POPULATION CONNECTIVITY OF *STYLOPHORA PISTILLATA* AND *SINULARIA BRASSICA* BETWEEN KWAZULU-NATAL MARINE PROTECTED AREAS

by

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As the candidate's supervisor, I have approved this dissertation for submission.

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Helping people to care for our ocean

PREFACE

The experimental work described in this thesis was carried out in the Oceanographic Research institute (ORI), which is an affiliated institute of the School of Biological and Conservation Sciences at the University of KwaZulu-Natal, Westville, and the South African Institute for Aquatic Biodiversity (SAIAB). Field work was conducted at Sodwana Bay, Durban, Aliwal Shoal and Pondoland, South Africa from April 2018 to September 2020, under the supervision of Doctor D.J Pearton and Professor A.H.H Macdonald. This thesis represents original work by the author and has not otherwise been submitted in any form for any degree or diploma to any tertiary institute. Where use has been made of the work of others, it is duly acknowledged in the text.

I certify that the above statement is correct:

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Jessica Gilmore, January 2021

DECLARATION 1 – PLAGIARISM

I, Jessica Rose Gilmore, declare as follows:

(i) the research reported in this thesis, except where otherwise indicated or acknowledged, is my original work.

(ii) this thesis has not been submitted in full or in part for any degree or examination to any other university.

(iii) this thesis does not contain other persons' data, pictures, graphs, or other information, unless specifically acknowledged as being sourced from other persons.

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(v) where I have used material for which publications followed, I have indicated in detail my role in the work.

(vi) this thesis is primarily a collection of material, prepared by myself, published as journal articles, or presented as a poster and oral presentations at conferences. In some cases, additional material has been included.

(vii) this thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed: ___[redacted]____ January 2021

Jessica Gilmore

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I would like to thank my parents Sandra and Samuel Gilmore for instilling a love for the ocean in me and for helping me to fulfil my childhood dream of becoming a marine scientist. Furthermore, to the rest of my immediate family, extended family, and friends, I appreciated your support during this journey. The cups of coffee, meals, pep talks, and prayers did not go unnoticed.

“We keep moving forward, opening up new doors and doing new things, because we're curious...and curiosity keeps leading us down new paths.” — Walt Disney

TABLE OF CONTENTS

Preface ..... **i**

Declaration 1 – Plagiarism ..... **ii**

Acknowledgements ..... **iii**

Table of Contents ..... **iv**

Table of Figures ..... **vi**

List of Tables ..... **viii**

Abstract ..... **ix**

Chapter 1: Introduction **1**

Coral Reefs and Their Threats *1*

Coral Reefs in South Africa *2*

Marine Protected Areas *4*

Study Locations *5*

Genetic Connectivity *6*

Coral Recruitment *7*

Study Species *8*

Genetic Background *8*

Key Questions and Research plan *11*

References *13*

Chapter 2: Genetic Diversity and Biogeographic Distribution of *Stylophora pistillata* in Kwa Zulu-Natal **27**

Abstract *27*

Introduction *27*

Aims and Objectives 30

Hypotheses 31

Methods *31*

Collection 31

Tissue Extraction 33

PCR Amplification and Sequencing 33

Sequence Data 34

Sequence Alignment and Data Analysis 34

Results and Discussion *36*

Phylogenetic Results 36

Discussion *51*

References *57*

Chapter 3: ddRADSeq of *Stylophora pistillata* Demonstrates Ecologically Driven Population Structure Along the East Coast of South Africa **66**

Abstract *66*

Introduction *66*
Aims and Objectives 68

Materials and Methods *69*
Collection 69
Tissue Extraction 69
Library Preparation and MiSeq 70
Bioinformatics and Analysis Pipeline 71

Results and Discussion *73*
Determination of Loci Under Selection 81

Conclusion *85*

References *87*

Chapter 4: A Preliminary Assessment of Population Connectivity of *Sinularia brassica* in the Northern Complex of iSimangaliso Wetland Park **91**

Abstract *91*

Introduction *91*
Aims and Objectives 95
Hypotheses 95

Methods *95*
Tissue Extraction 96
PCR Amplification and Sequencing 96
Sequence Data 97
Sequence Alignment and Data Analysis 97

Results and discussion *99*
Phylogenetic Results 99

Discussion *109*

Conclusion *110*

References *112*

Chapter 5: Final Discussion and Conclusion **119**

Management Implications 124

References *125*

Appendix A: QuaddRad Protocol (Adapted From Franchini *et al*., 2017) **127**

Equipment and Materials *127*
A: DNA extraction 128
B: Adapter preparation 128
C: Double restriction digestion and adapter ligation 128
D: Indexing PCR 129
E: Size selection 130
F: Illumina MiSeq Run 130

Appendix B: Supplementary Information for Chapter 3 ..... **131**

Appendix C: Supplementary Information for Chapter 4 138

TABLE OF FIGURES

Figure 1.1: The circulation of the Agulhas Current showing its features (Duncombe Rae, 1991; Lutjeharms, 2006, 2007; Lutjeharms et al., 2012). 3

Figure 1.2: The nine bioregions of Southern Africa as described by Lombard et al. (2004). (From Griffiths et al., 2010) 4

Figure 1.3: Marine Protected Areas (MPAs) in the study region along the coastline of South Africa. Image created using Google Earth 6

Figure 2.1: Map showing the locations from which *Stylophora pistillata* samples were obtained by Keshavmurthy, *et al*. (2013) (shown as red dots). The division of four regions based on the four clades of *Stylophora* is marked in colours. Clade 1 - Pacific-Western Australia clade (pink), clade 2 - Chagos-Madagascar-South Africa Clade (green), Clade 3 – Gulf of Aden-Zanzibar-Madagascar Clade (blue) and Clade 4 - Red Sea-Persian/Arabian Gulf-Kenya Clade (brown). Adapted from Keshavmurthy *et al*. (2013). 29

Figure 2.2: Sampling locations for this study including the MPAs and depths of the reefs sampled. MPA boundary information was downloaded from https://egis.environment.gov.za/data_egis/, and loaded in Google Earth Pro 31

Figure 2.3: Image of the ACEP Remotely Operated Vehicle (ROV) on the ACEP small craft, MV Phakisa. 33

Figure 2.4: Phylogenetic analysis of COI sequences of *Stylophora pistillata* with references derived from (Keshavmurthy *et al.,* 2013). Bootstrap values are based on 1000 repetitions (ML) analyses. Individual haplotypes found in each location are indicated by name-number and the numbers in the brackets indicate the total number of each haplotype obtained from each location. 38

Figure 2.5 Phylogenetic analysis of a 372bp subsequence of ITS2 sequences of *Stylophora pistillata* with references from Flot *et al*. (2011) and retrieved from GenBank (Appendix A). Bootstrap values are based on ML analyses. Different haplotypes sequenced from the same individual are designated as A or B and are joined by curves to form a haploweb (Flot, 2010). 43

Figure 2.6: Haplotype network of the COI sequences of Stylophora sp. In KZN, the median spanning network was drawn using the location sampled as a trait created using POPArt (Bandelt, Forster and Röhl, 1999; Leigh and Bryant, 2015). 44

Figure 2.7: COI haplotypes per locations. Showing distribution of Clade 3 haplotypes along the sample coastline. MPA boundary information was downloaded from HTTPS://EGIS.ENVIRONMENT.GOV.ZA/DATA_EGIS/ AND loaded in Google Earth. 45

Figure 2.8: Haplotype network of the full 14xx ITS region of *Stylophora*, the median spanning network was drawn using the location sampled as a trait created using POPArt (Bandelt, Forster and Röhl, 1999; Leigh and Bryant, 2015). 48

Figure 2.9: Haploweb generated using Haplowebmaker showing the connection between Flot 2011 Species A and B compared to the comperable region of the its region (xxxbp) of Clade 2 and Clade 3 of study samples 49

Figure 2.10: A, B, E. "mordax-like" form of Clade 2 found in IWP. C F clade 3 "madagascarensis-like" found in Aliwal Shoal D, G. Flat anastomosing form as clade 3 found in Pondoland. All images taken by members of ORI. 53

Figure 2.11: A model for clade 3 population connectivity based on the pairwise ϕPT values for the ITS marker, adapted using Google maps (Mannheim *et al.*, 2018). 54

Figure 3.1: Graphic representation of the varying total number of read and retained reads per individual accomplished using *process_radtags* in STACKS. 74

Figure 3.2: Selection of assembly parameters in a *de novo* analysis. The M And n parameters are kept equal, and the M parameter is fixed to 3. 75
Figure 3.3: PCA using SNPS. Each point represents an individual colour coded by sampling origin 78
Figure 3.4: Tree of 37 individuals in four populations based on SNPs in >80% samples, computed using dapc analysis in Adegenet. 78
Figure 3.5. Plot of admixture proportions at the population level, at K = 2-5, identified using structure is shown in the top panel. shown is the probability of membership (*Y*-Axis) In a given cluster for each sample (*X*-Axis) assuming values of K = 2, K = 3, K = 4 and K = 5. Analysis of 3622 SNPs (analysis included only one snp per locus) after combining read 1 and read 2. The most probable K was 3 (b. reefs within geographic regions are sorted by latitude. 79
Figure 3.6: Figure shows the Delta k for all values 2 to 5 and shows inflection point at K=4.Plot of Δk values from four runs of STRUCTURE on the dataset, varying K from 1 to 10 are shown in the top panel. The most likely value for K was identified at K = 4. Plot. 80
Figure 3.7: Scatter plot if the distribution of Fst values across individual loci. 82
Figure 3.8: SNP outlier analysis in diversity set using Bayescan. utlier locus analysis, blue vertical line indicates threshold (False discovery rate (FDR) ≤ 0.01). Markers on the right site of the threshold are significant outlier (red dots). 83
Figure 4.1: Sampling locations for this study including the MPAs of the reefs sampled. MPA boundary information was downloaded from https://egis.environment.gov.za/data_egis/, and loaded in Google Earth Pro 93
Figure 4.2: Phylogenetic tree of COI to illustrate the division of the samples collected and also highlight Kosi Bay sample numbers 22, 24 and 32 that are separate to the rest of the collection. 100
Figure 4.3: Haplotype network of COI sequences collected in this study. This indicates similarities between the sampled haplotypes. 101
Figure 4.4 Phylogenetic tree of mtMutS to illustrate the division of the samples collected and also highlight Kosi Bay sample number 22 (italics) and 24 that are separate to the rest of the collection, as well as Leadsman Shoal samples 5 and 14. 103
Figure 4.5: Haplotype network of samples collected in this study. This indicates similarities between the sampled haplotypes. 104
Figure.4.6 Concatenated phylogenetic tree of COI + mtMutS to illustrate the division of the samples collected and also highlight Kosi Bay sample number 22 (italics) movement alongside Leadsman Shoal and 32 that are separate to the rest of the collection. 105
Figure 4.7: Specimen images from each sample location including A representative of each clade from Kosi Bay. A. image of the Kosi Bay clade 1 representative (KB021). B. image of the Kosi Bay clade 2 representative (KB022). C. image of the Nine Mile reef clade 1 representative (NM122). D. image of the Leadsman Shoal clade 1 representative (LS115). E. *S.brassica* representative of Two Mile reef reference from Benayahu *et al*., 1998 (ZMTAU 27832). F. Image of the Two Mile reef clade 1 representative (TM139). 108
Figure 5.1: Blood Reef, Aliwal Shoal and Aliwal Deep in relation to the coastline and within the MPA. The map was generated in Google Maps and MPA zoning generated by SAAMBR. 122
Figure S1: Representative restriction enzyme digestion using *PSTI* and *MSPI*. Run at a 2% gel, 20ul total volume, 3-hour digestion, run for 45 mins at 130v 131
Figure S2: Bioanalyzer results 132

LIST OF TABLES

Table 2.1: Sample sites, their coordinates and depth.....32
Table 2.2: Primers used in study 34
Table 2.3: Number and proportion of individuals from each clade per sampling location and percentage proportion in relation to location. 39
Table 2.4 Comparative morphology of samples collected in their sites and corresponding clade designation.....40
Table 2.5: Estimates of evolutionary divergence over sequence pairs between groups. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). 41
Table 2.6: Nucleotide diversity of *Stylophora pistillata* COI marker using Arlequin.....455
Table 2.7 Nucleotide diversity of *Stylophora pistillata* ITS marker using Arlequin 46
Table 2.8: P-distance between alleles of Clade 3, number of alleles, Indels and SNPs.....47
Table 2.9: Matrix table of pairwise population ϕPT values for COI. Significant values (P < 0.05) are highlighted above the diagonal. 49
Table 2.10: Matrix table of pairwise population ϕPT values for ITS. Significant values (P < 0.05) are highlighted and bolded above the diagonal.....50
Table 2.11: ITS AMOVA results generated using GenALEx ver. 6.41 (Peakall, 2006). The table displays the amount of interpopulation variance explained and (2) the ϕPT value (and associated p) in decreasing order.....51
Table 3.1: Populations comparisons of *de novo*, BWA and Gsnap. 75
Table 3.2 Population summary statistics of *ref_map.pl of Stylophora Pistillata*.....77
Table 3.3: Fst evaluations between parameters of geographical distribution 77
Table 3.4: Summary K and Delta K between 1 To 5. Generated in STRUCTURE 80
Table 3.5: Fst of populations based on genetic population structure and --*write-single-snp* parameter in STACKS 81
Table 3.6: Outlier Loci with SNP data, Stylophora referenced contig GenBank ID, Position of contig in relation to the genome and associated gene to determine selection. 83
Table 4.1 Sample sites and their coordinates.....93
Table 4.2: Primers used in study 96
Table 4.3: Estimates of evolutionary divergence over sequence pairs between groups. evolutionary analyses were conducted in MEGA X (Kumar *Et Al.*, 2018).....101
Table 4.4: Nucleotide diversity of COI using Arlequin. 106
Table 4.5: Nucleotide diversity of mtMutS using Arlequin. 106
Table 4.6: Matrix table of pairwise population ϕPT values for COI. Significant values (p < 0.05) are highlighted. 106
Table 4.7 Matrix table of pairwise population ϕPT values for mtMutS. Significant values (P < 0.05) are highlighted 107
Table S1: Barcode adapters used per individual and the corresponding indexing barcodes.....133
Table S2: Total number of raw and filtered reads per group 134
Table S3: Total number of raw and filtered reads per individual.....135
Table S4: Global statistics of *Stylophora pistillata* genome from GenBank 137
Table S5: GenBank sequences for voucher specimens from previously published authors used in phylogenetic analyses of COI and mtMutS 138
Table S6 Results generated using BarcodingR showing the assigniment of samples to references using bp-based and Fuzzy Method.....139

ABSTRACT

Marine Protected Areas are a valuable tool for ecosystem protection and to enhance resilience in the face of global stressors such as global warming and ocean acidification. There is currently an incomplete understanding regarding the level to which MPAs in KwaZulu-Natal protect existing biodiversity and provide benefits beyond their boundaries. The focus of this study was to determine the extent to which the current MPA network acts to facilitate connectivity of sessile benthic species, the role of oceanographic processes, and whether these processes will persist under global change. The population connectivity of two coral species, a hard coral (Scleractinia), *Stylophora pistillata*, and a soft coral (Alcyonacea), *Sinularia brassica*, within and between the MPA network on the east coast of South Africa was studied using both traditional markers and RADSeq, a reduced representation genomic sequencing technique. Sampling locations were selected in three existing MPAs and on a representative reef located in the "gap" in between. These MPAs span the tropical Delagoa and subtropical Natal Bioregions. *Stylophora pistillata* in South Africa is split into two non-hybridizing clades with clear differences in distribution. Clade 2 was confined to the subtropical Delagoa bioregion whereas clade 3 was found throughout the study region from the tropical Delagoa bioregion down to the southern boundary of the warm temperate Natal bioregion. ITS data indicated that there is a complex population structure of the clade 2 potentially driven by a combination of currents, ecological selection, and distance. A subset of *Stylophora* clade 2 samples was analysed using a RADSeq approach which clarified the structure suggested by the ITS data and clearly identified three distinct populations across four reefs (Leadsman Shoal, Blood Reef, Aliwal Shoal and Aliwal Deep) spanning the Delagoa/Natal biogeographic break. These populations did not appear to be structured solely by geographic distance, with one population comprising samples from two sites (Blood Reef and Aliwal Deep) that were non-adjacent and at different depths (12-18 m vs >30 m), while a geographically adjacent population (Aliwal Shallow) at 12-18 m constituted a distinct population. This suggests that ecological selection might be involved in structuring the population over short distances for this coral. *Sinularia brassica* was not found south of the iSimangaliso Wetland Park in this study, despite it being recorded in the southern sites in previous surveys. Analyses of COI and mtMutS sequences revealed that there are potentially multiple clades present in the IWP population and that there is a poleward decrease in genetic diversity. Neither of these clades showed any clear geographical or genetic population structure between the reef complexes but additional studies using RADSeq may help to clarify the situation.

CHAPTER 1: INTRODUCTION

CORAL REEFS AND THEIR THREATS

The ability of a species to effectively exchange genetic information between populations through exchange of propagules, gametes or adults is critical to the persistence, growth, and resilience of those populations (Sale *et al.*, 2010; Montoya-Maya, Schleyer and Macdonald, 2016). Coral reef species begin populating reefs as larvae and, in doing so; they may utilize the currents of the ocean as vectors. It is possible that the larvae may settle on their parental reef (self-recruitment), move only as far as the neighbouring reefs, or that they may remain viable to settle after dispersing over a large distance. Genetic analysis provides a method to shed some light on these processes by studying the genetic 'footprint' of traveling larvae or migrating adults within a population gene pool. Additionally, it is possible to track the exchange of individuals between populations. Genetic diversity yields an indication of population size and helps identify very reduced, isolated, or vulnerable populations. Such knowledge on dispersal distances and connectivity provides baseline information for conservation efforts and efficient marine protected area (MPA) network design.

Organismal thermal tolerance is a major driver of biogeographic variation in the distribution of plants and animals on land and in the sea (Somero, 2005; Rodríguez *et al.*, 2019), and, as the earth is warming through climate change, these distributions are changing. Range shifts are an example of such change, and they occur through persistent extensions of organismal range in some areas. However, there are also contractions occurring within historical range limits in some locations as well (Green *et al.*, 2015; Rodríguez *et al.*, 2019).

Coral reefs can be found in both tropical and subtropical oceans (Hallock and Schlager, 1986). Threats and stress to coral reefs will result in varying degrees of damage (Obura, 2005; Schleyer, Kruger and Celliers, 2008; Mc Clanahan, 2014; Baums *et al.*, 2019; Robinson *et al.*, 2019). The need for a strategic approach to reef conservation and restoration to maximize the effectiveness of Marine Protected Areas (MPAs) has become an urgent issue, particularly due to climate change that has led to previously suitable habitats becoming inhospitable (Baker, Glynn and Riegl, 2008; Beheregaray, 2008; Griffiths *et al.*, 2010; Andras, Rypien and Harvell, 2013; Le Saout *et al.*, 2013; Richards and Day, 2018; Kuempel *et al.*, 2019). A combination of effective management and reef restoration techniques, where required, should be implemented to ensure the protection of coral reef ecosystems (Chavanich and Viyakarn, 2016).

The increase in ocean temperatures and in the duration and severity of marine increasing sea surface temperatures (SST) has resulted in coral reefs being increasingly threatened by a condition known as bleaching. This is referred to as thermally induced bleaching which is caused by a disassociation between coral hosts and symbionts (Edmunds, 1994; Van Oppen and Gates, 2006; Arif *et al.*, 2014; Moustafa *et al.*, 2014; Ferrari *et al.*, 2016; Parrin *et al.*, 2016; Muller, Bartels and Baums, 2018). Under stress, symbiotic relationship between coral hosts and symbiont breaks down and the symbionts may be expelled from the coral tissue (Schleyer, Kruger and Celliers, 2008; Ferrari *et al.*, 2016). This causes the coral host to become pale or, if more severe, bleached. The primary cause of mass bleaching in recent times is thermal stress, potentially exacerbated by other local stressors (Parrin *et al.*, 2016).

As with tropical corals, high-latitude corals suffer coral bleaching under thermal conditions that exceed long-term local averages (Cook *et al.*, 1990; Celliers and Schleyer, 2002; Dalton and Carroll, 2011; Hughes *et al.*, 2017; Kim *et al.*, 2019). The effects of bleaching in the tropics have been well-documented over the past three decades, and recent global warming-driven increases in the frequency of mass bleaching events have caused large-scale mortality among corals on tropical reefs (Hughes *et al.*, 2017). While high-latitude coral assemblages along the coasts of Australia, the Atlantic, Japan and South Africa have also experienced varying degrees of bleaching in recent years, the overall frequency of mass bleaching events has been lower at high latitude than in the tropics (Kim *et al.*, 2019). As with tropical reefs (Hughes, Kerry, et al., 2018), bleaching at high latitudes is characterised by taxonomic differences in bleaching susceptibility and mortality, which can lead to changes in community structure (Floros, Samways and Armstrong, 2004; Dalton and Carroll, 2011; Kim *et al.*, 2019). Unfortunately, high-latitude regions are predicted to experience greater heat stress than the tropics in the coming decades.(Kim *et al.*, 2019)

CORAL REEFS IN SOUTH AFRICA

Biogeographic patterns are determined by past and present-day ocean currents and dispersal barriers that define connectivity between sites, along with historical patterns of speciation, extinction, and immigration. The major oceanographic features in the Southwest Indian Ocean are the South Equatorial Current (SEC), Mozambique Channel Eddies (MCE), East Madagascar Current (EMC) and the Agulhas Current (AC) systems. The South Equatorial Current bifurcates and feeds both the MCE and the East African Coastal Current (EACC), which splits both south and north (Figure 1.1). The poleward Agulhas Current appears to originate between 25° and 30° South where waters from the MCE and the EMC meet (Lutjeharms, 2006; Lutjeharms *et al.*, 2012). The current speeds in this region vary, yet research suggests that it has a high potential for larval dispersal and reef connectivity (Bryden et al. 2005). Therefore, it is possible that northern

South African reefs may be acting as "landing-sites or sinks" for larvae that are transported from other locations by the MCE and EMC systems (Bryden et al. 2005; Roberts 2006; Morris 2009) before they radiate to the rest of the south-east African reefs. Figure 1.1 illustrates the flow of these currents relative to the eastern coastline of South Africa and the potential movement of larvae in a poleward direction. These reefs flourish primarily due to the effects of the warm Agulhas Current, comprising a mix of Subtropical Surface Water (STSW) and Tropical Surface Water (TSW) (temperatures range between 20 °C to 25 °C) coming from the currents previously mentioned. Because of this, reefs can flourish at depths beyond 30 m (Maggs, 2011).

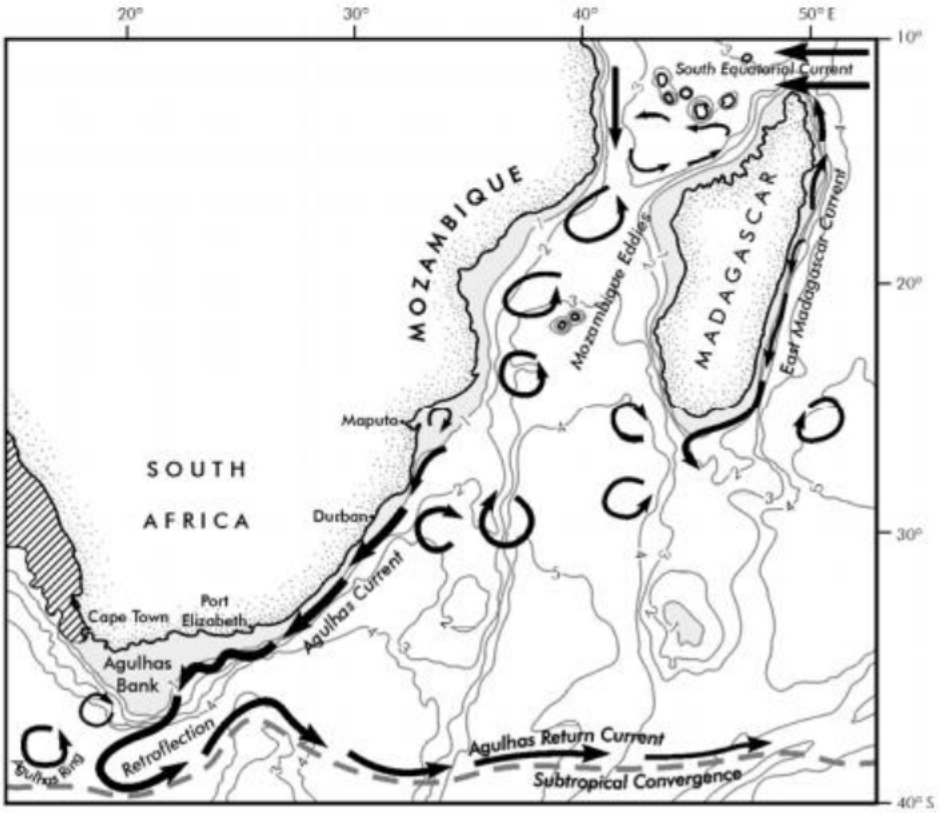

Figure 1. 1: The circulation of the Agulhas Current showing its features (Duncombe Rae, 1991; Lutjeharms, 2006, 2007; Lutjeharms et al., 2012).

Environmental conditions and species assemblages along the South African coastline change considerably from east to west. Most authors have accepted the division of the coast into four major, temperature-defined, coastal marine biogeographical regions, including the cool-temperate Namaqua bioregion in the west (upwelling-dominated system), the (nutrient-poor) warm-temperate Agulhas Bioregion in the south, the sub-tropical Natal Bioregion in the east, and the tropical Delagoa Region above (Wright *et al.*, 2015; Jooste *et al.*, 2018). The identification of unique marine biogeographical regions (Figure 1.2) has shaped our understanding of biodiversity

as we know it (Griffiths *et al.*, 2010; Beger *et al.*, 2014; Wright *et al.*, 2015; Jooste *et al.*, 2018). Within this study, we wish to examine if adaptation and change are present in the reef systems across two phylogeographic breaks. The first break occurs in the north-eastern part of the country near Saint Lucia, between the tropical and sub-tropical lineages (Teske *et al.*, 2008, 2019; Jooste *et al.*, 2018). The second is in the Wild Coast region (shown as Agulhas Bioregion in Figure 1.2), between the subtropical and warm-temperate provinces (Teske *et al.*, 2008; Jooste *et al.*, 2018).

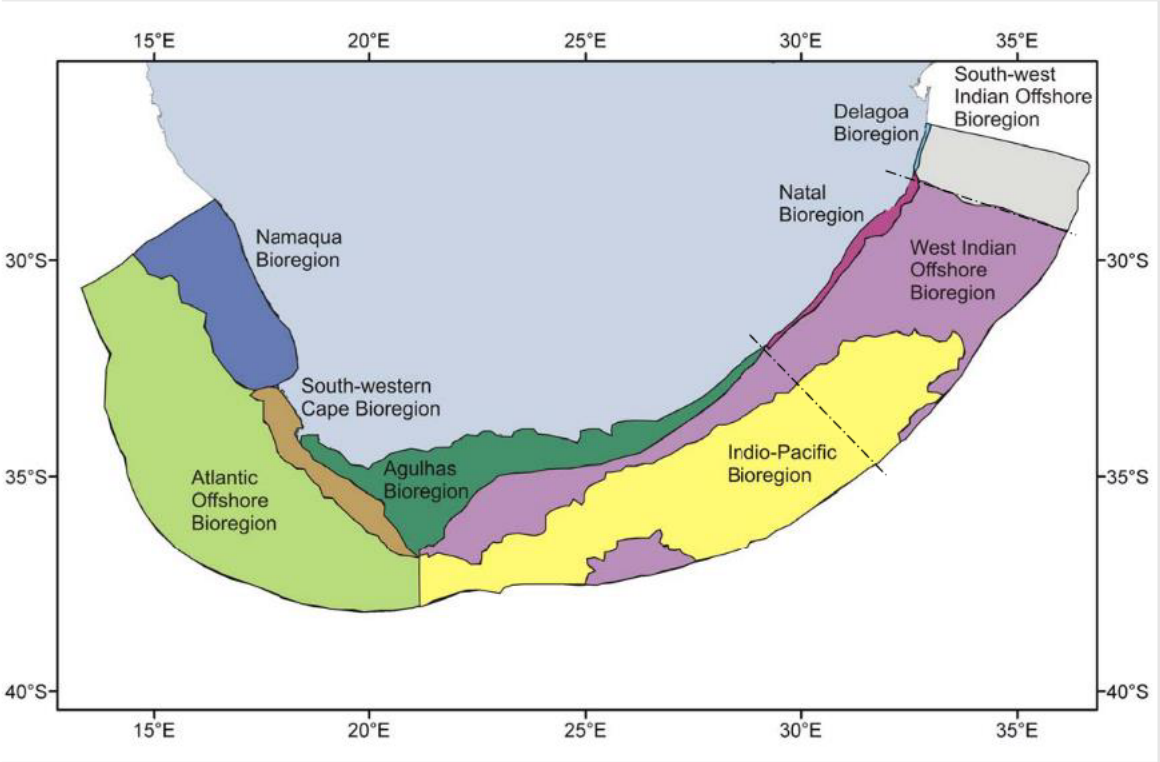

Figure 1.2: The nine bioregions of Southern Africa as described by Lombard et al. (2004). (From Griffiths *et al.*, 2010)

In South Africa, corals are found in shallow water along the east coast from the Mozambique border down to the Wild Coast of the Eastern Cape (Schleyer and Celliers, 2003; Schleyer *et al.*, 2018), although their biodiversity decreases from the warm subtropical regions in the north to the colder temperate waters further south.

MARINE PROTECTED AREAS

Marine Protected Areas (MPAs), as defined by the IUCN, are 'a clearly defined geographical space, recognised, dedicated, and managed, through legal or other effective means, to achieve the long-term conservation of nature with associated ecosystem services and cultural values' (Day *et al.*, 2012; Findlay, 2020). MPAs are an important management tool to protect critical habitats and preserve their ecosystem services. In the case of coral reefs, such services might include acting as a barrier to wave action, as a source of sediment (Schleyer *et al.*, 2018; Dames *et al.*, 2020), or

creating spatial havens for marine life replenishment and improved fishery management (Soulé, 1991). MPAs can, for example, increase the diversity, density, biomass, body size and reproductive potential of coral reef fishes, and provide conservation benefits for fisheries (Soulé, 1991; Attwood *et al.*, 1997; Almany *et al.*, 2009; Green *et al.*, 2015; Dames *et al.*, 2020).

A protected area should include resilient source populations that can ‘‘rescue’’ other populations in cases where they are reduced or eliminated. Protecting this connectivity should be considered in MPA network design (Green *et al.*, 2015; Bhattacharya *et al.*, 2016; Porter and Schleyer, 2017; Schleyer *et al.*, 2018). Populations benefit greatly when they receive genetic input from multiple populations as this provides an influx of genes that reduces gene fixing. Such populations have the genetic potential to respond more robustly to environmental fluctuations and better tolerate environmental pressures, compared to island populations that have low connectivity (Griffiths *et al.*, 2010; Rodríguez *et al.*, 2019). These parameters must be considered in MPA design.

STUDY LOCATIONS

There are two bioregions along the South African East Coast that serve as biogeographical breaks within the study area, and three MPAs are under consideration within this area. The first is the iSimangaliso Wetland Park (IWP) that is located on the east coast of KwaZulu-Natal, stretching from the Mozambique border to Cape St. Lucia, 150 km. It includes the conjoined Maputaland MPA (established in 1986) and St. Lucia MPA (established in 1979) (Dames *et al.*, 2020) both having marginal, high-latitude coral reefs with a rich Scleractinian and Alcyonacean fauna (Schleyer and Celliers, 2003, 2005; Schleyer and Benayahu, 2008; Schleyer *et al.*, 2018). The Agulhas Current, which flows in a south-westerly direction, is a powerful western boundary current, which generates sub-tropical conditions along this coastline (Lutjeharms, 2006; Schleyer *et al.*, 2018). The average seawater temperatures range between 22 °C and 26 °C, with a lack of significant riverine input and low levels of turbidity and nutrient input (Porter and Schleyer, 2017; Schleyer *et al.*, 2018).

Aliwal Shoal is an MPA located five km offshore from the Umkomaas River Mouth and extends 18.3 km along the coast between the Mzimayi and Umkomaas River mouths, and seven km out to sea. Aliwal Shoal is host to a mixture of tropical and subtropical species and it lies between the tropical/subtropical IWP and the Pondoland MPA to the south of Aliwal Shoal. The reef possesses a few zooxanthellae corals in turbid waters and is characterised by low light and cool temperatures with a strong riverine input (Olbers, Celliers and Schleyer, 2009).

The Pondoland MPA was declared a protected area in 2004 and is characterized by submerged palaeo-dune cordons forming reef complexes with few corals. It extends from the Mtamvuna River in the North (KwaZulu-Natal) to the Mbashe River in the south (Eastern Cape), with reefs

ranging in width of 5-50 km; it has a strong riverine input (Lombard *et al.*, 2004; Celliers *et al.*, 2007). The Pondoland MPA forms a unique transition zone with elements from subtropical and warm temperate ecosystems, between the Natal and Agulhas Biogeographic regions, with reefs reaching a depth beyond 30 m. During periods of heavy rain, the exposed coastline experiences silt-laden river flows into the sea and reduced visibility underwater (Jooste *et al.*, 2018).

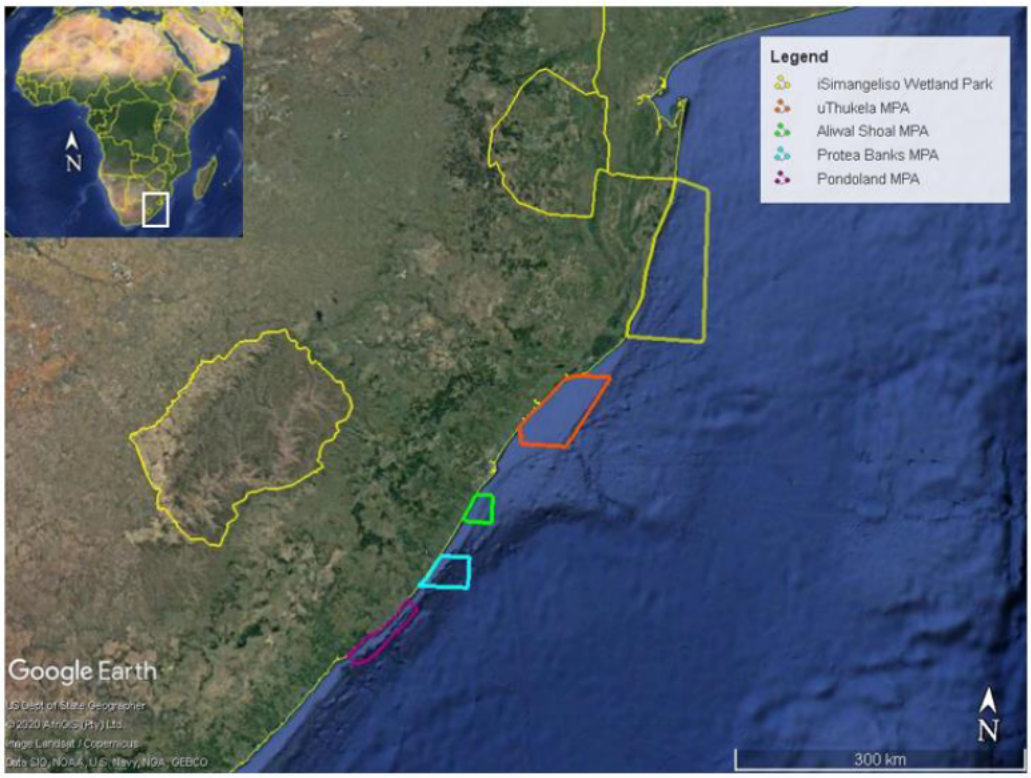

Figure 1.3: Marine Protected Areas (MPAs) in the study region along the coastline of South Africa. Image created using Google Earth

GENETIC CONNECTIVITY

The genetic make-up of organisms determines, in part, their vulnerability or resilience to global change by bounding their behavioural traits, physiological climate tolerance, evolutionary potential, and dispersal ability (Baker, Glynn and Riegl, 2008; Beger *et al.*, 2014; Mc Clanahan, 2014). Furthermore, observed genetic diversity can provide information about a species' long-term persistence and may indicate adaptive potential or evolutionary resilience (Beger *et al.*, 2014; Richards and Day, 2018; Sadek *et al.*, 2018). Genetic methods can provide information on these traits, which is otherwise not available in the conservation decision-making toolbox (Beger *et al.*, 2014; Hughes *et al.*, 2017), as well as establishing connectivity patterns.

