

A multidisciplinary study to assess the ecology of the Cape sea urchin, *Parechinus angulosus*, and its emerging use as a bioindicator to monitor coastal resiliency

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By

Suzanne Redelinghuys

ORCID ID

<https://orcid.org/0000-0002-0464-2858>

Supervisor: Professor Francesca Porri

NRF-South African Institute for Aquatic Biodiversity (NRF-SAIAB)

Co-supervisor: Dr Gwynneth Matcher

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Abstract

The resilience of marine species in the face of unpredictable climate change stands as a paramount concern for the maintenance of ecological stability. Under such fluctuating conditions, it is critical to understand how organisms mitigate these effects in physiological, genetic, and morphological terms. To that end, this thesis focused on the Cape sea urchin, *Parechinus angulosus*, employing a multidisciplinary approach encompassing morphology, genomics, and gut microbial diversity to assess its potential as a bioindicator species and elucidate its adaptive strategies in response to varying environmental conditions along the South African coastline. This was achieved through studying their anatomy in order to link observed variation to prevailing local environmental conditions, aided by the species' wide distribution range which allows insight into adaptations across broad geographic regions and ecological settings.

The first empirical chapter, Chapter 3, focusing on eight key morphometric traits of test, Aristotle's lantern and spines, revealed distinct variation in the Cape sea urchin's morphology between the east and west coasts of South Africa, suggesting the presence of some level of local adaptation to the prevailing environmental factors found on the east and west coasts of South Africa. This points to potential bioindicator capabilities of the species, reflecting adaptive divergence amidst contrasting environmental conditions. Further analysis is however necessary in order to isolate specific physiological trends that may be associated with these morphometric differences, thereby enhancing and tightening their ecological implications.

The second experimental chapter, Chapter 4, delved into the genetic structure of the Cape urchin by investigating genome-wide diversity, the presence of cryptic population structure, and spatial patterns of genomic diversity. Moderate genomic differentiation was detected among populations along the eastern and western coasts of South Africa by outlier loci that may undergo natural selection, which could indicate local adaptation to environmental conditions. This pattern hints at adaptive differentiation and cryptic genetic structures within the Cape sea urchin populations and emphasises the species' potential adaptive responses to localised (in this case regional) environmental pressures. Assigning functional significance to these genetic variations will require a comprehensive annotated reference genome, a limitation acknowledged in the current study.

Chapter 5 explored the gut microbial diversity and revealed significant compositional variations between the east and west coast populations of South Africa, confirming regional and inter-regional variation. This chapter also highlighted the essential biochemical pathways critical to the survival of the host which is crucial for assessing the health of the urchin host. Together, the functional content of the gut bacteria and microbial diversity showcases its potential as a bioindicator for coastal ecosystem health. Logistical challenges and confounding factors like host physiology will need to be fully considered for its effective application.

Overall, the findings of this doctoral research suggest that the Cape sea urchin displays promising characteristics as a bioindicator species due to its morphological, genetic, and gut microbial variations in response to environmental differences, providing a diverse array of means in which urchins could be used as bioindicators, from their uses to assess water quality and detect pollution, to ecosystem health monitoring and biodiversity studies in which sea urchin abundance, distribution, and presence are monitored. Further research, integrating these multidisciplinary approaches is recommended to validate and refine its bioindicator potential. Additionally, the development of a comprehensive annotated reference genome is imperative to harness the species' genetic information effectively. This study underscores the significance of integrating multiple disciplines in understanding how species respond to environmental change and their potentials contributions to monitor ecological resilience. The original multidisciplinary approach, combined with high computational outputs presents a promising framework for a comprehensive ecological monitoring in marine ecosystems.

Declaration

I, Suzanne Redelinghuys, hereby declare that the work described in this thesis was carried out at the South African Institute for Aquatic Biodiversity under the supervision of Professor Francesca Porri and Dr Gwynneth Matcher. The components of this thesis comprise original work by the author and have not been submitted to any other university.



Suzanne Redelinghuys

Dedication

To my incredible mother. This degree is yours.

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Research findings contained in this dissertation represent the candidate's original work, except where the work of others has been acknowledged in the text. Research findings have not been submitted in part or in full towards any other degree at any other universities or tertiary institutions. Conclusions arrived at are those of the candidate and are not necessarily to be attributed to Rhodes University or donors and funders.

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I had never encountered anything so hard, for which I was so ill prepared.

– A Walk in the Woods, Bill Bryson

List of Figures

Figure 2.1: The South African map indicating all sampling locations from which individuals of the Cape sea urchin, *Parechinus angulosus*, were collected. On the main top map, the easternmost collection site, Port St John's, is indicated by a red pin, as is the westernmost site, McDougall's Bay found just below the blue pin. The remaining pins represent the 41 other sites used in the data set. In order to make discerning between populations that are densely packed easier, two sections of the map were blown up. Populations blocked in turquoise represent a portion of the west and south coasts and populations blocked in red represent a portion of the south east coast of South Africa (GoogleMaps 2023).

Figure 2.2: South Africa's coastal and offshore bioregions as defined and published by Lombard 2004).

Figure 2.3: South African map indicating the modified bioregions used in this thesis. The blue indicates the east coast, consisting of the south Natal and Agulhas bioregions; the red indicates the west coast, consisting of the Namaqua and south-western Cape bioregions; and the green indicates the south-western Cape bioregion.

Figure 3.1: Denuded sea urchin test indicating the madreporite (red), apical system (yellow), interambulacral zone 1 (green), and ambulacral zone 1 (blue) (photographed with a Pentax DSLR camera, 2X magnification).

Figure 3.2: Denuded sea urchin test with axes along which the five width measurements were taken (blue) (photographed with a Pentax DSLR camera, 2X magnification).

Figure 3.3: Graphic illustration of the K nearest neighbour machine-learning method (Hardik Jaroli 2019). The star at the center of the diagram denotes the unclassified individual, with the circles denoting the individuals used to attempt to place the unclassified individual. Using the inner circle, the unclassified individual is classified as belonging to the West Coast population. Using the outer circle places the unclassified individual as originating from the East Coast.

Figure 3.4: A principal component ordination plot of populations (i.e., sites) from which individuals of *Parechinus angulosus* were collected, showing clusters generated at a Euclidean distance of 3.4. Each population is represented using a combination of different colours and symbols (see legend in figure).

Figure 3.5: A principal component ordination plot constructed using the same data of *Parechinus angulosus* individuals as in Figure 3.4 above, but of the coast factor level, also showing clusters at a Euclidean distance of 3.4. Blue triangles represent individuals from the east coast, red triangles individuals from the west coast.

Figure 3.6: Boxplots of the three indices calculated for the sea urchin, *Parechinus angulosus* compared for the east and west coasts of South Africa: (A) Globosity index; (B) Volumetry index; (C) Density index. The horizontal line inside the box represents the median value, and the box spans from the 25th percentile to the 75th percentile, while the vertical lines extend to the 5th and 95th percentiles. Note different scales on Y-axes.

Figure 3.7: Boxplots of the four characters measured in the sea urchin, *Parechinus angulosus*: (A) Small spine length (SL); (B) Small spine width (SW); (C) Large spine length (LL); (D) Large spine width (LW). The horizontal line inside the box represents the median value, and the box spans from the 25th percentile to the 75th percentile, while the vertical lines extend to the 5th and 95th percentiles.

Figure 3.8: Principal component analysis of the average values of each of the four measures collected from 46 individuals (SL, SW, LL, LW). A total of 99% of the data's variance is captured between the three axes.

Figure 3.9: Principal coordinate analysis of the average of the four metrics collected from 46 individuals (SL, SW, LL, LW). The axes capture 97.99% of the variance of the data.

Figure 3.10: Boxplots displaying the four measured characteristics of the sea urchin *Parechinus angulosus*: (A) Small spine length (SL); (B) Small spine width (SW); (C) Large spine length (LL); (D) Large spine width (LW). The median value is represented by the horizontal line in the box, which extends from the 25th to the 75th percentiles. The vertical lines span the distance from the 5th to the 95th percentiles.

Figure 3.11: Principal component analysis of the mean values of the four measures taken from 46 *Parechinus angulosus* individuals (SL, SW, LL, LW), capturing 99% of the data's variance between the three axes.

Figure 3.12: Principal coordinate analysis of the mean of the four measurements taken from 46 *Parechinus angulosus* individuals (SL, SW, LL, LW), accounting for 96.67% of the variance of the data between the three axes.

Figure 3.13: A graphical representation of the Bayesian model coefficients, showing that the estimated coefficient for Large Width (LW) and its 95% credible interval does not include zero.

Figure 3.14: The posterior distribution of model parameters (left panels) and a caterpillar graph (right panels) for the four morphometric traits ((SL, SW, LL, LW) that show multiple replicate simulations of the model has converged successfully on similar values, thus these estimates are robust.

Figure 4.1: Bar plot depicting the genome regions where variant sites are situated.

Figure 4.2: Vertical bar plot showing the number of discarded (-) and retained (+) individuals per population. The figure shows that none of the individuals were removed due to low-quality or low-coverage sequences.

Figure 4.3: The proportion of three different genotypes (homozygous major, heterozygous, and homozygous minor), across sea urchin populations. Red corresponds to individuals from the west coast, green to individuals from the border zone (i.e., Kommetjie, see Figure 2.1 for map reference), and blue to individuals from the east coast.

Figure 4.4: Boxplots of the estimated value for Tajima's D and the SNP divergence between and within populations of *P. angulosus*. Blue, green, and red colours show the east coast, south-western cape, and west coast, respectively.

Figure 4.5: Heatmap showing the genetic distance between individuals within different bioregions. Blue, green, and red colours show the east coast, south-western cape, and west coast, respectively. Values and shading on the plot indicate the extent of the genetic distance between individuals.

Figure 4.6: Graphical results of discriminant analysis of principal components on the generated SNP data sets. Blue, green, and red colours show the east coast, southwestern cape, and west coast, respectively, corresponding with the colours in 4.4 to delineate the same populations, which do not overlap. The four plots generated for the four top axes indicate the highest proportion of the variability within populations.

Figure 4.7: Cross-entropy graph indicating the number of clusters identified from the data set. A clear optimum is obvious at value $K = 3$.

Figure 4.8: Co-ancestry plot showing the proportions of each individual genome that are most likely to originate from three putative clusters identified in the study. Colours here do not represent the three populations as in previous graphs, but the three putative clusters identified in Figure 4.7 above.

Figure 4.9: Q-matrix plot showing the proportion of co-ancestry of *P. angulosus* population superimposed over the coastal map of South Africa. From west to east these sites read McDougall's Bay, Kleinsee, Hondeklip, Groenrivier, Doringbaai, Kommetjie, Betty's Bay, Arniston, Jongensfontein, Gouritz, Mossel Bay, Victoria Bay, Buffels Bay, Beacon Island, Natures' Valley, St Francis, Cannon Rocks, Kasouga, Spinning Reel, Rietrivier, Fort D'Acre, Mtati, Birha, Kidd's Beach, Glengariff, Cintsa West, Kei Mouth, Coffee Bay, Presley Bay, Port St John's. The colours correspond to the ancestry coefficients in Figure 4.8.

Figure 4.10: A Manhattan plot of the outlier loci detected by the OutFLANK and pcadapt analyses. The red colour shows outlier SNPs and the values on the y-axis are $-\log p$ -values of test statistics for the significance of these loci. Note that values on the y-axis are not on the same scale between the two methods.

Figure 4.11: Allele frequency changes in the identified outlier loci between populations of *P. angulosus* across South Africa. Green indicates allele frequency changes identified by pcadapt, and orange that of OutFLANK. The values at the top of the figure are SNP indices based on the *Lytechinus variegatus* genome.

Figure 4.12: Two loci identified (blue circles) based on genome-wide differentiation scan (GWDS) that significantly differ between the east and west coasts of South Africa.

Figure 4.13: Scatter plots indicating loci significantly associated (above the horizontal blue line) with test height (A), test width (B), and temperature (C).

Figure 4.14: Circus graph indicating the location of the 37 mitogenomic features in the representative mitogenome of Cape sea urchin.

Figure 4.15: Schematic representation of the alignment of 51 partially complete mitogenomes using sequences generated using the ezRAD protocol. Different colours represent different nucleotides (yellow is guanine, red is thymine, green is adenine, and dark blue is cytosine), and the horizontal bars of continuous light blue are the segments that are missing from that assembly.

Figure 4.16: Phylogenetic tree representing individuals from different populations of the Cape sea urchin, *Parechinus angulosus* along the South African coastline. The red and blue colours show branches leading to individuals from the east and west coasts, respectively. The x-axis is time to most recent common ancestor in millions of years.

Figure 4.17: Initial eastward expansion of the Cape sea urchin, *Parechinus angulosus*, from the root point at Kommetjie ($n = 30$, 1 cm = 50 000 cm). The green line indicates the dispersion of the species.

Figure 4.18: Secondary expansion of the distribution range of *Parechinus angulosus* westwards from the east coast sites colonised in the initial wave of eastward expansion ($n = 30$, 1 cm = 50 000 cm). The green line indicates the dispersion of the species.

Figure 5.1: Example of correlation clustering tree constructed using example data to describe the concept of balances. The values on different nodes are proportional to the abundance of bacterial taxa in all branches descending from that node. Different colours show presence of different bacterial taxa. The log-ratio highlighted in red are balances that are different between two biogeographical regions (Source: ‘Differential abundance analysis with gneiss’ in ‘Qiime2docs’ 2018, <https://docs.qiime2.org/2018.8/tutorials/gneiss/>).

Figure 5.2: Bacterial diversity reported at phylum level from the gut microbiota of the Cape sea urchin, *Parechinus angulosus*, from individuals collected from the east and west coasts of

South Africa. Abbreviations on the x-axis represent population (sampling site) names which can be viewed in Chapter 2; Figure 2.1.

Figure 5.3: Rarefaction curves of Observed sequence features (top) and the Shannon diversity (bottom) indices of the gut bacterial taxa from 101 individuals of *Parechinus angulosus* collected from 37 sites along the South African coastline. For better visualisation, the sequencing depth up to only 3000 was plotted.

Figure 5.4: Boxplots of the α -diversity measure of evenness among populations (top) and between coasts (bottom). The horizontal line inside the box represents the median value, and the box spans from the 25th percentile to the 75th percentile, while the vertical lines extend to the 5th and 95th percentile.

Figure 5.5: Principal coordinate analysis ordination plot of weighted-UniFrac (A) and unweighted UniFrac (B) distances generated on gut bacterial sequences of the sea urchin *Parechinus angulosus* collected from the east (red diamonds) and west (blue circles) coasts of South Africa.

Figure 5.6: Heatmap showing the differential abundances (log-scale abundance ratio) of gut bacterial taxa of individuals of *Parechinus angulosus* collected from the east and west coasts of South Africa. In the bar plot on the far left, the bright red bars represent a combination of taxa in the denominator of the balance, while the paler red bars indicate the subset of taxa found in the numerator. In the heat map on the right, different colours show the log-scale abundance ratio of different taxa.

Figure 5.7: Proportion plot of the y0 balance analysis from Gneiss showing differentially abundant bacterial groups between the east and west coasts of South Africa. Bacteria in the light-blue section of the graph are the bacteria found in the numerator of the balance, and those in the dark-blue section are bacteria found in the denominator of the balance.

Figure A2.1: Number of sequences per phylum per site for the four most dominant phyla, Bacteroidota, Campylobacterota, Fusobacteriota, Proteobacteria, and a class consisting of all the remaining less dominant, and unknown/unclassified phyla found in the gut microbial community of *Parechinus angulosus* individuals collected from 32 sites on the South African coastline.

Figure A2.2 A principal component analysis ordination plot of populations (i.e., sites) from which individuals of *Parechinus angulosus* were collected for analysis of their gut microbiota, showing clusters generated at 40% similarity. Each site is represented using a combination of different colours and symbols.

Figure A2.3: A principal component analysis ordination plot constructed using the same gut bacterial data of *Parechinus angulosus* individuals as in Figure A2.3 above, but of the coast factor level, clustering together individuals from the east and west coasts based on 40% similarity. Blue triangles represent individuals from the east coast, red triangles individuals from the west coast.

Figure A2.4: A clustered heatmap displaying the frequency of the four dominant gut microbial phyla in the sea urchin *Parechinus angulosus*, revealing clusters of populations irrespective of their east or west coast locations.

List of tables:

Table 3.1: A summary of studies utilising morphometric trait measurements in studies on various sea urchin species.

Table 3.2: PERMANOVA main test results examining the effects of coast and population (nested in coast) on the morphometric characters of individuals of *Parechinus angulosus* collected at sites along the South African coastline [Degrees of Freedom, Df: Sum of Squares, SS: Mean Squares; Pseudo F-Ratio; P-value (permutation); Monte Carlo P-values].

Table 3.3: Results of the PERMANOVA pairwise and SIMPER analyses showing the percentage contribution of each morphometric trait collected from individuals of *Parechinus angulosus* between pairs of populations within the east coast.

Table 3.4: PERMANOVA and SIMPER analyses results showing the percentage contribution of each morphometric trait collected from individuals of *Parechinus angulosus* to the difference between pairs of populations within the west coast.

Table 3.5: Results from SIMPER analyses results indicating which morphometric traits collected from individuals of *Parechinus angulosus* contributed towards dissimilarity observed between the coasts, and the percentage contribution of each trait towards the dissimilarity.

Table 3.6: Select summary of the Mann-Whitney tests for the globosity, volumetry and density indices calculated using test and lantern metric data collected from individuals of *Parechinus angulosus* from the east and west coasts of South Africa.

Table 3.7: Mean ($\pm$ SD) of the four spine character measurements (SL, SW, LL, LW) taken from individuals of *Parechinus angulosus* at individual level (unit = μm).

Table 3.8: Spearman's correlation coefficient calculated for each of the characters (SL, SW, LL, LW) measured from spines collected from *Parechinus angulosus* individuals, at individual level.

Table 3.9: An error matrix (confusion matrix) generated using mean values from four measurements (SL, SW, LL, LW) of 46 *Parechinus angulosus* individuals, representing the 16 subset populations, the two coasts, the total number of individuals per population, the number of individuals assigned to the east and west coasts, the number of individuals correctly assigned per coast, and the analysis' overall success rate (69.57%).

Table 3.10: An error matrix (confusion matrix) generated using mean values from four measurements (SL, SW, LL, LW) of 44 *Parechinus angulosus* individuals from 14 populations, indicating a 65.91% success rate in the assignment of individuals to the coast from which they were collected.

Table 3.11: Mean ($\pm$ SD) of the four spine characters (SL, SW, LL, LW) measurements collected from *Parechinus angulosus* individuals, at population level (unit = μm).

Table 3.12: Spearman's correlation coefficient computed for each of the characters measured (SL, SW, LL, LW) at population level.

Table 3.13: An error matrix (confusion matrix) generated from individual spine measurements (SL, SW, LL, LW) collected from *Parechinus angulosus* individuals for each of the two coasts ($n = 13\,609$), indicating the total number of data points per population, the number of data points assigned to the east and west coasts, the number of data points correctly assigned per coast, and the analysis's success rate (69.43%).

Table 3.14: A summary of the Bayesian model coefficients. The estimated coefficient for Large Width (LW) and its 95% credible interval does not include zero.

Table 3.15: A summary of the classificatory analysis of the Bayesian model created using four sea urchin spine characters (SL, SW, LL, LW) measured from *Parechinus angulosus* individuals collected from the east and west coasts of South Africa.

Table 4.1: Number of mutations by classed by function.

Table 4.2: Counts and percentages of mutations classed by the impact of the mutation on the genome.

Table 4.3: Counts and percentages of mutations by type.

Table 4.4: The base changes (SNPs) caused by mutations.

Table 4.5: Average heterozygosity estimated for each population based on the total number of assigned SNPs.

Table 4.6: Alignment summary table.

Table 5.1: Summary statistics of the data set containing the merged forward and reverse sequences of gut bacteria from the sea urchin *Parechinus angulosus*.

Table 5.2: Summary statistics of sequences collapsed to 1842 unique sequencing features.

Table 5.3: Results of PERMANOVA on the weighted (the abundance of bacteria) and unweighted-UniFrac (presence or absence of bacteria) diversity indices generated on gut bacterial sequence feature data of the Cape sea urchin, *Parechinus angulosus* collected from the east and west coasts of South Africa.

Table 5.4: Summary table of the functional content of gut bacterial taxa of individuals of the Cape sea urchin, *Parechinus angulosus* collected from the east and west coasts of South Africa, reported as a number of biochemical pathways.

Table A1.1: Codon changes in the genome of the Cape sea urchin, *Parechinus angulosus* collected from sites along the South African coastline.

Table A1.2.: PERMANOVA results on unweighted and weighted UniFrac indices of β -diversity.

Table A1.3: The results of simplicial linear regression for all balances.

Table A1.4: The functional content of gut bacterial taxa of individuals of the Cape sea urchin, *Parechinus angulosus*, collected from the east and west coasts of South Africa, reported as a number of biochemical pathways.

Table A1.5: The functional content of gut bacterial taxa of individuals of the Cape sea urchin, *Parechinus angulosus*, collected from the east and west coasts of South Africa, reported as a number of genetic information processing pathways.

Table A1.6: The functional content of gut bacterial taxa of individuals of the Cape sea urchin, *Parechinus angulosus*, collected from the east and west coasts of South Africa, reported as a number of environmental information processing pathways.

Table A1.7: The functional content of gut bacterial taxa of individuals of the Cape sea urchin, *Parechinus angulosus*, collected from the east and west coasts of South Africa, reported as a number of cellular processing pathways.

Table A1.8: The functional content of gut bacterial taxa of individuals of the Cape sea urchin, *Parechinus angulosus*, collected from the east and west coasts of South Africa, reported as a number of organismal system processing pathways.

Table A2.1: PERMANOVA main test results examining the effect of coast, or populations within coast, on bacterial phyla from the gut of *Parechinus angulosus* individuals collected at sites along the South African coastline [Degrees of Freedom, Df: Sum of Squares, SS: Mean Squares; Pseudo F-Ratio; P-value (permutation); Monte Carlo P-values].

Table A2.2: Results of the PERMANOVA pairwise and SIMPER analyses showing the percentage contribution of each morphometric trait collected from individuals of *Parechinus angulosus* between pairs of populations within the east coast.

Table A2.3: Clusters with the highest number of sequence features of sites from both the east (pink) and west (blue) coasts per bacterial phylum according to Figure A2.5.

Contents

A multidisciplinary study to assess the ecology of the Cape sea urchin, <i>Parechinus angulosus</i> , and its emerging use as a bioindicator to monitor coastal resiliency	i
Abstract.....	i
Declaration.....	iii
Dedication.....	iv
Acknowledgements:.....	v
Personal thanks	vi
List of Figures.....	ix
List of tables:.....	xv
Chapter one: General introduction.....	1
1.2 Structure of thesis	6
1.3 Ethical clearance and collection permits.....	8
Chapter two: An introduction to the Cape sea urchin, <i>Parechinus angulosus</i> , and its distribution range along the coast of South Africa.....	9
2.1 Coastal zones	10
2.1.1 Sea urchins.....	13
2.2 Study region.....	15
2.3 Study species.....	16
2.4 Study sampling locations	17
Chapter three: Assessing ecological resilience in <i>Parechinus angulosus</i> through morphometric trait analysis.....	24
3.1 Introduction.....	25
3.1.1 Background and significance	25
3.1.2 Morphometric analysis in ecological studies on sea urchins	32
3.1.3 Rationale and Aims.....	34
3.1.4 Hypotheses and Research Questions.....	40
3.2 Materials and Methods.....	41
3.2.1 Collection.....	41
3.2.1.1 Processing	42
3.2.2 Statistical analysis	45
3.2.2.1 Test and Aristotle's lantern metrics	45
3.2.2.2 Spine metrics.....	47
3.2.2.2.1 Classificatory discriminant analysis.....	48
3.2.2.2.2 Bayesian multi-level modelling for assigning individuals to their population of origin	51

3.3 Results.....	52
3.3.1 Test and Aristotle's lantern metrics	52
3.3.2 Spine metrics.....	77
3.3.2.1 Classificatory discriminant analysis.....	77
3.3.2.1.1 Individual-level data set	77
3.3.2.1.2 Population-level data set	84
3.3.2.2 Bayesian multi-level modelling for assigning individuals to their population of origin...	90
3.4 Discussion	93
3.4.1 Limitations	99
3.5 Conclusion	100
4.1 Introduction.....	103
4.1.1 Rationale and aims	107
4.1.2 Hypotheses and research questions.....	109
4.2 Materials and methods	109
4.2.1 Collection & Processing.....	109
4.2.2 Genomic DNA extraction and preparation of metabarcoding libraries	110
4.2.3 Bioinformatic analysis	111
4.2.3.1 Quality control and sequence assembly	111
4.2.3.2 Reference-based assembly	112
4.2.3.3 De novo assembly	114
4.2.3.3.1 De novo assembly of <i>P. angulosus</i> mitogenome from ezRAD sequences	114
4.2.3.3.2 Estimation of summary statistics	115
4.2.3.3.3 Detection of population structure.....	116
4.2.3.3.4 Detecting signals of local adaptation	116
4.2.3.3.5 Reconstruction of the sea urchin mitogenome phylogeography	117
4.3 Results.....	119
4.3.1 Reference-based assembly	119
4.3.2. Mitogenome assembly	134
4.3.3 De novo assembly of ezRAD sequences.....	141
4.4 Discussion	142
4.5 Conclusion	147
Chapter five: An ecological study of the gut microbiota of the Cape sea urchin, <i>Parechinus angulosus</i>	149
5.1 Introduction.....	150
5.1.1 Bacteria in the environment	150
5.1.2 Importance of bacteria	151

5.1.3 Bacterial symbioses.....	152
5.1.4 Rationale and aims.....	155
5.1.5 Hypotheses and research questions.....	155
5.2 Materials and methods	155
5.2.1 Collection & Processing.....	156
5.2.2 Genomic DNA extraction and preparation of metabarcoding libraries	156
5.2.3 Computational analysis	157
5.3 Results.....	161
5.4 Discussion.....	172
5.5 Conclusion	177
Chapter six: General discussion.....	178
References.....	187
Appendix 1 (Supplementary material for Chapter 4 and 5).....	312
Appendix 2 - Mothur (Supplementary material for Chapter 5)	381
A2.1 Processing	381
A.2.2 Results.....	381

Chapter one: General introduction

Anthropogenically driven climate change has profound effects on the planet's ecosystems, emerging as a powerful force reshaping climate patterns (Halpern *et al.* 2008). This phenomenon refers to the vast spectrum of shifts in the Earth's climatic patterns, primarily driven by human-induced factors such as greenhouse gas emissions (Ledley *et al.* 1999), deforestation (Shukla *et al.* 1990), and industrial practices (Galbreath 2010). These climatic shifts range from rising global temperatures (Walther *et al.* 2002) to fluctuating patterns of precipitation (Dore 2005) and intensified extreme weather events such as heatwaves (Ebi and Meehl 2007), cold spells (Meehl *et al.* 2000), and overall unpredictable severe climatic fluctuations (Bryant 1997).

The effects of these shifts are acutely felt across various ecosystems, particularly impacting the functioning of both terrestrial (Field *et al.* 2007) and marine habitats (Beaugrand *et al.* 2010). Just as with terrestrial ecosystems, marine ecosystems face unprecedented challenges due to the compounded negative effects of over-exploitation of marine resources, pollution, rising temperatures, changes in salinity, and ocean acidification which threaten ecological assemblages and functioning across the globe (Brierley and Kingsford 2009, Doney *et al.* 2012, Constanza *et al.* 2014, Bland *et al.* 2018). The speed of destruction is so drastic that in 2015, the protection of oceanic habitats became one of the most important targets in the agenda of the UN Sustainable Development Goals (SDG14: Life Below Water). Unfortunately, it is expected that unpredictable changes in climatic patterns will still continue and in fact exacerbate in coming decades (McMichael, 2001). An increase of even 2°C in global temperature is sufficient to drive 5% of all species to the brink of extinction (Waldvogel *et al.* 2020).

The destruction of marine ecosystems has a disproportionate impact on the planet and its inhabitants, with approximately 38% of the world's population living within coastal areas, which are directly or indirectly reliant on the ocean for their livelihoods (UNEP 2014b). Additionally, maritime transport of goods, accounted for an estimated 11 billion tons in 2021, with growth recorded in all developing regions: Africa, Latin America, the Caribbean, and Asia (collective growth of 14.6% of the growth observed in the industry), the latter of which remained the world's leading maritime cargo handling centre (UNCTAD 2022). Crucially, the world's oceans sustain biodiversity by providing diverse habitats and ecosystems (Palumbi *et*

al. 2009), supporting an array of species and genetic diversity required by marine organisms (Armbrust and Palumbi 2015). Anthropogenic activities such as those listed above, and overfishing and habitat destruction, put marine biodiversity at risk by disrupting ecosystems and causing the collapse of fisheries stocks (Bland *et al.* 2018). A further pivotal role by oceans is in the carbon cycle and production of oxygen, significantly influencing the entire global population (Nedoluzhko 2023).

Within ecosystems, habitat degradation and the breakdown of essential functions can trigger a cascading effect leading to vulnerable species' extinction (Thomsen *et al.* 2010, Gribben *et al.* 2019), of particular concern for keystone species. Such species are defined through the impact their presence or absence has on the ecological community (Paine 1969). The loss of keystone species further disrupts nutrient cycling and intensifies the collapse of functional ecosystems (Armesto *et al.* 2009). These ecological breakdowns destabilise ecosystems by compromising resources and habitats, highlighting the need to preserve functional integrity to protect biodiversity and prevent species loss (Gonzales *et al.* 2011, Sridharan *et al.* 2021). The speed and extent of changes in climate patterns does not affect all species equally owing to the fact that marine populations differ in terms of vulnerability (Zacharias and Gregr 2005). Consequently, marine organisms have developed various adaptive strategies to mitigate the adverse impacts of climatic challenges on their long-term survival (Brierly and Kingsford 2009, Roberts *et al.* 2017). Among these strategies, three stand out. First, a species may experience a shift in their distribution areas into more suitable habitats (Wernberg *et al.* 2013, Vergés *et al.* 2014), termed adaptive migration (Mougi 2019). Secondly, a species may develop a complex set of phenotypic plasticities in response to challenging environmental conditions (Westneat *et al.* 2019).

Among the different strategies describing species' adaption to (especially) unpredicted and extreme climate events, phenotypic plasticity and evolutionary adaptation are particularly important (Chevin and Hoffmann 2017, Burton *et al.* 2022). Both of these mechanisms improve an animal's overall ability to cope with challenging conditions, its fitness status and the overall fitness of a population (Acasuso-Rivero *et al.* 2019), with adaptive genomic mechanisms to respond to changing environment often in place (Lancaster *et al.* 2022). The underlying molecular mechanisms involving these two strategies, however, differ. Phenotypic plasticity refers to the adaptive responses of species to environmental stressors (Xue *et al.* 2018). The development of phenotype plasticity has been documented in response to rapid temperature changes in various species (Denver *et al.* 1998, Bonamour *et al.* 2019). While the underpinning

genetic makeup that modulates phenotypic plasticity and evolutionary adaptation is the same, a growing body of evidence points to the presence of strong genetic signals that are involved in plastic gene expression and its heritability (Duncan *et al.* 2014). By contrast, adaptive evolution typically takes place over a longer time scale and points to the gradual, heritable changes in the genetic makeup which favour the spread of genotypes with superior fitness under a range of environmental conditions (Rosenberg *et al.* 1998, Perrier and Charmanier 2019). Detecting signals of natural selection in wild populations and linking these to abiotic environmental drivers is challenging, as different demographic dynamics and evolutionary forces, such as the presence of population structures due to random genetic drift and isolation by distance (IBD), or patterns of distribution of genetic variation across land- or seascapes, can leave footprints in the genome that are very similar to those left behind by local adaptations, necessitating confirmation through controlled, long-term experiments (Crispo 2008, Brandon 2014, Williams 2018).

Organisms have undergone diverse adaptations that affect population fitness, functionality, and connectivity that may prevent local extinction (Hansen *et al.* 2006). One key aspect of adaptation lies in the genetic underpinnings of traits that confer fitness advantages in specific environments (Stapley *et al.* 2010). Maintaining and preserving genetic variance is crucial for adaptation and population persistence in a changing environment (Bernatchez 2016). Furthermore, local adaptation, which refers to the process by which populations become better suited to their local environments, is a fundamental mechanism for long-term population maintenance (Lenormand 2012). Local adaptation can play a critical role in the persistence of populations, even if it remains undetected or underestimated in short-term experiments (Bennington *et al.* 2012). Traditionally, the response of populations to predicted scenarios of climatic change has been investigated using ecological niche modelling and reconstruction of the species' distribution and range shift (Holt 2009, Thuiller *et al.* 2019). The presence of variation within a species and among individuals in a population and the ability to adapt to new environments are, however, still largely ignored by these predictions, which limits the power of these methods to efficiently predict and when possible, mitigate, the negative impacts of climatic change (Mawdsley *et al.* 2009, Berg *et al.* 2010, Bush *et al.* 2016, Garzon *et al.* 2019). The shortcomings of predicting scenarios using modelling are mostly due to technical (including, but not limited to the necessity of core expertise in multiple fields, e.g., ecology, genomic, computational) and logistical challenges that researchers face when investigating marine organisms over the required long periods of time (Peterson 2001, Warren 2012).

The recent development in genomic technologies and high throughput computational platforms, coupled with the availability and accessibility of genomic resources for marine species, enable researchers to investigate the eco-evolutionary mechanisms involved in local adaptations in a great level of detail (Lancaster *et al.* 2022). Understanding the genetic foundations of morphometric traits across diverse marine populations allows for a better understanding of the evolutionary forces that shape adaptation, and consequently, biodiversity (Lotze *et al.* 2006). An emerging suite of new multi-omic (an integrated approach using multiple disciplines) bioindicators that harness the combined power of advances in genomics, proteomics, epigenomics, and microbiomics (Pomfret *et al.* 2020, Machuca-Sepulveda *et al.* 2023, Tseng *et al.* 2023), in addition to the latest developments in morphometric and physiological trait analysis (Kolozsvari *et al.* 2020, Sabri *et al.* 2020), show great promise to monitor the vulnerability of marine populations and, where possible, effectively moderate the negative effects of climate change (Bonanno and Orlanda-Bonaca 2018, Fossi *et al.* 2018, Alves *et al.* 2022). Through uncovering the genetic variations essential for adaptations, essential insights into the resilience or vulnerability of marine species to environmental shifts are gained (Gupta *et al.* 2020). While these methods can potentially identify the species at greatest risk of extinction, and vulnerable ecosystems, they can also provide the baseline for bioprospecting, which is the discovery of novel enzymes, antibiotics, or other biochemical compounds produced by non-model organisms (Mock *et al.* 2022). Furthermore, an in-depth understanding of the evolutionary potential and predicting of the resiliency of marine populations in the face of unpredictable changes in the world's climatic patterns (Hogan *et al.* 2011, Munday *et al.* 2013) can help in the establishment of more comprehensive, effective, and multidisciplinary management strategies to address these issues by providing conservation groups, government agencies, environmental departments, legislators and lawmakers, scientific communities, and research institutions with more tangible ecological, knowledge-based evidence (Levin *et al.* 2009, Hamilton and Caselle 2015, Pirodda *et al.* 2022).

Alongside genetic traits, ecological traits, particularly morphometric characters, play a large role in defining the fitness (Laughlin *et al.* 2020), resiliency (Gladstone-Gallagher *et al.* 2019), and vulnerability (Leclerc *et al.* 2020) of (marine) populations separate and distinct from the effects of environmental fluctuations (Kroeker *et al.* 2020). Morphometric traits are those physical and measurable characters related to the shape and size of an individual (Klingenberg 2016). Many, if not all, of these traits directly inform on the fitness of populations as they allow

individuals to, among other functions, outcompete conspecifics or other species for resources, as well as confer some advantage that could aid in predator evasion and reproduction (Kishida *et al.* 2010, Schmitz 2017, Tocts *et al.* 2016, Silber *et al.* 2017). These traits highlight the importance of certain morphological attributes that contribute towards increased survival rates and reproductive success in changing environments by serving as indicators of fitness and resilience (Tuliozi *et al.* 2023). Temporal or spatial changes in characters could signal changes in the immediate environment, offering cues for potential species resiliency in the face of anthropogenic and other stressors (Canale and Henry 2010, Michalczyk *et al.* 2018).

Recent advancements in genomics and genome-wide population studies have garnered significant attention due to their crucial role in deciphering rapid species adaptations in response to diverse environmental pressures (Radwan and Babik 2012, Tigano and Friesen 2016, Lee *et al.* 2019). A population's genome encompasses a diverse genetic reservoir that dynamically reacts to environmental cues and can act as a buffer against environmental disturbances (Ledoux *et al.* 2015, DuBois *et al.* 2022). Studies exploring diverse populations have found genetic variations to be critical in facilitating relatively quick adaptations to changing environments (Walter *et al.* 2023). Investigating genetic variations in different populations can provide insights into how populations respond to stressors, which can serve as an indicator of vulnerability or resiliency to environmental changes (Mack *et al.* 2013, Prentice *et al.* 2020). The application of genome-wide studies to be used in assessing the health and resilience of ecosystems is gaining traction. Not only are genome-wide studies used as a bioindicator tool to monitor environmental health (Bourlat *et al.* 2013), but also to inform conservation efforts and management strategies (Kardos *et al.* 2021) in an attempt to preserve biodiversity in a changing climate (Supple and Shapiro 2018).

The significance of host-associated microbial communities, commonly known as microbiomes, in promoting quick local adaptations to extreme environmental changes has recently drawn scrutiny. Compared to eukaryotic animals, microbes have faster generation times, and so their response to environmental stimuli take place at shorter time scales (Houwenhuyse *et al.* 2021, Voolstra and Ziegler 2020). In a pioneering study in 2019, Qiu and co-workers exposed kelp (*Ecklonia radiata*), which is a major foundational species in marine ecosystems, to the projected scenarios of increased temperature and oceanic acidity progressing at the current rate of climatic change. Their results showed that kelp's associated microbiome composition responded to changes long before the host's physiological systems responded to the stressful

stimuli, confirming the early indicator nature of bacteria. The study of the intricate interplay between host-associated microbiomes and host marine organisms further adds insight into how microbial communities influence the metabolic responses of marine life to projected climate change scenarios (Cox *et al.* 2022). Studies show that the host-associated microbial diversity and its functional content may buffer the host against extreme changes in the marine environment until natural selection gradually creates adaptive genetic variation in populations over time (Voolstra and Ziegler 2020). This is due to the multitude of biochemical processes that the microbial community produces which could benefit the host (Rosenberg 2021). The first step in determining the power of using the microbiome as a predictor for the health of marine ecosystems is to provide the baseline inventory of bacterial taxa that are associated with different organs (Trevathan-Tackett *et al.* 2019). In this regard, the diversity of gut microbiota in the alimentary canal is of particular interest, since it harbours the largest and the most diverse microbial community in all organisms (Reynoso-Garcia *et al.* 2022), and so the impact of stressful environmental stimuli is more likely to be reflected in its compositional diversity (Karl *et al.* 2018). Integration of the knowledge derived from the fields of genomics, morphology, and microbiomics can provide a comprehensive understanding of marine ecosystems, facilitating effective ecosystem management (Heymans *et al.* 2019).

1.2 Structure of thesis

Sea urchins, recognised as keystone species due to their pivotal role in ecosystem regulation (Byrnes *et al.* 2013, Farina *et al.* 2020, Lourenço *et al.* 2022), face considerable pressure under the current climate change scenario (Listiawati and Kurihara 2020). As urchins play a crucial part in maintaining ecological stability, changes to their population sizes or distribution ranges can have severe, negative impacts on ecosystems. The Cape sea urchin, *Parechinus angulosus* is the most widespread species among the South African echinoids (sea urchins). The known distribution of this sexually dimorphic, inter-tidal and subtidal species (Schultz 2015) ranges from Port St John's on the east coast of South Africa to the cold-water environment found along the west coast from the Cape of Good Hope into Angola (Figure 2.1 in Chapter 2). Individuals of this species are relatively small as compared to *Diadema setosum*, *Tripneustes gratilla* and other South African species, and are one of the toxic species of urchin found along this coastline (Cannone 1970). The wide distribution of *P. angulosus* along the South African coastline that is known for its remarkable variability in oceanic forces between the east and west coasts makes

this species an ideal candidate to investigate the impact of environmental factors in shaping spatial variation in the morphology, microbiome, and genomic structure of this species.

