the natural mortality of a range of prey and alters the distribution and abundance of many other species (Ferretti *et al.* 2010). Without sharks a cascading effect that compromises marine biodiversity is set in motion, affecting all tiers of the trophic scale (Heithaus *et al.* 2008). This has become the reality with the increase of shark fishing spurred on by the rapid increase of East Asian interests in shark related products (Camhi *et al.* 2009). For example, a once rare and expensive delicacy, shark fin soup became available to a financially empowered middle class in China and Hong Kong, creating a steady demand for the product (Asia, 2004). Hammerhead sharks are among the top exploited species for their fins, comprising 6% of all fins in the Hong Kong shark fin market. This market had a severe impact on shark populations globally with 16, 000 tonnes of fins being exported annually from 2000 to 2011, a period which also saw major bans in the trade of shark fins (Dent & Clarke, 2015). Unfortunately, fishermen have largely ignored these bans and have been forced to intrude in international waters, and even World Heritage Sites (Charles *et al.* 2016). Since the decline of shark fins, an upward trend was seen in the demand and supply of shark meat, which can succeed the shark fin trade as the market for shark meat is more available across regions (Dent & Clarke, 2015). There is a paradox however in that shark meat has a relatively low economic value and the higher value of fins still encourages some exploitation and substantial waste of sharks, as fins are harvested and the rest of the shark is discarded (Davidson *et al.* 2015). With many areas seeing declines of sharks, there will also been a decrease in the quality and economic returns of dive tourism (Gallagher & Hammerschlag, 2011; Cisneros-Montemayor *et al.* 2013). For instance, in the Galapagos, hammerhead sharks are a popular attraction and a significant decrease in the number of sharks in this area will have a negative impact economically. Also, shark eco-tourism worldwide has demonstrated that sharks can be worth more alive than dead with the

ability to generate up to US$50 million annually (Bonfil, 1994; Stevens *et al.* 2000; Buckley & Hile, 2007).

Hammerheads are targeted for their fins because of their size and high number of cartilaginous "needle" structures within the fin, but large numbers of other species including *S. zygaena* are also killed through incidental bycatch (Rose, 1996). The majority of fisheries in which they are captured have little to no regulations on practices regarding finning and the use of bycatch (Oliver *et al.* 2015). Fishing records show that the prevalence of *S. zygaena* has diminished over the years and has virtually disappeared in the northwest Atlantic within the last 50 years (Myers *et al.* 2007). They are caught with a variety of fishing gear including pelagic and bottom longlines, drift and set gillnets, hand lines, shrimp trawls, and shrimp trammel nets (CITES, 2015). Even with the availability of diagnostic morphological markers, there is continued difficulty in species level identification between the three large bodied hammerheads and they are often lumped into the generic category of "hammerhead" (Abercrombie *et al.* 2005). This identification issue is compounded when considering the state in which the shark body parts are presented in the marketplace. It is standard practice to detach the fins at sea and process them to the point where they are indistinguishable. Molecular and morphometric methods have been developed to be able to distinguish between the main constituents of the fin trade (Abercrombie *et al.* 2013; Fields *et al.* 2015).

1.2.2 Conservation Status

Inadequate governance can hamper the success of conservation efforts aimed at alleviating the threats facing chondrichthyans. It has been recognized that the rate of consumption of shark products has far exceeded the rate required for stock maintenance (Evans, 2001). IUCN has listed *S. zygaena* as a vulnerable species and although data towards population trends is for the most part lacking, it is apparent there are declines in its global biomass (Fowler, 2005). Further investigation may warrant the species be placed in a higher category with the status of congeners *Sphyrna lewini* and *Sphyrna mokarran* that are listed as 'Endangered'. Based on this, Convention on International Trade in Endangered Species of wild fauna and flora (CITES) Proposal 43 called for *S.*

zygaena to be annexed in CITES Appendix II, which protects over-exploited species from unsustainable trade. The Convention on the Conservation of Migratory Species of Wild Animals (CMS) has put forth an intergovernmental treaty that includes *S. zygaena* in the conservation plan to protect endangered and vulnerable species within the signatory party and range states involved in the organization. These initiatives require the cooperation of all nations in order to optimize efficient management schemes aimed at protecting these sharks (Casper *et al.* 2005). A more comprehensive approach targeting species, stocks, location, and fisheries will facilitate the highest success towards shark conservation (Maguire, 2006; Bräutigam *et al.* 2015; Shiffman & Hammerschlag, 2016). Also, the conservation of sharks and rays necessitates a better understanding of the state of populations as well as the integration of different management tools. A global checklist has been compiled of all living chondrichthyans with a focus on their biogeographic diversity (Weigmann, 2016). Working with these tools can enable fisheries managers, other governmental authorities and the general public to be informed on the appropriate courses and mechanisms that will promote species protection and diversity.

1.3 Molecular Ecology

The application of molecular techniques in fisheries science is a recognized approach in further describing aspects surrounding the identification, phylogeography, biogeography, reproductive behaviours, and demographics of marine species. The tools used have direct implications in understanding the ecology and biology of chondrichthyans, of which over a quarter of the global stocks have been depleted (Stevens *et al.* 2000). Conventional molecular approaches have successfully been used in elucidating a number of basic genetic properties of chondrichthyan populations including kinship, population structure, and identifying cryptic species (Dudgeon *et al.* 2012). Also, with the advent of high throughput technologies more extensive and complicated issues such as cryptic speciation, hybridization and functional genomics can be addressed.

1.3.1 Molecular Species Identification

Taxonomy is the key tool towards assessing and quantifying biodiversity, which in itself is an extension of conservation biology that seeks to classify the fundamental units of biology- the species. DNA barcoding functions on the principle that a universal gene region can be used to identify a species, if the sequence variation is low between conspecifics (Moritz & Cicero, 2004). An initiative to DNA barcode all living organisms was established and led to the development of the Barcode of Life Data System (BOLD), which serves as a curated repository for publically available reference sequences (Ratnasingham & Hebert, 2007). The utility of this tool is reliant on comprehensive sampling and established taxonomic data in order to accurately assign species identity (Meyer & Paulay, 2005). The cytochrome oxidase subunit I (CO1) gene has most often been used to DNA barcode fish, while several other genes such as the NADH dehydrogenase subunit 2 (ND2) and the internal transcribed spacer 2 (ITS2), have also been successful in identifying a range of chondrichthyans, mostly in species composition studies (Ward *et al.* 2009; Naylor *et al.* 2012). Several studies have also applied DNA barcoding to reveal shark landings and potentially illegal activities in the marketplace by identifying shark products in various processed forms (Jabado *et al.* 2015; Velez-Zuazo *et al.* 2015).

The practical use of the DNA barcoding method alone has been scrutinized for the limitations of the CO1 species identification technique. A more holistic approach incorporating alternative gene regions and other types of molecular markers for species identification could alleviate this issue (DeSalle *et al.* 2005; Collins & Cruickshank, 2013). Due to a lack of reliable documentation and fraudulent activities, the degree of exploitation for many chondrichthyans is difficult to assess with precision (Holmes *et al.* 2009; Barbuto *et al.* 2010). The application of DNA barcoding has been useful as a tool for conservation biology for its ability to delimit taxa on a species level (Meusnier *et al.* 2008). Morphological identification schemes however have struggled to delimit species based on developmental stage, cryptic speciation, and degree of disfigurement (Rock *et al.* 2008; Ward *et al.* 2008). Several studies have been successful in producing unambiguous results from DNA barcoding, rapidly discerning a wide array of chondrichthyan taxa with limited specimen material. The information garnered from

these studies can assist in fisheries management and conservation for a cheap and rapid method of identifying commercially exploited and internationally protected species (Ward *et al.* 2005; Holmes *et al.* 2009; Fields *et al.* 2015; Chuang *et al.* 2016). This also holds particular importance for food traceability, where the origin of shark products can also be determined in a range of foods and other seafood products (Wallace *et al.* 2012; Galimberti *et al.* 2013). Given the low degree of error rates (Schlick-Steiner *et al.* 2010) and the demonstrated high performance, molecular identification through DNA barcoding is an effective tool for exploring biodiversity.

1.3.2 Population Genetic Analysis

Fisheries management schemes have sought to investigate the number and composition of fisheries stocks in order to determine the degree of exploitation certain species can tolerate (Carvalho & Hauser, 1995). In general, these management structures have been geared towards sustainable yields in fisheries by promoting the abundance of stocks (Hilborn *et al.* 2004; Worm *et al.* 2009). Population genetic assessments can improve the efficacy of stock management by detecting population differentiation and the number of discreet genetic stocks present for a particular species. This gives an indication of the levels of genetic variation and the ability of a species to adapt to environmental changes (Beheregaray & Caccone, 2007; Ovenden *et al.* 2009).

Several types of molecular markers have successfully uncovered genetic structure in order to reflect contemporary and historical processes influencing the population dynamics of chondrichthyans. To address a biological question on a biogeographic (contemporary) or phylogeographic (historical) scale, the correct molecular markers must be employed. In short, the feasibility of the molecular technique and the degree of variability of a specific gene region must be considered to best answer the question at hand (Sunnucks, 2000). For detecting population subdivision for example, the utility of highly polymorphic markers such as microsatellites is required, while mitochondrial genes can be used to infer genealogical lineages (Templeton, 1998). Population differentiation, inferred from genetic structure analysis, can help identify distinct genetic stocks, while measures of gene flow can indicate the directionality of dispersal or

migration between populations. Subsequent measures of subdivision can also assist in exposing the underlying biological processes such as philopatry or gender-specific dispersal that affects the distribution of alleles between sampling populations (Freeland *et al.* 2011).

A number of mitochondrial gene regions have been used in population level studies of sharks, including the mitochondrial control region (Chapman *et al.* 2009b; Pinhal *et al.* 2012), NADH dehydrogenase subunit 2 (Veríssimo *et al.* 2012; Bernard *et al.* 2016), NADH dehydrogenase subunit 4 (Dudgeon *et al.* 2009; Tillet *et al.* 2012a; Maduna *et al.* 2016), and cytochrome b (Castro, 2009; Pereyra *et al.* 2010). All of these mitochondrial loci have been useful in determining population genetic structure through haplotype analysis and historical demographic analysis, while the mitochondrial control region (mtCR) has been the most widely used. Previously, the mtCR was considered to be the most variable (Duncan *et al.* 2006; Keeney & Heist, 2006; Schultz *et al.* 2008; Gubili *et al.* 2010), but the use of this gene has been questioned by a recent mitogenomic study that revealed a significant lack of variability in the speartooth shark *Glyphis glyphis* (Feutry *et al.* 2014). This highlights the need to determine rates of intraspecific variance and informativeness for each mitochondrial marker in consideration.

Microsatellites are nuclear genome sequences that are a favored molecular marker in population studies due to their highly polymorphic nature, simplicity in genotyping, and wide distribution within genomes. They have a high resolving power to successfully infer population genetic structure, contemporary rates of migration, and contemporary demographics (Selkoe & Toonen, 2006). Microsatellite markers are comprised of tandem repeats of mono-, di-, tri-, or tetranucleotide motifs. Polymorphisms occur at a high rate with each allele being characterized by the number of repeated motifs (Tagu & Moussard, 2006). Microsatellite markers has become widely applied as well as cost efficient through the application of multiplexing and cross-species transferability of primers (Barbara *et al.* 2007). Multiplexing co-amplifies several microsatellite loci in a single PCR reaction to reduce the number of genotyping rounds, which also reduces time and cost. This is accomplished by combining several fluorescently labeled primer sequences in a single

PCR reaction (Neff *et al.* 2000). Mining microsatellite loci *de novo* is time-consuming and expensive, which could also result in a majority of monomorphic loci not suitable for population genetic analyses (Hoffman & Nichols, 2011). The use of cross-species amplification has alleviated the time and costly effort of *de novo* isolation of markers, by using loci developed for congeners or closely related taxa that target homologous loci (Wilson *et al.* 2004; Maduna *et al.* 2014).

Within the carcharhiniformes order, it has been noted that phylogeographic barriers generally restrict gene flow across oceanic basins, even amongst species with high dispersal potential (Dudgeon *et al.* 2012). From the global population genetic analysis of *S. zygaena* and *S. lewini*, there is significant matrilineal differentiation between basins with a lack of differentiation in the biparentally inherited microsatellites within biogeographic regions (Daly-Engel *et al.* 2012; Testerman, 2014). In contrast, an intra-regional evaluation of *S. lewini* in its Eastern Pacific range found a lack of structure in the mitochondrial DNA with highly significant structure in the microsatellite markers. This was attributed to population declines and isolation caused by anthropogenic pressures (Nance *et al.* 2011). These findings highlight the relevance of regional phylogeographic analysis, in conjunction with global phylogeographic analysis, using multiple markers for fisheries management. Regional studies within the southwest Indian Ocean have shown that even with a high degree of matrilineal structure and segregation in populations of *Carcharhinus brachyurus* and *Galeorhinus galeus*, there still exists a high degree of contemporary gene flow across various biogeographic barriers (Chabot & Allen, 2009; Benavides *et al.* 2011). The known biogeographic barriers around the South African coastline, such as ocean currents and thermal fronts, are the primary influences that determine the degree of gene flow for a number of species in this region (Teske *et al.* 2013). It is likely that these barriers play a similar role and determinant of population genetic structure and gene flow in the South African shark populations of *Galeorhinus galeus* and *Mustelus mustelus* (Bitalo *et al.* 2015; Maduna *et al.* 2016). In these studies, highly significant differentiation was seen between oceanic basins of the southeast Atlantic Ocean and the southwest Indian Ocean. Levels of connectivity were significantly higher in the *G. galeus* populations, which could be attributed to their higher dispersal

capabilities (Hernández *et al.* 2015). Populations of *Carcharias taurus* within the South African coastline have also experienced reductions in connectivity and gene flow that are linked to impediments from the cold Benguela Current (Ahonen *et al.* 2009). Owing to a physiological constraint, this significant drop in temperature is unsuitable for *C. taurus* (Dicken *et al.* 2006). As demonstrated, the presence of a single biogeographic barrier can present a myriad of scenarios that influence gene flow and population connectivity. It is therefore critical to use population specific data to manage each of these stocks to its best ability while keeping multiple species fisheries and eco-system requirements into account (Bester-Van der Merwe & Gledhill, 2015).

1.3.3 Kinship Analysis

Analysis of kinship among juveniles can allow for a deduction of the male and female contribution to the sampled populations (Owens, 2006; Davies *et al.* 2012). This is often accomplished through the application of microsatellite loci to explore relatedness through parentage and siblingship analysis (Ribolli *et al.* 2016). Studies of relatedness can also determine evolutionary potential by understanding the breeding potential for populations in the wild, which experience different selective pressures than those under domestication or some form of selection (DeWoody, 2005). Where data on broodstock are unavailable or unassigned, fairly simple procedures can allow for the reconstruction of parental genotypes from offspring genotypes (Feldheim *et al.* 2004). An indication of mating behaviour, often related to polyandry, can also be garnered from this information, however it is often imperative that litters are sampled and not just juveniles that share the same localities (Portnoy *et al.* 2007; DiBattista *et al.* 2008a).

Philopatry in sharks is often characterized by the habitual return or extended stay of pups to inshore areas during a period of their life history. These localities provide optimal environmental conditions that foster growth, prior to the using areas for mature sharks (Reyier *et al.* 2008; Chapman *et al.* 2009a). Studies focused on juvenile offspring, rely on preliminary assessments of siblingship between juvenile individuals over several years in order to detect patterns in diversity (Nance, 2010; Tillet *et al.* 2012b; Larson *et al.* 2015). Among these studies, the probability of half sibling and full sibling relationships over the

years ranges from 0.4 – 0.8 and 0.0 – 0.1 respectively. Subsequent profiling (>10 years) of breeders and/or offspring can allow for a characterization of reproductive behaviours in sharks, like reproductive or natal philopatry (Mourier & Planes, 2013; Feldheim *et al.* 2014).

Telemetry is a powerful tool in resolving the presence of philopatry amongst shark species with high dispersal capabilities (Howey-Jordan *et al.* 2013). In order to adequately designate species as philopatric, the genetic data must be complemented by telemetric data to distinguish between a philopatric species rather than a species with a limited home range (Dudgeon *et al.* 2012). This will allow for the most informative assessments of spatial use in order to infer philopatry for use in fisheries management schemes.

1.4 Research Aims and Objectives

The overall aim of this study is to address issues related to the conservation requirements of *S. zygaena* in South Africa, through the use of molecular approaches.

The first step was to validate the utility of molecular techniques in species identification. Thus, chapter 2 is a case study that examined species composition through molecular based identification of different shark species sampled opportunistically. Through this, a verification of morphological versus molecular identification schemes was made to determine any incongruence for the taxa investigated.

At a regional scale, the primary objective of Chapter 3 was to use microsatellite and mitochondrial sequence data to examine population genetic structure and demographics of *S. zygaena* sampled across to major biogeographic regions, the southern warm temperate and the eastern subtropical regions, along the South African coastline.

More locally, in Chapter 4, the relatedness of *S. zygaena* within the sampling area of Mossel Bay was investigated to determine temporal genetic variation. This serves as a

precursor to an on-going effort to investigate possible philopatry in this highly migratory hammerhead species.

CHAPTER 2

DNA barcoding of chondrichthyans caught by fisheries in South Africa: a case study of molecular species identification

Abstract

South Africa has one of the highest chondrichthyan species diversities in the world, with more than 35% affected by regional fisheries. In order to evaluate levels of diversity and impact of fisheries, accurate data on species occurrence and fisheries composition are needed. In this study, samples were collected from trawl, recreational, and line fisheries across three bioregions in South Africa. A subsample (76 specimens) from five different sampling efforts was sequenced for the mitochondrial cytochrome oxidase subunit 1 (CO1) gene. Species identity was inferred through sequence similarity testing using barcoding sequences available in the Barcode of Life Data (BOLD) database. A total of 18 species from ten different families of sharks and rays were identified. Tree-based identification and molecular diversity statistics were implemented to validate taxonomic ranking through a distance-based method. Within the triakid and Carcharhinid families, sequence similarity values for some specimens did not contain a satisfactory barcoding gap, which demonstrates the limitations of the CO1 gene to delimit species identity for all taxa involved. This study provides updated knowledge on the regional chondrichthyan biodiversity affected by South African fisheries as well as the utility of DNA barcoding for species identification. Sequences from species with specimen vouchers could also provide novel reference sequences for BOLD.