Connectivity refers to the movement of individual genotypes between populations; for sessile organisms in the sea, this occurs primarily through dispersal of planktonic larvae or propagules (Wright *et al.*, 2015). Coral reef seascapes are inherently patchy and fragmented, consisting of

spatially distinct subpopulations connected to an unknown degree and distance by larval dispersal (Almany *et al.*, 2009). Ocean currents can carry larvae long distances and assist in overcoming physical barriers to increase range and achieve colonization (Caley *et al.*, 1996; McKibbin *et al.*, 2010; Eble *et al.*, 2011; Wright *et al.*, 2015). Designing reserve networks (e.g., reserve location, size and spacing) that protect connectivity requires an understanding of larval dispersal. However, many existing MPA networks have not incorporated empirical estimates of larval dispersal in their design (Green *et al.*, 2015) and may, therefore, fail to protect connectivity, ensure population persistence, or protect biodiversity. Given high variability in dispersal distances, connectivity should be maintained over a broad range of between-reserve distances. Conservatively, between reserve distances of 50 km (Griffiths *et al.*, 2010; Mead *et al.*, 2011; Bhattacharya *et al.*, 2016) should ensure sufficient demographic connectivity for most species.

CORAL RECRUITMENT

Recent research has revealed a trend of decreasing coral recruitment in low-latitude regions and an increase on high-latitude reefs (Maida, Coll and Sammarco, 1994; Green *et al.*, 2015; Porter and Schleyer, 2017). In sessile marine organisms such as corals, temperature-mediated changes in distribution are difficult to detect. These difficulties arise from the lag in time between the dispersion of larval stages and the time needed for them to mature to adulthood (Price *et al.*, 2019). Sexually produced larvae are also the only means by which corals can become re-established where adult populations have been driven to local extirpation or become established in novel biogeographic locations where they historically did not exist (Ritson-Williams *et al.*, 2009; Price *et al.*, 2019).

The introduction of new, sexually produced individuals to a population will contribute towards community dynamics. Coral species have a pelagic larval phase, which is vital to their reproductive success, dispersal, and recruitment. There are two main forms of sexual reproduction in sessile marine life such as corals: brooding and broadcast spawning (Bujang, Harrison and Su, 2011). Correct timing and variations in environmental conditions can produce large variations in dispersal and recruitment and influence the connectivity between populations in different localities (Fan *et al.*, 2006).

Broadcast spawning occurs as individual coral polyps release gametes into the water column with fertilization occurring externally (Hoeksema, 2015; Goodbody-Gringley and de Putron, 2016). These develop into planula larvae that take several days to weeks to find a suitable substratum for settlement and to metamorphose into primary coral polyps. Broadcasters typically undergo one or two gametogenic cycles each year, producing many gametes (Rinkevich and Loya, 1979; Chavanich and Viyakarn, 2016). In brooding corals, fertilization is internal and space-

constrained, and brooding corals allocate energy to fewer offspring in multiple reproductive cycles per year (Ayre and Hughes, 2004; Glassom, Celliers and Schleyer, 2006; Wright *et al.*, 2015; Goodbody-Gringley and de Putron, 2016). In addition, when the planulae are released, they already contain maternal *Symbiodinium*, therefore constraining their symbiont cladal complement (Harii *et al.*, 2009).

STUDY SPECIES

Stylophora pistillata (Scleractinia) and *Sinularia brassica* (Alcyonacea) are both brooding spawning corals (Rinkevich and Loya, 1979; McFadden *et al.*, 2014) that are widely distributed in the research area (Ofwegen, 2000; McFadden *et al.*, 2009; Arrigoni *et al.*, 2016), and were chosen as subjects for this study. The first is well studied; the second less so; both have a wide Indo-Pacific distribution. *Stylophora pistillata* constitutes a species complex (near threatened. Hoeksema, 2015) that is found along the east coast of KwaZulu-Natal from Kosi Bay in the iSimangaliso to Pondoland in the Eastern Cape, in a range of depths, and it has a highly plastic morphology (Meroz *et al.*, 2002; Zvuloni *et al.*, 2008; Olbers, Celliers and Schleyer, 2009; Flot *et al.*, 2011; Arrigoni *et al.*, 2016).

The soft coral *S. brassica* is consistently found on all east coast reefs, although its abundance diminishes further south (Schleyer and Celliers, 2003). This species is typically found on reef flats and shallow slopes but has a high dominance across a wide depth gradient (Shoham and Benayahu, 2017; Quattrini *et al.*, 2019). It demonstrates morphological variability and is a suitable candidate for connectivity studies (Shaish, Abelson and Rinkevich, 2007; Flot *et al.*, 2011; Keshavmurthy *et al.*, 2013). The genus *Sinularia* is considered highly diverse and there is a limited understanding of how many species currently exist and how they are distributed. *Sinularia* is the dominant alcyonacean genus along the East African Coast with 15 currently recognised species (Benayahu, 1998; Pena-Rodríguez, 2015).

GENETIC BACKGROUND

Fundamental research has been undertaken on the reefs of KZN in a number of disciplines, such as the local oceanography (Morris, 2009; Painter, 2020), and coral systematics, distribution, reproduction, settlement and diseases (Riegl *et al.*, 1995; Van Ofwegen and Schleyer, 1997; Benayahu, 1998; Kruger, Schleyer and Benayahu, 1998; Schleyer and Celliers, 2003; Glassom, Celliers and Schleyer, 2006; Séré *et al.*, 2015; Porter and Schleyer, 2017).Previous studies on coral genetics have been undertaken in a number of genera, but most species have yet to be looked at in detail using modern genetic methods (Montoya-Maya, 2014; Montoya-Maya, Schleyer and Macdonald, 2016; Chiazzari *et al.*, 2019).

The above research has yielded background and baseline information on the reefs, their extent and condition. (Schleyer and Porter, 2018). Global genetic surveys on *Stylophora* have revealed cryptic diversity in both sympatric and allopatric populations throughout their distribution (Arrigoni *et al.*, 2016). Field identification is difficult due to their phenotypic plasticity, with environmental factors affecting the shape of coral colonies. In consequence, there is considerable intraspecific morphological variation (Hofmann *et al.*, 2010; Keshavmurthy *et al.*, 2013; Sawall *et al.*, 2015; Arrigoni *et al.*, 2016). Previous genetic diversity studies based on mitochondrial and nuclear loci have indicated the presence of cryptic divergence and four evolutionary lineages within the global range of *Stylophora pistillata* (Flot *et al.*, 2011; Keshavmurthy *et al.*, 2013; Arrigoni *et al.*, 2016; Voolstra *et al.*, 2017).

There is a great interest in elucidating the ecology and function of *Sinularia* spp. on reefs that are susceptible to bleaching-induced mortality (Bruno *et al.*, 2001; Quattrini *et al.*, 2019). However, studies have been hampered by the uncertainty of species identification based on morphology, and the difficulty in obtaining high quality DNA hence barcoding approaches have only been partially successful (McFadden *et al.*, 2009; Quattrini *et al.*, 2019). Additionally, information yielded by these studies has revealed that some well-known morphotypes actually constitute cryptic species-complexes (van Ofwegen, Benayahu and Mcfadden, 2013; Van Ofwegen, McFadden and Benayahu, 2016; Quattrini *et al.*, 2019).

Rates of octocoral mitochondrial gene evolution are slow compared to other animals (McFadden *et al.*, 2011). Mitochondrial genes are informative for genus and family level phylogenetic analyses (Ayre and Hughes, 2004; McFadden *et al.*, 2006; Bhattacharya *et al.*, 2016), but lack sufficient resolution to discriminate species within many genera (Ayre and Hughes, 2004; Bhattacharya *et al.*, 2016; Goodbody-Gringley and de Putron, 2016).

Recent systematic revision of this speciose genus revealed additional diagnostic characters that exhibited congruence with the phylogeny of the mitochondrial bacterial MutS homologue found exclusively in octocorals (Pont-Kingdon *et al.*, 1998, France and Hoover 2002 McFadden *et al.*, 2014). It has been widely applied in systematic and phylogenetic studies providing substantial taxonomic insight (McFadden, France, *et al*., 2006, McFadden *et al*., 2009, McFadden & van Ofwegen 2012, Reijnen *et al*., 2014, Bryce *et al*., 2015, Etsabeth, 2018). Some studies have been conducted on *S. brassica* along the KZN coastline, but these have not fully resolved the taxonomic status of the genus, nor have they looked at population structure (Benayahu, 1998; Ofwegen, 2000; Schleyer and Celliers, 2003; Olbers, Celliers and Schleyer, 2009; McFadden *et al.*, 2014; Pena-Rodríguez, 2015; Schleyer and Benayahu, 2018; Etsabeth, 2018).

Early approaches for studying coral species determination have included DNA barcoding for analyses of mitochondrial and nuclear phylogenetic trees (Keshavmurthy *et al.*, 2013; Arrigoni *et al.*, 2016), while more recent methods include haplowebs (Flot, Couloux and Tillier, 2010; Keshavmurthy *et al.*, 2013; Terraneo *et al.*, 2016), and next-generation sequencing (NGS). The application of these techniques, known as DNA taxonomy (Tautz *et al.*, 2003; Terraneo *et al.*, 2016), has proven to be fast, objective, and rigorous in the assessment of biodiversity (Flot *et al.*, 2011; Ferrari *et al.*, 2016; Terraneo *et al.*, 2016) although it is not without pitfalls. It is essential to integrate morphological analyses to ensure more accurate species delimitation (Schlick-Steiner *et al.*, 2010), particularly when dealing with corals where large population sizes and dispersal abilities increase the likelihood of incomplete lineage sorting (Pinzón and Lajeunesse, 2011; Terraneo *et al.*, 2016; Voolstra *et al.*, 2017; Chiazzari *et al.*, 2019). On the one hand, the accuracy of genetic identification depends on the availability of fixed species-specific mutations within the analysed DNA markers. On the other hand, these molecular data should always be coupled with information from other fields, such as morphology, ecology, and reproduction, with the aim of a correct and broad formulation of species boundaries (Stapley *et al.*, 2010; Montoya-Maya, Schleyer and Macdonald, 2016; Terraneo *et al.*, 2016; Jooste *et al.*, 2018)

Previous studies have emphasized the use of multiple lines of genetic evidence to reduce the effects of limitations such as introgression, incomplete lineage sorting, retained ancestral polymorphisms, pseudogenes, or intragenomic variations (Flot, 2010; Arrigoni *et al.*, 2016; Terraneo *et al.*, 2016). Restriction-site associated DNA sequencing (RADSeq) is becoming widely used in ecological, evolutionary and conservation genomics (Reitzel *et al.*, 2013). This approach provides a large amount of information at a fraction of the cost compared to other next-generation or conventional sequencing techniques (Keshavmurthy *et al.*, 2013; Voolstra *et al.*, 2015; Bhattacharya *et al.*, 2016).

In this project, we used RADSeq to target a subset of the genome for *S. pistillata* in a single, simple, cost-effective manner to compliment traditional barcoding techniques. We used a double-digest RADSeq methodology that employs two restriction enzymes: one rare cutter and one more frequent cutter, with adapters specific to each enzyme, which will tailor the number of loci produced (Kess *et al.*, 2016; Toonen *et al.*, 2013; Schweyen, Rozenberg and Leese, 2014). The designed adapters bind to characteristic single-stranded sticky-ends that remain at restriction cut sites after digestion to ensure that only fragments with the adapter combinations that are required for sequencing are PCR-amplified. This ensures that the data set produced is comparable between the samples, thus reducing the chance of different sets of loci appearing in different libraries (Bhattacharya *et al.*, 2016). The protocol requires high molecular weight DNA to ensure that the

loci are intact in all the samples after enzyme digestion (Bracken-Grissom *et al.*, 2014; Andrews *et al.*, 2016). It is also necessary to minimise any contaminants, such as symbiont DNA, to maximise the signal from the coral DNA.

KEY QUESTIONS AND RESEARCH PLAN

Scleractinian corals are the primary building blocks of coral reefs and hence form the basis of one of the most biodiverse ecosystems in the world. They harbour a significant fraction of global marine species, up to 25% by some estimates (Plalumbie, 2003; Takabayashi *et al.*, 2003; Macdonald, Schleyer and Lamb, 2008; Meyer and Holland, 2008; Sale *et al.*, 2010; Obura, 2012; Keshavmurthy *et al.*, 2013; Goffredo and Dubinsky, 2016; Porter and Schleyer, 2017; Richards and Day, 2018). Changes in climate (Ayre and Hughes, 2004; Drury, 2020), overfishing (Kennedy *et al.*, 2013; Sumaila and Tai, 2019), development (Griffiths *et al.*, 2010) and pollution (Kennedy *et al.*, 2013; Schleyer *et al.*, 2018) have impacted reefs and compromised the existing biodiversity and structure among established populations (Baird, Guest and Willis, 2009; Hofmann *et al.*, 2010; Hoadley *et al.*, 2015; Porter and Schleyer, 2017; Drury, 2020). Ocean warming due to climate change is predicted to lead to a significant loss of corals in lower latitudes as the temperatures exceed their range of thermal tolerance. Conversely, increasing temperatures at higher latitudes might open new habitat for corals and facilitate poleward range expansion (Takabayashi *et al.*, 2003; Zvuloni *et al.*, 2008; Andras, Rypien and Harvell, 2013; Couce, Ridgwell and Hendy, 2013; Van Oppen *et al.*, 2015; Porter and Schleyer, 2017).

Preliminary studies conducted on *Stylophora pistillata* has shown that, while it displays considerable morphological variability, it is divided into only two clades in South Africa. The alcyonacean soft coral *Sinularia brassica* is consistently found on all east coast reefs in South Africa, although its abundance diminishes further south. It too demonstrates morphological variability and is a suitable candidate for connectivity studies. Despite being widely distributed in the Indo-West Pacific region, nothing has yet been done on the population genetics of these species in South Africa and work on their connectivity will break new ground and reveal the level to which these species may or may not be polyphyletic.

In summary, the dual aims of this study were to determine the connectivity of:

- *Stylophora pistillata* along the KZN coastline using traditional genetics methods and next-generation sequencing, examining the connectivity between MPAs as well as gaps in between.
- Similarly, for *Sinularia brassica* along the KZN coastline.

Key questions for this thesis included:

a. What is the extent of genetic connectivity in *S. pistillata* and *S. brassica* populations along the east coast of South Africa and is the level of connectivity ecologically relevant?
b. Does the existing MPA network adequately cover the population diversity of these corals?
c. Is there any evidence of adaptation within populations of *S. pistillata* and *S. brassica* exposed to different environmental stressors, such as light, temperature, depth, or turbidity?
d. Can we develop an in-house RADSeq protocol that could be used to economically study multiple non-model species or groups?

Based on these questions we propose the following hypotheses.

The null hypothesis: H_0 There is no significant ecological connectivity of these species between MPAs. There are no migrants exchanged between populations, and they are completely independent from each other, comprising isolated populations on the east coast of southern Africa.

H1 There is ecological connectivity between all three MPAs on the Kwa Zulu-Natal coast. There is an exchange of individuals that can reach reproductive age and contribute to the local gene pool, to a point that there is no clear genetic differentiation. The direction of population movement is from north to south, due to the prevailing current regime.

H2 Population structure is present and restricted to the biogeographical regions, one population being found in the IWP and the other further south due to the biogeographic break between the subtropical and temperate regions.

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CHAPTER 2: GENETIC DIVERSITY AND BIOGEOGRAPHIC DISTRIBUTION OF *STYLOPHORA PISTILLATA* IN KWA ZULU-NATAL

ABSTRACT

This study aimed to examine the population connectivity of a coral species, within and between east coast reefs in the newly expanded MPA network. The hard coral (Scleractinia), *Stylophora pistillata*, was chosen for this study. Sampling locations were in existing MPAs (iSimangaliso, Aliwal Shoal, and Pondoland) and a representative site (Blood Reef) located in between. Two distinct clades were identified on the KZN coastline with one clade being restricted to the Delagoa Bioregion. The morphotypes of both clades within the Delagoa Bioregion resembled one another but differed from the morphotypes found in the Natal Bioregion, even with the same clade, likely due to environmental pressures. The ITS marker showed that Clade 3 individuals in the Natal bioregion were similar to the previously described variation in *Stylophora madagascarensis* with some small digitate individuals but also flattened morphotypes in sites where there is limited light penetration. Mitochondrial and nuclear markers showed evidence of population structure which was structured by both ecological and biophysical factors. Ecological factors included biogeographic breaks, with distinct populations in the tropical Delagoa and subtropical Natal bioregions. Further structure was evident within the Natal bioregion where analysis showed that the Blood Reef and Aliwal Deep populations were most genetically similar despite not being the geographically closest populations, suggesting that environmental factors, rather than distance limited dispersal, might be responsible for some of the genetic structure in this region. Due to this, populations did not manifest a classical stepping-stone pattern driven by the prevailing southward flowing Agulhas current. Despite this, all populations are represented within the expanded MPAs which therefore currently offers adequate protection to existing genetic diversity and provides a clear path for genotypes to migrate along the coastline.

INTRODUCTION

Species of reef-building corals typically have very broad distribution ranges (Hudson, Slatkin and Maddison, 1992; Report, 2000; Meroz *et al.*, 2002; Takabayashi *et al.*, 2003; Glassom, Celliers and Schleyer, 2006; Macdonald, Schleyer and Lamb, 2008; Floros, 2010; Carpenter *et al.*, 2011; Flot *et al.*, 2011; Andras, Rypien and Harvell, 2013; Keshavmurthy *et al.*, 2013; McFadden *et al.*, 2014; Voolstra *et al.*, 2015; Hoadley *et al.*, 2015; Montoya-Maya, Schleyer and Macdonald, 2016; Porter and Schleyer, 2017; Olguín-López *et al.*, 2018; Schleyer *et al.*, 2018; Rodríguez *et al.*, 2019; Kirkman *et al.*, 2019). Environmental conditions and marine species assemblages change considerably from east to west over the entire South African coastline. Most authors have accepted four major temperature defined coastal marine biogeographical regions, including the cool-temperature Namaqua Bioregion in the west (upwelling dominated system), the (nutrient-

poor) warm-temperate Agulhas bioregion in the south, the sub-tropical Natal Bioregion with an average SST at 23.5 °C (Roberson *et al.*, 2017) in the east, and the tropical Delagoa Bioregion above with an average SST of 24.1 °C (Wright *et al.*, 2015; Roberson *et al.*, 2017; Jooste *et al.*, 2018). The transition from the tropical Delagoa Bioregion to the sub-tropical Natal Bioregion has a relatively poorly defined and porous biogeographical barrier (Griffiths *et al.*, 2010; Bhattacharya *et al.*, 2016). The identification of unique marine biogeographical regions (Figure 1.1) is important in that these current-dominated systems have helped to shape present biodiversity as we know it and have also affected the genetic structure of marine species (Griffiths *et al.*, 2010; Beger *et al.*, 2014; Wright *et al.*, 2015; Jooste *et al.*, 2018). Each bioregion may harbour endemic species or populations, which might require individual management within the bioregions in which they occur. There is considerable risk of overexploiting these locally adapted populations or cryptic species, particularly if they are not recognised to be unique populations. With the warming of the oceans, species distributional changes are expected because of increasing temperatures (Kennedy *et al.*, 2013; Hughes *et al.*, 2017; Porter and Schleyer, 2017; Jooste *et al.*, 2018). Tropical, warm water-adapted species are predicted to gradually appear in warming waters, and more temperate species be displaced southwards (Floros *et al.*, 2012).

Stylophora pistillata (Esper, 2016) is a member of the robust clade (Pocilloporid group) coral species that is widely distributed throughout the Indo-Pacific region and is prevalent throughout the West Indian Ocean (WIO) (Glassom, Celliers and Schleyer, 2006; Sunde and Isaacs, 2008; Flot *et al.*, 2011; Arrigoni *et al.*, 2016). Many studies have been conducted on this species due to its ubiquity in different environments and its molecular and morphological plasticity (Gattuso, Pichon and Jaubert, 1991; Ayre and Hughes, 2000; Takabayashi *et al.*, 2003; Shaish, Abelson and Rinkevich, 2007; Zvuloni *et al.*, 2008; Flot *et al.*, 2011; Stefani *et al.*, 2011; Keshavmurthy *et al.*, 2013; Arrigoni *et al.*, 2016; Voolstra *et al.*, 2017). Traditional coral taxonomy has been based on skeletal morphology but recent advances in genetics which have allowed more detailed examination of traditional phylogenies and species have demonstrated that many of the established phylogenetic relationships and accepted species are, in fact, not supported by genetic evidence (Combosch and Vollmer, 2015; Quattrini *et al.*, 2019). Recent work by Kevashamurthy *et al.* (2013) and Flot *et al.* (2011) based on multiple genetic markers has started to resolve the conundrum and suggests the presence of more than one species in the *S. pistillata* complex. The map in Figure 2.1 was adapted from Keshavmurthy *et al.* (2013) and illustrates the distribution of the four clades that they identified using mitochondrial genetic markers (Keshavmurthy *et al.*, 2013). The WIO region harbours three of the four currently identified clades of *S. pistillata* and appears to be the centre of *Stylophora* diversity (Flot *et al.*, 2011; Keshavmurthy *et al.*, 2013; Arrigoni *et al.*, 2016; Voolstra *et al.*, 2017). These distribution maps show clades 2 and 3 co-

occurring in South Africa, with the distributions of both extending deep into the sub-tropical regions (Glassom, Celliers and Schleyer, 2006; Griffiths *et al.*, 2010; Obura, 2012; Bhattacharya *et al.*, 2016; Porter, Branch and Sink, 2017). Flot and his co-authors (2011) detailed how the genus *Stylophora* also displayed species boundaries, which are not congruent with morphological traits. They determined that the probable cause for this is the western Indian Ocean is the hub for *Stylophora*'s molecular phylogenies and in doing so, displays a higher biodiversity than in the "Coral Triangle" (Flot *et al.*, 2011).

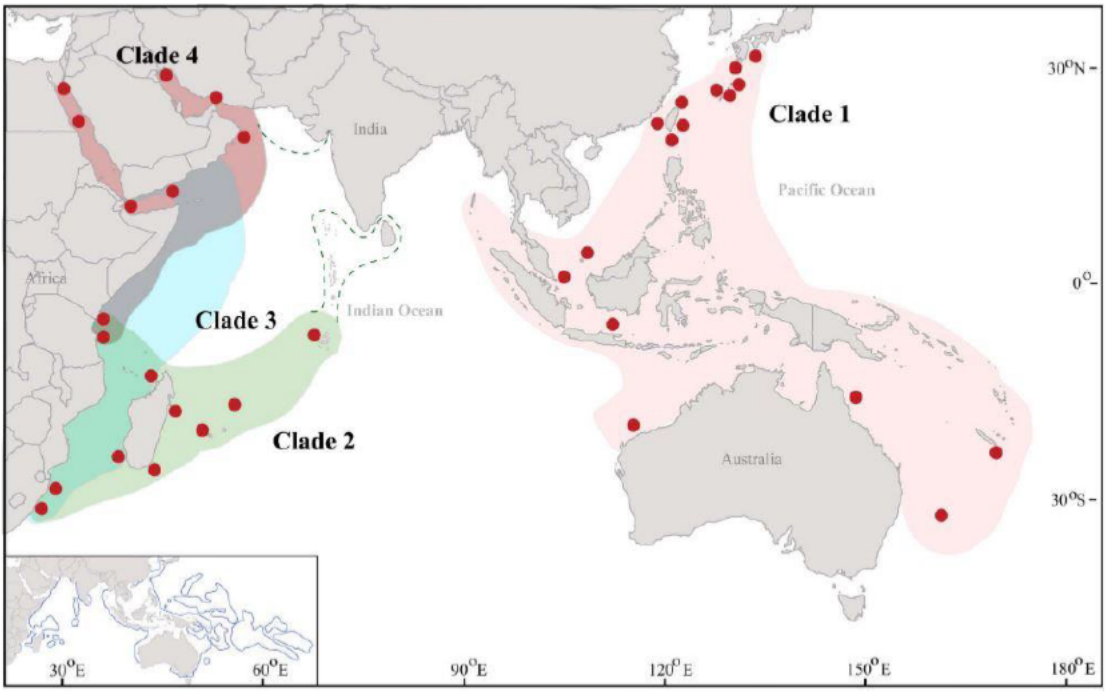


Figure 2.1: Map showing the locations from which *Stylophora pistillata* samples were obtained by Keshavmurthy, *et al.* (2013) (shown as red dots). The division of four regions based on the four clades of *Stylophora* is marked in colours. Clade 1 - Pacific-Western Australia clade (pink), clade 2 - Chagos-Madagascar-South Africa Clade (green), Clade 3 – Gulf of Aden-Zanzibar-Madagascar Clade (blue) and Clade 4 - Red Sea-Persian/Arabian Gulf-Kenya Clade (brown). Adapted from Keshavmurthy *et al.* (2013).

It is important to note the difficulty of definitively identifying species underwater based on morphology (Flot *et al.*, 2011). Multiple morphotypes have been discovered with no indication of interspecific hybridization. Coupling genetic connectivity studies with oceanographic models can help reveal barriers to larval dispersal and reveal source (upstream) versus sink (downstream) population dynamics that will be helpful in establishing functional networks of marine protected areas (Carpenter *et al.*, 2011; Keshavmurthy *et al.*, 2013). The knowledge and understanding of the extent of larval connectivity and gene flow between neighbouring and distant populations is important. It gives an understanding of how coral reefs might fare under increasing levels of

disturbance and will be critical to design and implement reef conservation strategies (Takabayashi *et al.*, 2003; Macdonald, Schleyer and Lamb, 2008; Carpenter *et al.*, 2011; Obura, 2012; Keshavmurthy *et al.*, 2013; Beger *et al.*, 2014; Richards and Day, 2018; Schleyer *et al.*, 2018). This larval recruitment aids in the recovery of previously depleted populations (Hoegh-Guldberg, 1999; Takabayashi *et al.*, 2003; Macdonald, Schleyer and Lamb, 2008; Burgess, Treml and Marshall, 2012; Schill *et al.*, 2015; Wright *et al.*, 2015; Roterman *et al.*, 2016). Interpreting the population ecology of these species ranges is important to understand coral reef ecosystems but can be difficult given the highly variable local environments spanning the large distances and complex underlying factors such as suitable settlement territory (Griffiths *et al.*, 2010; Souter, 2010; Arrigoni *et al.*, 2016). Understanding population structure and connectivity over multiple geographic scales is of concern for authorities managing ecosystems and Marine Protected Areas (MPAs). Genetic connectivity over larger distances is of growing interest given the global decline of coral reefs (Hoegh-Guldberg, 1999; Van Oppen and Gates, 2006; Stefani *et al.*, 2011).

Prior research has confirmed the distribution of *S. pistillata* along the KZN coastline from Kosi Bay to Pondoland with a reduction of abundance and change in morphology as it approaches the edge of reef-building coral distribution (Celliers *et al.*, 2007; Macdonald *et al.*, 2008; Olbers, Celliers and Schleyer, 2009; Flot *et al.*, 2011; Keshavmurthy *et al.*, 2013; Arrigoni *et al.*, 2016). The change in morphology has been seen in Blood Reef, Aliwal Shoal and Pondoland, has been thought to be attributed to the lower sea temperatures, and reduced light availability caused by riverine run-off and increase sediment loads (Macdonald, Schleyer and Lamb, 2008; Olbers, Celliers and Schleyer, 2009). The change in morphology is a southward transition and is seen to be from digitate to submassive forms (Macdonald *et al.*, 2008; Flot *et al.*, 2011). The extent to which the biogeographical break between IWP and MPAs southward affects the genetic connectivity of *S. pistillata* is yet to be determined. Some genetic studies have been conducted on other corals (*Pocillopora damicornis, P. verrucosa, Stylophora pistillata* and *Acropora austera*) on the KZN coastline and have expressed limited genetic exchange and a non-typical stepping-stone model with isolated populations and little hybridisation (Ridgway *et al.*, 2008; Souter, 2010; Montoya-Maya, Schleyer and Macdonald, 2016; Schleyer *et al.*, 2018; Chiazzari *et al.*, 2019). In the case of *S. pistillata*, the role genetic connectivity has on the of the geographical isolation and morphological changes it is limited and requires more research (Macdonald, Schleyer and Lamb, 2008; Chiazzari *et al.*, 2019).

Aims and Objectives

The objective of this study was to determine the connectivity of the reefs along the KZN coastline. The aim of this study was to confirm the presence of two clades of *Stylophora pistillata* and

distribution of said clades along the two bioregions and the population genetics along the KZN coastline using traditional mitochondrial (COI) and nuclear (ITS) markers based on reference data from Keshavmurthy *et al.* (2013).

Hypotheses

H1 The two clades identified by Keshavmurthy *et al.* (2013) occur in South Africa and constitute genetically distinct entities that do not exchange genetic material.

H2 Each of the clades shows clear population structure along the KZN coast.

H3 The population structure within each of the clades is based on the biogeographical features of the coast, resulting in ecologically driven population structure.

$H4_a$ The population structure within each clade is based on an isolation by distance (IBD) model driven by the prevailing current.

METHODS

Collection

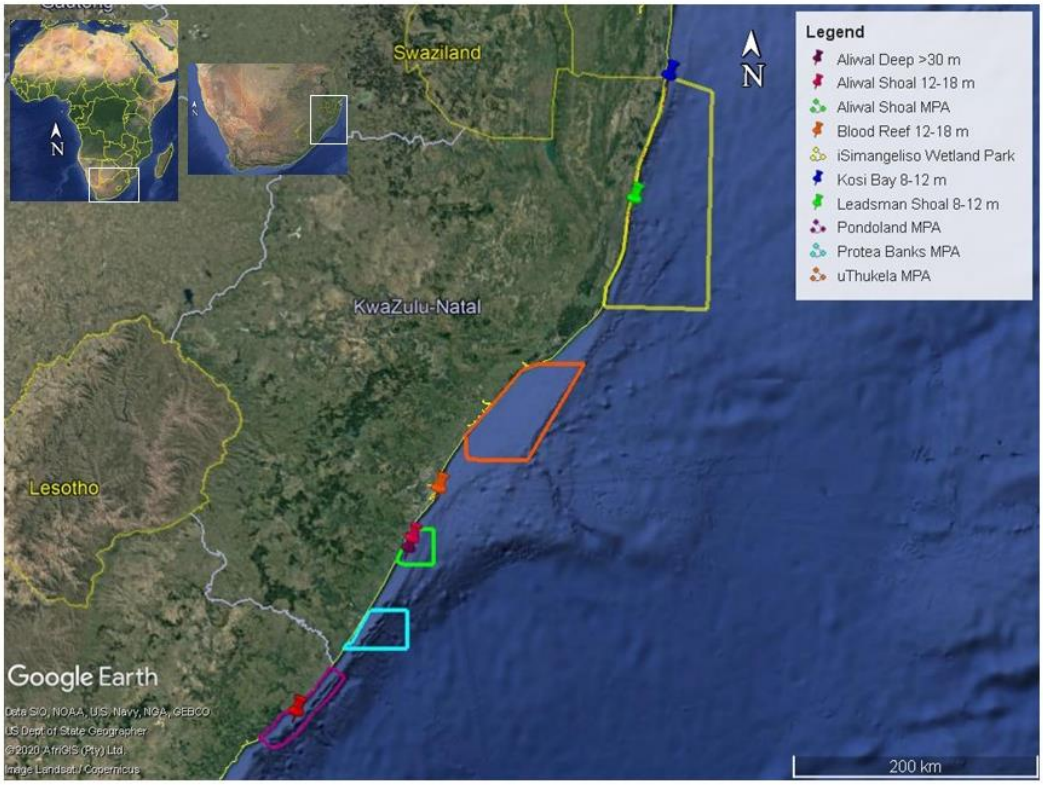


Figure 2.2: Sampling locations for this study including the MPAs and depths of the reefs sampled. MPA boundary information was downloaded from https://egis.environment.gov.za/data_egis/, and loaded in Google Earth Pro

Table 2.1: Sample sites, their coordinates and depth

Location	Latitude (DDM)	Longitude (DDM)	Depth	Biogeographic region
Kosi Bay	-26 55.680	32 53.100	8-12 m	Delagoa
Leadsman Shoal	-27 49.140	32 37.194	8-12 m	Delagoa
Blood Reef	-29 54.319	31 02.886	12 - 18 m	Natal
Aliwal Shoal	-30 15.950	30 49.510	12 - 18 m	Natal
Aliwal Deep	-30 20.095	30 47.595	>30 m	Natal
Pondoland	-31 16.026	30 02.820	12 - 18 m	Natal

All corals were collected under research permits from the Department of Agriculture Forestry and Fisheries (DAFF) and, where necessary, the iSimangaliso Wetland Park Authority and Ezemvelo-KZN Wildlife. *Stylophora* samples were collected from each of the following sites: Kosi Bay, Leadsman Shoal, Blood Reef and Aliwal Shoal, and Pondoland at depths and geographic locations indicated on Figure 2.2, details of which are provided in Table 2.1. Colonies were sampled by removing a small (2-5cm-sized) nubbin from each colony, during SCUBA dives mounted from the African Coelacanth Ecosystem Programme (ACEP) small craft, MV Phakisa (Durban and Aliwal Shoal) or from a rigid inflatable boat (RIB). Colonies were identified based on morphology and photographed underwater. The ACEP Remotely Operated Vehicle (ROV) (Fig 2.3) was used to identify suitable sampling localities at as-yet unexplored sites beyond commercial SCUBA diving depth limits on Aliwal Shoal at depths greater than 30 metres. Each sample was individually packaged in a sterile Whirl-Pak® bag (Nasco, WI, USA), preserved with a DMSO–EDTA–salt solution (DESS: 20% DMSO, 0.25M EDTA, pH 8.0 saturated with NaCl) (Gaither *et al.*, 2011) and stored at 4 °C for short periods and -20 °C long term. Harvesting was accomplished using either a chisel and hammer, side cutters or a knife to remove a subsection of the coral or by robotic arm of the ROV for retrieval to the surface.