Following this framework, this thesis consists of six chapters. The study species, its biology, and its distribution range are discussed in Chapter Two, to give a general insight into the unique oceanic conditions of the South African coastline and simultaneously the rationale for choosing *P. angulosus* as the study species.

This is followed by Chapter Three, which describes some key morphometric characters in *P. angulosus* that used machine learning and Bayesian modelling, among other techniques, to investigate whether there is significant variation in the morphometric traits between individuals collected from the east and west coasts of South Africa that can point to the presence of fine-scale adaptations and link to ecological resiliency.

Chapter Four investigates genomic diversity, population structure, and population connectivity, adaptive variation, and environmental association in the population of *P. angulosus* using data generated using the ezRAD library preparation protocol. Furthermore, in the conclusive section of this chapter, population-level changes in allele frequency in response to predicted scenarios of climate change and the vulnerability of different populations are assessed. This chapter concludes with the discovery that the digestion enzyme that was used to prepare a reduced representation of *P. angulosus* genome also cut frequently through the mitogenome. Because of this, it was possible to assemble a significant number of partial mitogenomes of the study species, and the assembled mitogenomes were used to reconstruct the phylogeographic of *P. angulosus* along the South African coastline.

In Chapter Five, a survey of the gut microbiome in *P. angulosus* was conducted using metabarcoding of the 16S rRNA marker and Qiime2 v.2023.9. Gut microbial diversity, its functional content, and its compositional variation between the east and west coasts of South Africa were investigated. This chapter aimed to determine whether there is a core group of dominant bacterial taxa in individuals across the east and west coasts, and if variation, if any exists, could be ascribed to the differing environmental factors on each of the coasts.

Finally, Chapter Six, integrates the findings of the previous three chapters and explains the application of the results to understand the evolutionary biology and resiliency of *P. angulosus*

in response to rising ocean temperatures, and potentially other anthropogenically-driven oceanic disturbances. In this chapter, the importance of using multi-disciplinary bioindicators to better monitor the responses of marine populations to the environmental challenges they face is further discussed. This chapter concludes with highlighting some of the shortcomings and opportunities and provides a future direction for research in the field of climate resilience of marine species.

1.3 Ethical clearance and collection permits

Ethical clearance for this study was granted by the South African Institute for Aquatic Biodiversity Ethics Committee (25/4/1/7/5 SAIAB-ETHICS-01) and the Rhodes University Ethics Committee (2020-2753-4784). Specimen collections were carried out by me with the help of up to three other students (acknowledged above) under the research permit issued by the Department of Environment, Forestry and Fisheries (DEFF) of the Republic of South Africa, under the Integrated Environmental and Fisheries Research and Development Permit (RES2020/73).

Chapter two: An introduction to the Cape sea urchin, *Parechinus angulosus*, and its distribution range along the coast of South Africa

2.1 Coastal zones

Coastal zones are transitions between the terrestrial and marine environments (Ray 1991), which perform key ecological functions as well as provide important ecosystem services (Ehrlich and Ehrlich 1981, Ehrlich and Mooney 1983, Turner and Schaafsma 2015). Coastal zone functioning is governed by many factors, including the physical environment, organism adaptations to the physical environment, species composition, nutrient cycling, and energy sources within a system (MacGillivray *et al.*, 1995, Grime *et al.*, 1997). A synthesised report on the findings of the Millenium Ecosystem Assessment, an initiative employed to assess the impact on human well-being of climate change, separates ecosystem services into four categories: provisioning, regulating, supporting, and cultural and amenity services (UNEP 2006), the most pertinent of which to this project is regulating services. Regulating services include flood prevention and erosion through shoreline stabilisation and storm protection (UNEP 2006, Barbier *et al.*, 2011), nutrient regulation, detoxification of polluted waters, and the regulation of the climate through carbon sequestration (UNEP 2006, Daba and Dejene 2018). A relevant example of supporting services is habitat provision for countless species, linking to provisioning, such as the harvesting of commercially important fish, shellfish, and crustaceans for food (UNEP 2006). Fishing activities in coastal zones provide the primary source of protein for many, and in some cases are the only means of generating income (Clark *et al.*, 2002, Sowman 2006, Schumann and Macinko 2007). These ecosystem services also have the secondary benefit of supporting local and regional economies through tourism (Honey and Krantz 2007), recreational activities (Paudel *et al.*, 2011), and cultural practices (Khakzad *et al.*, 2015).

It is broadly acknowledged that ecosystem functioning, and biodiversity are closely linked concepts (Balvanera *et al.* 2006, Dudgeon *et al.* 2006). A system's biodiversity relies on its productivity, based on its local conditions, species composition, and ecological dynamics (Worm and Duffy 2003). Rocky shores are good examples of this setting as they are highly dynamic habitats, hosting great biodiversity owing to the microhabitats within, such as pools, crevices, caves, and algal beds (Clark and Taylor 1980). Alternation of tides is one of the key features of rocky shores worldwide and the derived high physical and chemical heterogeneity also mirrors the high biodiversity characteristic of these systems (Ewel *et al.* 2001). Tidal variation also exposes rocky shore organisms to environmental extremes (Denny *et al.*, 2009). Aside from tidal regimes, on exposed shores, the most extreme of these factors are perhaps the

mechanical forces organisms experience as a result of repeated wave action (Jones and Demetropoulos 1968), a factor that also shapes the physical structure of the shore. Additionally, exposure of the rocky shore to air, as the tides retreat, exposes biota to solar radiation (Burnaford and Vasquez 2008). Organisms on the rocky shores can experience temperatures of up to 40°C, an environmental stressor that may lead to death by heat stress (Glynn 1968, Cartwright and Williams 2014); or atmospheric chilling events in the high latitudes (Tokio 1963, Zamir *et al.* 2018); or if exposure is prolonged, death by desiccation, if the organism is unable to prevent water loss under these long, air-exposed conditions (Glynn 1968, Theriault and Kosala 2001). Similar to rocky shores, rock pools host rich biodiversity due to the various selective pressures experienced by the organisms as they compete for space and resources (Aspbury and Juliano 1998, Blaustein *et al.*, 1999), resulting in highly adapted organisms. At high tide, organisms are exposed to a drastic decrease in ambient temperature, bringing relief from solar radiation and thermal extremes (Glynn 1968), as well as changing the chemical properties of the seawater in the pool, though water chemistry is less variable at high tide as compared to that found in rock pools during low tide (Smit and Glassom 2017). Evaporation (Williams 1994) and/or precipitation (Chan 2000) also affect the salinity, temperature, dissolved oxygen, and nutrient concentrations of rock pools. Shallow or small tidal pools that experience large amounts of water loss through evaporation, can experience increases in salinity, creating a hypersaline environment (Van Dam 1988) and exposing organisms to the negative physiological effects associated with osmotic stress (Giese and Farmanfarmaian 1963, Branch and Cherry 1985). Further affecting the biodiversity of rock pools, are the location of the rock pool within the intertidal zone, the amount and frequency of freshwater input, the duration of inundation and exposure to air, and the depth, volume, surface area, and substrate of the pool (Ganning, 1971, Morris and Taylor 1982, Huggett and Griffiths 1986, McMahon 1990, Metaxas and Scheibling 1993, Little *et al.*, 2009).

Aside from a characteristic sets of physico-chemical properties and nutrient cycling in rock pools, rocky shore ecology is dictated by biotic factors such as predation, competition, grazing, and (biological and physical) disturbance (Connell 1972, Underwood and Denley 1984, Sousa 1985, Petraitis 1987, Johnson and Hawkins 1998, Petraitis and Dudgeon 1999). Intense predation pressure can trigger trophic cascades where changes in the abundance of one species affect distribution of species on the shore and population sizes at other trophic levels, resulting in altered community composition (Schultz *et al.* 2016, Dudgeon *et al.* 2019). Space is a critical resource on rocky shores, where available substrate for attachment or occupation is limited

(Robles 1997, Underwood 2000). Sessile/sedentary invertebrates such as mussels and barnacles hence compete with each other (Underwood and Denley 1984), but also vegetation (Chapman 1992), which is the food source for many rocky shore species and supports the trophic food web (Won *et al.* 2007). Other food sources competed for include plankton and edible detritus, a shortage of which results in intense competition, especially in areas with high population densities (Cliff 1982, Vinagre *et al.* 2015, Ding *et al.* 2018). The competition for shelter resources, alongside that of space and food, drives niche differentiation where resources are partitioned between species to reduce direct competition (Aguilera *et al.* 2014). One of the primary drivers of species distribution on rocky shore is grazing, which shapes community composition, maintains biodiversity and regulating primary productivity (Connell 1972, Lindegarth *et al.* 2001, Hawkins 2020). Most rocky shore invertebrates, such as limpets, snails, and sea urchins engage in herbivory (Cubit 1984, Benedetti-Cecchi and Cinelli 1995, Coleman *et al.* 2006), feeding on primary producers such as macroalgae (kelps), and microalgae (diatoms and green algae), helping to regulate algal biomass and preventing over-proliferation of any one species in the intertidal zone (Kaehler and Williams 1998, Bokn *et al.* 2003). One of the drivers of niche differentiation is grazing intensity which varies spatially and temporally (Xu *et al.* 2019), influenced by factors such as grazer abundance and diversity, which employ different feeding strategies as a result of dietary preferences (Lee and Bruno 2009, Wickham *et al.* 2022). These grazers play a critical role in nutrient cycling (Bracken *et al.* 2014), primarily through decomposition (digestion), but also through mineralisation and uptake, playing a key role in regulating nutrient dynamics on the rocky shore (Sauchyn and Schiebling 2009, Pedersen and Johnsen 2017). The nutrient-rich waste products of rocky shore species contribute to primary productivity by making available the key nutrients vegetation requires, thereby contributing to the nutrient cycling within this ecosystem (Turner 2002, Sauchyn *et al.* 2011).

Rocky shores, just as all other ecosystems, experience many different types of disturbance, heavily influencing the species and types of organisms found there, shaping community structure (Schiel 2004, Micheli *et al.* 2016). The primary physical disturbance experienced by organisms on the rocky shores is wave action, along with tidal fluctuations, the intensity of which may be influenced by such factors as substrate type and geographic location (Gaylord 1999, Kraufvelin 2007, Micheli *et al.* 2016). These physical pressures can dislodge, break, bury or otherwise harm organisms, altering habitat structure and complexity (Hicks 1982). Rocky shores also experience the biological disturbances given above, where predation, herbivory,

and competition help shape rocky shore communities (Connell 1972, Menge 1978, Underwood and Denley 1984, Petraitis 1987). One of the increasing pressure on rocky shores is anthropogenic disturbances, such as fishing, pollution, coastal development, trampling and the overarching climate change, which impact community structure to often an irreversible state (Addessi 1994, Deepananda and Macusi 2012, Deepananda and Macusi 2013, Cabral-Oliveira *et al.* 2014, Micheli *et al.* 2016, Pardal-Souza *et al.* 2017, Mendez *et al.* 2018).

2.1.1 Sea urchins

Sea urchins are invertebrates abundantly found in rock pools and crevices on rocky shores, occurring from tropical (Lawrence and Agatsuma 2013) to temperate regions (Scheibling and Hatcher 2013). They are acknowledged globally as keystone species, due to their disproportionally large effect on the environment relative to their low biomass (Power *et al.*, 1996). For example, sea urchins play a crucial role in maintaining the health of marine ecosystems (Bertness *et al.* 2004) as they consume a variety of (foundational) marine algae, through which they help regulate algal populations, preventing over-proliferation (Steneck 2013), resulting in alternative stable states (Ling *et al.* 2009). Similarly, through grazing, sea urchins influence algal species composition, contributing to species diversity (Hereu 2006). Sea urchins may even promote the recruitment of new and/or juvenile algal settlement by clearing the substrate of present algal species, making space available for spore settlement (Norderhaug and Christie 2013). As such, sea urchins are known as ecosystem engineers, owing to their modification of resource availability ‘by causing physical state changes in biotic or abiotic materials’ (Jones *et al.*, 1994), which is to say changes in the properties of the space they occupy. To further underline the ecosystem engineering role, through grazing, sea urchins help to control algal growth, impacting the resource availability for other herbivores (Livore and Connell 2012). Additionally, by preventing algal overgrowth, sea urchins simultaneously protect coral reefs by eliminating or lessening competition for resources like space and light, promoting assemblage diversity of those species dependent on coral reefs for survival (Ogden and Lobel 1978, Edmunds and Carpenter 2001). Furthermore, the grazing activities of sea urchins contribute to nutrient cycling by producing waste containing nutrients like ammonium (Koike *et al.* 1987), which then bacterial processes make bioavailable. Sea urchins themselves contribute to energy transfer to higher trophic level organisms (Schley and Roper 2003) when preyed upon by a range of organisms like fish (Figueiredo *et al.* 2005), crabs (Tegner 2000), lobsters (Ling *et al.* 2009), and otters (Pearse 2006).

Even in their absence sea urchins remain ecosystem engineers. Due to a lack of grazing pressure on algal species in areas where sea urchins have been removed, competitive interactions between different algal species also result in shifts in species composition of algae (Schultz *et al.* 2016). Additional to vegetation control, the ecosystem engineering capabilities of the sea urchin operates via modification of the physical characteristics of the substrate in their environment, through the formation of small pits in the rocky substrate, or the introduction of microbe-rich faecal pellets in the environment they live in (Fletcher 1987, Sauchyn and Scheibling 2009, White *et al.*, 2018). Furthermore, sea urchins contribute to ecosystem management through bioturbation, which refers to the biotic processes of sea urchins that impact on sediment stability, such as burrowing, feeding, and waste deposition (Higgins 1975, Thompson *et al.* 1987, Ghiold 1989, Widdicombe and Austen 1998). These processes may destabilise sediments as intense sea urchin grazing pressure can reduce the binding efficacy of macroalgae on the sediments.

The grazing behaviour of sea urchins can regulate the productivity and structure of benthic algal communities but may also have deleterious effects if over-grazing occurs (Carr and Bruno 2013). A distinctive example of the extreme effects of their grazing is the barrens left behind after urchins have over-grazed algal turfs (Connell *et al.* 2018) or kelp stipes and holdfasts, which result in the detachment of the kelp fronds from the substrate (North 1965, Leighton *et al.*, 1966). Such occurrences have been reported on as far back as 1961 (Kitching and Ebling 1961), when kelp forest loss was described in Great Britain. Interestingly, South African sea urchins, particularly *Parechinus angulosus*, also graze on kelp holdfasts, but this grazing action does not sever kelp stipes, in part because this species preferentially feeds on kelp sporelings (Fricke 1979), but also potentially because the urchins feed on small invertebrates on the holdfasts and not on the holdfasts themselves (Scheibling and Lauzon-Guay 2007). Overgrazed algal turfs and kelp forests can, however, be restored, through changes in predation pressure of sea urchins (Mann 1977, Estes and Palmisano 1974, Estes *et al.*, 1978) and the mechanical removal of sea urchins by divers (Paine and Vadas 1969, Watanuki *et al.*, 2010) or storm occurrences (Harris *et al.*, 1984). Other causes of sea urchin mass mortality (Williams *et al.*, 2021) include environmental catastrophes like oil spills (Carthy and Arthur 1968) or disease (Pearse and Hines 1979, Miller and Colodey 1983), as with the amoeba *Neoparamoeba branchiphilia* in 2009-2010 in the Canary Islands (Dykova *et al.*, 2011, Sangil and Hernandez 2021).

A more contemporary and pervasive stressor of urchins that affects grazing pressure is ocean acidification (Byrne *et al.* 2013, Dworjanyn and Byrne 2018) resulting from diffusive uptake of atmospheric carbon dioxide by seawater (Caldeira and Wickett 2003, IPCC 2007, 2014, 2022). Multiple laboratory-based studies (e.g., Dupont *et al.*, 2013, Wolfe *et al.*, 2013, Chan *et al.*, 2016) have shown that increases in oceanic carbon dioxide levels diminish survival rates, growth, and the abundance of calcifying organisms (Kroeker *et al.*, 2013), but paradoxically, and simultaneously, boost growth in primary producers (Connell *et al.*, 2018), the combination of which may lead to restoration of algae and kelp in overgrazed areas. Specifically, this anthropogenic-driven phenomenon affects sea urchin reproductive success by delaying gametogenesis and spawning (Kurihara *et al.*, 2013), and reducing sperm swimming speed and motility (Havenhand *et al.*, 2008), as well as affecting growth (Kurihara *et al.*, 2008), physiology (Melzner *et al.*, 2009), immune responses (Bibby *et al.*, 2008), and a reduction in calcification rates of larvae (Sheppard Brennan *et al.*, 2010).

Dependent on the health of the ecosystem and the health of sea urchins are the industries that harvest this echinoderm for their uni. Uni, the gonads of the sea urchin, have gained popularity in the northern hemisphere, where they are included in sushi (Robinson *et al.*, 2001), sauces and bread (Stefánsson *et al.*, 2017), ice cream and custards (Pols 2014), and pasta and pizza (Furesi *et al.*, 2016). A 2022 article reported the price of 200 g of sea urchin uni at 100 USD, though noted that the same product may fetch up to five times that amount in Japan (Varricchio and Morgan, Business Insider 2022). Unfortunately, the high economic return on uni has led to over-harvesting and the depletion of natural stocks (Juinio-Menez *et al.*, 2008). This pressure has further led to the global development of echinoculture operations to meet the demand for sea urchin uni in many countries such as China (Liu and Chang 2015), Russia (Dvoretzky and Dvoretzky 2020), Japan (Unuma *et al.*, 2015), Norway (James *et al.*, 2015), Scotland (Kelly *et al.*, 2015) and Australia (Williamson 2015). A prominent exception from this list is South Africa around which a knowledge gap was noted.

2.2 Study region

The South African marine environment is dominated by two currents – the Agulhas and Benguela currents (Darbyshire 1966). On the east coast and originating in the Indian Ocean, the Agulhas current flows from a north-easterly equatorial in a south-westerly direction

(Shannon 1985, Lutjeharms and de Ruijter 1996, Beal and Bryden 1997, Reason *et al.*, 2006). Reaching speeds in excess of 2.4 meters per second (Boebel *et al.*, 2003), this current is characterised by warm water that is lower in oxygen and nutrient concentrations when compared to the Benguela Current on the west (Lutjeharms 1988, Meeuwis and Lutjeharms 1990, Griffiths *et al.*, 2010). On the west coast, driven by the south easterly trade winds flows the Benguela Current northwards, from Antarctica towards the equator (Shannon 1985). This direction of wind is responsible for upwelling, which brings cold, oxygen- and nutrient-rich water from the depths of the Atlantic Ocean to the surface, where it drives productivity (Siesser 1980, Meeuwis and Lutjeharms 1990, Diester-Haass *et al.*, 2002). Bordered by Namibia and the Atlantic Ocean on the west, and Mozambique and the Indian Ocean on the east, South Africa's coastline stretches for approximately 3650 kilometres (Griffiths *et al.*, 2010), and with a 200-nautical mile economic exclusive zone, South African waters support a diverse range of organisms within its approximately 1 million square kilometres (Griffiths *et al.*, 2010).

Rock pools are found in sections all along the South African rocky shore coastline (Branch and Branch 2018). Rocky shores and rock pools in South Africa are not exceptions to the abiotic physical factors of aerial (Huggett and Griffiths 1986) and solar exposure (Smit and Glassom 2017), inundation at high tide (Kemp 2003), temperature extremes within the tidal regime (Huggett and Griffiths 1986), the changing chemical properties of salinity (Kemp 2003), and dissolved oxygen concentrations (Huggett and Griffiths 1986). The geology and rock type also varies between the east and west coasts. The east coast has predominantly sedimentary rock (Miller and Mason 1994), especially sandstone and shale (Baiyegunhi *et al.* 2020), whereas the west coast is dominated by granitic and metamorphic rock such as granite, gneiss, and schist (Walker 1917, Waters 1986, Belcher 2003).

2.3 Study species

Parechinus angulosus (Leske, 1778), the Cape urchin was chosen as the study species of this research. This species was chosen owing to its regional cosmopolitanism and the resultant wide range of environmental factors *P. angulosus* is exposed to, namely the physicochemical factors associated with the Benguela and Agulhas Currents. Furthermore, sea urchins are classified as indicator species, which is to say, species that offer insight into the health of the ecosystem through examination of their body condition (Johnstone *et al.*, 2019). This makes this

cosmopolitan species an ideal candidate for studies assessing the health of sea urchins and potential resiliency in the face of anthropogenically-induced or -driven events.

The study undertaken by Filander (2017) on the taxonomy, systematics, and biogeography of South African echinoids describes the distribution of *Parechinus angulosus* as extending from Angola around the tip of Africa and into Mozambique. Though the distribution range for this species extends beyond the borders of South Africa, for logistical challenges, the current project undertook sampling efforts at sites starting at McDougall's Bay, approximately 80km south of the Namibian-South African border, around the Cape and into the northern reaches of KwaZulu-Natal (bordering Mozambique), however, no specimens were found in rock pools at any locations north of Port St John's (Figure 2.1). Filander (2017) and Mertens (2021) note that this species can be found up to 180m and 30m in depth respectively, so the absence of this species during this study does not indicate a change in its distribution since the last survey was conducted, because sample areas in the current research were limited to rock pools only.

2.4 Study sampling locations

The target region for this thesis was the entirety of the South African coastline, encompassing the whole of the South African distribution range of *Parechinus angulosus*. A total of 43 sites were chosen, specifically (from west to east) McDougall's Bay, Kleinsee, Hondeklip, Groenrivier, Doringbaai, Lambert's Bay, St Helena Bay, Jacob's Bay, Yzerfontein, Silwerstroom, Bloubergstrand, Kommetjie, Betty's Bay, Arniston, Jongensfontein, Still Bay, Gouritz, Mossel Bay, Herrold's Bay Victoria Bay, Beacon Island, Buffels Bay, Nature's Valley, St Francis, Schoenmakerskop, Cape Padrone, Cannon Rocks, Kenton, Kasouga, Spinning Reel, Kleinemonde, Rietrivier, Fort D'Acre, Mtati, Birha, Kidd's Beach, Glengariff, Cintsawest, Kei Mouth, Coffee Bay, Presley Bay and Port St John's (Figure 2.1) from which 20 individuals per site were collected. These sites were selected as they are mostly rocky shores, and though this species is intertidal to subtidal, the decision was made to standardise the method of sampling to hand collection from rock pools. Sampling was conducted during late spring/early summer (October 2019 to January 2020) to eliminate the confounding factors of season and the resultant changes in ocean water chemistry. To note, however, the number of sites selected for inclusion in each of the chapters varied owing to logistical challenges in optimising protocols while ensuring the most comprehensive coverage of the coastline in the time available.



Figure 2.1: The South African map indicating all sampling locations from which individuals of the Cape sea urchin, *Parechinus angulosus*, were collected. On the main top map, the easternmost collection site, Port St John's, is indicated by a red pin, as is the westernmost site, McDougall's Bay found just below the blue pin. The remaining pins represent the 41 other sites used in the data set. In order to make discerning between populations that are densely packed easier, two sections of the map were blown up. Populations blocked in turquoise represent a portion of the west and south coasts and populations blocked in red represent a portion of the south east coast of South Africa (GoogleMaps 2023).

Owing to the interaction between the prevalent current systems, the geographical position, and resulting differences in biota, the South African marine environment can be divided into eight bioregions, namely the Namaqua, south-western Cape, Agulhas, Natal, Delagoa, West Indian Offshore, Indo-Pacific, and the Atlantic Offshore Bioregions (Lombard 2004). The collection of samples was done according to Lombard's (2004) classification in the four coastal bioregions of the Namaqua, south-western Cape, Agulhas, and Natal Bioregions (Figure 2.2). Though site selection was dependent on the availability of suitable habitat and site accessibility, where possible, the distance between adjacent sites was maintained at approximately 50-100km, with the furthest distance between sites recorded as 123km. The sites were chosen to allow for the most comprehensive sampling effort to compare (genetic and/or morphometric) differences between and among the various populations. The broad distribution of this species exposes populations to the warm Agulhas and the cold Benguela currents, offering an excellent setting to identify potential options for resiliency of populations, owing to the presence of contrasting physicochemical properties, especially temperature.

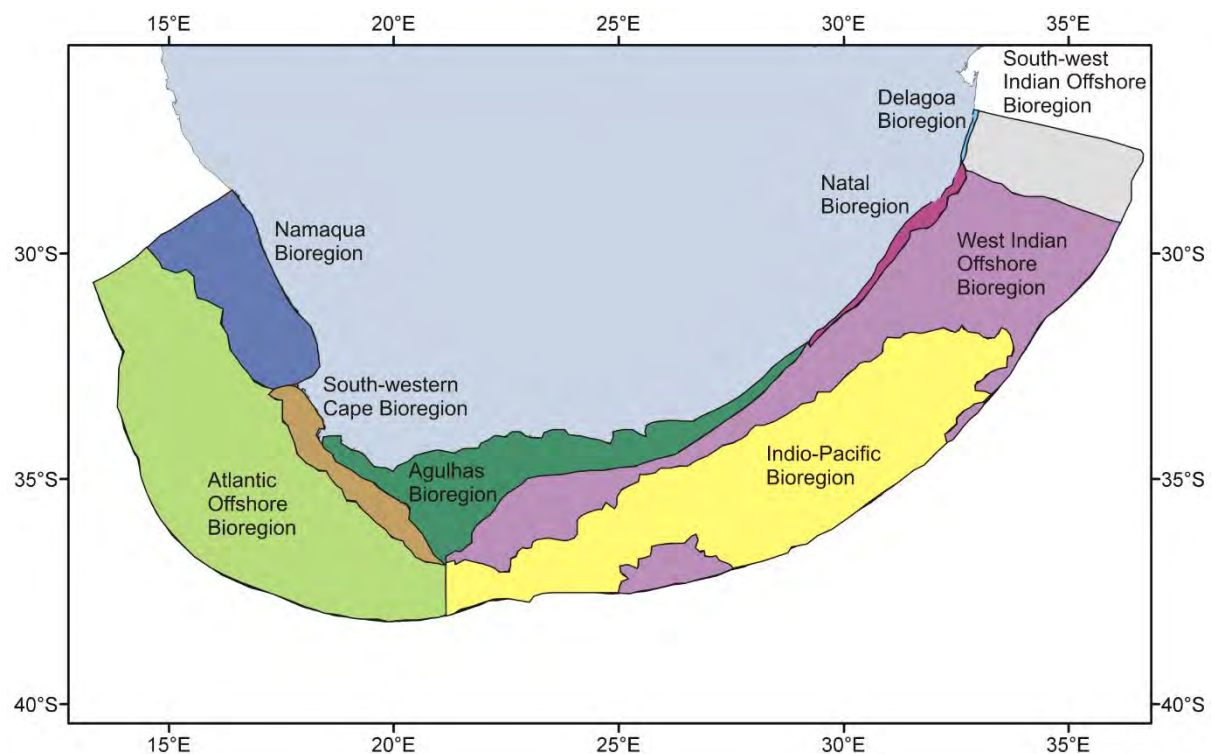


Figure 2.2: South Africa's coastal and offshore bioregions as defined and published by Lombard (2004).

Due to the absence of sea urchins from most of the Natal bioregion, for each empirical chapter, the focus was funnelled to three bioregions (Lombard 2004, for further details please see Chapters 3, 4, and 5, Figure 2.3). Sample sites were classified as belonging to either the Namaqua bioregion (Namaqua and south-western Cape bioregions), which lies on the west coast and is described as cool-temperate; the warm-temperate Agulhas Bioregion (consisting of south Natal and Agulhas bioregions), which is found on the east coast; or the south-western Cape bioregion, in which elements of both the cool- and warm-temperate regions can be found as it separates the Namaqua and Agulhas bioregions (Lombard 2004; Figure 2.3).

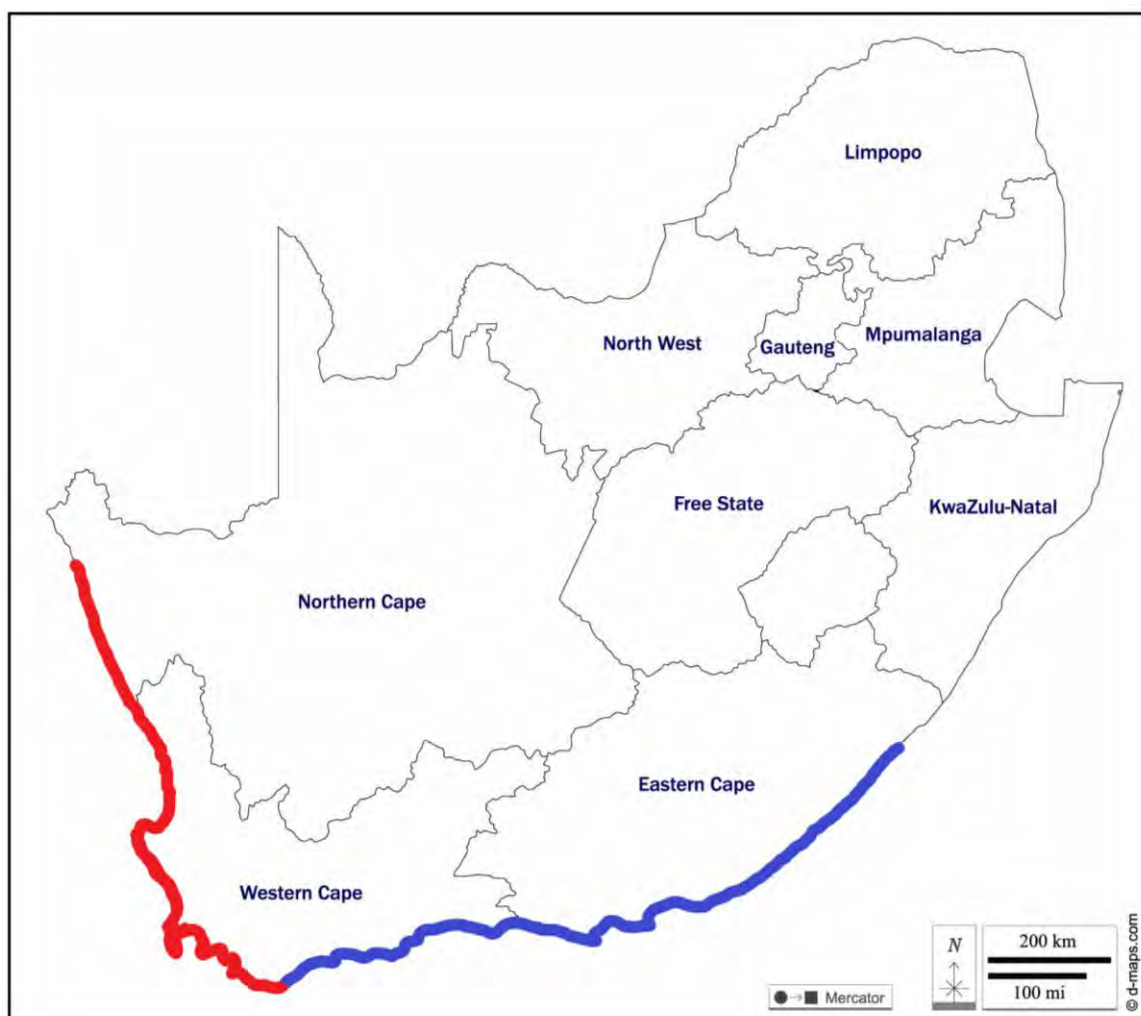


Figure 2.3: South African map indicating the modified bioregions used in this thesis. The blue indicates the east coast, consisting of the south Natal and Agulhas bioregions; the red indicates the west coast, consisting of the Namaqua and south-western Cape bioregions; and the green indicates the south-western Cape bioregion, which is nested in the west coast.

The physical parameter that characterises the three bioregions is temperature, and since temperature is the primary factor limiting species distribution (Thomas 2010), it is significant that *P. angulosus* appears to maintain connectivity among its populations, where populations on the eastern coast experience water temperatures up to 22 °C (Lutjeharms 2007) and populations on the western coast experience temperatures as low as 10 °C (Mholongo *et al.* 2015). This geographical setting allowed for comparison of different genetic and morphometric traits among and between sites, but also bioregions as governed by one of the two dominating South African currents or a combination of the two. Such a broad occurrence of species over a large thermal range is, however, not unique in sea urchins, as evidenced by the extreme temperatures experienced by *Lytechinus semituberculatus* around the Galapagos Islands, ranging from 11 °C to 31 °C due to upwelling events (Carr and Bruno 2013), *Paracentrotus lividus* which includes in its distribution range Scotland and Ireland, which range from 4 °C to 29 °C, and southern Morocco and the Canary Islands and is therefore exposed to temperatures as low as 4 °C and temperatures exceeding 30 °C depending on geographic location (Boudouresque and Verlaque 2013), and *Strongylocentrotus droebachiensis*, which adjusts its metabolism to accommodate temperatures as low as -1 °C and up to 20 °C (Scheibling and Hatcher 2013). Less extreme ranges are evident in the Philippines for the sea urchin species *Echinometra mathaei* and *Echinometra oblonga*, which experience temperature ranges between 26.8 °C and 34.3 °C (Ubaldo *et al.*, 2007), *Psammechinus miliaris*, which has a thermal tolerance range between just above 0 °C and 11 °C (Kelly *et al.*, 2013), and the very narrow thermal tolerance range of *Tripneustes gratilla* from 23.6 °C to 26.8 °C (Lawrence and Agatsuma 2013). These studies did not attempt to find a relationship between temperature and differences in either morphometric or genetic profiles, but in a separate study, smaller individuals of *Tripneustes gratilla* were found at the species' northern distribution limit on the Miura and Izu Peninsulas in Japan (Shigei 1970), a finding that is consistent with the known effects of cooler water slowing the metabolic rate of sea urchins and therefore somatic growth (Brockington and Clarke 2001, Détrée *et al.* 2020). Temperature has also been found to affect the expression of genes responsible for aerobic metabolism in *Loxechinus albus*, and the interaction of diet, temperature, and species had variable effects on the expression of genes related to lipid metabolism, ATP, and oxidative stress (Détrée *et al.* 2020).

Chapter three: Assessing ecological resilience in *Parechinus angulosus* through morphometric trait analysis

3.1 Introduction

3.1.1 Background and significance

Ecology is a branch of biology that deals with the relations between organisms and their environment (Haeckel 1866, King and Russel 1909, Eggleton 1939), and assists with the understanding of ecosystem functioning (Levin 1992), population dynamics (Botsford *et al.* 2019), interactions among species in ecosystems. This branch subdivides into community ecology (Webb *et al.* 2002), behavioural ecology (Kreb's and Davies 2009), and microbial ecology (Prosser *et al.* 2007). The interaction between organisms and the environment is affected by the interplay between various abiotic factors, such as habitat characteristics, temperature, moisture, humidity, ultraviolet radiation (Ment *et al.* 2017), and light availability (Bornette and Puijalon 2011). These interactions are further influenced by biotic factors, such as predation, competition (Benton 2009), parasitism (Borgsteede 1996), and the organism's adaptations to its environment (Hoffmann and Sgrò 2011). Adaptations to an environment can be anatomical (Reidenberg 2007) physiological (Schubert *et al.* 2017), behavioural (e.g., migration, hibernation; Geiser 2013, Martin *et al.* 2014), and ecological (e.g., competition, mutualism; Mazancourt *et al.* 2005, Good *et al.* 2018), and arise through natural selection over time (Futuyma 2009).

One iconic and pioneering study that investigated interactions between organisms and their environment is that of Charles Darwin on beaks of finches found on the Galápagos islands, in which changes in beak shape and size enabled different species of finches to occupy distinct feeding niches (Lack 1983, Sato *et al.* 2001, Grant 2017). Aquatic examples of adaptation to the selective pressures of the environment include the case of the 380-million-year-old Tiktaalik tetrapod fossils, which show evidence of early limb-like fins. This adaptation allowed the Tiktaalik tetrapods to move along water margins and into other aquatic environments, where they were only partly submerged (Shubin *et al.* 2006). Similarly, a present-day case of impacts of environmental stimuli on marine organisms that caused drastic changes in their normal life cycles is the example of coral reefs, which expel their commensal photosynthetic algae, zooxanthellae, when thermally stressed (Glyn 1984). To better understand this organism's reaction to environmental stressors, researchers have investigated the physiological and underlying genetic adaptations which may have allowed targeted coral reef populations to better tolerate changes in their environment (Thomas and Palumbi 2017, Weiss 2019, Fine *et*

al. 2019) and ultimately minimise the negative impacts of such stressors on coral biodiversity. Through harnessing the underlying mechanisms of adaptations, corals that exhibit these adaptations could be used to restore dead reefs by introducing pieces of these genetically modified or naturally resilient corals (Van Oppen *et al.* 2017).

There are several methods and tools that can be used in ecology to understand organism adaptation at population-level, including behavioural observations (Owens 2006, Davies *et al.* 2012), genomics (Tautz *et al.* 2010), (eco)physiology (Bozinovic and Portner 2015), community- (Morin 2009) and ecosystem- level ecology (Jorgensen 2009), stable isotope analysis (Fry 2006), and experimental ecology (Lewis and Taylor 1967). The latter of these, experimental ecology, often uses morphometric analysis, which is a comprehensive method to determine the links between an organism and its environment (Hollander *et al.* 2006, Frederick *et al.* 2008, Monteiro and Nogueira 2009, Parsons *et al.* 2011, Watson *et al.* 2018, Grinang *et al.* 2019). Morphometric analysis is defined as ‘the quantitative analysis of form and covariances with form’ (Bookstein 1991), or alternatively, morphology is the quantitative analysis of form (Ashburner *et al.* 2000). Morphometry plays a significant role in ecological research, providing a series of tools and advanced methodologies for a better understanding of the ecological relationships, species adaptations, and variations within populations (Adams and Otarola-Castillo 2013).

As a concept, morphometric analysis in science was first explored as far back as 1662 (Graunt 1662) and applied to characterise the ‘average man’, but it has since been implemented in the natural sciences focussing on biological organisms (Macleod 2017) through to geographical features like volcanoes on earth (Grosse and Kervyn 2018) and even to uplands or islands on Mars (Baker and Kochel 1978). Morphometric analysis has been applied to a wide range of terrestrial organisms, including invertebrates (Van Rensburg *et al.* 2003), reptiles (Kaliontzopoulou 2011), birds (Woodall 1991), and mammals (Muñoz-Muñoz *et al.* 2016). In freshwater and marine environments, studies on morphometrics studies have specifically addressed adaptive and non-adaptive changes in morphometric traits among a wide array of organisms including marine mammals, such as the Chilean blue whale (Pastene *et al.* 2020), fish (Mojekwu and Anumudu 2015), and marine invertebrates (Blank and Trench 1985, Scapini *et al.* 1999, Halliday 2001, MacCord and Amaral 2005, Saunder and Mayfield 2008, Tlig-Zouari *et al.* 2010, Chen *et al.* 2013, Inoue *et al.* 2013, Vaux *et al.* 2018, Grinang *et al.* 2019, Nicolau *et al.* 2022), including cephalopods (Anderson *et al.* 2008, Tajika *et al.* 2021). Short-

and long-term transplant studies in the form of exposure, common gardens, feeding assay, are often used to assess (morphological) response to varying environmental stressors such as light (Blank and Trench 1985), salinity (Kautsky *et al.*, 1990), ocean acidification (Del Pasqua *et al.*, 2019). Studies such as these can be used to investigate the plasticity and adaptive capabilities of marine invertebrates, providing insight into their ecological and physiological responses to environmental change (Calosi *et al.*, 2013; DuBois *et al.* 2019).