2.1. Introduction

There has recently been a downward trend in the trade of shark fins, however the trade of shark meat has increased at a rate of 4.5% from 2000 – 2011 globally. During this time, South Africa ranked at 20th in the world for the export of shark meat with an average of 942 tonnes (worth more than US$ 3,000,000) per annum (Dent & Clarke, 2015). It has been demonstrated that habitat degradation and climate change can amplify the effects that overfishing has on fish stocks (Waples & Audzijonyte, 2016). Due to low productivity rates, chondrichthyans are more vulnerable towards these rapid environmental changes (Stevens *et al.* 2000). This factor in conjunction with a high demand for shark and ray products places chondrichthyan populations at high risk (Field *et al.* 2009; Hutchings *et al.* 2012). Recent declines in shark and ray landings were thought to be a result of more effective monitoring leading to reduced catches. However, the possibility exists that the declines may be a result of an increased demand and catch effort, which has debilitated the stocks to a point of no recovery (Dulvy *et al.* 2014; Davidson *et al.* 2015).

The South African coastline encompasses some 3650 km and an Exclusive Economic Zone (EEZ) of just over 1 million km that includes two oceans, the South-East Atlantic and South-West Indian Ocean, spanning all nine marine bioregions (Griffiths *et al.* 2010). Chondrichthyans face high fishing pressures from direct and indirect (bycatch) fisheries, with approximately 25% of all chondrichthyans in the region being threatened with extinction according to the International Union for the Conservation of Nature (IUCN 2015). A major concern regarding the sustainability of chondrichthyan fisheries in South Africa is inaccurate catch data on species composition, in part due to species misidentification (da Silva *et al.* 2015). A number of issues related to identification can hamper the success of chondrichthyan stock assessments. Individuals are often categorized as the same species or as a member of the same genus, with generic names like “vaalhaai” (grey shark) and “hondhaai” (hound shark) that used to classify members of the *Mustelus*, *Galeorhinus*, and *Carcharhinus* genera. This issue is compounded when catches are processed at sea and many distinguishing features are compromised, resulting in misidentifications and inaccurate catch composition reports. This could also allow

fraudulent activities to go unregulated. In addition to this, hybridization and cryptic speciation suspected for a number of genera with the South African chondrichthyan biodiversity can compound the accurate designation of specimen identity.

Alpha taxonomy has provided the foundation for barcoding studies, while barcoding in turn has highlighted the necessity for an integrative taxonomic approach drawing from several fields of species biology, ecology, morphology, as well as genetics. The combination of each of these systems provides a complementary approach allowing character traits to be assigned with greater accuracy (Schlick-Steiner *et al.* 2010; Ebert & van Hees, 2015; Sukumaran & Gopalakrishnan, 2015). With this taken into account, efforts are being made to describe new species by complementing morphometrics with DNA barcoding as an important tool to distinguish congeneric animals based on the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene (Laurito *et al.* 2013; Chan *et al.* 2014; Sumruayphol *et al.* 2016). The CO1 gene is currently the most favourable gene for DNA barcoding in animals, because of its comparatively high inter-species (7.0-8.0%) and low intra-species (0.2-0.5%) variation (Hebert *et al.* 2003a; Hebert *et al.* 2003b; Ward *et al.* 2005; Ratnasingham & Hebert, 2007; Liu *et al.* 2013). Although the effectiveness of CO1 in barcoding of fish species has been validated, there are a number of studies showing some limitations of the CO1 gene in chondrichthyan identification (Naylor *et al.* 2012; Bester-van der Merwe & Gledhill, 2015). Accuracy of the barcoding data is dependent on a clear delineation between intraspecific variation and interspecific divergence. When there is an overlap between the genetic variation within a species and divergence between closely related or congeneric species, the DNA barcode cannot be assigned with confidence. This is especially the case for some cryptic taxa that have not been extensively sampled and/or have limited taxonomic data (Meyer & Paulay, 2005).

In this study, species composition and incongruences in molecular versus morphological identification surrounding chondrichthyan species caught in South African fisheries were examined using a DNA barcoding approach. Shark species composition was assessed in three different shark fisheries to validate existing identification schemes and identify unrecognized taxa. A combination of morphological and molecular data could provide a

more accurate account of fisheries data relating to chondrichthyans in South Africa and provide novel sequences from species identified with specimen vouchers for public databases such as the BOLD system and GenBank.

2.2 Methods

2.2.1 *Ethics Statement*

This study was carried out in accordance with the guidelines of the *Permit for the Purposes of a Scientific Investigation or Practical Experiment in Terms of Section 83 of the Marine Living Resources Act, 1998 (Act No. 18 of 1998)* established by the South African Department of Agriculture, Forestry and Fisheries (DAFF). The sampling protocol was approved by DAFF under the permits RES2014/01 and RES2015/74. Under these conditions, sharks were acquired for sampling for *bona fide* research and returned to their habitat with efforts taken to minimize stress and mortality. Samples taken from fisheries were acquired opportunistically.

2.2.2 *Study area & Sampling*

Chondrichthyan catches at various fish landing sites and during recreational fishing in South Africa were recorded on five occasions between November 2013 and April 2015. Samples were collected from various fishery methods such as trawl, rod/handline, and longline fishing gear across different coastal regions in South Africa (Fig. 1). Samples included in this study were binned according to the fishing method used and the sampling region. Specimens were morphologically identified using the keys of Compagno *et al*. (2005), da Silva (2007) and Mann (2013). Owing to logistical constraints, measurements and biological data could not be taken from all individuals, but where possible, total length of each individual was measured to the nearest 1 mm and its sex recorded. Fin clip samples were collected and the number of each species landed or caught per fishing effort was recorded.

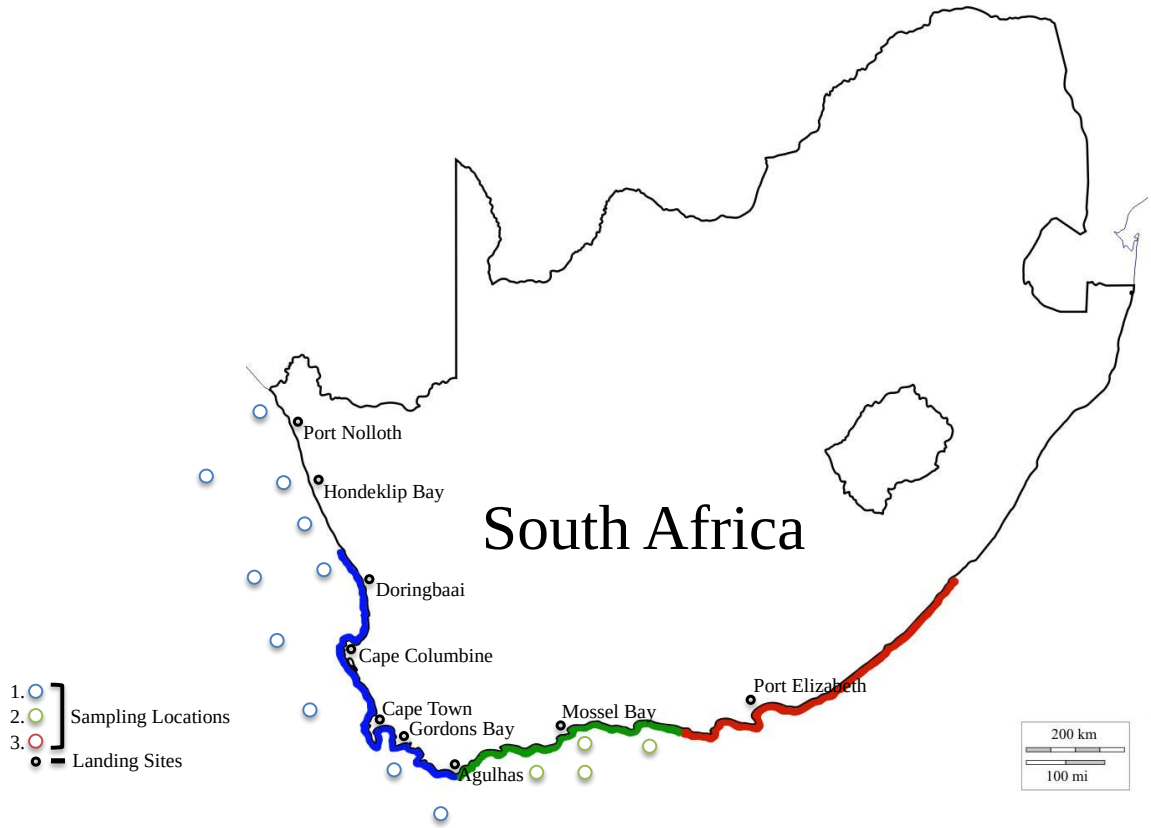

Figure 2.1. Map of South Africa with coloured lines demarcating the three biogeographic areas from where genetic samples were sourced; Western Cape (1), southern Cape (2), Eastern Cape (3).

The Fisheries Research group at the DAFF collected samples from the eastern coast through a longline fishing operation. Shark landings were reported (including sex, total length) from the east coast during January and February 2015. The catches comprised mostly pelagic sharks and skates from three different families: Carcharhinidae, Triakidae, and Rajidae. With 78 chondrichthyans sampled in total, a sub-sample of 13 morphologically ambiguous or cryptic individuals were selected for DNA barcoding. Along the southern Cape, chondrichthyans were targeted for recreation and sport, and were captured using rod and handline fishing methods. Data was collected with tissue and basic biological data such as species, date and locality of capture, size, sex, and tag number, and photographs of morphology. These photographs provided a visual identification data set, which was compared to the key identifying features in Compagno *et al*. (2005) and da Silva (2007). Additional samples were taken from sport fisherman

and fishing competitions. A total of 508 shark and ray, fin and disc tissue were obtained representing over 20 different species from Vleesbaai all the way to Plettenberg Bay. Samples were stored in absolute ethanol and 27 were selected for barcoding.

In addition, a total of 407 samples were collected for a demersal hake biomass survey from mid-2013 to mid-2014, in areas on the west coast from landing sites between Cape Agulhas and Port Nolloth. In these operations, catches are typically offloaded and identified to the lowest possible taxonomic level, however catches are often mislabelled. The ten individuals selected for barcoding were comprised solely of sharks and identification was narrowed down to two orders, Carcharhiniformes and Squaliformes. Sharks captured were mostly as a result of by-catch.

2.2.3 *Molecular Species Identification*

Genomic DNA was extracted from 250mg of fin clip or muscle tissue using the standard cetyltrimethylammonium bromide (CTAB) method of Saghai-Maroof *et al*. (1984). The DNA concentration and quality were determined with a NanoDrop ND 2000 spectrophotometer (Thermo Fisher Scientific; www.thermofisher.com). Subsequently each DNA sample was diluted to a working stock concentration of 50 ng/µL and stored at -20ºC until further analysis.

The mitochondrial cytochrome c oxidase I (CO1) gene fragment was amplified using the primers FishF1 and FishR1 according to the PCR conditions outlined in Ward *et al*. (2005). Amplicons were sequenced in both directions using standard Sanger sequencing chemistry (BigDye® terminator v3.1 cycle sequencing kit, Life Technologies) and capillary electrophoresis conducted at the Central Analytical Facility, Stellenbosch University. Sequences were manually edited for sequential errors using 4Peaks (Griekspoor & Groothuis, 2005) and assembled in MEGA 7.0.14 (Kumar *et al*. 2016). Edited sequences were aligned using the MUSCLE algorithm with default parameters (Thompson *et al*. 1994) implemented in MEGA 7.0.14 and trimmed to equal lengths of 550bp. The public database BOLD was used to identify all unique sequences within the

Animal Species Level Barcode records for a minimum sequence length of 500bp. Sequences not able to be identified in BOLD were identified in GenBank using the Basic Local Alignment Search Tool (BLAST) function.

For genetic distance calculations, the evolutionary model was selected and applied through MEGA. The Kimura Two-Parameter (K2P) distance model was selected on the basis of the Akaike information criterion, as the best-fit model (Nei & Kumar, 2000). A Neighbor-Joining (NJ) tree was constructed to visualize the relationships between the CO1 haplotypes of all the species, genera and families represented in the samples sequenced (Collins & Cruickshank, 2013). In this analysis, nodal support was assessed through a 1000 bootstrap iterations (Saitou & Nei, 1987; Kimura, 1980; Kumar *et al.* 2016). Nucleotide diversity statistics were generated using DnaSP 5.10 (Librado & Rozas, 2009).

2.3 Results

2.3.1 *Catch Statistics*

Longlining - (Eastern cape)

The landing comprised mostly *Carcharhinus obscurus* and *Mustelus mustelus* though the samples were taken from a wider array of fish. The catches associated with the longline seemed to target all areas of the water column. This is evidenced by the large number of the demersal *M. mustelus* and the pelagic *C. obscurus* catches. There were a number of species from the Rajidae family in the cohort, which indicates that there may have been a fishing effort targeting demersal fish, or perhaps that there was incidental by-catch from the longline trail.

Fishing Charters – (southern Cape)

Recreational fishing ventures normally seek game fish and exotics for sport, normally employing the catch and release method. Sites targeted are normally comprised of complex reef systems or even aggregation sites known to be highly productive with large bony fish, sharks, and rays. This was reflected in the catch data that showed a high

quantity of reef-based chondrichthyans such as catsharks, houndsharks, and batoids with pelagic/semi-pelagic carcharhinid and lamnid sharks comprising the majority. As a preliminary identification method, the morphological features could be used to identify the sharks to the most likely species through key diagnostic features, like dorsal fins, caudal fins, and rostrum. For congeneric species however, there was a broad overlap of morphological features, while subtle discriminating criteria still allowed for species assignment.

Trawling – (Western Cape)

South Africa has a large fishing industry with the largest fisheries focused within the Western Cape. This is due to the upwelling phenomenon concentrated in the oceanic transitional zone that results in high biological activity supporting species like pilchard, anchovy, hake, and mackerel (Griffiths *et al.* 2010). With only two months of catching effort, the trawling managed to capture 349 chondrichthyans, which represented a minimum of ten different species. Most of these species occur in depths ranging from 100-1000m, indicating that operators were bottom trawling. The ten species represented three families, Triakidae, Squalidae and Scyliorhinidae, of which two include commercially important species (dogfish and houndsharks).

2.3.2 *Species Identification*

Species level matches were made in BOLD using 550bp of the CO1 gene (Table 2.1). All barcoded chondrichthyan individuals included details regarding initial identification, best sequence match, and second best sequence match. Among all data sets, 48 out of 50 representative samples gave a minimum 97% sequence similarity match with an average of 99%. The two sequences with less than 97% sequence similarity matches, failed to obtain a positive match in BOLD and was compared against the GenBank database instead. The alignment matches are detailed in Table 2.1. Sequences from 23 specimens produced a sequence match with 14 species matching with 100% sequence similarity. Only three individuals matched below 98%.

Table 2.1. Samples from fisheries sequenced and barcoded for species identification. The best matches, as well as the second best match with corresponding percentage similarities are listed. Secondary matches were only made if it was different from the best match.

Initial Identification	Location	Best matched species	Similarity %	2nd Best matched species	Similarity %
Raja straeleni	Eastern Cape	*Raja straeleni**	99.84	*Raja clavata*	98.59
Raja wallacei	Eastern Cape	*Leucoraja wallacei*	99.68	*Dipturus pullopunctata*	99.36
Rostroraja alba	Eastern Cape	*Rostroraja alba****	100	N/A	
Rostroraja alba	Eastern Cape	*Rostroraja alba****	100	N/A	
Carcharhinus obscurus	Eastern Cape	*Mustelus mustelus***	100	N/A	
Galeorhinus galeus	Eastern Cape	*Galeorhinus galeus***	99.84	N/A	
Mustelus mustelus	Eastern Cape	*Galeorhinus galeus***	100	N/A	
Raja straeleni	Eastern Cape	*Sphyrna zygaena***	100	N/A	
Raja straeleni	Eastern Cape	*Etmopterus brachyurus**	99.49	*Etmopterus lucifer*	99.12
Mustelus mustelus	Eastern Cape	*Mustelus mustelus***	99.66	N/A	
Galeorhinus galeus	Eastern Cape	*Mustelus mustelus***	99.5	N/A	
Mustelus mustelus	Eastern Cape	*Galeorhinus galeus***	98.61	N/A	
Unlabelled	Eastern Cape	*Mustelus mustelus***	99.67	N/A	
Unlabelled	Southern Cape	*Haploblepharus edwardsii*	98.25	*Halaelurus lineatus**	90.02
Unlabelled	Southern Cape	*Haploblepharus edwardsii*	97.3	*Halaelurus lineatus**	89.23
Unlabelled	Southern Cape	*Poroderma africanum*	100	*Poroderma pantherinum**	99.23
Unlabelled	Southern Cape	*Poroderma africanum*	100	*Poroderma pantherinum**	99.21
Unlabelled	Southern Cape	*Poroderma pantherinum**	100	*Poroderma africanum*	99.2
Unlabelled	Southern Cape	*Poroderma pantherinum**	99.59	*Poroderma africanum*	99.18
Carcharias taurus	Southern Cape	*Carcharias taurus***	99.61	N/A	
Carcharias taurus	Southern Cape	*Carcharias taurus***	99.43	N/A	
Triakis megalopterus	Southern Cape	*Traikis megalopterus*	100	*Scylliogaleus quecketti*	99.84
Dasyatis chrysonota	Southern Cape	*Dasyatis chrysonota*	100	*Dasyatis marmorata*	98.5
Dasyatis chrysonota	Southern Cape	*Dasyatis chrysonota*	99.84	*Dasyatis pastinaca**	98.51
Carcharhinus obscurus	Southern Cape	*Carcharhinus obscurus***	87	*Carcharhinus galapagensis*	87
Pteromylaeus bovinus	Southern Cape	*Myliobatis aquila**	82	*Myliobatis tobijei**	80
Carcharhinus obscurus	Southern Cape	*Carcharhinus galapagensis*	99.66	*Carcharhinus longimanus***	99.66
Torpedo marmorata	Southern Cape	*Dasyatis chrysonota*	100	*Dasyatis marmorata**	98.59
Carcharhinus brevipinna	Southern Cape	*Carcharhinus brevipinna*	98.2	N/A	
Unknown8	Southern Cape	*Carcharhinus brachyurus*	99.8	N/A	
Unknown11	Southern Cape	*Carcharhinus brachyurus*	99.84	N/A	
Unknown13	Southern Cape	*Carcharhinus brachyurus*	100	N/A	
Unknown14	Southern Cape	*Carcharhinus brachyurus*	100	N/A	
Unknown23	Southern Cape	*Mustelus lenticulatus*	99.68	*Mustelus asterias*	99.68
Unknown15	Southern Cape	*Mustelus palumbes**	100	*Mustelus asterias*	99.69
Unknown18	Southern Cape	*Mustelus palumbes**	100	*Mustelus asterias*	99.69
Sphyrna zygaena	Southern Cape	*Sphyrna zygaena***	100	N/A	
Sphyrna zygaena	Southern Cape	*Sphyrna zygaena***	100	N/A	
Unknown3	Southern Cape	*Sphyrna zygaena***	100	N/A	
Unknown20	Southern Cape	*Sphyrna zygaena***	99.82	N/A	
Holohalaelurus regani	Western Cape	*Holohalaelurus regani*	99.53	*Holohalaelurus punctatus*	90.57
Holohalaelurus regani	Western Cape	*Holohalaelurus regani*	99.53	*Holohalaelurus punctatus*	90.77

Table 2.1 (continued). Samples from fisheries sequenced and barcoded for species identification. The best matches, as well as the second best match with corresponding percentage similarities are listed. Secondary matches were only made if it was different from the best match.