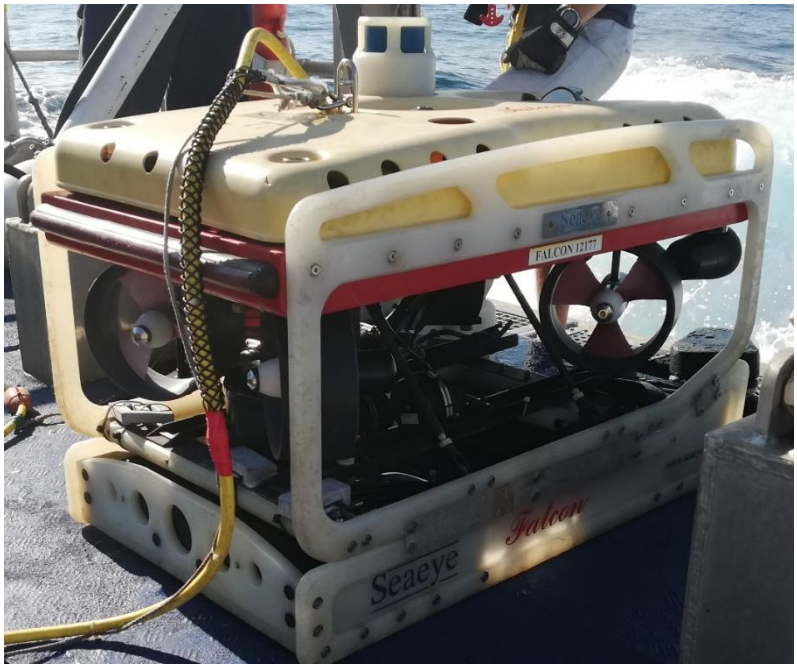


Figure 2.3: Image of the ACEP Remotely Operated Vehicle (ROV) on the ACEP small craft, MV Phakisa.

Tissue Extraction

Samples were subdivided to allow for multiple extractions if necessary. Sub-fragments of approximately 1 cm in length were used for genomic DNA extraction. They were washed with PBS to remove excess DESS and then placed in petri dish with 20 mL of 0.2 M EDTA. Tissue was then scraped from the skeleton using a sterile razor blade. The cell suspension was transferred to a 50 mL falcon tube and left on ice for 2 hours. The suspension was passed through a 100 µm and 30 µm sequential MACS SmartStrainers (Miltenyi Biotec Inc. Auburn, CA, USA) to reduce the number of *Symbiodinium* cells present in the extracts (Woodley *et al.*, 2015). Genomic DNA was extracted using a Zymo universal gDNA MiniPrep kit (Zymo Research, Irvine, CA, USA) using the tissue extraction buffer and performed as per the user manual except for an overnight digestion step. DNA concentration was determined using the Broad Range kit for the Qubit fluorescence reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

PCR Amplification and Sequencing

Polymerase Chain Reaction (PCR) was performed using TEMPase Hot Start 2x Master Mix BLUE (Ampliqon, Denmark) (master mix containing TEMPase Hot Start DNA polymerase, the ammonium buffer system, inert blue dye, stabilizer, dNTPs, and $MgCl_2$). Primers utilized in the PCR reactions for *Stylophora* for the "Folmer" region of the Cytochrome Oxidase subunit I (COI) gene, which is commonly used for barcoding (Folmer *et al.*, 1994) and for the Internally Transcribed Spacer (ITS) (White *et al.*, 1990) as shown in Table 2.2. The PCR amplification

reaction was carried out in a total reaction volume of 25 µL. A gradient PCR was used to determine the optimal annealing temperature, which resulted in the following successful protocol.

Table 2.2: Primers used in study

Primer Name	Primer Sequence	Program	Reference
COI: LCO1490/ HC02198	GGTCAACAAATCATAAAGATATTGG/ TAAACTTCAGGGTGACCAAAAAATCA	95 °C 15 min, 35 cycles of (95 °C 30s, 46 °C 45s, 72 °C 60s), 72 °C 10 min	(Folmer *et al.*, 1994)
ITS: ITS4/ ITS5	TCCTCCGCTTAGATATGC/ GGAAGTAAAAGTCGTAACAAGG	95 °C 15 min, 35 cycles of (95 °C 30s, 52 °C 45s, 72 °C 60s), 72 °C 10 min	(White *et al.*, 1990)

Sequence Data

Five microliters of each PCR product were separated on a 1.5% agarose gel immersed in sodium borate buffer at 120 V for 30 min and visualized with Pronasafe dye (Ampliqon, Denmark) on a Biorad Gel Doc™ XR+ to determine if the amplification was successful. If the bands were clear and distinct, the remainder of the PCR product was sent to the Central Analysis Facility (CAF) at Stellenbosch University or the KZN Research Innovation & Sequencing Platform (KRISP). BigDye terminator chemistry (Applied Biosystems) was used to generate sequences on an ABI Sanger 3730xl Genetic Analyzer automated sequencer. Samples that appeared as distinct bands when visualized on the gel were sequenced in a single direction (using the forward primer) and for those whose bands appeared dull but still visible, bi-directional sequencing was used (both forward and reverse primers). Any sequences that gave unclear or ambiguous base calls after unidirectional sequencing were re-sequenced in the reverse direction.

Sequence Alignment and Data Analysis

COI

Sequences were visually inspected using a Geneious software version 9.1.5 (https://www.geneious.com), adjusted by eye, using the quality scores and bidirectional sequences when available All sequences were then used in a BLAST search against the non-redundant (nr) GenBank database to confirm that they correspond to the *S. pistillata* COI region. Sequences were then inspected using GEAR, a web server for molecular biology applications (https://www.gear-genomics.com) (Rausch *et al.*, 2019, 2020). Within GEAR, Teal was used to visually inspect each sequence given multiple sequences could be used to align as well as a

reference sequence for greater confidence and determine a representative sample from each sample location that was then used to compare with all other sequences where ambiguities were present. Pearl was used to assemble the chromatograms to visually compare sequences and deliver a consensus sequence to suggest the correct base that may present a questionable peak. Sequences were aligned using the ClustalW algorithm implemented in MEGA X (Kumar *et al.*, 2018). Alignments were manually trimmed so that all sequences were 547 bp long. Phylogenetic trees were constructed from the COI sequences using the Maximum Likelihood (ML) method (performed using MEGA X), with 1000 bootstraps using the Tamura and Nei substitution model (Tamura and Nei, 1993). Phylogenetic trees were edited using TreeGraph 2 (Stöver and Müller, 2010). The reference sequences for *S. pistillata* clades 1-4 and *Seriatopora hystrix* were retrieved from the supplementary material from Keshavmurthy, *et al.* (2013) in the Dryad Repository: http://dx.doi.org/dryad.n2fb2. Pairwise distances were calculated in MEGA X (Appendix A) and median joining haplotype networks using locations as traits were created using POPArt (Leigh and Bryant, 2015; Gargan *et al.*, 2016).

ITS

Sequences were visually inspected using Geneious software version 9.1.5 (https://www.geneious.com), adjusted by eye, using the quality scores and bidirectional sequences when available. Sequences are inspected using GEAR, a web server for molecular biology applications (https://www.gear-genomics.com) (Rausch *et al.*, 2019, 2020). Teal was used to visually inspect each sequence and determine a representative sample used to compare all other sequences of poorer quality. Briefly, Pearl was used to assemble the chromatograms to visually compare sequences with the presence of ambiguities. Once ambiguities have been identified, Indigo was then used to determine the presence of insertion-deletion mutations (indels) and single nucleotide polymorphisms (SNPs) by presenting alignments of the two identical alleles with respect to a reference which is saved in FASTA format and represented as A or B. A BLAST search against the GenBank non-redundant nucleotide database was used to confirm that each sequence was most closely related to existing *S. pistillata* sequences. Sequences were aligned using ClustalW algorithm implemented in MEGA X (Kumar *et al.*, 2018). Alignments were then manually trimmed to increase the overall similarity by removing leading and trailing nucleotides to form a 635bp long data matrix. Phylogenetic trees were constructed and analysed using Maximum Likelihood (ML) (performed using MEGA X), with 1000 bootstrap using the Hasegawa, Kishino and Yano 1985 (HKY) (Hasegawa, Kishino and Yano, 1985) substitution model including gamma distributed rate variation among sites (+G) but no defined proportion of invariant sites (-I). This model was selected based on the lowest BIC score. Phylogenetic trees were drawn using MEGA X and edited using TreeGraph 2 (Stöver and Müller, 2010) and availble

in Supplementary Information SI2. Additionally, to compare the results of our study with those previously published data from Flot *et al.* (2011), the ITS2 regions of 113 representative individuals of ITS2 from the molecularly defined species A, B and C were sequenced and aligned with sequences retrieved from a public deposit onto GenBank (JN558840-JN559111) and aligned with the sequences generated in this study. Alignments for ITS2 were only 353bp due to the length limitations of the published sequences. Phylogenetic trees were constructed and analysed using Maximum Likelihood (ML) performed using MEGA X, with 1000 bootstraps using the Tamura 3-parameter model with gamma substitution and no invariant sites. The best substitution model was chosen on the basic of the lowest BIC score generated in MEGA X (Tamura, 1992). Phylogenetic trees were drawn using MEGA X (Kumar *et al.*, 2018) and edited using TreeGraph 2 (Stöver and Müller, 2010). Pairwise distance models were calculated in MEGA X and median joining haplowebs were constructed with the online tool HaplowebMaker (https:// eeg-ebe.github.io/HaplowebMaker/) with default settings (Spöri and Flot 2020). In the networks, curves show alleles found co-occurring in heterozygous individuals.

Population Genetics

Statistical population genetic analysis was carried out using COI and ITS sequences belonging to the individuals identified as belonging to Clade 3. Sequences were aligned and trimmed using MEGA X and exported in FASTA format. The FASTA files were transformed into Arlequin format using to DNAsp (version 6.12.3) and population genetics parameters were analysed in Arlequin version 3.5.2.2 (Excoffier and Lischer, 2010). An Analysis of Molecular Variance (AMOVA) test was performed (Excoffier, Smouse and Quattro, 1992) using alternative potential population structures and the results are shown in Table 2.8 and 2.9. Its significance was tested by 9,999 random permutations of individuals among populations. Arlequin (version 3.5), (Excoffier and Lischer, 2010) was also used to calculate the nucleotide diversity index

RESULTS AND DISCUSSION

Phylogenetic Results

Phylogenetic reconstructions were carried out with the COI haplotypes recovered in this study together with the global *Stylophora* and *Seriatopora* sequences described by Keshavmurthy *et al.* (2013). Figure 2.4 show the same phylogenetic tree generated in MEGA X and are sampled and colour coordinated to the sample locations shown in Figure 2.2. The sequences clearly segregate into two of the clades (clades 2 and 3) described by Keshavmurthy *et al.* (2013) which appear to correspond to the species A (clade 3) and B (clade 2) as described by Flot *et al.* 2011. For convenience, we will henceforth refer to them as clade 2 and clade 3. This corroborates results from previous studies containing representative sequences originating from South Africa (Flot,

Couloux and Tillier, 2010; Keshavmurthy *et al.*, 2013). In this case, differential geographical distributions of the clade 2 and 3 samples from South Africa are clear. As evident in Figure 2.4, the new South African samples from this study are assigned molecularly to either clade 2 or clade 3 based on the COI and ITS sequences. Clade 2 individuals are only found in Kosi Bay (13 of 18 individuals) and Leadsman Shoal (1 of 23 individuals), while clade 3 individuals were found in all sample sites. Additionally, the reference genome for *Stylophora pistillata* found on GenBank (assembly accession: GCA_002571385.1) (Shinzato *et al.*, 2011) is aligned to clade 1. Figure 2.5 shows how all the sequences and morphotypes from this study align to the *Stylophora* phylogeny and indicates the geographical distribution of sequences and morphotypes.

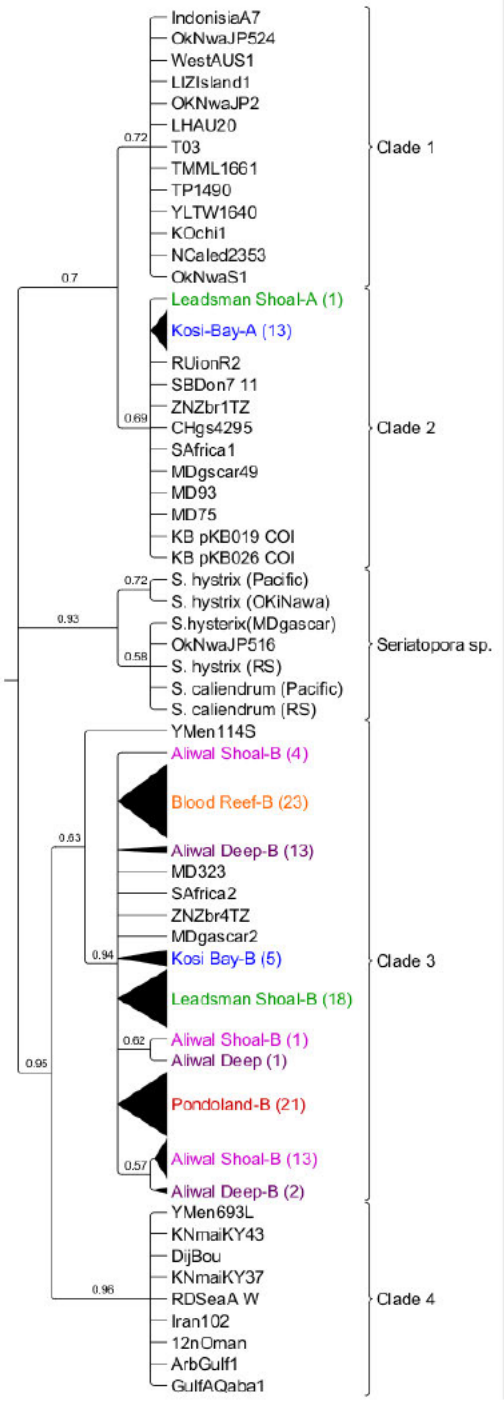


Figure 2.4: Phylogenetic analysis of COI sequences of *Stylophora pistillata* with references derived from (Keshavmurthy *et al.,* 2013). Bootstrap values are based on 1000 repetitions (ML) analyses. Individual haplotypes found in each location are indicated by name-number and the numbers in the brackets indicate the total number of each haplotype obtained from each location.

Table 2.3: Distribution of clade 2 and 3 individuals across sample sites.

	Clade 2		**Clade 3**	
Sample Site	**Number**	**Percentage**	**Number**	**Percentage**
Kosi Bay	13	72	5	18
Leadsman Shoal	1	5	22	95
Blood Reef	0	0	23	100
Aliwal Shoal	0	0	20	100
Aliwal Deep	0	0	16	100
Pondoland	0	0	20	100

Figure 2.4 shows the COI-derived phylogenetic tree of the samples from this study together with their location (full tree in Supplementary Information SI1). The proportions of samples that are assigned to clade 2 and 3 in each sample location is summarised in Table 2.3 above. As seen in this Table, 72% (13/18) of samples from the northernmost site near the Mozambique border (Kosi Bay) belonged to clade 2 with a single individual (1/23 = 4%) from clade 2 occurring at the southern site in the iSimangaliso MPA. No individuals belonging to clade 2 (0/79) were found in sites south of iSimangaliso, i.e., in the Natal Bioregion. This distribution pattern is clearly illustrated in the distribution maps in Table 2.3. Table 2.4 shows a comparative morphology of a subset of individuals within each site and their assigned clade. Based on the images (E), fused corallites immersed in the cœnosteum can be seen in clade 2. As shown by Keshavmurthy and co-authors (2013), clade 3 appeared to have poorly developed primary septa which are separated. Corallite hoods are found in individuals from both clade 2 and 3 in the Delagoa bioregion (IWP) while clade 3 samples from the Natal Bioregion completely lack corallite hoods. In addition, corallites from clade 2 and 3 in the Delagoa bioregion are relatively closely spaced and deep while those from clade 3 in the Natal Bioregion are more widely dispersed and shallower. On a gross morphological level, samples in the IWP (Delagoa bioregion) all have a digitate morphology, although clade 3 in this region tend to have thicker, “club-like” branches similar to what has previously been identified as a “*mordax*” form (Veron, 2000). In contrast many of the clade 3 individuals in the Natal bioregion, particularly from the deep Aliwal, Blood Reef and Pondoland sites, have a flat, non-digitate, anastomosing morphology and are more brittle than the Delagoa individuals, suggesting a less dense skeleton. Other clade 3 individuals in the Natal bioregion, particularly from Aliwal Shoal and Blood Reef grow in a compact digitate form with relatively thin branches.

Table 2.4 Comparative morphology of samples collected in their sites and corresponding clade designation

Site	Sample name	Clade	Primary Septum	Secondary septum	Columella	Hoods present	Spinules	Colony	Branch
Kosi Bay	KB001	2	Thick, Joined,6	Absent	Present fused	Present	Absent	Digitate	Thick
	KB003	2	Thick, Joined,6	Absent	Present fused	Present	Present	Digitate	Thick
	KB004	3	Thick, Joined,6	Absent	Present fused	Present	Present	Digitate	Thick
	KB005	3	Thick, Joined,6	Absent	Present fused	Present	Absent	Tabular	Thin
	KB018	3	Thick, Joined,6	Absent	Present fused	Present	Present	Digitate	Thick
Leadsman Shoal	LS315	2	Thick, Joined,6	Absent	Present fused	Present	Present	Digitate	Thick
	LS023	3	Thick, Joined,6	Absent	Present fused	Present	Present	Tabular	Thin
	LS027	3	Thick, Joined,6	Absent	Present fused	Present	Present	Tabular	Thin
	LS037	3	Thick, Joined,6	Absent	Present fused	Present	Present	Tabular	Thin
	LS055	3	Thick, Joined,6	Present	Present fused	Present	Present	Tabular	Thin
	LS056	3	Thick, Joined,6	Absent	Present fused	Present	Absent	Digitate	Thick
	LS061	3	Thick, Joined,6	Absent	Present fused	Present	Present	Tabular	Thin
	LS062	3	Thick, Not Joined	Present	Present fused	Present	Present	Tabular	Thin
Blood Reef	BR012	3	Thin, joined,6	Absent	Present fused	Absent	Present	Digitate	Thick
	BR027	3	Thin,6	Absent	Present, not fused	Absent	Present	Digitate	Thick
	BR028	3	Thin,6	Absent	Present, not fused	Absent	Present	Tabular	Thin
Aliwal Shoal	AS088	3	Thin,6	Absent	Present, not fused	Absent	Present	Digitate	Thick
	AS110	3	Thin,6	Absent	Present, not fused	Absent	Present	Tabular	Flat, Anastomosing
Aliwal Deep	AD004	3	thick, joined,6	Present	Present fused	Absent	Present	Tabular	Flat, Anastomosing
	AD023	3	thick, joined,6	Absent	Present fused	Absent	Present	Tabular	Flat, Anastomosing
Pondoland	PD004	3	thick, not joined	Absent	Present fused	Absent	Present	Tabular	Flat, Anastomosing
	PD005	3	thick, not joined	Absent	Present fused	Absent	Present	Tabular	Flat, Anastomosing

Table 2.5: Estimates of evolutionary divergence over sequence pairs between groups. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

	Clade_1	Clade_2	KB-sp1	LS-sp1	Seriatopora	Clade_3	KB-sp2	LS-sp2	BR-sp2	AD-sp2	AS-sp2	PD-sp2
Clade_1												
Clade_2	0.004											
KB-sp1	0.004											
LS-sp1	0.004											
Seriatopora	0.009	0.009	0.009	0.009								
Clade_3	0.015	0.015	0.015	0.015	0.017							
KB-sp2	0.015	0.015	0.015	0.015	0.017	0.002						
LS-sp2	0.015	0.015	0.015	0.015	0.017	0.002	0.000					
BR-sp2	0.015	0.015	0.015	0.015	0.017	0.002	0.000	0.000				
AD-sp2	0.015	0.015	0.015	0.015	0.017	0.002	0.000	0.001	0.001			
AS-sp2	0.016	0.016	0.016	0.016	0.016	0.003	0.001	0.001	0.001	0.001		
PD-sp2	0.015	0.015	0.015	0.015	0.017	0.002		0.000	0.000	0.000	0.001	
Clade_4	0.014	0.013	0.013	0.013	0.015	0.013	0.013	0.014	0.014	0.014	0.015	0.013

Table 2.5 illustrates the pairwise distance between the four clades described by Keshavmurthy and co-authors (2013) and the study samples that aligned within the four clades. As seen the South African samples appear to be cluster very closely with the designated clades. There is insignificant variation within the clades yet clear separation between the clades. Clade 1 and clade 2 appeared to me more connected between on another, clades 3 and 4 are more connected to one another than to clade 1 and 2, with *Seriatopora* situated in between the clade pairs.

ITS

Phylogenetic reconstruction based on the ITS sequences are shown in Figure 2.5 (full tree in Supplementary Information SI2). Certain individuals had multiple ITS haplotypes, and these were mapped into a haploweb (Figure 2.9) (Spöri and Flot 2020). All nuclear ITS haplotypes from clade 3 individuals map to the clade 3 portion of the tree which suggests that there is no, or limited, gene exchange between clade 2 and 3 and confirms supports the hypotheses that these *Stylophora* clades represent true species. A caveat is that no ITS sequences from clade 3 individuals from Kosi Bay, where most of the geographic overlap between the two clades occurs, were unsuccessfully sequenced. Phylogenetic analysis of ITS sequences (Figure 2.5) showed the same tree topology as obtained using COI, further supporting the separation of the clades and lack of inter-clade hybridisation (Flot *et al.*, 2011) and the species complex separation of two species A and B. The three clades as described by Flot *et al.* (2011) remain intact. The ITS sequences presented as a multi-copy nuclear marker throughout, with each individual consisting of either one or two sequence types (Flot *et al.*, 2011).

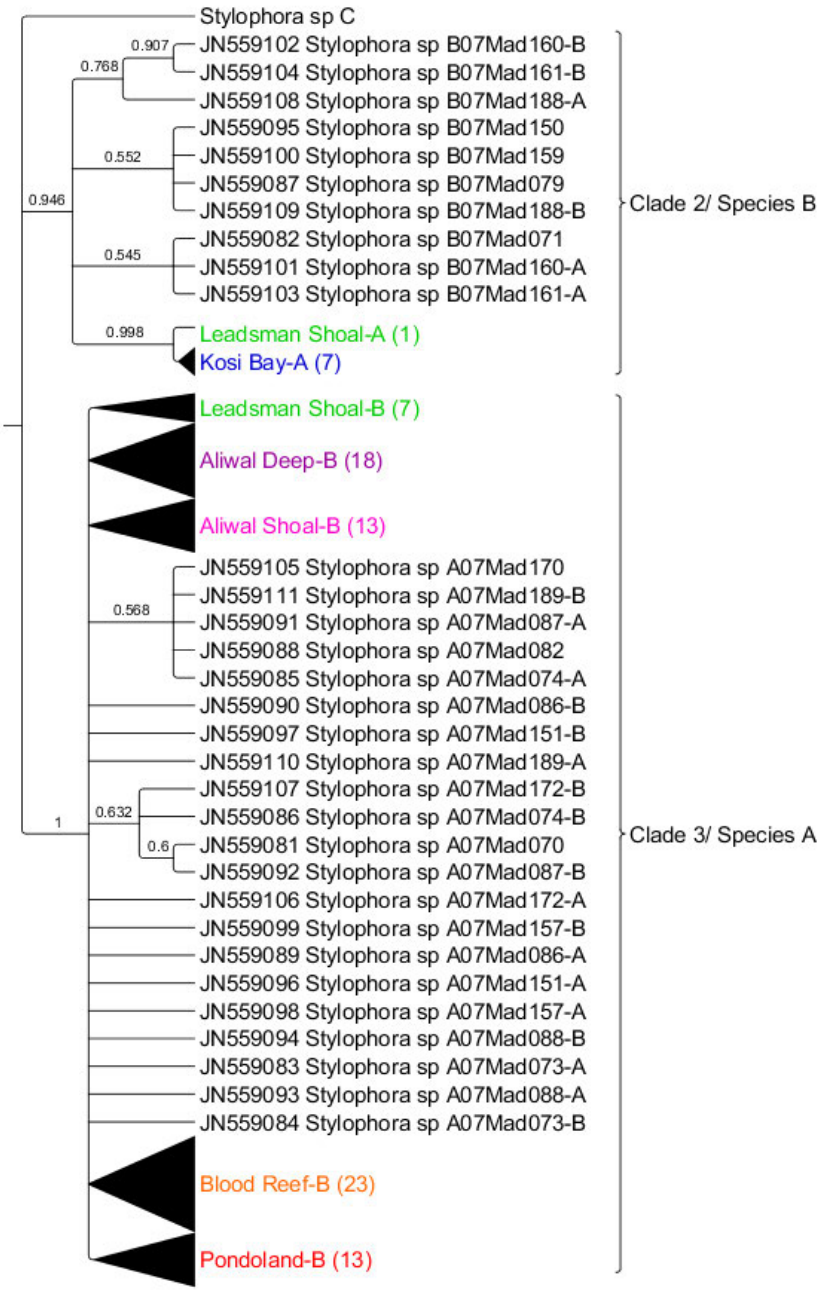


Figure 2.5 Phylogenetic analysis of a 372bp subsequence of ITS2 sequences of *Stylophora pistillata* with references from Flot *et al*. (2011) and retrieved from GenBank (Appendix A).

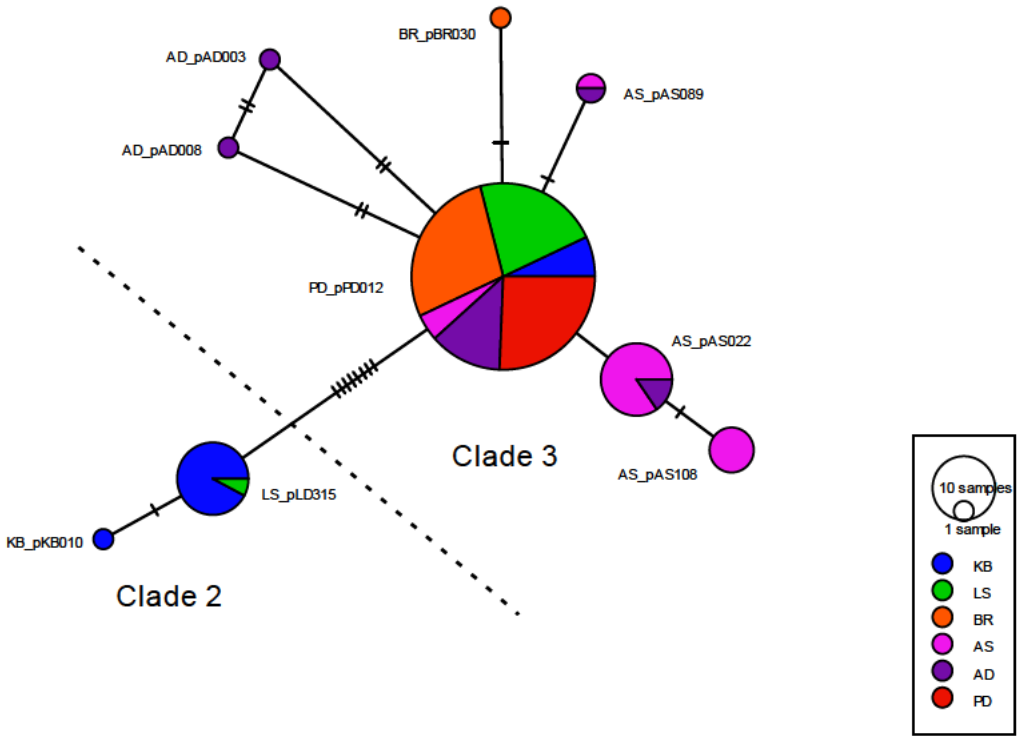


Figure 2.6: Haplotype network of the COI sequences of Stylophora sp. In KZN, the median spanning network was drawn using the location sampled as a trait created using POPArt (Bandelt, Forster and Röhl, 1999; Leigh and Bryant, 2015).

The COI haplotype network (Figure 2.6) best displays that there is little genetic variance between individuals and populations. This Figure also reflects the separation of Clade 2 and 3. Within each clade the genetic diversity is exceptionally low, the mean genetic diversity as measured in MEGA X (Kumar *et al.*, 2018) using the estimates of the mean evolutionary diversity within subpopulations and diversity for the entire population method was 0.00 ±0.00.

Figure 2.7 shows the geographic distribution of haplotypes of clade 3. A single COI haplotype dominates and is present in all the populations. There are, however, some unique alleles present in Aliwal Shoal and Blood Reef. The sample sizes are not equal between locations.

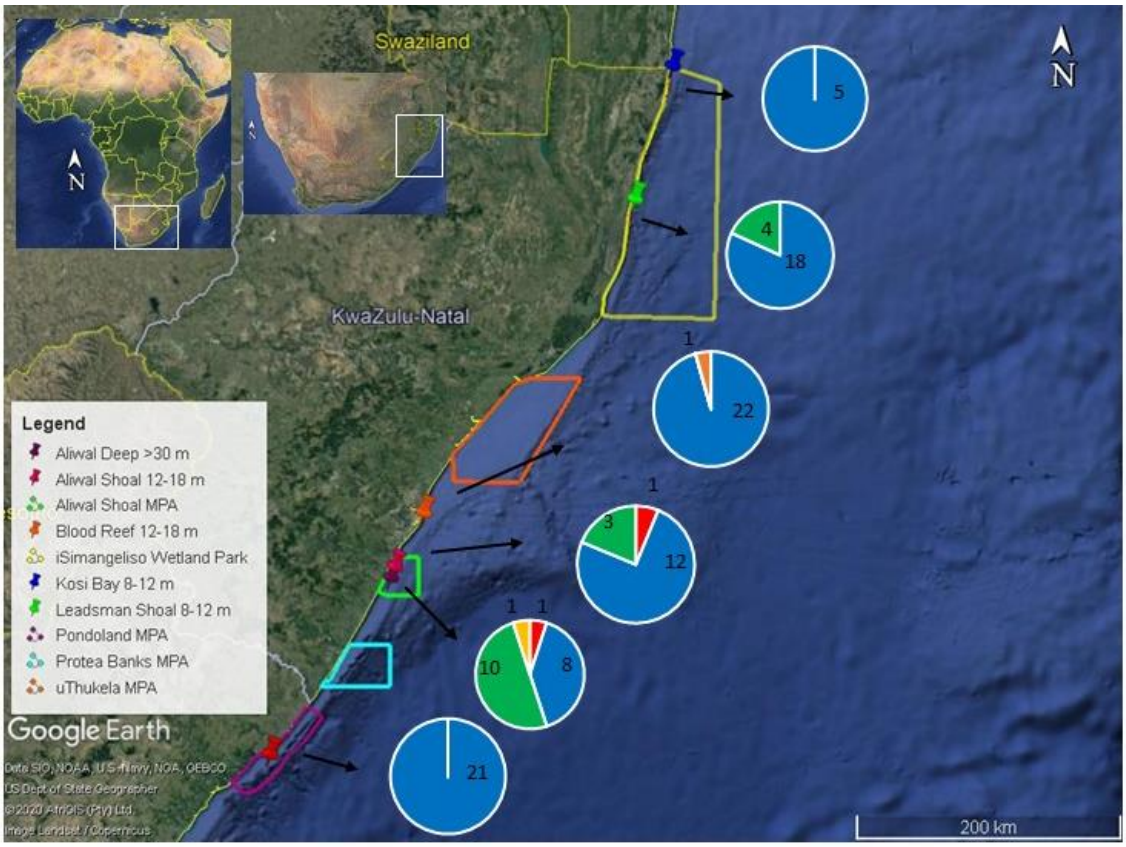


Figure 2.7: COI haplotypes per locations. Showing distribution of Clade 3 haplotypes along the sample coastline. MPA boundary information was downloaded from HTTPS://EGIS.ENVIRONMENT.GOV.ZA/DATA_EGIS/ AND loaded in Google Earth.

Table 2.6: Nucleotide diversity of *Stylophora pistillata* COI marker using Arlequin

Site	*n*	n_h	Nucleotide diversity (±SD)	Mean pairwise differences (±SD)
Kosi Bay	5	2	0.246 +/- 0.178	3.200 +/- 1.979
Leadsman Shoal	22	2	0.024 +/- 0.028	0.312 +/- 0.332
Blood Reef	23	2	0.013 +/- 0.020	0.174 +/- 0.238
Aliwal Shoal	20	4	0.055 +/- 0.048	0.721 +/- 0.562
Aliwal Deep	16	3	0.035 +/- 0.036	0.450 +/- 0.420
Pondoland	21	1	0.000 +/- 0.000	0.000 +/- 0.000

n = number of individuals, n_h = number of haplotypes. Nucleotide diversity is and standard deviations (SD) are provided with mean pairwise differences.

The haplotype and nucleotide diversity of COI is low within the population (Table 2.6). There are only a few haplotypes present within the populations. It is expected given that the region is slowly

evolving. Table 2.7 reveals the ITS marker is more diverse as expected with more individual haplotypes present. However, the nucleotide diversity remains low with Leadsman Shoal showing the most diversity (0.291).

Table 2.7 Nucleotide diversity of *Stylophora pistillata* ITS marker using Arlequin

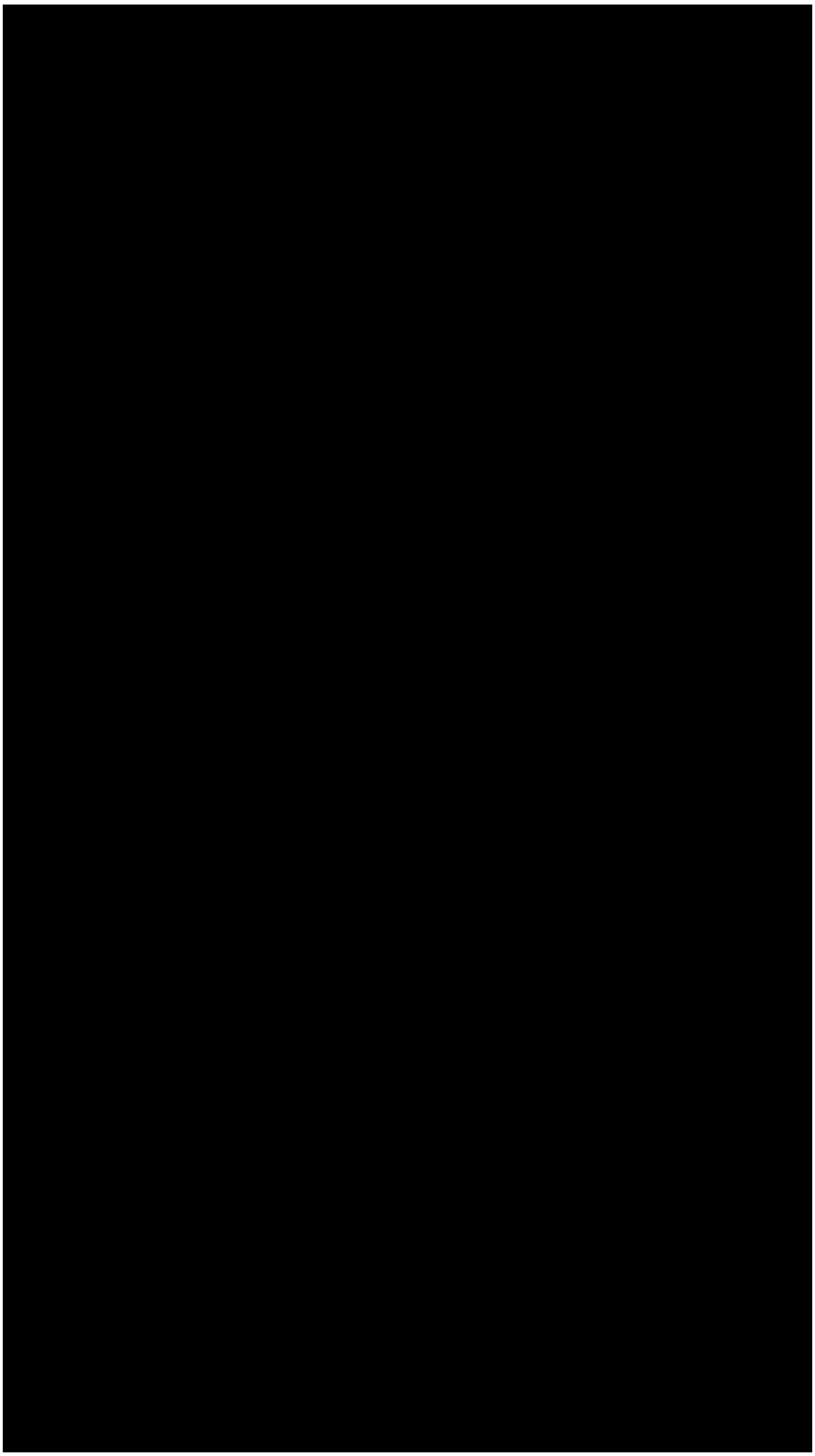
Site	***n***	n_h	**Nucleotide diversity (±SD)**	**Mean pairwise differences (±SD)**
Leadsman Shoal	7	7	0.291 +/- 0.174	9.905 +/- 5.167
Blood Reef	25	8	0.046 +/- 0.033	1.577 +/- 0.971
Aliwal Shoal	9	4	0.138 +/- 0.085	4.556 +/- 2.470
Aliwal Deep	17	4	0.015 +/- 0.016	0.463 +/- 0.426
Pondoland	12	8	0.155 +/- 0.091	4.970 +/- 2.600

n = number of individuals, n_h = number of haplotypes. Nucleotide diversity is and standard deviations (SD) are provided with mean pairwise differences.