Among the different marine invertebrates, one taxon that has drawn considerable attention while investigating adaptations to anthropogenically driven environmental changes is sea urchins. A large part of research on echinoderms has been centred on their morphological and physiological adaptations to the impacts of climate change (Lawrence 1973, Lawrence and Cowell 1995, Ling *et al.* 2009, Sherman 2015), particularly to the rapid chemical modification in ocean chemistry largely resulting from the excess of the increased atmospheric carbon dioxide absorbed by the ocean (Caldeira and Wickett 2003, Doney *et al.* 2009). Changes in oceanic chemistry pose a significant risk to those marine invertebrates that have hard body parts consisting of calcium carbonate, like sea urchins (Wickett 2012, Dubois 2014). A decrease of available carbonate ions lowers the saturation rate of calcium carbonate in the sea water, reducing the efficacy with which calcium carbonate precipitates or dissolves (Bates *et al.* 2014). A lower calcium carbonate saturation rate impedes the ability of larval urchins to form shells, called tests (Sheppard Brennan *et al.* 2010). This results in weak or malformed tests unable to protect the larva as it enters its settler and juvenile stages respectively (O'Donnell *et al.* 2010). This diminished saturation rate also affects adult urchins, hindering the assimilation of calcium carbonate to replenish what is lost from the test, resulting in reduced somatic/test growth (Mos *et al.* 2016, Uthicke *et al.* 2014). Test growth may further be decreased when individuals are exposed to temperatures approaching, but not exceeding, their upper thermal limit (Moore and McPherson 1965), a scenario that is predicted with rising ocean temperatures (Deutsch *et al.* 2008). A smaller body, with a relatively bigger surface area, as compared to a large body, dissipates heat more effectively (Hamilton 1960, Berman 2003). This, together with the higher metabolic rate observed in temperature-stressed individuals may result in slower somatic growth in urchins in response to the predicted higher ocean temperatures (Uthicke *et al.* 2014). These complex interactions between ocean chemistry and sea urchin development demonstrate the profound implications of environmental changes on adult sea urchins' morphology (Emerson *et al.* 2017). Alongside water chemistry, variation in the morphology of sea urchins is due to their habitat-specific environment, particularly temperature (Hughes *et al.*

2012, Norderhaug and Christie 2013, Spyksma 2016, Pretorius *et al.* 2019). Various sea urchin species have been the subject of morphometric trait analysis to study the taxonomy, ecology, and adaptations of urchins under different experimental setups, most of which test the response of urchins to environmental changes brought about by anthropogenic pressures. Species include *Strongylocentrotus purpuratus* (Manier and Palumbi 2008, Smith and Garcia 2021, Strader *et al.* 2022), *Arbacia punctulata* (Lessios 1981, Courville *et al.* 2023), *Lytechinus variegatus* (Bishop and Watts 1992, McEdward and Herrera 1999, O'Donnell *et al.* 2010, Challener *et al.* 2013), *Echinometra mathaei* (Kurihara and Shirayama 2004, Uthicke *et al.* 2016, Keshavarz *et al.* 2017), *Diadema antillarum* (Leviton 1991, Rodriguez *et al.* 2013, Francis-Floyd 2020), *Paracentrotus lividus* (Antoniadou and Vafidis 2009, Küçükdermenci and Lök, 2014, Nicolau *et al.* 2022), *Strongylocentrotus intermedius* (Naidenko 1997, Kalachev *et al.* 2018), *Heliocidaris tuberculata* (Abessa 2001, Soars *et al.* 2009), *Eucidaris tribuloides* (Lessios 1981, Pfaller *et al.* 2008) and *Tripneustes gratilla* (Muthiga 2005, Byrne *et al.* 2008, Radjab *et al.* 2020). Common traits measured on sea urchins include test height (Santos and Flammang 2008), test diameter (McShane and Anderson), peristomial diameter (Deli *et al.* 2017), spine length (Rogers-Bennett *et al.* 1995), spine width (Küçükdermenci and Lök 2014), and measurements of the Aristotle's lantern, the mouth structure of the sea urchin, including lantern height (Deli *et al.* 2017), and lantern weight (Pecorino *et al.* 2012). In a comparison between deep-sea and coastal sea urchins, deep-sea species have been found to have longer and thinner spines than their coastal counterparts, which aid in locomotion across soft sediments (Serafy and Fell 1985). In general, deep-sea species also have flattened tests (Minin 2012) to facilitate burrowing in soft sediments (David 1988, Mironov 1975, David *et al.* 2003, Mironov 2008), and colours close to those in the red wavelength (e.g., crimson, vermillion, 'purplish velvet', 'chocolate', and orange; Nutting 1898), to better camouflage in low-light environments where the red colour wavelength is no longer present, as it is the first colour wavelength to be lost underwater as depth increases (Wicksten 1989). By contrast, coastal species have shorter spines (Rogers-Bennett *et al.* 1995) to more effectively protect against wave action (Strathmann 1981) and predation (Moureaux *et al.* 2010), a rounder (Gonzales-Irusta 2009) and thicker test (Dix 1970) to better withstand hydrodynamic forces, and brighter colouring, which may play a role in intraspecific communication (Rowland 2018) and in predator deterrence (aposematic colouring; Wicksten 1989). It is clear that morphometric trait analysis holds significant implications for understanding sea urchins' biology and ecology (Oxnard and O'Higgins 2009, Chiaverano *et al.* 2016).

There are multiple approaches to analyse morphometric data of species, for example, linear distances analysis, boundary outlines analysis, landmarks analysis, and digital images analysis (Macleod 2017). Linear distances analysis relies on establishing the relationship between two-dimensional linear distances on a specimen or a major subregion on the specimen (Fisher 1936, Rao 1948), taken in parallel or at right angles (Macleod 2017). This approach draws some criticism, however, owing to the fact that these linear measurements lack information relating to shape (Mosimann 1970). Because of this and paired with the fact that shape is linked to complexity, the effectiveness of linear distance in precisely characterising shapes is limited (Bookstein *et al.* 1985). Additionally, owing to these limitations, when linear measurements are used to calculate different morphological indices (e.g., volumetry, globosity, density in sea urchins), redundancy and uneven coverage of the original form is introduced. This can affect the accuracy, reproducibility and reliability of morphometric analyses based on linear distances (Albrecht *et al.* 1993).

The use of linear distances in morphometric studies is, however, still widely applied due to the comparatively straight forward standardisation of measures, which reduces the chance of introducing errors into data sets and thus allows for comparison between samples (Dryden and Mardia 1998). In addition, linear distances, such as perimeter, area, and volume, are effective when used to represent variations in the size of the object being measured, as these measurements are scalar (Monteiro *et al.* 2000), and therefore makes the quantification and comparison of differing object sizes easier. Lastly, the use of linear measurements is a well-established method in morphometric traits analysis and the study of relationships between the traits (Galton 1888), giving rise to a series of advanced statistical methods that use linear distance as opposed to the more complex Manhattan, Minkowski, and Hamming distances (Merigo and Casanovas 2010, Greche *et al.* 2017).

Morphometric analyses can be used in a variety of applications such as quantitative measurements (Bondesan *et al.* 1992), shape analyses (Lohmann 1983), taxonomy and species identifications (Amin 1986), evolutionary studies (Oxnard 1978), ecological adaptations (Atchley 1983), population studies (Crouau-Roy 1989) and functional morphology (Zaaf *et al.* 1999). Morphometry plays a pivotal role in taxonomic classification, aiding in species (de Queiroz 2005) and subspecies (Haig *et al.* 2006) delimitation through the comparison of morphological traits. Moreover, morphometric analyses are essential in studying the variation found in the phenotypes of organisms, providing critical insights into how the underlying

genotypes are expressed and how variation in genotypes in turn result in phenotypic variation (Klingenberg 2002, Lawing and Polly 2010, Xu and Bassel 2020). As an example, a study on the common cuttlefish, *Sepia officinalis*, showed the relationship between its genotype and observed phenotype (Turan and Yaglioglu 2010) and the ability of an individual to change their colour and appearance to camouflage or indicate sexual maturity and readiness for mating (Gibson-Hall and Wilson 2018). Although this is a good example of the genotype-phenotype relationship, colour should not be considered in isolation when investigating distinctions between sexes or species (Ralls and Mesnick 2009). Intraspecific variation is also a component of phenotypic variation that is most often observed between male and female counterparts of a species (Shine 1989). Termed sexual dimorphism, such adaptations can be used to infer how selection pressures have created observed phenotypic variations (Baker and Parker 1979, Slatkin 1984). Finally, morphometric analysis can be used to study or reveal geographic variation within a species or between closely related species (Saboriso-Ray and Nedreaas 2000, Jefferson 2002). These morphological changes would arise in response to local geographic conditions within a species' distribution range or that of closely related species (Thorpe 1987). In the same way, morphometric analysis can reveal the impact of interactions between species, such as the coevolution of prey and predator species (e.g., Pitcher and Butler 1987, Wirsing 2003, Sarma *et al.* 2011).

Sea urchins function as indicator species (i.e., a species that can be used to infer the health status of the ecosystem in which it is found by considering their physiological or health condition, Zhao *et al.* 2019). The methods to assess population health of aquatic and terrestrial species alike range from haematological and biochemical or plasma chemistry parameter analysis (Dunbar *et al.* 1997, Lloyd *et al.* 2016, Cakmak *et al.* 2020), and physiology through assessments of respiration and metabolic rates (Kirby *et al.* 2006, Tomšić *et al.* 2010, Elliot *et al.* 2012, Küçükdermenci and Lök 2014, Nicolau *et al.* 2022). An alternative to these methods is morphometric analysis, which shares a common origin with the varied fields of geometry and biometry (Macleod 2017). Morphometric analysis plays a critical role in assessing population health by revealing how the morphological traits of individuals are shaped by, and in turn influence, their environment, resource utilisation, reproductive success, predator-prey dynamics, genetic diversity, adaptation to changing climate, population demographics, and responses to anthropogenic impacts (Skeljo and Ferri 2012, Muiocha and Onming 2017, Albarran-Melzer *et al.* 2020, de Melo *et al.* 2020, Kang *et al.* 2020). Understanding these

relationships is fundamental to gauging the overall well-being and resilience of populations in various ecological contexts.

In morphometrics, quantitative measurements and analysis of body metrics of an organism, such as length, width, counts, and ratios are used to calculate different indices (Kirby *et al.* 2006, Elliot *et al.* 2012). In sea urchins, a number of indices can be calculated based on morphometric characters of the species, examples of which include: volumetry index, based on test volume and test diameter used to calculate the volume of a spherical bottle cap (Pomory and Lares 2011); globosity index, which is a measure of the roundness of a sea urchin test and is calculated using test height and diameter; and density index, which determines the density of the sea urchin by dividing wet weight of the sea urchin by test diameter (Nicolau *et al.* 2022). Furthermore, shape analysis of an organism allows for the investigation of how different ecological factors, such as habitat, diet, and behaviour, affect the organism's underlying physiology that modulates variation in shapes (Rastogi 2007). There is a relationship among quantitative measurements, shape analysis, and functional morphology (Clark 1981, de Stefano and de Stefano 2005, Jones *et al.* 2012), the latter of which links an organism's structural form to the function of the form (Wake 1992). Functional adaptations, which include changes in the morphology (Vermeij *et al.* 1985), physiology (Lockwood 1976), and behaviour of organisms, in response to environmental factors (Lauder *et al.* 1993), are crucial for a species' long-term survival since changes in an organism's function ultimately defines its fitness (Wainwright 2007) and resilience to change (De Battisti 2021). Understanding the dynamics and links of adaptation to ecological niches provides insight into the factors that result in those observed adaptations (Levins 1968). In this instance, analysing morphological traits can be used to infer for which function the adaptation exists (Wainwright *et al.* 2008). Morphometrics can be used to assess how different populations within a species have adapted to their ecological niches (Allen and Bekoff 1995) and help establish the ecological biotic and abiotic interactions of a species with its environment (Kirwan *et al.* 2009). For example, the adaptive radiation of the Hawaiian humbug damselfish, *Dascyllus aruanus*, is a good case study, examining how changes in the morphometric traits of body shape and colouration relate to the ecological niches occupied by the fish in the coastal waters of different Hawaiian islands (Frederich *et al.* 2013, Stieb *et al.* 2017). This sets the foundation for population-level studies, where morphometrics are used to assess variations within populations (Mohaddasi *et al.* 2013), information which can then be used in the monitoring of population health (e.g., Reif *et al.* 2004, Kershaw *et al.* 2017, Hillcoat *et al.* 2021).

Adaptations through natural selection improve the fitness of a species which, over time, impact an organism's survival (Gadgil and Bossert 1970) and its reproductive success (Pianka 1976, Feller 1967). Within this context, for instance, adaptations may enable an organism to outcompete others for available resources, aid in predator avoidance, and better manage changes in environmental conditions or threats (Dawkins and Krebs 1979, Butzer 1980). Associated with morphological adaptations are the underlying physiological and genetic adaptations, which involve changes in the internal biological processes of an organism to optimise various biochemical processes regulating temperature, metabolism and/or energy utilisation (Asres and Amha 2014; e.g., Codron *et al.* 2019, Phillips *et al.* 2020, Jia *et al.* 2021).

3.1.2 Morphometric analysis in ecological studies on sea urchins

Morphometric trait analyses provide significant insight into sea urchins' adaptive potential, such as optimal resource utilisation (Contretas and Castilla 1987), habitat specialisation (Rose and Cross 1993), predator-prey dynamics (Voulgaris *et al.* 2021), reproductive strategies (Ourens *et al.* 2011), phenotypic plasticity (Donelson *et al.* 2018), responses to environmental changes (Uthicke *et al.* 2019), ecosystem functioning (Kelso 1971), and evolutionary history (Kinjo *et al.* 2008). Similarly, morphometric trait analysis makes it possible to investigate adaptations of different species of sea urchin that arose in response to filling a specific ecological niche (Lessios 2010).

Sea urchin spines are the most important defence mechanism sea urchins have, protecting them from predators and competitors alike (Fricke 1974, Moureaux *et al.* 2010). Spine morphology varies in length, thickness and shape (Dix 1970), and is dependent on the environment in which the individual is found (David and Neraudeau 1989). Besides environmental influences, in adults these traits, as well as spine density on the test, are affected by the test size and shape (Grossmann and Nebelsick 2013). Spines are brittle and can break off in a predator's mouth or other feeding appendages, increasing the chances that a sea urchin would survive such a predation event (Strathmann 1981). Furthermore, spine breakage prevents predators from properly gripping an urchin, and may simultaneously produce injuries, further acting as a deterrent (Tsafnat *et al.* 2012). Besides acting as a defence or protective mechanism, spines also serve a function in locomotion, allowing an individual to cover more distance than through locomotion achieved by tube foot movement alone (Prouho 1888, Moureaux *et al.* 2010).

Another character exhibiting high plasticity in morphology is the Aristotle's lantern. The Aristotle's lantern is the mouth structure of the urchin and is a pentaradially symmetric jaw structure found within the urchin test and attached to the test by ligaments (Bolt 1963). The lantern tapers to five calcareous, regenerative teeth that extend beyond the ventral opening of the test (Holland 1965, Ma *et al.* 2007). These teeth allow sea urchins to grasp and scrape food material from rocks, as well as being used for the mastication of food for easier chemical digestion and nutrient uptake (Ebert 1980, Lawrence and Jangoux 2013). Surrounding the lantern and connecting it to the sea urchin test is the peristomial membrane, which also covers the ventral opening of the test (Wilkie *et al.* 1994). The lantern is connected to the test by ligaments that attach to the calcareous processes, called apophyses, found inside the test (Candia Carnevali *et al.* 1992). These five apophyses make up the perignathic girdle inside the test of the urchin (Dolmatov *et al.* 2007) which, by acting as an anchor point for the ligaments, allows stability, yet some flexibility, in the movement of the lantern thereby allowing the urchin to manipulate or recover otherwise inaccessible food items (Stauber 1993). The morphology of the lantern is determined by the amount of energetic resources allocated towards the growth and maintenance of the structure (Black *et al.* 1982, Ebert 1996). Individuals with larger lanterns relative to their test size, and therefore larger ligaments attaching the lantern to the test (Andrietti *et al.* 1990), are better able to scrape food material from harder rocky substrate (Stock 2014) or feed on tougher algae and algal fronds (Agatsuma *et al.* 2018).

Linked to grazing efficiency is the attachment strength of sea urchins to substrate through their tube feet (Santos *et al.* 2009). Tube foot length varies among species (Santos and Flammang 2006) and may be associated with specific morphological traits such as larger body size or longer spines (Gallien 1986). Other features of the tube feet that vary depending on species include size, shape and the arrangement of the tube feet in the ambulacral zones in which they are found (Sharp and Gray 1962, Flammang *et al.* 2005). Tube feet arrangement is partly influenced by the density of these structures which, akin to tube foot length, size, and shape, is a function of the substrate where urchins are located. This because tube feet serve different functions depending on the substrate characteristics (Markel and Titschack 1965). Urchins inhabiting rocky substrates have shorter, sturdier tube feet on the adoral surface as opposed to the aboral surface, to provide better attachment and stability in environments with intense wave action or strong currents (Smith 1978).

Sea urchins are keystone species (Fletcher 1987) as well as ecosystem engineers (Graham 2004, Ling 2008) and these roles have been largely reviewed in Chapter 2. The analysis of morphometric traits can provide insights into the pivotal role sea urchins play in the ecosystem as ecosystem engineers. For example, the grazing behaviour of urchins result in the modification of their habitat through their influence on species abundance and community composition as discussed previously (White *et al.* 2018), but also through the creation of pits in rock through bioerosion (Krumbein and Pers 1974, Spencer 1992). Laboratory-based evidence shows that sea urchins can modify rocky substrata by excavating hollows with the Aristotle's lantern while grazing, the depth of which varies in relation to rock type (Russel *et al.* 2018). Inside the pit and encased in rock apart from the ventral surface, movement of the urchin causes abrasion against the interior pit surfaces by spines (Fewkes 1890). Similarly, the movement of tube feet inside the pit as they attach and detach to the pit's surface is a further source of bioerosion (Valenciennes 1854). Collectively, the attachment of the tube feet to the inner surfaces of the pit, as well as the ability of sea urchins to arrange their spines in such a manner that they are wedged in the pit, makes pit-dwelling very advantageous (Hernandez and Russel 2009). Pit-dwelling individuals typically have a taller test as sideward test growth is limited (Baron 1991, Grupe 2006). Pits that are too shallow or small for sea urchins to shelter in may be used by small invertebrates or juvenile fish for shelter, thereby creating microhabitats for use by other species (Davidson and Grupe 2015).

3.1.3 Rationale and Aims

Sea urchins have been extensively studied with regard to morphometric traits (Table 3.1). These studies have investigated: growth in *Centrostephanus rodgersii* to increase understanding of the species' population biology (Pecorino *et al.* 2012); the adhesive capacities of *Colobocentrotus atratus* with three sympatric species (Santos and Flammang 2008); current population density of *Diadema africanum*, and compared observed spatio-temporal differences in the Aristotle's lantern (Cabanillas-Terán *et al.* 2015); the spatial variation in growth rates of *Evechinus chloroticus* at localities with different levels of food availability (McShane and Anderson 1997); the influence of differing densities of fish predators on *Evechinus chloroticus* using density and size structure measures (Cole and Keuskamp 1998); morphometric features of legal-size *Paracentrotus lividus* (Pais *et al.* 2006); the attachment strength of tube feet of *Arbacia lixula*, *Paracentrotus lividus*, and *Sphaerechinus granularis* (Santos and Flammang 2007); the temporal variation of body condition and the length-weight relationship of *P. lividus*

(Küçükdermenci *and* Lök 2014); plastic responses in the attachment capacity of *P. lividus* to different hydrodynamic conditions (Cohen-Rengifo *et al.* 2017); patterns of population differentiation within *P. lividus* along a wide distribution where environmental factors differ along the distribution range, possibly delineating morphometric subunits (Deli *et al.* 2017); morphological patterns of *P. lividus* and if any of these could be linked to environmental conditions (Caill-Milly *et al.* 2020); morphometric relationships and relative growth in *P. lividus* (Nicolau *et al.* 2022); spatial variability of growth in *Pseudechinus huttoni* at a standardised depth, growth rates between shallow- and deep-water populations (Kirby *et al.* 2006); reproductive potential and sheltering behaviour of *Strongylocentrotus franciscanus* (Rogers-Bennet *et al.* 1995).

Table 3.1: A summary of studies utilising morphometric trait measurements in studies on various sea urchin species.

Species	Country	Sampling location(s)	Year	Authors	Traits measured	With test ornamentation (Y/N)
<i>Centrostephanus rodgersii</i>	New Zealand	Mokohinau Islands	2012	Pecorino <i>et al.</i>	Test diameter (mm)	N
					Eviscerated wet weight (g)	Y
					Lantern weight (g)	N/A
<i>Colobocentrotus atratus</i>	Reunion Island	Cap La Houssaye	2008	Santos and Flammang	Test height (mm)	Y
					Test diameter (mm)	Y
					Wet weight (g)	Y
					Tube feet (counts)	N/A
<i>Diadema africanum</i>	Canary Islands	Gran Canaria	2014	Cabanillas-Terán <i>et al.</i>	Aristotle's Lantern diameter (mm)	N/A
<i>Evechinus chloroticus</i>	New Zealand	Dusky Sound	1997	McShane and Anderson	Test diameter (mm)	Y
		Arapawa Island			Wet weight (g)	Y
					Eviscerated wet weight (g)	Y
<i>Evechinus chloroticus</i>	New Zealand	Tawharanui Marine Park	1998	Cole and Keuskamp	Test diameter (mm)	N
		Kawau Island			Eviscerated wet weight (g)	Y
		Cape Rodney - Okakari Point Marine Reserve				
		Coastal Leigh				
<i>Paracentrotus lividus</i>	Mediterranean	Alghero	2006	Pais <i>et al.</i>	Test diameter (mm)	N
					Test height (mm)	N
					Wet weight (g)	N/A

Species	Country	Sampling location(s)	Year	Authors	Traits measured	With test ornamentation (Y/N)
<i>Paracentrotus lividus</i>	France	Mediterranean Sea	2007	Santos <i>and</i> Flammang	Test diameter (mm)	N
<i>Sphaerechinus granularis</i>					Test height (mm)	N
<i>Arbacia lixula</i>					Tube feet (counts)	N/A
<i>Paracentrotus lividus</i>	Turkey	South Aegen Sea	2014	Küçükdermenci <i>and</i> Lök	Test diameter (mm)	N
					Wet weight (g)	Y
					Test height (mm)	N
					Spine width (mm)	N/A
					Spine length (mm)	N/A
<i>Paracentrotus lividus</i>	France	Crozon Peninsula	2017	Cohen-Rengifo <i>et al.</i>	Tube feet (counts)	N/A
<i>Paracentrotus lividus</i>	Algeria	Littoral zone	2017	Deli <i>et al.</i>	Test diameter (mm)	N
	Tunisia				Test height (mm)	N
	Libya				Aristotle's Lantern diameter (mm)	N/A
					Lantern height (mm)	N/A
					Peristomial diameter (mm)	N/A
					Spine length (mm)	N/A
					Wet weight (g)	Y
<i>Paracentrotus lividus</i>	France	Bay of Biscay	2020	Caill-Milly <i>et al.</i>	Test diameter (mm)	N
					Test height (mm)	N
					Dry mass (g)	Y

Species	Country	Sampling location(s)	Year	Authors	Traits measured	With test ornamentation (Y/N)
<i>Paracentrotus lividus</i>	Portugal	Carreço Coast	2022	Nicolau <i>et al.</i>	Test diameter (mm)	N
		Aljezur Coast			Test height (mm)	N
					Wet weight (g)	Y
<i>Pseudechinus huttoni</i>	New Zealand	Doubtful Sound, Fiordland	2006	Kirby <i>et al.</i>	Test diameter (mm)	N
		Otago Peninsula			Wet weight (g)	Y
					Test height (mm)	N
<i>Strongylocentrotus franciscanus</i>	USA	Bodega Bay, California	1995	Rogers-Bennett <i>et al.</i>	Test diameter (mm)	Y
					Test height (mm)	Y
					Peristomial diameter (mm)	N/A
					Spine length (mm)	N/A
					Wet weight (g)	Y
					Tube feet (counts)	N/A

A knowledge gap, however, remains in the morphometric analyses of sea urchins, specifically in the use of traits to determine if there is variation within and among populations which may inform on the ecological resiliency of one population over another, with relevance going beyond the specific scope of this study on urchins. As widely addressed in Chapter 2, the marine environment along the coast of South Africa offers an ideal region to address such an ecological gap, owing to the contrasting current systems found on east and west coasts, namely the warm Agulhas Current on the east (Lutjeharms 2007), and cold Benguela Current on the west (Garzoli and Gordon 1996). The presence of such divergent current systems can influence the diversity of shapes and forms in marine organisms in South Africa (Teske *et al.* 2019). Aside from water temperature, salinity is lower on the west coast in surface waters owing to the water's place of origin (initially the Antarctic Circumpolar Current) that becomes the South Atlantic Current and which in turn becomes the Benguela current (Stramma and England 1999, Boehme *et al.* 2008). The warm water of the east coast originates from the Indian Ocean around the equator, developing into the westward fast-flowing Agulhas current, which is comparatively also more saline than the west coast waters, owing to evaporation (McCartney and Talley 1984, Maslin *et al.* 1995).

As a result of this physical divergence, the current regime heavily influences the ecosystem on each of the coasts where the west coast is characterised by kelp forests (Field *et al.* 1977, Velimirov *et al.* 1977) as opposed to the east coast which mainly supports estuarine mangrove forests (Adams *et al.* 2004). Though the rocky shores on the east and west coasts are largely the same in terms of ecosystem functioning, the primary difference between the east and west coasts of South Africa is the presence of the oyster belt on the east coast, and the cochlear zone on the west coast (Branch and Branch 2018). Among many species of vegetation found exclusively on the west coast, sea bamboo (*Ecklonia maxima*), split fan kelp (*Laminaria pallida*), slippery orbits (*Aeodes orbitosa*), and ribbon-weed (*Grateloupia doryphora*) are notable, supporting west coast exclusives such rock lobsters (*Jaysus lalandii*), the Mediterranean mussel (*Mytilus galloprovincialis*) and granite limpets (*Cymbula granatina*), whereas the east coast features jelly weeds (*Gelidium spp*), wedge weed (*Halimeda cuneata*), the green fan (*Udotea orientalis*) and the turkey tail (*Padina boryana*, Branch 1994) support such diverse invertebrates as the Natal rock oyster (*Saccostrea cucullata*, Dye 1989), zoanthids (Biseswar *et al.* 2002), and the needle urchin (*Diadema setosum*, Filander 2014).

Populations in these ecosystems are subjected to different biotic and abiotic factors which can result in subtle fine scale adaptations that usually remains unnoticed to broad/general sampling. For instance, the sea urchins found on the west coast could be predated on by sea birds (Schneider 1985, Irons *et al.* 1986, Bustnes 1996, Erlandson and Moss 2001), fish, lobsters, and larger marine mammal predators such as the Cape fur seals (Kirkman *et al.* 2011). Though the distribution of the Cape fur seal is historically accepted as from Algoa Bay to Namibia, sea urchins are more likely to be predated upon by fur seals on the west coast, where the colonies are largest (David 1987). This environmental contrast between the east and west coast of South Africa created a unique opportunity to study how morphometric traits change within a species across a wide range of environmental variables. For this reason, the cosmopolitan Cape sea urchin species, *Parechinus angulosus*, was selected for this study, owing to its wide distribution range from the southern reaches of Kwa-Zulu Natal along and around the south and west coasts of South Africa. Any coast-specific morphological adaptations seen between the east and west coast populations of *P. angulosus* is likely due to the interaction of these factors.

Like all echinoids, *Parechinus angulosus* is characterised by a hard, calcareous test that is covered in spines called radioles (Ziegler *et al.* 2008, Neige 2015). The strictly green test varies in dimension and shape from round to pentagonal and provides structural support for the internal organs of the sea urchin (Aizenberg 2006, Ziegler *et al.* 2008, Filander 2014). The test comprises ten zones, five ambulacral, and five interambulacral zones (Rose and Cross 1993). All ten zones contain relatively long, slender primary spines and short secondary spines which attach to the test at tubercles (David and Neraudeau 1989). These spines vary markedly in size and colour from deep purple to bright red, subtle pink or purple, and beige at the tips, with the base of the spine remaining green (Mertens 2021). Filander (2014) notes that individuals found in the littoral zone have shorter and more robust spines than their sublittoral counterparts. Similarly, all ten zones contain pedicellariae, stalked, jaw-like organs that are used for the manipulation of small organisms and detritus that may pose a threat to the health of the urchin if allowed to settle on the test (Von Uexkull 1899, Campbell 1973, Nagelkerken *et al.* 1999, Bauer and Young). Wider than the interambulacral zones are the ambulacral zones in which tube feet are exclusively found (Rose and Cross 1993). Based on these characteristics, measurements of several traits were considered (detailed in section 3.2).

3.1.4 Hypotheses and Research Questions

The research objectives for this study were to determine if morphometric traits vary in response to the different environmental conditions of the east and west coasts of South Africa and if morphometric trait variation can be used to assess ecological resiliency. Alongside the other measures used in this project (see the following chapters for the microbiome and genomic approaches), morphometric analysis was explored as an approach to assess resiliency. It was hypothesised that there is a difference in the morphometric traits of individuals collected from the east and west coasts of South Africa owing to the different currents dominating each of the coasts and that this variation can be quantified, such that individuals can be said to originate from either the east or west coast. Specifically, it was hypothesized that the test and Aristotle's lantern would exhibit regional patterns and differ significantly among populations found within the east and west coasts bioregions. Furthermore, it was hypothesised that there would be a significant difference observed in the spine metrics of individuals collected from the east and west coasts, with larger (and therefore sturdier) spines found on the west coast as a result of the intense wave action experienced by the west coast. A secondary hypothesis linked to this trait was that spine morphometric measurements can be used to accurately classify individuals as originating from either the east or west coasts which are defined a priori based on non-morphometric data using classificatory discriminant analysis (the K- nearest neighbour method) and Bayesian multi-level modelling.

3.2 Materials and Methods

3.2.1 Collection

During the late spring and summer of 2019 – 2020 (October 2019 – January 2020), ten *Parechinus angulosus* individuals were collected from each of 37 populations along the South African coastline, from McDougall's Bay to Port St. John's (Chapter 2; Figure 2.1), with a minimum of 6 km and a maximum of 123 km distance between sites. The largest distance (i.e., 123 km) between two adjacent populations corresponded to a lack of suitable rocky habitat which this species of sea urchin inhabits. The decision was made to collect ten individuals owing to the feasibility in terms of the time it would take to process the samples for morphometric measurements (Lakens 2022). Due to the time taken for processing the number of individuals ultimately used for this study was much lower than 10 individuals per site. These populations were categorised into bioregions based on Lombard's (2004) classification (Chapter 2; Figure 2.2). Urchins were collected from rock pools by hand during low spring

tides and immediately placed into jars containing 99% ethanol to cover the specimens completely. Individuals were collected from as many rock pools that were found to contain them in a 14 m² section of rock. Although urchins ranging between 40-60 mm in width (spine-to-spine) were targeted, at some localities individuals smaller than 30 mm were collected owing to a shortage of larger-size individuals. Upon return to the laboratory, individuals were kept at -27 °C in freezers located at the South African Institute for Aquatic Biodiversity (SAIAB). Of the 430 individuals of *Parechinus angulosus* that were collected specifically for the morphometric study, the test and lantern metrics described below were collected from 286 urchins that were processed for collection of body tissues for chapters four and five. A further 54 individuals were processed for the same metrics, but also for the collection of spine metrics from each urchin, but eight samples had to be removed from the data set due to missing data. The sample sizes used in this study therefore, were as follows: test weight, test height, test width, peristomial width, lantern weight, lantern height, and lantern width (n = 333); spine measures (n = 46).

3.2.1.1 Processing

Individual sea urchins which had been stored in absolute (99%) ethanol at -27 °C were individually removed from the ethanol, blotted on tissue paper and left to drain in a Petri dish for 1 minute. Individuals were left in a Petri dish to drain as opposed to tissue paper in order to prevent excessive loss of coelomic fluid (Kirby *et al.* 2006) which would have impacted the accuracy of weight measurement. After 1 minute, individual urchins were weighed (g) to two decimal points using an Adam Equipment® PGL 3002 precision balance. The colour of each urchin was recorded, and a photograph taken for later comparative purposes. For the subset of individuals used for the measurement of spine metrics, a Leica MZ75 dissecting microscope fitted with a graduated eyepiece was used to collect the spines and pedicellariae from each ambulacral and interambulacral zone using fine forceps and a dissecting needle into a Petri dish, starting with the interambulacral zone adjacent to the madreporite when the madreporite is at the 12 o' clock position when viewed ventrally. The test ornamentation from each interambulacral zone was collected in a clockwise order (Figure 3.1) with zones labelled IAZone1 – IAZone5 (Interambulacral Zone 1 – 5), and AZone1 – AZone5 (Ambulacral Zone 1 – 5) while taking note of any damage to a large amount of spines, or any evidence of healed over injury or trauma to the test. Spine characters were further investigated by measuring the length and diameter of the 300 longest spines and the 300 shortest spines per individual.

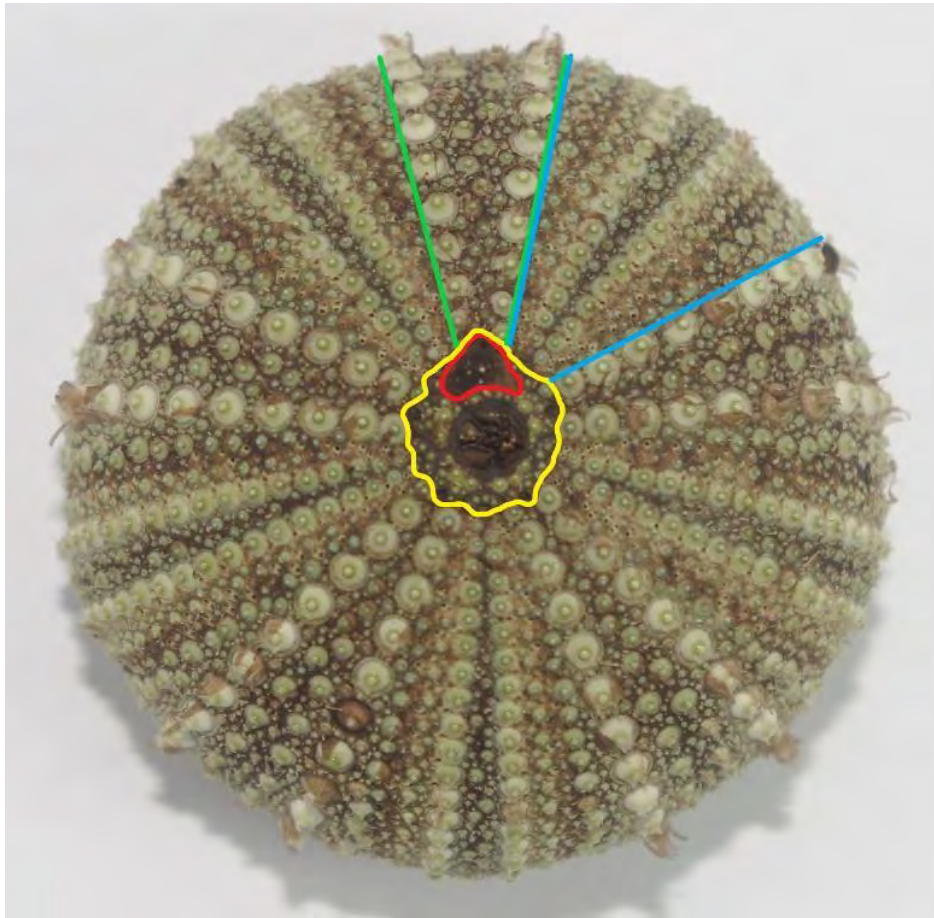


Figure 3.1: Denuded sea urchin test indicating the madreporite (red), apical system (yellow), interambulacral zone 1 (green), and ambulacral zone 1 (blue) (photographed with a Pentax DSLR camera, 2X magnification).

For the same subset of data, one height measurement of the urchin was taken along the oral-aboral axis from the meeting point of the teeth on the oral/ventral surface of the urchin to the anus, at the centre of the genital plate system on the aboral/dorsal surface of the urchin, using a 150 mm Tork Craft Vernier carbon steel calliper. Thereafter, five measurements of horizontal test diameter (perpendicular to the oral-aboral axis) were taken at the ambitus, starting with the madreporite at the 12 o'clock position (Figure 3.2) and the urchin rotated clockwise. The diameter measurements were taken with the fixed jaw of the calliper placed in the centre of each of the interambulacral zones and the movable jaw of the calliper placed in the centre of the opposite ambulacral zone. Five peristomial diameter measurements were taken in the same manner, with the fixed jaw of the calliper placed in the centre of each interambulacral zone and the movable jaw of the calliper fixed in the centre of the opposite ambulacral zone.

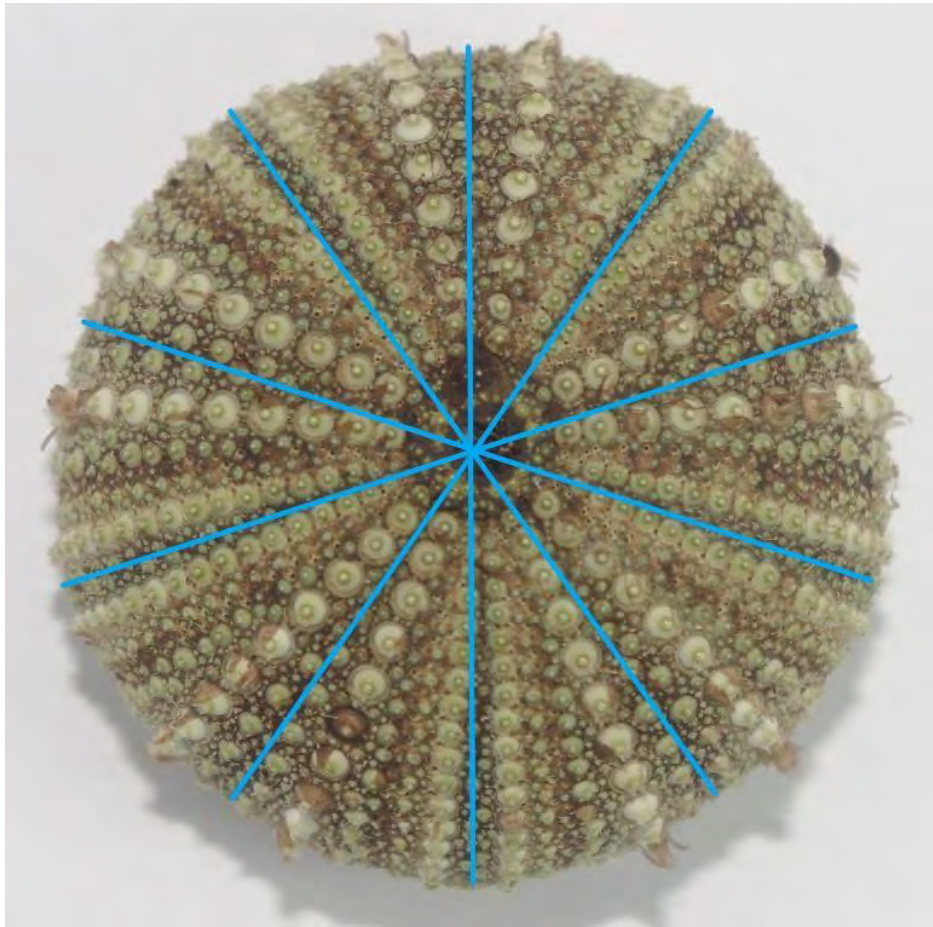


Figure 3.2: Denuded sea urchin test with axes along which the five width measurements were taken (blue) (photographed with a Pentax DSLR camera, 2X magnification).

After removing the Aristotle's lantern from the ethanol, all excess tissue was removed from the external surfaces of the lantern after which it was blotted dry, and a wet weight (g) was measured. Height was measured from the centre of the oesophageal opening at the top of the lantern to the point where the teeth meet. Five width measurements of the lantern were taken across the aboral surface.