Holohalaelurus regani	Western Cape	*Holohalaelurus regani*	100	*Cyclophorus affinis*	82
Holohalaelurus regani	Western Cape	*Holohalaelurus regani*	100	*Holohalaelurus punctatus****	91.14
Scyliorhinus capensis	Western Cape	*Scyliorhinus capensis*	99.53	*Scyliorhinus retifer*	95.58
Scyliorhinus capensis	Western Cape	*Scyliorhinus capensis*	99.84	*Scyliorhinus retifer*	95.89
Scyliorhinus capensis	Western Cape	*Scyliorhinus capensis*	100	*Scyliorhinus retifer*	96.06
Squalus megalops	Western Cape	*Squalus blainville**	99.64	*Squalus acanthias***	99.1
Squalus megalops	Western Cape	*Squalus megalops**	100	*Squalus mitsukurii**	100
Squalus megalops	Western Cape	*Squalus blainville**	100	*Squalus megalops**	99.8

* IUCN rating is Data Deficient
** IUCN rating is Near Threatened or Vulnerable
***IUCN rating is Endangered

Longlining – (Eastern Cape)

Match percentages in BOLD for the Eastern Cape specimens were high (> 99%) across all individuals sequenced. The field identifications were consistent with the sequence matches for *Mustelus* spp. and *Galeorhinus galeus*, indicating accurate identifications within the houndsharks. The sequence matches for *Rostroraja alba* were well referenced in BOLD, as all matches to *R. alba* were 100% without a secondary match. Of the species caught in the Eastern Cape, only *L. wallacei* and *E. brachyurus* are endemic to Southern Africa. *E. brachyurus* is normally considered a very rare catch as it inhabits an upper depth limit of 400m (McCormack *et al.* 2015).

Fishing Charters – (southern Cape)

The majority of the sequences attained species level matches that confirmed their initial identification based on field guides, with only a few misidentifications. The matches made from the fins taken from a previously identified *Carcharhinus obscurus* were matched to *Carcharhinus galapagensis*, *Carcharhinus longimanus*, and *Carcharhinus obscurus* all at 99.66%. The number of matches for *C. galapagensis* outranked the other two species and based on sequence similarity in BOLD, the specimens matched closest to *C. galapagensis*. With the added benefit of species vouchers and photographs, the likelihood that the sequence belonged to *C. longimanus* was low due to a lack of white pigmentation on the first dorsal fin. However, *C. galapagensis* have not been described

within Southern African waters and highlights the limitations of the CO1 gene to resolve such identification issues in chondrichthyan groups that are slow-evolving and possibly also experience hybridization (Radulovici *et al.* 2010)

Trawling – (Western Cape)

All samples selected for sequencing were morphologically identified as either dogfish or catsharks. For the endemic catsharks, *Holohalaerus regani* and *Scyliorhinus capensis*, species level matches were all above 99% most likely due to a sufficient number of representative sequences of the species available in BOLD. On the other hand, *Squalus megalops* and *Squalus blainville* had matches with other congenerics with sequence similarities above 99% but alludes to the taxonomic issues previously highlighted for the genus and those two species in particular (Veríssimo *et al.* 2016). Overall, it does not appear that misidentifications in the field are particularly prevalent, but some inconsistencies may arise from species that are morphologically difficult to distinguish.

2.3.3 *Diversity Analysis*

From the tree-based identification, it was evident that a number of species showed intra-specific variation based on the number of CO1 haplotypes present (Figure 2.2). Although bootstrap values were low (40% – 50%) for some nodes, the tree was congruent with similar regional barcoding studies on chondrichthyans at the genera level (Ribeiro *et al.* 2012; Liu *et al.* 2013; Moura *et al.* 2015; Bineesh *et al.* 2016). Diversity statistics generated from DnaSP revealed the highest nucleotide diversity (π) values for species belonging to the Scyliorhinidae family. With values for the CO1 in animals ranging from $\pi = 0.0\text{-}0.1993$ with an average of $\pi = 0.00388$ (Goodall-Copestake *et al.* 2012), this was unexpected as previous studies have demonstrated the inability of the CO1 to distinguish between species from the *Haploblepharus* genus for example (Bester-van der Merwe & Gledhill, 2015). According to the phylogenetic tree, *Triakis megalopterus* clustered within the *Mustelus* grouping. Based on alignments in BOLD, individuals for *T. megalopterus* specimens had cryptic secondary matching with high similarity to *Scylliogaleus quecketti*. This however is most likely due to a lack of available reference

sequences for *T. megalopterus* in BOLD. It has previously been noted that *T. megalopterus* do reside with other non-congeneric houndsharks and that the *Mustelus* grouping is not monophyletic (López *et al.* 2006; Vélez-Zuazo & Agnarsson, 2011). Additionally, Maduna (2014) found that the houndsharks formed a polyphyletic grouping with other *Mustelus* spp. in South African fisheries. Within the catsharks, two separate groupings were observed for the Scyliorhinidae family, one comprising *Haploblepharus* and *Holohalaelurus* species and the other *Poroderma* spp. and *Schyliorhinus capensis*.

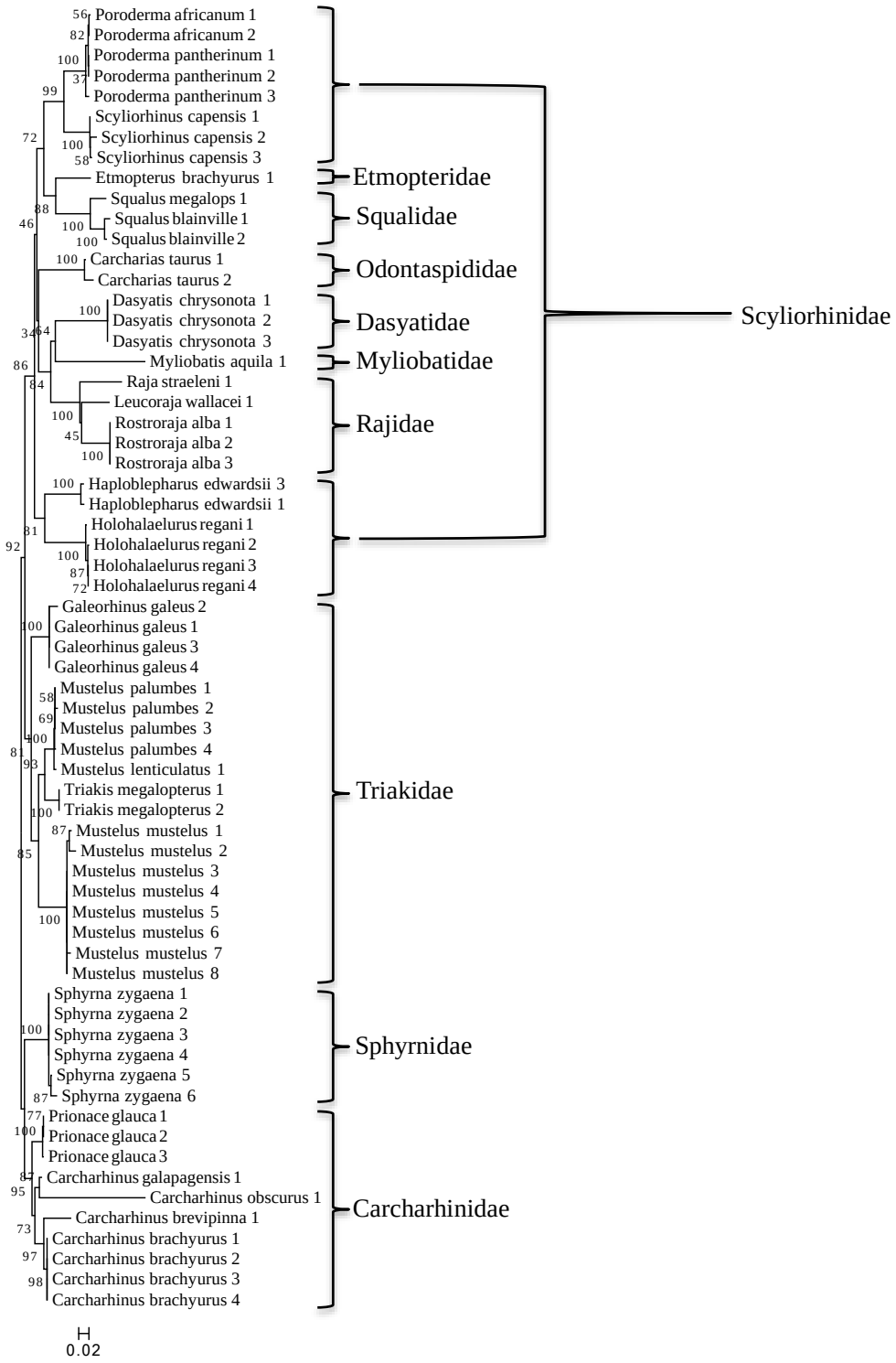

Figure 2.2. Tree based identification of chondrichthyans sampled in Southern Africa. A Neighbor-Joining tree based on Kimura's genetic distance model from a 550bp fragment of the CO1 gene. Groupings based on family are listed in the brackets. The Scyliorhinidae family is represented by two paraphyletic clades indicated by the overarching bracket.

2.3.4 *Species Composition and Conservation*

Overall, the molecular designation of sequences to species helped in verifying the sharks and rays commonly targeted in the different South African fisheries. Identifications made through field guides proved to correctly assign species more so than the conventional method employed for commercial purposes. Samples taken from commercially targeted and bycatch shark species provided accurate DNA barcodes that could be designated up to the genus level particularly for *Holohalaelurus*, *Scyliorhinus*, and *Squalus* spp. which comprised 31.52%, 12.61%, and 27.22% of the catches respectively. A number of the *Squalus* spp. individuals were however misidentified. Misidentification or inaccurate labelling mostly occurred within the Triakidae family with names such as *vaalhaai* and *hondhaai* being used to describe *Mustelus* spp. and *Galeorhinus galeus*. The IUCN RedList designates these commercially important species of houndsharks as Threatened. Among the rest of the species, *Rostroraja alba*, is the only one listed as Endangered while five species are listed as Near Threatened or Vulnerable and six as Data Deficient (Dulvy *et al*. 2006).

2.4 Discussion

The South African coastline is subjected to various anthropogenic and environmental changes that can negatively affect the productivity and recovery of many fish stocks. Alleviating the pressures that threaten the survival of marine life is essential for maintaining the existing biodiversity. Compared to other vertebrates, chondrichthyans face the highest threat of extinction and considering that they are top predators, their ecological role in regulating the structure and composition of the lower trophic levels should not be compromised (Fowler, 2005; Dulvy *et al.* 2014). A priority in addressing these compounding issues requires an understanding of the biodiversity and the extent to which the regional fauna is affected by these external factors such as fisheries.

In order to improve the ability of a DNA barcode to identify a species, accurate and comprehensive morphological data must accompany the specimen to be barcoded. The fishing data collected from the different fishing efforts revealed the capacity of each method to impact biodiversity in a unique way as well as highlighted the role that recreational fisheries can play in providing species information. As expected, the quantity of individuals captured from the commercial fishing trawls was the highest, while the diversity of species captured (and subsequently released) from the recreational fisheries far outweighed the longline and trawl records. Due to an inability to be selective, trawling indiscriminately catches large quantities of fish and could be particularly harmful for species not yet assessed. Trawling productivity was highest when a dense biomass was encountered, but its depth limit only saw certain types of fish being captured while other areas were under-utilized. This can potentially be remedied by the expansion of the longline trade in hard to reach areas (Grekov & Pavlenko, 2011). Accurate catch statistics is essential for the assessment of fisheries impact on specific species and the wider ecosystem (Bester-van der Merwe & Gledhill, 2015). This is especially paramount for non-targeted fisheries or large catches of unidentified sharks and rays. The majority of the specimens captured in the trawls were accurately identified by their scientific name. Misidentifications in recreational fishing were most common among rare species or species with sparse abundance, but the fairly extensive catch records allowed for misidentifications to be clarified. Understandably, recreational fishing appears to be the least invasive and also the most useful in capturing data on species diversity. It has been shown that recreational anglers are equipped with the best practices for the safe catch and release of sharks (Heard *et al.* 2016). The utility and success of DNA barcoding in species identification is dependent on the representation of sequences characterized and curated from the different geographical regions in which the target species occur (Hajibabaei *et al*. 2007). In this study, the CO1 was unable to provide a species level match for certain taxa, which could be due to limitations of CO1 in barcoding of chondrichthyans or a lack of reference sequences for endemic species available in the BOLD database. Supplementing the database with sequences of voucher specimens from this region could increase the success of the CO1 gene as a barcoding tool for regional fisheries. Still, the discrepancy seen within the members of the Scyliorhinidae family for

example could be an artefact of the gene itself in that for this family, the CO1 gene is merely not informative enough (no barcoding gap) to be used for species identification. These results were similar to a previous phylogenetic evaluation of the molecular phylogeny of scyliorhinid sharks using three mtDNA genes (cytochrome *b*, NADH-2, and NADH-4) (Human *et al.* 2006). The latter study also indicated that the scyliorhinidae family is also paraphyletic, which sharply contrasted the morphological evidence found in the field identification guides (Human *et al.* 2006). Ambiguities were also seen in specimens originally identified as *C. obscurus*, with a match made to *C. galapagensis* in BOLD. Previous studies have observed shared similarities in haplotypes as well as SNPs (single-nucleotide polymorphisms) that may be plesiomorphic, where the state of this SNP was inherited from a recent common ancestor (Chuang *et al.* 2016). Another explanation for this may be that *C. galapagensis* is the oceanic ecotype of *C. obscurus* (Naylor *et al.* 2012). Nonetheless, the CO1 gene has previously shown to be useful in clarifying a number of misidentifications amongst skate species, which is most likely due to a very similar morphological appearance in patterning and shape. For closely related or cryptic species, a combination of mitochondrial and nuclear genes can provide a means for distinguishing between such taxa (Moore *et al.* 2011). By combining systematic practices with molecular tools, the efficacy of the available identification tool can be maximized. Taxonomists and geneticists can play a vital role in describing the allelic diversity belonging to a species that can establish the link between taxonomy and DNA barcoding (Radulovici *et al.* 2010). As stand alone fields, both DNA barcoding and taxonomy have a paucity of information, and a complementary approach where both approaches are applied can help clarify the ambiguity in species identity. Species misidentification or mislabelling can only be detected if there are a sufficient number of correctly determined sequences available for those specimens. In addition, the cryptic sequences can only be defined if there is available specimen information, which will require an increase of new and curated sequences to be added to public sequence databases.

The use of DNA barcoding as a taxonomic tool has burgeoned recently and a number of studies have been able address species delineation problems for fisheries management

(Chuang *et al.* 2016; Jabado *et al.* 2015). As reflected in this study, there is often discordance between the various common names of chondrichthyans and the actual species identity. Commercially exploited species most often carry a variety of common names, which impedes effective record keeping for individual species (Sembiring *et al.* 2015; Velez-Zuazo *et al.* 2015). Several recent studies have found that species identification for chondrichthyans will be most effective only once augmented with a wide array of DNA sequences and taxonomic records. Without this integrative approach, current identification issues surrounding cryptic, or frequently misidentified chondrichthyans will continue to be problematic (Rodriguez-Cabello *et al*., 2013; Moura *et al.* 2015). In addition to identification, DNA barcoding can add clarity to the phylogeny and evolutionary history of chondrichthyan taxa. Though the phylogenetic positioning of many taxa in the original classification scheme proposed by Compagno (Compagno, 1973) was validated, revisions had to be made for a number of species with the discovery of new species (Vélez-Zuazo and Agnarsson, 2011; Henderson *et al.* 2016). The application of DNA barcoding for well-established species in targeted fisheries is advantageous for conservation of vulnerable and endangered species. From this, the degree of exploitation can be inferred to understand the trade dynamics that threatens marine biodiversity (Fields *et al.* 2015; Glaus *et al.* 2015).