Table 2.8 shows the heterozygous individuals from the sampled populations. The table shows the levels of intraindividual polymorphism in the form of p-distance between the alleles. There is little variation between the alleles.

The ITS haplotypes (Figure 2.8) are more variable and hence the haplotype network is more dispersed. The Leadsman Shoal population appears to separate out with a high proportion of private alleles. The network suggests a higher intra-group genetic diversity for ITS than in COI and for Leadsman Shoal samples, while the sequences from more southern regions are much more tightly clustered.

Table 2.8: P-distance between alleles of Clade 3, number of alleles, Indels and SNPs



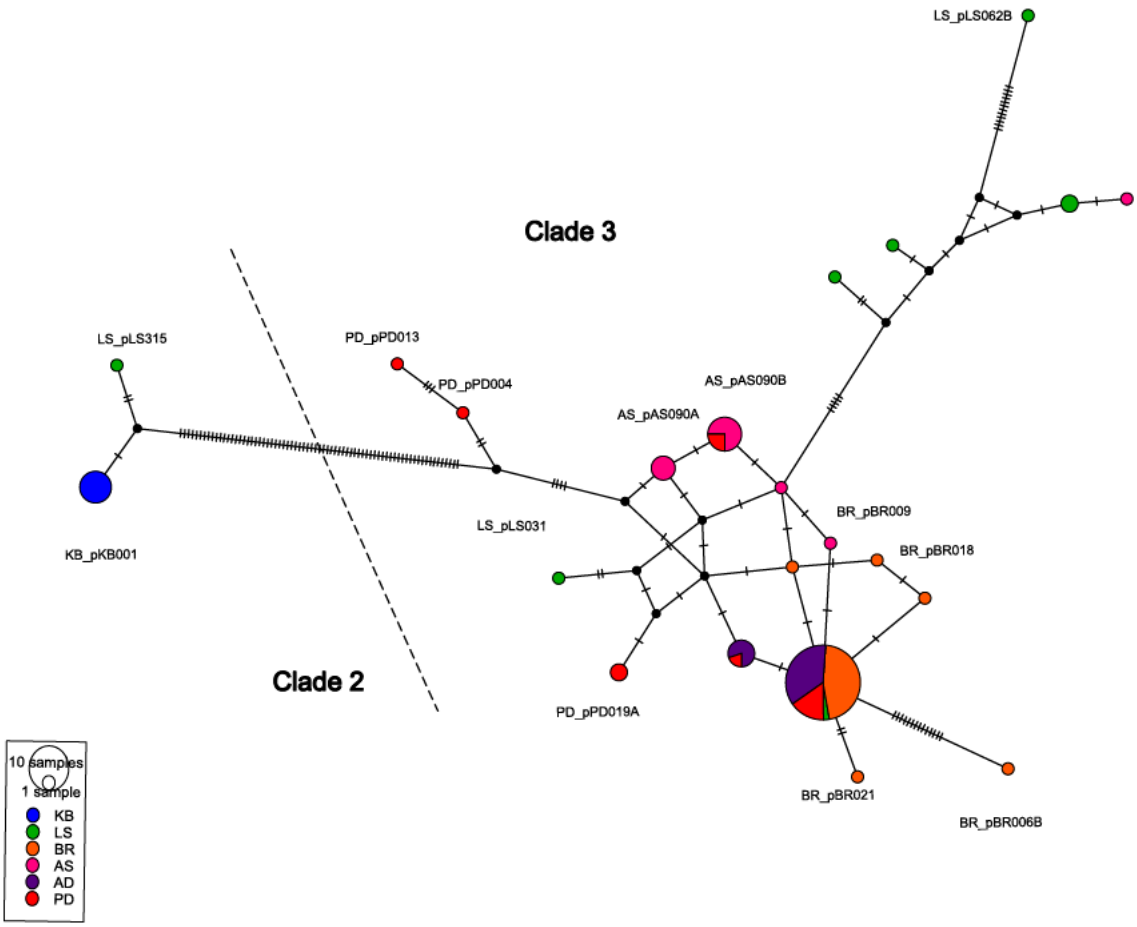


Figure 2.8: Haplotype network of the full 635 bp dataset ITS region of *Stylophora*, the median joining network was drawn using the location sampled as a trait created using POPArt (Bandelt, Forster and Röhl, 1999; Leigh and Bryant, 2015).

Using HaplowebMaker, figure 2.9 corresponds to the phylogenetic tree of ITS and indicates that there is no hybridisation between clades and species as defined by Spöri and Flot (2020). Interestingly the clade 2 and 3 in South Africa appear to be more closely related to each other than the corresponding species A and B from Madagascar are to each other.

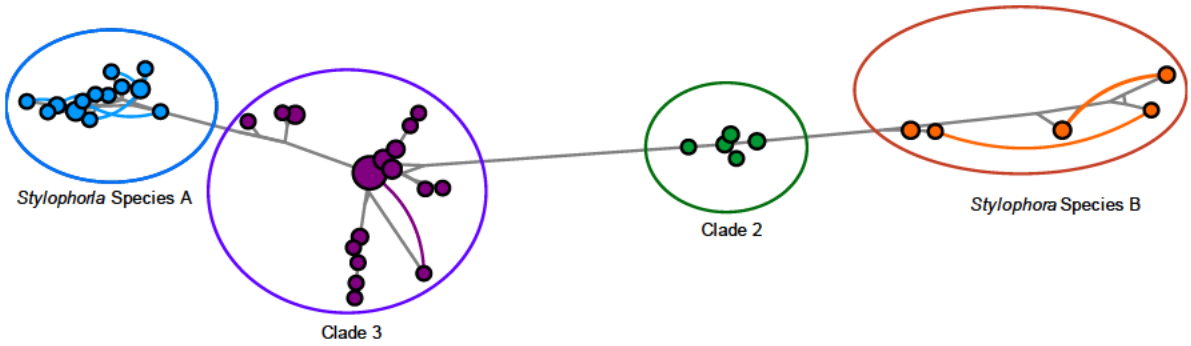


Figure 2.9: Haploweb generated using HaplowebMaker showing the connection between Flot 2011 Species A and B compared to the comparable region of its region (385bp) of Clade 2 and Clade 3 of study samples

Population Genetics for Clade 3

Table 2.9: Matrix table of pairwise population ϕPT values for COI. Significant values (P < 0.05) are highlighted above the diagonal.

Pairwise Population ϕPT Values for COI						
	KB	**LS**	**BR**	**AS**	**AD**	**PD**
KB		0.570	0.160	0.238	0.052	**0.001**
LS	0.004		0.051	0.189	**0.010**	0.109
BR	0.000	0.098		**0.046**	**0.001**	0.442
AS	0.000	0.000	**0.088**		**0.042**	**0.020**
AD	0.281	**0.187**	**0.405**	**0.143**		**0.001**
PD	#N/A	0.138	**0.000**	**0.129**	0.453	
ϕPT Values below diagonal. Significant ϕPT values are highlighted						

Pairwise ϕPT values (Table 2.9) for the COI marker using GenALEx ver. 6.41 (Peakall and Smouse, 2012) show there are significant pairwise differences between several populations. The highest significant ϕPT values are between the Pondoland and Aliwal Deep (ϕPT = 0.453, p = 0.001) and Blood Reef and Aliwal Deep (ϕPT = 0.405, p = 0.001), indicating they are the most genetically divergent. Aliwal Deep is the most different with relative pairwise high ϕPT values with all other sites, particularly Blood Reef. The COI marker poorly performs as a population genetic marker in coral.

Table 2.10: Matrix table of pairwise population ϕPT values for ITS. Significant values (P < 0.05) are highlighted and bolded above the diagonal.

Pairwise Population ϕPT Values for ITS					
	LS	**BR**	**AS**	**AD**	**PD**
LS		**0.002**	0.068	**0.002**	**0.011**
BR	**0.538**		**0.001**	0.405	**0.019**
AS	0.172	**0.435**		**0.001**	0.094
AD	**0.609**	0.000	**0.559**		**0.001**
PD	**0.240**	0.120	0.089	**0.188**	
ϕPT Values below diagonal. Significant ϕPT values are highlighted					

Pairwise ϕPT values (Table 2.10) for the ITS marker using GenALEx ver. 6.41 (Peakall and Smouse, 2012) show strong population structure particularly between Aliwal Deep and Leadsman (ϕPT = 0.609, p = 0.002), Leadsman and Blood reef (ϕPT = 0.538, p = 0.002), Blood Reef vs. Aliwal Shoal (ϕPT = 0.435 p = 0.001) and Aliwal Deep and Aliwal Shoal (ϕPT = 0.559, p = 0.001). Interestingly with ITS, in contrast to COI, there is no significant difference between Aliwal Deep and Blood Reef.

Given the disparities of the pairwise comparisons and lack of any obvious patterns we used a hierarchical model method to assess which potential population structure best describes the structure and connectivity between sample sites along the KZN coast. Using AMOVAs performed using GenALEx ver. 6.41 (Peakall and Smouse, 2012) on the data from the ITS (Table 2.10) sequences.

Table 2.11: ITS AMOVA results generated using GenALEx ver. 6.41 (Peakall, 2006). The table displays the amount of interpopulation variance explained and (2) the ϕPT value (and associated p) in decreasing order.

Population combinations	**Source of Variation**	**df**	**Sum of squares**	**Variance components**	**Percent of total variation**	**Fixation indices**
(LS +AS + PD) vs (BR+AD)	**AMOVA grouping by population**					
ITS pairwise	**Among Populations**	1	20.099	0.602	26%	ϕPT: 0.259
	Within Populations	61	105.059	1.722	74%	P= 0.001
(LS) vs (BR+AS+AD+PD)	**AMOVA grouping by population**					
	Among Populations	1	19.369	1.624	48%	ϕPT: 0.484
Delagoa vs Natal	**Within Populations**	61	105.789	1.734	52%	P= 0.001
Population combinations	**Source of Variation**	**df**	**Sum of squares**	**Variance components**	**Percent of total variation**	**Fixation indices**
LS vs BR vs AS vs AD vs PD	**AMOVA grouping by population**					
All separate	**Among Populations**	5	485.379	8.808	78%	ϕPT: 0.784
	Within Populations	61	147.695	2.421	22%	P=0.001

The ITS AMOVA (Table 2.11) models were considered given the greater potential resolution afforded by greater genetic diversity in the ITS sequences. These models include isolated populations, and a separation by biogeographical regions (LS vs AS +AD +BR+ PD combined) and the suggested population structure based on the pairwise ϕPT values of ITS, (LS +AS + PD) vs (BR+AD). AMOVA results shows the ϕPT of the ITS marker indicate that the most likely population structure is the populations derived from ϕPT results. The population structure that has the lowest ϕPT differentiation index had a value of 0.259 (p = 0.000), which is significant. Finally, the model in which all populations are separate indicates the populations are isolated with limited gene flow amongst populations ϕPT of 0.784 (p = 0.001).

DISCUSSION

Phylogenetic results confirm that two of the five currently identified *Stylophora* clades identified by Keshavmurthy and co-authors (2013) are found in South Africa. This previous study stated that clades 2 and 3 existed in South Africa but did not discuss any “fine-scale” distribution in

South Africa. These results clearly show a difference in the biogeographic distribution of clades 2 and 3 in South Africa with clade 2 being restricted to the warmer Delagoa region and clade 3 being more widely distributed, extending all the way down to the southern end of the cooler Natal Bioregion. Previous studies have shown that clade 2 is widely distributed across the central and southern Indian Ocean including South Africa, Zanzibar, Reunion, Madagascar, and the Chagos Archipelago, while clade 3 appears to be restricted to the east coast of Africa and Madagascar from Yemen down to South Africa but covers a much wider range of latitude. However, morphologically it is difficult to distinguish between clade 2 and 3 species complex in the field within the IWP. As described by Veron (Veron, 2000), specimens collected in the Delagoa Bioregion exhibit morphology described as *Stylophora madagascarensis* (Veron, 2000, 2002). However, whilst it has been suggested that the genus should undergo taxonomic revision, the clades are also referred to as *Stylophora* sp. A and *Stylophora* sp. B. There is dedicated support for *S. madagascarensis* to belong in sp. A as suggested by Flot *et al.* (2011). Morphologically, however, some samples collected in this study resemble that of species A, which has small columellae with six primary septa fused with the columella (Flot *et al.*, 2011). Species B, which was only seen in the Delagoa Bioregion, presented in both clades with *mordax*-like flattened stout branches as originally described from Fiji in the Pacific Ocean (Flot *et al.*, 2011). Within the IWP, both clades have hooded corallites with and digitate branches with closely spaced corallites. The presence of hoods was only seen in the northern populations and appeared to become scarce in specimens from higher latitudes and importantly, hoods were present in both clade 2 and 3 as reflected in Figure 2.10. Within the Natal Bioregion, samples are more varied in appearance between a digitate structure and colonies that have varying degrees of flattened anastomosing or thin digitate branches, and more widely spaced, hoodless corallites as seen in image c of Figure 2.10. None of the samples presented to be submassive as described by Macdonald *et al.* (2008), all samples had a single attachment point to the substrate. Additionally, Flot described a contrasting morphology of *S. madagascarensis* found at a depth of 30 meters. Colonies at these deeper depths are described as having thicker, less compact branches.

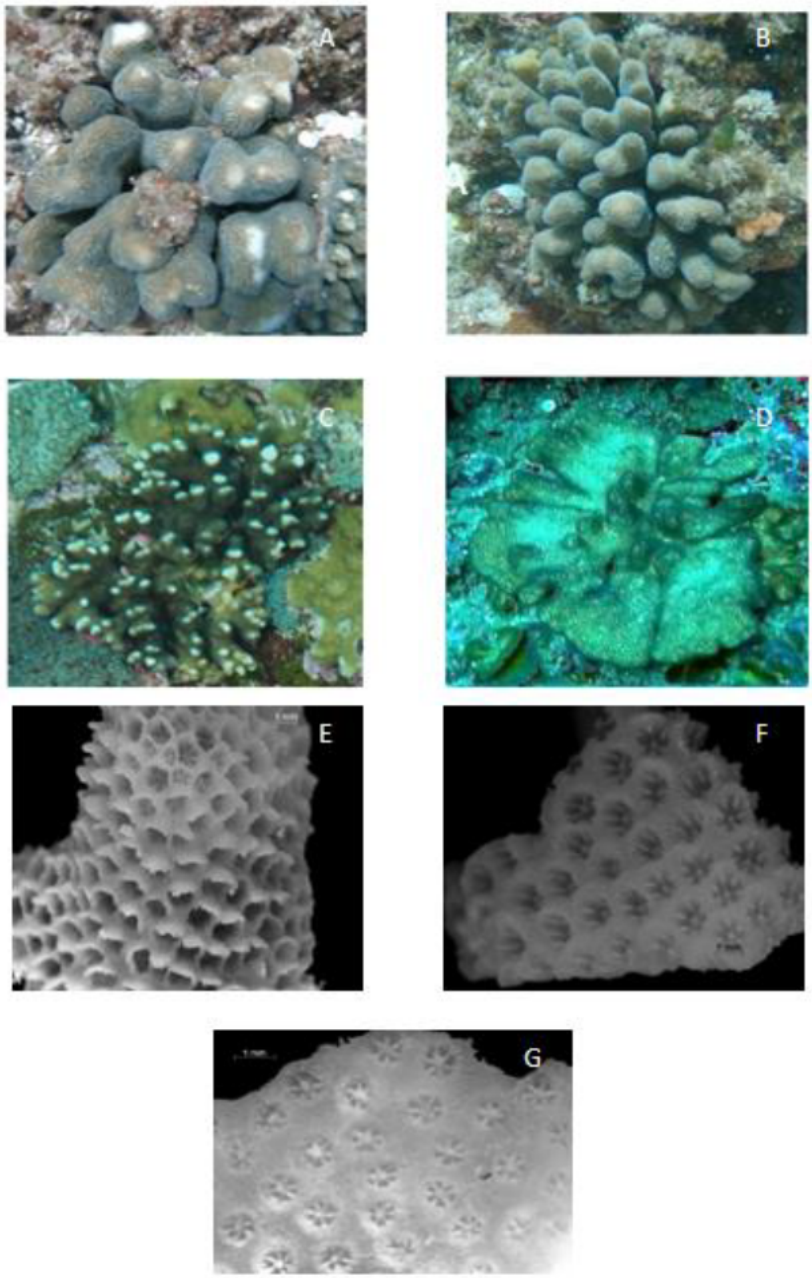


Figure 2.10: A, B, E. "mordax-like" form of Clade 2 found in IWP. C F clade 3 "madagascarensis-like" found in Aliwal Shoal D, G. Flat anastomosing form as clade 3 found in Pondoland. All images taken by members of ORI.

Given that the Leadsman population is more phylogenetically diverse, it suggests that the Leadsman population might be a source population. Populations at the extremes of the distributional range of a species tend to have lower genetic diversity and this is clearly observed moving further away from Leadsman Shoal. The clear separation of mitochondrial and nuclear haplotypes in both the phylogenetic trees, haplotype networks and haplowebs strongly suggest

that, in South Africa, there is no recent hybridization between the two clades. Whether this is because of geographic or genetic separation mechanisms remains to be determined. Given that these morphotypes are only found in the northernmost region of the study area and are also recorded in the literature, this supports the hypothesis that the origin of the *Stylophora* species lies within the Indian Ocean (Veron, 2002; Hoeksema, 2007; Flot *et al.*, 2011).

To assess the level of genetic exchange between populations with the use of multiple molecular approaches, it is extremely important to thoroughly analyse population structures of coral reefs (Takabayashi *et al.*, 2003). Approaches such as the techniques employed in this study are considered vital to assess the impacts of environmental pressures and damage (e.g., bleaching).

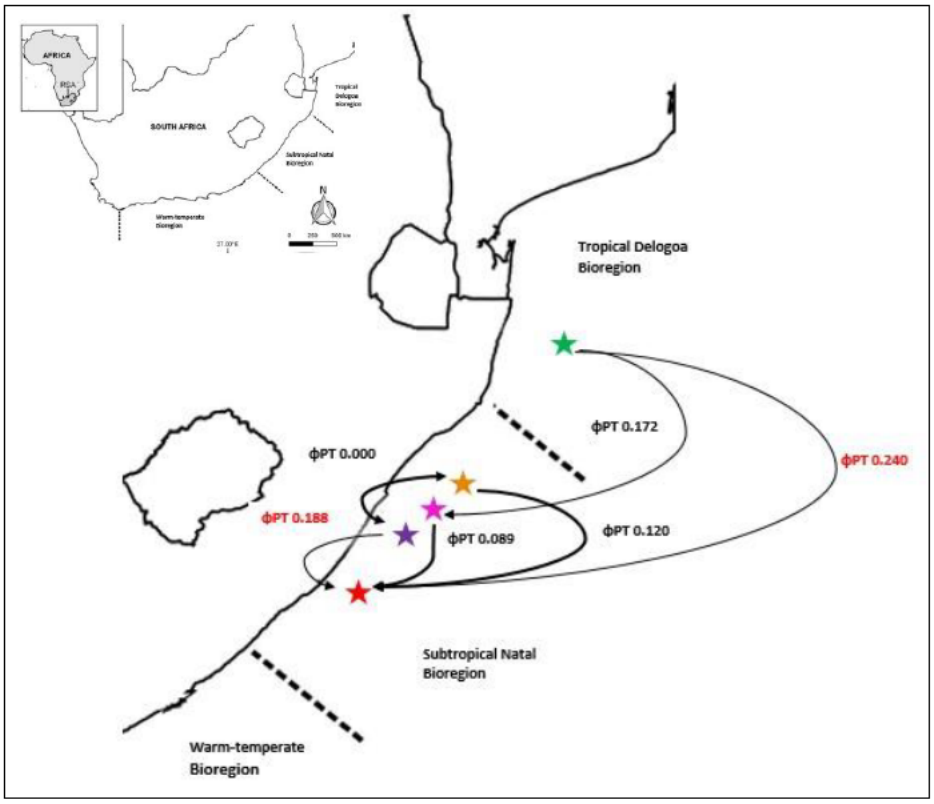


Figure 2.11: A model for clade 3 population connectivity based on the pairwise ϕPT values for the ITS marker, adapted using Google maps (Mannheim *et al.*, 2018).

Analysis of COI revealed few signals of population structure, but it is a poor marker for cnidarians because it evolves very slowly in this group. The more variable (faster evolving) ITS marker does suggest some degree of population structure. Pairwise comparisons between reefs in each location revealed the population structure of best fit for COI and ITS which was confirmed by analysis of different population models and revealed that populations of similar depths are connected. The "best-fit" population model is illustrated on Figure 2.11. This shows the strong connection

between Blood Reef and Aliwal Deep and weaker connections between Leadsman Shoal, Aliwal Shoal and Pondoland. The reasons for the strong connectivity between Blood Reef and Aliwal Deep and lack of connections between the geographically close (8kms) sites is unclear but might be related environmental factors, potentially light availability. Blood Reef is an unprotected inshore reef with high boat traffic and increased sedimentation and turbidity causing low light penetration (Macdonald, Schleyer and Lamb, 2008). The shallower reefs of Aliwal Shoal are further offshore and has a strong current that flows over and allows waters to be clear, but the deeper reefs are not as fortunate as they too have limited light penetration causing a potential environmental pressure for selection. Pondoland is a sink population as it receives input from more northern reefs. Overall, the clade 3 population does not display a classical stepping-stone model of gene flow or isolation by distance model (Chiazzari *et al*., 2019), as there is no genetic exchange between Blood Reef and Leadsman Shoal. Previous studies have determined a similar distribution among other coral species along the KZN coastline (Montoya-Maya, Schleyer and Macdonald, 2016; Chiazzari *et al.*, 2019). A similar study was also done was conducted on *Seriatopora hystrix* whereby it was also determined that populations had clustered caused by unique environmental conditions (strong wave action and low-light conditions) (Bongaerts *et al*., 2010). This proves to be more relevant considering *Seriatopora hystrix* is potentially paraphyletic with *Stylophora*.

CONCLUSION

Two genetically distinct clades of *Stylophora pistillata* have been identified along the KZN coastline and clade 2 is isolated within the Delagoa Bioregion in the study area. These two clades do not share the most recent common ancestor as shown the Figure 2.4. Clade 2 is more closely related to the Pacific clade (1) and clade 3 is more closely related to clade 4 than to clade 1 or 2. Clade 2 and 3 from the Delagoa Bioregion resembled each other more closely morphologically than other clade 3 individuals from the Natal Bioregion resembled clade 3 individuals from the Delagoa Bioregion. This suggests that morphological characteristics are strongly influenced by environmental context and are an unreliable method of determining species identity for these two clades. Population genetic analysis suggests there is population structure across the region. This is based more on ITS than on COI evidence. Leadsman Shoal is a source population and appears the most genetically diverse. Aliwal Shoal and Pondoland populations were the least diverse. Aliwal Deep and Blood Reef appeared more connected than Aliwal Shoal and Aliwal Deep, despite the proximity of the Aliwal Shoal and Aliwal Deep sites. Pondoland appears to be a sink population that is connected to most of the other sites. This is most likely due to its southernmost position and influence of the Agulhas Current. Morphologically, clade 2 and 3 populations of the

Delagoa Bioregion present tubular and digitate morphotypes. Clade 3 of the Natal Bioregion present a more varied morphology including a flat anastomosing form with digits that have grown out, flattened, and fused once contact is made. Findings displayed a non-classical stepping-stone model or isolation by distance model as the results for Leadsman Shoal, Aliwal Shoal and Pondoland conformed with these models, while Blood Reef and Aliwal Deep did not. Overall, the populations manifested a decrease in biodiversity with increasing latitude and proximity to the species' distributional limits. The MPA network protected three of the four populations assessed in this study; the Blood Reef population is unprotected but appears to be continuous with the protected Aliwal Deep population.

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CHAPTER 3: DDRADSEQ OF *STYLOPHORA PISTILLATA* DEMONSTRATES ECOLOGICALLY DRIVEN POPULATION STRUCTURE ALONG THE EAST COAST OF SOUTH AFRICA

ABSTRACT

Population genetic analysis is a crucial tool for estimating the degree of ecological connectivity in marine organisms. Many organisms are expanding and/or contracting their ranges in response to changing environmental conditions and understanding the patterns of genetic diversity and adaptation is crucial to assessing a species' potential for long-term success. Identifying genetic connectivity and discrete population boundaries is an important objective for management of corals along the KZN coastline. We explored the use of ddRADSeq to determine the population structure of scleractinian coral *Stylophora pistillata,* (Clade 3) sampled from existing MPAs (iSimangaliso and Aliwal Shoal) and a non-protected site (Blood Reef) located in the "gap". Interestingly the populations did not appear to be primarily structured by geographic distance with one population consisting of samples from two sites (Blood Reef and Aliwal Deep) that are both geographically distant and at different depths (12-18 m vs >30 m) while a geographically adjacent population (Aliwal Shoal) at 12-18 m formed a distinct population. The Leadsman Shoal population appears to be most closely linked to the shallow Aliwal population.

INTRODUCTION

Based on the previous chapter, we discovered that there are two distinct species of *Stylophora* in KwaZulu-Natal. Clade 2 was found exclusively in the iSimangaliso Wetland Park (IWP) MPA and most specimens were found at a single site (Kosi Bay) with only one individual found in the Leadsman's Shoal, there are not sufficient samples of this clade to determine if there is any meaningful population structure within the MPA. Clade 3 however, had a greater sample size and presented a wide geographical distribution across three widely separated MPAs. Although the mitochondrial marker gave weak evidence for population structure, the use of the nuclear marker ITS showed quite compelling evidence of population structure. With the clade 3 population as a focus group, it was decided to undertake a more in-depth study of this population using a greater density of markers, specifically Single Nucleotide Polymorphisms (SNPs) generated by reduced representation next-generation sequencing using a RADSeq methodology that allows us to maximise data output in a cost-effective manner.

The use of high throughput-sequencing-based methods have become increasing popular as the technology has become cheaper, more customisable to projects, more accessible to scientists and user-friendly (Flot *et al.*, 2011; Ferrari *et al.*, 2016; Terraneo *et al.*, 2016). Next-generation sequencing (NGS) has allowed us to sample genomes much more densely and to observe the

patterns of genetic variation that result from the full range of evolutionary processes acting across the genome (Narum *et al.*, 2013; Shinzato *et al.*, 2015). Using restriction site associated DNA sequencing (RADSeq), it is possible to identify thousands of single nucleotide polymorphisms (SNPs) throughout a genome, and to acquire vast quantities of genomic information from a sample, whereas formerly it was only possible to examine small numbers of neutral molecular markers (e.g., allozymes, microsatellites) covering a very relatively small sampling of the genome (Shinzato *et al.*, 2015). Application of RADSeq to coral population genetics (Combosch and Vollmer, 2015; Shinzato *et al.*, 2015) and linkage mapping (Wang, Meyer, Mckay, *et al.*, 2012; Shinzato *et al.*, 2015) have been reported. RADSeq gives us the ability to look at allele frequencies of thousands of markers and look at any potential population structure in depth and, in addition, determine what the potential drivers of any population structure or selective pressures might be. These can be either ecological drivers (temperature, light, salinity, food availability) or physical drivers (currents, distance, physical barriers, etc.) (O'Leary *et al.*, 2018).

RADSeq can maximise a greater depth of coverage per locus than whole-genome sequencing and sequencing greater numbers of samples within a given budget (Andrews *et al.*, 2016). The evolution and refinement of RADSeq protocols allows increased numbers of loci to be assayed at decreased cost for ecological and evolutionary genomics studies (Miller *et al.*, 2007; Peterson *et al.*, 2012; Wang, Meyer, McKay, *et al.*, 2012; O'Leary *et al.*, 2018). Some RADSeq protocols include the original RAD protocol (mbRAD) (Miller et al. 2007 & Baird et al. 2008), double digest RAD (ddRAD) (Peterson et al. 2012), ezRAD (Toonen et al. 2013) and 2bRAD (Wang et al. 2012).

DdRADSeq produces sequencing libraries which consist of a subset of genomic restriction digest fragments generated by cuts with two restriction enzymes which fall within a selected size-selection window (Peterson *et al.*, 2012; Franchini *et al.*, 2017; O'Leary *et al.*, 2018). Some studies have found certain enzyme pairs work more consistently than others have across a wide range of taxonomic groups after optimizing the ddRADSeq protocol (Burford Reiskind *et al.*, 2016; Yang *et al.*, 2016). An advantage of ddRADSeq over conventional RADSeq protocols, such as ezRAD, is that no shearing or end repair is required, which reduces library development costs (Peterson *et al.*, 2012; Franchini *et al.*, 2017; Holliday, Hallerman and Haak, 2018). ddRADSeq is the most customable protocols compared to ezRAD and 2bRAD as it allows for tuning of fragment number by employing two different enzymes and size selection, and the process of constructing a library is quite simple. Because of their small size, 2bRAD fragments cannot be used to build genome contigs and are less likely to be mapped and cross-referenced across large genetic distances, such as across varied species. 2bRAD produces 36-bp tags that may not be long

enough to be highly unique in a genome as the predicted size of Stylophora pistillata has an estimated haploid genome size of 434Mb.

Limitations of ddRADSeq are that it is particularly susceptible to allele dropout (Arnold et al. 2013) and requires higher quality genomic DNA is than for all other RAD methods (Puritz *et al.*, 2014; Yang *et al.*, 2016).

The double-digest RADSeq approach of Peterson et al. (2012) and modified by Franchini *et al.* (2017) is robust, highly scalable, repeatable, and replicable. High molecular weight DNA is extracted from tissue samples, quantified, and digested with two restriction enzymes. The protocol uses two restriction enzymes: one rare cutter and one more frequent cutter, with adapters specific to each enzyme which will tailor the number of loci produced (Kess *et al.*, 2016; Toonen *et al.*, 2013; Schweyen, Rozenberg and Leese, 2014). The protocol from Franchini *et al.* (2017) is different in more traditional RADSeq preparation models in that restriction enzyme digestion and ligation are combined into a single reaction. This modification thereby limiting the per-sample DNA quantification steps and becoming a more streamlined protocol that reduces hands-on time (Franchini *et al.*, 2017). The use of short four-base tail at the sequencing distal region of each Illumina adapter (i5 and i7) and incorporating two inner (one per paired end) and two outer (one per adapter) six-base barcodes have a two-fold benefit. This method facilitates the removal of PCR duplicates as well as reducing cost per individual by sequencing several dozen uniquely barcoded individuals in a sequencing lane (Franchini *et al.*, 2017). The process involves a small number of barcoded adapters that would be ligated separately to individual samples in microplate format, minimizing the required input DNA per individual. These samples are then pooled following ligation, but before size selection. The size selection step can consist of single pool of hundreds of individuals in the same gel lane of either a hand-prepared electrophoresis lane or an automatic machine and are amplified with a primer that introduces an index that will be read off in a separate multiplexing read per the standard Illumina multiplexed paired-end sequencing protocol. This allows for quality control between steps and ensure equal quantities between pools and increasing the number of overlapping loci among individuals by eliminating the random interlane size variation (Franchini *et al.*, 2017).

Aims and Objectives

The aim of this study is to establish a ddRADSeq, which can be repeatable and used for multiple species that are not often considered a “laboratory-rat’ species. The genome wide SNPs generated using this protocol can also be used to identify non-neutral genomic areas potentially under selection, which might be involved in adaptation to ecological drivers. The use of the coral *Stylophora pistillata* clade 3 is based on the distribution of this coral along the coastline that has

been established to some degree in chapter 2. *Stylophora pistillata* additionally already has a reference genome for a closely related species, clade 1 from the Pacific (Voolstra *et al.*, 2017), which was used as a scaffold during analysis. We aimed to determine what genetic evidence could be found for population structure in *S. pistillata* clade 3 along the east coast of KZN and how it relates to the MPA network. Additional aims of this study include:

1. Develop and optimise a robust and replicable ddRADSeq workflow and analytical pipeline for the generation and analysis of Single Nucleotide Polymorphism (SNP) data for *Stylophora pistillata* that is transferable to other marine species.

2. Determine the population structure and gene flow of these two species using high-density genetic markers.

3. Determine potential environmental drivers of clade distribution and loci under selection.

MATERIALS AND METHODS

Collection

All corals were collected under research permits from the Department of Agriculture Forestry and Fisheries (DAFF) and, where necessary, the iSimangaliso Wetland Park Authority and Ezemvelo-KZN Wildlife. *Stylophora* samples were collected from each of the following sites: Kosi Bay, Leadsman Shoal, Blood Reef, Aliwal Shoal and the Pondoland MPA. Colonies were sampled by taking a small (2-5cm-sized) nubbin from each colony on SCUBA, either from the ACEP small craft, MV Phakisa (Durban and Aliwal) or from the ORI rigid inflatable boat (RIB). Colonies were identified underwater based on morphology and detailed photographs were taken. We were able to use the ACEP Remotely Operated Vehicle (ROV), to identify suitable sampling localities at as-yet unexplored sites beyond commercial SCUBA diving depth limits and were therefore able to collect *Stylophora* samples from Aliwal Shoal up to 20 m on SCUBA and at depths greater than 30 metres (identified as AWL and AWD respectively). Each sample was individually packaged in a sterile Whirl-Pak® bag (Nasco, WI, USA) and preserved with a DMSO–EDTA–salt solution (DESS: 20% DMSO, 0.25M EDTA, pH 8.0 saturated with NaCl) (Gaither *et al.*, 2011) and stored at 4 °C for short periods and -20 °C long term. Harvesting was accomplished using either a chisel and hammer, side cutters or a knife to remove a subsection of the coral or by robotic arm of the ROV and brought to the surface.