Based on Nicolau *et al.* (2022), morphometric indices were calculated from the measurements described above using the following formulae:

$$\text{Globosity index (Gi)} = \frac{\text{Test height (Th)}}{\text{Test diameter (Td)}}$$

$$\text{Density index } (Di) = \frac{\text{Test weight } (Tw)}{\text{Test diameter } (Td)}$$

$$\text{Volumetry index } (Vi) = \frac{\text{Test volume } (Tv)}{\text{Test diameter } (Td)}$$

Test volume was calculated using the formula of a spherical cap which was first used for estimating test volume in urchins by Pomory and Lares (2011):

Test volume (Tv)

$$= \left(\pi \times \frac{\text{Test height } (Th)}{6} \right) \times \left[3 \times \left(\frac{\text{Test diameter } (Td)}{2} \right)^2 + \text{Test height } (Th)^2 \right]$$

These indices were selected owing to their wide-spread use among studies on sea urchins, and the unambiguity in measuring the traits, enabling the replication of trait measurements used in the calculation of these indices. Neither the gonadal (Bennett and Giese 1955) nor gonad somatic (Shpigel *et al.* 2005) indices were considered for this study because the aim was not to quantify reproduction or reproductive cycles as this study did not include a temporal component to compare indices seasonally or annually. These indices vary greatly with time whereas indices attempting to quantify growth exhibit a much smaller scale of variation among seasons. Second, this study also did not consider condition index (Rouane-Hacene *et al.* 2018) owing to the subjective nature of collecting the flesh wet weight which serves as the numerator for this equation, as the authors did not describe how meticulously this was done (e.g., if the ligaments attaching the lantern to the test or the peristomial membrane were included in the weight measurement). Finally, the repletion index was also not included in this study as this is highly subjective to the food availability at the collection site, and similar to the gonadal and gonad somatic indices, is better suited to a temporal study as opposed to the current cross-sectional regional study (Belkhedim *et al.* 2014).

3.2.2 Statistical analysis

3.2.2.1 Test and Aristotle's lantern metrics

Prior to determining if there was a population or regional effect reflected in the morphometric data sets on test and the Aristotle's lantern, samples with missing morphometric data were removed. Thereafter, the metric data were analysed using a two-way nested design, with coast (fixed effect) and population (nested within coast) using several non-parametric multivariate statistical permutation tests, PERMANOVAs. Prior to analyses, the data were normalised due to the different units of the metrics measured (g vs mm; Clarke and Gorley 2015). A resemblance matrix was created based on Euclidean distances as this is the appropriate statistic for continuous data (Anderson 2017). Subsequently, a PERMDISP test was performed for the two factors of population and coast to test for deviations from the assumption of homogeneity of variances. Based on the outcome of this test, the data were $\text{Log}(X+1)$ transformed. Even after transformation, the data sets (for population only) still violated the homogeneity assumption. The decision was made to continue with the transformed data as this transformation resolved the non-homogeneity of data for the factor of coast. PERMANOVAs were nevertheless applied to the transformed data set as it is a robust test against non-homogenous data (Anderson 2017) and were followed by pairwise comparisons of significant results. Primer v7 (Clarke and Gorley, 2015) was used to generate principal coordinate analysis ordination plots (PCOs) and for PERMANOVAs. Traits that were found to vary significantly among populations within either the east- or west coast were further investigated using similarity percentage (SIMPER) analyses dissimilarity based on Euclidean distance to assess which traits contributed to the observed difference in the significant PERMANOVA pairwise outcomes.

These analyses were originally repeated on three data subsets consisting of all traits, test traits, and lantern traits. This was due to the much larger data sets for test and lantern (428 and 422 data points for test and lantern traits respectively) than for the combined all traits (287 data points that remained). The results of the subset analyses were, however, not included, owing to the fact that the PERMANOVA results remained unchanged for the all traits data set. Furthermore, while originally the two bioregions of Agulhas and Natal (Chapter 2: Figure 2.2), were investigated as separate, they were subsequently merged into 'east coast', owing to the fact that the Natal bioregion did not significantly differ from the Agulhas Bioregion. The Namaqua and south-western Cape Bioregions were merged into 'west coast', because similarly, the south-western Cape Bioregion did not differ significantly from the Namaqua Bioregion but was found to be significantly different to both the pooled Natal and Agulhas Bioregions.

Finally, three Mann-Whitney U (Wilcoxon Rank Sum) tests were performed on each of the globosity, density and volumetry indices.

3.2.2.2 Spine metrics

For this component of the study, morphometrics from sixty spines (thirty of the smallest and thirty of the largest) per ambulacral zones ($n = 5$) and interambulacral zones ($n = 5$) were measured and organised into two separate data sets. The number of spines per urchin in this study ranged from 854 – 6804, making it infeasible to measure every spine, and so the decision was made to measure only 150 of the largest and smallest spines ($n = 5$ IAZ and 5 AZ multiplied by 30).

The first data set (hereafter individual-level data set) consisted of the average values of each measurement for each spine metric (short spine length – SL, short spine width – SW, large spine length – LL, large spine width – LW) per individual for each of the 46 individuals in the data set that had been collected across a subset of 16 populations. In the second data set (hereafter population-level data set), an average was not taken, and the spine measurements for each morphometric character for all individuals collected from each population ($n_{pop} = 16$) were combined to create a pooled data set for each subset of the 16 populations. Both data sets were analysed in parallel using R package MorphoTools2 v1.0 (Šlenker *et al.* 2022).

First, the distribution of spine morphometrics was visually inspected using a combination of the `summary()` function in R, and the `histCharacter()` function in the MorphoTools2 package. This step is necessary to investigate those morphometric characters with extremely high or extremely low values (outliers) that potentially point to errors made during measurement reading or recording of the data. In addition, the pairwise relationship between characters (that is $SL > SW$; $LL > LW$; $LL > SL$; $LW > SW$) was verified in the R package `dplyr()` (Wickham *et al.* 2019) and in the case of any unusual measurement, the raw data were investigated for a second time. When a conclusive correction was not possible, those data points were removed, leaving a total of 13 609 data points for downstream analysis. To select the best statistical method to analyse this data set, the normality of the morphometric data was tested using the Lilliefors (Kolmogorov-Smirnov) test implemented in the `nortest v.1.0` R package (Gross *et al.* 2009).

A total of 189 data points were filtered because it was not possible to conclusively resolve uncertainty in these measurements. Visual inspection of the data, as well as the statistical test of Lilliefors (Kolmogorov-Smirnov), confirmed that the data were not normally distributed, therefore, the following results were obtained using non-parametric statistical tests. Prior to the implementation of machine-learning methods, it first had to be determined if there was a difference in the measures of central tendency from the east and west coasts. The results of the Mann-Whitney U Test (not presented) highlighted that there were significant differences for all the spine characters of short spine length (SL), short spine width (SW), large spine length (LL), and large spine width (LW, $p < 0.05$), confirming a difference in the measure of central tendency between the east and west coasts.

Since the number of data points for the population-level data set exceeded the maximum number of data points recommended ($n = 5\,000$) for a Shapiro-Wilk test (Shapiro and Wilk 1965), a limit beyond which there is no consensus about the statistical power of this test, this MorphoTools2 built-in function was not used. In cases where the data were not normally distributed, different transformations recommended in the same R package were applied to investigate whether any improvement in the shape of the distribution can be observed, and since only minor improvement in the normality was observed after transforming data, non-parametric statistical methods were chosen for downstream analysis.

To visually inspect the distribution of data points in both data sets, two principal component analyses (PCAs) were performed to summarise multivariable data in a lower number of dimensions. In the first PCA analysis, the average value of each character per individual ($n = 46$) was estimated and subsequently visualised. In the second PCA analysis, the total number of metrics ($n = 13\,609$) per individual ($n = 300$) were combined and visualised. Additionally, two principal coordinate analyses (PCoA) were performed on the data sets in order to visualise distances between data points. In all analyses, Euclidean distance among data points was selected, unless stated otherwise.

3.2.2.2.1 Classificatory discriminant analysis

The purpose of this analysis was to investigate whether spine morphometric measurements from sea urchins can be used to classify individuals into separate groups that have been defined a priori, based on non-morphometric data (e.g., genetics or biogeography). Such classifications

could point to underlying fine-scale physiological adaptation that different populations develop in response to variations in environmental conditions. To discern from the original three-region design, the data sets were divided into the following two broad regions: the east coast, which encompassed sites/populations to the east of and including Arniston, and the west coast, encompassing sites/populations to the west of and including Kommetjie (Chapter 2: Figure 2.2).

There are several methods for classificatory discriminant analysis. Each has assumptions that need to be validated prior to analysis, otherwise, such results could be biased and less informative. The first step is to make sure that morphometric characters are not highly correlated ($r > 0.95$). To check for this assumption, a correlation analysis was performed using the Spearman correlation coefficient (Spearman 1904). The second assumption is that no single character may be a linear combination of any other characters. For this purpose, a stepwise discriminant analysis was performed using MorphoTools2 to ensure that the incorporation of the four measurements (SL, SW, LL, LW) in the following analyses is justified (i.e., it identifies the optimal number of independent characters that need to be retained without sacrificing the level of information in the data). At each step of the discriminant analysis, the method evaluates all variables and identifies which variables contribute most to the discrimination between populations. More importantly, for the total number of taxa (g), which in this case represents the two coastal regions, the number of morphometric parameters (p), and total number of samples (n), the following equation needs to be satisfied: $0 < p < (n-g)$ (MorphoTools2 v1.0.1.0, Šlenker *et al.* 2022). Finally, there must be at least two taxa and within each taxon there needs to be at least two distinguishable groups.

The K nearest neighbour classificatory discriminant analysis (K-NN; Fix and Hodges 1951, Cover and Hart 1967), which is a supervised (i.e., the number of groups are known a priori) machine-learning method that does not require the underlying data to be normally distributed, was used. The successful classification of individuals to either the east or west coast could provide evidence for potential associations between the spine morphometrics and the environmental conditions on the east and west coasts. Briefly, this method measures distances (e.g., Euclidean) between an unknown object and K number of its closest neighbours and then tries to classify unknown objects based on a majority vote into the number of groups that have been defined a priori (i.e., from biogeographic or genetic results).

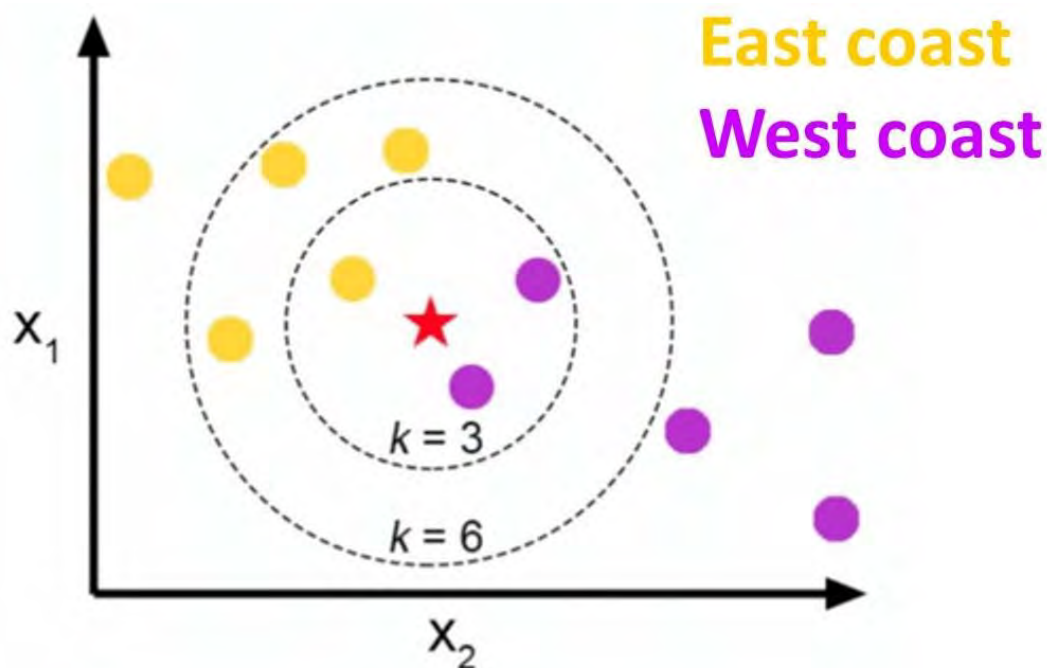


Figure 3.3: Graphic illustration of the K nearest neighbour machine-learning method (Hardik Jaroli 2019). The star at the centre of the diagram denotes the unclassified individual, with the circles denoting the individuals used to attempt to place the unclassified individual. Using the inner circle, the unclassified individual is classified as belonging to the West Coast population. Using the outer circle places the unclassified individual as originating from the East Coast. Figure 3.3 provides a hypothetical graphic explanation, where the red star at the centre of the plot could be considered as an unclassified individual, the yellow individuals as those collected from the east coast, and purple individuals as those collected from the west coast. If $K = 3$, the nearest neighbours are one yellow (east coast) and two purple (west coast) individuals, in which case the unclassified individual is assigned to the purple group, the west coast. If, however, $K = 6$, then nearest neighbours consist of two purple and four yellow-coloured individuals, in which event the unknown individual is assigned to the yellow group, the east coast.

This method is sensitive to the optimal number of neighbours to be considered when classifying an unknown object. The value of K needs to be estimated empirically, based on the data sets being analysed (Šlenker *et al.* 2022). For class validation, this method uses leave-one-out criteria which is based on building a model using $n-1$ individuals and testing the model to classify the left-out individual(s). This method was tested to quantify the combined diagnostic power of the four morphometric measurements to assign an individual to a given group (i.e.,

population and/or coast). The analysis was repeated on the average measurements per morphometric character per individual (individual-level data set), as well as on the data set that includes combined measurements from all individuals within a population (population-level data set). The aim of the population-level classificatory analysis was merely to show whether the closest morphometric measurements of an individual belongs to the individual from the same population that shows the presence of some level of structure or alternatively, the closest morphometric measurements of an individual show a mixed pattern from individuals located on the east and west coasts.

While there is no recommendation for the minimum number of individuals required for optimal performance of this machine-learning algorithm, it is commonly suggested that these methods perform considerably better when they are trained on a large number of data points (Šlenker *et al.* 2022). Since collecting more specimens in this study was not possible due to a combination of logistic challenges (section 3.4.1), the level of uncertainty in the performance of the K-NN classificatory method that could stem from the comparatively small number of data points that were available for training the method was quantified. Moreover, the number of data points for the east and west coasts of South Africa was slightly unbalanced. Thus, quantifying the effect of this unbalanced data set on the performance of the programme was also necessary. To address this issue, in both individual- and population-level data sets, the east coast, which included a comparatively larger number of data points, was subsampled to the same number of morphometric characters comparable to that of the west coast population (the population with the smallest number of data points). This procedure of subsampling with replacement was repeated for a total of 99 replicates, and the model parameters, as well as the average over 99 replicates, were estimated for each step.

3.2.2.2.2 Bayesian multi-level modelling for assigning individuals to their population of origin

To test whether spine morphometrics could be used to assign individuals to different populations and to evaluate the statistical power of each morphometric character to distinguish between individuals from the east and west coasts of South Africa, a Bayesian multi-level or hierarchical model was built in the brms v.2.16 R package (Bürkner 2017). Briefly, in a Bayesian model, a series of simulations estimate the posterior probability of different model parameters from a set of user-defined values summarised into a prior distribution. As simulations progress, the estimated values are optimised, such that after running simulations

for a large number of iterations, the whole distribution of the parameters of interest can be achieved. It is recommended that multiple independent replicates, also known as a chain, be simulated simultaneously to achieve more accurate estimates. There are several statistics such as $\hat{R}$ (a potential scale reduction statistic, also known as the Gelman-Rubin statistic) that test whether all independent replicates have converged on similar results. It is customary to remove a user-defined number of simulations as a warm-up or ‘burn-in’ since the initial simulations are unlikely to reflect the true parameter estimation.

In the fitted model, the collection site for each individual sea urchin was used as a response variable, and the four spine morphometrics (SL, SW, LL, and LW) were set as predictors. To account for the fact that morphometric measurements from individuals within the same population might be related, a group-level (also known as random effects in some literature; Gillies *et al.* 2006) term was added to the model that considered the presence of such correlation. This model was fitted using a categorical distribution implemented in the same package, and other model parameters were set to their recommended default values. The only exception to this was that the prior distributions on predictors coefficients were set to a normal distribution of $N(0, 8)$. The model was run for 24 chains, each 200 000 interactions with 50 000 initial warm-ups. The convergence and the model performance were evaluated in the same package.

3.3 Results

3.3.1 Test and Aristotle’s lantern metrics

The results of the PERMDISP tests performed post- $\text{Log}(X+1)$ transformation on population and coast within the nested design respectively revealed that population still significantly deviated from the assumption of homogeneity of variance, at $p = 0.001$, but coast did not ($p = 0.225$). All analyses were therefore performed on a resemblance matrix constructed using the $\text{Log}(X+1)$ transformed data. Principal component ordination plots of population and coast showed that the first two axes explained 89.5% of the variance observed in the data set (Figures 3.4 and 3.5, respectively).

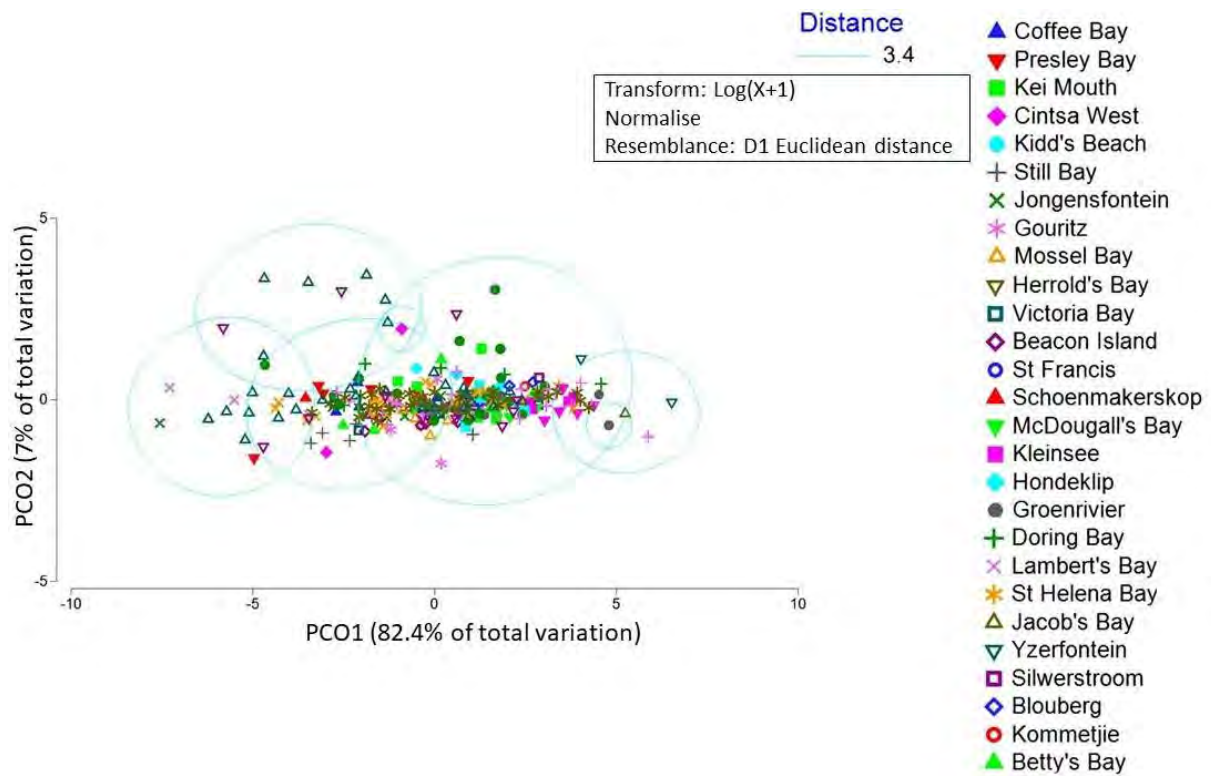


Figure 3.4: A principal component ordination plot of populations (i.e., sites) from which individuals of *Parechinus angulosus* were collected, showing clusters generated at a Euclidean distance of 3.4. Each population is represented using a combination of different colours and symbols (see legend in figure).

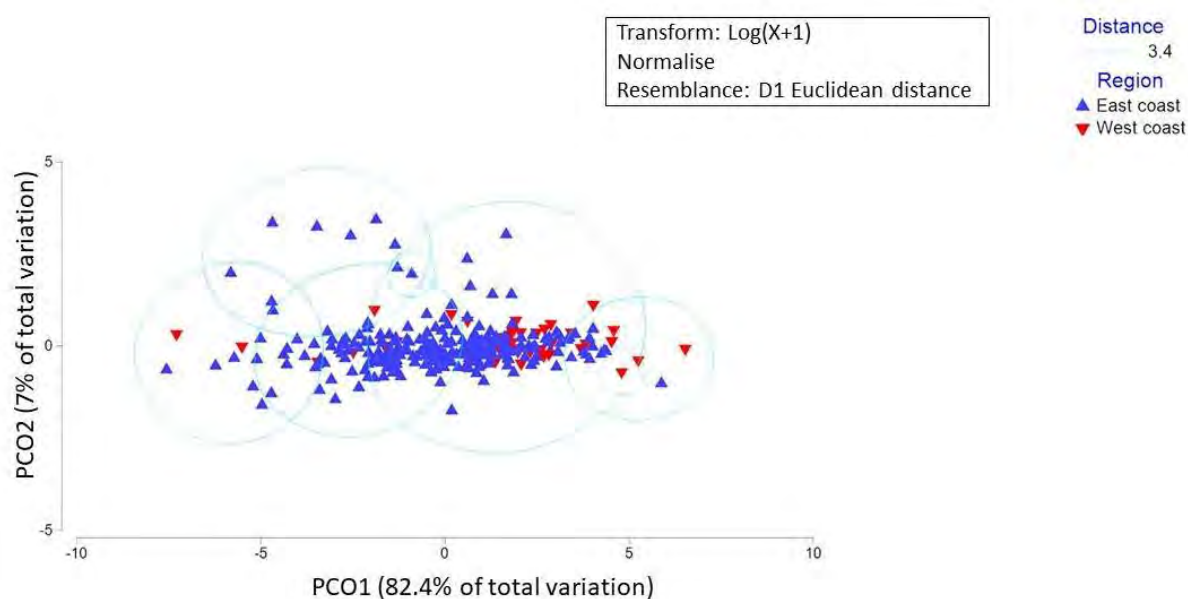


Figure 3.5: A principal component ordination plot constructed using the same data of *Parechinus angulosus* individuals as in Figure 3.4 above, but of the coast factor level, also showing clusters at a Euclidean distance of 3.4. Blue triangles represent individuals from the east coast, red triangles individuals from the west coast.

The PERMANOVA results highlighted a statistically significant difference among populations ($p = 0.001$, unique perms = 999) and between coasts ($p = 0.003$, unique perms = 999; Table 3.2).

Table 3.2: PERMANOVA main test results examining the effects of coast and population (nested in coast) on the morphometric characters of individuals of *Parechinus angulosus* collected at sites along the South African coastline [Degrees of Freedom, Df: Sum of Squares, SS: Mean Squares; Pseudo F-Ratio; P-value (permutation); Monte Carlo P-values].

Pairwise PERMANOVA and SIMPER analyses performed on populations revealed that individuals collected from Arniston were consistently the most dissimilar to all other populations within the east coast ($n = 23$; Table 3.3), whereas Lambert's Bay was found to have the highest dissimilarity when compared to all other populations within the west coast ($n = 8$; Table 3.4). At population level, the character found to contribute the most to the dissimilarity of Arniston with all the other populations on the east coast was lantern weight, ranging in

percentage contribution from 18.11 – 44.98%, and with an average of 27.36%. No conclusive patterns were observed in characters contributing the greatest amount towards the percentage dissimilarity between Lambert's Bay and all other populations within the west coast data set.

Table 3.2: Results of the PERMANOVA pairwise and SIMPER analyses showing the percentage contribution of each morphometric trait collected from individuals of *Parechinus angulosus* between pairs of populations within the east coast.

Groups	t	P(perm)	Unique perms	Variable	Average value	Percentage contribution	Cumulative percentage
Coffee Bay, Arniston	6,7487	0,01	126	Lantern weight (g)	2,06	28,36	28,36
				Test weight (g)	1,33	17,06	45,42
				Test width (mm)	1,47	14,89	60,31
				Lantern width (mm)	1,48	12,4	72,71
Presley Bay, Arniston	4,7297	0,004	570	Lantern weight (g)	2,06	22,68	22,68
				Test width (mm)	1,47	18,73	41,41
				Test weight (g)	1,33	16,17	57,58
				Test height (mm)	0,915	13,21	70,79
Kei Mouth, Arniston	3,641	0,003	565	Lantern weight (g)	2,06	26,54	26,54
				Test weight (g)	1,33	18	44,54
				Test width (mm)	1,47	15,65	60,19
				Perisistomal width (mm)	1,15	13,58	73,77
Cintsa West, Arniston	3,1026	0,044	21	Lantern weight (g)	2,06	23,18	23,18
				Test weight (g)	1,33	20,21	43,39
				Test width (mm)	1,47	14,66	58,05
				Test height (mm)	0,915	13,75	71,8
Still Bay, Arniston	6,0907	0,001	687	Lantern weight (g)	2,06	25,71	25,71
				Test width (mm)	1,47	18,83	44,55
				Test weight (g)	1,33	17,21	61,75

Groups	t	P(perm)	Unique perms	Variable	Average value	Percentage contribution	Cumulative percentage
				Test height (mm)	0,915	12,17	73,92
Jongensfontein, Arniston	4,3892	0,002	787	Lantern weight (g)	2,06	23,02	23,02
				Test width (mm)	1,47	17,22	40,24
				Lantern height (mm)	1,21	14,83	55,07
				Test weight (g)	1,33	14,2	69,28
				Lantern width (mm)	1,48	13,1	82,37
Gouritz, Arniston	3,9367	0,003	406	Lantern weight (g)	2,06	29,85	29,85
				Test width (mm)	1,47	16,48	46,33
				Lantern width (mm)	1,48	16,39	62,72
				Test weight (g)	1,33	15,55	78,27
Mossel Bay, Arniston	6,9537	0,002	412	Lantern weight (g)	2,06	26,37	26,37
				Test width (mm)	1,47	16,34	42,72
				Test weight (g)	1,33	15,94	58,66
				Lantern width (mm)	1,48	13,67	72,33
Herrold's Bay, Arniston	3,1601	0,036	21	Lantern weight (g)	2,06	44,98	44,98
				Test weight (g)	1,33	15,47	60,45
				Lantern width (mm)	1,48	11,41	71,85
Victoria Bay, Arniston	3,3745	0,006	126	Lantern weight (g)	2,06	26,42	26,42
				Test width (mm)	1,47	24,28	50,7
				Test weight (g)	1,33	14,07	64,77
				Lantern width (mm)	1,48	10,8	75,56
Beacon Island, Arniston	6,4898	0,002	787	Lantern weight (g)	2,06	31,85	31,85

Groups	t	P(perm)	Unique perms	Variable	Average value	Percentage contribution	Cumulative percentage
				Test weight (g)	1,33	13,9	45,75
				Test width (mm)	1,47	13,72	59,47
				Lantern width (mm)	1,48	13,23	72,69
St Francis, Arniston	4,6092	0,015	126	Lantern weight (g)	2,06	42,23	42,23
				Test width (mm)	1,47	15,81	58,05
				Test weight (g)	1,33	12,65	70,7
Schoenmakers kop, Arniston	4,3193	0,024	56	Lantern weight (g)	2,06	22,26	22,26
				Lantern width (mm)	1,48	19,03	41,29
				Test width (mm)	1,47	16,17	57,46
				Lantern height (mm)	1,21	13,1	70,56
Betty's Bay, Arniston	3,1603	0,004	404	Lantern weight (g)	2,06	32,89	32,89
				Test width (mm)	1,47	14,21	47,1
				Test weight (g)	1,33	13,46	60,56
				Lantern height (mm)	1,21	13,28	73,84
Arniston, Nature's Valley	4,0057	0,004	407	Lantern weight (g)	0,4	33,88	33,88
				Test width (mm)	0,19	19,78	53,66
				Test weight (g)	0,24	14,19	67,85
				Lantern width (mm)	0,436	13,46	81,31
Arniston, Spinning Reel	4,7327	0,013	56	Lantern weight (g)	0,466	22,53	22,53
				Lantern width (mm)	-0,153	22,14	44,67
				Test height (mm)	-0,494	16,68	61,35
				Lantern height (mm)	0,0493	12,66	74,01
Arniston, Birha	3,0711	0,001	992	Lantern weight (g)	0,111	26,96	26,96

Groups	t	P(perm)	Unique perms	Variable	Average value	Percentage contribution	Cumulative percentage
				Lantern width (mm)	-0,102	19,8	46,76
				Perisistomal width (mm)	-0,143	14,47	61,23
				Lantern height (mm)	0,0524	13,29	74,52
Arniston, Rietrivier	2,4249	0,016	996	Lantern weight (g)	0,483	26,4	26,4
				Lantern width (mm)	0,128	20,79	47,19
				Lantern height (mm)	0,324	13,68	60,87
				Test width (mm)	0,468	13,52	74,39
Arniston, Kleinemonde	3,7754	0,001	996	Lantern weight (g)	0,0057	23,43	23,43
				Lantern width (mm)	-0,201	18,11	41,54
				Test width (mm)	-0,137	15,34	56,87
				Lantern height (mm)	-0,0242	12,19	69,07
				Test weight (g)	-0,0321	11,65	80,71
Arniston, Mtati	3,674	0,001	999	Lantern weight (g)	0,13	26,61	26,61
				Lantern width (mm)	-0,0645	18,37	44,99
				Test width (mm)	0,0556	15,21	60,2
				Lantern height (mm)	0,0527	12,61	72,81
Arniston, Kasouga	4,3097	0,001	995	Lantern weight (g)	-0,747	21,67	21,67
				Perisistomal width (mm)	-1,1	16,98	38,65
				Lantern width (mm)	-0,715	14,59	53,24
				Test width (mm)	-0,711	14,24	67,49
				Lantern height (mm)	-0,694	12,21	79,69
Arniston, Cape Padrone	3,0128	0,007	845	Perisistomal width (mm)	-0,837	20,54	20,54
				Lantern weight (g)	-0,215	19,39	39,93
				Test weight (g)	-0,7	15,91	55,84

Groups	t	P(perm)	Unique perms	Variable	Average value	Percentage contribution	Cumulative percentage
				Test width (mm)	-0,531	15,32	71,16
Coffee Bay, Kei Mouth	2,6288	0,013	578	Lantern weight (g)	0,328	22,33	22,33
				Test height (mm)	0,393	16,59	38,92
				Test width (mm)	0,187	15,79	54,71
				Lantern width (mm)	0,553	14,15	68,86
				Test weight (g)	-0,171	11,23	80,09
Presley Bay, Kei Mouth	2,1526	0,033	753	Test height (mm)	0,393	26,02	26,02
				Test width (mm)	0,187	18,86	44,89
				Lantern weight (g)	0,328	14,25	59,13
				Lantern width (mm)	0,553	11,91	71,04
Kei Mouth, Still Bay	2,6159	0,005	926	Test height (mm)	-0,896	22,2	22,2
				Test width (mm)	-0,845	19,39	41,59
				Lantern weight (g)	-0,685	17,05	58,65
				Test weight (g)	-0,866	11,85	70,5
Kei Mouth, Jongensfontein	1,9944	0,019	958	Lantern height (mm)	-0,698	18,84	18,84
				Test width (mm)	-0,707	17,96	36,81
				Test height (mm)	-0,704	17,05	53,86
				Lantern weight (g)	-0,604	14,13	67,99
				Lantern width (mm)	-0,465	13,68	81,67
Coffee Bay, Gouritz	2,7226	0,016	410	Perisistomal width (mm)	0,652	20,59	20,59
				Lantern weight (g)	0,178	18,67	39,26
				Test height (mm)	0,157	17,17	56,42
				Test weight (g)	-0,0194	15,39	71,82

Groups	t	P(perm)	Unique perms	Variable	Average value	Percentage contribution	Cumulative percentage
Presley Bay, Gouritz	2,0982	0,037	758	Test height (mm)	0,157	26,81	26,81
				Perisistomal width (mm)	0,652	18,4	45,22
				Test width (mm)	0,0804	16,28	61,49
				Test weight (g)	-0,0194	13,65	75,15
Still Bay, Gouritz	2,3893	0,02	855	Test height (mm)	0,157	24,29	24,29
				Test width (mm)	0,0804	18,02	42,32
				Test weight (g)	-0,0194	17,02	59,34
				Lantern weight (g)	0,178	15,31	74,65
Jongensfontein, Gouritz	1,8476	0,042	907	Lantern height (mm)	0,318	19,75	19,75
				Test height (mm)	0,157	17,99	37,74
				Test width (mm)	0,0804	16,24	53,98
				Perisistomal width (mm)	0,652	12,87	66,85
				Test weight (g)	-0,0194	12,78	79,63
Coffee Bay, Mossel Bay	2,5279	0,013	400	Lantern weight (g)	-0,0151	22,63	22,63
				Perisistomal width (mm)	0,299	22,09	44,71
				Test weight (g)	-0,321	14,49	59,2
				Test width (mm)	-0,191	14,04	73,24
Mossel Bay, Herrold's Bay	2,531	0,033	28	Test height (mm)	0,526	34,74	34,74
				Test width (mm)	0,535	19,82	54,56
				Lantern width (mm)	0,564	14,6	69,16
				Lantern height (mm)	0,404	11,55	80,71
Coffee Bay, Victoria Bay	2,071	0,039	126	Lantern height (mm)	0,548	21,38	21,38

Groups	t	P(perm)	Unique perms	Variable	Average value	Percentage contribution	Cumulative percentage
				Lantern weight (g)	0,15	18,54	39,92
				Test weight (g)	-0,0691	14,18	54,1
				Test height (mm)	-0,0316	12,21	66,31
				Lantern width (mm)	0,333	11,82	78,13
Coffee Bay, Beacon Island	2,5894	0,012	793	Perisistomal width (mm)	0,353	20,3	20,3
				Test weight (g)	-0,18	19,07	39,37
				Lantern weight (g)	-0,224	17,33	56,7
				Test width (mm)	-0,0446	16,2	72,9
Kei Mouth, Beacon Island	1,8013	0,031	954	Test height (mm)	-0,498	23,43	23,43
				Lantern weight (g)	-0,224	19,61	43,04
				Perisistomal width (mm)	0,353	14,18	57,22
				Lantern height (mm)	-0,0381	12,68	69,9
				Test width (mm)	-0,0446	11,69	81,59
Still Bay, Beacon Island	1,9836	0,047	977	Test width (mm)	-0,0446	22,87	22,87
				Test weight (g)	-0,18	21,86	44,73
				Lantern weight (g)	-0,224	14,89	59,62
				Test height (mm)	-0,498	14,76	74,38
Coffee Bay, St Francis	3,0942	0,027	126	Test weight (g)	0,151	18,73	18,73
				Test width (mm)	0,193	15,25	33,98
				Test height (mm)	0,233	14,67	48,65
				Perisistomal width (mm)	0,413	14,12	62,77
				Lantern height (mm)	0,564	13,86	76,63
Still Bay, St Francis	2,5157	0,03	425	Test height (mm)	0,233	21,64	21,64

Groups	t	P(perm)	Unique perms	Variable	Average value	Percentage contribution	Cumulative percentage
				Test width (mm)	0,193	20,52	42,15
				Test weight (g)	0,151	19,21	61,36
				Lantern height (mm)	0,564	17,34	78,71
Mossel Bay, St Francis	2,2919	0,006	208	Test height (mm)	0,233	27,55	27,55
				Lantern height (mm)	0,564	26	53,55
				Test width (mm)	0,193	13,43	66,98
				Test weight (g)	0,151	11,1	78,08
Coffee Bay, Nature's Valley	3,8386	0,003	404	Lantern weight (g)	0,4	19,96	19,96
				Test weight (g)	0,24	16,75	36,7
				Test height (mm)	0,469	16,29	53
				Perisistomal width (mm)	0,56	14,2	67,19
				Test width (mm)	0,19	11,85	79,04
Presley Bay, Nature's Valley	2,6805	0,017	760	Test height (mm)	0,469	25,49	25,49
				Test width (mm)	0,19	15,68	41,16
				Test weight (g)	0,24	14,94	56,1
				Perisistomal width (mm)	0,56	13,67	69,77
				Lantern weight (g)	0,4	12,88	82,65
Still Bay, Nature's Valley	3,3298	0,009	855	Test height (mm)	0,469	23,39	23,39
				Test weight (g)	0,24	17,49	40,88
				Test width (mm)	0,19	16,32	57,21
				Lantern weight (g)	0,4	16,06	73,27
Jongensfontein, Nature's Valley	2,4234	0,006	918	Lantern height (mm)	0,578	22	22
				Test height (mm)	0,469	17,39	39,39

Groups	t	P(perm)	Unique perms	Variable	Average value	Percentage contribution	Cumulative percentage
				Test width (mm)	0,19	15,07	54,47
				Test weight (g)	0,24	13	67,47
				Lantern weight (g)	0,4	12,92	80,39
Mossel Bay, Nature's Valley	2,6352	0,009	410	Test height (mm)	0,469	30,95	30,95
				Lantern height (mm)	0,578	19,03	49,98
				Lantern weight (g)	0,4	12,61	62,6
				Test weight (g)	0,24	11,92	74,52
Beacon Island, Nature's Valley	2,5316	0,017	904	Test height (mm)	0,469	31,85	31,85
				Lantern weight (g)	0,4	21,1	52,95
				Lantern height (mm)	0,578	15,46	68,42
				Test weight (g)	0,24	10,54	78,96
Schoenmakers kop, Nature's Valley	2,2567	0,035	84	Lantern width (mm)	0,436	21,35	21,35
				Lantern height (mm)	0,578	19,74	41,09
				Test height (mm)	0,469	17,36	58,45
				Perisistomal width (mm)	0,56	12,36	70,81
Coffee Bay, Spinning Reel	2,8063	0,016	56	Test weight (g)	0,473	30,64	30,64
				Lantern weight (g)	0,466	28,33	58,97
				Test width (mm)	0,305	17,42	76,39
Still Bay, Spinning Reel	2,1444	0,044	164	Test weight (g)	0,473	31,3	31,3
				Test width (mm)	0,305	24,38	55,68
				Lantern weight (g)	0,466	22,23	77,91

Groups	t	P(perm)	Unique perms	Variable	Average value	Percentage contribution	Cumulative percentage
Mossel Bay, Spinning Reel	2,225	0,011	84	Test weight (g)	0,473	36,91	36,91
				Lantern weight (g)	0,466	17,99	54,9
				Test width (mm)	0,305	16,16	71,06
St Francis, Spinning Reel	2,2527	0,049	35	Test height (mm)	-0,494	28,85	28,85
				Lantern weight (g)	0,466	19,59	48,44
				Lantern height (mm)	0,0493	18,4	66,84
				Lantern width (mm)	-0,153	15,2	82,03
Nature's Valley, Spinning Reel	1,9235	0,036	84	Test height (mm)	-0,494	38,67	38,67
				Lantern height (mm)	0,0493	16,16	54,83
				Lantern width (mm)	-0,153	14,29	69,12
				Lantern weight (g)	0,466	9,07	78,19
Coffee Bay, Birha	2,1106	0,023	983	Test weight (g)	0,365	21,58	21,58
				Lantern weight (g)	0,111	17,48	39,06
				Test width (mm)	0,279	14,97	54,03
				Test height (mm)	0,353	14,91	68,94
				Perisistomal width (mm)	-0,143	10,84	79,77
Presley Bay, Birha	2,1978	0,024	999	Test height (mm)	0,353	22,78	22,78
				Test weight (g)	0,365	18,49	41,27
				Test width (mm)	0,279	17,81	59,07
				Lantern weight (g)	0,111	12,29	71,36
Still Bay, Birha	2,4377	0,007	999	Test weight (g)	0,365	20,17	20,17
				Test height (mm)	0,353	18,76	38,93
				Test width (mm)	0,279	17,61	56,53