2.5 Conclusions

The primary initiative for marine management and biodiversity conservation should be to DNA barcode all fish species. Without effective identification of a taxonomic grouping, management structures cannot function properly and the data becomes misleading. Accomplishing this goal requires expert taxonomic identification so that species referencing can be designated accurately. In this study, the mitochondrial CO1 region was successful for the purpose of chondrichthyan species identification of most species caught in the South African fisheries. It also provided evidence for the existence of identification problems particularly within the genera Carcharhinus and Squalus. Even though it will not always allow for unambiguous identification (in the sense that matches are not always made with 100% confidence), it readily equips fisheries management with

a functioning identification tool. Taking this into account, DNA barcoding can also be useful in keeping commercial fisheries accountable.

CHAPTER 3

Genetic diversity, population connectivity and demographics of smooth hammerhead sharks (*Sphyrna zygaena*) in South Africa

Abstract

Due to the high market value of their fins, the smooth hammerhead shark (*Sphyrna zygaena*) is highly vulnerable due to fishing pressure. Significant population declines have been noted in areas across its circumglobal range, while species-specific data for their western Indian Ocean range are very limited. In this study, the population genetics of *S. zygaena* was investigated along the South African coastline using the mtDNA NADH dehydrogenase subunit 2 ND2 gene (n = 40) as well as seven polymorphic nuclear microsatellite loci (n = 96). Analysis of the ND2 gene revealed a recent population expansion using neutrality analysis, mismatch distribution analysis, and a Bayesian skyline plot. A very low degree of differentiation, indicating uniformity, was seen with the mtDNA (Φ_{ST} = 0.2667) across the sampled region. A total of 12 mtDNA haplotypes were identified, with two major haplotypes shared within the region and ten being unique to sampling sites. Analysis of population differentiation based on the microsatellites showed significant subdivision between the two biogeographic regions represented by the southern coast and eastern coast, with high connectivity within either grouping (F_{ST} = 0.066, P = 0.001). The restricted gene flow found between the populations in the southern coast and eastern coast of South Africa should be taken into account in order to make useful recommendations for biodiversity conservation and the future protection of the species. The lower 95% CI effective population size estimates were an order higher than the sample sizes indicating no contemporary declines, while neutrality tests, mismatch distribution analysis, and a Bayesian Skyline Plot (BSP) revealed a historical expansion most likely during the Holocene (~0.05 mya).

3.1 Introduction

The South African coastline stretches over 3000 km and houses three major biogeographical regions determined by oceanographic conditions and ecological factors: the cool-temperate West Coast, the warm-temperate South Coast and the subtropical East Coast (Heydorn *et al*. 1978). *Sphyrna zygaena* is found seasonally in coastal areas, and normally found in non-polarized aggregations during the summer months (Bass *et al.* 1975; Harrison *et al.* 2002; Spalding *et al.* 2007). Migrations are often made towards the equator in the winter and away from the equator during the summer (Casper *et al.* 2005; Hayes, 2007). Through sighting and anecdotal data, *S. zygaena* has been seen utilizing a number of inshore areas in South Africa from Delagoa Bay to False Bay (Bass *et al.* 1975; Compagno, 1984). Warmer waters and an increase in species diversity and abundance typify this seasonal shift within the Agulhas bioregion (Lombard *et al.* 2005). This provides the optimal conditions for *S. zygaena* individuals to migrate into, with a substantial amount of resources for growth and development. During the young-of-the-year stage of life, the *S. zygaena* individuals are more vulnerable to the already recognized anthropogenic threats. Given this, nursery areas should be a main priority for conservation efforts as to provide a critical haven for the juveniles and survival of the species as a whole (Feldheim *et al.* 2001b; Heupel *et al.* 2007). Two current systems, the eastern warm Agulhas current and western cold Benguela currents dominate the oceanographic regime around the southern tip of Africa. It has been suggested that there are structural differences among overall faunistic and floristic groupings corresponding to the bioregions of the southern cape and Eastern Cape (Lubke & Moore, 1998; Spalding *et al.* 2007; Kirkman *et al.* 2016). These differences also sometimes correlate to genetic stock structure, which can be assessed via molecular tools. Previous studies have found that a change in region associated with oceanic regime, disrupts population connectivity in a number of elasmobranch and other marine species. This often comes in the form of restrictions from biogeographic barriers, such as cold-water upwelling, freshwater discharge, currents, and dunefields preventing gene flow (Teske *et al*. 2011; Chabot *et al.* 2015; Larson *et al.* 2015; Camargo *et al.* 2016; Maduna *et al.* 2016). Throughout the South African coastline, faunal biodiversity is rich with a range of biota occurring

exclusively within bioregions, while other span across several bioregions and comprise multiple evolutionary significant units. Using molecular markers, these units can be distinguished (Teske *et al.* 2011) based on spatial distribution. Though there have not been any fine-scale genetic assessments for this species, mark-recapture data studies have indicated that *S. zygaena* individuals are likely to return to utilize certain areas within their home ranges (Bass *et al*. 1975). Individuals recaptured on the East coast were mostly limited to the areas they were originally tagged in (Diemer *et al.* 2011). However, there is a lack of crucial information regarding the distribution of *S. zygaena* along the South African coastline.

This study therefore aims to understand the population genetic structure of *S. zygaena* along the South African coastline. This will be achieved by assessing the levels of genetic diversity and genetic differentiation between populations sampled across the South and East coast. Through mtDNA analysis, matrilineal structure will be determined as well as the long-term demographic history. Additionally, microsatellites will be used to assess gene flow and identify possible discreet genetic stocks as well as to detect recent population declines. This will aid in providing valuable data for the future conservation of this vulnerable species in South Africa by allowing fisheries managers and nature conservationists to make informed decisions regarding possible genetically distinct populations.

3.2 Materials & Methods

3.2.1 *Study Site*

In this study, a total of 219 *S. zygaena* young-of-the-year specimens were collected from three coastal areas of South Africa. Sampling in Mossel Bay (MB) was carried out under the *Permit for the Purposes of a Scientific Investigation or Practical Experiment in Terms of Section 83 of the Marine Living Resources Act, 1998 (Act No. 18 of 1998)* established by the South African National Department of Agriculture, Forestry, & Fisheries (DAFF). The sampling protocol was approved by DAFF under the permits RES2014/01 and RES2015/74. These sharks were caught using handlines. Fin clips were

taken from the trailing edge of the dorsal fin. Individuals from Mossel Bay were sourced during the summer months starting in December 2013. Five additional samples from Mossel Bay were caught in August 2014 during an out-of-season period (MBO) in order to uncover overlap of maternal lineages in other sampling sites. Samples from the eastern coast were sourced from Algoa Bay (AB) and various locations along the KwaZulu Natal (KZN) coastline. A subset of 96 individuals was selected for fragment analysis and of those, 40 were selected for sequencing (Figure 3.1).

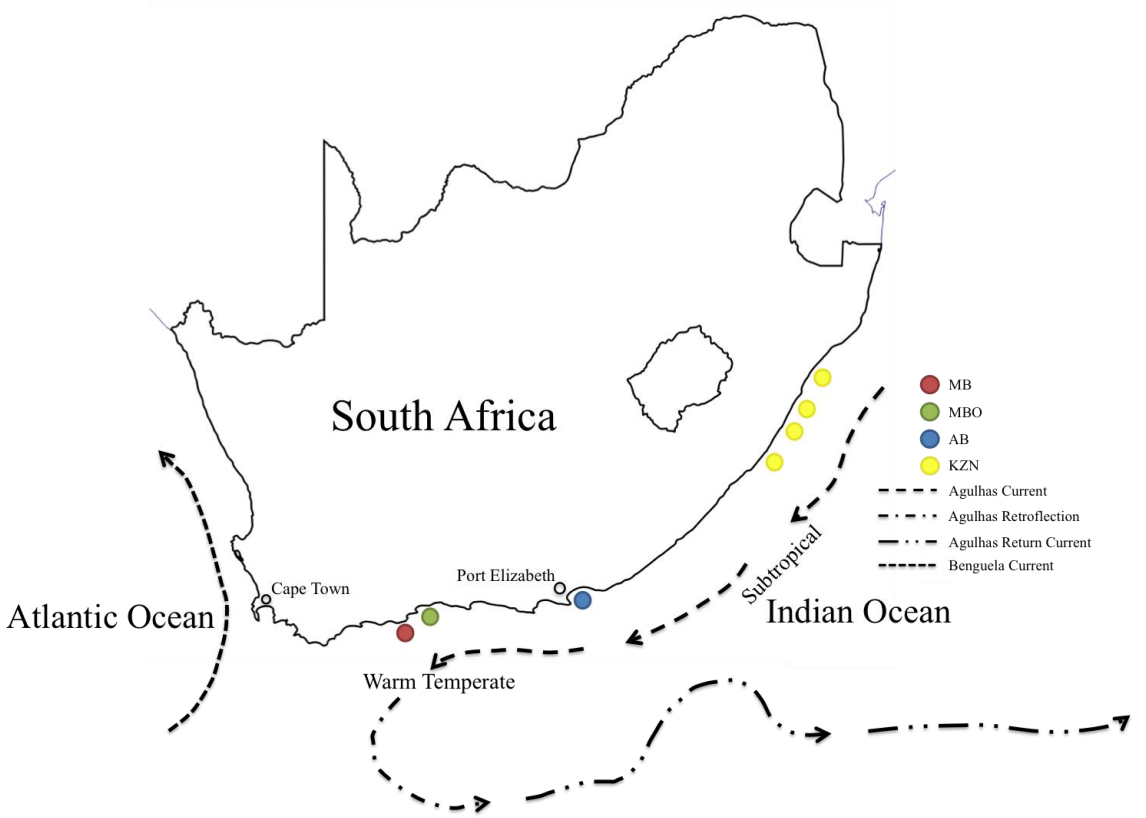

Figure 3.1. Map of all sampling localities for *Sphyrna zygaena* individuals included in this study.

3.2.2 *DNA Extractions and Marker Optimisation*

Genomic DNA extractions were performed using the standard CTAB method of Saghai-Maroof *et al.* (1984) followed by DNA quantification on the NanoDrop ND-2000, UV-Vis spectrophotometer (NanoDrop®). Genomic DNA was stored in Milli-Q water at 20°C until further use.

Microsatellite markers were amplified for 96 individuals (Table 3.1). A total of seven loci designed for the congener *S. lewini* (Nance *et al.* 2009) were cross-amplified to *S. zygaena* and successfully yielded amplicons (Table 3.2). The loci were evaluated based on size and subsequently grouped into multiplexes based on size and annealing temperature. A 10µL PCR was performed using a Veriti® 96-Well Thermal Cycler with 50ng template DNA, 0.2µM of each primer set (forward and reverse), and 1X KAPA2G Fast Multiplex Kit. The PCR cycling conditions consisted of: 95°C for 3min; 35 cycles of 95°C for 15s, 60°C for 30s, 72°C for 60s, and a final extension step of 72°C for 10min. Amplification products were subjected to a post PCR clean-up performed using a Nucleofast 96 well PCR plate (Macherey Nagel). The protocols supplied by the manufacturer are implemented on a Tecan EVO150 robotic workstation prior to capillary electrophoresis. Two microliters of purified PCR product was mixed with the LIZ600 internal size standard (Life Technologies) and Hi-Di prior to denaturation for 5 minutes at 95°C. Subsequent to this, samples were run through capillary electrophoresis performed on an ABI3730xl (all supplied by Life Technologies).

Table 3.1. Properties of two microsatellite multiplex assays optimized for *Sphyrna zygaena* cross species amplification. The dye color, allelic range, and annealing temperature were listed for all seven loci.

Multiplex	Locus	Dye	Primer Sequence	Allelic Range	Ta (˚C)
Multiplex 1	SLE027	FAM	F: GAGACCAGCCAAAGGAAAAA	180–220	60
			R: ATGCCATATTCATCCAGGCAC		
	SLE089	NED	F: TTACCACAGTTTGTGTGGGTG	280–320	60
			R: AAGTTTCAGTGTCAGTGTGC		
	SLE018	VIC	F: ACAGAAACAGAACGAGGGACA	180–220	60
			R: TGGGTTGGCATTGAACAGAA		
Multiplex 2	SLE054	FAM	F: CTGACACTGCCAATTTGCAT	280–320	60
			R: CCAACTGGAGTTGTCAATCCA		
	SLE071	NED	F: TCAGACGGTGGTACGTACACA	300–340	60
			R: TGACCCTTTTGGATTGAAGGA		
	SLE081	VIC	F: ATGTTCATCATCCGAGACAGG	360–400	60
			R: CCAAACACACGTATCTGCACCCA		
	SLE038	FAM	F: AGCCTACTTCTGCCACATTTT	390–430	60
			R: AATCAAAGTTCCTGCAGTCCT		

The mitochondrial NADH dehydrogenase subunit 2 (ND2) gene was amplified using a set of universal primers (Ilem-Mustelus-F: 5'-AAGGACCACTTTGATAGAGT-3'; Asn-Mustelus-R: 5'-AACGCTTAGCTGTTAATTAA-3') (Naylor *et al.* 2005). A total of 40 individuals were selected for amplification (Table 3.1). Amplification reactions were carried out in 15µL volumes consisting of 1X Invitrogen Buffer (Thermo Fisher Scientific), 200µM dNTPs, 2mM $MgCl_2$, 0.5µM of each primer, 0.125U of Invitrogen Platinum Taq Polymerase (Thermo Fisher Scientific), and 2 µL of 50ng/µL DNA template. Cycling conditions for all primer pairs consisted of 95°C (2 min), 35 cycles of 95°C (30 sec), 55°C (30 sec), and 72°C (60 sec) with a final extension at 72°C for 10 min. Bidirectional sequencing was performed using the ABI Big Dye Terminator Cycle Sequencing Kit (Life Technologies) with a cycling regime of 96°C (1 min), 25 cycles of 96°C (10 sec), 50°C (5 sec), and 60°C (4 sec). All amplified PCR products were electrophoresed on an automated sequencer using POP 7 and 50cm capillaries and run against a LIZ600® size standard on a ABI 3730 XL DNA Analyzer (Life Technologies).

3.2.3 *Genetic Data Analysis*

Microsatellite Marker Data

Following fragment analysis, allele sizes were determined in PEAK SCANNER 2.0 software (Life Technologies). Genotypes were exported in Excel and binned using AUTOBIN (Salin, 2010). Quality control was performed in order to determine marker utility in MICROCHECKER 2.2.3. The microsatellite loci were evaluated for possible genotypic errors due to large allelic dropouts, null alleles, and stuttering (van Oosterhout *et al.* 2004). Null allele frequencies were calculated using the Brookfield 2 method (Brookfield, 1996). A deviation from Hardy-Weinberg Equilibrium (HWE) using the exact test was calculated in GENEPOP 4.2 (1000 dememorization, 100 batches, and 1000 iterations per batch). Additionally, the presence of genotypic linkage disequilibrium and the fixation index (F_{IS}) was also quantified (Raymond and Rousset, 1995). Significant levels were adjusted with sequential Bonferroni corrections at the $p < 0.05$ level. Genetic diversity statistics were generated in GENALEX 6.501 and all loci were characterized by the number of alleles (Na), the effective number of alleles (Ae), and expected and observed heterozygosity (Ho and He). The polymorphic information content (PIC) was calculated in MICROSATELLITE TOOLKIT (Park, 2001; Peakall and Smouse, 2006).

Pairwise F_{ST} and significance values were calculated in GENALEX to test for population differentiation between sampling sites. The Discriminant Analysis of Principal Components (DAPC) was applied using the Adegenet package for R (version 3.3.2: R Core Team 2014) with the optimal number of principal components set to 19 following an optimized alpha-score indication (Jombart *et al.* 2010). To infer the most likely number of distinct populations present, a Bayesian multi-locus clustering method was implemented through STRUCTURE 2.3.4. An admixture model was used with no *a priori* assumption, with K ranging from 1 to 4 and 1,000,000 Markov Chain Monte Carlo (MCMC) iterations with 100,000 burn-ins. In order to determine convergence based on the number of clusters (K), the ΔK metric was employed using the Evanno method in STRUCTURE HARVESTER 0.6.94, which statistically determines the most likely number of clusters supported by the data (Pritchard *et al.* 2000; Evanno *et al.* 2005; Earl

& vonHoldt, 2012). In order to determine the regional level of differentiation between MB, AB, and KZN, an Analysis of Molecular Variance (AMOVA) was performed using GENALEX and statistical significance was determined by 999 permutations. Patterns of gene flow in terms of migration rates between MB, AB, and KZN were investigated using the Bayesian Inference approach implemented in MIGRATE v.3.2.6. Due to the mutational properties of the microsatellite markers, the stepwise mutation model (SMM) was selected. Additional parameters included 100,000 recorded steps, with a burn-in of 200,000, 5 long chains, with 20 replicates (Beerli, 2006; Beerli & Felsenstein 2001).

Effective population size (Ne) for each population was estimated in NEESTIMATOR 2.0 (Do *et al.* 2014) through the linkage disequilibrium (LD) and the heterozygote excess (HE) method (Waples & Do, 2008). In addition to this, the parameter for monogamy was selected as genetic monogamy has been identified in a congeneric species (Chapman *et al.* 2004). The random mating parameter was also tested. The LD method accurately reflects local Ne, but it does assume a population closed to immigration in addition to non-overlapping generations and the presence of unlinked alleles. With this taken into account for a migratory species, should there be any admixture with a genetically distinct population, local Ne estimates will be downwardly biased (Waples & England, 2011; Do *et al.* 2014).