Tissue Extraction

Samples were subdivided to allow for multiple extractions if necessary. Sub-fragments of approximately 1 cm in length were used for genomic DNA extraction. They were washed with

PBS to remove excess DESS and then placed in petri dish with 20 mL of 0.2 M EDTA. Tissue was then scraped from the skeleton using a sterile razor blade. The cell suspension was transferred to a 50 mL falcon tube and left on ice for 2 hours. The suspension was passed through a 100 µm and 30 µm sequential MACS SmartStrainers (Miltenyi Biotec Inc. Auburn, CA, USA) to reduce the number of *Symbiodinium* cells present in the extracts (Woodley *et al.*, 2015). Genomic DNA was extracted using a Zymo universal gDNA MiniPrep kit using the tissue extraction buffer and performed as per the user manual except for an overnight digestion step. Quantification of the DNA was determined using Qubit Broad Range kit

Library Preparation and MiSeq

Double digestions of 200 ng high molecular weight DNA template from each of 42 samples, using the frequent-cutting restriction enzyme *PstI* (ThermoFisher) and the rare-cutting restriction enzyme *MspI* (ThermoFisher), and ligation of the modified and barcoded Illumina adapters were conducted simultaneously. Each of the 12 unique combinations of the quaddRAD-i5 and quaddRAD-i7 adapters were ligated to each batch of samples (three batches of 12 and one batch of six samples, where six combinations were used), independently (Appendix A). This was accomplished in 40 mL reactions, as detailed by Franchini et al. (2017), but with 0.5 mM ATP to facilitate the simultaneous digestion and ligation using ThermoFisher T4 DNA ligase, and with the concentration of *PstI* doubled to allow the double digestion in 1 x Tango buffer (ThermoFisher). Incubation was at 30 °C for four hours, with deactivation by the addition of EDTA to 20 mM (Gel image in Appendix B Figure S1). Each batch of 12 samples with differing inner barcode/adapter combinations was pooled, purified and size selected with a double-sided selection using Ampure XP (Beckman Coulter, Indianapolis, Indiana) beads at 0.5 × and 0.8 ×. Products were eluted in in 40 mL ddH2O, and concentrations determined with a Qubit High Sensitivity assay.

Outer indexes were added through an indexing PCR, with each of the four pooled samples being indexed with a unique combination of the four D5xx and four D7xx indexes, using each index combination only once. PCRs were set up in 100 mL volumes, as in Franchini et al. (2017), using AccuPOL DNA Polymerase and 1 × ammonium buffer (15 mM MgCl2) (Ampliqon A/S, Odense, Denmark). The thermocycling regime followed Franchini et al. (2017), but with denaturing performed at 95 °C and using 15 cycles. Indexed products were purified with Ampure XP beads at 0.8 ×, eluted as above and concentrations determined with the Qubit Broad Range assay. Fragments were sized and concentrations determined using a Bioanalyzer 2100 chip (Agilent Technologies, Santa Clara, California), with a High Sensitivity kit. Indexed samples were pooled equimolarly (at 8.5 nM/L, according to the BioAnalyser determined concentration of the 279 –

281 bp peak) and concentrated using an additional Ampure XP purification at 1.8 ×. A tight size-selection of the targeted fragments (252 to 308 bp) was accomplished using a PippinPrep (Sage Science) by the Central Analytical Facility at the University of Stellenbosch (South Africa) to remove improper ligations and ensure for on completely intact overhangs. The concentration of the eluted, size-selected library was determined using a High Sensitivity BioAnalyser analysis [Mean size: 302 bp; 1379 pM, eluted in 40 mL]. Sequencing was conducted at the NRF-SAIAB Aquatic Genomics Research Platform on an Illumina MiSeq, using a v3 150-cycle kit. The standard library preparation and loading was followed, with a PhiX spike of only 1% because of the high complexity library.

The size selected RADSeq library was checked using an Agilent BioAnalyser (Appendix B Figure S) and sequenced on an Illunima MiSeq instrument using the v3 150-cycle kit (Thermo Scientific) giving 2 X 75 bp paired-end sequences.

Bioinformatics and Analysis Pipeline

Sequences were retrieved in fastq format from the sequencing facility and transferred to the lengau cluster of the Centre for High Performance Computing (CHPC) run by the National Research Foundation (NRF, South Africa). Sequence processing from raw fastq to list of SNPs was performed using the STACKS (version 4.21) package (Catchen *et al.*, 2011, 2013). In STACKS (version 4.21), sequence groups were demultiplexed "on the fly" according to the unique i5 – i7 indexes to give four groups (Appendix B Table S3). Each of these groups was processed using the *process_radtaqs* module from STACKS with inline-inline barcodes to remove the inline-inline barcode. 5' and 3' internal indexes and split each group into its constituent individuals. This step also removed the *PstI* and *MspI* overhangs and the sequence were trimmed to 65 bp for uniformity and to facilitate stack creation.

There are two main pipelines depending on the availability of a reference genome, to build loci either de novo (*denovo_map.pl*; hereafter de novo map) or aligned to an existing reference genome belonging to the same or a closely related sequence (*ref_map.pl*; hereafter refmap). Both *de novo* and reference-based analysis was conducted for comparison.

Parameters *M* (controls the number of mismatches allowed between the two alleles of a heterozygote sample), and *n* (controls the number of mismatches allowed between any two alleles of the population) are set between 1-9 to ensure that a large number of reads are used and to also reduce the proportion of artificial loci, alignments, or calls. Using small sample subset, the *denovo_map.pl* was used to test the effects of varying m=n= 1 to m=n=9 for the purpose of optimization the calling of SNPs per locus and loci (Figure 3.2) while other parameters were kept at the default values (M, the number of identical reads required to initially define a stack during

loci construction was set to 3). Once the optimal m and n values were determined the denovo.pl pipeline was run with the whole dataset. Individuals AWD011, AWD024, LDS102, BRF015 and BRF016 were removed because of very low coverage (less than 10X) and full population analysis was repeated.

The reference genome was retrieved from GenBank is from a sample found in the Pacific (clade 1) and has a total scaffold length of 400 million bp and consisting of 5,000 scaffolds with a scaffold N50 of 457 kb (Appendix B Table S6) (Voolstra *et al.*, 2017). Reference loci were indexed using BWA version 0.7.7 (Li & Durbin, 2009) or Gsnap (version 2015-12-31; Wu & Nacu 2010) which aligns the consensus sequence of the catalog loci from the optimal *denovo* map runs for comparison. BAM files for each individual in the analysis were created by mapping their cleaned, trimmed reads to the reference using bwa-mem (Li, 2013), sorting the reads using samtools. We compared the number of uniquely mapping loci that were assembled using an alignment from *refmap*, to those aligned using *denovo* map consensus sequences (Appendix B Table S4 and S5).

This catalog made with optimal M=n=3 was then analysed using the populations module with the different population assignments. In the *populations* module of STACKS, all individuals were initially grouped into their respective sampling localities. Additional parameters used included monitoring the number of polymorphic RAD loci found in 80% of samples or more (the r80 loci), the Minimum Allele Frequency (MAF) a variable site must possess to be included (--min_maf) set to 0.05, and the maximum level of heterozygosity a variable site can possess to be included (--max_obs_het) set to 70% (Peterson *et al.*, 2012; Franchini *et al.*, 2017). To identify potential markers in genomic regions subject to selection, we used the FST outlier method (Beaumont & Nichols 1996). The final output files included –fstats, to calculate a nucleotide-level FST value, using an analysis of molecular variance framework (Slatkin, Maddison and Weir, 1991), data was exported in genepop (--genepop) and structure (--structure) format in order to facilitate downstream analysis (Reitzel *et al.*, 2013)

To test for potential population structure, we used Bayesian clustering analysis as implemented in STRUCTURE 2.3.4 (Pritchard, Stephens, & Donnelly, 2000), which detects significantly genetically differentiated clusters or populations (K). We ran the program for 500,000 MCMC steps following a burn-in of 100,000 steps using the admixture model with correlated allele frequencies. We set sampling locations as a priori (LOCPRIOR model) because we expected structure signal to be weak (Hubisz, Falush, Stephens, & Pritchard, 2009) and included admixture in order to measure potential geneflow between populations (Leydet & Hellberg, 2015). We performed 10 iterations for each inferred number of genetic clusters, K=1–6, and used the Evanno

method (Evanno, Regnaut, & Goudet, 2005) implemented in STRUCTURE HARVESTER (Earl & von Holdt, 2012) to determine the most likely number of genetic clusters. Figures show the barpolt of k-value 2 to 5. One individual, AWD023 indicated an identical genetic profile to the samples collected in Leadsman Shoal of the tropical bioregion.

In addition to the STRUCTURE analysis, we used principal component analysis (PCA) using both the find groups and DAPC approach (60 retained eigenvectors and two PCs) to explore spatial relationships in two dimensions using the ADEgenet, version 2.1.3 (Jombart, 2008; Jombart and Ahmed, 2011), package in R. PCA on all sets of loci split north and south samples along the first principal component, similar to the division in the STRUCTURE analysis. The distance measures obtained from this approach were used to build a Neighbour-joining tree (Figure 3.3) which showed clear separation into three clusters: with only one individual from the Aliwal Deep not clustering with the northern group consisting primarily of Leadsman Shoal individuals. There seems to be little admixture between the clusters.

Finally, we used a Bayesian method (Beaumont and Balding, 2004) implemented in the program BayeScan version .2.1 (Foll and Gaggiotti, 2008) to determine loci that appear to have remarkably high or low Fst values and thus might be under selection. This was conducted with a burn-in period of 50,000 followed by 100,000 iterations, subsequently identifying outliers in R with a false discovery rate q- value of .01 (i.e., 1%) to determine which loci were putatively under selection (Van Wyngaarden *et al.*, 2017). We used our BLASTn and BLASTx searches of the contigs representing the SNPs (Table 3.4) to identify possible genes and functions associated with loci putatively under selection. Loci identified as potentially under selection were mapped back to the reference genome and any corresponding genes were identified and functionally characterised by reference to the literature and gene function databases (Table 3.6)

RESULTS AND DISCUSSION

The RADSeq generated 32,314,222 raw reads with a mean of 8 million reads per library. In total, 3,030,583 (9%) reads with low quality, ambiguous barcode and short length were discarded. On an average, there were 575,177 reads per individual (range: 19,142–1,630,315 reads). We used the *clone_filter* program to remove any read having the same combination of random tag and RAD sequence, which probably represent duplicates created during PCR amplification. Table S7 and Table S8 in Appendix A show the number of retained reads per group as well as per individual. We ran *process_radtags* to truncate reads to 65 bp and demultiplex reads by the unique inner adapter combinations, which produced paired-end files for each individual in each group. Figure 3.1 displays graphically the varying total number of read and retained reads per individual.

Individuals of low retained reads were noted and the whole dataset was still used in *denovo_map.pl*.

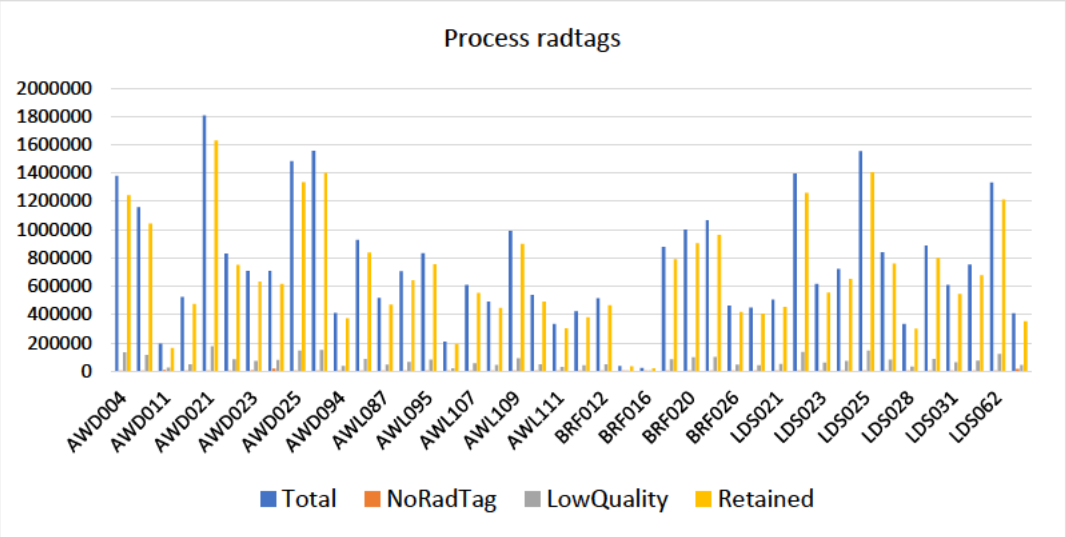


Figure 3.2: Graphic representation of the varying total number of read and retained reads per individual accomplished using *process_radtags* in STACKS.

Iterated de novo runs of a subset of individuals were run to determine the optimal M and n parameters. After visual inspection (Figure 3-2), it was determined than M=3 and n=3 would be the optimum parameters for a full analysis. Running the de novo pipeline with these parameters generated a total of 430,130 loci in the 42 individuals (from 491 to 24,692 per individual; mean: 10241 SD: 7 972) with an average coverage of 36.69 (from 10.39 to 54.89; SD: 9.19).

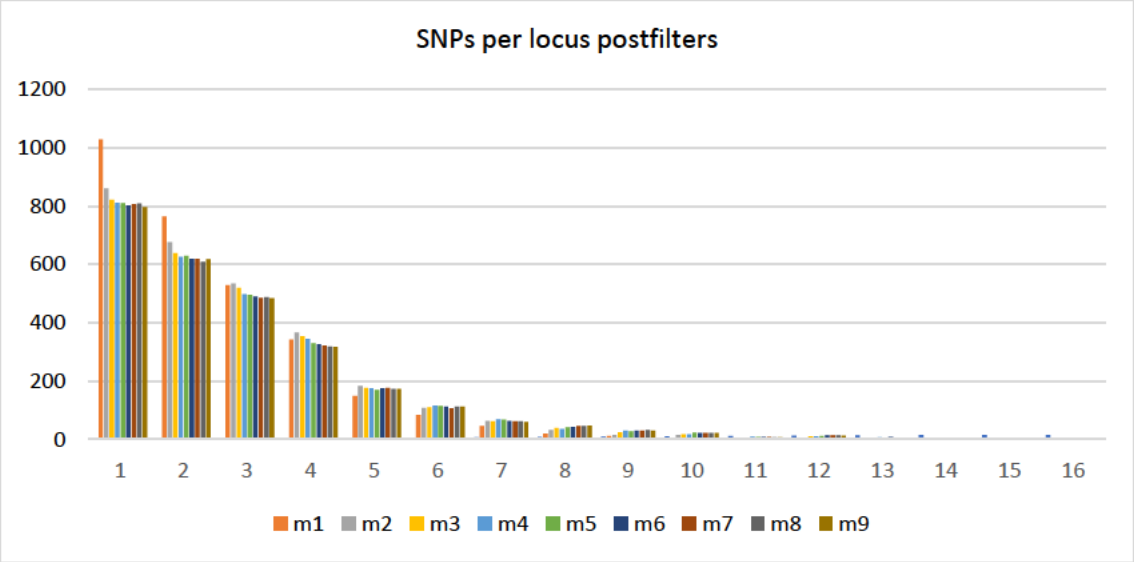


Figure 3.3: Selection of assembly parameters in a *de novo* analysis. The M and n parameters are kept equal, and the M parameter is fixed to 3.

Table 3.1: Populations comparisons of *de novo*, BWA and Gsnap.

	Denovo	**m=3**	**BWA**		**Gsnap**	
Individual	**n_loci**	**mean_cov**	**n_loci**	**mean_cov**	**n_loci**	**mean_cov**
LS_LDS021	12065	9.008	8710	8.456	8231	8.677
LS_LDS022	23085	14.757	11099	22.308	10542	22.403
LS_LDS023	13663	10.268	8915	10.567	8446	10.903
LS_LDS024	12683	13.161	10012	13.010	9479	13.097
LS_LDS025	24692	15.332	11550	22.160	10746	23.053
LS_LDS027	15500	12.578	10691	13.591	9918	13.909
LS_LDS028	6184	10.459	8408	6.907	7911	7.363
LS_LDS030	16725	11.964	10659	13.549	10053	13.947
LS_LDS031	12325	11.047	9387	10.616	8883	10.991
LS_LDS037	13354	13.062	10073	13.390	9477	13.479
LS_LDS062	11114	28.998	11555	25.773	11014	25.942
LS_LDS102	6287	18.137	5864	1.840	5941	1.965
BR_BRF010	6753	14.874	9161	10.355	8600	10.684
BR_BRF012	7622	15.580	9802	11.738	9264	11.931
BR_BRF015	629	6.013	-	-	-	-
BR_BRF016	491	6.151	-	-	-	-
BR_BRF019	10077	20.865	10732	18.471	10170	18.680
BR_BRF020	10518	22.809	10989	20.692	10410	20.889
BR_BRF022	10460	24.048	10940	22.250	10390	22.557

	Denovo	m=3	BWA		Gsnap	
Individual	**n_loci**	**mean_cov**	**n_loci**	**mean_cov**	**n_loci**	**mean_cov**
BR_BRF026	8700	11.099	9231	9.065	8683	9.383
BR_BRF028	7667	14.042	9909	10.354	9317	10.558
AS_AWL071	10502	19.897	10522	17.014	9853	17.708
AS_AWL087	7183	17.019	10111	11.398	9490	11.639
AS_AWL088	8382	20.740	10066	15.794	9533	16.028
AS_AWL095	8827	21.719	10424	16.876	9697	17.338
AS_AWL097	4956	10.447	-	-	-	-
AS_AWL107	7370	20.100	10105	13.809	9386	14.244
AS_AWL108	6883	17.533	9452	12.053	8846	12.212
AS_AWL109	9298	26.266	10691	20.990	10097	21.380
AS_AWL110	7141	18.493	9630	12.815	9036	13.117
AS_AWL111	5980	13.878	8765	8.707	8112	8.886
AD_AWD004	11671	26.760	11985	25.012	11348	25.604
AD_AWD006	11350	24.990	11146	23.756	10721	23.510
AD_AWD011	5458	7.239	-	-	-	-
Individual	**n_loci**	**mean_cov**	**n_loci**	**mean_cov**	**n_loci**	**mean_cov**
AD_AWD017	8220	13.846	9287	10.880	8712	11.157
AD_AWD021	15595	25.699	12099	30.551	11411	31.140
AD_AWD022	11418	16.529	10672	15.553	10061	16.027
AD_AWD023	13683	13.168	8505	7.600	8003	7.808
AD_AWD024	12624	13.436	5635	1.789	4910	1.809
AD_AWD025	14412	23.659	11615	26.371	11013	26.914
AD_AWD093	11871	31.412	11876	29.676	11330	29.632
AD_AWD094	6709	13.460	9247	9.199	8731	9.234

Table 3.2 shows a summary of the "populations" module after the stacks analysis is referenced onto the reference genome using *ref_map.pl*. Interestingly, the number of private alleles is another measure of the genetic variation and isolation, as well as potentially population size and/or selection. Aliwal Deep displays the highest number of private alleles of 434, this can indicate that this reef is either isolated from other reefs or appears that it has a source of gene flow that is not shared with other reefs. In this case, it suggests that the populations have been largely separated for an extended period of time (sufficiently long for different alleles to have evolved). This provides the first evidence those three distinct populations with truly little gene flow between them. Interestingly, Blood Reef is the most closely related to Aliwal Deep and presents with the lowest number of private alleles. This suggests that either the direction of gene flow is from

Aliwal Deep to Blood Reef and/or that the effective population size for the Aliwal reefs is higher than Blood Reef.

Table 3.2 Population summary statistics of *ref_map.pl* of *Stylophora Pistillata*

	Samples per locus	**Pi value**	**All sites**	**Variant sites**	**Polymorphic sites**	**Private alleles**
LDS	10.49	0.222	587576	5065	3490	400
BRF	8.268	0.142	365853	2200	1078	64
AWL	9.483	0.202	587645	5058	3123	344
AWD	8.689	0.212	592800	5094	3676	434

Fstats results were run using the 'populations' module with the following parameters: a minimum call rate of 80% (−r 0.80), a maximum observed heterozygosity of 70% (--max_obs_het 0.70), 4914 loci were retained. Pairwise Fst values showed Blood Reef and Aliwal Deep populations are most closely linked (Fst 0.049) (Table 3.3). Leadsman Shoal is most different to Blood Reef despite this being the closest site in geographical terms (~280 kms) while the more distant Aliwal Shoal site (~320 kms) is more closely related to Leadsman Shoal. The geographically proximal sites Aliwal Shoal and Aliwal Deep are relatively less connected (~8kms) (Fst 0.146).

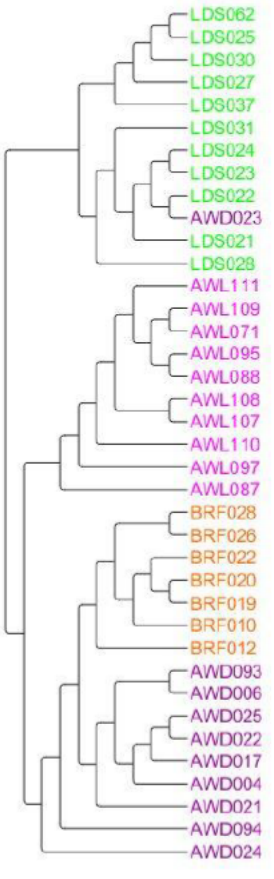
Table 3.3: Fst evaluations between parameters of geographical distribution

Geographical Origin			
	AWL	**BRF**	**LDS**
AWD	0.146	0.049	0.140
AWL		0.165	0.114
BRF			0.161

Fst values were calculated between population in Stacks using *populations* and –fstats command. The first table is based on full haplotypes and with individuals grouped by geographical origin (-r 0.8 –min_maf 0.05 –max_obs_het 0.7), (Table 3.3). Initial Fst revealed that Aliwal Deep and Blood Reef are most closely related.

To evaluate the validity of putative populations defined by their sampling location, we inferred population structuring through a principal component analysis (PCA). A PCA with the two primary eigenvectors (axes of variation) comprising greater than 80% of variation revealed the presence of three distinct clusters (Figure 3.3). Figure 3.4 shows the assignment of clusters between the sample locations. The northern population (Leadsman Shoal and AWD023) appear

to be the most genetically distant, Aliwal Shoal is closer to Blood reef and Aliwal Deep than to Leadsman Shoal, and Blood Reef and Aliwal Deep appear to be genetically the closest.



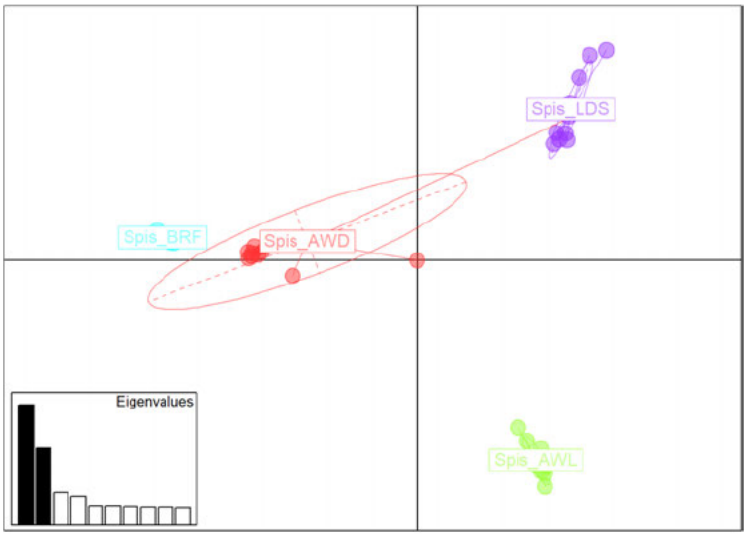


Figure 3.3: PCA using SNPS. Each point represents an individual colour coded by sampling origin

Figure 3.4: Tree of 37 individuals in four populations based on SNPs in >80% samples, computed using dapc analysis in ADEgenet.

We also inferred population structuring (historical lineages) by maximizing the posterior probability of the genotypic data, given a set number of clusters (K). This method is known as Bayesian population clustering and is implemented in the program STRUCTURE v2.3.2 (Pritchard et al. 2000; Falush et al. 2003). The admixture model was used with uncorrelated allele frequencies. The MCMC was run for 100,000 repetitions (burnin period 100,000). Values of K from 1 to 5 (10 replicates each) were evaluated. The optimal value of K was selected using the program STRUCTURE HARVESTER v0.6.92 (Earl & Vonholdt 2012) according to the ad hoc Delta K statistic (Table 3.5) (Evanno et al. 2005), which is the second-order rate of change of the likelihood function.

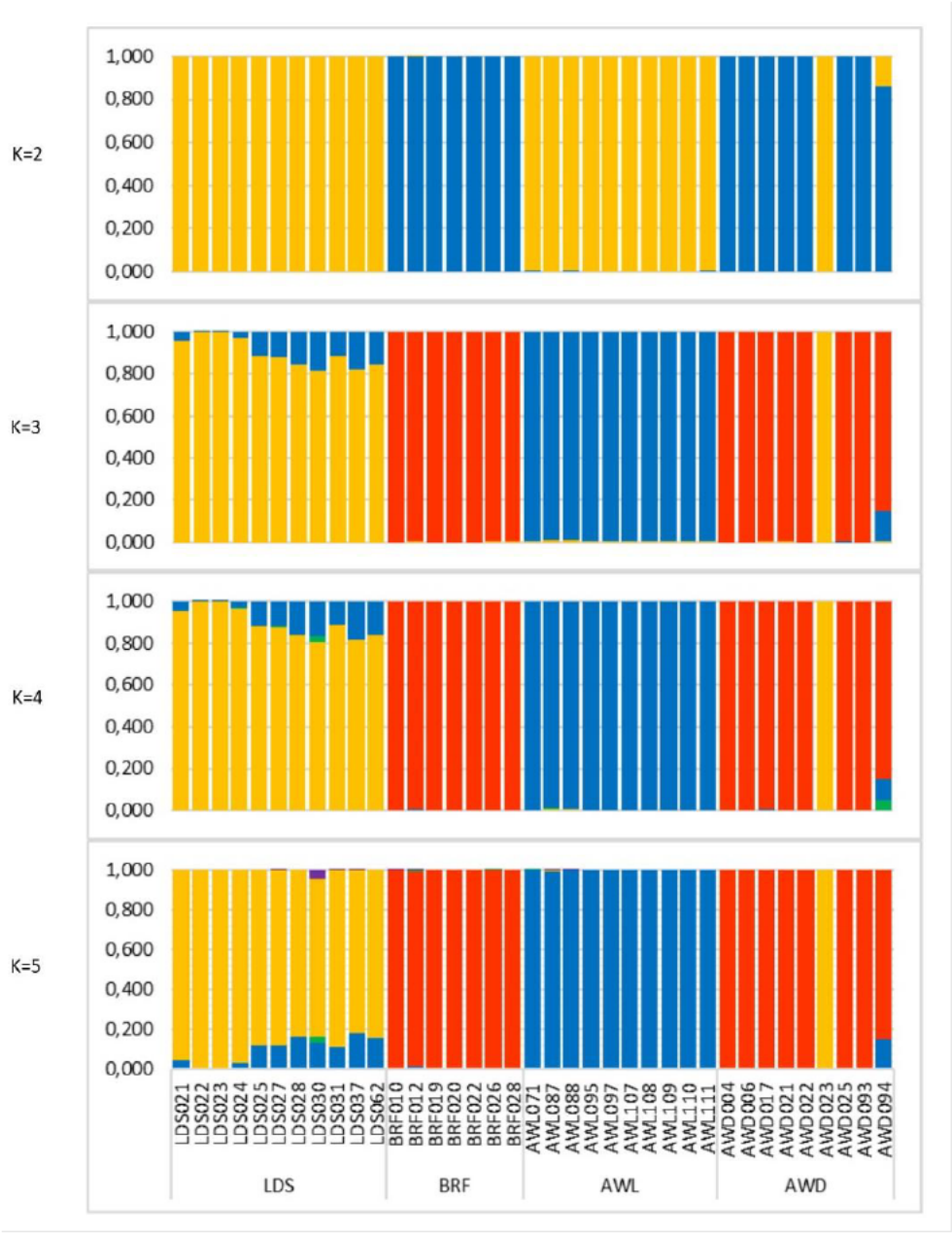


Figure 3.5. Plot of admixture proportions at the population level, at K = 2-5, identified using structure is shown in the top panel. shown is the probability of membership (*Y*-Axis) In a given cluster for each sample (*X*-Axis) assuming values of K = 2, K = 3, K = 4 and K = 5. Analysis of 3622 SNPs (analysis included only one snp per locus) after combining read 1 and read 2. The most probable K was 3 (b. reefs within geographic regions are sorted by latitude.

Table 3.4: Summary K and Delta K between 1 To 5. Generated in STRUCTURE

K	Reps	Mean LnP(K)	Stdev L	nP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	5	-85661.6200	13.2422	NA	NA	NA	
2	9	-70924.7000	26.3979	14736.	920000	10020.208889	379.583323
3	9	-66207.9889	25.1571	4716.7	11111	4754.377778	188.987673
4	9	-66245.6556	73.6016	-37.66	6667	31.744444	0.431301
5	9	-66251.5778	62.1037	-5.922	222	29.320000	0.472114
6	5	-66286.8200	76.5774	-35.24	2222	NA	NA

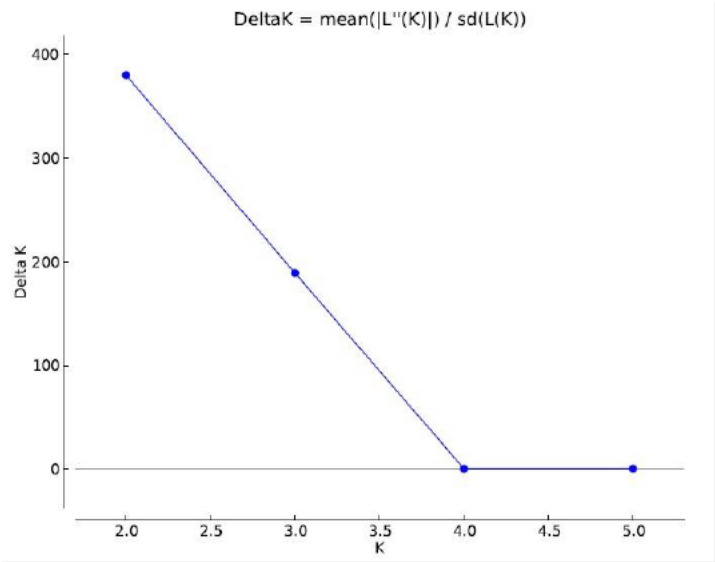


Figure 3.6: Figure shows the Delta k for all values 2 to 5 and shows inflection point at K=4.Plot of Δk values from four runs of STRUCTURE on the dataset, varying K from 1 to 10 are shown in the top panel. The most likely value for K was identified at K = 4. Plot.

With the information gathered using STUCTURE and STRUCTURE HARVESTER (Figure 3.3, 3.4 and 3.5), individuals were assigned populations based on their genetic structure. Aliwal Deep and Blood Reef were placed into a population and one individual (AWD 023) was grouped with the Leadsman Shoal individuals. The Aliwal Shoal individuals all group together. Fst calculations statistics showed significant differences between populations. Fst values were calculated using both single SNPs per locus and full haplotypes given that Fst based on haplotypes is known to underestimate Fst values, individuals are assigned to populations based their genetic assignment firstly with the full haplotypes, and finally using a single SNP per locus. These are: (Pop1) a northern population consisting of all the clade 3 Leadsman Shoal individuals plus a single

individual from the Aliwal Deep sites; (Pop2) all the Aliwal Shoal populations and (Pop3) all the remaining Aliwal Deep individuals and all the Blood Reef individuals. Measures of genetic differentiation (Fst) between these distinct populations are high (Table 3.5), particularly for Pop2 (AD+BR) vs the other two populations suggesting that there is little exchange of genetic material between these populations, despite the close physical proximity (AWD and AWS are separated by only ~8kms).

Table 3.5: Fst of populations based on genetic population structure and --*write-single-snp* parameter in STACKS

Genetic Population Structure – full alleles			
	AWD+BRF	**LDS+1**	**AWL**
AWD+BRF		0.166	0.161
LDS+1			0.120
Genetic Population Structure – SNP			
	AWD+BRF	**LDS+1**	**AWL**
AWD+BRF		0.171	0.166
LDS+1			0.122

Determination of Loci Under Selection

BayeScan (version 2.1) was used to determine outlier SNPs based on their Fst values related to a postori model of Fst value distribution. Figure 3.7 shows a manhattan plot of the Fst as calculated from BayeScan against the locus. If we had a more complete genome, we could use this to show how the areas with extremely high Fst values are distributed physically across the chromosomes. As we have a disjointed reference assembly (considerable number of small contigs) we can just use this to show that the high Fst loci (i.e., potential areas under selection) are randomly distributed. However, there does appear to be a number of significant Fst values that may be under selection.

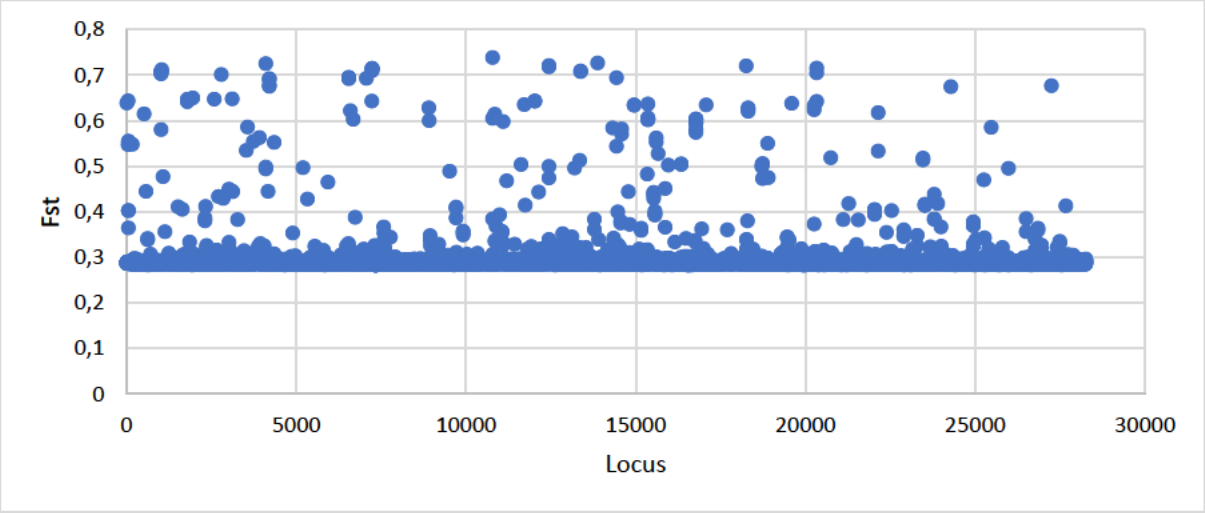


Figure 3.7: Scatter plot if the distribution of Fst values across individual loci.

The vast majority of Fst values are low but some high Fst values are present indicating possible signals of selection at those loci. In order to identify outlier loci, a modified Fdist and BayeScan likelihood method was used to identify outlier loci with both a 5% and 1% false discovery rate (FDR) threshold (Storey and Tibshirani, 2003; Lotterhos and Whitlock, 2014). The false-positive rate is the number of significant neutral loci (false positives) divided by the total number of neutral loci tested. With an FDR of 1%, 21 outlier SNPs were identified and 49 were identified at the 5% level. Many of the SNPs were located in the same, or adjacent loci and a total of 30 loci were identified using the combined outlier SNPS.