Groups	t	P(perm)	Unique perms	Variable	Average value	Percentage contribution	Cumulative percentage
				Lantern weight (g)	0,111	13,88	70,42
Jongensfontein, Birha	2,1048	0,024	998	Lantern height (mm)	0,0524	17,37	17,37
				Test width (mm)	0,279	17,12	34,49
				Test weight (g)	0,365	16,49	50,98
				Test height (mm)	0,353	15,71	66,69
				Lantern weight (g)	0,111	12,64	79,33
Coffee Bay, Rietrivier	2,6548	0,009	994	Test weight (g)	0,59	21,07	21,07
				Lantern weight (g)	0,483	19,97	41,04
				Test width (mm)	0,468	15,52	56,57
				Test height (mm)	0,326	12,95	69,51
				Perisistomal width (mm)	0,425	11,1	80,61
Presley Bay, Rietrivier	2,8062	0,005	999	Test height (mm)	0,326	19,92	19,92
				Test weight (g)	0,59	18,67	38,59
				Test width (mm)	0,468	18,62	57,21
				Lantern weight (g)	0,483	14,3	71,51
Still Bay, Rietrivier	2,9405	0,007	998	Test weight (g)	0,59	20,89	20,89
				Test width (mm)	0,468	19,24	40,13
				Test height (mm)	0,326	17,07	57,21
				Lantern weight (g)	0,483	16,8	74
Jongensfontein, Rietrivier	2,756	0,006	999	Test width (mm)	0,468	17,93	17,93
				Lantern height (mm)	0,324	17,51	35,44
				Test weight (g)	0,59	16,72	52,16
				Lantern weight (g)	0,483	14,71	66,87

Groups	t	P(perm)	Unique perms	Variable	Average value	Percentage contribution	Cumulative percentage
				Test height (mm)	0,326	14,38	81,25
Rietrivier, Kleinemonde	1,9939	0,049	998	Lantern width (mm)	-0,201	16,69	16,69
				Lantern height (mm)	-0,0242	15,75	32,44
				Test height (mm)	-0,284	15,43	47,87
				Test width (mm)	-0,137	14,91	62,78
				Lantern weight (g)	0,0057	14,56	77,34
Coffee Bay, Mtati	2,2569	0,017	999	Test weight (g)	0,175	20,95	20,95
				Lantern weight (g)	0,13	19,96	40,91
				Test width (mm)	0,0556	14,9	55,81
				Test height (mm)	0,0542	13,8	69,61
				Lantern width (mm)	-0,0645	10,5	80,11
Presley Bay, Mtati	2,3982	0,011	999	Test height (mm)	0,0542	23,25	23,25
				Test weight (g)	0,175	17,89	41,14
				Test width (mm)	0,0556	17,33	58,47
				Lantern weight (g)	0,13	13,43	71,9
Still Bay, Mtati	2,4547	0,021	998	Test weight (g)	0,175	20,85	20,85
				Test height (mm)	0,0542	18,28	39,12
				Test width (mm)	0,0556	18,23	57,35
				Lantern weight (g)	0,13	16,27	73,62
Jongensfontein, Mtati	2,2692	0,022	999	Lantern height (mm)	0,0527	18,83	18,83
				Test width (mm)	0,0556	17,39	36,22
				Test weight (g)	0,175	16,32	52,54
				Test height (mm)	0,0542	15,02	67,56
				Lantern weight (g)	0,13	13,84	81,39

Groups	t	P(perm)	Unique perms	Variable	Average value	Percentage contribution	Cumulative percentage
Kei Mouth, Kasouga	2,2554	0,017	998	Perisistomal width (mm)	-1,1	21,35	21,35
				Lantern width (mm)	-0,715	16,61	37,95
				Lantern height (mm)	-0,694	14,04	51,99
				Lantern weight (g)	-0,747	13,96	65,95
				Test width (mm)	-0,711	13,41	79,37
Gouritz, Kasouga	2,191	0,016	999	Perisistomal width (mm)	-1,1	30,65	30,65
				Lantern height (mm)	-0,694	13,68	44,33
				Test height (mm)	-0,56	12,8	57,14
				Lantern weight (g)	-0,747	11,52	68,66
				Test width (mm)	-0,711	11,25	79,91
Victoria Bay, Kasouga	1,86	0,045	999	Perisistomal width (mm)	-1,1	23,41	23,41
				Lantern height (mm)	-0,694	21,9	45,31
				Lantern width (mm)	-0,715	14,21	59,51
				Lantern weight (g)	-0,747	11,7	71,22
Beacon Island, Kasouga	2,0378	0,023	999	Perisistomal width (mm)	-1,1	31,39	31,39
				Lantern width (mm)	-0,715	13,39	44,78
				Lantern height (mm)	-0,694	13,22	58
				Test width (mm)	-0,711	12,04	70,04
St Francis, Kasouga	1,8805	0,037	988	Perisistomal width (mm)	-1,1	26,21	26,21
				Lantern height (mm)	-0,694	17,82	44,03
				Lantern width (mm)	-0,715	14,23	58,26
				Test width (mm)	-0,711	12,38	70,63

Groups	t	P(perm)	Unique perms	Variable	Average value	Percentage contribution	Cumulative percentage
Betty's Bay, Kasouga	1,9341	0,045	998	Perisistomal width (mm)	-1,1	24,37	24,37
				Test height (mm)	-0,56	15,04	39,4
				Lantern width (mm)	-0,715	14,98	54,39
				Test width (mm)	-0,711	13,49	67,88
				Lantern height (mm)	-0,694	12,07	79,95
Nature's Valley, Kasouga	2,5852	0,008	997	Perisistomal width (mm)	-1,1	25,5	25,5
				Lantern height (mm)	-0,694	15,82	41,32
				Lantern width (mm)	-0,715	13,08	54,4
				Lantern weight (g)	-0,747	12,52	66,92
				Test height (mm)	-0,56	12,32	79,24
Birha, Kasouga	2,9588	0,003	997	Perisistomal width (mm)	-1,1	20,25	20,25
				Lantern height (mm)	-0,694	13,86	34,11
				Test weight (g)	-0,688	13,85	47,97
				Test width (mm)	-0,711	13,7	61,66
				Lantern width (mm)	-0,715	12,94	74,61
Rietrivier, Kasouga	4,1654	0,001	999	Perisistomal width (mm)	-1,1	20,96	20,96
				Lantern weight (g)	-0,747	14,27	35,23
				Test width (mm)	-0,711	14,07	49,31
				Test weight (g)	-0,688	13,73	63,03
				Lantern height (mm)	-0,694	13,7	76,73
Kleinemonde, Kasouga	2,5244	0,008	999	Perisistomal width (mm)	-1,1	21,14	21,14
				Lantern height (mm)	-0,694	15,78	36,92
				Lantern width (mm)	-0,715	14,37	51,28
				Test width (mm)	-0,711	12,64	63,93

Groups	t	P(perm)	Unique perms	Variable	Average value	Percentage contribution	Cumulative percentage
				Lantern weight (g)	-0,747	12,29	76,21
Mtati, Kasouga	3,8682	0,001	999	Perisistomal width (mm)	-1,1	21,94	21,94
				Lantern height (mm)	-0,694	14,07	36,01
				Lantern weight (g)	-0,747	13,41	49,43
				Test width (mm)	-0,711	13,01	62,44
				Lantern width (mm)	-0,715	13,01	75,44
Rietrivier, Cape Padrone	2,235	0,022	997	Perisistomal width (mm)	-0,837	24,58	24,58
				Test weight (g)	-0,7	16,73	41,3
				Test width (mm)	-0,531	13,97	55,28
				Lantern weight (g)	-0,215	12,52	67,8
				Lantern height (mm)	-0,101	10,98	78,77
Mtati, Cape Padrone	1,8181	0,047	999	Perisistomal width (mm)	-0,837	25,74	25,74
				Test weight (g)	-0,7	15,49	41,23
				Test width (mm)	-0,531	12,62	53,85
				Lantern weight (g)	-0,215	12,26	66,1
				Lantern height (mm)	-0,101	11,44	77,55

Table 3.3: PERMANOVA and SIMPER analyses results showing the percentage contribution of each morphometric trait collected from individuals of *Parechinus angulosus* to the difference between pairs of populations within the west coast.

Groups	t	P(perm)	Unique perms	Variable	Average value	Percentage contribution	Cumulative percentage
McDougall's Bay, Hondeklip	1,9	0,017	56	Test weight (g)	0,653	29,84	29,84
				Test width (mm)	0,555	24,07	53,92
				Lantern weight (g)	0,452	15,76	69,68
				Perisistomal width (mm)	0,445	9,99	79,67
Kleinsee, Hondeklip	2,83	0,023	126	Test weight (g)	0,653	20,72	20,72
				Lantern weight (g)	0,452	20,03	40,75
				Lantern width (mm)	0,759	16	56,75
				Lantern height (mm)	0,513	15,03	71,78
Kleinsee, Lambert's Bay	7,548	0,033	35	Lantern height (mm)	-2,63	18,77	18,77
				Test weight (g)	-2,05	16,42	35,19
				Lantern width (mm)	-2,02	15,96	51,15
				Lantern weight (g)	-2,09	13,9	65,05
				Test width (mm)	-1,85	12,74	77,79

Groups	t	P(perm)	Unique perms	Variable	Average value	Percentage contribution	Cumulative percentage
Hondeklip, Lambert's Bay	6,75	0,014	56	Lantern height (mm)	-2,63	20,25	20,25
				Test height (mm)	-1,99	16,42	36,67
				Lantern width (mm)	-2,02	15,32	51,99
				Test weight (g)	-2,05	14,22	66,21
				Lantern weight (g)	-2,09	12,54	78,74
Groenrivier, Lambert's Bay	4,995	0,012	84	Lantern width (mm)	-2,02	19,52	19,52
				Lantern height (mm)	-2,63	16,88	36,4
				Lantern weight (g)	-2,09	13,71	50,11
				Test width (mm)	-1,85	13,64	63,75
				Test weight (g)	-2,05	12,82	76,57
Doring Bay, Lambert's Bay	3,183	0,011	84	Test height (mm)	-1,99	20,24	20,24
				Lantern height (mm)	-2,63	20,21	40,45
				Test weight (g)	-2,05	13,81	54,26
				Test width (mm)	-1,85	13,34	67,6
				Lantern width (mm)	-2,02	12,48	80,08
Lambert's Bay, St Helena Bay	3,255	0,03	35	Lantern height (mm)	0,00718	20,97	20,97
				Test height (mm)	0,361	16,1	37,07

Groups	t	P(perm)	Unique perms	Variable	Average value	Percentage contribution	Cumulative percentage
				Test weight (g)	0,218	15,33	52,4
				Test width (mm)	0,236	13,43	65,83
				Lantern width (mm)	0,0648	12,99	78,83
Hondeklip, Yzerfontein	3,554	0,049	21	Test weight (g)	2,48	21,92	21,92
				Lantern weight (g)	2,04	21,7	43,61
				Test width (mm)	2,31	18,99	62,61
				Test height (mm)	2,27	10,67	73,28

Additionally, pairwise PERMANOVA and SIMPER analyses that were performed to assess differences between the coasts revealed that individuals collected from the east coast were significantly different ($p < 0.05$) to those collected from the west coast in terms of test and lantern characters. This was followed by SIMPER analysis which revealed that the characters found to contribute the greatest amount towards the dissimilarity between the two coasts were lantern width, followed by test width, weight, and height and lantern height (Table 3.5).

Table 3.4: Results from SIMPER analyses results indicating which morphometric traits collected from individuals of *Parechinus angulosus* contributed towards dissimilarity observed between the coasts, and the percentage contribution of each trait towards the dissimilarity.

Variable	Average value east coast	Average value west coast	Percentage contribution	Cumulative percentage
Lantern width (mm)	-0,103	0,637	15,64	15,64
Test width (mm)	-0,115	0,71	15,15	30,79
Test weight (g)	-0,095	0,585	15,01	45,8
Test height (mm)	-0,116	0,719	14,67	60,47
Lantern height (mm)	-0,040	0,249	14,02	74,49

The globosity, volumetry and density indices were examined for significant differences among individuals collected from the east and individuals from the west coasts using the non-parametric Mann-Whitney U (Wilcoxon Rank Sum) test since this test does not require that the underlying data be normally distributed, nor that variances around the means being tested be equal, and it is the appropriate statistic for unbalanced data sets (Mann and Whitney 1947;). All three indices (globosity, volumetry and density) were found to have significant differences for individuals collected from the east and west coasts (Figure 3.6).

Table 3.5: Select summary of the Mann-Whitney tests for the globosity, volumetry and density indices calculated using test and lantern metric data collected from individuals of *Parechinus angulosus* from the east and west coasts of South Africa.

Index	Coast	Globosity Index (GI)	Volumetry Index (VI)	Density Index (DI)
Median	East	0,531	344,54	0,565
	West	0,556	531	0,738
p-value		0,026	1,66E-07	0,002

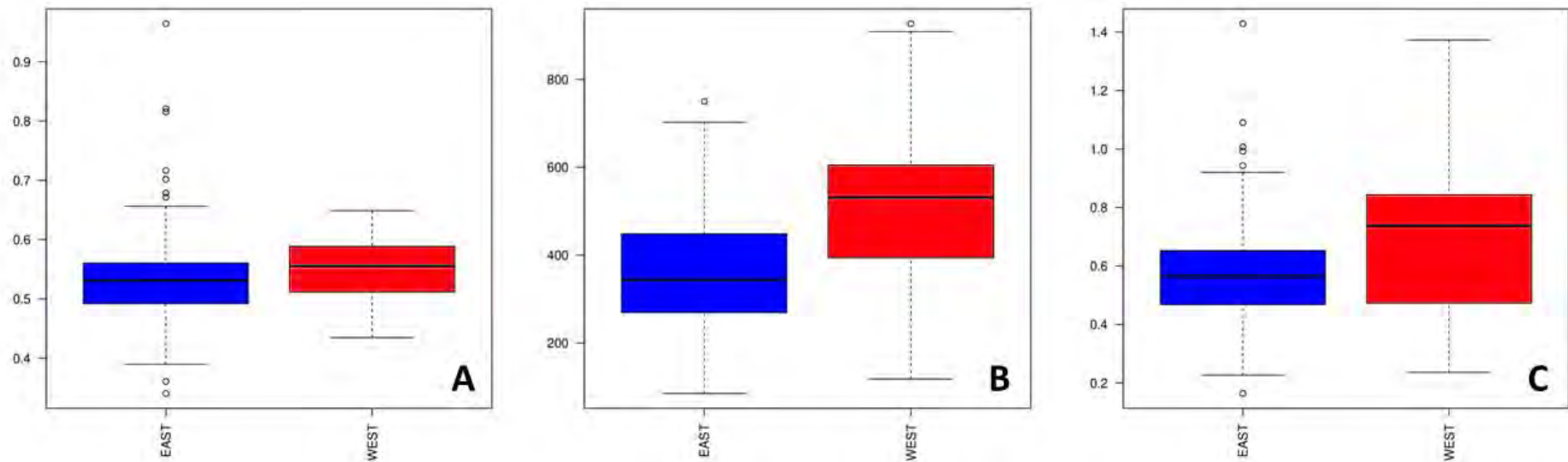


Figure 3.6: Boxplots of the three indices calculated for the sea urchin, *Parechinus angulosus* compared for the east and west coasts of South Africa: (A) Globosity index; (B) Volumetry index; (C) Density index. The horizontal line inside the box represents the median value, and the box spans from the 25th percentile to the 75th percentile, while the vertical lines extend to the 5th and 95th percentiles. Note different scales on Y-axes.

The medians of the populations for each of the indices were considered as opposed to the means because the data were not normally distributed. The median globosity index for the west coast (0.556) was significantly higher than that of the east (GI = 0.5312; p-value = 0.025). Similarly, for the volumetry index, the median for the west coast (531) was significantly higher than for the east coast (344.54; p-value = 1,66E-07), as well as for the density index, with the median for the west coast at 0.7378 being significantly higher than that of the east coast (0.5645; p-value = 0.001).

3.3.2 Spine metrics

3.3.2.1 Classificatory discriminant analysis

3.3.2.1.1 Individual-level data set

Taking the average from each of the spine morphometric characters resulted in four morphometric measurements: small spine length (SL), small spine width (SW), large spine length (LL), and large spine width (LW) for each of the 46 individuals. The average of SL and SW for the east coast population was 1261.36 and 213.47 μm respectively. Similarly, the average SL and SW for the west coast population was 1396.3 and 228.7 μm . For the east coast population, LL and LW were estimated at 8240.68 and 869.15 μm , and those of the west coast were estimated at 7164.89 and 815.42 μm , respectively (Table 3.7, Figure 3.7).

Table 3.6: Mean ($\pm$ SD) of the four spine character measurements (SL, SW, LL, LW) taken from individuals of *Parechinus angulosus* at individual level (unit = μm).

	East Coast (n = 34) Mean $\pm$ SD	West Coast (n = 12) Mean $\pm$ SD
Small spine length	1261.36 $\pm$ 168.28	1396.3 $\pm$ 154.25
Small spine width	213.47 $\pm$ 17.27	228.37 $\pm$ 21.8
Large spine length	8240.68 $\pm$ 1479.61	7164.89 $\pm$ 905.84
Large spine width	869.15 $\pm$ 119.98	815.42 $\pm$ 103.12

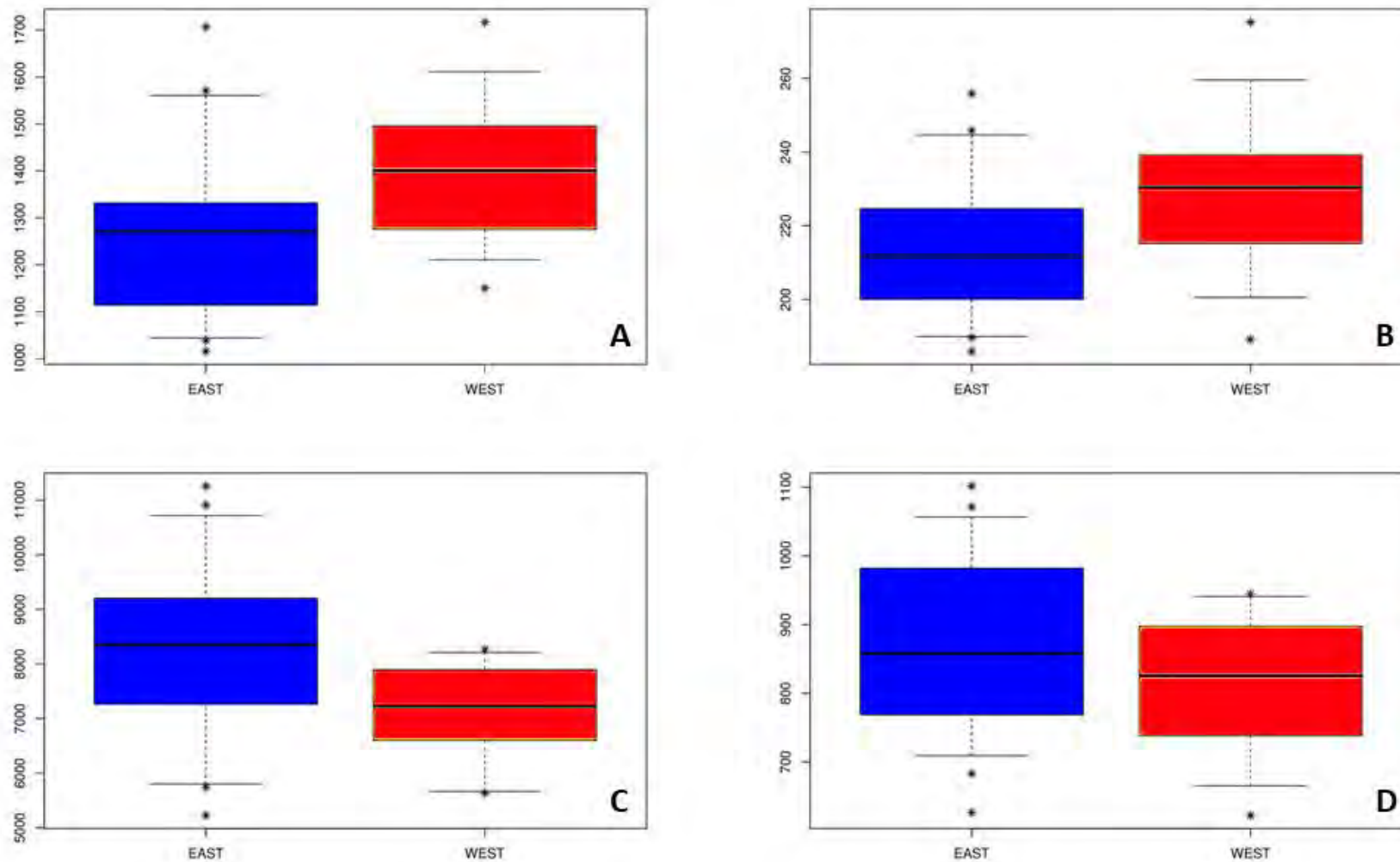


Figure 3.7: Boxplots of the four characters measured in the sea urchin, *Parechinus angulosus*: (A) Small spine length (SL); (B) Small spine width (SW); (C) Large spine length (LL); (D) Large spine width (LW). The horizontal line inside the box represents the median value, and the box spans from the 25th percentile to the 75th percentile, while the vertical lines extend to the 5th and 95th percentiles.

The Spearman correlation analysis shows none of the characters were correlated with a coefficient larger than 0.95 that the author considers the threshold for reconsidering incorporation of those variables in the analysis (Šlenker *et al.* 2022; Table 3.8), all metrics were considered for the subsequent analyses as the requirement of non-correlation between any two measures was met.

Table 3.7: Spearman's correlation coefficient calculated for each of the characters (SL, SW, LL, LW) measured from spines collected from *Parechinus angulosus* individuals, at individual level.

Character	Short spine length	Short spine width	Large spine length	Large spine width
Short spine length	N/A	0.813	0.019	-0.014
Short spine width	0.813	N/A	0.260	0.258
Large spine length	0.019	0.260	N/A	0.903
Large spine width	-0.014	0.258	0.903	N/A

Stepwise discriminant analysis confirmed that all characters could be retained for down-stream classification analysis because no single character was identified as a linear combination of the other characters. Neither principal component analysis (Figure 3.8), nor principal coordinate analysis (Figure 3.9) showed a clear pattern that could conclusively separate individuals from different coasts. Rather, the west coast individuals were dispersed within the bigger east coast data set.

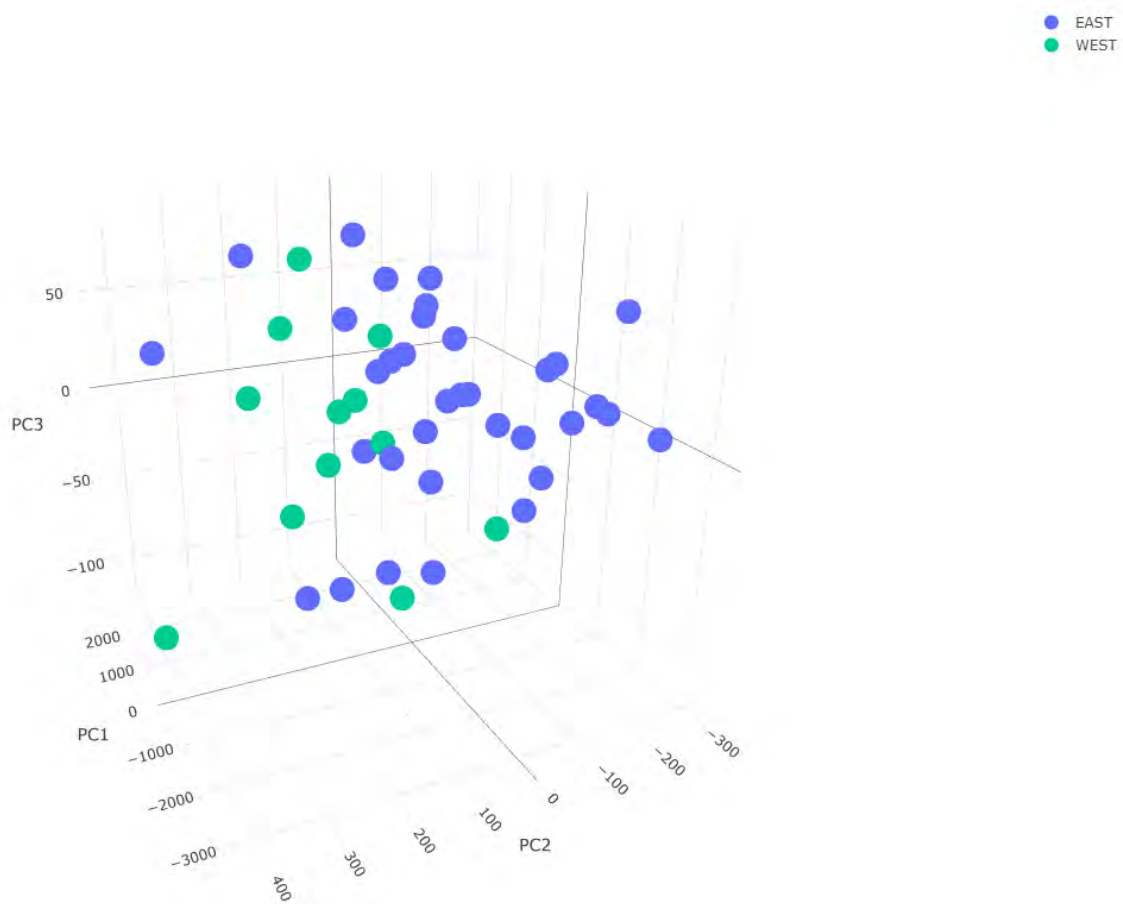


Figure 3.8: Principal component analysis of the average values of each of the four measures collected from 46 individuals (SL, SW, LL, LW). A total of 99% of the data's variance is captured between the three axes.

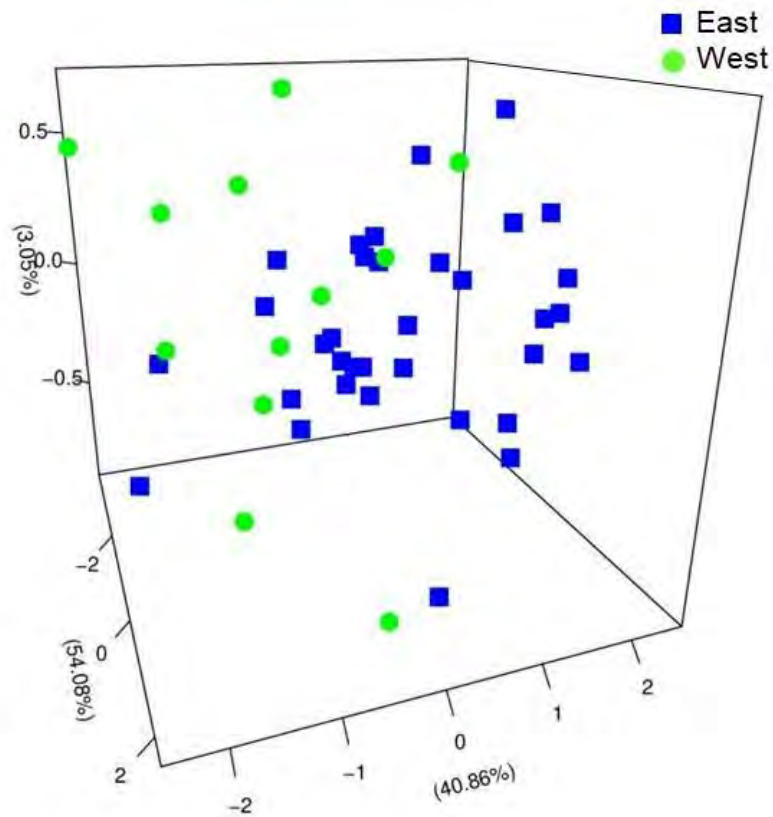


Figure 3.9: Principal coordinate analysis of the average of the four metrics collected from 46 individuals (SL, SW, LL, LW). The axes capture 97.99% of the variance of the data.

Since the smaller of the two data sets represented 12 individuals collected from west coast, compared to 34 individuals from the east coast, $k = 12$ closest neighbour of each individual was used to assign individuals to an a priori-defined number of groups for the K-NN classificatory discriminant analysis. Based on this, 69.57% of individuals were correctly assigned to their population of origin (Table 3.9).

Table 3.8: An error matrix (confusion matrix) generated using mean values from four measurements (SL, SW, LL, LW) of 46 *Parechinus angulosus* individuals, representing the 16 subset populations, the two coasts, the total number of individuals per population, the number of individuals assigned to the east and west coasts, the number of individuals correctly assigned per coast, and the analysis' overall success rate (69.57%).

Population	Coast	Number of individuals in population	Number of individuals assigned to the east coast population	Number of individuals assigned to the west coast population	Number of correctly assigned individuals	Percentage correctly assigned individuals
Arniston	East	2	2	0	2	100
Port St John's	East	3	3	0	3	100
Buffels Bay	East	2	2	0	2	100
Coffee Bay	East	1	1	0	1	100
Jongensfontein	East	3	3	0	3	100
Kei Mouth	East	3	3	0	3	100
Kenton	East	3	3	0	3	100
Kidd's Beach	East	1	1	0	1	100
Presley Bay	East	4	4	0	4	100
St Francis	East	3	3	0	3	100
Cannon Rocks	East	6	5	1	5	83.33
Schoenmakerskop	East	3	2	1	2	66.67
Blouberg	West	2	2	0	0	0
Kleinsee	West	4	4	0	0	0
Kommetjie	West	3	3	0	0	0
St Helena	West	3	3	0	0	0
Total individuals from east coast	34					
Total individuals from west coast	12					
Total		46	44	2	32	69.57

Since two of the populations, Kidd's Beach and Coffee Bay, were represented by only one individual each, which is unsuitable for optimal classificatory analysis, these populations were removed from the data set and the K-NN analysis was repeated. After removing those populations, the overall performance dropped to 65.91% (Table 3.10).

Table 3.9: An error matrix (confusion matrix) generated using mean values from four measurements (SL, SW, LL, LW) of 44 *Parechinus angulosus* individuals from 14 populations, indicating a 65.91% success rate in the assignment of individuals to the coast from which they were collected.

Population	Coast	Number of individuals in population	Number of individuals assigned to the east coast population	Number of individuals assigned to the west coast population	Number of correctly assigned individuals	Percentage correctly assigned individuals
Arniston	East	2	2	0	2	100
Buffels Bay	East	2	2	0	2	100
St Francis	East	3	3	0	3	100
Jongensfontein	East	3	3	0	3	100
Kei Mouth	East	3	3	0	3	100
Kenton	East	3	3	0	3	100
Port St John's	East	3	3	0	3	100
Cannon Rocks	East	6	5	1	5	83.33
Presley Bay	East	4	3	1	3	75
Schoenmakerskop	East	3	2	1	2	66.67
Blouberg	West	2	2	0	0	0
Kleinsee	West	4	4	0	0	0
Kommetjie	West	3	3	0	0	0
St Helena	West	3	3	0	0	0
Total		44	41	3	29	65.91

To assess the accuracy of the K-NN classificatory analysis and to correctly assign individuals to the location of origin, the two removed individuals, each representing one population, were used as a query (type specimen). This method takes an individual of known origin and presents it to the algorithm as unclassified and needing to be assigned. Thereby the success of the assignment of individuals to their coast of origin can be evaluated. Using this method, K-NN classificatory analysis correctly assigned the removed/type specimen individuals from Kidd's Beach and Coffee Bay to their population of origin on the east coast, thereby demonstrating the ability and success of this classificatory method.

In general, this classification method had a significantly higher success rate on data collected from the east coast of South Africa compared to samples collected from the west coast. These

results were not surprising, because all machine-learning algorithms perform significantly better when they are trained on a large number of data points. Generally, a smaller data set reduces the reliability and robustness of the statistical results, which in the case of K-NN, results in the algorithm being unable to conclusively delineate the boundaries of the classes (coasts). This algorithm can perform reasonably well if trained on a data set that is clearly separated and accurately represents the underlying data distribution. Too few samples per class, however, even with well-separated data, leads to comparatively lower performance and can cause overfitting of the model. To quantify the impact of the unbalanced sample size on the performance of the algorithm, when the east coast population was subsampled to a number of individuals comparable to that of the west coast ($n = 12$), predictably the average success rate over 99 independent replicates for the east coast was 56.89%, a lower percentage compared to that successfully assigned from full data set. In the reduced data set, however, 44.75% of individuals from the west coast were correctly assigned to their population of origin.

3.3.2.1.2 Population-level data set

A total of 300 spine measurements for each character of the 46 individuals was generated, namely small length (SL), small width (SW), large length (LL), and large width (LW). As explained above, in section 1.3.2 ‘Spine metrics’, measures with missing or inconclusive data points were removed, leaving 13609 data points for analysis. The mean values for each of the measures remained the same as for the individual-level data set (please see above; Table 3.11, Figure 3.10). Nevertheless, the boxplots show that data points in the population-level data set have comparatively and expectedly higher variance around the mean.

Table 3.10: Mean ($\pm$ SD) of the four spine characters (SL, SW, LL, LW) measurements collected from *Parechinus angulosus* individuals, at population level (unit = μm).

	East Coast (n = 10 058) Mean $\pm$ SD	West Coast (n = 3 551) Mean $\pm$ SD
Small spine length	1261.72 $\pm$ 425.04	1396.26 $\pm$ 418.37
Small spine width	213.45 $\pm$ 57.84	228.45 $\pm$ 53.04
Large spine length	8240.94 $\pm$ 2688.67	7162.11 $\pm$ 1829
Large spine width	869.39 $\pm$ 266.96	815.21 $\pm$ 228.59

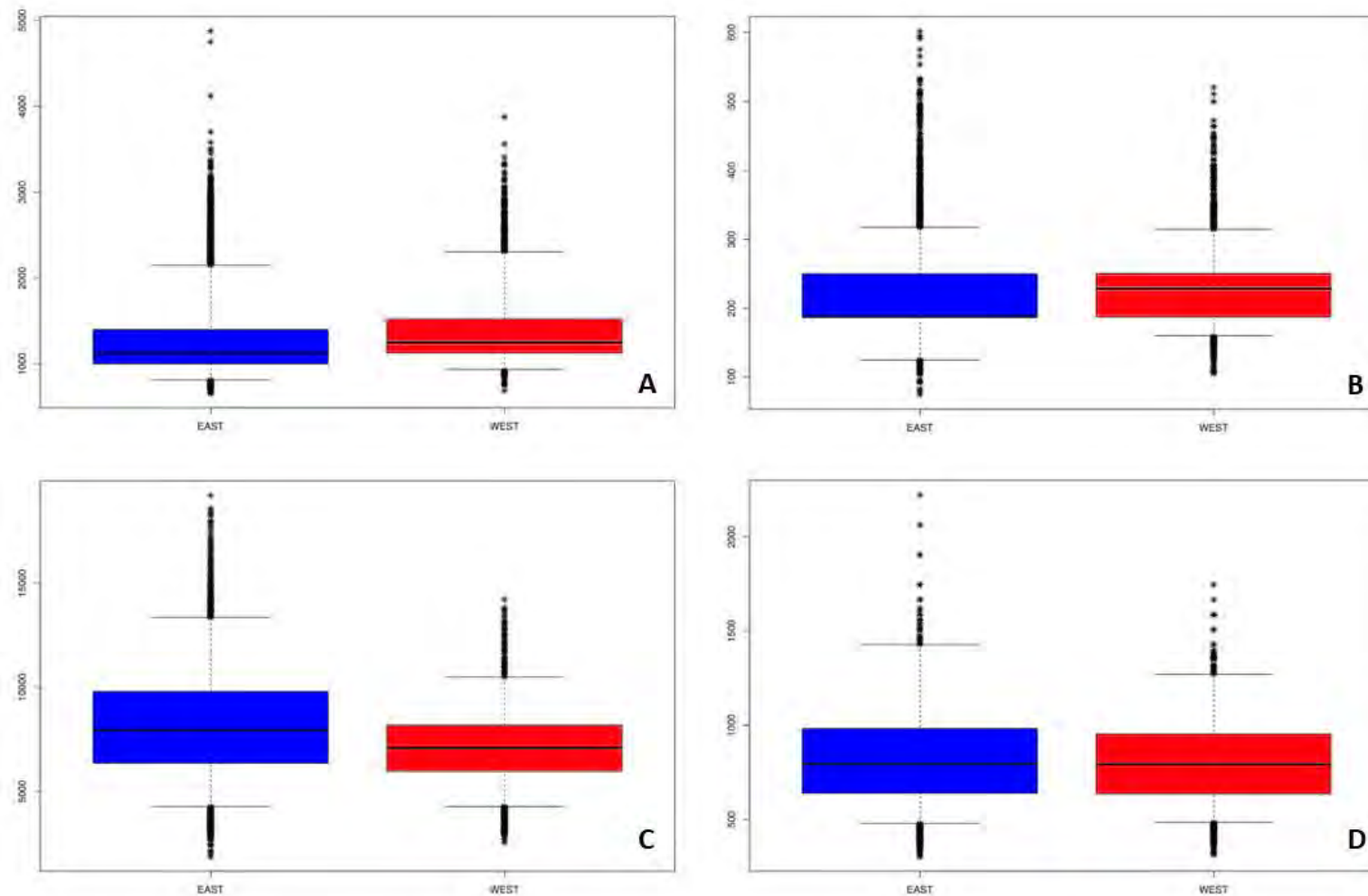


Figure 3.10: Boxplots displaying the four measured characteristics of the sea urchin *Parechinus angulosus*: (A) Small spine length (SL); (B) Small spine width (SW); (C) Large spine length (LL); (D) Large spine width (LW). The median value is represented by the horizontal line in the box, which extends from the 25th to the 75th percentiles. The vertical lines span the distance from the 5th to the 95th percentiles.

The Spearman correlation analysis did not reveal correlation between any characters ($r < 0.95$; Šlenker *et al.* 2022; Table 3.12), and therefore no characters were omitted from the analysis as the requirement of non-correlation between any two characters was met.

Table 3.11: Spearman's correlation coefficient computed for each of the characters measured (SL, SW, LL, LW) at population level.

Character	Short spine length	Short spine width	Large spine length	Large spine width
Short spine length	N/A	0.694	-0.161	-0.159
Short spine width	0.694	N/A	0.02	0.012
Large spine length	-0.161	0.02	N/A	0.857
Large spine width	-0.159	0.012	0.857	N/A

Similarly, stepwise discriminant analysis confirmed that all characters could be retained for subsequent classification analysis, as no single character was identified as a linear combination of other characters. Both principal component analysis (Figure 3.11) and principal coordinate analysis (Figure 3.12) did not reveal distinct patterns that could clearly identify individuals as belonging to either the east or west coast. Similar to the individual-level data set, population-level data from the west coast were dispersed within the larger eastern coast data set.