Mitochondrial Sequence Data

Where appropriate, sequence data was manually edited in 4PEAKS and subsequently imported into MEGA 7.0.14 for sequence alignment applying the ClustalW multiple alignment algorithm (Thompson *et al.* 1994; Griekspoor & Groothuis, 2005; Kumar *et al.* 2016). Individuals were grouped according to sampling site (MB, MBO, AB, and KZN) and haplotypic (h) and nucleotide diversity (π) were calculated in DnaSP 5.10 (Rozas *et al.* 2003). An analysis of molecular variance (AMOVA) was conducted in ARLEQUIN 3.5.1.2 (3000 permutations) to test for the significance and the degree of differentiation between the sampling sites (Excoffier & Lischer, 2010). A haplotype genealogy was constructed in HAPLOVIEWER following the method of Salzburger *et al.* (2011) using a phylogenetic tree derived in PhyML v3.0 (Guindon & Gascuel 2003, Guindon *et al.*

2010) following 1000 bootstraps. The TN93 evolutionary model was selected as the best-fit for the sequence data, based on nucleotide substitution, using a series of likelihood ratio tests in JMODELTEST (Posada 2008). Neutrality tests for Fu's Fs, Ramon-Onsins & Rozas R^2, and Tajima's D were generated to test for change in population size. Mismatch distribution analysis, also performed in ARLEQUIN, was used to determine ancestral θ_0, actual θ_1, and τ. This determines goodness of fit between haplotype pairs, assuming a sudden population expansion. Where θ_0 and θ_1 are the expected pairwise differences, and τ is the mutational timescale. The mismatch distribution is estimated by θ_k, which evaluates variance on the basis of the observed number of alleles, or the infinite-allele equilibrium relationship (Ewens, 1972). Analyses of demographic history were conducted in BEAST 1.8.3 through generating Bayesian Skyline Plots (BSP), which infers change in historical effective population size. A TN93 model was used, with a chain length of 60,000,000 iterations and thinning every 10,000 iterations. The associated software packages BEAUTI 1.8.3 and TRACER 1.6.0 were used to generate experimental parameters and visualize the logarithmic files respectively (Ho & Shapiro, 2011). The MBO cohort was incorporated into the ND2 analysis in order to test for the overlap of matrilineal ancestry.

3.3 Results

For the microsatellite loci, all multiplexes were successfully characterized in a panel of 96 *S. zygaena* individuals from MB, AB, and KZN (Figure 3.2). The number of alleles per locus ranged from seven to 86 alleles with a mean effective number of alleles of 17.4. The observed heterozygosity ranged from 0.063–0.851, with a mean Ho = 0.428. The average PIC values across all loci were 0.705, ranging from 0.474–0.983. Highly significant deviations from the HWE were detected across all loci, except for locus SLE089 ($p = 0.300$). This is likely due to the presence of null alleles. In addition to this, the markers conformed to linkage equilibrium expectations for the majority of pair-wise comparisons.

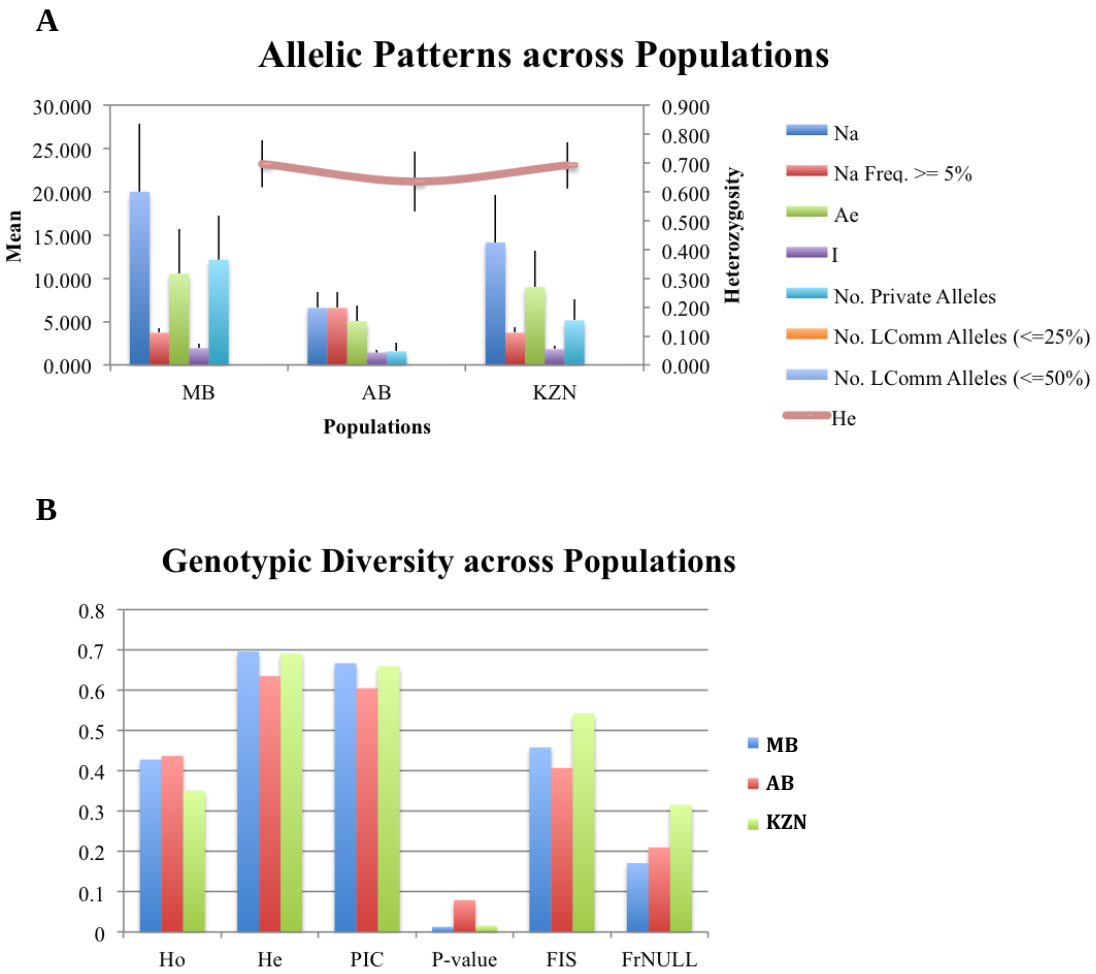

Figure 3.2. Allelic (A) and genotypic (B) diversity statistics for *S. zygaena* sampled populations characterized for seven microsatellite loci. Number of alleles per locus (Na); effective number of alleles (Ae); observed heterozygosity (Ho); expected heterozygosity (He); polymorphic information content (PIC); significant deviations from HWE as determined by the exact test (P-values <0.05); inbreeding coefficient (F_{IS}); null allele frequency (Fr_{NULL}).

The pairwise F_{ST} values for the microsatellite loci indicated a high degree of differentiation. Overall, the values were low (< F_{ST} = 0.09) but were significant for all pair-wise comparison with an average of F_{ST} = 0.066, p = 0.001. This showed genetic structure amongst the sampled populations with most significant differentiation observed between the southern coast (MB) and the eastern coast population groupings (AB/KZN) (Table 3.2).

Table 3.2. Pairwise F_{ST} values of *Sphyrna zygaena* populations sampled along the South African coastline (*indicates significance $p < 0.05$ after Bonferroni correction)

Microsatellite F_{ST} and p-Values			
	MB	**AB**	**KZN**
MB		0.001*	0.001*
AB	0.081		0.437
KZN	0.077	0.000	

The DAPC defined two primary clusters with substantial overlap between AB and the KZN, while MB individuals indicated no overlap with the Algoa Bay/KZN sampled populations (Figure 3.3).

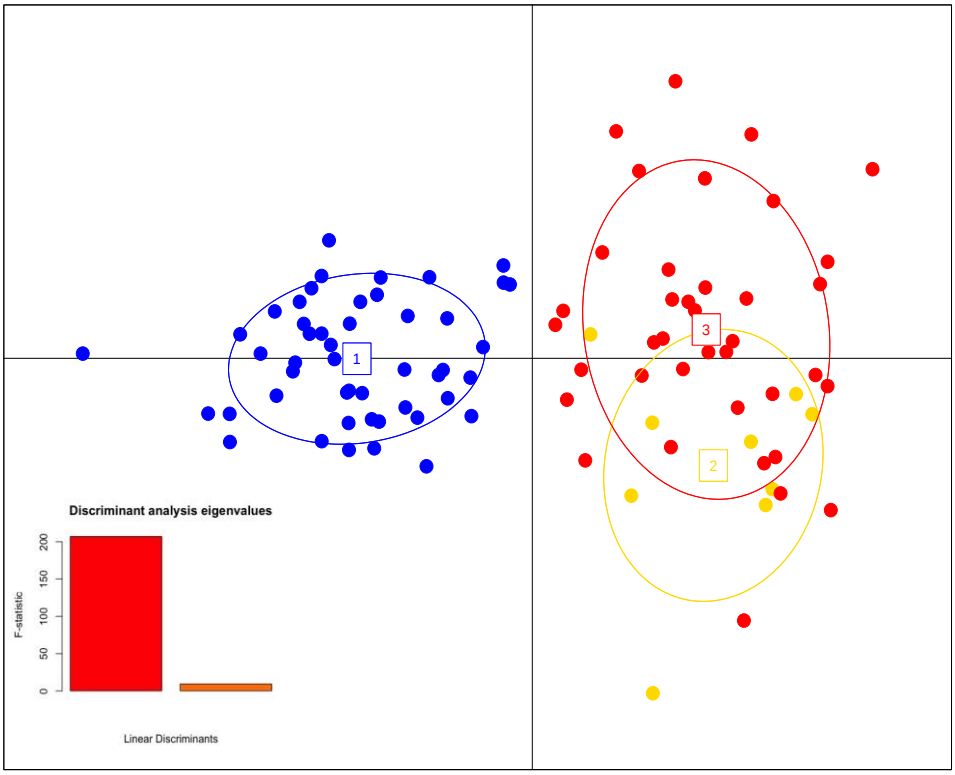

Figure 3.3. A Discriminant Analysis of Principle Components (DAPC) scatterplot generated from seven microsatellite loci for *Sphyrna zygaena* individuals from MB (1), AB (2), and KZN (3); a total of 19 PCAs were retained based on an optimized alpha score. Each dot is a representation of an individual's genotype and the inertia ellipses are a representation of the group. Ellipses in the DAPC represent the center of gravity around the cloud of points. Two main clusters were seen with MB in one and AB/KZN in the other.

The visualization of the multilocus genotypes performed in STRUCTURE presented significant subdivision between the south coast sampling locality of MB and the AB/KZN sampled populations from the east coast (Figure 3.4). The MB population comprised mainly two ancestral clusters, while the AB/KZN showed assignment to three ancestral clusters. There was no clear distinction between the two east coast populations (AB/KZN), although there appeared to be a difference in the degree of admixture represented by the two populations. Overall, the correlation to two geographic regions corroborated the results presented by the DAPC scatterplot.

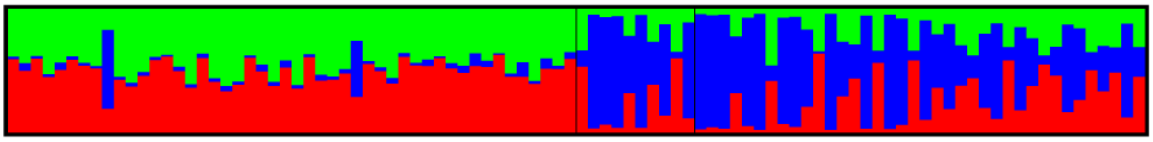

Figure 3.4. Bayesian clustering analysis performed amongst *S. zygaena* individuals in STRUCTURE (Pritchard et al 2000); each bar column is representative of a single individual and its apparent constituency to a particular cluster. The groupings are as follows: (1) Mossel Bay, (2) Algoa Bay Group, and (3) KZN Group. The ΔK method as inferred by Evanno *et al.* (2005) indicated three clusters (K=3).

Overall, contemporary gene flow estimates demonstrated evidence of asymmetric gene flow within the different bays across the coastline, with gene flow predominantly from the South coast to the East coast. The highest contributions came from MB to KZN (M=408.67) with AB and KZN each contributing to MB at rates of M=214.00 and M=218.67 respectively. Gene flow towards AB was minimal from either sampling locality (Figure 3.5).

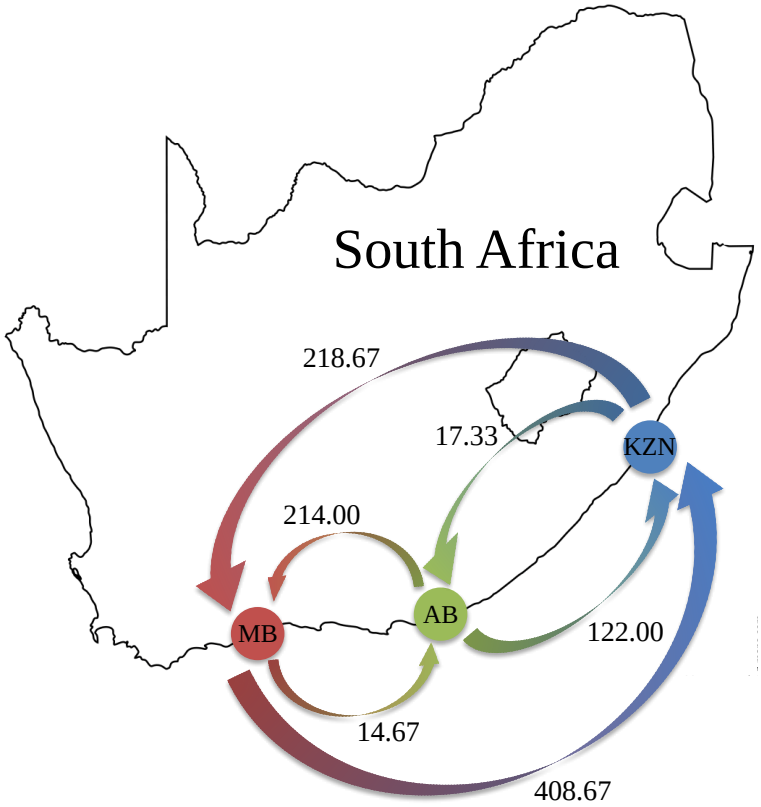

Figure 3.5. Summary of gene flow estimates based on a Bayesian Inference of migration among *S. zygaena* clusters from each sampling site. The gene flow parameters have been estimated by the number of immigrants per generation, scaled by 4Nm, for diploid data. Each arrow represents the direction of gene flow, where the value associated with the arrow represents the magnitude.

Analysis with MICROCHECKER did not detect evidence of large allelic dropouts or large allelic gaps, but null alleles were present at all loci. Although, the presence of null alleles generally has little effect on population structure analysis, it can influence effective population size estimates, particularly when using the heterozygote excess test (O'Leary *et al.* 2015). Effective population sizes as approximated by NeEstimator2.0 (Table 3.3) using the LD method showed infinite values for the point estimate and upper 95% CI (Ne = ∞; upper 95% CI = ∞) with the lower 95% CIs at 1354.3 for MB, 61.3 for AB, and 730.6 KZN. With the populations combined Ne = 6783.3 with a lower 95% CI = 1704.8. As the markers were overall not in LD, the given estimates may be downwardly biased (Hollenbeck *et al.* 2016). The ranges of the 95% CI values, were high, and must be interpreted conservatively. The HE method for estimating Ne provided values of infinite

for all 95% CI and the Ne point estimate for all population groupings. The random mating model was also tested, however overall values for Ne were lower.

Table 3.3. The values for effective population size as estimated by the linkage disequilibrium (LD) and heterozygote excess (HE) methods with the associated 95% CIs and point estimates listed below title headings.

	LD		HE	
	95% CI	**Estimated Ne^**	**95% CI**	**Estimated Ne^**
MB	1354.3 - ∞	∞	∞ - ∞	∞
PE	61.3 - ∞	∞	∞ - ∞	∞
KZN	730.6 - ∞	∞	∞ - ∞	∞
Combined	1704 - ∞	6783.3	∞ - ∞	∞

The mitochondrial ND2 region was successfully amplified, sequenced and aligned for a total of 40 individuals. The 750 bp alignments contained a GC content of 38.6% and a transition/transversion bias (*R*) of 0.254 with 18 segregating sites (*S*), this resulted in 12 different haplotypes. . Overall, values for haplotype diversity (*h*) and nucleotide diversity (π) were 0.603 ($\pm$0.088) and 0.002 ($\pm$0). The *h* and π values for the sampling sites were moderate to high except for MB that were considerably lower ($h = 0.133$; $\pi = 0$) than the other groups (Table 3.4).

Table 3.4. Nucleotide and haplotype diversity values, with number of haplotypes and GC content, for four *Sphyrna zygaena* sampled populations.

Population	***n***	**Haplotypes**	***h***	***π***	**GC**
MB	15	2	0.133	0.000	0.386
MBO	5	4	0.900	0.005	0.388
AB	9	4	0.694	0.002	0.386
KZN	11	7	0.873	0.004	0.386
All	**40**	**12**	**0.603**	**0.002**	**0.386**

Pairwise Φ_{ST} values of the mtDNA ND2 gene showed significant heterogeneity between MBO and all the other sampled populations except KZN. Mean pairwise differences were low (Φ_{ST} = 0.2667) and showed no to little differentiation between the sampled populations (Table 3.5).

Table 3.5. Population pairwise Φ_{ST} values and associated p-values on the upper diagonal for four *Sphyrna* zygaena populations based on the mtDNA ND2 gene.

mtDNA Φst and p-Values				
	MB	**MBO**	**AB**	**KZN**
MB		0.008*	0.286	0.043*
MBO	0.3428		0.021*	0.266
AB	0.02924	0.18729		0.570
KZN	0.07218	0.02844	-0.01766	

*indicates significance $p < 0.05$

A parsimony reconstruction based on the ND2 gene revealed one common haplotype shared amongst 62.5% individuals from different sampled populations with a secondary, less frequent haplotype representing 12.5% of the individuals. In addition to the two common haplotypes, there were ten private haplotypes each represented by a single individual. The sampled population MB presented no unique haplotypes (Figure 3.6).