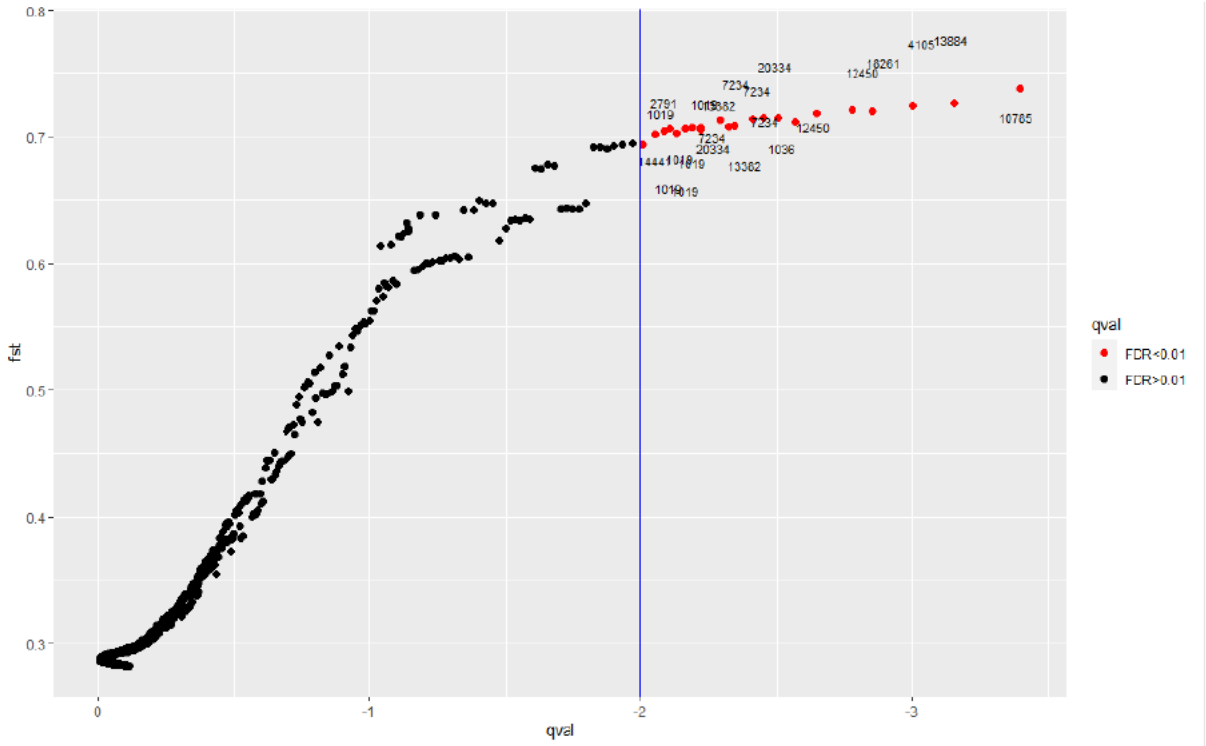


Figure 3.8: SNP outlier analysis in diversity set using Bayescan. utlier locus analysis, blue vertical line indicates threshold (False discovery rate (FDR) ≤ 0.01). Markers on the right site of the threshold are significant outlier (red dots).

Significant outliers and determined in Figure 3.8, loci were noted with SNPs associated to each locus. Table 3.6 shows the 30 loci that were then cross referenced to the reference genome of *S. pistillata* to determine a contig GenBankID using NCBI, and again cross-referenced to determine whether there are any genes associated with the loci under selection. The GenBank genomic viewer was used to visualise the contigs and identify genomic features, such as genes, associated with the selected loci.

Table 3.6: Outlier Loci with SNP data, Stylophora referenced contig GenBank ID, Position of contig in relation to the genome and associated gene to determine selection.

Locus	SNP	FDR 0.01	FDR 0.05	log10 (qval)	Stylophora contig genbankID	Nucleotide position in contig	Associated genes
44	108		x	-1,724067	NW_019217784.1	399302	E3 ubiquitin-protein ligase MARCH6-like
1019	34	x	x	-2,1897608	NW_019217789.1	1033611	Cation-independent mannose-6-phosphate receptor
	39	x	x	-2,1319201			
	40	x	x	-2,2217619			
	41	x	x	-2,1061495			
	45	x	x	-2,1610628			
	61	x	x	-2,0830831			

Locus	Snp	FDR 0.01	FDR 0.05	log10 (qval)	Stylophora contig genbankID	Nucleotide position in contig	Associated genes
1036	89	x	x	-2,5662622	NW_019217789.1	1347690	mucin-5AC-like isoform X1
2791	93	x	x	-2,0536411	NW_019217804.1	326599	protein O-GlcNAcase-like
3111	137		x	-1,7966951	NW_019217807.1	513081	myotubularin-related protein 1-like isoform X1
4105	108	x	x	-2,9999131	NW_019217817.1	658141	Phosphonoacetaldehyde hydrolase
4210	46		x	-1,608324	NW_019217818.1	729567	uncharacterized protein LOC111322078 isoform X2
	48		x	-1,6534514			uncharacterized protein LOC111322078 isoform X2
4212	36		x	-1,8252136	NW_019217818.1	735446	uncharacterized protein LOC111322078 isoform X2
	54		x	-1,8744862			uncharacterized protein LOC111322078 isoform X2
6546	23		x	-1,8502576	NW_019217848.1	523930	angiopoietin-1-like
	24		x	-1,9685107			
	140		x	-1,9327803			
7065	164		x	-1,901945	NW_019217856.1	140567	none
7231	134		x	-1,7462986	NW_019217858.1	370758	importin-13-like
7234	118		x	-2,4088463	NW_019217858.1	372508	importin-13-like
	119	x	x	-2,2887075			
	120	x	x	-2,4517335			
	121	x	x	-2,3450922			
8906	47		x	-1,5022275	NW_019217887.1	483089	GATOR complex protein DEPDC5-like
10782	95		x	-1,3628206	NW_019217925.1	189962	glycerol-3-phosphate transporter 1
10785	9	x	x	-3,3978532	NW_019217925.1	203706	ankyrin repeat, PH and SEC7 domain containing protein secG-like
11716	39		x	-1,5880269	NW_019217945.1	207245	neuropeptide FF receptor 2
12035	45		x	-1,7706254	NW_019217952.1	312243	caspase-3-like
	50		x	-1,7023489			
12450	18	x	x	-2,6445293	NW_019217963.1	435527	glycosyltransferase-like domain-containing protein 1
	19	x	x	-2,7746132			
13382	5		x	-2,3214634	NW_019217990.1	223148	none
	120	x	x	-2,3450922			none
13884	15	x	x	-3,1548151	NW_019217990.1	235304	none
14441	71	x	x	-2,0083	NW_019218018.1	259478	None
14954	89		x	-1,5189015	NW_019218034.1	220288	Meckel syndrome type 1
	138		x	-1,5513246			

Locus	Snp	FDR 0.01	FDR 0.05	log10 (qval)	Stylophora contig genbankID	Nucleotide position in contig	Associated genes
15361	26		x	-1,3102828	NW_019218046.1	125604	None
	124		x	-1,5693607			
16775	30		x	-1,3266473	NW_019218091.1	232304	peroxidase mlt-7-like
17082	62		x	-1,5345279	NW_019218132.1	59971	uncharacterized LOC111337884
18261	107	x	x	-2,8537789	NW_019218150.1	64012	death-associated protein kinase 2
20334	19	x	x	-2,5050666	NW_019218247.1	109334	E3 ubiquitin-protein ligase HERC2-like
	28		x	-1,3827375			
	96	x	x	-2,2217619			
22148	44		x	-1,4756428	NW_019218369.1	159509	uncharacterized LOC111342762
24285	37		x	-1,6303019	NW_019218588.1	33949	TNFAIP3-interacting protein 1-like/Tnip1
27254	11		x	-1,6773466	NW_019219460.1	17278	None

CONCLUSION

Results of the RADSeq analysis of a subset of our *S. pistillata* clade 3 samples showed noticeably clear population structure along the KZN coast. STRUCTURE and PCA analyses of 1,999 independent loci show that the samples from the four different sites partition cleanly into three different populations.

The analyses showed that there are three distinct clusters among the clade 3 individuals. Individuals sampled from Leadsman Shoal are closely related to one another with minor variation among them. Analysis of the STRUCTURE results using the evanno method suggest that the best fitting model is K=4. This is surprising as there are no individuals assigned to the putative fourth group and it appears to contribute little to any particular individual and only to individuals from the Leadsman group. Aliwal Deep and Blood Reef individuals form one panmictic population. Interestingly, based on the PCA and structure analysis, there is one individual sampled from Aliwal Deep (AWD023) that clusters with the Leadsman Shoal population. This individual appears to be a first-generation migrant that has no admixture from other clusters. This implies that, while migration of individuals does occur over relatively long distances (>300 km) and those individuals can establish and grow in their unfamiliar environment, they do not appear to contribute meaningfully to the genetics of the local population. A greater number of individuals sampled will help clarify this situation.

There are 30 loci that are associated with outlier 49 SNPs (FDR of 0.05). Genes associated to these loci are involved in a large number of processes but appear to be enriched for proteins involved in protein degradation and apoptosis (E3 ubiquitin-protein ligase MARCH6-like, caspase-3-like) as well as cytoskeleton and cell adhesion (mucin-5AC-like isoform X1, myotubularin-related protein 1-like isoform X1 and ankyrin repeat, PH and SEC7 domain containing protein secG-like). However, care needs to be taken in interpreting this as no functional data in corals is available for these proteins, their functions are inferred based on their homology to proteins from other, evolutionarily distant, organisms. Additional functional studies would need to be carried out to confirm these findings.

These results suggest that there is strong population structure along the KZN coast and that this appears to be mediated by environmental parameters as well as by distance. The two most closely related sites (Blood Reef and Aliwal Deep) are not the closest together suggesting that there is an environmental filter that prevents propagules from more proximal sites becoming established in these sites. The environmental parameter that drives this is currently unknown but empirical observation indicates that both Blood Reef and the Aliwal Deep Reefs have very low light penetration, Blood Reef because of its proximity to the shore and several sources of sediment (Durban Harbour and the Mlazi River canal) and the deeper Aliwal sites due to light attenuation through the relatively turbid water.

It also suggests that, particularly in the case of the Aliwal Shoal MPA, the deeper reefs are not capable of acting as refugia to replenish the shallower population. In terms of management recommendations, more care needs to be taken to protect the deep reefs as this forms a distinct population. The evidence of limited population connectivity between geographically close, but environmentally distinct sites means that this coral needs to be managed and protected on a local level and that source and sink populations need to be identified through further study.

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CHAPTER 4: A PRELIMINARY ASSESSMENT OF POPULATION CONNECTIVITY OF *SINULARIA BRASSICA* IN THE NORTHERN COMPLEX OF ISIMANGALISO WETLAND PARK

ABSTRACT

As coral populations decline worldwide in the face of ongoing environmental change, documenting their distribution, diversity and conservation status is now more important than ever. Accurate delimitation and identification of species is a critical first step. The tropical soft coral genus *Sinularia* has traditionally been divided into five intrageneric taxonomic groups based on variation in a single morphological character: the shape of the club sclerites (calcite skeletal elements) embedded in the surface tissues of the colony. Based on the traditional barcoding marker COI, *Sinularia brassica* in the northern complex of the iSimangaliso Wetland Park might represent two clades of the IWP. Overall, *S. brassica* appears to be monophyletic with *S. brassica* from the Pacific although there may be some degree of differentiation. Genetic diversity of COI is limited and decreases from north to south. The inclusion of mtMutS has shed further light on the potential divergence of *S. brassica* across the IWP. There is potentially more than one species present, although the present molecular evidence does not allow for unambiguous determination of species boundaries given the conflicting topologies of phylogenies derived from the two sequenced markers and, as such, no claims regarding the number of species or the assignment of samples to species is made. Additional data in the form of SNPs derived from RADSeq were, unfortunately, not received in time for inclusion into this study due to covid related delays in sequencing of the RADSeq libraries. The samples from this study were compared to another recent study conducted on South African *Sinularia* and support the results from that study. The limited data does not support any significant population structure within the reef complex.

INTRODUCTION

The genus *Sinularia* is highly variable in growth form and occurs in waters as shallow as one meter and as deep as 35 metres in various reef habitats (Fabricius, 1995; Ofwegen, 2000; Benayahu *et al.*, 2019). The southernmost reefs of the east African coast are marginal and cover an area of ~40 km². In terms of coral coverage, soft corals predominate with overall cover of ~32%, albeit with relatively few species present, while hard corals cover ~27% but are apparently more diverse (Schleyer *et al.*, 2018; Porter and Schleyer, 2019). Studies around the world suggest that temperate marine ecosystems are becoming more tropicalized due to poleward range expansion caused by global warming (Richards *et al.*, 2016; Porter and Schleyer, 2017; Schleyer *et al.*, 2018; Vetrimurugan *et al.*, 2018).

The high-latitude coral communities found along South Africa's east coast are small, marginal reefs that lack the typical traits of coral reef geomorphology (Montoya-Maya, 2014; Porter and Schleyer, 2017, 2019; Schleyer *et al.*, 2018). The reefs are bathed by the Agulhas Current originating primarily from the East Madagascar Current (EMC) and Mozambique Current eddies, forming one of the strongest warm, western boundary currents. These coral assemblages are found within the Delagoa Bioregion tropical, a subtropical transition zone in which the UNESCO World Heritage Site known as the iSimangaliso Wetland Park (IWP) is located. The IWP is noted as an area of exceptional species diversity and a primary objective of the World Heritage Site designation is preserving the genetic integrity of these populations (Souter, Obura and Lindén, 2000; Maggs, 2011; Montoya-Maya, Schleyer and Macdonald, 2016; Benayahu *et al.*, 2019). The reefs focused on in this study are grouped in three complexes (Fig. 4.1): a northern complex adjoining Kosi Bay, a central complex within the Sodwana Bay bight, and a southern complex, which includes Leadsman Shoal. In area, they total approximately 50 km² (Montoya-Maya, Schleyer and Macdonald, 2016; Schleyer *et al.*, 2018). Sections of the Northern complex are accessible to the public. The central complex, considered to be where *Sinularia* sp. is most abundant, is the most accessible and is the most important node for tourism in the area; visitor pressure at this location presently constitutes a cause for concern (Sunde and Isaacs, 2008; Schleyer and Porter, 2018; Schleyer *et al.*, 2018; Sowman and Sunde, 2018). Lastly, the southern complex falls within a sanctuary area and is closed to the public (Schleyer and Tomalin, 2000; Porter, Branch and Sink, 2017; Sowman and Sunde, 2018).

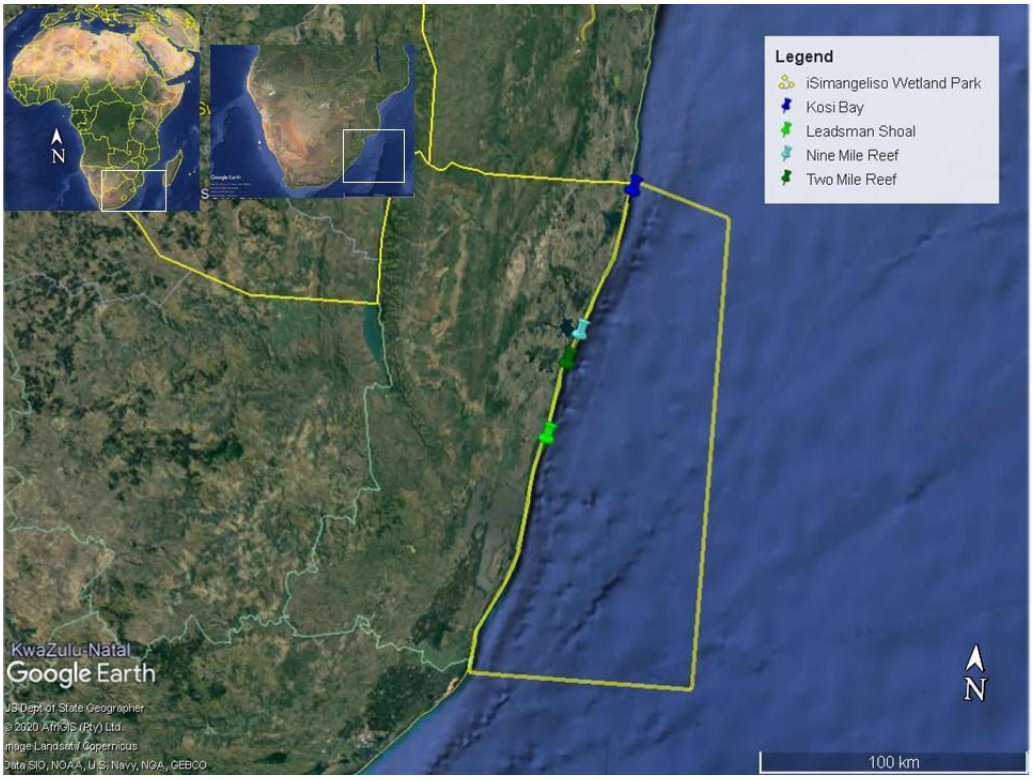

Figure 4.1: Sampling locations for this study including the MPAs of the reefs sampled. MPA boundary information was downloaded from https://egis.environment.gov.za/data_egis/, and loaded in Google Earth Pro

Table 4.1 Sample sites and their coordinates

Location	Latitude (DDM)	Longitude (DDM)
Kosi Bay	-26 55.680	32 53.100
Nine-Mile Reef	-27 24.897	32 43.598
Two-Mile Reef	-27 30.776	32 41.173
Leadsman's Shoal	-27 45.890	32 37.836

In the case of the genus *Sinularia*, as with many other corals, newly elucidated taxonomy based on phylogenetics frequently differs substantially from traditional morphology-based taxonomy (Ofwegen, 2000; McFadden *et al.*, 2009; Quattrini *et al.*, 2019). Ecological and evolutionary studies of soft corals have been previously seen as challenging, with difficulties in identifying and classifying them due to phenotypic plasticity and unreliable diagnostic morphological characters (Beheregaray, 2008; McFadden *et al.*, 2009; Teske *et al.*, 2009; Kirkman *et al.*, 2019; Drury, 2020).

Initially, taxonomic work on the genus *Sinularia* has followed the protocol described by Verseveldt (1980). This protocol included a key in which *Sinularia* is sub-divided into five groups based on characteristics of the club sclerites and this classification is still commonly used. *Sinularia brassica* is assigned to Clade IV, which has club sclerites that lack a central wart and are distinguished from one another by size, i.e., >0.12 mm long (Vennam and van Ofwegen, 1996). A study conducted by McFadden (2009) has recently expanded this distinction by sub-dividing *Sinularia* into four Clades with *S. brassica* and *S. dura* assigned to Clade 1. These species have both been synonymized (Benayahu, 1998; McFadden *et al.*, 2009) but present two distinct haplotypes which is suggestive of two species (McFadden *et al.*, 2009). As described by McFadden *et al.*, 2009; "The shape of the club sclerites (very wide heads with no central wart) is unique within the genus, as is the enormous variation in colony growth form that these species exhibit (Benayahu, 1998)". Clade 1 is further distinguished from the other four major clades by the presence of scales in the tentacles (Shoham and Benayahu, 2017).

MPAs had been established to protect basic biodiversity conservation and promote sustainability over the long term. This growing concern among scientists has led to the establishment of marine protected areas and conducting biological surveys to examine the diversity an abundance of marine flora and fauna along the KwaZulu-Natal (KZN) coastline (Attwood *et al.*, 1997; Almany *et al.*, 2009; Richards *et al.*, 2016). Since the reefs within the IWP are all protected and they have been largely unaffected by coral bleaching and disease, they are considered to be largely pristine (Schleyer and Porter, 2018). More information is needed to reveal how the systems are structured, whether there are prominent levels of self-recruitment, since this will affect their recovery from large-scale damage and may cause it to be gradual and localised. Little work has been done *Sinularia brassica* on the KZN coastline, one limiting factor being the population decrease in a poleward direction. Molecular systematics studies have been conducted on other Indo-Pacific *Sinularia*; however, the studies suggest that clade 1 (*S. brassica* / *S. dura*) fall outside of *Sinularia* studies indicated clade 1 is slightly more genetically distant from the other clades (McFadden *et al.*, 2009). For this, further phylogenetic analyses are required. The use of the mitochondrial cytochrome oxidase I gene (COI) and the octocoral-specific mitochondrial gene mtMutS has commonly been used for species identification on multiple taxa (Hebert *et al.*, 2004; Smith, Fisher and Hebert, 2005; Ward *et al.*, 2005; Hajibabaei *et al.*, 2006; Smith, Poyarkov Jr and Hebert, 2008; Tavares and Baker, 2008; Baker, Tavares and Elbourne, 2009; Mcfadden *et al.*, 2011; Mcfadden *et al*., 2014). The use of COI with octocorals has not worked for positive identification purposes as octocorals are known that to possess a slow evolving mitochondrial genome (Shearer *et al.*, 2002; Mcfadden *et al.*, 2011). The mtMutS gene region is exclusive to the octocoral genome (Pont-Kingdon *et al*., 1998), and has been extensively applied in phylogenetic studies of these

taxa and has been useful in elucidating lower taxonomic relationships, while COI allows comparison to other taxa for which an extensive database already exists (McFadden *et al*., 2006; McFadden *et al*., 2009, McFadden *et al*., 2011; McFadden *et al*., 2014; Bryce *et al*., 2018). Previous studies have shown that the lack of variation within the COI barcodes means that it cannot adequately resolve species boundaries and therefore there are limits to the use of COI to explore octocorals on a population level and it should not be used alone to discriminate morphospecies (Calderon, Garrabou and Aurelle, 2006; Shearer and Coffroth, 2008; Mcfadden *et al.*, 2011; Benayahu *et al.*, 2012, 2018). In the case of mtMutS, the marker has been considered the most rapidly evolving mitochondrial region and therefore the most promising candidate in combination with COI to unravel the *Sinularia brassica* species complex within the IWP (van der Ham *et al*., 2009; McFadden *et al*., 2011; Etsabeth, 2018).

Aims and Objectives

The objective of this study is to use the mitochondrial COI and mtMutS barcode markers to establish if it is possible to resolve the true species identity of *S. brassica* and the corresponding morphology across the three reef complexes of the IWP. Additionally, whether we can distinguish biogeographical population structure within the IWP.

Hypotheses

H1 There is limited genetic difference of *S.brassica* between the three complexes within the IWP

H2 The diversity of *S.brassica* decreases in a poleward direction.

METHODS

All corals were collected under research permits from the South African Department of Agriculture Forestry and Fisheries (DAFF), the iSimangaliso Wetland Park Authority and Ezemvelo-KZN Wildlife. Sample sites were Kosi Bay in the Northern reef complex, Nine-mile and Two-mile Reef in the Central complex and Leadsman Shoal in the Southern complex, as indicated in Figure 3.1. Colonies were sampled on SCUBA by taking a small subsection of each colony using either a knife or a pair of side cutters to remove a sample of the coral. Each 1 cm^2 subsample was individually packaged in a Whirl-Pak® (Nasco, 2018) underwater and subsequently preserved in DMSO–EDTA–salt (DESS) (20% DMSO, 0.25M EDTA, pH 8.0 saturated with NaCl) (Gaither *et al.*, 2011) and stored at 4 °C for short periods and -20 °C long term (Arif *et al.*, 2014).

Tissue Extraction

Samples were subdivided to allow for multiple extractions if necessary. Sub-fragments of approximately 1 cm in length and width were used for genomic extraction. They were washed with PBS to remove excess DESS and then were then placed in petri dish with 20mL of 0.2M EDTA. Tissue was then scraped from the skeleton using a sterile razor blade. The suspension was transferred to a 50 mL falcon tube and left on ice for two hours. The suspension was passed through a 100 µM and 30 µM sequential MACS SmartStrainers (Miltenyi Biotec Inc. Auburn, CA, USA to reduce the relative proportion of *Symbiodinium* symbiont content present in the extracts (Woodley *et al.*, 2015; Reyes-Becerril *et al.*, 2017). Extraction was done using a Zymo DNA MiniPrep kit and performed as per the user manual except for a 2-hour proteinase K digestion time. Quantification of the DNA was determined using Qubit Broad Range kit

PCR Amplification and Sequencing

Polymerase Chain Reaction (PCR) was performed using TEMPase Hot Start 2x Master Mix BLUE (master mix containing TEMPase Hot Start DNA polymerase, the ammonium buffer system, inert blue dye, stabilizer, dNTPs, and magnesium chloride). A gradient PCR was used to determine the optimal annealing temperature which resulted in the successful protocol indicated in Table 4.2 below. Primers utilized in the PCR reactions for the Cytochrome Oxidase subunit I (COI) (Sinniger, Reimer and Pawlowski, 2010) and mtMutS (France and Hoover 2002, Sanchez et al. 2003, Brugler and France 2008) are indicated in Table 4.2. The PCR amplification reaction was carried out in a 25µl reaction volume.

Table 4.2: Primers used in study

Primer Name	**Primer Sequence**	**Program**	**Reference**
LCOant/ COIantR	TTTTCYACTAATCATAAAGATAT/ GCCCACACAATAAAGCCCAATAYYCCAAT	95°C 15 min, 35 cycles of (95°C 30s, 46°C 45s, 72°C 60s), 72°C 10 min	(Sinniger, Reimer and Pawlowski, 2010)
mtMutS ND42599F or ND4L2475F /MUT3458R	ND42599F: GCC ATT ATG GTT AAC TAT TAC or ND4L2475F: TAG TTT TAC TGG CCT CTA C MUT3458R: TSG AGC AAA AGC CAC TCC	95°C 15 min, 35 cycles of (95°C 30s, 51°C 45s, 72°C 60s), 72°C 10 min	(Brugler & France 2008) (France & Hoover 2002) (Sanchez et al. 2003)

Sequence Data

Five microliters of each PCR product were separated on a 1.5% agarose gel immersed in sodium borate buffer at 120 V for 25 min and visualized with Pronasafe dye on a Biorad Gel Doc™ XR+ to determine if the amplification was successful. If the bands were clear and distinct the remainder of the PCR product was sent to the Sequencing Unit at Stellenbosch University (CAF) or KZN Research Innovation & Sequencing Platform (KRISP). BigDye terminator chemistry (Applied Biosystems) was used to generate sequences on an ABI Sanger 3730xl Genetic Analyzer automated sequencer. Samples that appeared as distinct bands when visualized on a 1% agarose gel were sequenced in a single direction (using the forward primer).

Sequence Alignment and Data Analysis

COI

Sequences were visually inspected using Geneious software version 9.1.5 (https://www.geneious.com), adjusted by eye, using the quality scores. Sequences are inspected using GEAR, a web server for molecular biology applications (https://www.gear-genomics.com) (Rausch et al., 2019, 2020). Within GEAR, Teal was used to visually inspect each sequence given multiple sequences could be used to align as well as a reference sequence for greater confidence and determine a representative sample from each sample location that was then used to compare with all other sequences where ambiguities were present. Pearl was used to assemble the chromatograms to visually compare sequences and deliver a consensus sequence to suggest the correct base that may present a questionable peak. Sequences were verified to represent the corresponding regions from *S. brassica* by a BLAST search implemented in Geneious and used in later analyses. Sequences were aligned using ClustalW algorithm implemented in MEGA X (Kumar *et al.*, 2018). Alignments were then manually adjusted to increase the overall similarity in the form of a 685 bp long data matrix for COI. COI Genetic distance calculations implemented in MEGA X (Kumar *et al.*, 2018) were used to estimate sequence divergence, using the method of Tamura and Nei (Tamura and Nei, 1993). The phylogeny of the COI sequences was analysed using Maximum Likelihood (ML) method (performed using MEGA X), with 1000 bootstrap and the Tamura 3 parameter model (Tamura and Nei, 1993) was selected as the substitution model. Phylogenetic trees were drawn using MEGA and edited using TreeGraph 2 (Stöver and Müller, 2010). Sequences were compared to references on GenBank. *S. brassica* references were retrieved from McFadden *et al.* (2014) and Benayahu *et al.* (2018). Pairwise distance models were conducted in MEGA and median joining haplotype networks using locations as traits were created using POPArt (Leigh and Bryant, 2015; Gargan *et al.*, 2016).

MtMutS

Sequences were visually inspected using Geneious software version 9.1.5 (https://www.geneious.com), adjusted by eye, using the quality scores and bidirectional sequences when available. Sequences with unresolved area were disambiguated using GEAR, a web server for molecular biology applications (https://www.gear-genomics.com) (Rausch et al., 2019, 2020). Teal was used to visually inspect each sequence given multiple sequences could be used to align as well as a reference sequence for greater confidence and determine a representative sample from each sample location that was then used to compare with all other sequences where ambiguities were present. Pearl was used to assemble the chromatograms to visually compare sequences and deliver a consensus sequence to suggest the correct base that may present a questionable peak. *S. brassica* reference sequences were retrieved from McFadden *et al*. (2014), Benayahu *et al*. (2018) and Etsabeth (2018). Pairwise distance models were conducted in MEGA and median joining haplotype networks using locations as traits were created using POPArt (Leigh and Bryant, 2015; Gargan et al., 2016). Sequences were aligned using ClustalW algorithm implemented in MEGA X (Kumar et al., 2018). Alignments were manually trimmed so that all sequences were 1474 bp long. Phylogenetic trees were constructed using the mtMutS sequences in MEGA X using Maximum Likelihood (ML) with the TN93 Tamura-Nei (1993) model and 1000 bootstraps. Phylogenetic trees were edited using TreeGraph 2 (Stöver and Müller, 2010).

For molecular analysis, a fasta file was exported from MEGA X to DNAsp (version 6.12.3) and converted into Arlequin format and population genetics parameters were analysed in Arlequin version 3.5.2.2 (Excoffier and Lischer, 2010) including an Analysis of Molecular Variance (AMOVA). Significance was addressed using 9,999 permutations of the original dataset. Pairwise FST (Jost, 2008) were calculated to address genetic structure and differentiation between locations. Arlequin (version 3.5), (Excoffier and Lischer, 2010) was used to calculate nucleotide diversity index.

Additionally, the use of species identification using DNA barcodes and a comprehensive reference library has become a core task in biodiversity studies (Mitchell 2015; Saslis-Lagoudakis et al. 2015; Schmidt et al. 2015; Versteirt et al. 2015). BarcodingR is an R package for species identification using DNA barcodes that is designed to be user-friendly. BarcodingR includes implementations of BP-based (Zhang et al. 2008; Zhang & Savolainen 2009), fuzzy-set based (Zhang et al. 2012) algorithms for species delineation. The function barcoding. *spe.identify* was used for species identification using protein coding barcodes with BP-based method (Zhang et al. 2008) and fuzzy-set based method (Zhang et al. 2012), given reference and query DNA sequences.

The fuzzy-theory based approach had proven to aid avoiding false-positive identifications. With the option 'fuzzyId', the function assigns each potential identification an 'FMF value' in the range of 0–1, indicating likelihood of the assignment (Zhang et al. 2012).

RESULTS AND DISCUSSION

Phylogenetic Results

COI

Preliminary results using the COI and mtMutS markers are presented. It was originally planned to use a large suite of additional markers generated by RADSeq similarly to what was shown for *Stylophora* in chapter three. However, the ongoing covid-19 pandemic meant that this part of the study was unable to be completed in time.

No *S. brassica* was found south of Leadsman Shoal in the Natal Bioregion, despite extensive searches across multiple expeditions, so no results across biogeographic barriers are available. In addition, only a limited number of samples were obtained and successfully sequenced from the northern reef complex.

The phylogenetic tree based on the mitochondrial COI gene shown in Figure 4.2 reveals that the majority of the individuals collected in the study form a monophyletic clade that is similar to the *S. brassica* obtained from the Pacific (McFadden *et al.*, 2014; Benayahu *et al.*, 2018). The majority of individuals form a tightly clustered, well-resolved clade (0.95) distinct from the Pacific reference sequences. Two individuals from Kosi Bay (KB022 and KB032) cluster separately and appear to be more distantly related to the other South African individuals than those are to the Pacific individuals, although support for this is not strong (0.57). They still apparently fall within the *S. brassica* clade. To determine pairwise distance within populations, the three individuals (KB022, KB024 and KB032) were analysed separately.

Figure 4.2: Phylogenetic tree of COI to illustrate the division of the samples collected and also highlight Kosi Bay sample numbers 22, 24 and 32 that are separate to the rest of the collection.

Table 4.3 shows the pairwise genetic distances of the COI sequences between populations as suggested by the phylogeny (Figure 4.2) seen in the tree in Figure 2. Based on the table, the two individuals (KB022 and KB032), marked on this table as KB_Clade 2, are marginally more similar to the reference *S.brassica* derived from the Pacific than to rest of the samples collected in this study. Within Clade 1, the locations display little difference with a genetic distance between 0.001 and 0.003 and can be considered part of the same population. The table also shows that

there is remarkably little genetic difference between *S. brassica* found in South Africa and the *S. brassica* reference sequences that originate from the Pacific.

Table 4.3: Estimates of evolutionary divergence over sequence pairs between groups. evolutionary analyses were conducted in MEGA X (Kumar *Et Al.*, 2018).

	S.bra_Pacific	KB_Clade 2	KB_Clade 1	NM_Clade 1	TM_Clade 1
S.bra_Pacific					
KB_Clade 2	0.002				
KB_Clade 1	0.005	0.007			
NM_Clade 1	0.007	0.009	0.002		
TM_Clade 1	0.006	0.008	0.001	0.003	
LS_Clade 1	0.005	0.007	0.000	0.002	0.001

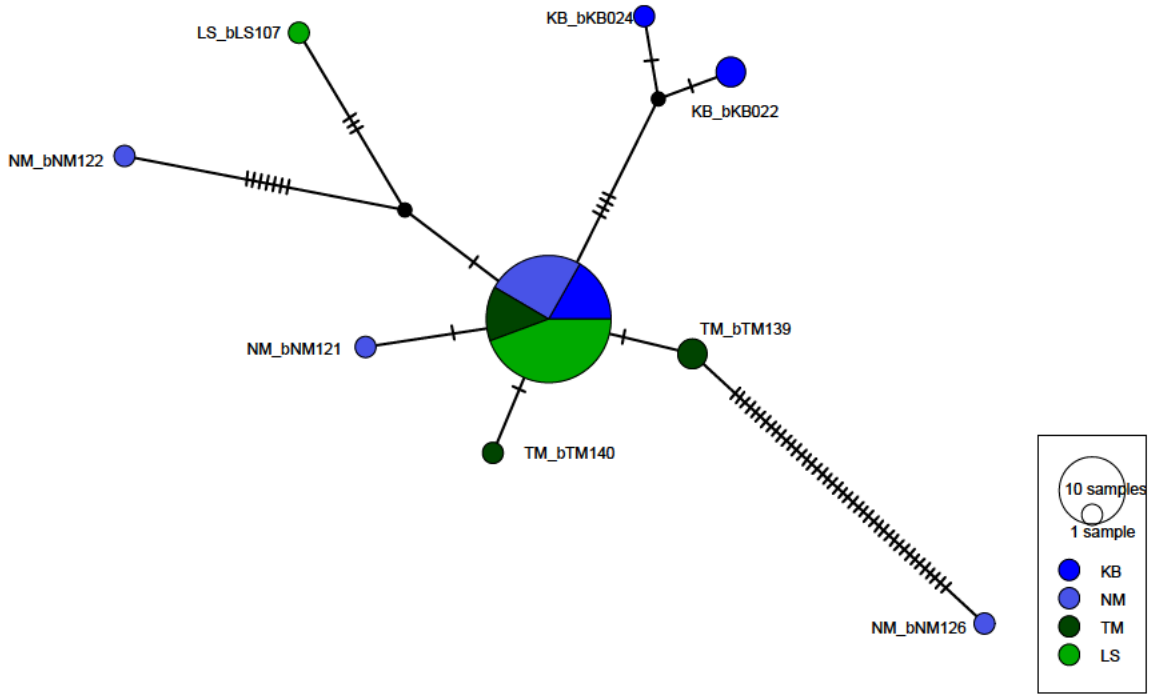

Figure 4.3: Haplotype network of COI sequences 685bp collected in this study. This indicates similarities between the sampled haplotypes.