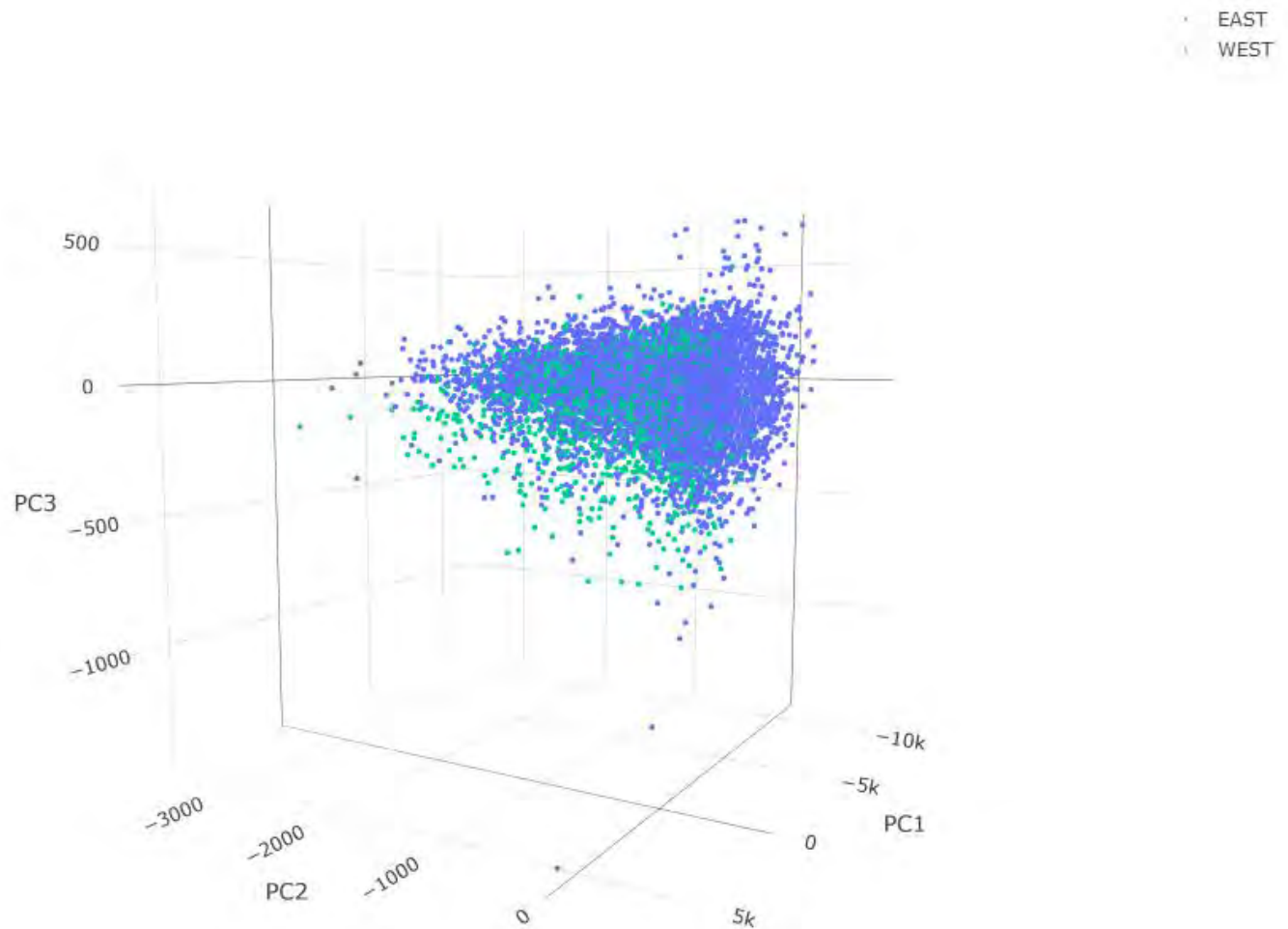


Figure 3.11: Principal component analysis of the mean values of the four measures taken from 46 *Parechinus angulosus* individuals (SL, SW, LL, LW), capturing 99% of the data's variance between the three axes.

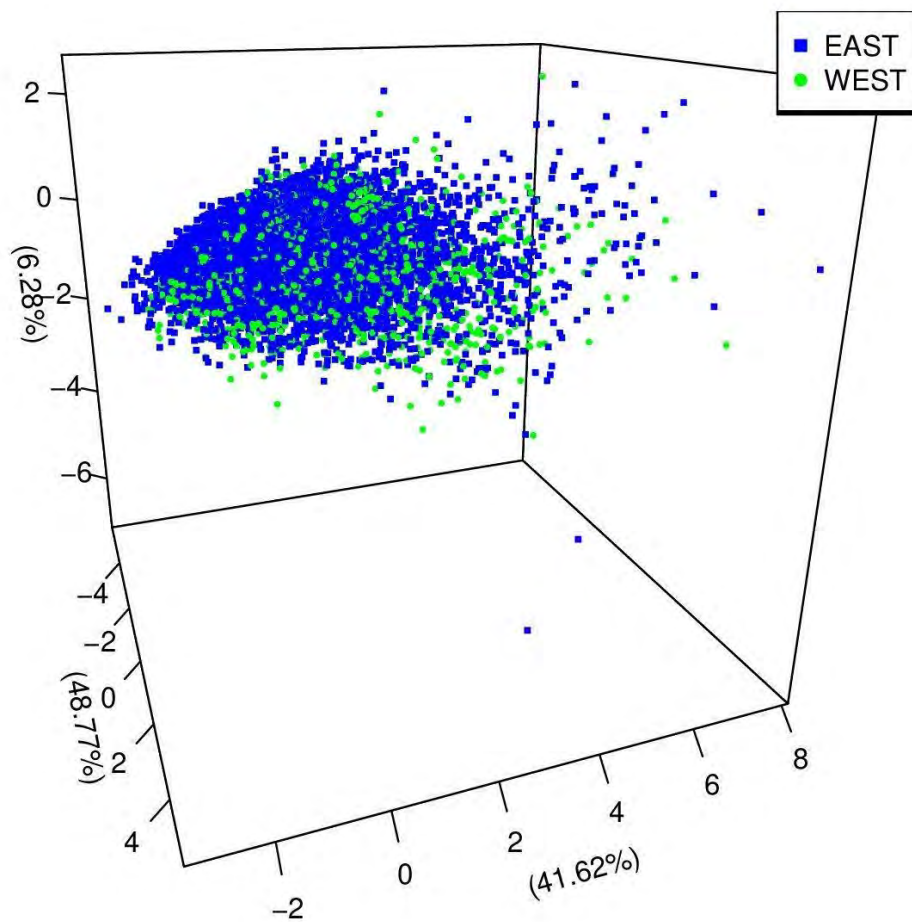


Figure 3.12: Principal coordinate analysis of the mean of the four measurements taken from 46 *Parechinus angulosus* individuals (SL, SW, LL, LW), accounting for 96.67% of the variance of the data between the three axes.

The K nearest neighbour classificatory discriminant analysis was repeated on the population-level data set ($n = 13\,609$). The method had the highest success rate at $K = 29$ nearest neighbours, and so this value was therefore used to assign observations to a priori defined groups, which is the east and west coasts. The success rate of assigning individuals to the coast from which the observations were measured was on average 69.43% (Table 3.13). Similar to the individual-level data set, the success was higher for the individuals originating from east coast. In the reduced and subsampled data set (not presented), the east coast data set ($n = 9\,463$)

was reduced to a number of data points comparable to that of the west coast (east coast $n = 4\ 146$; subsampled $n = 3\ 000$), for a total of 99 repetitions, for which a mean value was calculated. This mean revealed that, for the east coast, the algorithm's success rate in assigning individuals to their population of origin dropped to 56.14%, however, an increase in the percentage of correctly assigned individuals to their population of origin was seen for the west coast (55.36%).

Table 3.12: An error matrix (confusion matrix) generated from individual spine measurements (SL, SW, LL, LW) collected from *Parechinus angulosus* individuals for each of the two coasts ($n = 13\ 609$), indicating the total number of data points per population, the number of data points assigned to the east and west coasts, the number of data points correctly assigned per coast, and the analysis's success rate (69.43%).

Population	Coast	Number of individuals in population	Number of individuals assigned to the east coast population	Number of individuals assigned to the west coast population	Number of correctly assigned individuals	Percentage correctly assigned individuals
Kidd's Beach	East	294	287	7	287	97.62
Buffels Bay	East	593	542	51	542	91.4
Schoenmakerskop	East	889	809	80	809	91
Kei Mouth	East	894	813	81	813	90.94
Jongensfontein	East	889	796	93	796	89.54
Port St Johns	East	892	788	104	788	88.34
Cannon Rocks	East	1760	1549	211	1549	88.01
Presley Bay	East	1188	1041	147	1041	87.63
Kenton	East	886	775	111	775	87.47
St Francis	East	876	753	123	753	85.96
Arniston	East	597	500	97	500	83.75
Coffee Bay	East	300	243	57	243	81
St Helena	West	892	729	163	163	18.27
Kommetjie	West	889	731	158	158	17.77
Blouberg	West	597	496	101	101	16.92
Kleinsee	West	1173	1042	131	131	11.17
Total		13609	11894	1715	9449	69.43

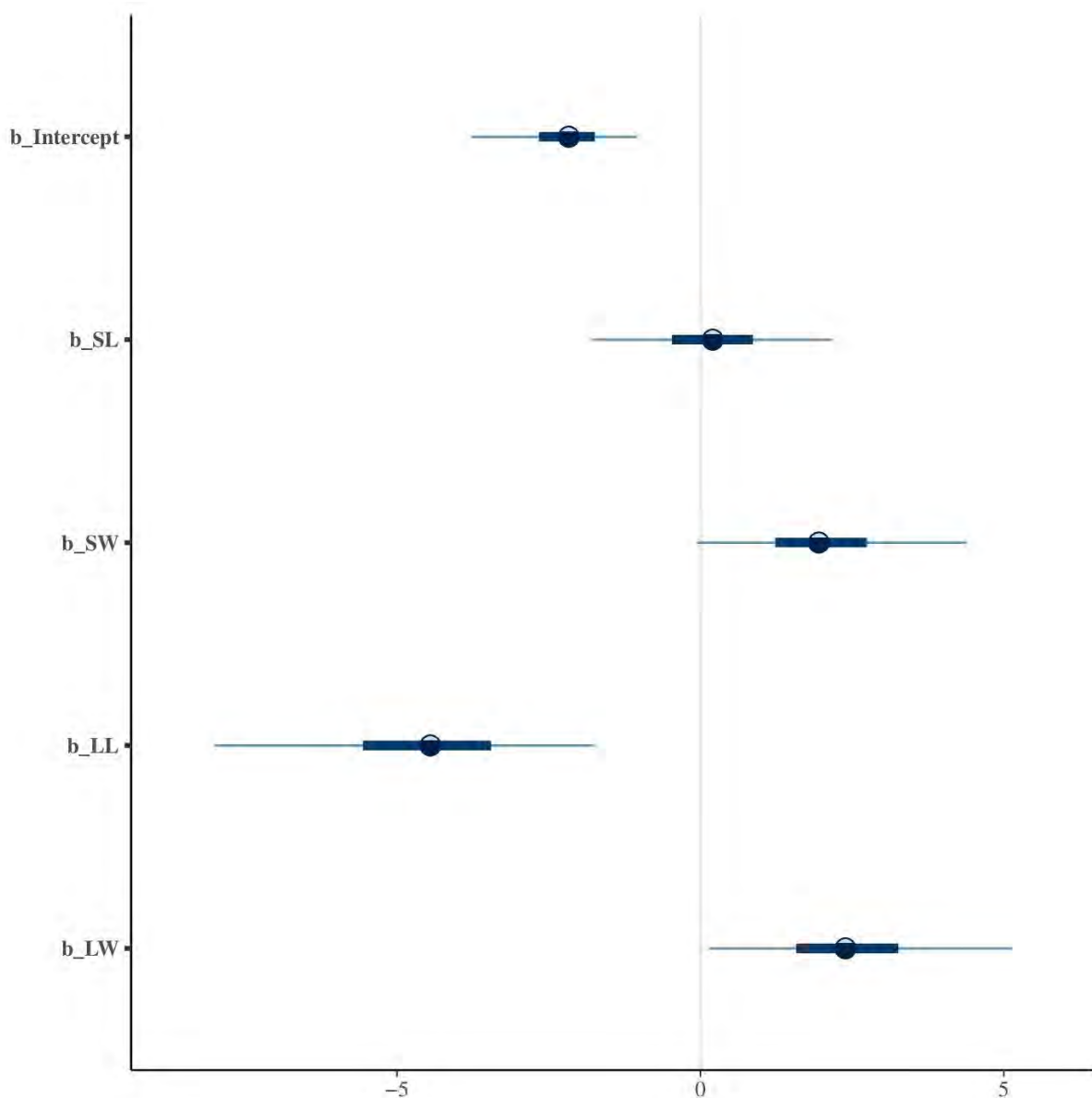


Figure 3.13: A graphical representation of the Bayesian model coefficients, showing that the estimated coefficient for Large Width (LW) and its 95% credible interval does not include zero.

When the model's summary was considered in more detail, the group-level effect showed that there are considerable morphological variations among individuals in each population. It is suspected that the poor performance of this method is due to a combination of a relatively small sample size ($n = 46$), and high variability in the individual character measurements. A new, simpler model that does not include the nested structure of individuals within each population was fitted. Similarly, the summary statistic of the second fitted model showed that the model converged successfully, since the posterior distributions are almost normally distributed, and all chains are oscillating around the same estimates (Figure 3.14).

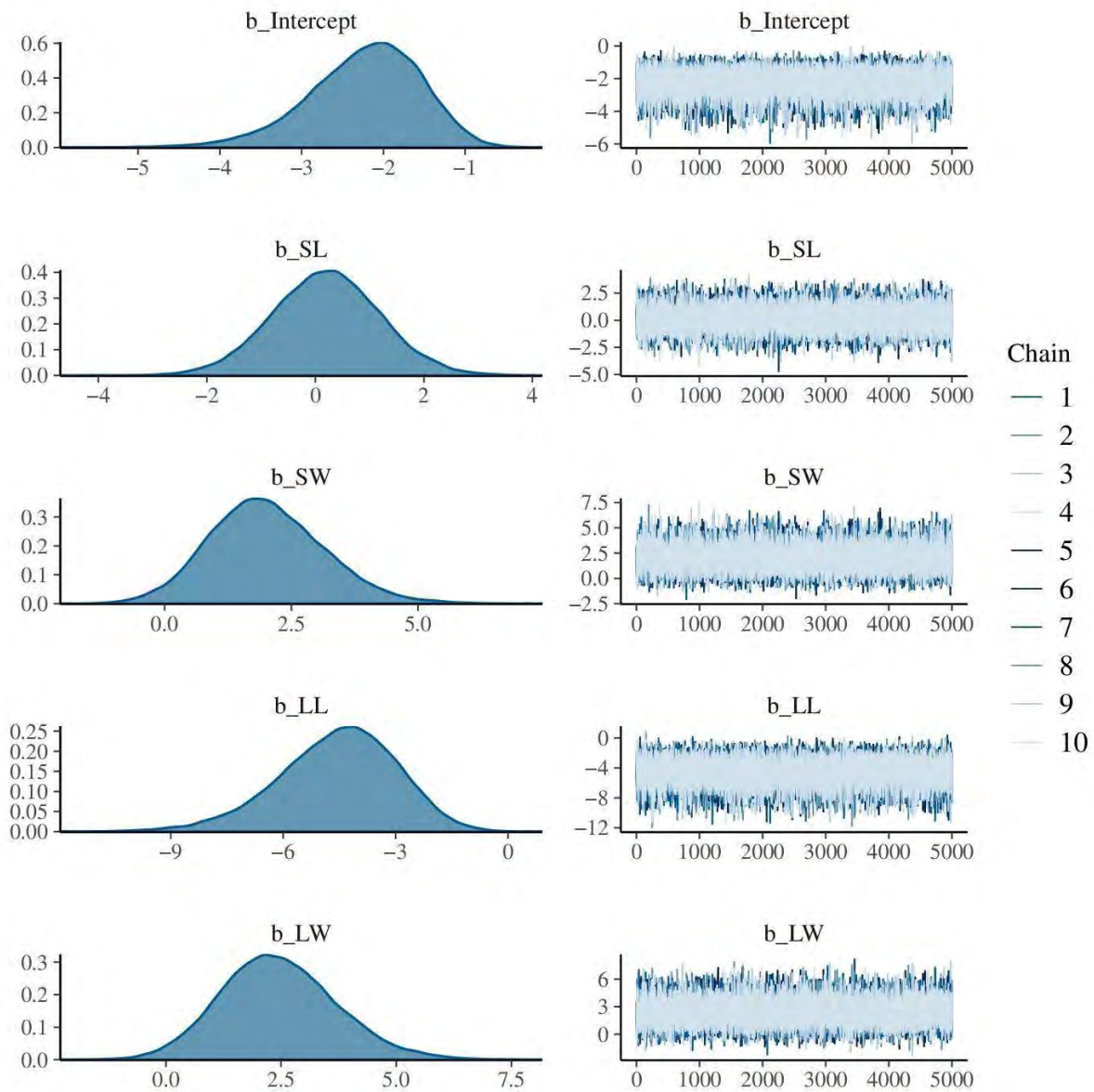


Figure 3.14: The posterior distribution of model parameters (left panels) and a caterpillar graph (right panels) for the four morphometric traits ((SL, SW, LL, LW) that show multiple replicate simulations of the model has converged successfully on similar values, thus these estimates are robust.

The investigation into the simpler model, particularly focusing on the coefficient for large width, is paramount as its 95% Credible Interval excludes zero values, indicating significance, (Figure 3.14), and therefore the simpler model is worth investigating in more detail. In the next step, the `confusionmatrix()` function in R was used to assess the classification power of this model (Table 3.14). The estimated sensitivity (the proportion of true positive assignment of

individuals to their collection site on the east or west coast), and specificity (the proportion of true negative, which means excluding individuals depending on whether they belong to the east or west coast) were 91% and 58%, respectively. The accuracy of the Bayesian classifier was 82.61%, CI 0.6858 - 0.9218, and it was approximately 10% higher than the 74% estimated for a No Information Rate model (NIR), which is the accuracy of the model by invariably assigning individuals to the majority class, the east coast in this case. The large credible interval around the estimates, however, made the p-value insignificant (p-value = 0.11, Table 3.15).

Table 3.14: A summary of the classificatory analysis of the Bayesian model created using four sea urchin spine characters (SL, SW, LL, LW) measured from *Parechinus angulosus* individuals collected from the east and west coasts of South Africa.

Statistic	Value
Accuracy	0.826
95% CI	(0.686, 0.922)
No Information Rate	0.739
p-Value [Acc > NIR]	0.117
Kappa	0.523
McNemar's Test P-Value	0.724
Sensitivity	0.912
Specificity	0.583
Pos Pred Value	0.861
Neg Pred Value	0.700
Prevalence	0.739
Detection Rate	0.674
Detection Prevalence	0.783
Balanced Accuracy	0.748

3.4 Discussion

The results of this study show that *Parechinus angulosus* individuals collected from the east and west coasts of South Africa exhibit variation in various aspects of their phenotype owing to the drastically different ecological conditions found on each of the coasts. This was demonstrated using both the approaches of classificatory discriminant analysis and Bayesian multi-level modelling. This study first investigated whether a difference in the test and Aristotle's lantern traits among individuals of *Parechinus angulosus* from (a) the east and west coasts, and (b) different populations (i.e., sites

of collection) along the entire range of distribution of this species exists. Subsequently, the power of spine morphometrics, as distinguishing characters that could potentially separate individuals from the eastern and western coasts of South Africa, was also evaluated.

The results indicated that individuals from the west coast differed significantly from those on the east coast. Out of the six characters measured, test width, weight, and height, and lantern width and height were found to contribute the most towards the dissimilarity observed. The study of morphometric traits is a powerful tool in the characterisation and comparison of sea urchin populations from different regions, driven by their capacity for phenotypic plasticity in response to the environmental conditions at a site (Ourens *et al.* 2011, Nicolau *et al.* 2022). Although various studies have explored these same traits as those recorded in this study (Table 3.1), regional comparisons, such as those made in the present research, remain limited.

One noteworthy exception demonstrated that morphometric analyses of certain traits (test diameter, spine length, and total dry weight) of the purple sea urchin, *Paracentrotus lividus*, showed sufficient variation among individuals collected from the Western Mediterranean (Algeria, Tunisia, Libya, and Palermo, Italy) that contributed to delineate between two morphologically differentiated groups on the eastern and western sides of the Mediterranean basin (Deli *et al.* 2017). The authors note that additional work would be required to disentangle the driving mechanisms behind the different morphologies of individuals from the separate coasts but suggest that the observed differences may be due to regional differences in temperature, salinity, habitat/substrate texture (muddy vs pebbly), and perhaps even recruitment patterns of urchins. Similarly, Nicolau *et al.* (2022) found that morphometric relationships between specimen size (test diameter, height, and volume) and weight were significantly different between two sites along the western coast of Portugal separated by 490 km. These authors postulated that environmental factors such as nutrient availability from river discharge and energetic coastal hydrodynamics are likely the most influential factor contributing to the differences observed.

The various species that *P. angulosus* competes against have many commonalities between the two coasts. For example, sea stars (Duggins 1983), molluscs (Pequeno and Matthews-Cascon 2010), fish (Cowen 1983, McClanahan 1998, Sala and Zabala 1996), and other sea urchin species (McClanahan 1988) are common competitors on both coasts, though it must be noted that very seldom the same competitor species are cosmopolitan around the entire South African

coastline (Branch and Branch 2018) and/or exercise the same competitive strength. The competitive pressure exerted on sea urchin populations on the two coasts can depend, in part, on some of the factors described above, but the geology and rock type of the west vs east coast, and the resulting sand grain size can also contribute to the observed morphometric differences. The east coast has predominantly sedimentary rock (Miller and Mason 1994), especially sandstone and shale (Baiyegunhi *et al.* 2020), whereas the west coast is dominated by granitic and metamorphic rock, such as granite, gneiss, and schist (Walker 1917, Waters 1986, Belcher 2003). It is reasonable to consider that sea urchins' populations on different coasts respond to this varied range of environmental and ecological pressures differently and, as a consequence, develop slightly dissimilar morphologies. A combination of these factors likely influences the shape and weight of the sea urchin test, the lantern, and spine morphometrics as test and lantern traits were observed drivers of the regional divergence.

During this study, the globosity, volumetry, and density indices of the test were all found to be significantly larger in individuals collected from the west coast as compared to the east coast. While there is no direct comparison between round and pentagonal test shapes (when viewed dorsally and considered as a two-dimensional shape, i.e., excluding the third dimension of depth which is used to calculate globosity), spherical shapes have lower drag coefficients (0.5; The Engineering Toolbox 2004) as compared to pentagonal prisms (0.912; Shanmugasundaram 2021). Dislodging a spherical urchin should therefore theoretically be more difficult than an urchin with a pentagonal test shape. This is important when considering wave which is on average higher on the west coast when compared to the east coast, although the difference between these averages is slight (0.4 m; Branch and Branch 2018). The globosity, which links to the roundness or flatness of an urchin, may be smaller in individuals collected from the east coast due to greater exposure of individuals to wave action as supported by the findings of Gonzalez-Irusta (2009), or more likely, current speed, as the east coast is dominated by the fast flowing Agulhas current (Lutjeharms 2007). Consequently, the volumetry index would also be smaller in individuals on the east coast owing to the flatter tests of which globosity is a measure, leaving less space internally for structures in the test. Finally, a lower density would also be logical for individuals observed with lower globosity and volumetry indices, such as those found on the east coast, since a smaller, flatter body would logically be less dense than a rounder and consequently more voluminous test.

Additionally, linked to urchin shape and weight is the controlling factor of test size. While a larger body size has many benefits, one overlooked benefit of a smaller body size is that it dissipates heat more effectively (Hamilton 1960, Berman 2003), owing to its relatively larger surface area as compared to a larger body size. Since test weight did reflect as one of the characters contributing towards the observed dissimilarity between populations and region, it may be that smaller test sizes could be due to result of the warmer Agulhas Current found on the east coast. The opposite argument, however, also holds true, that a smaller body size gains heat more quickly than a larger body size (Cain *et al.* 2006), which may partly explain why smaller urchins are found under rocks and in crevices. Juvenile green sea urchins (*Strongylocentrotus droebachiensis*), for example, find refuge under and between rocks to avoid predators (Keats *et al.* 1985) and only begin to move out onto open surfaces in pursuit of food once reaching 15 mm in diameter (Himmelman 1986). It may be that this behaviour serves two purposes, however, to protect against predators, but also to limit solar exposure to avoid overheating.

Another body metric that holds ecological importance is the Aristotle's lantern, the mouth apparatus of the sea urchin. Studying the dimensions of the lantern in isolation or in relation to the dimensions of the test can provide insights into the feeding ecology of urchins.

A larger lantern relative to test size is more likely to be present in individuals settled on hard rock as are those specimens of *P. angulosus* in South Africa found on the west coast, a finding supported by those of Black *et al.* (1984). This is likely due to the additional effort required, and therefore stronger ligaments attaching the lantern to the test, to rasp algae from hard rock as opposed to sandstone or shale (Ebert 1980). Additionally, certain species of sea urchin like *Strongylocentrotus purpuratus* have been found to reallocate energy resources toward lantern growth and away from somatic growth when food material is scarce and increased grazing efforts are required (Ebert 1980, 1996, Russel 1987, Edwards and Ebert 1991). This may be a reason for a larger lantern in individuals collected from the west coast if one considers that the cold water, such as that of the Benguela current, is a directive factor of the level of activity individuals engage in (Reynolds 1979). Indeed, feeding trials of *Lytechinus variegatus* at different temperatures approaching their lower thermal limit (11°C; Beddingfield 1997) found that feed intake slowed when decreased to 16°C from the acclimating temperature of 22°C (Watts *et al.* 2011). Additionally, though cold water is associated with high levels of primary productivity (Kone *et al.* 2005), it has been found that macroalgal growth slows down in cold waters (Ras *et al.* 2013), which could further explain why individuals from the west coast may

have larger lantern, as macrophytes form a large part of the diet of sea urchin since they are primarily detritivores (Frantzis 1992)

In the second part of this study, spine morphometrics were intensively investigated, though separately from test and lantern metrics owing to the two data sets being generated from a different number of individuals, and not directly comparable. Though some level of variation in spine morphometrics was observed among and within specimens collected from east and west coasts, neither the traditional dimensionality reduction PCA nor PCoA ordination plots revealed obvious clustering as it was otherwise hypothesised. This high level of data spread was expected given the high intra-population variability. Though it is not possible to directly link any spine morphometric character to an individual adaptive response to environmental conditions, the identification of a genetic or epigenetic association with spine length and width of an individual could point to the potential selective advantage of those traits that can pass on through generations (Gerber and Griffen 2003).

Fine-scale variation in spine morphometrics of *P. angulosus* was expected, pointing to the presence of some level of local adaptation, perhaps at an individual level, to prevailing environmental factors. This is because urchins individual variation in spine morphometrics is likely driven by local environmental conditions. It was found that individuals on the west coast had longer and thicker small spines whereas individuals on the east coast had longer and thicker large spines. This may be due to the fact that the cold water environment on the west coast slows a predator's metabolism (Johnston 1990), making the breaking open of sea urchin tests against rocks an energetically expensive exercise and so from the perspective of the sea urchin, there is little selective pressure to grow more robust large spines. Longer large spines can therefore be "sacrificed" to break in the event of hard contact with an unyielding surface such as rocks, reefs, and the like. Spines are also the primary defensive trait for sea urchins (Fricke 1974, Moureaux *et al.* 2010) and longer, thicker spines would translate into better defence against predators and competitors. Growing (and regrowing) spines is, however, energetically expensive (Edwards and Ebert 1991). It may therefore be that individuals with longer and thicker spines have more access to abundant food sources and defend themselves and their resources more efficiently. Low food availability for sea urchins is often a result of high levels of competition with other herbivorous and/or grazing species like snails, limpets, and other urchin species (McClanahan 1992). Spines have also been found to assist in locomotion alongside the tube feet (Prouho 1888). Urchins move to escape from predators like starfish or

other slow-moving predatory organisms (Nelson and Vance 1979, Carpenter 1984), for seaward retreat at low tide to avoid solar exposure and the risk of desiccation (Gibson 2003), or in pursuit of better foraging grounds (Mattison *et al.* 1977, Andrew and Stocker 1986). Longer spines result in more effective locomotion, resulting in individuals covering larger distances than they otherwise would using tube feet action only (Prouho 1888, Moureaux *et al.* 2010).

In this study, the performance of the K-NN algorithm on spines was significantly better for the east coast populations for which the method was trained on a comparatively larger number of data points. This was more obvious in the individual-level data set that was comparatively smaller in size, such that the method failed to correctly assign west coast individuals. This poor performance was expected since when the number of data points is small; the algorithm has a known tendency to assign individuals to the classes with comparatively larger data sets since the method had more data points to learn a pattern from. When a balanced number of data points was, however, used following subsampling of data, the performance of the methods on individuals from west coast increased to 44%. Subsampling this population to a comparable size, however, indicated that training the algorithm on a balanced data set could result in similar performances.

There is no doubt that incorporating more data points will improve the classificatory power of the applied method, however, due to logistic challenges, including restrictions associated with the global COVID pandemic in 2020 and the closure of the interprovincial borders in South Africa, collecting more specimens was not feasible within the time frame of this study. As such, it was decided to quantify the level of uncertainty in classificatory analysis using a novel method that in its essence is very similar to widely used permutation approaches. The results of components of this study show that there is some level of variation in spine morphometrics among populations between the east and west coasts of South Africa. To train a model that can conclusively assign individuals to their population of origin however, such a model needs to be trained on a sample data set larger than what was used in the current study. As such, while the impact of unbalanced data sets, the intra-population variability, and the classificatory power of the morphometric characters cannot conclusively be disentangled, the relevance and potential of this approach should be recognised, even for a reduced model. This is especially relevant given the reasonable performance of this method when trained on the large number of data points collected from the east coast of South Africa and the relatively comparable performance of this method on the subsampled databases. The combination of the K-NN

classificatory analysis and the collection of a larger number of data points from the eastern and western coasts of South Africa could provide conclusive evidence for the performance of this method. Such a conclusion is not possible given the current unbalanced data sets, since it is believed that the performance of the algorithm could have been affected by the lower number of measurements collected from the west coast on which the algorithm was trained.

3.4.1 Limitations

Going forward, one suggestion to improve the methodology of morphometric measurements would be to consider using measurement techniques other than linear distances. Though all the following methods are somewhat differently limited, one could consider using boundary outlines, a landmark-based approach, or even digital images (Macleod 2017). Alternatively, one could consider an altogether different morphometric approach in the form of geometric morphometrics, which relies on geometric shape space (Kendall 1981, 1984). This approach also aims to understand the variation in form, size, and shape, but by placing landmark coordinates on a set of axes. This approach has been refined since its introduction in the 1980s (Kendall 1981, 1984) and ultimately aims to provide a comprehensive, quantitative framework for studying variation in form, size, and shape. While this study may have benefitted from such an approach, the time limits of this doctoral project did not make this approach a viable option, and linear distance measurements and the indices that can be calculated as a result remain a good proxy measurement for shape and size of an urchin as the most well-established of the approaches of morphometric studies as discussed in Macleod (2017).

As with most studies, a larger number of individuals per population, as well as a fully balanced number of populations between the two coasts would have increased the classificatory power. The incorporation of additional spine measurement data, as well as pedicellariae, and tube feet data would have resulted in a more holistic study and allowed for the comparison of traits that may, at first glance, be thought to be unrelated. An example of this might have been the relationship between spine length and the size of the Aristotle's lantern, or potentially the length of the teeth, since longer spines would require greater flexibility of the lantern to reach edible material as compared to those individuals with shorter spines (Hagen 2008). Owing to the various purposes for which the urchins were collected for the studies described in this thesis, it was not possible to create data sets that included all test and lantern and spine metrics, and count data for spines, tube feet and pedicellariae from the same set of individuals. For

example, not all spines could individually be removed from the individuals from which test metrics were collected, as the majority of these latter urchins were used for the genetic and gut microbiome studies described in chapters 4 and 5, and the amount of time the urchin would have to spend outside of the preservative ethanol for spine collection would have compromised the quality of the tissues used for further molecular analyses. Other methods of collecting spines to minimise the amount of time the urchin is not kept in ethanol, such as soaking in a solution of diluted bleach (Kemp and Smith 2005) would similarly impact the biological material used in chapters four and five as bleach degrades DNA. Nevertheless, the data sets generated for this study were sufficiently comprehensive to ensure that the key traits associated with feeding, reproduction and growth were effectively encompassed, providing a holistic understanding of spatial patterns of traits that included the primary functions of sea urchins.

Furthermore, this study adds to the growing body of evidence that emphasises the impact of having a large number of data points for the optimal performance of machine-learning methods. While there is no consensus about the minimum sample size required for this algorithm, the accuracy and reliability of the predictions made by the algorithm are dependent on the quality and complexity of the training data (Guo *et al.* 2003). For optimal performance of K-NN, each class must be represented by an adequate number of samples to capture the variability in the class. A statistical method of determining the requisite sample size to correctly identify boundaries between classes is cross-validation of K-NN with varying sample sizes, ultimately to select the sample size that yields the most reliable result (Guo *et al.* 2003). Although a larger sample size could improve the reliability and robustness of results, this study show that K-NN can perform well with fewer samples if the data are well-separated and accurately represent the underlying distribution of the data such as the data set used for the east coast. Having very few samples in a class, such as the west coast data set, however, inhibits the ability of the algorithm to accurately classify or assign samples to a class in an error called overfitting.

3.5 Conclusion

This study presents a comprehensive analysis of the morphometric characteristics and indices derived from the sea urchin, *Parechinus angulosus*, sampled from the east and west coasts of South Africa. The analyses undertaken in this study revealed variations in the measured characters and indices between these two distinct regions. These findings underscore the significance of regional environmental factors and their influences when examining the

morphological traits of sea urchins. The observed differences between the two coasts provide a foundation for the following genetic and microbiological studies aimed at elucidating the underlying mechanisms driving these morphometric disparities. Furthermore, this work stands alongside that of others, emphasising the potential for morphometric analyses to serve as a valuable tool for understanding how phenotypic plasticity and, by extension, the dynamics of their populations in distinct ecological contexts are driven by prevailing environmental conditions.

Chapter four: Population genomic structure and the presence of fine-scale local adaptation in populations of the Cape sea urchin, *Parechinus angulosus*

4.1 Introduction

Evolutionary genetics is a branch of biology that addresses the molecular underpinning of the phenotypic changes in life forms through time and which specifically targets both short and long-term spatial and temporal genetic variation in response to various environmental stimuli among groups of living organisms (Hellenthal and Stephens 2006). The major focus of this discipline is to investigate how this variation is created and subsequently maintained within populations (Huang *et al.* 2016). It is known that the ultimate source of genetic variation is mutation (Barton and Keightley 2002). Additionally, in animals that reproduce sexually, recombination between homologous chromosomes plays a crucial role in introducing genetic variation into the population (Spencer *et al.* 2006). Studies show that even in the absence of new mutations, populations can still maintain a significant level of genetic variation through recombination (Watt 1972). Effective gene flow also introduces new genetic variants into a population, however, compared to the other two forces, it is typically less significant. After the emergence of new genetic variations within populations, an intricate interplay between natural selection, random genetic drift, and migration shapes the spatial and temporal distribution of genetic variations among populations (see Charlesworth and Charlesworth 2016 for a complete review).

Marine organisms are of particular interest in population genetics and evolutionary biology as it was originally believed that marine populations are demographically open, due to the lack of obvious environmental barriers across oceanic basins (Hauser and Carvalho 2008). Comparatively to terrestrial systems, it was therefore expected that marine populations would show low levels of population differentiation due to the unimpeded homogenising effect of gene flow over generations (Graves 1998). This has not always been the case, however, since the presence of ocean currents, geomorphological features of the coastline, and the physico-chemical gradients of primarily temperature, salinity and acidity act as significant barriers to gene flow between marine populations (Hauser *et al.* 2008, Sanford *et al.* 2010, da Silva *et al.* 2016). The presence of both fine and coarse mosaics of environments in marine habitats play a crucial role in determining the distribution of genetic variation, since the spatial distribution of species is strongly influenced by local conditions (Williams *et al.* 2018), and habitat structure over time ultimately creates the observed pattern of biodiversity across the globe (Godbold *et al.* 2011).

Local adaptation is a combination of behavioural, physiological, and genetic changes or modifications that species develop in response to the environment in which they live to survive and reproduce successfully (see Savolainen *et al.* 2013 for a review). Studying local adaptation in experimental biology has traditionally relied on two major experimental setups known as common garden experiments, which are conducted in the laboratory, and reciprocal transplant studies, which are typically conducted under field conditions. Briefly, in common garden studies, individuals from differing populations are exposed to closely monitored biotic or abiotic stimuli to evaluate their fitness (Conover 1998). These experiments aim to identify, among others, the underlying physiological adaptations of organisms by controlling for any confounding effects resulting from exposure to different abiotic factors under field conditions (Nuismer and Gandon 2008). In reciprocal transplants, individuals from the study populations are relocated or exchanged between habitats, allowing for an assessment of the fitness of local genotypes compared to that of translocated individuals (Kawecki and Ebert 2004). Such studies can provide confounding results as noted by Chapman (1986) who reported that the pattern of movement of translocated individuals were not as a result of the characteristics of the snail test subjects, but rather as a result of the area in which the experiment was done. In addition to this, maternal effects can also introduce confounding variables in the context of translocation. When breeding individuals in controlled environments over several generations, the experimental conditions can help mitigate these confounding effects (Wolf and Wade 2009). Such a solution is, however, logistically and financially challenging, and at times not feasible for wild species. The expression of a particular phenotype is a complex interaction between the genotype of an individual and its environment (Shimelis and Shiringani 2010). As such, near translocation of individuals between habitats in species like *P. angulatus*, with little information about the underpinning processes of genetics and epigenetics, could suffer from many underexplored genetic processes. Moreover, phenotypic plasticity of traits that point to the development of different phenotypes from the same underlying genomic background can also negatively affect such experiments (Etheridge *et al.* 2010). That is, differences in fitness can be wrongly attributed to genetic differences while it is not the case, and the critical role of other processes such as gene expression or epigenetics from the same underlying genomic makeup needs to be taken into consideration.

A significant shortcoming of these experimental designs, however, is the limited ability to minimise the confounding effects of biotic and abiotic factors that an individual is exposed to prior to the experiments (Hall and Willis 2006). Various approaches have been implemented to

minimise these undesired interferences, including long acclimatisation to the conditions of the experimental setup, typically from weeks to potentially a year, prior to the start of the experiment, or the breeding of multiple generations of the study species under controlled laboratory conditions (Sanford and Worth 2010). In both approaches, a combination of direct measurements of fitness, such as the survival and successful reproduction, or indirect measurements, such as growth rate, respiration rate, enzyme activity, and changes in behavioural traits such as prey defences or active habitat selection are used to assess species' adaptability (Walsh and Somero 1981, Reusch 2014). While these methods provide valuable information about a species potential to respond to environmental stimuli, the underlying molecular mechanisms involved in the process, its heritability across generations, and its significance for the long-term survival of the species cannot be directly addressed.

Conducting common garden and reciprocal transplants in marine organisms is labour intensive and logistically challenging. Coastal invertebrates are, however, exceptional since implementation of these methodologies is comparatively easier, due to coastal species being generally easier to track than pelagic species (Olivares-Zambrano *et al.* 2022). As such, coastal invertebrates are ideal candidates for studying the molecular bases of local adaptation, owing to the fact that the primary method of reproduction in these species is broadcast spawning, and that most of these species are benthic and sessile/sedentary during their adult phase (e.g., Lawrence and Soame 2004, Lange *et al.* 2018, Melzner *et al.* 2020). Juveniles settle onto the benthos in response to cues like temperature and substrate type, to which these invertebrates are adapted (Cameron and Schroeter 1980, Chase *et al.* 2016). Moreover, the prevailing environmental conditions experienced by such benthic species can easily be assessed, allowing researchers to directly link molecular and physiological changes to environmental conditions at different ecological niches. By integrating evidence from molecular, ecological, physiological or physical (i.e., distribution or range changes) responses of organisms to environmental changes, the significance of the environment in shaping population structure, and the spatial distribution of biodiversity can be studied in more detail (Koen *et al.* 2014).

Recent advancements in massively parallel sequencing technologies, along with the increased accessibility and affordability of high-throughput computing resources, have resulted in a significant shift in the way in which adaptations in marine organisms are studied (see Cordier *et al.* 2021 for review). It is now possible to investigate the molecular basis of local adaptation and its spatial variation due to an unprecedented resolution of the genomic and computational

tools. In a similar way, the association between genotypes and the marine environmental gradients, such as temperature, salinity, and pH, can be established (Teske *et al.* 2019, Stracke and Hejnol 2023). With the application of these new methodologies, a large body of research shows that fine-scale local adaptation and population structure in marine organisms is widespread and occurs across both large- and small-scale marine ecosystems (see Hauser and Carvalho 2008 for a review). This finding further sparked an old debate in population genetics, questioning if terrestrial and marine ecosystems require distinct investigative approaches.