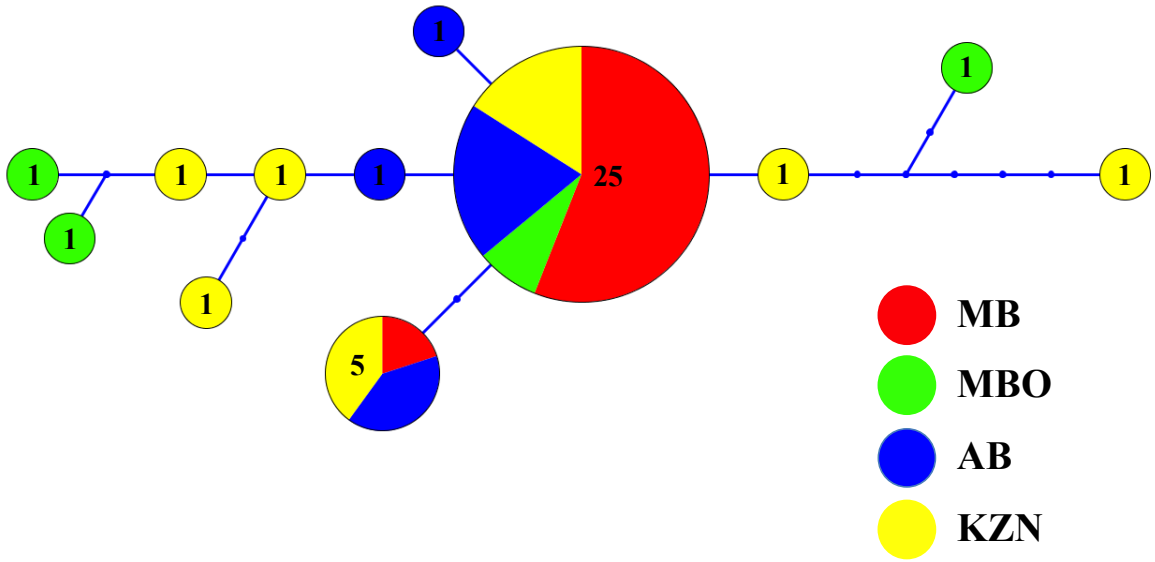

Figure 3.6. Haplotype network for *Sphyrna zygaena* individuals sampled along the South African coastline; the network was constructed by a statistical parsimony in HAPVIEW. Size of the circle is scaled to the frequency of occurrence for a haplotype. Small blue dots indicate mutational events.

A population expansion, detected by a high and negative Fs, a low and positive R^2, and a low and negative D, showed departure from the neutral theory (Tajima, 1989; Fu, 1997; Ramos-Onsins & Rozas 2002). Values obtained for the neutrality analysis were indicative of a population expansion (Fs = -5.184, R^2 = 0.107, and D = -1.920) and a slight departure from the neutral model characterized by an expansion. The mismatch analysis produced a θ_0 = 1.543 and θ_1 = 33.076 with a τ = 3.000. A visual representation of demographic history within the region was represented by a BSP (Figure 3.7), which showed overall population stability, but a relatively recent expansion (< 5 kya).

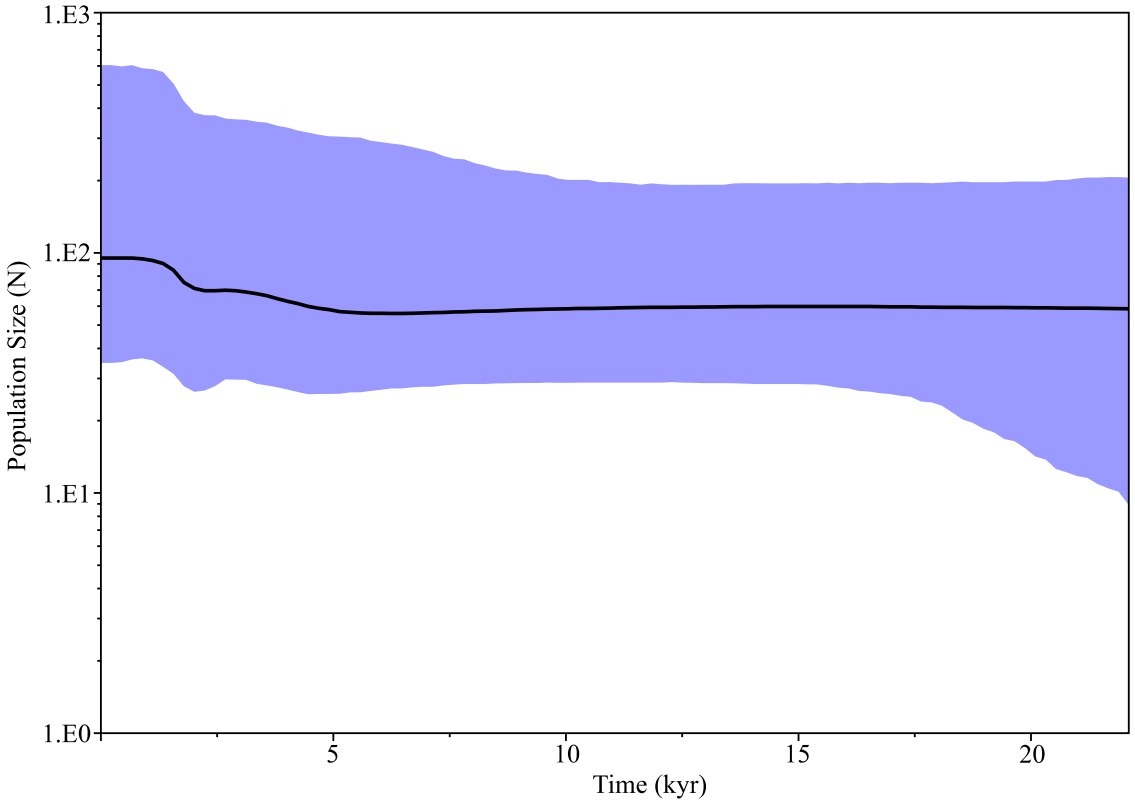

Figure 3.7. Bayesian Skyline Plot (BSP) generated based on the mtDNA ND2 gene for the pooled *S. zygaena* individuals sampled from the South African coastline. The black line represents the median estimate and the shaded area represents the 95% high posterior limits.

3.4 Discussion

3.4.1 *Genetic Diversity*

Overall haplotype and nucleotide diversity estimates based on the mitochondrial ND2 gene were moderate and comparable to similar studies employing mtDNA for population genetic analysis in other shark species (Nance *et al.* 2011; Blower *et al.* 2012; Vignaud *et al.* 2014; Testerman, 2014; Chabot *et al.* 2015). The values for h and π in the MB sampled population were significantly lower than the rest of the sampled populations. In comparison to other studies estimating mitochondrial diversity for other shark species, average values were $h = 0.665$ and $\pi = 0.002$ (Verissimo *et al.* 2010; Ashe, 2011; Boomer *et al.* 2012; Verissimo & Graves, 2012; Ashe *et al.* 2015). The MB values significantly skewed the overall values, where the average values without MB were $h = 0.822$ and $\pi =$

0.004 at a range of h = 0.694—0.900 and π = 0.002—0.005. Across all loci, observed heterozygosities (Ho) were lower than the expected heterozygosity (He) values, with an overall F_{IS} value of 0.5245. Recent studies employing microsatellites for chondrichthyans diversity analysis on a regional scale found the Ho values were comparable, if not higher than the He values (Bitalo *et al.* 2015; O'Leary *et al.* 2015; Andreotti *et al.* 2016; Maduna *et al.* 2016). This excess of homozygotes may be due to population sub-structuring, but not necessarily due to inbreeding caused by a decrease in population size, as the sampling regime was carried out across two major biogeographic regions.

3.4.2 *Genetic Connectivity*

The relationship between genetic populations and geographic populations can be elucidated through polymorphic microsatellite loci and mitochondrial (mtDNA) DNA sequences. Due to nature of either of these genomes, combined with reproductive behaviour, one can expect an incongruent pattern of inheritance if there is female philopatry or sexual segregation. This is represented by homogeneity for microsatellites and heterogeneity in mtDNA. The effects of population subdivision are difficult to evaluate when spatial scales are large and movement patterns are complex. The degree of dispersal that affects the distribution of alleles between sampled cohorts, determines the genetic connectivity and structure within and between each cohort (Lowe & Allendorf, 2010). Pairwise F_{ST} values for the microsatellites respectively revealed moderate population differentiation with values indicative of significant population structure. This structure could be attributed to a biogeographic division between the southern coast and the eastern coast. From the results it is evident that oceanic currents and thermal fronts may impact the scale and direction of gene flow between the sampling sites (Griffiths *et al.* 2010; White *et al.* 2010). The MIGRATE-N analysis corroborated this finding further, showing asymmetric gene flow between geographic regions primarily flowing against the Agulhas current. Previous studies show that fish can disperse against the Agulhas current, when stimulated to move in a northeast direction to avoid cooler waters (Dicken *et al.* 2007; Hussey *et al.* 2009; Reid *et al.* 2016). The haplotypic analysis revealed 12 haplotypes with one major maternal lineage and a smaller, less common lineage comprising 62.5% and 12.5% of all the individuals. This result shows similarity to the

mitochondrial control region (mtCR) analysis of *S. lewini* in which Testerman (2014) elucidated genetic structure, gene flow, and stock structure for *S. zygaena* on a global scale. Similarly, Daly-Engel *et al.* 2012 observed comparable results for the congener species *S. lewini* with a few major mtDNA haplotypes shared among the majority of individuals while major divisions were seen in the microsatellites. These studies showed a general lack of mtDNA structure, with higher degrees of structure seen in the microsatellites on average for the sampling region investigated. This occurrence known as mitonuclear discordance, which is common in animals and is most often attributed to demographic asymmetries as the driving factor e.g. sex-biased dispersal (Toews & Brelsford, 2012). Though these assessments are available, a fine scale study revealing nuances in intra-population variation have not been conducted for *S. zygaena*. Nance (2010) did conduct a regional population genetic study for *S. lewini* along its eastern Pacific range showing no net gene flow between sampled populations, as well as a low degree of mtDNA structure (Nance *et al*. 2011). In the current study, analysis of microsatellites showed significant structure as appose to the mtDNA, with distinct subdivisions in the microsatellites being correlated to the different biogeographic regions divided by phylogeographic barriers. A comparison between the DAPC plot and the STRUCTURE plot both showed a combined grouping of the AB/KZN sampled populations on the eastern coast and both groups showed separation from the Mossel Bay sampled population situated on the southern coast. Within these two sub-groups, considerable levels of admixture were evident while limited shared genotypes between the sub-groups were observed. Increased levels of relatedness within regions could also in part explain this pronounced subdivision between south and east coast *S. zygaena* sampled populations. Within the Mossel Bay sampled cohort, all the individuals were sampled over the course of three months from December 2013 to February 2014. Considering the seasonal occurrence of the neonate/juvenile pups during the Mossel Bay summer, there is a high likelihood that MB samples may belong to similar litters stemming from the two maternal lineages as seen in the haplotype network, which may cause a sampling bias. There was little overlap of maternal lineages between the MBO sampled population and the other sampled sites. Kinship has been shown to inflate population differentiation parameters as well drift, in the absence of migration-drift

equilibrium. This migration-drift disequilibrium is described as an "ephemeral, finely spatio-temporal pattern of genetic structure," (otherwise known as chaotic genetic patchiness) that may occur in shark species with temporary residence in nursery grounds prior to dispersal (Roderick & Navajas, 2003; Efremov, 2005; Toonen & Grosberg, 2011; Messier *et al.* 2012; Iacchei *et al.* 2013).

3.4.3 *Demographics*

Departures of mtDNA from the neutral theory are particularly important for conservation genetics, as they are useful for indicating processes unique to species population ecology. Neutrality tests can help determine demographic scenarios regarding population expansions and contractions. The most functional tests for estimating sudden population growth in contrast to constant population size are Fs for large sample sizes and R^2 for small ones (Rand, 1996; Ramon-Onsins *et al.* 2002). The large difference between the θ_k values exhibited a population expansion. The results for either of these tests for the ND2 gene substantiate the results for the BSP for the same loci, showing that the *S. zygaena* population across the South African coastline has experienced a slight population expansion during the Holocene (~0.05 mya). Amongst several carcharhinid species, substantial historical expansions have been noted during the mid-to-late Pleistocene, however few expansions occurred as recently as *S. zygaena* in South Africa (Duncan *et al.* 2005; Keeney *et al.* 2005; Geraghty *et al.* 2013).

Contemporary demographics measured through Ne revealed that the population groupings combined had properties of a larger population with respect to genetic drift. This indicates that the sampled cohorts from around the coast should have adequate evolutionary potential to resist effects of genetic drift caused by population declines (Kilman *et al.* 2008). The measure of Ne is a staple parameter estimate in population genetic studies in determining the magnitude of genetic drift, however, considerations must be made to prevent biased reflections of contemporary demographics. When obtained on a regional scale, sampled populations should be contained within the same biogeographic cohort (i.e. single, panmictic population) and same generation (i.e. similar age structure). This will reduce the variables that may affect the target grouping (Hare *et*

al. 2011; Palstra & Fraser, 2012). Estimates of Ne in this study, with the associated 95% CI values, were comparable to the estimates from previous studies on sharks. In cases where sampled individuals or loci are too few, the point estimates and the upper confidence intervals are often inflated. Although the lower 95% CI apparently provide the most consistent bounds for describing the lowest possible effective population size estimate (Portnoy *et al.* 2009; Nance *et al*. 2011 Karl *et al.* 2011; Blower *et al.* 2012; O'Leary *et al.* 2015), the all too often infinity found at the upper bound CI and point estimate is in most cases due to insufficient number of loci and/or sample size included in Ne estimation (Blower *et al.* 2016).

3.5 Conclusions

Each of these assessments has shown that the *S. zygaena* individuals in South Africa belong to two sub-populations with moderate to high levels of diversity. As a commercially utilized fish, knowledge of its species biology will aid in postulating the specific conservation actions, should they need to be implemented. As a primary priority, stock delineation is an integral part of fisheries management schemes in the Department of Agriculture, Forestry, and Fisheries (DAFF). This study is the first to evaluate the population genetics of *S. zygaena* within South Africa using molecular tools. Similar patterns of structure were seen in the microsatellites and mtDNA in comparison to other carcharhinid shark species, indicating a biological significant adaptation related to reproductive behaviour. Gravid females are the driving force behind the lack of regional structure seen in the mtDNA as they are likely returning to their origin site for parturition. The discontinuity in genetic connectivity seen in the microsatellite data was related to the respective bioregions, however there still seems to be significant gene flow between the biogeographic barriers. It is likely that *S. zygaena* individuals are moving against the Agulhas Current to reach the warmer subtropical region for more adequate foraging conditions (Dudley & Cliff, 2010). With regards to population viability, there is a sufficient amount of contemporary variation and conservation schemes should aim to maintain this diversity within these connected populations.

CHAPTER 4

Assessing temporal genetic variation in juvenile *Sphyrna zygaena* sharks sampled in Mossel Bay, South Africa

Abstract

The seasonal use of nursery sites for purposes surrounding parturition has provided the basis for inferring philopatry in sharks. Understanding the population genetic dynamics behind their spatio-temporal occurrence in the inshore regions can present data useful for prioritizing certain areas for better protection of the species. In this study, *S. zygaena* juveniles were sampled in Mossel Bay across two different sampling seasons to assess temporal variation in genetic diversity based primarily on seven polymorphic microsatellite markers and the mitochondrial ND2 gene. Overall, F-statistics (F_{ST} = 0.123; $P < 0.001$), Analysis of Molecular Variance (AMOVA) and Bayesian analysis revealed significant variation between sampling seasons. Analysis of the ND2 gene revealed very little temporal differentiation and an overlap of maternal lineages between seasons. Kinship analysis presented a high degree of half-siblingship within and between the sampling seasons with a low degree of full-siblingship. Inbreeding estimates (F_{IS}) were higher in the parental generation (MBP1 = 0.678; MBP2 = 0.709) than in the respective offspring (MB1 = 0.458; MB2 = 0.509). This study is the first to investigate temporal variation and kinship among *S. zygaena* juveniles of an inshore area in South Africa. Overall, these results are indicative of different temporal stocks showing site fidelity to Mossel Bay and should be taken into consideration when conservation management schemes are formulated for the species.

4.1 Introduction

The spatio-temporal demographics amongst shark populations can be influenced by reproductive and/or mating strategies. Studies have shown female sharks displaying strong site fidelity to areas that act as nurseries, which promote the successful development of young (Feldheim *et al.* 2004; Duncan, 2005; Parsons & Hoffmayer, 2007; DiBattista *et al.* 2008a). These reproductive strategies can be investigated through the molecular analysis of population genetic structure and relatedness (Dudgeon *et al.* 2012). Migrations can introduce gene flow, which alters the distribution of alleles in juvenile sharks and therefore the genetic composition of the overall population. In the case of sharks or any marine species, it means that during the inshore phase, reproduction can lead to homogenization of the bi-parentally inherited genome (Clark, 2015). This is characteristic in sharks displaying natal homing, where females return to site of origin for parturition (Chapman *et al*. 2009). This is often not mirrored in the mitochondrial genome, which is maternally inherited and shows heterogeneity across space and time (Tillet *et al.* 2012; Portnoy *et al.* 2016; Bernard *et al.* 2016). The genetic structure seen in mitochondrial DNA, with no structure detected with microsatellites, could be an indicator of site fidelity (Driggers *et al.* 2014). In addition to this, assessing relatedness between individuals through kinship analyses can provide details of pedigree as well as parental contribution. Estimates of kinship can reveal sibling relationships among sampled juveniles and the recurrence of these relationships across sampling years (Nance, 2010). The recurrence of genetic profiles over consecutive years can reveal the degree of usage of specific sites by a population or cohort, allowing for the further inference of a philopatric association or site fidelity to these areas (Feldheim *et al.* 2013; Mourier *et al.* 2013; Mourier & Planes, 2013).