The COI haplotype network (Figure 4.3) revealed that there is little genetic distance between individuals and populations. The two individuals from Kosi Bay are separated from the rest of the

samples although the number of variant sites is small, which corroborates the findings shown in Appendix Table S4. It appears that Kosi Bay and Nine Mile reef are more genetically diverse and diversity decreases moving southward. Sample NM126 appears to be more different compared to the phylogenetic tree. This could be due to the lack of other reference sequence that could resolve the tree to a greater degree.

mtMutS

The phylogenetic tree based on the mitochondrial mtMutS gene shown in Figure 4.4 reveals that all the individuals collected in the study form a polyphyletic clade with *S. brassica* obtained from the Pacific (McFadden *et al.*, 2014; Benayahu *et al.*, 2018). Branches also reposition between trees in comparison to COI with an increase of diversity among populations and the increased number and variance of reference sequences from within the study locations. Most individuals form a tight cluster with southern African reference sequences with one Leadsman Shoal sample, LS 005, clusters the closest. Only three individuals appear more resolved to distinct from the Pacific reference sequences although not with strong support (0.5). Two individuals (KB 022 and LS 014) cluster separately and appear to be more distantly related to the other the Pacific individuals with extraordinarily strong support (0.91), they still fall within the *S. brassica* species complex. Samples LS106, LS107 and NM116 appear to form a separate branch with reference samples collected from Leadsman Shoal and Seven-Mile Reef.

The haplotype network corroborates this as well as showing greater overall haplotype diversity in comparison to the COI marker. There is a greater divergence of samples with separation of what appears to be at species-level.

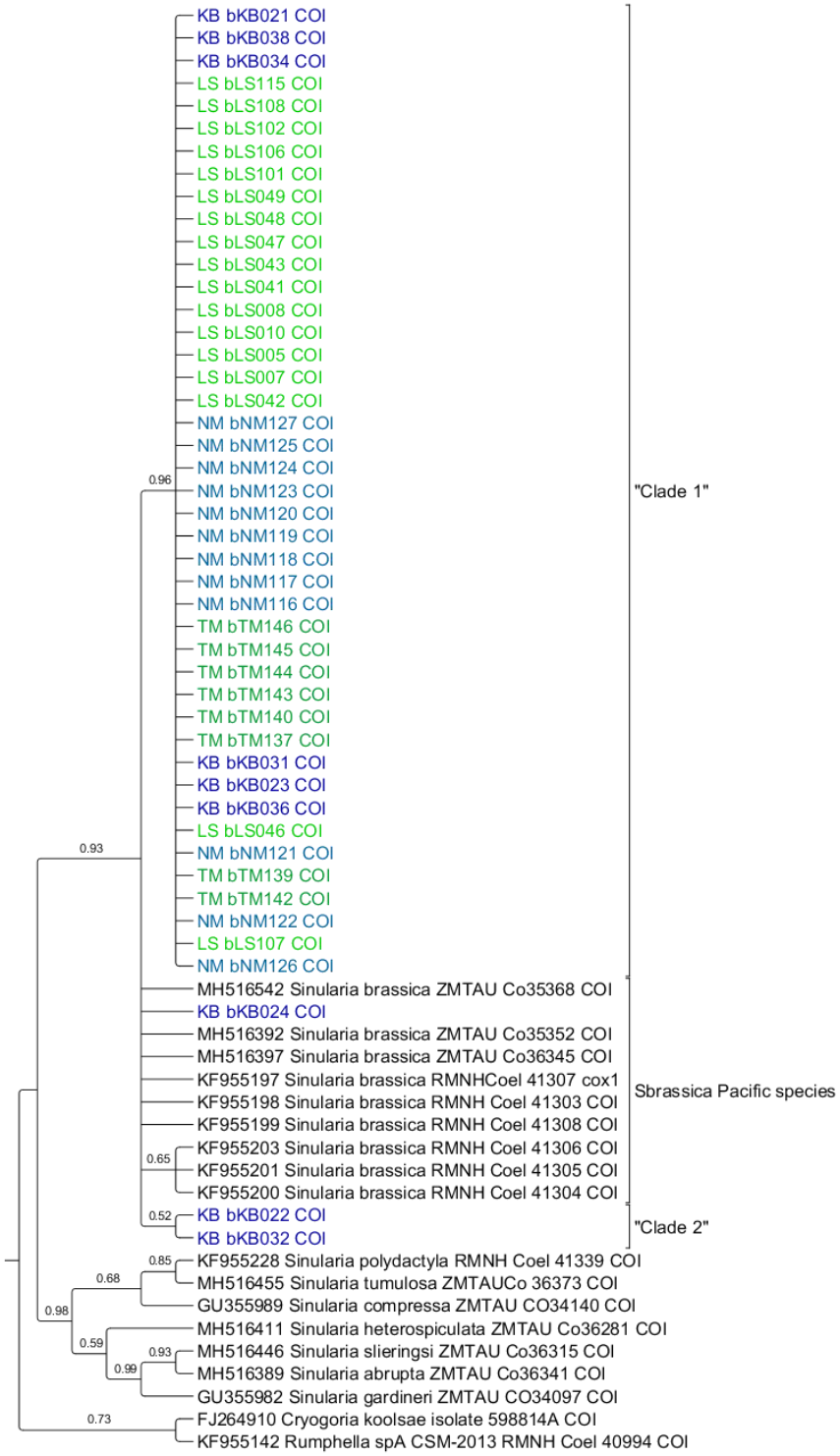

Figure 4.4 Phylogenetic tree of mtMutS to illustrate the division of the samples collected and highlight Kosi Bay sample number 22 (italics) and 24 that are separate to the rest of the collection, as well as Leadsman Shoal samples 5 and 14.

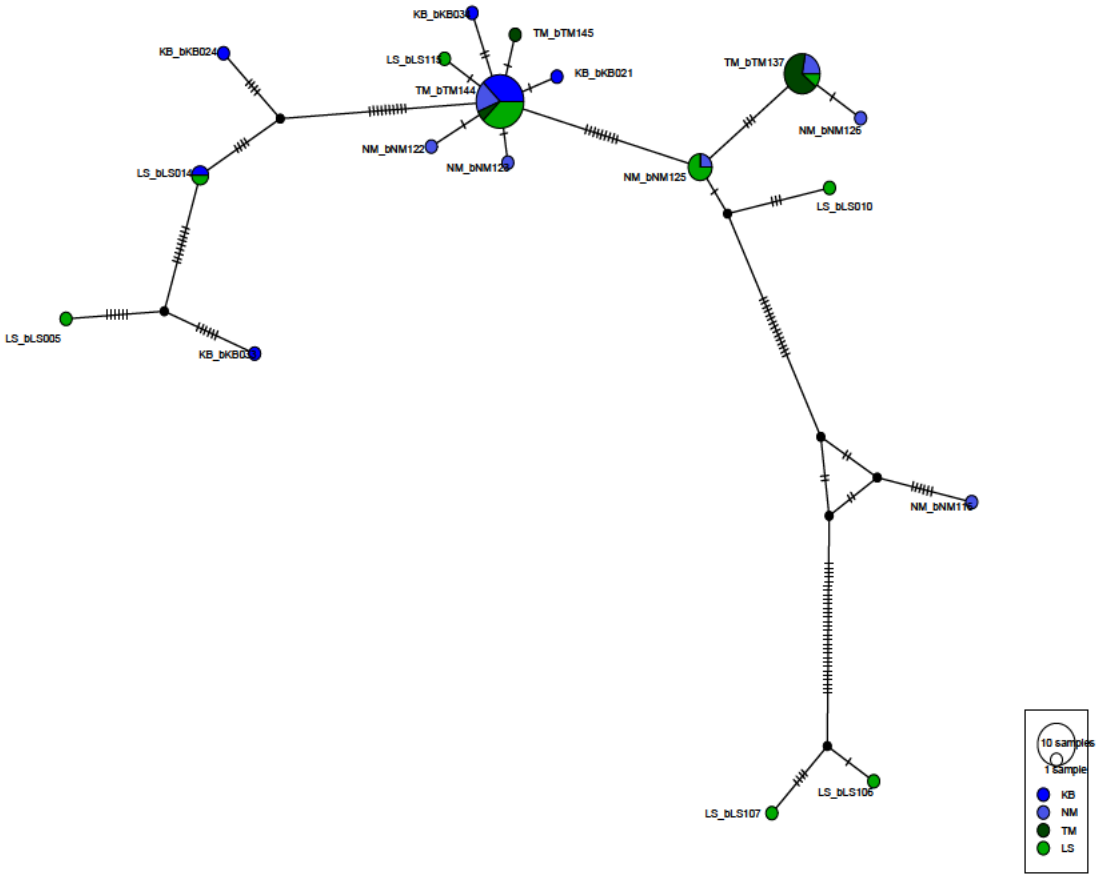

Figure 4.5: Haplotype network of samples collected in this study of 1472bp in length. This indicates similarities between the sampled haplotypes.

The concatenated tree below (Figure 4.6) shows a close kinship between two individuals from Kosi Bay (KB_bKB022, KB_bKB024) and one from Leadsman Shoal (LS_bLS005) with the Pacific reference samples. Additionally, the haplotype network and concatenated tree both show a separation of one individual from Nine-Mile reef (NM116) and two from Leadsman Shoal (LS106 and LS107) showing some genetic distance from the rest. There appears as if the species-level resolution of *S. brassica* still requires work in this region.

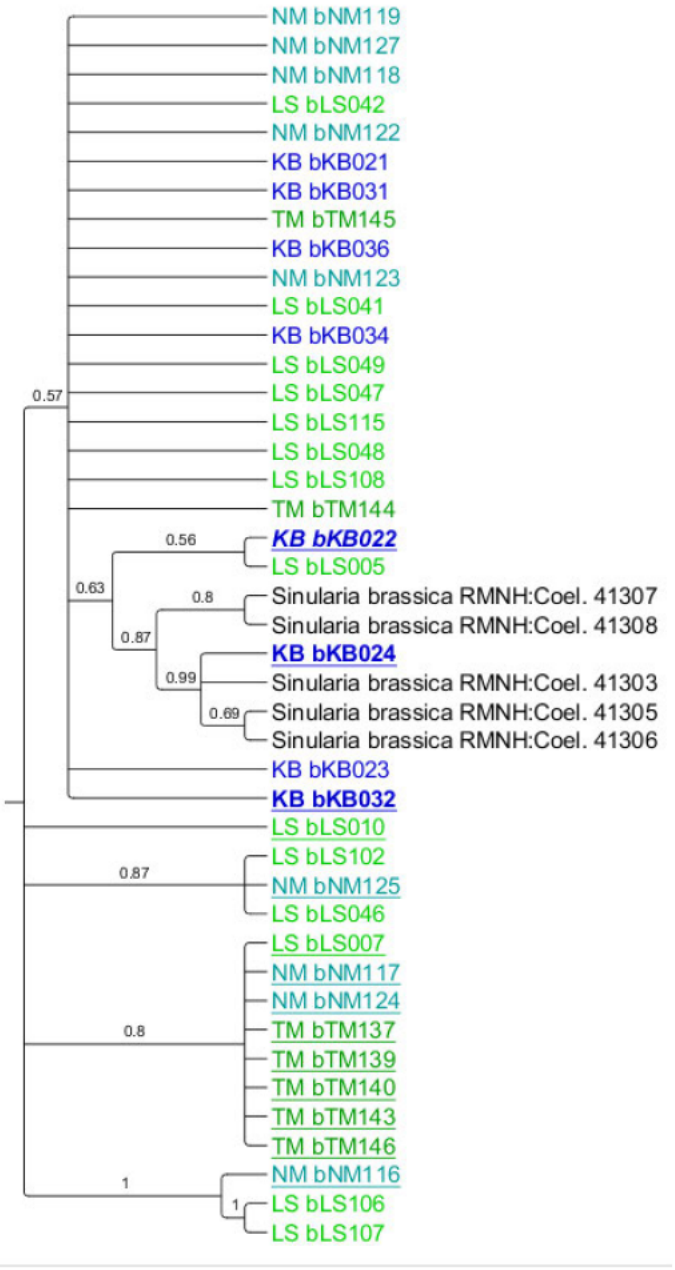

Figure.4.6 Concatenated phylogenetic tree of COI + mtMutS to illustrate the division of the samples collected and highlight Kosi Bay sample number 22 (italics) movement alongside Leadsman Shoal and 32 that are separate to the rest of the collection.

Table 4.4 shows the nucleotide and haplotype diversity generated using Arlequin (version 3.5) (Excoffier and Lischer, 2010). There was a gradient of genetic diversity from north to south indicating a general poleward movement of propagules. The number of samples from Kosi Bay is considerably smaller than other locations and the depth of sampling across the coastline would to be increased to get better resolution. Table 4.5 shows that the mtMutS diversity and pairwise differences of Nine- Mile Reef and Leadsman Shoal express a prominent level of diversity

(11.356 +/- 5.634 and 20.392 +/- 9.515) within their respective sites and suggest more than one "species."

Table 4.4: Nucleotide diversity of COI using Arlequin.

Site	***n***	***n*h**	**Nucleotide diversity (±SD)**	**Mean pairwise differences (±SD)**
Kosi Bay	4	3	0.228 +/- 0.183	2.500 +/- 1.685
Nine Mile Reef	25	8	0.140 +/- 0.111	1.533 +/- 1.060
Two Mile Reef	8	2	0.0227 +/- 0.032	0.250 +/- 0.311
Leadsman Shoal	14	3	0.026 +/- 0.033	0.285 +/- 0.322

Table 4.5: Nucleotide diversity of mtMutS using Arlequin.

Site	**n**	**nh**	**Nucleotide diversity (±SD)**	**Mean pairwise differences (±SD)**
Kosi Bay	11	6	0.099 +/- 0.055	0.208 +/- 0.109
Nine Mile Reef	10	7	0.116 +/- 0.065	11.356 +/- 5.634
Two Mile Reef	8	3	0.055 +/- 0.034	5.393 +/- 2.907
Leadsman Shoal	16	9	0.208 +/- 0.109	20.392 +/- 9.515

Pairwise ϕPT analysis in Table 4.6 shows that there is no indication of strong genetic structuring between the sites, although the power is small due to the small sample sizes from certain sites. The Leadsman shoal population appears to be the least connected but the only statistically significant difference (ϕPT = 0.175, p = 0.005) is between Two Mile Reef and Leadsman Shoal. Overall, the limited COI data showed that there was little genetic differentiation between populations from sample locations and suggests that there is good historical connectivity.

Table 4.6: Matrix table of pairwise population ϕPT values for COI. Significant values ($p < 0.05$) are highlighted.

Pairwise Population ϕPT Values for COI				
	KB	**NM**	**TM**	**LS**
KB		0.433	0.287	0.241
NM	0.000		0.177	0.099
TM	0.000	0.039		**0.005**
LS	0.187	0.024	**0.175**	
ϕPT Values below diagonal. Significant ϕPT values are highlighted				

Table 4.7 Matrix table of pairwise population ϕPT values for mtMutS. Significant values (P < 0.05) are highlighted

Pairwise Population ϕPT Values for mtMutS				
	KB	**NM**	**TM**	**LS**
KB		0,018	**0,001**	0,095
NM	0,115		0,273	0,435
TM	**0,227**	0,019		0,144
LS	0,057	0,000	0,053	
ϕPT Values below diagonal. Significant ϕPT values are highlighted				

BarcodingR results shown in Appendix Table S5 corresponded to the total number of species included from GenBank. However, none of these methods consistently delineated species in congruence with how each species was recovered in the mtMutS phylogeny. Two highly supported monophyletic clades with species delimitations were recovered that corresponded to two morphologically differentiated colony forms of *S. brassica* from South Africa. Only a few samples did not identify as Sinularia brassica. Ten samples were assigned to *S. dura* and only NM_bNM116 assigned to *S. compressa* using both bp-based and fuzzy membership function (FMF). Only one sample (MF817875.1 *Sinularia brassica* isolate 188 MutS) that is used as a reference from South Africa was assigned with the samples used in this study. Based only on a single or a few genes, and without ecological, behavioural or any other biological information, species identifications via DNA barcoding cannot be totally determined.

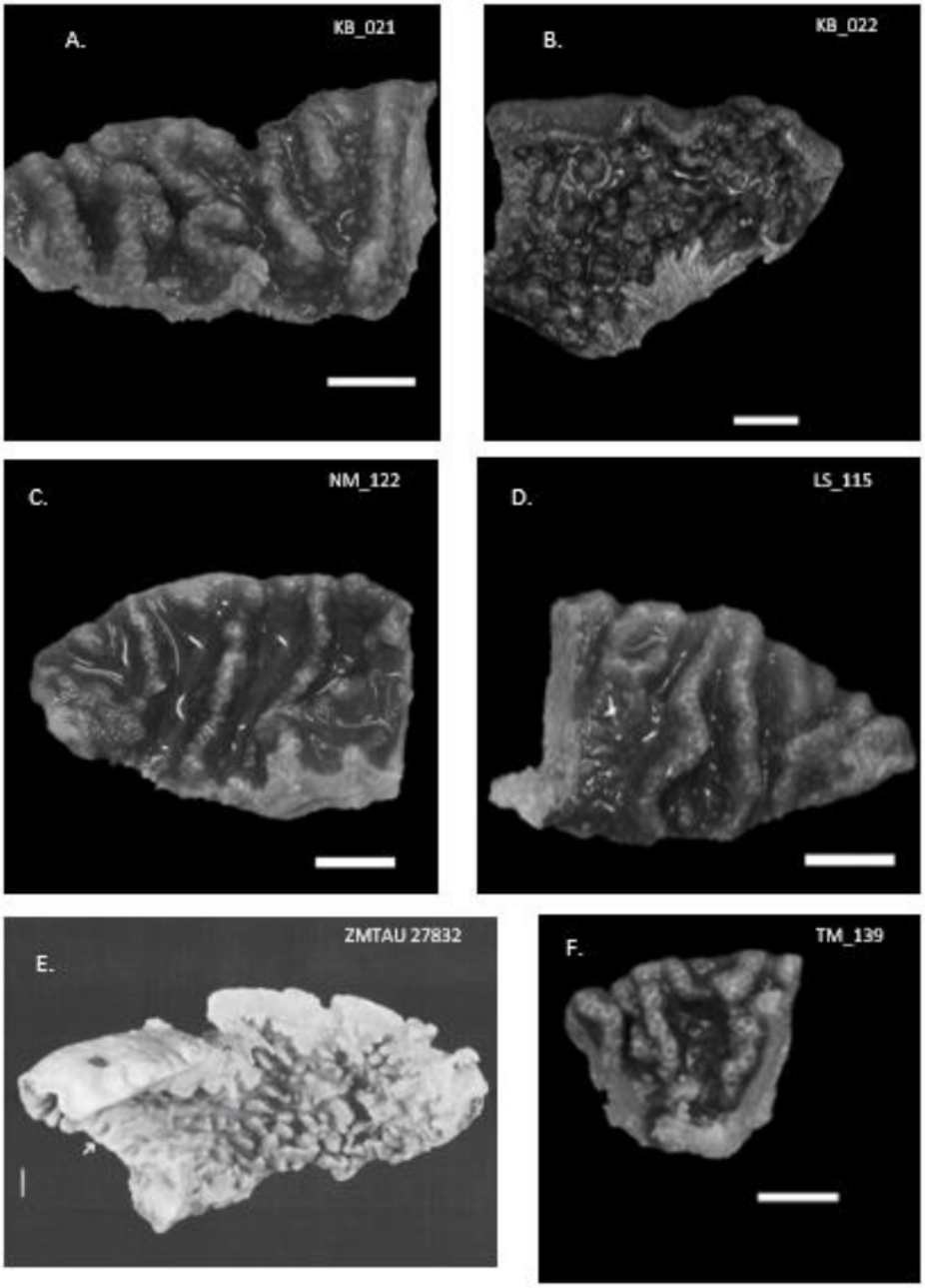

Figure 4.7: Specimen images from each sample location including A representative of each clade from Kosi Bay. A. image of the Kosi Bay clade 1 representative (KB021). B. image of the Kosi Bay clade 2 representative (KB022). C. image of the Nine Mile reef clade 1 representative (NM122). D. image of the Leadsman Shoal clade 1 representative (LS115). E. *S.brassica* representative of Two Mile reef reference from Benayahu *et al.*, 1998 (ZMTAU 27832). F. Image of the Two Mile reef clade 1 representative (TM139).

Figure 4.7 illustrates representative morphologies from samples across the reef complexes compared to a reference sample from Two Mile Reef by Benayahu *et al.* (1998). All colonies

presented with short or non-visible stalks and a flat capitumlum with small lobes. Surface ridges range from continuous (e.g., A, C, D, F) to short and discontinuous (B and the Two Mile Reef "type" specimen (Benayahu, *et al.*, 1998). All the morphologies fall within the range described in Benayahu, *et al.* (1998).

DISCUSSION

Intentions for this study were to include RADSeq data on *Sinularia brassica*. This was not possible due to complications surrounding the ongoing COVID-19 pandemic. This means that the conclusions are based on mitochondrial markers including the slowly evolving COI marker which can help to delineate historical connectivity and long-range phylogenetic relationships and the mtMutS marker which has been used for species-level studies, especially with octocorals (McFadden *et al.*, 2011).

Sinularia dura (Pratt, 1903) and *S. brassica* were previously regarded as separate species that were primarily distinguished by the shape of their capitula, where *S. dura* was distinctly cup-shaped and *S. brassica* primarily lobed (Verseveldt 1974, 1980). Benayahu (1993) noted the occurrence of both taxa at Sodwana Bay (South Africa). Benayahu *et al.* (1998) subsequently synonymized the two species based on considerable similarities between the sclerites of the two taxa and colonies comprising a spectrum of growth forms from cup-shaped to lobed. Based on the morphology observed in this study and shown in Figure 2.5, there is some degree if morphological difference between the representative of the Kosi Bay clade 2 and the bulk to the South Africa samples, particularly in the size and shape of the surface ridges. However, these variations fall well within the range of variability described by Benayahu *et al.* (1998).

We were unable to find any *S. brassica* south of Leadsman Shoal despite extensive attempts to locate it. Additionally, the sample size of the populations used in this study needs to be increased. For example, only five individuals from Kosi Bay could be sequenced, this number would need to be increased in the future for a more representative result. Of these samples, only three individuals from Kosi Bay appear to be separate from the bulk of the other South African sequences. Although they fall within the *brassica* clade, they are less closely related to their South Africa conspecifics, than those are to *brassica* from the Pacific. The bootstrap support for this branch is low (0.57) so additional markers would need to be analysed.

Overlap between intra and interspecific genetic distances were, however, comparable for the two gene regions, and in character-based analyses mtMutS was often no more effective than COI at distinguishing species. All the species' pairs that shared identical COI sequences in McFadden and co-authors (2011) and Etsabeth (2018) were also identical at mtMutS. However, some specimens that shared COI haplotypes did differ at mtMutS. Given the poor state of octocoral

taxonomy and our lack of understanding of morphological characters in many genera, the potential for identification of cryptic species and genera using mtDNA barcodes is still high, as evidenced by the outcome of some recent studies (McFadden *et al*., 2009; van der Ham et al. 2009; McFadden *et al.*, 2011).There appears to be little population structure between the three reef complexes in the IWP. Leadsman Shoal is seen to have the least connected population with the highest pairwise ϕPT values to other reefs. This give an indication that there is a north to south gradient of diversity which parallels the prevailing current direction suggesting that the movement of propagules follows the prevailing currents. Given that the reefs all fall within the IWP MPA, it can be said that the MPA is protecting the genetic diversity of *S.brassica* within its borders. The samples in this study do resemble those from the Pacific Region and more research could be conducted using a large SNP dataset or multiple barcode markers.

CONCLUSION

The power of this part of the study is limited due the small numbers of samples that were obtained and successfully sequenced as well as reliance on the slowly evolving COI region. No samples were found at any sites in the Natal Bioregion despite extensive sampling attempts. Previous surveys (Schleyer and Benayahu, 2008; Olbers, Celliers and Schleyer, 2009; Maggs, 2011) have identified *S. brassica* at multiple sites in the Natal Bioregion. More systematic and extensive surveys would have to be conducted to determine if the lack of samples represents a real change its distribution.

It is known that mtMutS lacks the resolution to delimit closely related species (McFadden *et al.* 2011; Etsabeth, 2018), yet in combination with COI, it is useful in reducing the number of possible species identifications (McFadden *et al*. 2009, 2010) The disparity between the success rates of molecular identifications in this study and that of others may be accredited to the lack of available representative sequences for each *Sinularia* species. Furthermore, the reference sequences represent taxa from distant tropical reefs in the Coral Triangle and the Red Sea (Benayahu *et al.*, 2012; McFadden *et al.*, 2014; Etsabeth, 2018), as opposed to the isolated, subtropical high latitudinal communities in this study. This integrated tool refined species assignments and facilitated express identifications with basic traditional taxonomy. Phylogenetically, all the sampled individuals fall within the *S. brassica* species complex and segregate with *S. brassica* from the Pacific. There is morphological and genetic evidence that suggests that two or more clades of *S. brassica* are present in the samples collected. However, more extensive analysis using additional genetic markers and detailed morphological analysis of diagnostic features such as sclerite shape and size needs to be conducted to confirm potential edges of species distribution. There is no significant genetic structure between the three reef complexes based on this mitochondrial data set. Previous studies using broadcast spawning species such as *Acropora*

austera and *Platygyra daedalea* have demonstrated some degree of genetic structure within the IWP, particularly in terms of self-seeding and local recruitment (Montoya-Maya, Schleyer and Macdonald, 2016) and it would be surprising if *Sinularia*, which is a brooding coral with a shorter pelagic larval duration, did not show a similar pattern that might be revealed using a larger number of rapidly evolving markers. There does appear to be a gradient of diversity from north to south implying that the northern complex acts as a source of propagules and hence should be looked at for stronger protection. Future studies would benefit the use of additional more rapidly evolving neutral markers from both mitochondrial and nuclear regions, for example using RADSeq, to determine the exact species delimitations and the extent and nature of any population structure.

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CHAPTER 5: FINAL DISCUSSION AND CONCLUSION

The research aimed to determine the connectivity of two coral species, a hard coral *Stylophora pistillata*, and a soft coral, *Sinularia brassica*, along the KZN coastline to determine if the newly expanded MPA network provides sufficient protection for populations of these corals along the coast. Examining connectivity of functionally important coral reefs in existing MPAs, determines whether these MPAs do more than just conserve the marine fauna and flora. Specifically, it aimed to establish whether MPAs of iSimangaliso Wetland Park (IWP), Aliwal Shoal and Pondoland form a coherent network, and whether they provide recruits to surrounding areas, such as Blood Reef, which thereby also benefit from shared genetic diversity. Also addressed is whether areas in-between existing or proposed MPAs act as stepping-stones to facilitate connectivity and might therefore need to be given some degree of protection. Traditional genetic population markers as well as next-generation sequencing techniques were used to determine the pattern of connectivity between MPAs as well as to identify potential gaps along the KZN coastline.

Networks of marine protected areas (MPAs) are required for the successful protection of coral reefs. In the case of this study, ecological partitioning appeared to have a strong influence on population structure as the reefs that are deeper or with more turbid water were more genetically connected than geographically more proximal reefs. Recent genetic studies of corals at higher latitudes have focused on several coral species using traditional markers along the KZN coastline, for example, *Pocillopora damicornis*, *P. verrucosa*, *Stylophora pistillata* and *Acropora austera* (Obura, 2005; Ridgway *et al.*, 2008; Souter, 2010; Macdonald, Schleyer and Lamb, 2011; Montoya-Maya, Schleyer and Macdonald, 2016; Chiazzari *et al.*, 2019). Within these species, there was some fine-scale structuring attributed to the isolated nature of high-latitude reefs nearing the edge of their geographical range (Ridgway *et al.*, 2008; Macdonald, Schleyer and Lamb, 2011; Chiazzari *et al.*, 2019), or there were spatio-temporal patterns enhanced by seasonal changes (der Bank *et al.*, 2019). The genetic and population structure of corals further south of IWP remained relatively understudied and provided a suitable study location for Next-Generation sequencing (NGS), which requires fewer samples (Macdonald, Schleyer and Lamb, 2011).

The candidate species, *S. pistillata* and *S. brassica*, were selected due to their widespread distribution, as determined in previous biodiversity surveys where they were found from Kosi Bay to Pondoland. Unfortunately, despite extensive efforts, no individuals of the soft coral, *S. brassica*, could now be found in the temperate Natal bioregion. Because of this, it was decided to concentrate on *S. pistillata* for the bulk of the research, including the RADSeq analysis.

An in-house ddRADSeq protocol that is economical for multiple non-model species was developed and utilised. The protocol produced high molecular weight DNA and, in turn, produced sequences with more than 10X mean coverage with a large number of loci (1999).

The distance between the northernmost sample sites at Kosi Bay to the most southern site in Pondoland is ~550 kms. Phylogenetic analysis of the COI and ITS loci of *S. pistillata*, covering the whole of the KZN coast and spanning two bioregions, confirmed that the population is comprised of two genetically distinct species/clades as described in previous studies (Flot *et al.*, 2011; Keshavmurthy *et al.*, 2013; Arrigoni *et al.*, 2016). Intra-clade p-distance based on COI was very low, but this is characteristic of the slow evolving cnidarian COI. The pattern of distribution for the *S.pistillata* clades revealed a clear biogeographical split between the Delagoa and Natal Bioregion. Clade 2, which is considered the Chagos-Madagascar-South Africa clade, was not found south of Leadsman Shoal. Clade 3 (Gulf of Aden-Zanzibar-Madagascar) was identified throughout the ~550 km distance in both bioregions. This suggests that clade 2 is limited to tropical regions, while clade 3 is more of a generalist and occurs in a much wider range of latitudes and climatic conditions, from the northern Red Sea to the temperate regions of South Africa. It would be remarkably interesting to examine the clade 3 population structure and loci under selection along its entire East African distribution.

There appears to be no hybridization between the two clades but needs further investigation with more markers or the use of next-generation sequencing (e.g., RADSeq).

The genus *Stylophora* remains paraphyletic based on COI as *Seriatopora sp.* is embedded between the Pacific-Western Australia clade and the Chagos-Madagascar-South Africa clade (Keshavmurthy *et al.*, 2013).

Morphologically *S. pistillata* presented a wide range of morphotypes and it proved difficult to definitively distinguish between clades in the field. Within the Delagoa Bioregion, the morphology of both Clade 2 and 3 resembled each other more than clade 3 colonies from the Natal Bioregion resembled each other or clade 3 from the Natal Bioregion resembled clade 3 specimens from the Delogoa Bioregion. Using ITS, clade 2, as described by Keshavmurthy *et al.* (2013), corresponds to the “species B” morphological described as of *S. mordax* published by Flot *et al.* (2011). The study by Flot *et al.* (2011) also included samples from < 30 m depth, which were similar to the rest of the study material but had thicker branches. Samples of Clade 3 across the sample locations corresponded to “species A” and the morphological description *S. madagascarensis*. It can thus be considered that the morphospecies boundaries are not congruent with genetic boundaries.

At the population genetics level, analysis of a mitochondrial gene (COI) and a nuclear locus (ITS) supported the hypothesis that there is some degree of evolutionary connectivity between all three MPAs on the Kwa Zulu-Natal coastline, and that the overall direction of population movement is from north to south, most likely due to the influence of the Agulhas Current. Within Clade 3, both traditional and RADSeq-generated SNP markers showed a clear signal of population structure along the KZN coastline. More samples were used for the traditional markers from more sites to examine the full scope of clade 3 along the coastline. The COI and ITS markers suggested an atypical stepping-stone model whereby there was a general north to south movement of genetic material, except for the Blood Reef population, which appeared to be isolated from the northern population, and shared more genetic connectivity with the Aliwal Deep population to form a distinct subpopulation. Analysis of a much larger set of ~2,000 markers generated by RADSeq clarified this image and showed clear, unambiguous population structure. Three distinct populations: (1) Leadsman Shoal; (2) Aliwal Deep plus Blood Reef; and (3) Aliwal Shoal. The presence of a single apparent first-generation migrant from Leadsman Shoal in the Aliwal Deep site did indicate some movement of propagules, yet there was no signal of admixture between the Aliwal Deep/Blood Reef population and Leadsman Shoal. The lack of any admixture suggests that migrants that can reach reproductive maturity but do not contribute meaningfully to the local gene pool. However, the relatively small sample size does not preclude rare hybridization events. The distance-based phylogenetic tree also showed noticeably clear separation of the populations.

Figure 5.1 shows the sampling locations of Blood Reef in relation to the coastline, Aliwal Shoal and Aliwal Deep within the Aliwal Shoal MPA. It indicates the level of protection between the three sampling sites. Blood reef is unprotected, Aliwal Shoal is the most protected within the Crown Offshore Restricted Zone, a restricted zone, and is in the Park Rhynie Offshore Controlled zone where line fishing is allowed. The deeper reefs, although within an MPA in the case of Aliwal Deep, are not afforded the same level of protection as the others, so it might be prudent to increase the protection of some of these more isolated reefs.

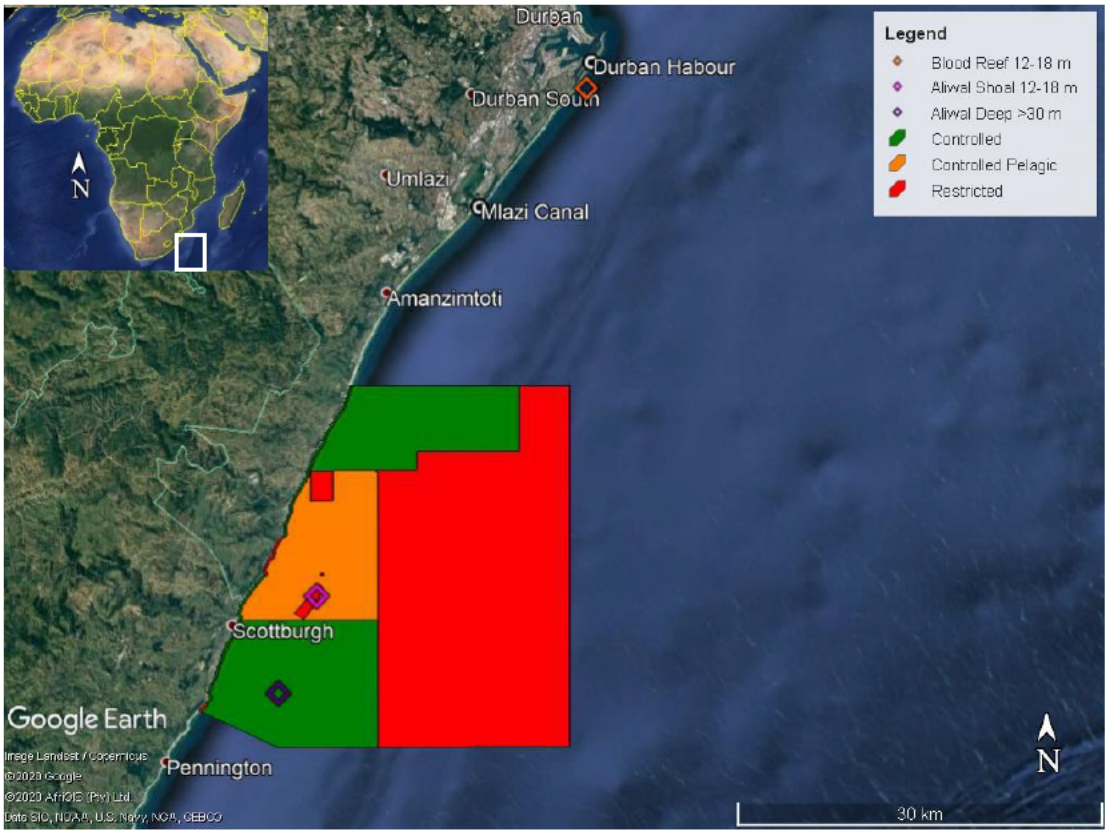

Figure 5.4: Blood Reef, Aliwal Shoal and Aliwal Deep in relation to the coastline and within the MPA. The map was generated in Google Maps and MPA zoning generated by SAAMBR.