Marine organisms demonstrate a wide array of demographic dynamics that range from highly isolated subpopulations (termed demes) within a larger interconnected population, to patterns of isolation by distance (IBD; which describes the inverse relationship of a decrease in genetic similarity among individuals, groups, or populations as the geographic distance between them increases), to complete panmixia in species that congregate in large numbers in breeding areas (Pascual 2019). Understanding and predicting the patterns of local adaptation under these diverse ranges of demographic scenarios is crucial, since the intricate balance among various evolutionary forces, including mutations, recombination, genetic drift, natural selection, and gene flow define the evolutionary potential of organisms to respond to current and predicted challenges in their life history and the long-term maintenance of populations (Lenormand 2002, Slatkin 1973). The diversity of demographic structures in marine organisms, however, makes the detection of genomic footprints left behind by local adaptations challenging, since a number of various demographic histories, that are not adaptive, can create genome-wide signatures that resemble local adaptations (e.g., genetic drift, isolation by distance or secondary contact after recolonisation from local refugia, see Dylan 2020 for a comprehensive review).

Making a conclusive distinction between the genomic signatures of natural selection and that of genetic drift as the causative agent of observed genetic structure is a challenging task (Millstein 2002). The consensus is that, since evolutionary forces, such as genetic drift, usually leave their diagnosing signature across an individual genome, it is reasonable to assume that loci across the genome will be more or less similarly affected. By contrast, natural selection is more focal and mainly impacts those loci that are involved in traits with consequences for an individual's survival and reproduction. The signature of these evolutionary forces is typically more obvious in target loci and in genomic regions or loci that are in linkage disequilibrium (LD) with the genomic regions, which are the direct targets of selection (Hoban *et al.* 2016).

These distinctive genomic signatures serve as the basic assumption of one of the most commonly used methods to identify the loci that are under selection, since those are the loci expected to stand out from the background level of variation across the genome. For this purpose, genomic summary statistics, such as fixation index (F_{st} , Wright 1949) or site frequency spectrum (SFS, Ganapathy and Uyenoyama 2009), are calculated throughout the genome, and those outlier loci that significantly deviate from the estimated background summary statistics can be considered as putatively under selection. The outlier detection method relies heavily on accurate simulations of a background null distribution that can serve as a frame of reference to identify those loci that appear in the extreme tail of the distribution as evidence of non-neutral evolution (Beaumont and Nichols, 1996). Another group of methods to identify putative loci under selection is collectively known as environmental association analysis (EAA) or genotype-phenotype associations (Manel and Holderegger 2013). The main focus of this group of methodologies is the identification of statistically significant changes in allele frequency that can be correlated to a selected subset of environmental conditions (e.g., temperature, salinity, and nutrient loading, Rellstab *et al.* 2015).

Understanding and predicting the adaptive responses of marine organisms to the environment has never been more important, since the combination of increased temperature, high acidity, low oxygen content, perturbed salinity, and anthropogenic pollutants threaten the survival of marine organisms at an unprecedented level. The extinction of vulnerable marine populations, the collapse of economically important stocks due to extreme climatic events and their negative impact on population structure, survival, and the free movement of individuals to more suitable marine habitats, not only pose a severe threat to the health and the biodiversity of the planet, but also holds profound negative socio-economic implications for the well-being of coastal communities worldwide (Halpern *et al.* 2007).

4.1.1 Rationale and aims

As an ideal set up to test adaptive responses to the environment, and as well detailed in Chapter 2, the South African coastline is shaped by the two major contrasting oceanic currents dominating the coastline: the warm Agulhas current on the east coast and the cold Benguela current on the west coast. As expected for such dynamic marine ecosystems, the identification of exact boundaries of the coastal bioregions (Lombard 2004), however, continues to be a subject of debate (Trembl and Halpin 2012). As a consequence, marine organisms living in these

contrasting environmental conditions have to fine-tune their physiological and ecological responses to the distinct environmental pressures they are exposed to. The focal species in this study, *Parechinus angulosus*, is the most widely distributed echinoderm in southern Africa. Despite its wide distribution in coastal ecosystems, the genetic diversity, potential local adaptations, and the extent of gene flow between populations on the east and west coasts of South Africa is not well understood. An earlier study, aiming to expand the understanding of the population structure and gene flow patterns of this species, examined the COI mitochondrial gene and the SpREJ9 gene of nuclear DNA in individuals collected from 18 sites from southern Namibia to Durban (Muller 2011). This study found a significant genetic break in the region of Cape Point. A more recent study assessed local adaptation across environmental gradients of *Parechinus angulosus* (Mertens 2021) using a Pool-seq approach. Employing Single Nucleotide Polymorphisms (SNPs), this study aimed to determine potentially adaptive loci and based on this, also adaptive population structure. This study, however, only sampled at a limited number of sites (Port Nolloth, Sea Point, Cape Agulhas, Knysna, Cape St Francis, and Haga Haga), and pooled DNA from multiple individuals collected at each site, limiting the power of the chosen methodology to detect rare alleles that may otherwise be present in only a few of the individuals within a population.

To complement the previous research, this study uses a reduced representation of *P. angulosus* genome generated using the ezRAD protocol (Toonen *et al.* 2013), and the genome-wide diversity, presence of cryptic population structure, and spatial pattern of distribution of genomic diversity across South African coastlines were investigated. The presence of a statistically significant correlation between genomic variation and temperature, as well as the association between individuals' genotypes and test morphometric traits (test height and width) in individuals from three different bioregions was also tested. Furthermore, the phylogeography of the species through time was reconstructed using the mitogenomic data in a Bayesian framework. Finally, shifts in allele frequency in response to projected extreme climatic conditions were investigated to assess the vulnerability of current populations. In the next section of this chapter, I briefly explain the laboratory steps to generate ezRAD markers, and the several high throughput bioinformatic pipelines that were used to analyse the generated data. Finally, I discuss the limitations of genomic studies in non-model marine organisms. This research aimed to assess (and characterise) the genetic diversity and population structure of *P. angulosus* using SNPs and investigate the evolutionary history of *P. angulosus* by

reconstructing phylogeographic relationships and estimating divergence times between populations.

4.1.2 Hypotheses and research questions

To investigate the level of genetic variation within the South African population of *Parechinus angulosus*, and to evaluate the potential link between an individual's genomic make-up and its expressed phenotype, and its association with the environment where they are found, the following hypotheses were tested: A) there exists two distinct lineages within the population of *P. angulosus*, and certain genetic loci are significantly associated with the measured phenotypic traits of *P. angulosus*, or marine environments on the east and west coasts of South Africa; and B) the contemporary population of *P. angulosus* are panmictic with little genetic differentiation between populations and, as such, the signal of local adaptation might have been diluted due to the extensive level of gene flow between these populations.

Then it was investigated whether different populations respond to the predicted scenarios of climate change in terms of temperature and pH homogenously, or there exist significant differences between the studied populations in terms of their adaptive responses that could reflect their resiliency to the changes in climatic conditions. I used changes in allele frequency as a proxy to predict population responses.

4.2 Materials and methods

4.2.1 Collection & Processing

Ten *Parechinus angulosus* individuals were collected from each of 43 populations along the South African coastline, from McDougall's Bay to Port St. John's (Chapter 2; Figure 2.1) from October 2019 to January 2020. Samples were collected from each of the four bioregions (the Namaqua, Southwestern Cape, Agulhas, and Natal Bioregions) as identified and published by Lombard (2004; Chapter 2; Figure 2.2). Urchins were collected from rock pools by hand during low spring tides and immediately placed into jars containing 99% ethanol to cover the urchin completely. Upon return to the laboratory, individuals were kept at -27°C in freezers located at the South African Institute for Aquatic Biodiversity (SAIAB) (please refer to Chapter 3 for full details on specimen collection). Intestinal tissue adjacent to the anus was dissected and

removed from each of 222 sea urchins and placed into Eppendorf tubes containing absolute sterile ethanol after which samples were once again kept at -27°C.

4.2.2 Genomic DNA extraction and preparation of metabarcoding libraries

Although the initial study design intended for the tissue of six individuals to be used per site, due to time constraints, only three samples per site could be extracted. Using the modified protocol of DNA extraction by ‘salting out’ (Sunnucks and Hales 1996), with the addition of 20 ng/μL RNase subsequent to the pelleting step, genomic DNA was extracted from the intestinal tissues of three individuals from each of the 43 sites. The DNA extracts were purified using Mag-Bind® Total Pure NGS SPRI beads (Omega Bio-Tek Inc), at a ratio of 20 μL DNA extract: 22.4 μL SP beads, completing the bead purification step according to manufacturer’s instruction manual. This was followed by a quantification step using Invitrogen™ Qubit™ High Sensitivity Assay (Thermo Fisher Scientific), also according to manufacturer’s instructions.

Prior to library preparation, the purified DNA extracts were digested using the enzyme MboI (5 000 units/ml, NEB). A master mix for the MboI restriction enzyme per reaction (50 μL) was prepared for approximately 1 μg of DNA by adding the following reagents in the following order: 5 μL of buffer (3.1, NEB), 19 μL HPLC grade water, and 1 μL of 1 unit of MboI/μL. Twenty-five microliters of this master mix was added to 25 μL of purified DNA extract, and the solution was incubated in the thermocycler for three hours at 37 °C, 20 minutes at 65 °C with a final holding step of 15 °C. A post-digestion sample purification step was performed at a ratio of 1:1.8 DNA to SPRI beads, following the same procedure as above, ultimately resuspending the purified DNA in 38 μL of buffer, 33 μL of which was kept for library preparation, and 5 μL of which was used for quality check. Three microliters of the purified digested sample were viewed using gel electrophoresis, with successful digestion resulting in smears across the gel.

Once DNA extracts of sufficient quality were identified, the Illumina TruSeq DNA library protocol was used to prepare libraries. First, the ‘repair end and select library size’ (ERP) step was performed using the End Repair Mix 2 (kit) by adding 20 μL of the mix to 30 μL of DNA extract for a final volume of 50 μL. The product was incubated in the thermocycler for 30 minutes at 30 °C. ERP products were purified using the SP beads. To retain fragments of 350

bp insert size, the bead solution required dilution which was achieved by adding 37.4 μL HPLC grade water to 54.6 μL SP beads per ERP product. Following this, 80 μL of the diluted SP bead solution was added to 50 μL of the ERP product, with a final elution volume of 125 μL . To this, 15 μL of undiluted SP bead solution was added for a second size selection step, which was eluted in 12 μL of elution buffer (kit). This was followed by the ‘adenylate’ or A-tailing step (ATL) during which 6 μL of A-tailing mix was added to 9 μL of the purified ERP product for a total volume of 15 μL . The ATL solution was incubated in the thermocycler for 30 minutes at 37 °C, 5 minutes at 70 °C, and 5 minutes at 4 °C. This was followed by the adapter ligation step (LIG) in which 1.4 μL of resuspension buffer, 1.3 μL of the ligation mix 2 (kit), and 1.3 μL of the DNA adapter indexes (in this case from the DNA adapter plate) were added in that order to 15 μL of the ATL product. The resultant LIG solution was incubated in the thermocycler at 30 °C for 10 minutes, after which 2.5 μL of the Stop Ligation buffer (kit) was immediately added. The bead purification step was repeated in a 1:1 ratio of SP beads to DNA, and the DNA was eluted in 12.5 μL resuspension buffer. The final step was the DNA enrichment step in which 2.5 μL of PCR Primer Cocktail (kit) and 10 μL Enhanced PCR Master Mix were added to 12.5 μL of DNA for a final volume of 25 μL . Thermocycling parameters for this step began with a three minute denaturation step at 95 °C, followed by eight cycles of denaturation for 80 seconds at 98 °C, annealing for 15 seconds at 60 °C, and extension for 30 seconds at 72 °C, with a final extension step of five minutes at 72 °C. A final bead purification step was performed at a 1:1 ratio of SP beads to DNA, at the end of which the purified DNA was eluted in 30 μL of resuspension buffer. Of this, 20 μL was kept for sequencing and 5 μL was used for library validation: 1 μL purified DNA product used in HS Qubit, and 3 μL used in gel electrophoresis with a 100 bp ladder which was viewed on a Uvitec Cambridge FireReader.

4.2.3 Bioinformatic analysis

4.2.3.1 Quality control and sequence assembly

The quality of raw sequences was checked using FastQC v.0.12 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and potential adapter contamination, leading and trailing low-quality base pairs, and low-quality reads, which were defined as sequences with an average Phred score less than 25 in a 4 bp window, were removed using Trimmomatic v.0.39 (Bolger 2014).

There are two major pipelines that are commonly used to assemble ezRAD or other restriction site-associated DNA sequences: ipyrad (Eaton and Overcast 2020) and stacks (Rochette *et al.* 2019). The performances of both methods were tested on the generated data sets. During this evaluation step, it was noticed that the digestive enzyme, MboI, used to cut the sea urchin genome resulted in a large number of singleton sequences. As a result, the de novo assembly was expected to be computationally extensive (more than 1 000 hours) and the issue was more pronounced for ipyrad since the underlying sequence clustering method in ipyrad is an implementation of commonly used VSEARCH pipeline (Rognes *et al.* 2016), which is extremely resource intensive when it comes to assembling data sets with a significant level of singletons. The stacks pipeline has the option for a reference-based assembly and a similar approach is also available in ipyrad. A pilot analysis of the generated data sets showed that neither of these methods performs optimally, and after direct communication with the developers, it was decided that given the complex nature of the generated data, a highly customised pipeline specifically for this type of data, with more flexibility and control over different assembly steps was needed. Consequently, a method that combines a reference-based and de novo assembly with more flexibility was selected.

4.2.3.2 Reference-based assembly

There is currently no reference genome for *Parechinus angulosus*, however, several sea urchins' genomes have been sequenced and are publicly available. Based on this, draft genomes for eight closely related species were downloaded from the National Centre for Biotechnology Information (NCBI) genome database, and all forward and reverse sequences were mapped against these surrogate genomes using short-read aligner, bwa-mem v.07.17 (Burrows-Wheeler Alignment, Li 2013). Following this, alignment summary statistics were estimated for each surrogate genome in the sequence alignment map (SAM) tools v.1.6 (Danecek *et al.* 2021) flagstat command. SAMtools is a suite of programmes appropriate for basic interactions with alignment files generated by high-throughput sequencing platforms. The primary application of this pipeline is to convert SAM files to BAM format (a binary version of a SAM file) and to sort the resulting BAM files based on their alignment position against the reference genome. This binary format is optimised in terms of memory usage and computational requirements in downstream variant calling pipelines.

The surrogate genome exhibiting the highest percentage of accurately mapped sequences – where paired-end sequences were mapped in the correct order within the expected range based on the insert size – was selected as the best pseudo genome to use prior to the variant calling step. The surrogate genome with the highest number of properly mapped sequences was first indexed using the bwa index command which enables more efficient sequence alignment. Then quality-filtered sequences were mapped against this genome using the bwa-mem algorithm. In the resulting SAM files, only a subset of sequences with a mapping quality higher than 20 (which is the probability of erroneous alignment at less than 0.01%) was converted to the BAM format in SAMtools and was subsequently sorted and compiled into mpileup format in the same package. An mpileup format is a summary of the coverage of mapped reads across a reference sequence at the resolution of single base pairs. Following the mpileup step, all unmapped sequences were extracted from the resulting SAM files for the de novo assembly step (see below).

Single nucleotide polymorphic sites (SNPs) were identified using Varscan2 (Koboldt *et al.* 2012), which is a commonly used software to detect variants from high throughput sequencing data. Only variable sites with a coverage of more than six reads and a p-value less than 0.01 (which is the probability of generating a false positive), were selected for population genomic analysis. In the resulting variant calling format (VCF) files, all putative indels (a general term referring to insertion, deletion, or insertion and deletion of nucleotides in genomic DNA), multi-allelic sites (locations in the genome containing three or more alleles), and all sites with more than 70% missing data and a minor allele frequency less than 1% in the population were filtered using a combination of VCFtools v.0.1.16 (Danecek *et al.* 2011) and BCFtools v.1.18 (Danecek *et al.* 2021), a suite of scripts designed to manipulate VCF files and their binary equivalent, BCF format, respectively. Similarly, all sites within an LD coefficient more than 60% were filtered prior to downstream analysis in the same packages.

Average read coverage per variable site assigned above, the percentage of missing genotypes per individual, and the percentage of transitions [defined as the number of mutations that results in the change of nucleotides from adenine (A) to guanine (G), or cytosine (C) to thiamine (T)] and transversions (defined as the number of mutations that results in the change of nucleotides from A to C, G to T, A to T and G to C) were estimated in the same packages using recommended commands in the manual (<https://vcftools.github.io/index.html>). The functional

annotation of variable sites and the mutation effect were summarised in SnpEff v.5.2 (Cingolani 2012).

4.2.3.3 De novo assembly

Paired-end unmapped sequences were extracted using picardtools v.3.1(<https://broadinstitute.github.io/picard/>), which is another set of tools for manipulating high-throughput sequencing data in the form of SAM and BAM files.

The extracted fastq files were examined in fastq-pair v.1.0.5 (Edwards and Edwards 2019) to make sure all forward and reverse reads were synchronised. Then all these reads were assembled de novo in MEGAHIT assembler v.1.2.9 (Li *et al.* 2015, Li *et al.* 2016). The aim of this step was to assemble a pseudo-reference that can serve as a template for the subset of sequences that have not been mapped against the surrogate reference genome similar to the methods tested in Pool-seq data (Nielsen *et al.* 2020, Emami-Khoyi *et al.* 2023). The variant calling and filtering steps were similar to the steps explained for the reference-based assembly in the previous section.

The two data sets (i.e., de novo SNPs and reference-based – see below for details on this assembly) were analysed in parallel. While it may be simpler to concatenate these two databases into a single database that include SNPs from both methods, concatenation might result in unpredicted behaviour during the estimation of the summary statistics since the reference allele in the reference-based assembly and that of de novo assembly were called based on different genomes. Moreover, genomic coordinates on the pseudo-genome do not have any particular functional meaning and merely represent locations across a long string of unannotated DNA. By using this hybrid method, the strength of assigning biological function to variable sites based on a well-annotated reference genome of a closely related species, and the statistical power of having a second independent subset of variable sites in a de novo approach, were simultaneously harnessed.

4.2.3.3.1 De novo assembly of *P. angulosus* mitogenome from ezRAD sequences

During the de novo assembly of ezRAD sequences, it was noticed that the MboI enzyme used to cleave nuclear DNA also cut through the mitogenome more than 24 times. The possibility

of assembling complete or partial mitogenomes as a source of extra information from the generated data set was therefore investigated. To this end, raw sequences from each sample were assembled de novo in MitoZ v.3.6 pipeline, which is a toolkit for the de novo assembly of the mitochondrial genome. MitoZ uses a local implementation of MEGAHIT, a next generation sequencing de novo assembler, to assemble all putative contigs (continuous segments of DNA made up of sets of overlapping DNA fragments) of mitochondrial origin. This pipeline allows users to select a desired taxonomic group such that other sequences that are identified as those originating from non-target taxa groups will be filtered and excluded. MitoZ was run with default settings, except for the target taxa group that was set to Echinodermata. The assembled contigs were first searched against NCBI nucleotide database to identify their origin, and then a combination of MITOS web server and MitoZ ('annotate' subcommand) was used to identify the location of different sequencing features (protein-coding genes, transfer RNAs, and rRNAs) on the assembled mitogenome with descriptive information about structure or function.

4.2.3.3.2 Estimation of summary statistics

Summary statistics such as the level of heterozygosity, Tajima's D (Tajima 1989), fixation index (F_{st} , Wright 1951, Weir 2012), and its analogous G_{st} and G'_{st} (Culley *et al.* 2002) were calculated using a combination of VCFtools and BCFtools and the VCFR v.1.15 (Knaus and Grunwald 2017) and SambaR v.1.9 (de Jong *et al.* 2021) R packages.

Briefly, Tajima's D is a commonly used population genetics parameter that compares the observed nucleotide diversity with the value expected when all polymorphisms are selectively neutral and population size is constant. Empirical studies show that Tajima's D statistics and its 95% confidence interval typically vary between -2 and 2. Positive values indicate an excess of variants and could point to the presence of balancing selection or population contraction. Negative values, on the other hand, indicate the presence of large numbers of rare variants that suggest positive selection or population growth (i.e., expansion after demographic bottlenecks). The values that exceed -2 or 2 are of particular interest (Tajima 1989).

The fixation index F_{st} and its two analogues, G_{st} and G'_{st} measure the level of genetic differentiation between populations, and varies between 0 and 1, where 0 is complete panmixia and value of 1 shows the maximal genetic differentiation between populations. The only

difference between the two methods is that F_{st} is a population parameter estimate while G_{st} is calculated directly from observed data (Verity and Nichols 2014).

4.2.3.3.3 Detection of population structure

The presence of the genetic structures among populations was first visually investigated using the discriminant analysis of principal components (DAPC) function implemented in adegenet v.2.1 (Jombart and Collins 2015), an R package used for the multivariate analysis of genetic markers. In parallel to this method, a minimum cross-entropy method, implemented in another R package LEA v.3.2.0 (Frichot and Francois 2015), was used to investigate the presence of population structure in more detail. The presence of isolation by distance (IBD), which is the positive correlation between genetic distance and geographic distance, was tested in adegenet by using Nei's genetic distance (Nei 1972) and Euclidean geographic distances among populations. Ultimately, three populations were decided on for this study. Throughout the rest of this chapter, 'Pop1' or 'population one' refer to what I define as the east coast, which merges the Natal and Agulhas Bioregions. Thus, population one encompasses all sites from Port St John's to Betty's Bay ($n = 64$). 'Pop2' or 'population two' refer to the south-western Cape which corresponds to the population Kommetjie ($n = 3$). 'Pop3' or 'population three' refers to the populations on the west coast, from Bloubergstrand to McDougall's Bay ($n = 14$). Population two is nested within population three and is investigated as a border area between populations one and three.

4.2 3.3.4 Detecting signals of local adaptation

There are two major categories of analytical methods to detect the putative loci under selection: A) outlier detection, and B) environmental association analysis, also known as genotype-phenotype association analysis.

To identify putative loci under local adaptation in *P. angulosus*, a combination of both methods was used. For outlier detection, OutFLANK v.0.2 (Whitlock and Lotterhos 2015), which is based on detecting extreme values of F_{st} that significantly differ from background levels, and pcadapt v.4 (Luu *et al.* 2017), which identifies loci that significantly contribute to population structure, were used. In both methods, the steps explained by developers in the public

repositories were closely followed, except for commands that need to modify the parameters based on the generated data set.

In genotype-phenotype association studies, the widely used R package, LEA v.3.1 (landscape and ecological association studies; Frichot and Francois 2015) was used. This method uses a minimum cross-entropy (Frichot and Francois 2015) approach to identify the underlying genetic structures within populations. This task is crucial since association studies are highly sensitive to the presence of concealed genetic structure within populations. Then LEA uses the estimated number of clusters as a latent variable in the model to find loci that are linked to the selected phenotype, or environmental conditions. In LEA, one environmental measurement, sea surface temperature (SST), and two morphometric traits (test height and test diameter) were used as a covariant. In addition to LEA, the Genome Wide Differentiation Scan (GWDS; de Jong *et al.* 2021) method was also tested. In this method, the population of origin for each individual (east coast vs west coast) was used as a categorical variable to model potential associations between location and the genetic makeup of individuals.

The predicted changes in allele frequency were analysed under projected scenarios of climatic changes for the year 2100 (Van der Walt *et al.* 2019) using a recently developed method implemented in the R package AlleleShift v.1.1.2 (Kindt 2021). This method uses a combination of redundancy analysis (RDA, Legendre and Anderson 1999) and generalised linear models (GLM) to model allelic changes through time and is especially useful when the functional significance of selected allele(s), based on an annotated reference genome, is known. Since this information is not available for *P. angulosus*, this method was first tested on a subset of 100 independent loci with no missing data across populations. Then, allelic shift in a subset of outlier loci, which could potentially point to the role these loci play in the development of adaptive divergence among sea urchin populations, was estimated. The aim here was to show that different populations may respond differently to the scenarios of climatic change and that shifts in the allele frequency of putatively adaptive loci can provide a prediction about the resiliency of populations in the face of changing environmental conditions.

4.2.3.3.5 Reconstruction of the sea urchin mitogenome phylogeography

To reconstruct the phylogeography of the species across the South African coastline, Bayesian Evolutionary Analysis Sampling Trees (BEAST) v.1.4 was used (Drummond and Rambaut

2007). Prior to running this pipeline, the input file was generated in BEAUti, a graphical user-interface application for generating files compatible for use in BEAST. There are two phylogeographic reconstruction models implemented in this package: A) a phylogeographic model across continuous space; and B) a phylogenetic model across discrete space. Given that this pipeline is developed to model the spread of airborne diseases across landscapes (i.e., epidemiology), its implementation for a marine organism that can only be dispersed across marine corridors was unconventional, and to the best of my knowledge, this study is the first to use this model in the context of marine ecosystems. Phylogeographic diffusion in discrete space can be simulated using both symmetric and asymmetric trait substitution models. The distinction is that the asymmetric method takes into account the fact that the dispersal of marine organisms typically follows marine gradients and consequently the rate of movement of individuals in different directions might not be equally likely, making this the appropriate model for use in this study. To provide a statistical support for the selection of the trait substitution model and to determine which of these models fit the data better, however, a stepping stone simulation with path sampling was performed in the BEAST package. Briefly, path sampling is the initial step of the Bayesian analysis to evaluate the fit of competing scenarios, also known as models. This provides a subjective statistical framework to select the model that explains the data most accurately. To this end, the probabilities of competing phylogeography models were evaluated based on 100 steps [which cover the probability space between the selected prior and the predicted posterior (i.e., the observed pattern in the data) of the model], each with 1 000 000 iterations. The remaining parameters in BEAST were set to their default values as explained in the programme tutorial (<https://www.beast2.org/tutorials/>). To estimate approximate divergence time, the prior for the molecular clock rate was set to a uniform prior probability with a minimum of 0.007 and a maximum of 0.02 divergence per lineage per million years, (which means the two sister taxa are believed to diverge in the entirety of their nucleotide from each other based on this rate) since this range includes the consensus value for the mitogenomic clock rate reported for other echinoderms (Loeza-Quintana 2017). The BEAST package was run for ten independent simulated chains, each starting from a random point to explore the probability space. The convergence of different chains provides strong support about the optimal performance of the estimates. In the simulation, the number of iterations for each chain was set to 500 000 000, with 40% burn-in steps, which correspond to the iterations at the beginning of the simulations, which are typically not close to the optimal value and are therefore discarded. The resulting files were combined and summarised in logcombiner, which is a part of the BEAST package. The convergence of

different chains in terms of effective sample size (ESS) was visually investigated in Tracer v.1.7.1 (Rambaut *et al.* 2018), a programme for analysing trace files generated by Bayesian statistics. A maximum clade probability consensus tree was generated in Treeannotator v.1.4 and the resulting tree was visualised in Figtree v1.4.4 (<https://github.com/rambaut/figtree/releases>). The series of events that lead to the colonisation of the South African coastline by *P. angulosus* was visualised using Spread3 v.0.9.7 (Beilejec *et al.* 2016), an interactive tool for visualising the spatio-temporal history and trait evolutionary processes of species.

4.3 Results

The sequencing run resulted in a total of 295 925 978 quality filtered sequences. Each sample produced an average of 1 720 499.87 sequences with an average length of 141.6 bp.

4.3.1 Reference-based assembly

The mapping of ezRAD-generated sequences against the surrogate reference genome resulted in a total of 149 623 891 properly mapped sequences. On average, 1 824 681.598 ezRAD tags were mapped per sample with a maximum probability of the erroneous mapping of 1%.

The reference-based variant calling identified a total of 133 624 variant sites across individuals. Among those variants, 134 894 were SNPs, 88 were insertions, and 56 were deletions. The ratio of transitions to transversions for the assigned variable sites was estimated at 1.3 (757.457/561.373). The functional annotation of SNPs identified that 81.95% of variant sites as silent (mutations that do not change the amino acid), 0.179% as nonsense (mutations that change the amino acid codon into a stop codon), and 17.86% as missense (mutations that changes the amino acid to a different amino acid, Table 4.1).

Table 4.1: Number of mutations by classed by function.

Type	Count	Percent
Missense	21 053	17.867%
Nonsense	211	0.179%
Silent	96571	81.954%

The number of effects by impact shows that the majority of the mutations are modifiers followed by low, moderate, and high impact mutations (Table 4.2).

Table 4.2: Counts and percentages of mutations classed by the impact of the mutation on the genome.

Type	Count	Percent
High	229	0.084%
Low	97.12	35.6%
Moderate	21.041	7.713%
Modifier	154.42	56.603%

The majority of the variable sites were synonymous variants (Table 4.3, Figure 4.1).

Table 4.3: Counts and percentages of mutations by type.

Type	Count	Percent
3 prime UTR variant	4 082	1.492%
5 prime UTR premature start codon gain variant	255	0.093%
5 prime UTR variant	1 932	0.706%
Downstream gene variant	18 089	6.611%
Frameshift variant	3	0.001%
Initiator codon variant	1	0%
Intergenic region	43 767	15.995%
Intron variant	67 853	24.798%
Missense variant	21 041	7.69%
Splice acceptor variant	2	0.001%
Splice donor variant	2	0.001%
Splice region variant	825	0.302%
Start lost	5	0.002%
Stop gained	211	0.077%
Stop lost	6	0.002%
Stop retained variant	2	0.001%
Synonymous variant	96 569	35.292%
Upstream gene variant	18 982	6.937%

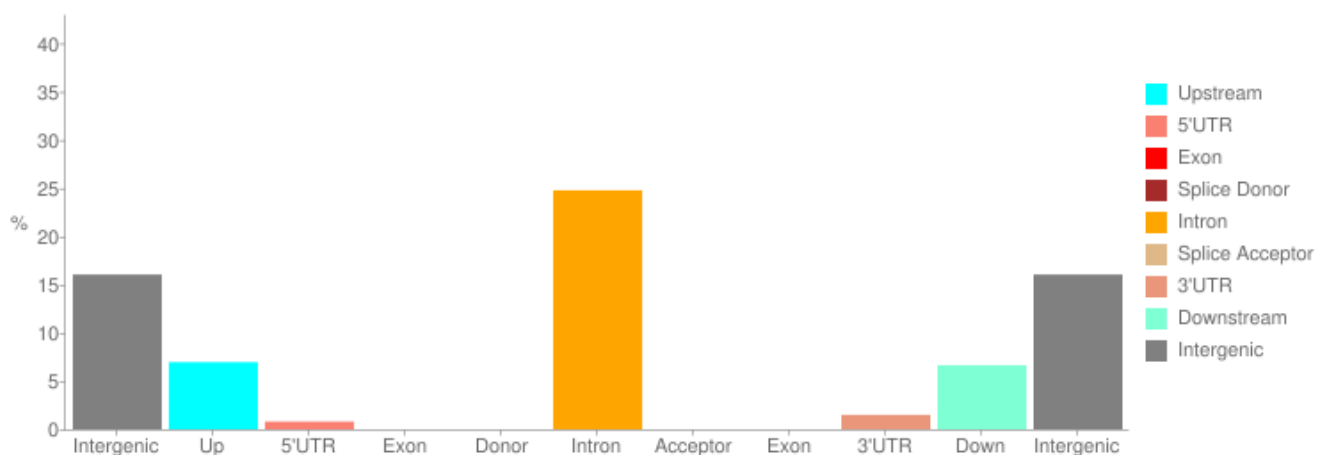


Figure 4.1: Bar plot depicting the genome regions where variant sites are situated.

The base changes (SPNs) show that mutations from A to G, from C to T, and from T to C were the most commonly observed mutations (Table 4.4).

Table 4.4: The base changes (SNPs) caused by mutations.

	A	C	G	T
A	-	7.023	1,497	10.45
C	6.668	-	4.99	18.858
G	18.758	4.973	-	6.668
T	10.443	19.39	7.176	-

Codon changes from GAA to TAT and from GAT to TAC were the most prevalent (Appendix 1: Table A1.1).

After filtering for multiallelic loci, indels, variable sites that were not shared between at least 70% of individuals, and those with a minor allele frequency less than 0.01 percent (i.e., singletons) that could point to false-positive variant assignment, a total of 3 032 high-quality SNPs were selected for downstream analysis. The average percentage of missing data was 0.11% and each variable site across the populations was covered by an average 45.2 sequences, pointing to the high quality of the generated SNP dataset. No correlation between the level of heterozygosity and read depth was observed, and this confirmed that genotype calling was consistent across populations. One can expect to see a significant association if the call of the heterozygote genotypes is significantly correlated to low sequence coverage, which means that

the risk of having only one of the two alleles sequenced will increase, hence, the genotyping of heterozygotes is reliable (Figure 4.2).

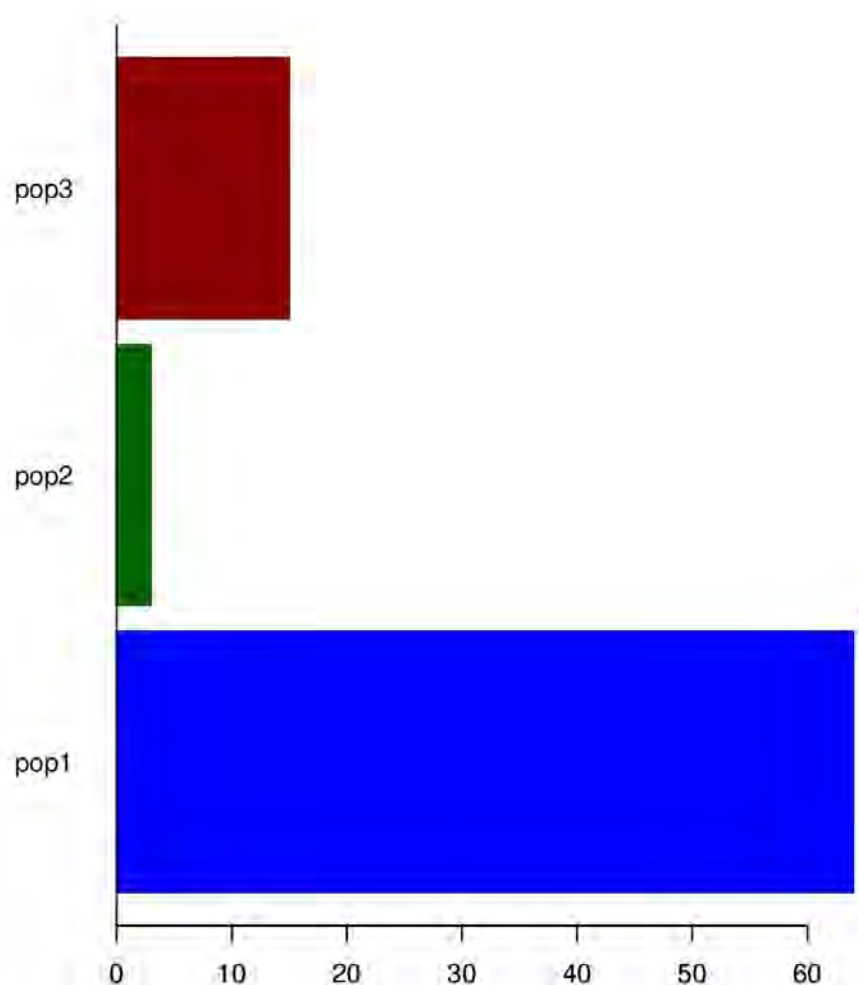


Figure 4.2: Vertical bar plot showing the number of discarded (-) and retained (+) individuals per population. The figure shows that none of the individuals were removed due to low-quality or low-coverage sequences.

The majority of called genotypes were homozygous for the major allele which was the most abundant allele within populations (Figure 4.3).

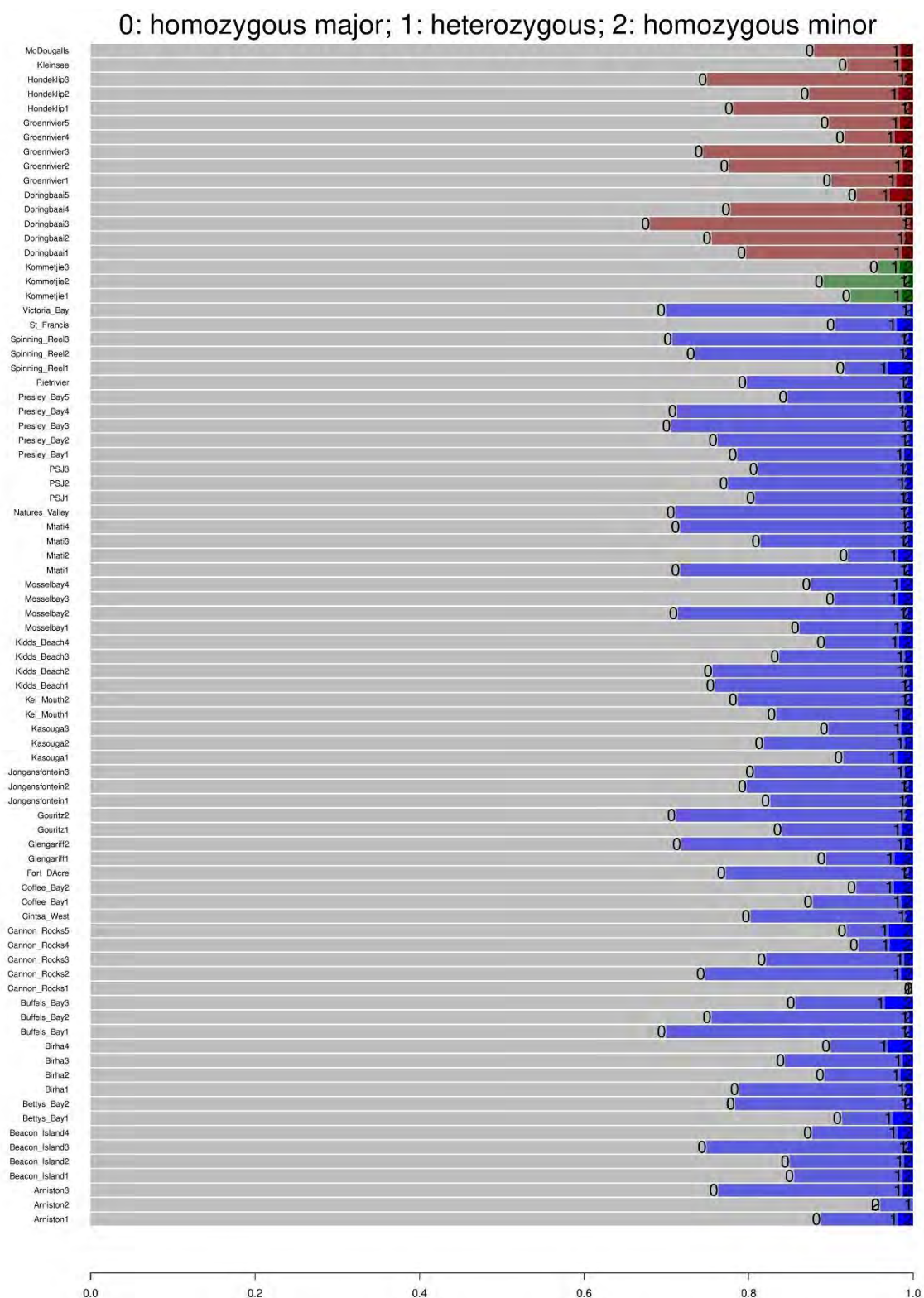


Figure 4.3: The proportion of three different genotypes (homozygous major, heterozygous, and homozygous minor), across sea urchin populations. Red corresponds to individuals from the west coast, green to individuals from the border zone (i.e., Kommetjie, see Figure 2.1 for map reference), and blue to individuals from the east coast.

The average heterozygosity ranged from 0.21 in Presley Bay to 0.06 in Kleinsee and St Francis Bay. The distribution of F_{st} showed that the majority of estimates were less than 0.10. Similarly, the other two indices of population differentiation, G_{st} , and G'_{st} were 0.14 and 0.17, respectively, pointing to the moderate level of population structure in *P. angulosus* populations throughout coastlines (Table 4.5).

Table 4.5: Average heterozygosity estimated for each population based on the total number of assigned SNPs.