Similar to the reproductive mode of most other carcharhiniformes, smooth hammerhead sharks (*Sphyrna zygaena*) are viviparous, and are likely polyandrous (Chapman *et al.* 2004; Portnoy *et al.* 2007; DiBattista *et al.* 2008b; Farrell *et al.* 2014). These sharks have a biennial reproductive cycle with 1-year recovery phase following parturition (Smale, 1991; Hazin *et al.* 2001; White *et al.* 2008). There is still very limited data available for

Sphyrna zygaena, while a number of studies have investigated movement patterns and reproductive behavior in congeners. Tracking data of habitat use in *Sphyrna lewini* for example revealed a seasonal occurrence of the species in coastal areas, leading to the identification of potential nursery areas (Bessudo *et al.* 2011; Zanella *et al.* 2011; Daly-Engel *et al.* 2012). For a philopatric species, it is expected that there would be low inter-annual genetic differentiation. This has not been observed in the hammerhead species *Sphyrna lewini* that showed temporal variation between four years of sampling (Nance, 2010). This variation described in *S. lewini* over multiple years, may be influenced by the biennial reproductive cycle, which prevents mature females from gestating and returning to nursery grounds. This non-parturient year is thought to promote the efficient use of reproductive resources so that progeny fitness is maximized (Rideout *et al.* 2005). Additionally, the spatial scale of site fidelity may present a scenario where females use a range of inshore areas that foster survival of juveniles (regional philopatry) rather than site-specific philopatry (Keeney *et al.* 2005).

Hammerheads are highly prized for the delicacy of shark fin soup in the Asian market and have likely been over-exploited, with catches declining in recent years (Dent & Clarke, 2015). Due to their conservation needs, the Convention on International Trade in Endangered Species (CITES) listed the three large bodied hammerhead species, *Sphyrna zygaena*, *Sphyrna mokarran*, and *Sphyrna lewini*, in Appendix II to protect them from unsustainable trade (CITES, 2015). Throughout its distribution, large inshore aggregations of *S. zygaena* juveniles have an increased incidence of capture, which in turn increases their vulnerability (Vooren *et al.* 2005). Understanding the dynamics behind the reproductive cycle and spatial use of *S. zygaena* in an inshore area such as Mossel Bay can aid in developing local management schemes and better protection for this putative threatened species (Casper *et al.* 2005).

The aim of this study was to assess (1) genetic differentiation and (2) relatedness among *S. zygaena* juveniles to determine the presence of temporal site fidelity to Mossel Bay. Microsatellite markers and mitochondrial DNA were employed in order to investigate variation and kinship between *S. zygaena* cohorts sampled across two seasons (MB1 and

MB2). If no variation is seen in the microsatellite markers or the mitochondrial DNA across years, this could be indicative of the same sharks using Mossel Bay each year, suggesting philopatric behavior. However, if temporal structure of *S. zygaena* is revealed, site fidelity of different parental stocks utilizing Mossel Bay is more likely, reflecting the biannual reproductive cycle of *S. zygaena* rather than philopatry *per se*. Additionally, the degree of relatedness within and amongst cohorts sampled by year could substantiate either hypothesis.

4.2 Materials and Methods

4.2.1 *Study Site*

A total of 95 *S. zygaena* samples were collected in Mossel Bay in 2014 and 2015 under the *Permit for the Purposes of a Scientific Investigation or Practical Experiment in Terms of Section 83 of the Marine Living Resources Act, 1998 (Act No. 18 of 1998)* established by the South African Department of Agriculture, Forestry and Fisheries (DAFF). The sampling protocol was approved by DAFF under the permits RES2014/01 and RES2015/74. These sharks were caught, and subsequently released, using handlines during which fin clippings were taken from the trailing edge of the dorsal fin. The sampling populations comprised 48 collected from December 2013 to February 2014 (MB1) and 46 collected from December 2014 to February 2015 (MB2). The sampled localities are presented in Figure 4.1.

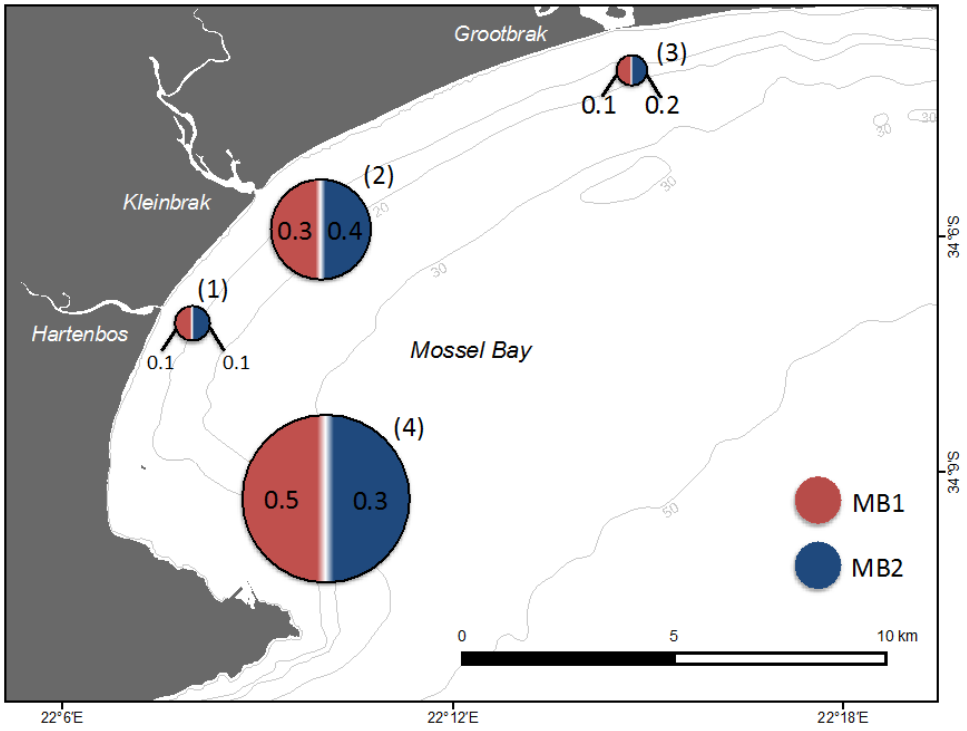

Figure 4.1. Sampling sites within Mossel Bay as demarcated by circles with numbers corresponding to specific areas: (1) Hartenbos River Mouth, (2) Kleinbrak River Mouth, (3) Grootbrak River Mouth, and (4) Mossel Bay Harbour Mouth. Values within the circles represent the frequency of *S. zygaena* individuals sampled at each site.

4.2.2 *DNA extraction, genotyping and sequencing*

Genomic DNA was extracted from samples using the CTAB method (Saghai-Maroof *et al*. 1984) and quantified using the NanoDrop ND-2000, UV-Vis spectrophotometer (NanoDrop®). A total of 48 and 47 from sampling year one and sampling year two were selected for cross-species amplification of seven microsatellite loci respectively. The mitochondrial ND2 gene was sequenced for a total of 15 individuals from both sampling years. These procedures follow the protocol as previously described in Chapter 3.

4.2.3 Microsatellite Analysis

Microsatellite genotypes were analyzed for marker utility using MICROCHECKER 2.2.3 (van Oosterhout *et al.* 2004) and analyzed for departures from the Hardy-Weinberg Equilibrium (HWE) using GENEPOP 4.2 (Raymond and Rousset, 1995). Genetic diversity statistics were generated in GENALEX 6.501 and the polymorphic information

content (PIC) was generated in MICROSATELLITE TOOLKIT (Park, 2001; Peakall and Smouse, 2012). These procedures follow the protocol as previously described in Chapter 3.

In order to characterize temporal genetic differentiation between *S. zygaena* MB1 and MB2 sampling seasons, an Analysis of Molecular Variance (AMOVA) was conducted in ARLEQUIN v 3.5.1.2 (Excoffier *et al.* 2005). Global and pairwise F_{ST} values between the two sampling seasons were calculated also in ARLEQUIN. Similarly, R_{ST} values were generated in GENALEX to determine differentiation by allelic size rather than allelic state (Goodman, 1997). The estimate for R_{ST} is an analogue of F_{ST} that assumes a stepwise mutational model (Balloux & Lugon-Moulin, 2002). A Bayesian multi-locus clustering method was implemented through STRUCTURE 2.3.4 to assign individuals to ancestral clusters. An admixture model was used with non *a priori* assumptions, with K ranging from 1 to 4 and 1,000,000 MCMC iterations with 100,000 burn-ins. In order to determine convergence based on the number of clusters (K), the ΔK metric was employed through the Evanno method in STRUCTUREHARVESTER, which statistically determines the most likely number of clusters supported by the data (Pritchard *et al.* 2000; Evanno *et al.* 2005; Earl & vonHoldt, 2012).

4.2.4 *Kinship Analyses*

The proportion of related individuals within and among sampling seasons was determined by COLONY (Jones & Wang, 2010). This was done by a search for the maximum likelihood configuration of siblingship assignments for individuals based on the microsatellite genotypes. All samples were assigned to their respective sampling season and for each sample, allelic frequencies were updated over one run, with medium, full-likelihood precision. The polygamous mating system was set for both males and females and no priors were established for the sibling groups. With the resulting assignments, the reconstructions of unsampled parental genotypes were simulated based on the siblingship relationships to determine the number of contributing parents. Duplicate genotypes were tested for in MICROSATELLITE TOOLKIT and inbreeding estimates (F_{IS}) were generated in GENEPOP for the unsampled, reconstructed parental

genotypes. Additional pairwise and mean relatedness analyses for the juvenile *S. zygaena* individuals were generated through MLRELATE (Kalinowsky, 2006) and GENALEX respectively. Maximum likelihood relatedness estimated by MLRELATE and the relatedness coefficient (*r)* was generated through the Queller & Goodnight (1989) method in GENALEX.

4.2.5 *Mitochondrial Haplotype Analysis*

Sequence data was manually edited in 4PEAKS (Griekspoor & Groothuis, 2005) and subsequently imported into MEGA 7.0.14 (Kumar *et al*. 2016) for sequence alignment applying the ClustalW multiple alignment algorithm (Thompson *et al.* 1994). Haplotype (*h*) and nucleotide diversity (π) were calculated in DnaSP 5.10 (Rozas *et al.* 2003). An analysis of molecular variance (AMOVA) was conducted in ARLEQUIN 3.5.1.2 (3000 permutations) through Φ_{ST}, to test for genetic differentiation and significance between the sampling seasons (Excoffier & Lischer, 2010). A haplotype genealogy was constructed in HAPLOVIEWER following the method of Salzburger *et al.* (2011) using a phylogenetic tree derived in PhyML v3.0 (Guindon & Gascuel 2003, Guindon *et al.* 2010) following 1000 bootstraps and the TN93 evolutionary model inferred by JMODELTEST (Posada 2008).

4.3 Results

4.3.1 *Microsatellite Diversity*

Quality control measures for the microsatellites revealed all loci showed evidence of null alleles but no genotyping errors (Table 4.1). The average expected heterozygosity (He) was 0.7 (range 0.250-0.972) and the average observed heterozygosity (Ho) was 0.41 (range 0.063-0.851). Both MB1 and MB2 significantly deviated from HWE ($p < 0.01$), possibly from inbreeding or the presence of the Wahlund effect, caused by sub-structuring. There was non-significant linkage disequilibrium (LD) amongst the loci.

Table 4.1. Microsatellite characterization of two *S. zygaena* cohorts sampled at Mossel Bay

Population	Na	Ae	Ho	He	PI	PIC	HWE	FIS	FrNULL
MB1	44	24.126	0.428	0.696	2.3E-03	0.667	0.013	0.458	0.171
MB2	38	24.142	0.392	0.704	2.0E-03	0.685	0.005	0.509	0.342
AVERAGE	**41**	**24.134**	**0.410**	**0.700**	**9.74E-04**	**0.676**	**0.009**	**0.483**	**0.257**

4.3.2 *Temporal Genetic Variation*

Results for a global AMOVA showed significant differentiation among the Mossel Bay sampling years (F_{ST} = 0.119; p < 0.001). This was also reflected with the F-statistics (pairwise F_{ST} = 0.123; p = 0.000), which showed high differentiation between groups. The values for R_{ST} = 0.159 (p < 0.001) also suggested highly significant differentiation between the sampling years. Bayesian clustering analysis in STRUCTURE revealed two distinct clusters corresponding to individuals from MB1 and MB2 respectively (Figure 4.2). With the ΔK metric set to K=2, both groupings had one primary ancestral cluster with a low membership coefficient and assignment to the second cluster. From this, strong temporal variation was evident between MB1 and MB2, indicating 'isolation by sampling year'.

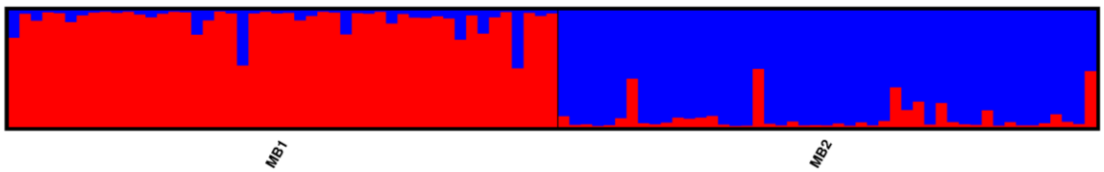

Figure 4.2. Bayesian clustering assignment of two *S. zygaena* cohorts sampled across different sampling seasons. Sampling seasons are indicated on the x-axis and each individual is represented by a single vertical column.

4.3.3 *Kinship Analysis*

Overall relatedness (Table 4.2) estimated with COLONY, MLRELATE and GENALEX, which estimated the sibling relationships within MB1 and MB2, revealed a high degree of siblingship in both seasons (MB1 = 0.620; MB2 = 0.643) as well as overall (MB1+MB2 = 0.632). Individuals within MB1 had low likelihood for full sibling matches. In order to detect sibling pairs between the sampling years, the populations were combined and 55.5% were found to have a degree siblingship. Reconstruction of parental

genotypes revealed equal proportion of contribution from males and females in each season (MB1: Males = 34, Females = 33; MB2: Males = 33, Females = 29). There were no shared genotypes for males or females between MB1 and MB2, indicating different parental genotypes across seasons. The inbreeding estimates as calculated in GENEPOP for the parental generation were significantly higher for MBP1 (F_{IS} = 0.678) and MBP2 (F_{IS} = 0.709) than in the respective offspring.

Table 4.2. Probability of relatedness within and between *S. zygaena* individuals sampled in Mossel Bay. Probability values indicate the proportion of related individuals.

COLONY				
Within	FS	HS	UR	R
MB1	0.009	0.059	0.932	0.688
MB2	0.021	0.063	0.916	0.660
Average	0.015	0.061	0.924	0.674
Between	0.023	0.066	0.910	0.611
ML-RELATE				
Within	FS	HS	UR	R
MB1	0.008	0.059	0.933	0.545
MB2	0.021	0.063	0.916	0.661
Average	0.015	0.061	0.924	0.603
Between	0.026	0.060	0.916	0.336
GENALEX				
Within	FS	HS	UR	R
MB1	0.009	0.263	0.728	0.628
MB2	0.019	0.229	0.751	0.609
Average	0.014	0.246	0.739	0.619
Between	0.022	0.239	0.741	0.719
Average MB1	0.009	0.127	0.864	0.620
Average MB2	0.021	0.118	0.861	0.643
Average Overall	0.015	0.123	0.862	0.632
Average Between	0.024	0.122	0.856	0.555

FS: Full Sibling; HS: Half Sibling; UR: Unrelated; R: Percent of Related Individuals

4.3.4 *Mitochondrial Haplotype Analysis*

The mitochondrial ND2 region was successfully sequenced and aligned for a total of 30 individuals. The 750 bp alignment contained a GC content of 38.6% and a transition/transversion bias (*R*) of 0.000 with 2 segregating sites (*S*). Overall, values for

haplotype diversity (*h*) and nucleotide diversity (π) were 0.239 (±0.092SD) and 0.001 (±0.000SD) respectively. The *h* and π values were relatively low for both temporal groups; MB1 (*h* = 0.133; π = 0.000) and MB2 (*h* = 0.343; π = 0.001). Pairwise Φ_{ST} values of the mtDNA ND2 gene showed no significant differentiation between MB1 and MB2 (Φ_{ST} = 0.008; P = 0.694 ± 0.047). A parsimony reconstruction based on the ND2 gene revealed only two haplotypes, with each haplotype represented by 26 and four individuals respectively. The two sampling seasons shared the same haplotypes and therefore presents a lack of temporal variation at the ND2 gene (Figure 4.3).

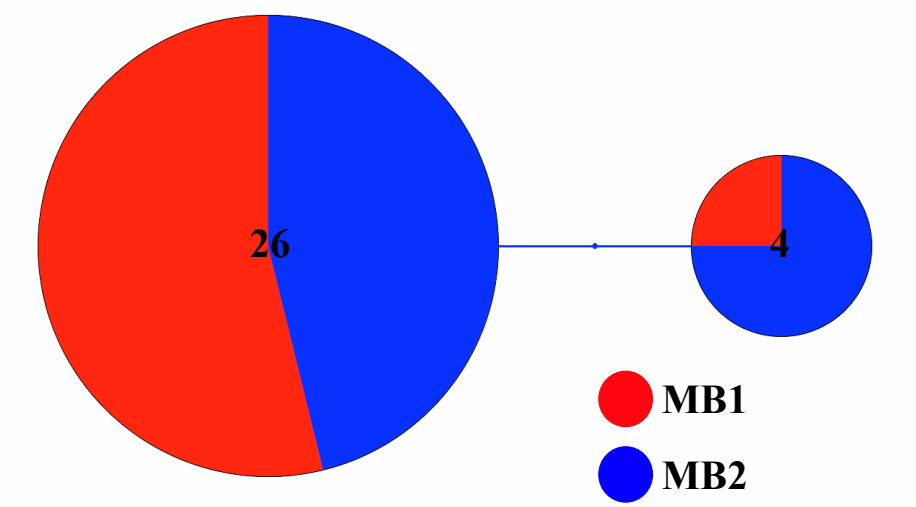

Figure 4.3. Haplotype network for *Sphyrna zygaena* individuals sampled in Mossel Bay. Size of the circle is scaled to the frequency of occurrence for haplotype. Small blue dots indicate hypothetical haplotypes.