Aliwal Deep and Blood Reef form a distinct, well-connected population, which is interesting as the Aliwal Shoal population is located in between. These relationships suggest that something apart from geographical distance is driving the population structure in this region. The relatively considerable number of private alleles indicates that the populations are relatively isolated from each other. Alternatively, the site-specific private alleles may have become fixed in response to environmental pressures driving their selection. When determining loci under selection, there does appear to an environmental pressure of light dependence and high turbidity limiting light penetration. Light penetration is considered an important parameter that allows for the development of biodiverse coral communities at a high latitude (Porter, Branch and Sink, 2017). Additionally, the high proportion of private alleles in Aliwal Deep and corresponding few private alleles of Blood reef, suggest the deeper reefs are a source population. The presence of a longshore current that could potentially connect these two reefs in a south to north direction against the prevailing north to south Agulhas flow_would support this model.

S. brassica was sampled from the three reef complexes of the iSimangaliso Wetland Park (IWP) and used to examine population connectivity within the park. The analyses of *S. brassica* were

limited in terms of sample numbers for various sites. However, it was determined with the use of COI, that all the sampled individuals are monophyletic with, but distinct from, *S. brassica* from the Pacific region (McFadden *et al.*, 2014; Benayahu *et al.*, 2018). Two individuals, although clearly related, appeared to separate from most of the samples, but the evidence was not strong due to the limited genetic distance. *S.brassica* did appear to exhibit a range of morphologies but remained within the previously published morphological range (Benayahu, 1998). Using mtMutS, showed a much higher diversity within the individuals sampled and suggest that there are multiple species as previously suggested based on the morphology. Two individuals, which are more related to the Pacific reference species and the larger proportion more, aligned to reference sequences from the sample location previously described, plus a well resolved group of three individuals. The trees are poorly resolved, and additional markers will be needed to clarify the number of genetically delimited species and the degree of hybridization between them. With the inclusion of mtMutS, we find some evidence of population structure within the IWP but, given the lack of clarity whether the individuals belong to a single breeding population or not, this is not considered to be robust evidence. Further investigation into the true species range of *S.brassica* along the KZN coastline is required, using a larger sample size over a wider geographical range. Given the conservative nature of COI in combination with the fast-evolving mitochondrial homolog mtMutS, it is possible assess the results at as an indication of species level differentiation, the use of additional molecular approaches that target nuclear genomic elements would be beneficial.

In previous studies, northern reefs within this MPA network had a “landing-site” function for migrants, given their geographical location and the prevailing currents (Montoya-Maya, Schleyer and Macdonald, 2016). In the case of this study, it is shown by the highest levels of genetic diversity situated in the northern reefs such as Leadsman Shoal and Kosi Bay. As a result, every effort must be made to maintain this transboundary network of MPAs and protect individual reefs, especially the “landing-site” reefs, to ensure the interdependence of the coral communities in terms of genetic exchange.

The present results revealed significant fixation of alleles on the reefs; moderate levels of genetic differentiation between reefs; a clear genetic subdivision at the largest spatial scale and considerable levels of self-recruitment on each reef; and the presence of temporal genetic structure. All these signs point toward populations that are less open (or more closed), or that the effective population sizes are smaller than was initially assumed.

Management Implications

The existence of multiple distinct populations and two distinct species of *Stylophora* has important management implications. Firstly, this suggests that the populations may need to be managed as individual units (Montoya-Maya, Schleyer and Macdonald, 2016; Schleyer *et al.*, 2018). It does appear, however, that the expanded network of MPAs includes representatives of all three populations of *Stylophora* and, as such, provides sufficient protection for this species. Secondly, the demonstration that multiple cryptic species of both corals are present indicates that there is an underestimation of the biodiversity present in these high latitude reefs. This suggests that their relative importance has been underestimated and that further research using newly developed techniques will uncover many more species and distinct local populations. Thirdly, the wide range of ecological niches occupied by *Stylophora* ranging from warm, shallow, clear reefs in the north to cold, deep reefs with poor light availability and wide range of morphotypes suggests that this species, especially clade 3, is an excellent model species to investigate morphological and ecological adaption in corals. The existence of a reference genome and high-quality genetic data (including this study) reinforce this conclusion.

Fourthly, the presence of first-generation migrants detected over long distances demonstrates that there is a potential route for poleward migration of this species in the face of continued warming and that the existing MPA network would be able to facilitate this.

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APPENDIX A: QUADDRAD PROTOCOL (ADAPTED FROM FRANCHINI *ET AL*., 2017)

Equipment and Materials

- 1.5 ml microcentrifuge tubes
- 96 well 0.2 ml PCR plates and Flat Cap Strips
- 0.2 ml PCR tubes
- Tips
- Micropipettes
- Thermomixer
- Vortex
- Microcentrifuge
- Thermocycler
- Pippin Prep DNA Size Selection System
- Fluorometer
- UV transilluminator
- Agilent 2100 Bioanalyzer

Reagents and Kits

- 80% Ethanol
- AmpureXP beads (Beckman-Coulter)
- ddH2O molecular biology grade
- 10x Cutsmart Buffer (NEB)
- PstI-HF (20,000 U/ml, NEB) – Other restriction enzymes can be used
- MspI (20,000 U/ml, NEB) – Other restriction enzymes can be used
- Individually Barcoded Single-stranded D5XX oligos top and bottom (100 µM in Zymo EB)
- Individually Barcoded Single-stranded D7XX oligos top and bottom (100 µM in Zymo EB)
- Buffer AB: 500 mM NaCl, 100 mM Tris-Cl, pH 7.5-8.0
- T4 ligase (400 U/µl, NEB)

· Pippin Prep Gel Cassettes and Reagents - CDS-2010 (2% Agarose with ethidium bromide for 100-600 bp; OMNI Life Science)

A: DNA extraction

1) Extract genomic DNA with whichever protocol gives good quality, high molecular weight, pure DNA

2) Quantify DNA extracts using Qubit HS assay

3) Dilute to working concentration (to determine) and quantify DNA with Qubit HS assay

B: Adapter preparation

This adapter preparation will provide a final concentration of 10 µM and is sufficient for 50 samples for each adapter. This is sufficient for the full quaddRAD run of 192 samples (16 unique inner barcoding combinations × 12 unique outer barcode combinations), providing there are no pipetting errors. Prepared adapters must be stored at -20 °C and used within 2 weeks.

For **each** adapter:

Adapter D5xx-top or D7xx-top (100 mM)	5 mL
Corresponding adapter D5xx-bottom or D7xx-bottom (100 mM)	5 mL
1 x Buffer AB	40 mL

In PCR machine/thermocycler:

1) 97.5 °C for 2.5 min

2) Cool at 3 °C per minute down to 21 °C

3) Hold at 4 °C

C: Double restriction digestion and adapter ligation

1) Digest 10 ng – 1 µg of DNA with two enzymes (a rare cutter, in this case *PstI*, and a frequent cutter, here *MspI*) and conduct ligation of adapters simultaneously. This is done in a 96 well plate or in 0.2 mL PCR tubes.

Reagent	**Working []**	**Required []**	**Volume**
ThermoScientific Tango buffer	10 ×	1 ×	4 mL
ThermoScientific *PstI*	10 U/mL	30 U	3.0 mL

ThermoScientific *Msp*I	10 U/mL	15 U	1.5 mL
ATP	10 mM	0.5 mM	2 mL
ThermoScientific T4 DNA ligase	1000 CEU/mL	400 CEU	0.4 mL
Adapter i5x	10 mM	0.1875 mM	0.75 mL
Adapter i7x	10 mM	0.1875 mM	0.75 mL
DNA template		10 ng to 1 mg	x
ddH_2O			To volume
Total volume			40 mL

In a PCR machine/thermocycler:

Incubate at 30 °C for 3 hours

Add 10 μL of 50 mM EDTA to stop the reaction

2) Check (all or selected) digestions by electrophoresis, including 100 bp and 1 kb ladders

3) Pool the 16 samples with different inner and outer barcode combinations

4) Purify and size select with Ampure XP beads at 0.5 ×

5) Repeat purify and size select with Ampure XP beads 0.8 ×

6) Elute in 30 μL EB

7) Measure concentrations with Qubit HS assay

D: Indexing PCR

Each of up to 12 pooled sets of 16 samples with unique inner barcode combinations is indexed with the 12 unique outer barcode combinations.

1) For each pooled set of 16 samples with unique inner barcode combinations:

Reagent	**Working []**	**Volume**
ddH_2O		To final volume
Buffer (ammonium buffer, 15 mM $MgCl_2$)	10 ×	10 mL
Primer i5xx	10 mM	4 mL
Primer i7xx	10 mM	4 mL

dNTPs	10 mM each	2 mL
AccuPol High Fidelity DNA Polymerase	2.5 U/mL	0.32
Digested and adapter-ligated DNA		
Total volume		100

2) In PCR machine/thermocycler: 95 °C for 2 min

10 to 20 cycles of 95 °C for 10 s

65 °C for 30 s

72 °C for 30 s

72 °C for 5 min

Hold at 4 °C

3) Purify with Ampure XP beads at 0.8 × and elute in 20 μL EB

4) Determine DNA concentration with Qubit Broad Range kit

5) Determine size distribution of fragments on the Agilent BioAnalyser. Select the size range that you wish to select and determine the concentration/amount of DNA for this range.

6) Combine samples from different indexing PCRs (Polymerase Chain Reaction) (i.e., differing outer barcode combinations) equimolarly according to (5).

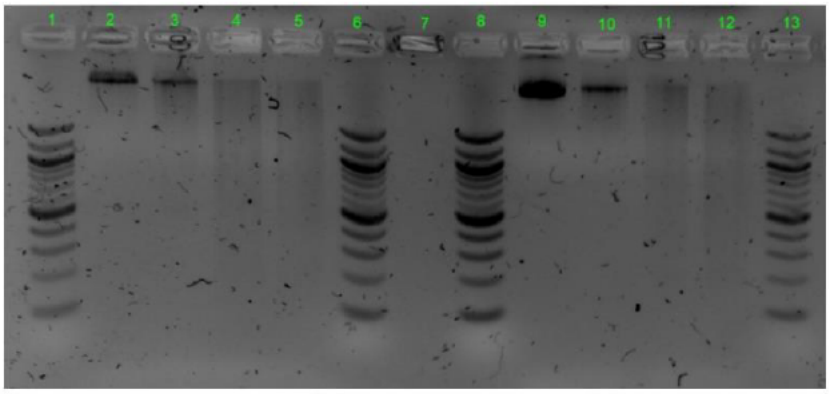
E: Size selection

Size selection and purification - either by PippinPrep or equivalent, or by gel extraction. If by gel-extraction,

F: Illumina MiSeq Run

Follow manufacturer's instructions for V3 kits

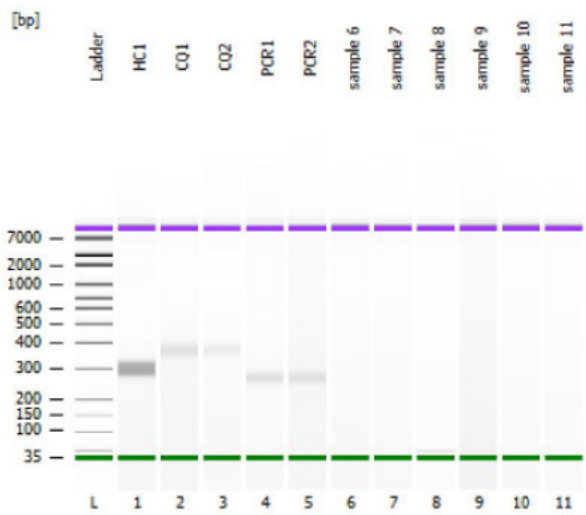
APPENDIX B: SUPPLEMENTARY INFORMATION FOR CHAPTER 3



Lane	Sample Name	Enzyme 1	Enzyme 2	Vol loaded in gel (µL)	DNA Conc ng/µL
1	Standard Ladder				
2	Bead purification Control (no enzyme) Spis_Aliwal_Shoal_AWL017D			20µL	100
3	Bead purification Spis_Aliwal_Shoal_AWL017D PstI	PstI 0.75µL		20µL	100
4	Bead purification Spis_Aliwal_Shoal_AWL017D MspI		MspI 1.5µL	20µL	100
5	Bead purification Spis_Aliwal_Shoal_AWL017D PstI+MspI	PstI 0.75µL	MspI 1.5µL	20µL	100
6	Standard Ladder				
7	Blank				
8	Standard Ladder				
9	Control (no enzyme) Spis_Aliwal_Shoal_AWL017D			20µL	100
10	Spis_Aliwal_Shoal_AWL017D PstI	PstI 0.75µL		20µL	100
11	Spis_Aliwal_Shoal_AWL017D MspI		MspI 1.5µL	20µL	100
12	Spis_Aliwal_Shoal_AWL017D PstI+MspI	PstI 0.75µL	MspI 1.5µL	20µL	100
13	Standard Ladder				

Figure S1: Representative restriction enzyme digestion using *PSTI* and *MSPI*. Run at a 2% gel, 20ul total volume, 3-hour digestion, run for 45 mins at 130v

Electrophoresis File Run Summary



Instrument Information:

Instrument Name:	DE04103806	Firmware:	C.01.069
Serial#:	DE04103806	Type:	G2939A

Assay Information:

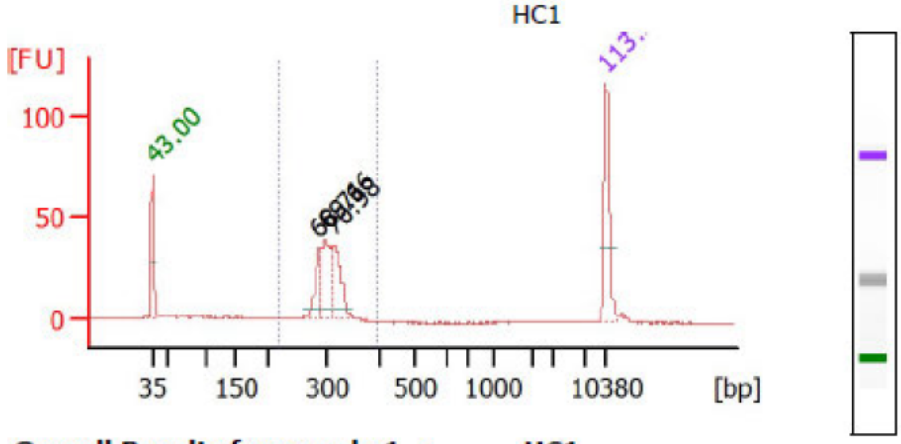
Assay Origin Path:	C:\Program Files (x86)\Agilent\2100 bioanalyzer\2100 expert\assays\dsDNA\High Sensitivity DNA.xsy
Assay Class:	High Sensitivity DNA Assay
Version:	1.03
Assay Comments:	Copyright © 2003-2010 Agilent Technologies

Chip Information:

Chip Lot #:

Reagent Kit Lot #:

Chip Comments:



Overall Results for sample 1 : HC1

Number of peaks found:	3
Noise:	0.3
Corr. Area 1:	251.0

Peak table for sample 1 : HC1

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5 411.3	Lower Marker
2	286	61.55	326.4	
3	296	96.33	493.4	
4	312	98.51	478.7	
5	10 380	75.00	10.9	Upper Marker

Figure S2: Bioanalyzer results

Table S1: Barcode adapters used per individual and the corresponding indexing barcodes

Sample	Species/locality	Inner adapter barcode		Outer index barcode
Group 1	BRF016	AACCCG	AGTCAT	D504-D701
	BRF012	AACCCG	GATCGT	D504-D701
	BRF010	AACCCG	GCATTG	D504-D701
	BRF026	AACCCG	TTAATG	D504-D701
	BRF022	AAGGGA	AGTCAT	D504-D701
	BRF020	AAGGGA	GATCGT	D504-D701
	BRF019	AAGGGA	GCATTG	D504-D701
	BRF028	AAGGGA	TTAATG	D504-D701
	BRF015	CAACTA	AGTCAT	D504-D701
	LDS021	CAACTA	GATCGT	D504-D701
	LDS031	CAACTA	GCATTG	D504-D701
	LDS028	CAACTA	TTAATG	D504-D701
Group 2	AWD024	AACCCG	AGTCAT	D503-D702
	LDS024	AACCCG	GATCGT	D503-D702
	LDS027	AACCCG	GCATTG	D503-D702
	AWD023	AACCCG	TTAATG	D503-D702
	LDS062	AAGGGA	AGTCAT	D503-D702
	AWD021	AAGGGA	GATCGT	D503-D702
	LDS037	AAGGGA	GCATTG	D503-D702
	LDS022	AAGGGA	TTAATG	D503-D702
	LDS025	CAACTA	AGTCAT	D503-D702
	LDS030	CAACTA	GATCGT	D503-D702
	LDS102	CAACTA	GCATTG	D503-D702
	LDS023	CAACTA	TTAATG	D503-D702
Group 3	AWL107	AACCCG	AGTCAT	D501-D704
	AWL108	AACCCG	GATCGT	D501-D704
	AWL110	AACCCG	GCATTG	D501-D704
	AWL111	AACCCG	TTAATG	D501-D704
	AWL095	AAGGGA	AGTCAT	D501-D704
	AWL071	AAGGGA	GATCGT	D501-D704
	AWL109	AAGGGA	GCATTG	D501-D704
	AWL088	AAGGGA	TTAATG	D501-D704
	AWL087	CAACTA	AGTCAT	D501-D704

Sample	Species/locality	Inner adapter barcode		Outer index barcode
	AWL097	CAACTA	GATCGT	D501-D704
	AWD017	CAACTA	GCATTG	D501-D704
	AWD094	CAACTA	TTAATG	D501-D704
Group 4	AWD093	AAGGGA	AGTCAT	D502-D703
	AWD006	AAGGGA	GATCGT	D502-D703
	AWD004	CAACTA	AGTCAT	D502-D703
	AWD022	CAACTA	GATCGT	D502-D703
	AWD025	CAACTA	GCATTG	D502-D703
	AWD011	CAACTA	TTAATG	D502-D703

Table S2: Total number of raw and filtered reads per group

File	Retained Reads	Low Quality	Barcode Not Found	RAD Cutsite Not Found	Total
Group-1	5673926	605014	99750	11992	6390682
Total Sequences	6390682				
Barcode Not Found	99750				
Low Quality	605014				
RAD Cutsite Not Found	11992				
Retained Reads	5673926				
Group-2	10540854	1134117	252144	54305	11981420
Total Sequences	11981420				
Barcode Not Found	252144				
Low Quality	1134117				
RAD Cutsite Not Found	54305				
Retained Reads	10540854				
Group-3	6430582	646726	134914	9824	7222046
Total Sequences	7222046				
Barcode Not Found	134914				
Low Quality	646726				
RAD Cutsite Not Found	9824				
Retained Reads	6430582				
Group-4	5932658	644726	124516	18174	6720074
Total Sequences	6720074				
Barcode Not Found	124516				
Low Quality	644726				
RAD Cutsite Not Found	18174				
Retained Reads	5932658				

Table S3: Total number of raw and filtered reads per individual and retained

Group 1					
Barcode	**Filename**	**Total**	**NoRadTag**	**LowQuality**	**Retained**
AACCCG-AGTCAT	BR016	21064	37	1885	19142
AACCCG-GATCGT	BR012	513508	1114	47761	464633
AACCCG-GCATTG	BR010	420370	614	40248	379508
AACCCG-TTAATG	BR026	461600	746	44585	416269
AAGGGA-AGTCAT	BR022	1065692	1908	100515	963269
AAGGGA-GATCGT	BR020	1001288	1789	96971	902528
AAGGGA-GCATTG	BR019	875762	1886	82794	791082
AAGGGA-TTAATG	BR028	448230	880	41895	405455
CAACTA-AGTCAT	BR015	37632	119	3775	33738
CAACTA-GATCGT	LS021	504198	1000	50587	452611
CAACTA-GCATTG	LS031	609254	1352	61086	546816
CAACTA-TTAATG	LS028	332334	547	32912	298875
Group 2					
Barcode	**Filename**	**Total**	**NoRadTag**	**LowQuality**	**Retained**
AACCCG-AGTCAT	AD024	707190	15644	76498	615048
AACCCG-GATCGT	LS024	721258	1349	70842	649067
AACCCG-GCATTG	LS027	839448	1970	80007	757471
AACCCG-TTAATG	AD023	707558	5560	69885	632113
AAGGGA-AGTCAT	LS062	1334314	3546	119702	1211066
AAGGGA-GATCGT	AD021	1807276	2821	174140	1630315
AAGGGA-GCATTG	LS037	750754	1518	73225	676011
AAGGGA-TTAATG	LS022	1395490	2633	133576	1259281
CAACTA-AGTCAT	LS025	1552932	1589	145920	1405423
CAACTA-GATCGT	LS030	888488	2521	87198	798769

CAACTA-GCATTG	LS102	407794	14021	43120	350653
Group 2					
Barcode	**Filename**	**Total**	**NoRadTag**	**LowQuality**	**Retained**
CAACTA-TTAATG	LS023	616774	1133	60004	555637
Group 3					
Barcode	**Filename**	**Total**	**NoRadTag**	**LowQuality**	**Retained**
AACCCG-AGTCAT	AS107	608128	820	55086	552222
AACCCG-GATCGT	AS108	491002	639	43379	446984
AACCCG-GCATTG	AS110	539718	654	48111	490953
AACCCG-TTAATG	AS111	332330	242	29850	302238
AAGGGA-AGTCAT	AS095	833976	1460	78617	753899
AAGGGA-GATCGT	AS071	926772	1258	86933	838581
AAGGGA-GCATTG	AS109	991856	1808	90893	899155
AAGGGA-TTAATG	AS088	704960	785	62887	641288
CAACTA-AGTCAT	AS087	516292	840	46474	468978
CAACTA-GATCGT	AS097	208900	184	19221	189495
CAACTA-GCATTG	AD017	522178	734	47877	473567
CAACTA-TTAATG	AD094	411020	400	37398	373222
Group 4					
Barcode	**Filename**	**Total**	**NoRadTag**	**LowQuality**	**Retained**
AAGGGA-AGTCAT	AD093	1554902	3081	150713	1401108
AAGGGA-GATCGT	AD006	1155978	2147	113030	1040801
CAACTA-AGTCAT	AD004	1377618	2424	131685	1243509
CAACTA-GATCGT	AD022	831302	1643	80792	748867
CAACTA-GCATTG	AD025	1482286	1943	144999	1335344
CAACTA-TTAATG	AD011	193472	6936	23507	163029

Table S4: Global statistics of *Stylophora pistillata* genome from GenBank

Global statistics of *Stylophora pistillata* genome	
Total sequence length	400,120,318
Total ungapped length	357,996,474
Gaps between scaffolds	0
Number of scaffolds	5,688
Scaffold N50	457,453
Scaffold L50	246
Number of contigs	37,615
Contig N50	20,604
Contig L50	4,779
Total number of chromosomes and plasmids	1
Number of component sequences (WGS or clone)	5,688

APPENDIX C: SUPPLEMENTARY INFORMATION FOR CHAPTER 4

Table S5: GenBank sequences for voucher specimens from previously published authors used in phylogenetic analyses of COI and mtMutS

Scientific Name	**Voucher Number**	**COI**
Sinularia brassica	RMNH:Coel. 41304	KF955200
Sinularia brassica	ZMTAU: Co36345	MH516397
Sinularia brassica	ZMTAU: Co35352	MH516392
Sinularia brassica	RMNHCoel_41307	KF955197
Sinularia brassica	ZMTAU: Co35368	MH516542
Sinularia slieringsi	ZMTAU: Co36315	MH516446
Sinularia abrupta	ZMTAU: Co36341	MH516389
Sinularia heterospiculata	ZMTAU: Co36281	MH516411
Sinularia variabilis	ZMTAU: Co36396	MH516461
Sinularia tumulosa	ZMTAUCo_36373	MH516455
Sinularia polydactyla	RMNH:Coel. 41339	KF955228
Sinularia gardineri	ZMTAU CO34097	GU355982
Sinularia compressa	ZMTAU CO34140	GU355989
Sinularia pavida	ZMTAU:CO33630	JX991269
Rumphella sp. A	RMNH:Coel. 40994	KF955142
Cryogorgia koolsae	5988-14A-1	FJ264910
Scientific Name	**Voucher Number**	**mtMutS**
Sinularia brassica	RMNH:Coel. 41306	KF915730.1
Sinularia brassica	RMNH:Coel. 41309	KF915729.1
Sinularia brassica	RMNH:Coel. 41305	KF915728.1
Sinularia brassica	RMNH:Coel. 41304	KF915727.1
Sinularia brassica	RMNH:Coel. 41308	KF915726.1
Sinularia brassica	RMNH:Coel. 41303	KF915725.
Sinularia brassica	RMNH:Coel. 41307	KF915724.1
Sinularia dura	NTM C13808	FJ621402.1
Sinularia compressa	RMNH Coel.38420	FJ621385.1
Sinularia polydactyla	RMNH:Coel. 41339	KF915753.1
Sinularia sp.		MF817841.1
Sinularia brassica		MF817879.1
Sinularia brassica		MF817870.1
Sinularia brassica		MF817881.1
Sinularia brassica		MF817882.1
Sinularia brassica		MF817886.1
Sinularia brassica		MF817891.1
Sinularia brassica		MF817827.1
Sinularia brassica		MF817833.1
Sinularia brassica		MF817891.1
Sinularia brassica		MF817877.1
Sinularia brassica		MF817875.1
Pterogorgia anceps		GQ342500.1

Table S6 Results generated using BarcodingR showing the assignment of samples to references using bp-based and Fuzzy Method

Sample IDs	Species Identified	bp.prob	Species Identified	FMF
TM bTM137	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.5404727	KF915728.1 Sinularia brassica voucher RMNH:Coel. 41305 MutS	0.000000000
TM bTM139	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.5404727	KF915725.1 Sinularia brassica voucher RMNH:Coel. 41303 MutS	0.000000000
TM bTM140	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.5404727	KF915728.1 Sinularia brassica voucher RMNH:Coel. 41305 MutS	0.000000000
TM bTM141	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.5404727	KF915725.1 Sinularia brassica voucher RMNH:Coel. 41303 MutS	0.000000000
TM bTM146	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.5404727	KF915728.1 Sinularia brassica voucher RMNH:Coel. 41305 MutS	0.000000000
TM bTM143	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.5404727	KF915728.1 Sinularia brassica voucher RMNH:Coel. 41305 MutS	0.000000000
TM bTM144	FJ621402.1 Sinularia dura voucher NTM C13808 MutS	0.6145973	MF817875.1 Sinularia brassica isolate 188 MutS	0.000000000
TM bTM145	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.3430635	KF915724.1 Sinularia brassica voucher RMNH:Coel. 41307 MutS	0.000000000
NM bNM117	FJ621402.1 Sinularia dura voucher NTM C13808 MutS	0.9372109	15753.1 Sinularia polydactyla voucher RMNH:Coel. 41339 MutS	0.000000000
NM bNM127	FJ621402.1 Sinularia dura voucher NTM C13808 MutS	0.9533382	KF915726.1 Sinularia brassica voucher RMNH:Coel. 41308 MutS	0.000000000
NM bNM116	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.6913299	FJ621385.1 Sinularia compressa voucher RMNH Coel.38420 MutS	0.000000000
NM bNM118	FJ621402.1 Sinularia dura voucher NTM C13808 MutS	0.9511465	FJ621402.1 Sinularia dura voucher NTM C13808 MutS	0.000000000
NM bNM119	FJ621402.1 Sinularia dura voucher NTM C13808 MutS	0.9529181	FJ621402.1 Sinularia dura voucher NTM C13808 MutS	0.000000000
NM bNM122	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.3059293	KF915724.1 Sinularia brassica voucher RMNH:Coel. 41307 MutS	0.000000000
NM bNM123	KF915729.1 Sinularia brassica voucher RMNH:Coel. 41309 MutS	0.1579941	KF915724.1 Sinularia brassica voucher RMNH:Coel. 41307 MutS	0.000000000
NM bNM124	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.5404727	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.000000000
NM bNM125	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.5144846	KF915729.1 Sinularia brassica voucher RMNH:Coel. 41309 MutS	0.000000000
NM bNM126	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.5411819	KF915728.1 Sinularia brassica voucher RMNH:Coel. 41305 MutS	0.000000000
LS bLS005	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.2596870	FJ621402.1 Sinularia dura voucher NTM C13808 MutS	0.000000000
LS bLS006	FJ621402.1 Sinularia dura voucher NTM C13808 MutS	0.9483175	FJ621402.1 Sinularia dura voucher NTM C13808 MutS	0.000000000
LS bLS007	FJ621402.1 Sinularia dura voucher NTM C13808 MutS	0.9404859	15753.1 Sinularia polydactyla voucher RMNH:Coel. 41339 MutS	0.000000000
LS bLS046	FJ621402.1 Sinularia dura voucher NTM C13808 MutS	0.9488669	FJ621402.1 Sinularia dura voucher NTM C13808 MutS	0.000000000
LS bLS102	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.5144846	KF915726.1 Sinularia brassica voucher RMNH:Coel. 41308 MutS	0.000000000
LS bLS106	MF817875.1 Sinularia brassica isolate 188 MutS	0.7433883	FJ621385.1 Sinularia compressa voucher RMNH Coel.38420 MutS	0.862068966
LS bLS107	MF817875.1 Sinularia brassica isolate 188 MutS	0.7402525	KF915753.1 Sinularia polydactyla voucher RMNH:Coel. 41339 MutS	0.009535988
LS bLS108	FJ621402.1 Sinularia dura voucher NTM C13808 MutS	0.9485787	FJ621402.1 Sinularia dura voucher NTM C13808 MutS	0.000000000
LS bLS115	FJ621402.1 Sinularia dura voucher NTM C13808 MutS	0.9501253	KF915753.1 Sinularia polydactyla voucher RMNH:Coel. 41339 MutS	0.000000000
LS bLS041	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.3053805	KF915726.1 Sinularia brassica voucher RMNH:Coel. 41308 MutS	0.000000000
LS bLS047	KF915729.1 Sinularia brassica voucher RMNH:Coel. 41309 MutS	0.1579105	KF915724.1 Sinularia brassica voucher RMNH:Coel. 41307 MutS	0.000000000
LS bLS014	KF915726.1 Sinularia brassica voucher RMNH:Coel. 41308 MutS	0.2472820	KF915726.1 Sinularia brassica voucher RMNH:Coel. 41308 MutS	0.000000000
LS bLS010	FJ621402.1 Sinularia dura voucher NTM C13808 MutS	0.1117410	KF915729.1 Sinularia brassica voucher RMNH:Coel. 41309 MutS	0.000000000
LS bLS042	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.3053805	KF915726.1 Sinularia brassica voucher RMNH:Coel. 41308 MutS	0.000000000
LS bLS048	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.3053805	KF915726.1 Sinularia brassica voucher RMNH:Coel. 41308 MutS	0.000000000
LS bLS049	FJ621402.1 Sinularia dura voucher NTM C13808 MutS	0.1419558	KF915726.1 Sinularia brassica voucher RMNH:Coel. 41308 MutS	0.000000000
KB bKB037	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.3053805	KF915724.1 Sinularia brassica voucher RMNH:Coel. 41307 MutS	0.000000000
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KB bKB031	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.3055601	KF915729.1 Sinularia brassica voucher RMNH:Coel. 41309 MutS	0.000000000
KB bKB032	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.3053805	KF915724.1 Sinularia brassica voucher RMNH:Coel. 41307 MutS	0.000000000
KB bKB033	KF915730.1 Sinularia brassica voucher RMNH:Coel. 41306 MutS	0.2587397	FJ621402.1 Sinularia dura voucher NTM C13808 MutS	0.000000000
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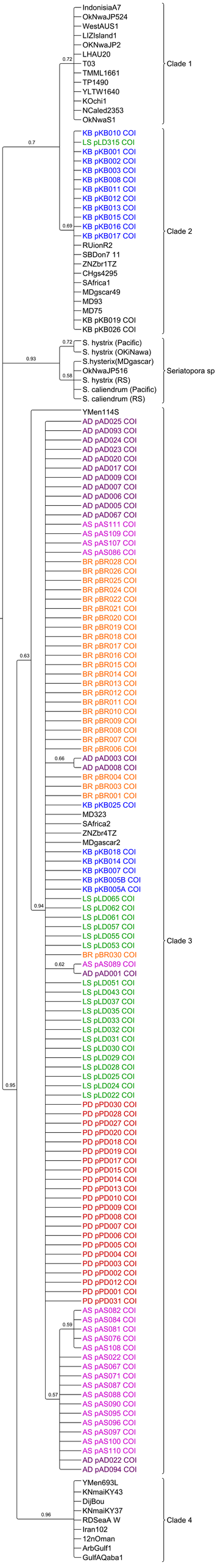
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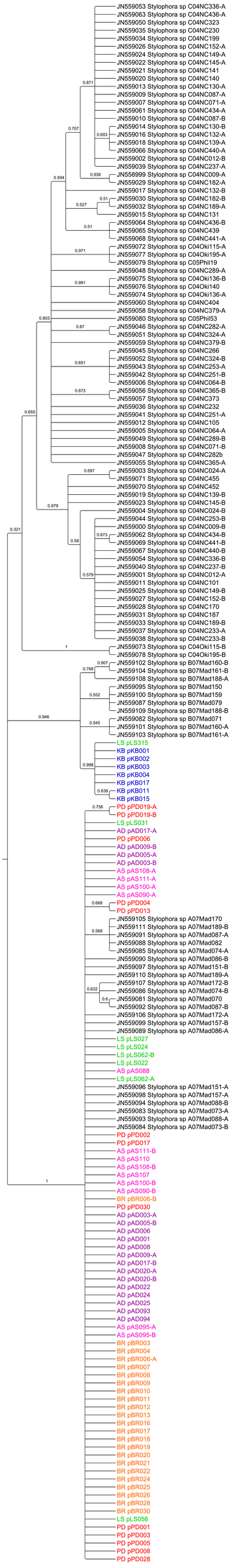
GulfAQaba1
Clade 4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JN559060 Stylophora sp C04NC404
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JN559042 Stylophora sp C04NC251-B
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JN559008 Stylophora sp C04NC071-B
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JN559040 Stylophora sp C04NC237-B
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JN559103 Stylophora sp B07Mad161-A
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KB pKB002
KB pKB003
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KB pKB011
KB pKB015
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LS pLS031
AD pAD017-A
PD pPD006
AD pAD009-B
AD pAD005-A
AD pAD003-B
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AS pAS100-A
AS pAS090-A
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PD pPD013
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JN559097 Stylophora sp A07Mad151-B
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JN559089 Stylophora sp A07Mad086-A
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LS pLS024
LS pLS062-B
LS pLS022
AS pAS088
LS pLS062-A
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PD pPD017
AS pAS111-B
AS pAS110
AS pAS108-B
AS pAS107
AS pAS100-B
AS pAS090-B
BR pBR006-B
PD pPD030
AD pAD003-A
AD pAD005-B
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AD pAD001
AD pAD008
AD pAD009-A
AD pAD017-B
AD pAD020-A
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AD pAD022
AD pAD024
AD pAD025
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AD pAD094
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BR pBR010
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BR pBR012
BR pBR013
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BR pBR018
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BR pBR020
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BR pBR022
BR pBR024
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LS pLS056
PD pPD001
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PD pPD005
PD pPD008
PD pPD028
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0.568
0.632
0.6
1