4.4 Discussion

Mitochondrial diversity was low between and within the samplings seasons based on haplotype and nucleotide diversity of the *S. zygaena* individuals sampled in Mossel Bay during 2014 and 2015. This was also evident from the low number of segregating sites that resulted in only two haplotypes shared between sampling seasons. The lack of mtDNA genetic structure across seasons implies that individuals share the same maternal lineages over sampling years. The microsatellite results from the F-statistics, AMOVA, and Bayesian clustering analyses showed that samples from the two seasons were

significantly different, though there was a similar level of genetic diversity within seasons. The temporal variation observed between the two seasons could most likely be attributed to the biennial reproductive cycle seen in *S. zygaena*, which also explains the lack of overlap with genotypes in non-reproductive years. This adds a new dimension to the management requirements of the species, as differentiation between years may indicate the presence of temporal stocks that utilize the same geographic regions. Subsequent sampling can aid in revealing if this is a trend that occurs across more than just two sampling years.

There was a high prevalence of sibling relationships within the sampling years, but also a significant degree of sibling relationships between the sampling years. This might indicate an overlap between years, where males and females are philopatric and return to Mossel Bay annually. Any overlap can be explained by the annual return of individuals that utilize Mossel Bay in consecutive years. This is apparent through the unrelated individuals within the sampling years were found to have half-sibling relationships between sampling years. It is also possible that some females might undergo a parturition event annually, returning to the area every year. From the microsatellite data, this does however not seem probable, as a higher degree of admixture between seasons would be expected as well as the recurrence of reconstructed maternal genotypes across seasons. A high degree of half sibling relationships may rather present a strong case for polyandry (females being sired by multiple males) within these hypothetical *S. zygaena* litters as seen in several other carcharhiniformes (Feldheim *et al.* 2004; Portnoy *et al.* 2007; Larson *et al.* 2011; Marino *et al.* 2015; Pirog *et al.* 2015). The presence of polyandry could be determined through the reconstruction of parental genotypes, where there would be a disproportionate increase in the paternal contribution to the maternal contribution among the litters, however this could not be ascertained from the current data set. Among carcharhinid sharks, multiple paternity is a result of polyandrous activities and produces litters with unequal distribution of male and female parents. Ratios of parentage between males and females can range between 2:1 and 7:1 among litters (Feldheim *et al.* 2001a; Saville *et al.* 2002; Feldheim *et al.* 2004; Daly-Engel *et al.* 2006; Rossouw *et al.* 2016). As there was a 1:1 ratio on average, with a greater maternal contribution than expected,

polyandry could not be confirmed in this study. An alternative scenario may be a physiological departure from the social reproductive norm that is meant to promote multiple paternity, rather than single paternity. For example, Chapman *et al.* (2004) described genetically monogamous litters in *Sphyrna tiburo*, and therefore did not support the presence of multiple paternity in this species. In Chapman *et al*. (2004), it was hypothesized that there was sperm selection on part of the mothers, where genetically compatible males would be preferred. The authors also suggested it could be an evolutionary development from the father's sperm to outcompete sperm from other males (Chapman *et al.* 2004). What is however evident from the current study is that the same maternal lineages are contributing to the juveniles in Mossel Bay and that the high degree of contemporary differentiation revealed with the microsatellites could be mediated by male contributions to the cohorts.

The presence of homozygous excess among the offspring and reconstructed parental genotypes, as estimated by F_{IS}, was detected in both sampling years. For the parental generation, this could be an artifact of the temporal Wahlund effect, arising from sampling bias where subpopulations are sampled rather than a true representation of the general population (Wahlund, 1928). The high F_{IS} values in the offspring may suggest that the appearance of inbreeding may be due to population differentiation and not necessarily due to inbreeding depression (Mourier & Planes, 2013).

4.5 Conclusions

These findings have implications for the conservation of this CITES protected species. The use of putative nursery sites can promote diversity of the *S. zygaena* sampled cohorts, by providing an environment that fosters growth and development. Though deficits in genetic diversity were not noted, preserving current levels is fundamental to preventing depletions of biodiversity within the Mossel Bay area. Harvesting these sharks during their juvenile phase of life carries more detriment to the sub-population groups, as individual temporal stocks are solely utilized per annual season, which alters the demographics of the whole population. Conversely, these temporal breeding populations

or ‘stocks’ may actually buffer the negative effects of localized overfishing in this region. It can be postulated that the temporally differentiated cohorts identified in this study are from separate populations that utilize Mossel Bay for pupping. Analyses of subsequent sampling seasons and other similar areas frequented by smooth hammerheads are therefore necessary to give a better indication of the vulnerability of the species on a temporal scale.

CHAPTER 5

Conclusions

The sustainable use of marine resources is a requirement for the survival of global fisheries stocks and studies have shown that the consequences of depleting biodiversity can impact the ability for the oceans to recover and support the three billion people requiring its resources (Worm *et al.* 2006; Pauly & Zeller, 2016; Worm, 2016). Fundamental research on species biology and ecology, combined with molecular investigations such as genetic diversity, population structure, historical demographics, genetic diversity and species composition in fisheries is a requirement for the long-term conservation of marine biodiversity (Ridgeway & Sampayo, 2005). Fisheries data have indicated that the global biomass of the smooth hammerhead shark *S. zygaena* has been on a downward trend (Fowler, 2005), but to date, there have only been a few molecular studies investigating *Sphyrna zygaena* (Lim *et al.* 2010; Testerman, 2014). In order to address potential threats, the population dynamics of this shark species can be evaluated through molecular methods and incorporated into shark biodiversity conservation schemes. The overall aim of this study was therefore to address some of these conservation concerns in South African chondrichthyan fisheries and more specifically for smooth hammerhead sharks, *S. zygaena*. The aims were achieved by outlining three primary research directions; (1) investigate species composition in South Africa fisheries and validate molecular identification tools, (2) describe spatial patterns of genetic diversity among *S. zygaena* individuals sampled along the coastline, and (3) to determine temporal variation of *S. zygaena* in Mossel Bay over two sampling years.

5.1 Research Findings

5.1.1 *Chondrichthyan Species Identification in South African Fisheries*

As a tool for fisheries management, DNA barcoding using the cytochrome oxidase subunit I (CO1) proved to be effective in identifying species in South African fisheries. Barcoding also showed that although species misidentification for *S. zygaena* does occur

in regional fisheries, the error rate seemed to be relatively low, which demonstrates the effectiveness of morphological identification schemes in differentiating *S. zygaena* from congener *S. lewini* which has an overlap in geographic range. The success of the DNA barcoding effort was in part dependent on the availability of reference sequences in the Barcode of Life Data System (BOLD), and in some cases failed to provide accurate hits for taxa analyzed. An example was *Triakis megalopterus,* which is easily identifiable through morphological identification schemes, but is one of the many endemic species for which reference sequences are still lacking on BOLD. The utility of morphological identification schemes did not always align with the molecular data. A few ambiguous matches were found for several Scyliorhinid sharks and were attributed to morphological conservatism as well as possible cryptic speciation events or hybridization proposed for the Scyliorhinidae family (Bester-van der Merwe & Gledhill, 2015). Morphological conservatism and cryptic speciation are often a result of convergent evolution or species that have experienced radiations, yet maintained their physical traits over evolutionary advancements (Quattro *et al.* 2013; Moen *et al*. 2013; Lindholm, 2014). This highlighted the need for a more robust identification scheme that combines morphological keys and multiple molecular markers types to be used in South African fisheries affecting chondrichthyans (Moore *et al.* 2011).

A total of 18 species from ten different families were identified using DNA barcoding, providing one of the first molecular assessments of chondrichthyan biodiversity in South Africa. As expected, most of the species found to be at the highest quantities, were species of commercial importance and in line with what was found in similar studies investigating species composition in other regions. The barcoding data also confirmed that recreational fishing might be an effective tool in exploring shark biodiversity as the highest number of species was encountered through this fishing method. The morphological and molecular data revealed a high degree of similarity between *Carcharhinus obscurus* and *Carcharhinus galapagensis,* and therefore may be considered as conspecifics. The latter highlighted once more that an approach combining DNA barcoding with alternative identification tools, such as morphological keys, could increase the accuracy of species identification. Given the individual shortcomings of each

of the disciplines, their combined scope can address biodiversity and identification issues more rigorously (Schlick-Steiner *et al.* 2010).

5.1.2 *Population Structure of Sphyrna zygaena in South Africa*

The main objective of this chapter was to investigate population genetic structure of *S. zygaena* with regards to connectivity of populations along the South African coastline. It was hypothesized that there would be high population connectivity, and minimal population subdivision based on the species dispersal ability. If there were to be any population substructuring, it was further postulated that it would most likely correspond to the two major biogeographic regions from where samples were sourced; the southern warm temperate and the eastern subtropical biogeographic regions. Throughout the South African coastline, it was proposed that contemporary restrictions to gene flow might be as a result of cold-water upwellings, shelf-breaks, and freshwater discharges. Given the major oceanographic currents at play, it was also predicted that the gene flow would potentially be in a southwesterly direction, moving with the Agulhas current. An additional priority was to evaluate levels of genetic diversity as predictions had been made indicating that the species may have experienced significant declines in their global population numbers (Casper *et al.* 2005).

The results rejected the original hypothesis of panmixia, showing very low connectivity between individuals sampled from the warm temperate (south coast) and the subtropical (east coast) biogeographic regions. The microsatellite dataset overall indicated asymmetric gene flow predominantly towards the south coast from the eastern Natal regions. Bayesian clustering analysis implemented in STRUCTURE and a discriminate analysis of principle components (DAPC) showed significant population subdivision between the southern and eastern coast populations, and was corroborated by significant pairwise F_{ST} values. This could be due to oceanic dispersal barriers, which divide the south and east coast (Teske *et al*. 2011). To the contrary, there was no genetic structure represented by the mitochondrial ND2 gene with 62.5% of the individuals sharing a single haplotype. In this study, it is likely that the heterogeneity seen with the microsatellites is attributed to contemporary oceanographic divisions in biogeographical

regions previously demonstrated to reduce migrations between sites (White *et al.* 2010). Significant asymmetric gene flow was observed predominantly in an eastward direction going against the Agulhas current. The former result did not substantiate the hypothesis that gene flow mainly occurs in the direction consistent with the principle current, the southward flowing Agulhas current, but rather in line with the Agulhas Return current that flows back in an eastward direction. Previous studies have shown that predominant gene flow against the principle current is not uncommon in fish with high dispersal ability (Dicken *et al.* 2007; Hussey *et al.* 2009; Reid *et al.* 2016). The Agulhas Return Current may provide these juveniles a medium to utilize warmer waters if temperatures in the temperate south coast become unfavourable (Teske *et al*. 2013). In addition to this, the subtropical biogeographic region on the east coast carries a richer diversity of biota (Lombard *et al*. 2004), which could be crucial for attaining foraging opportunities.

Analysis of contemporary demographics and effective population size in particular suggested that these sampling populations had properties similar to large, healthy populations not experiencing a high degree of genetic drift. Though this result represents a positive outlook of the South African *S. zygaena* populations, effective population sizes need to be interpreted with caution due to the wide confidence intervals obtained with both approaches of Ne estimation. Due to the life history stage of these juveniles, the likelihood of kinship between individuals in Mossel Bay is high and presents a scenario where the true population remains unsampled. This can inflate the appearance of genetic drift if only juveniles are sampled during their life history stage in an area such as Mossel Bay. Analysis of historical demographics showed that a population expansion possibly occurred during the Holocene, which began ~0.01 mya (Walker *et al.* 2009). Though similar expansions have been noted for many shark species, the majority occurred during the Pleistocene (~2.5-0.12 mya) deeming this as a more recent expansion (Duncan *et al.* 2005; Keeney *et al.* 2005; Geraghty *et al.* 2013). These expansions may have come in light of the recent warming after the last glacial period (Abell & Plug, 2000) and correlates to paleoenvironmental conditions in the past 10 kya necessary for populations to expand (Scott *et al*. 1995). Analysis of historical demographics in other oceanic basins can aid in uncovering trends that lead to the phylogeography of this species globally and

possibly reveal current climate trends that could influence the current distributions of *S. zygaena* populations.

5.1.3 *Kinship and Temporal Variation of S. zygaena in Mossel Bay*

This chapter sought to examine relatedness among the *S. zygaena* juveniles sampled in Mossel Bay over a two-year sampling period. Due to the annual use of Mossel Bay, it was hypothesized that there would not be temporal variation between seasons. Analysis of mitochondrial structure across the seasons showed no differentiation in matrilineal lineages within Mossel Bay while the microsatellites showed a high degree of differentiation between years. The temporal variation between the sampling years could be attributed to the biennial reproductive cycle, which sees *S. zygaena* females undergoing a gestational year followed by vitellogenesis, which is a period used to recover reproductive tissues (Castro, 2009; NOAA, 2015). Similar to the temporal variation that was found in Nance, 2010 for *S. lewini* (Nance, 2010), it is most likely that for *S. zygaena* there are different breeding stocks frequenting Mossel Bay each year.

The kinship analyses revealed that the degree of sibling relationships was within similar ratios for both seasons. The majority of *S. zygaena* juveniles had half-sibling kinship with one other individual sampled. If these sharks were pupped in Mossel Bay, then it can be assumed that some individuals may have come from the same litters, which may be a result of polyandrous mating (Nance, 2010). This is a life history trait that is common in sharks and may also be a remnant of philopatry related to nursery use (Heupel *et al.* 2007). Though the data could not reveal the presence (or absence) of polyandry, with the greater male contribution observed for each sampled cohort, it is likely that the presence of contemporary structure could be a result of male mediated gene flow. Genetic diversity estimates between the juveniles and reconstructed parental generation was insignificant and showed stability between seasons.

5.2 Implications for Biodiversity Conservation

Regarding species composition of South African fisheries, the data leads to the proposition that more resources be allocated to fundamental taxonomic and molecular research on species that may not be well understood, as that is a primary goal of the Shark Biodiversity Management Plan (Lombard *et al.* 2004; Ebert & Van Hees, 2015). Misidentification issues surrounding chondrichthyans can severely hamper the efficacy of conservation management, though this can be largely mitigated through the use of mitochondrial markers (Naylor *et al*. 2012). With generic terms being used for a range of species, the information regarding specific life history traits, stock structure, and phylogeography goes unaccounted for, while existing management schemes may not address species-specific conservation needs (Ovenden *et al.* 2015). The conservation of chondrichthyan biodiversity requires the implementation of effective management schemes to address concerns surrounding large depletions of chondrichthyans globally (Costello *et al.* 2016). From a conservation perspective, the findings of this study suggest that the south and east coast *S. zygaena* in South Africa potentially needs to be regarded as separate genetic units. This study is the first to present a regional population assessment of *S. zygaena* using molecular tools. The data showed compelling evidence that biogeography can have a significant effect on stock structure, showing high differentiation within the *S. zygaena* populations across the South African coastline. Analysis of genetic diversity and effective population size among the sampled cohorts revealed each cohort had properties of a relatively large and healthy population. This information comes in light of the recent annexing of *S. zygaena* into CITES Appendix II (CITES) to be protected from unsustainable trade. Hopefully, this is an indication that the negative effects of shark finning had not afflicted these sharks as severe as *Sphyrna lewini* and *Sphyrna mokarran* for example.

Additionally, the occurrence of variation across years could present a further temporal component to consider with regards to the conservation needs of this species. This indicates the presence of multiple breeding stocks utilizing Mossel Bay, with a degree of overlap in breeders as evidence by the occurrence of sibling relationships across the

years. Based on this, the temporal stock structure could either buffer or amplify the negative effects of over-fishing depending on the degree of panmixia or drift between the temporal stocks. As inshore areas are critical for the reproductive success of these sharks, there is an increased susceptibility to localized stock depletions that may occur on either a temporal or a spatial level (Hueter, 2004). Therefore, it is proposed that the spatio-temporal trends in *S. zygaena* be investigated further and managed based on these trends. The results do not reveal any significant declines in *S. zygaena* genetic diversity through historical and contemporary demographic analysis and the aim should be to monitor and preserve the existing biodiversity, while mitigating potential threats.

5.3 Project Limitations and Future endeavors

This study has implications that stretch beyond the hypotheses and research aims, however due to limited sample size and difficulty in securing samples throughout the species distribution range much remains to be investigated. Incorporating individuals of the southeast Atlantic in subsequent analyses can be informative regarding the ability *S. zygaena* to traverse the cold front created by the Benguela current. Nonetheless, the current study provides a platform that can lead to a multifaceted research endeavor that integrates molecular approaches with traditional management tools. A proposed methodology would be to incorporate telemetry to further reveal the patterns of movement and gene flow as well as population connectivity of *S. zygaena* along the South African coastline. In addition to this, telemetry can shed light on the temporal variation seen within Mossel Bay especially if genotyping over additional sampling seasons show a similar trend. This may also aid in confirming whether the temporal variation is due to the biannual cycle of *S. zygaena*. In future, high-throughput genomic technologies can help understand more about the adaptive potential of the species based on the identification of outlier loci possibly subject to selection. This will also allow macro-evolutionary questions related to species origin and evolutionary history to be addressed. Given the questions that still remain unanswered regarding fine-scale population structure and demographics of these sharks this study has been successful in

disseminating relevant research findings that can be useful for chondrichthyan biodiversity conservation and providing a basis for future research endeavors.

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