Population	Average heterozygosity
Presley Bay	0.21
Mossel Bay	0.204
Gouritz	0.199
Jongensfontein	0.199
Buffels Bay	0.198
Hondeklip	0.191
Groenrivier	0.185
Mtati	0.183
Port St John's	0.183
Kidd's Beach	0.182
Beacon Island	0.178
Doringbaai	0.178
Kei Mouth	0.178
Spinning Reel	0.177
Arniston	0.174
Cannon Rocks	0.174
Kasouga	0.174
Kommetjie	0.17
Glengariff	0.16
Bettys Bay	0.158
Cintsa West	0.158
Coffee Bay	0.158
Rietrivier	0.157
Natures Valley	0.156
Fort D'Acre	0.149
Victoria Bay	0.148
Birha	0.143
McDougall's Bay	0.124
St Francis	0.069
Kleinsee	0.064

Tajima's D was negative for all three populations pointing to an excess of rare variants within populations. The distribution of divergence within samples from one bioregion and that of the difference between bioregions showed a moderate level of variation. There was some variation in the coefficients of relatedness within each bioregion, however, relatedness between these populations was not significantly different (Figure 4.4).

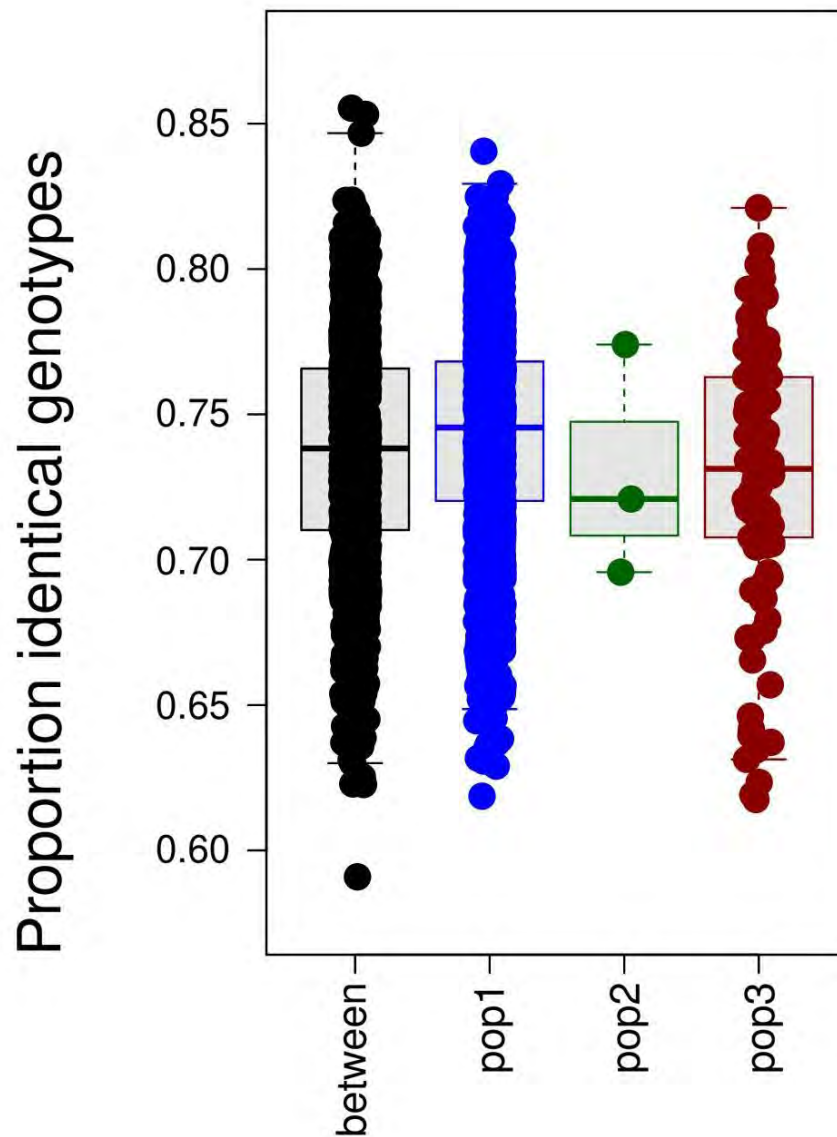


Figure 4.4: Boxplots of the estimated value for Tajima's D and the SNP divergence between and within populations of *P. angulosus*. Blue, green, and red colours show the east coast, south-western cape, and west coast, respectively.

When all populations within a bioregion were combined, the value of G_{st} and G'_{st} dropped to 0.013 and 0.02, respectively. The maximum estimated Nei genetic distance between populations was 0.17 (Figure 4.5).

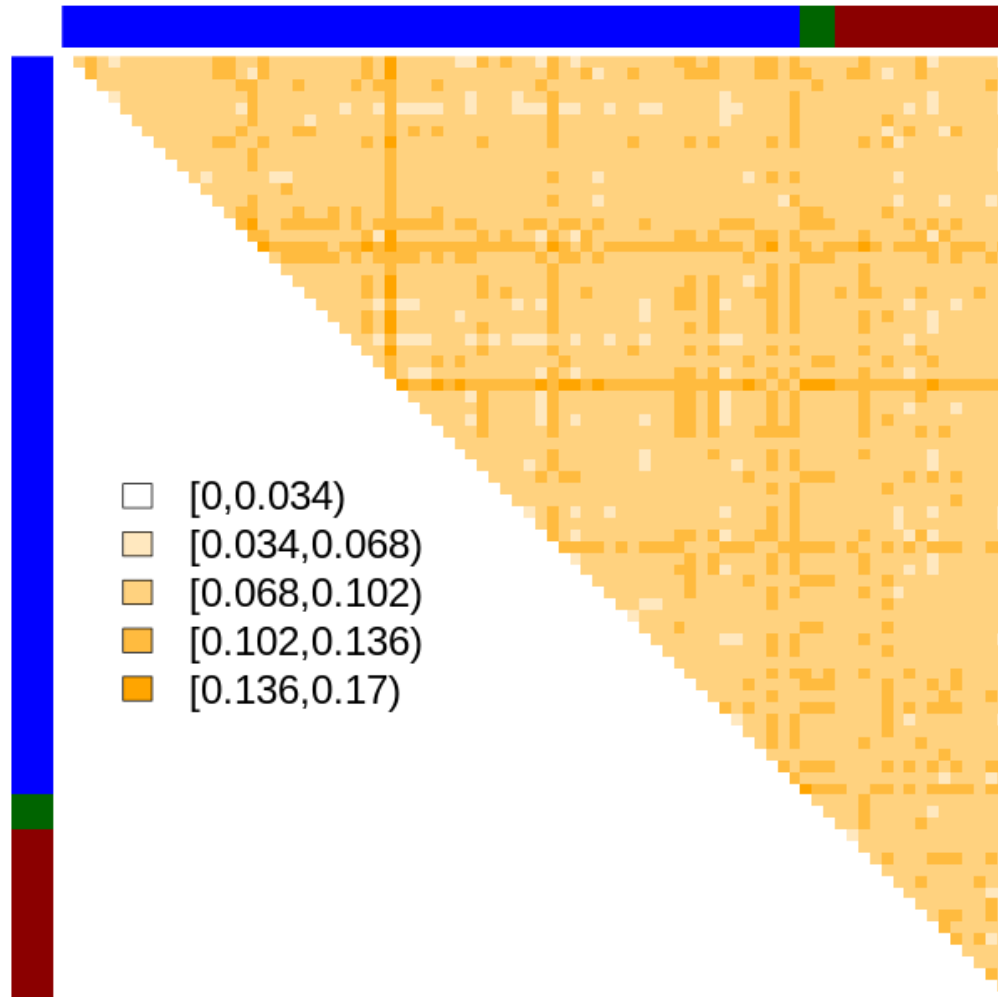


Figure 4.5: Heatmap showing the genetic distance between individuals within different bioregions. Blue, green, and red colours show the east coast, south-western cape, and west coast, respectively. Values and shading on the plot indicate the extent of the genetic distance between individuals.

The discriminant analysis of principal components did not identify clear clustering patterns within the data such that the individuals from different bioregions (west coast, south-western coast and east coast) did not cluster distinctively based on their population of origin in the eastern or western coast of South Africa (Figure 4.6).

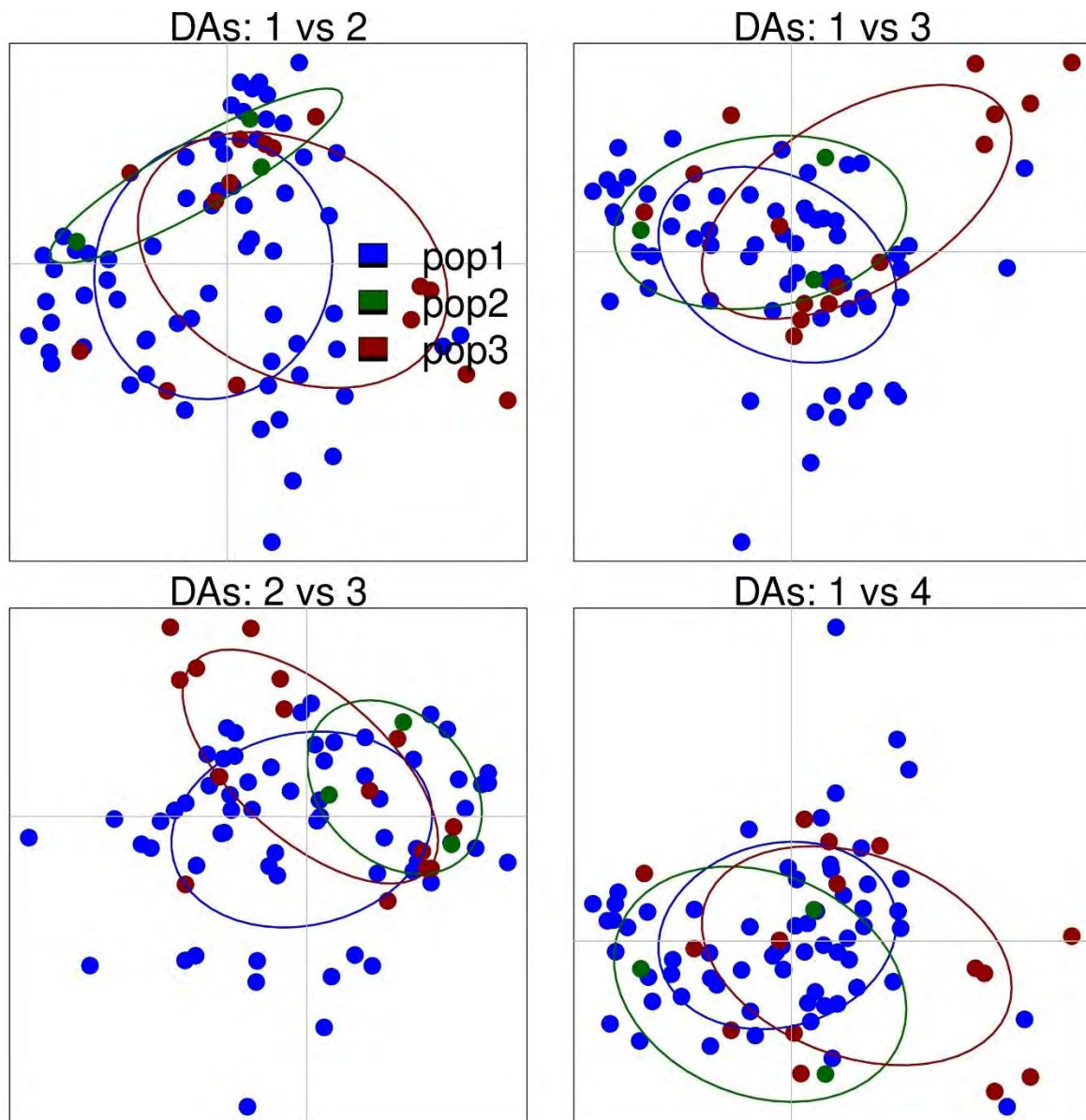


Figure 4.6: Graphical results of discriminant analysis of principal components on the generated SNP data sets. Blue, green, and red colours show the east coast, southwestern cape, and west coast, respectively, corresponding with the colours in Figure 4.4 to delineate the same populations, which do not overlap. The four plots generated for the four top axes indicate the highest proportion of the variability within populations.

The poor performance of the DAPC was also obvious in the resulting BIC graph (not presented), since no stable estimate for the putative number of clusters was calculated, and the graph descended in the complete range of the K-value tested, which is typical of this method that cannot perform optimally. The cross-entropy method implemented in the LEA R package, however, identified the presence of three putative population clusters conclusively, and this

result was strongly supported by a typical elbow shape of the generated graph that has a minimum at $K = 3$ (Figure 4.7 and 4.8). However, the three identified ancestral clusters do not show a correlation with geographical locations in contemporary populations and no geographic pattern in the coefficient of co-ancestry of individuals from different bioregions was observed. The ancestral clusters correspond to those identified in the phylogram in Figure 4.16.

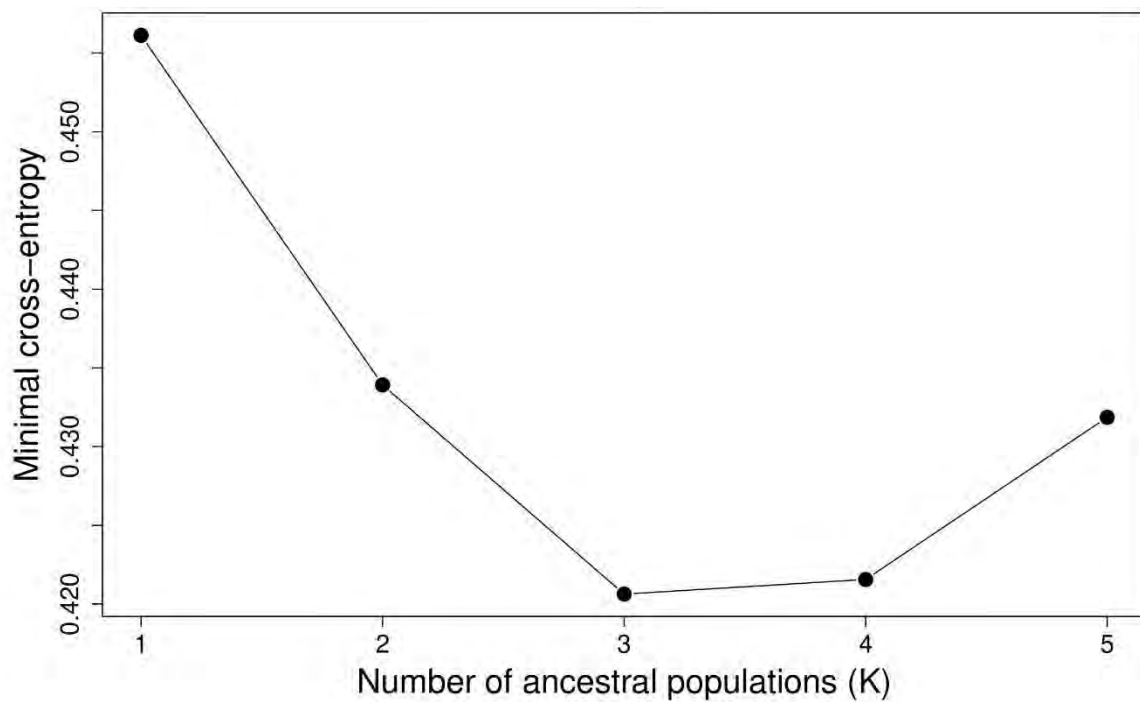


Figure 4.7: Cross-entropy graph indicating the number of clusters identified from the data set. A clear optimum is obvious at value $K = 3$.

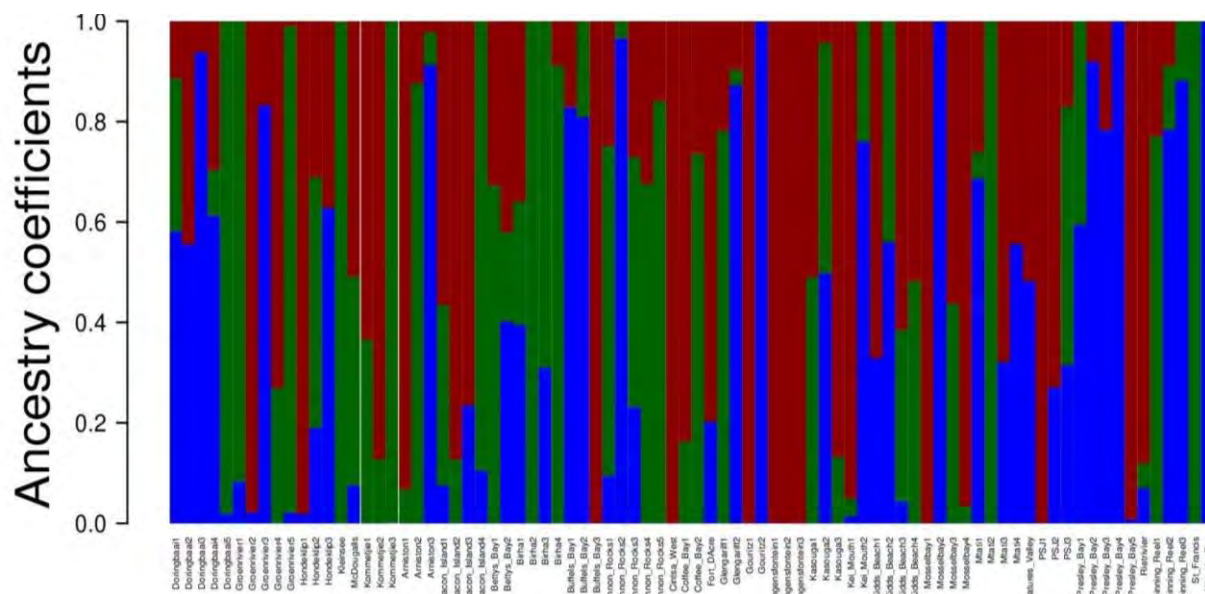


Figure 4.8: Co-ancestry plot showing the proportions of each individual genome that are most likely to originate from three putative clusters identified in the study. Colours here do not represent the three populations as in previous graphs, but the three putative clusters identified in Figure 4.7 above.

When the LEA admixture plot was superimposed over a geographic map of the South African coastline, no clear spatial patterns were observed (Figure 4.9) showing that individuals from three putative clusters are not restricted to one bioregion.

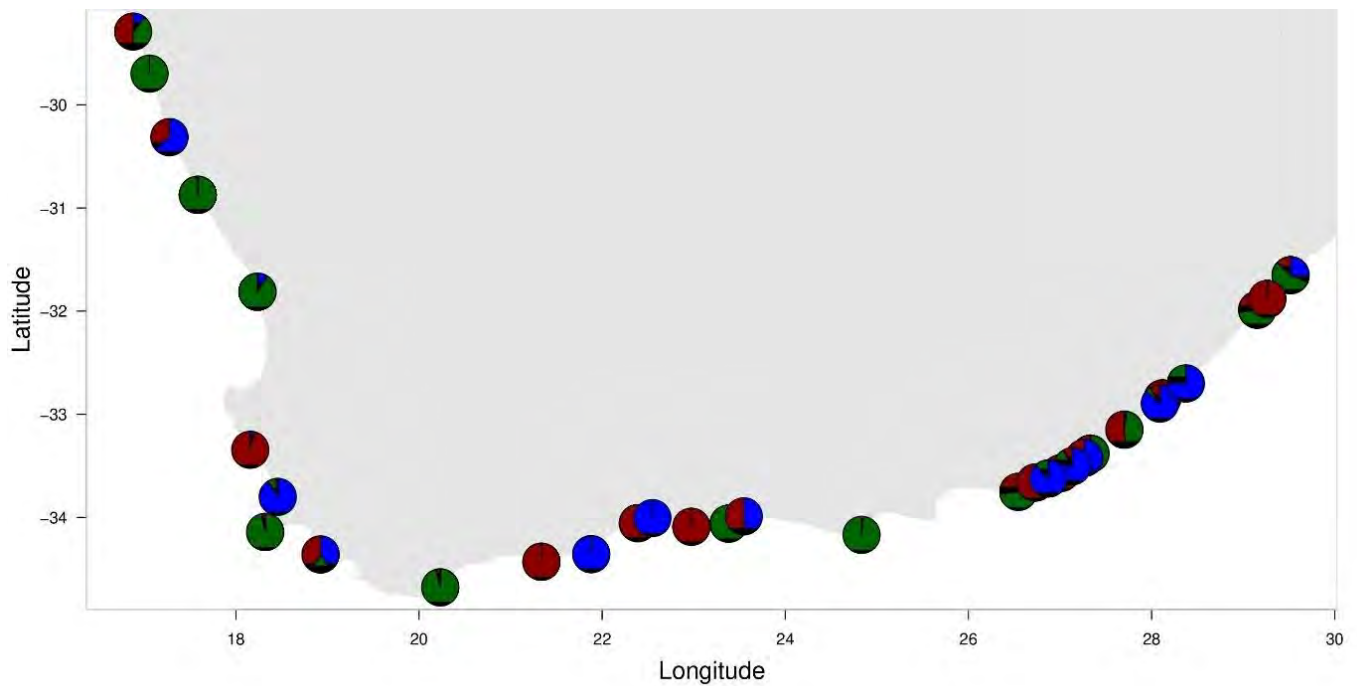


Figure 4.9: Q-matrix plot showing the proportion of co-ancestry of *P. angulosus* population superimposed over the coastal map of South Africa. From west to east these sites read McDougall's Bay, Kleinsee, Hondeklip, Groenrivier, Doringbaai, Kommetjie, Betty's Bay, Arniston, Jongensfontein, Gouritz, Mossel Bay, Victoria Bay, Buffels Bay, Beacon Island, Natures' Valley, St Francis, Cannon Rocks, Kasouga, Spinning Reel, Rietrivier, Fort D'Acre, Mtati, Birha, Kidd's Beach, Glengariff, Cintsa West, Kei Mouth, Coffee Bay, Presley Bay, Port St John's. The colours correspond to the ancestry coefficients in Figure 4.8.

The two tested methods of outlier detection identified a total of ten outlier loci that significantly deviated from the background rate across the genome and could point to the presence of fine-scale local adaptations in sea urchin populations (Figures 4.10 and 4.11). No outlier was shared between the two methods, highlighting the importance of including multiple methods of outlier detection for the maximum identification of putative loci under selection.

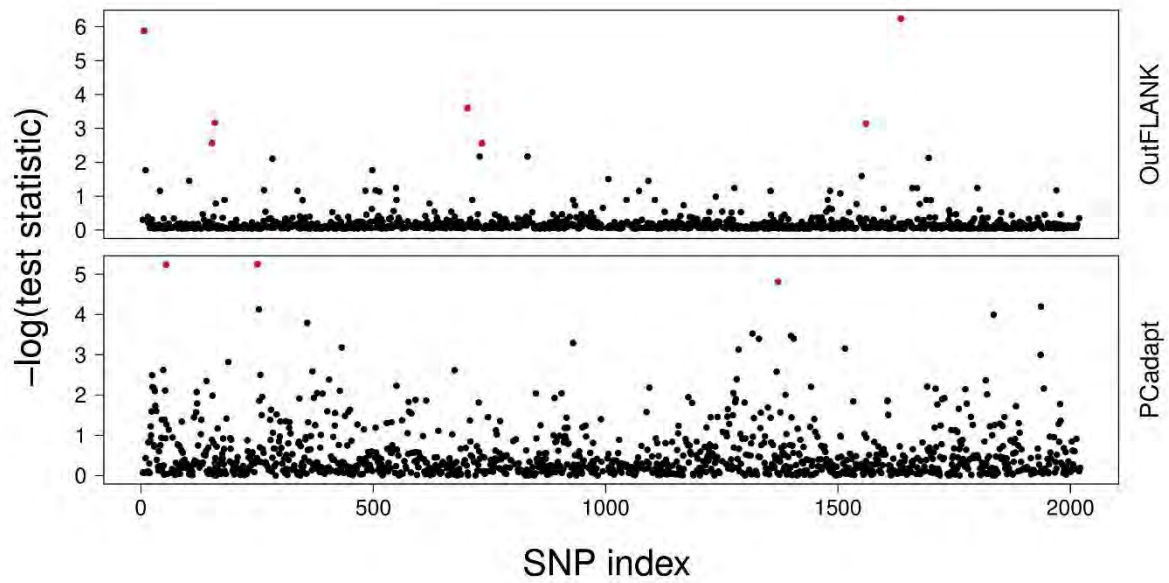


Figure 4.10: A Manhattan plot of the outlier loci detected by the OutFLANK and pcadapt analyses. The red colour shows outlier SNPs and the values on the y-axis are $-\log$ p-values of test statistics for the significance of these loci. Note that values on the y-axis are not on the same scale between the two methods.

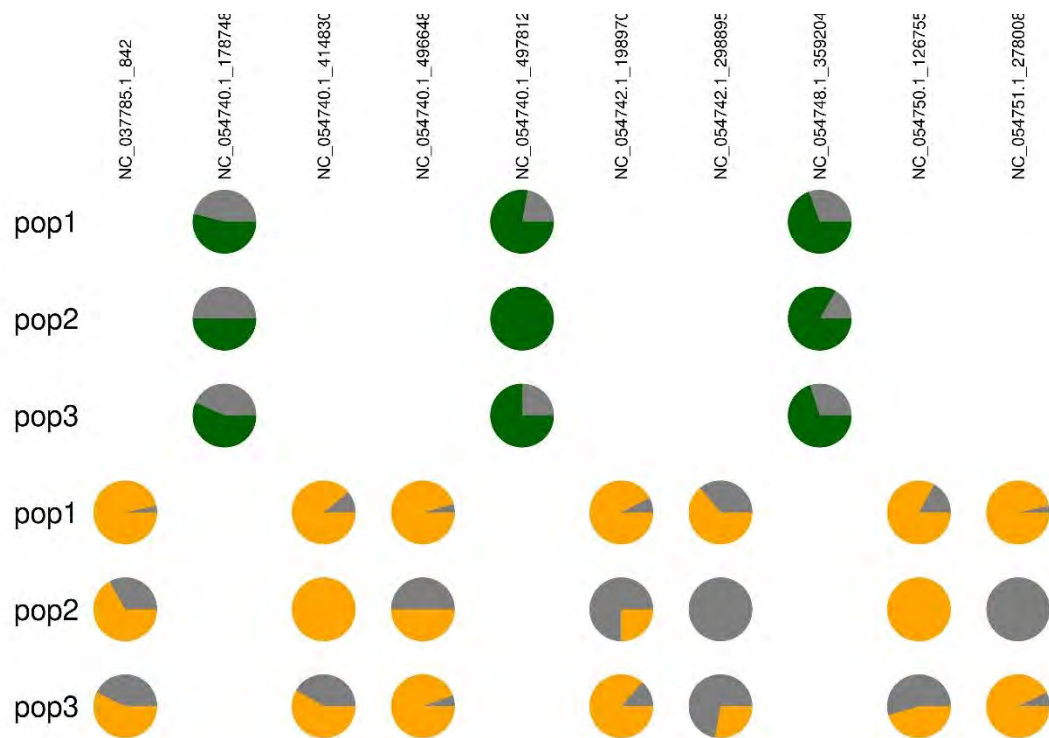


Figure 4.11: Allele frequency changes in the identified outlier loci between populations of *P. angulosus* across South Africa. Green indicates allele frequency changes identified by pcadapt, and orange that of OutFLANK. The values at the top of the figure are SNP indices based on the *Lytechinus variegatus* genome.

The functional annotation of these outlier loci showed that, among the 10 outliers identified using both pcadapt and OutFLANK, seven of these were situated in the non-coding region of the *Lytechinus variegatus* genome. Among the remaining three loci, one was within the boundaries of an uncharacterised protein reported from *L. variegatus* and one of the other outlier loci was within the mitogenome 12S rRNA. The last outlier was within an rRNA binding protein 26-like which is involved in RNA recognition motif. In a similar way, a genome-wide differentiation scan (GWDS) identified two loci that significantly vary in allele frequency between the coastlines of South Africa and could point to the presence of diversifying selection (Figure 4.12).

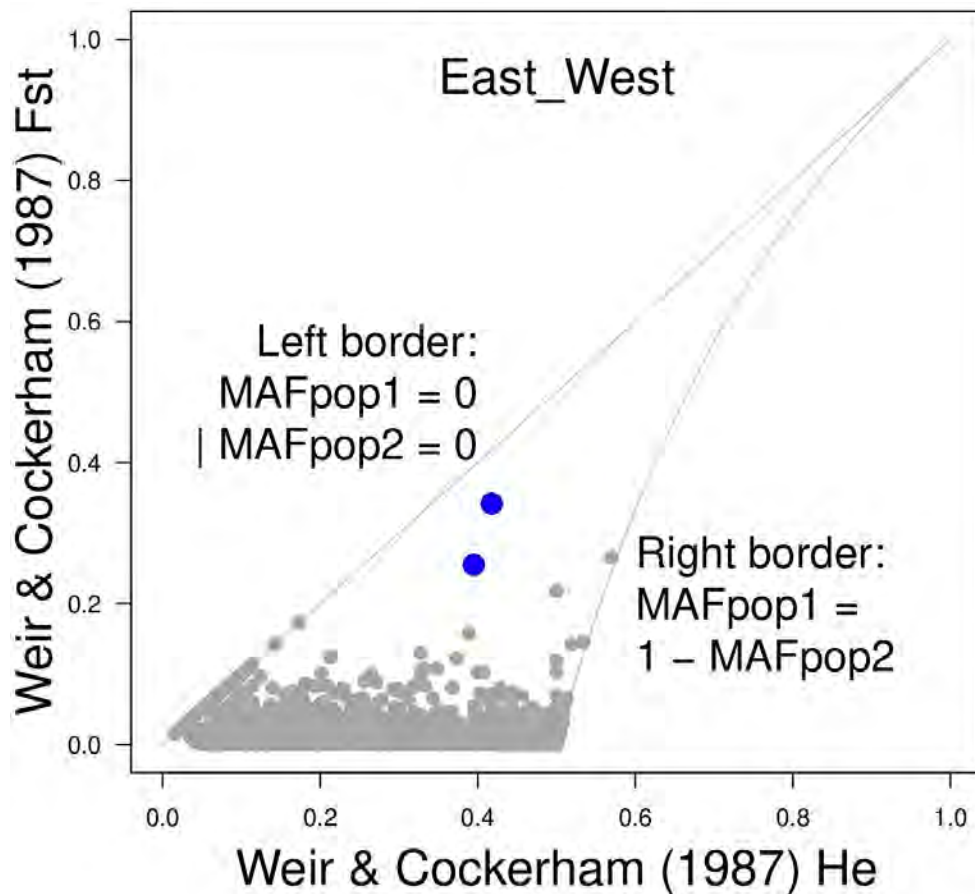


Figure 4.12: Two loci identified (blue circles) based on genome-wide differentiation scan (GWDS) that significantly differ between the east and west coasts of South Africa.

The simulation of allelic changes under predicted climate change scenarios, in terms of both temperature and pH, showed that, in the dataset of 100 subsampled loci with no missing data across populations, the absolute changes in the allele frequency ranged from 0.0203 to 0.0344. On the subset of loci that were putatively under selection, the allele frequency in Betty's Bay, Cintsa-West, Fort D'Acre, Glengariff, Groenrivier, Kommetjie, Hondeklip, Jongensfontein, Kasouga, Kei Mouth, Kidd's Beach, Kleinsee, McDougall's Bay, Nature's Valley, Rietrivier, Spinning Reel, St Francis and Victoria decreases, and that of Arniston, Beacon Island, Birha, Buffel's Bay, Cannon Rocks, Coffee Bay, Doringbaai, Gouritz, Mtati, and Presley Bay increased. The highest increase was in Coffee Bay and the lowest was estimated to be in Arniston. The genotype-phenotype association analysis identified several loci that were significantly ($p\text{-val} < 0.001$) associated with test height, test width, and temperature (Figure 4.13).

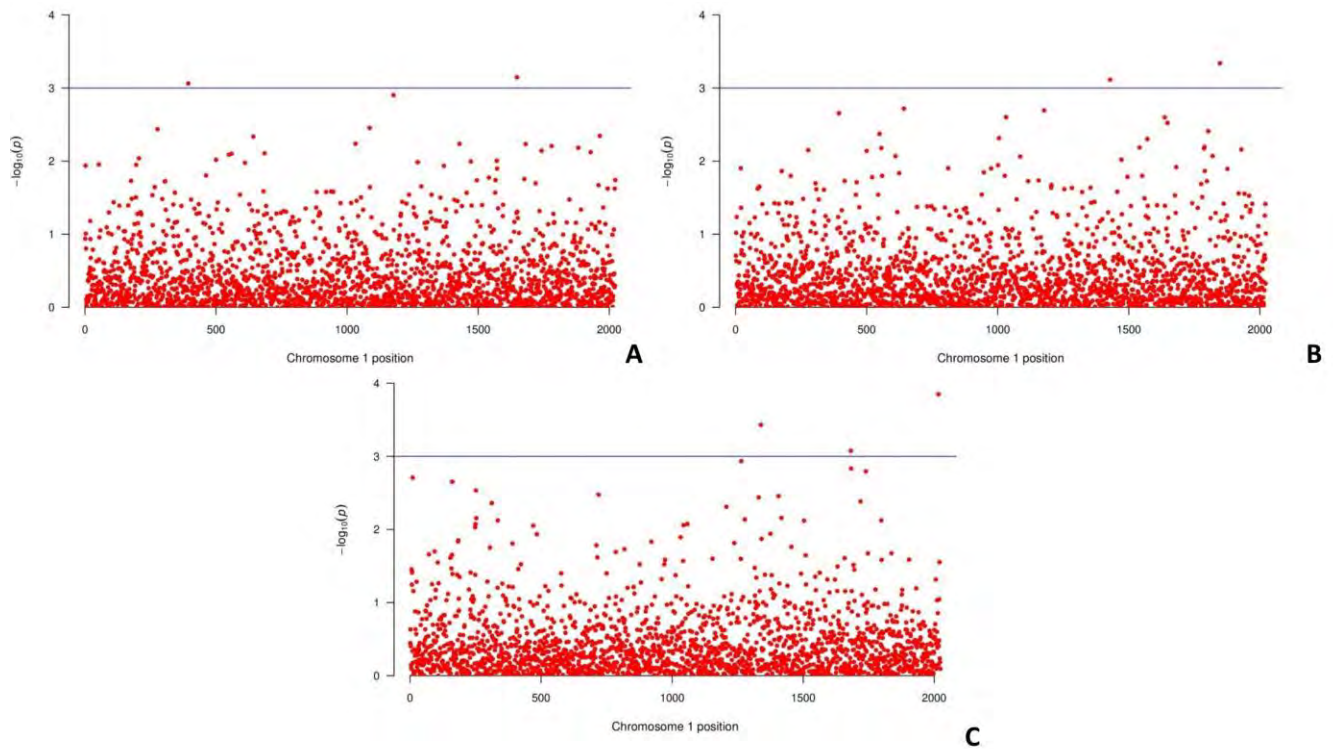


Figure 4.13: Scatter plots indicating loci significantly associated (above the horizontal blue line) with test height (A), test width (B), and temperature (C).

After correcting for a false discovery rate of < 0.05 , however, no significant association was found. This was expected since conservative corrections of p-values in multiple testing are a function of sample size, and such studies require sample sizes far larger (> 100 , Hong and Park 2012) than those used in this study to provide conclusive evidence.

4.3.2. Mitogenome assembly

The MitoZ assembly pipeline assembled a total of 51 partial mitogenomes among which 25 assemblies were complete in terms of having all 37 sequencing features (Figure 4.14, Table 4.6), which were 13 protein-coding genes, 22 tRNAs, and 2 rRNAs. The remaining 26 assemblies had the full sequences for the 13 protein-coding genes which are the most informative for phylogenetic analysis, and the missing sections were mainly some of the most highly conserved tRNAs.

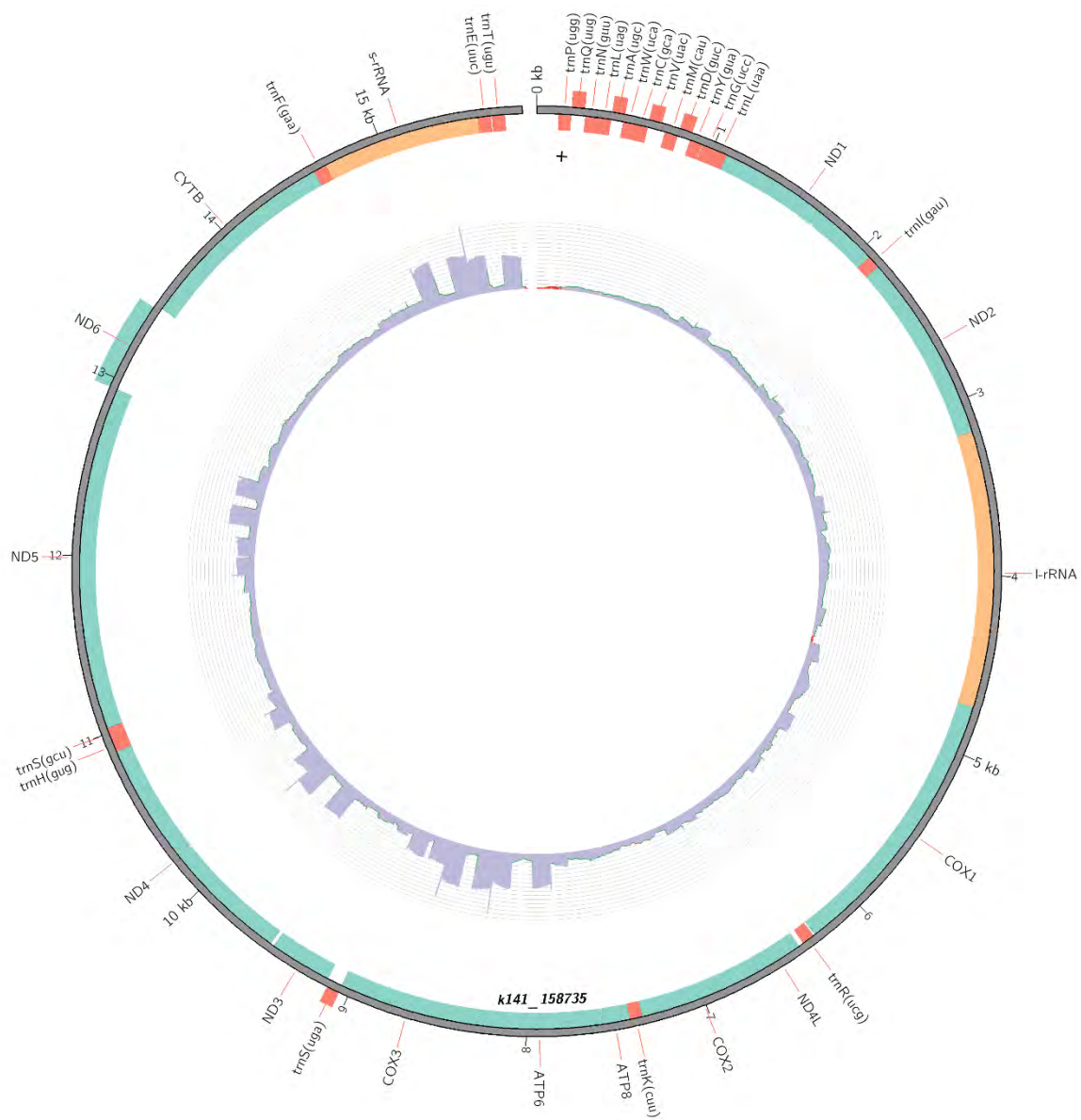


Figure 4.14: Circus graph indicating the location of the 37 mitogenomic features in the representative mitogenome of Cape sea urchin.

Table 4.6: Alignment summary table.

Alignment name	Confidence Interval align cleaned fasta
Number of mitogenomes	51
Alignment length	13949
Total matrix cells	711399
Undetermined characters	42610
Missing percent	5.99
Number of variable sites	840
Proportion of variable sites	0.06
Parsimony informative sites	602
Proportion parsimony informative	0.043
AT content	0.6
GC content	0.4
A	195307
C	151287
G	116338
T	205857
N	42610

When these sequences were used for the reconstruction of the phylogeography of *P. angulosus*, the combined length of the total alignments was 13 949 bp. A total of 840 variable sites and 602 parsimony informative sites were identified. As expected from an assembly that is based on digested sequences as opposed to assemblies based on whole genome sequencing, assembled mitogenomes had different levels of missing data (Figure 4.15, Table 4.6 above).

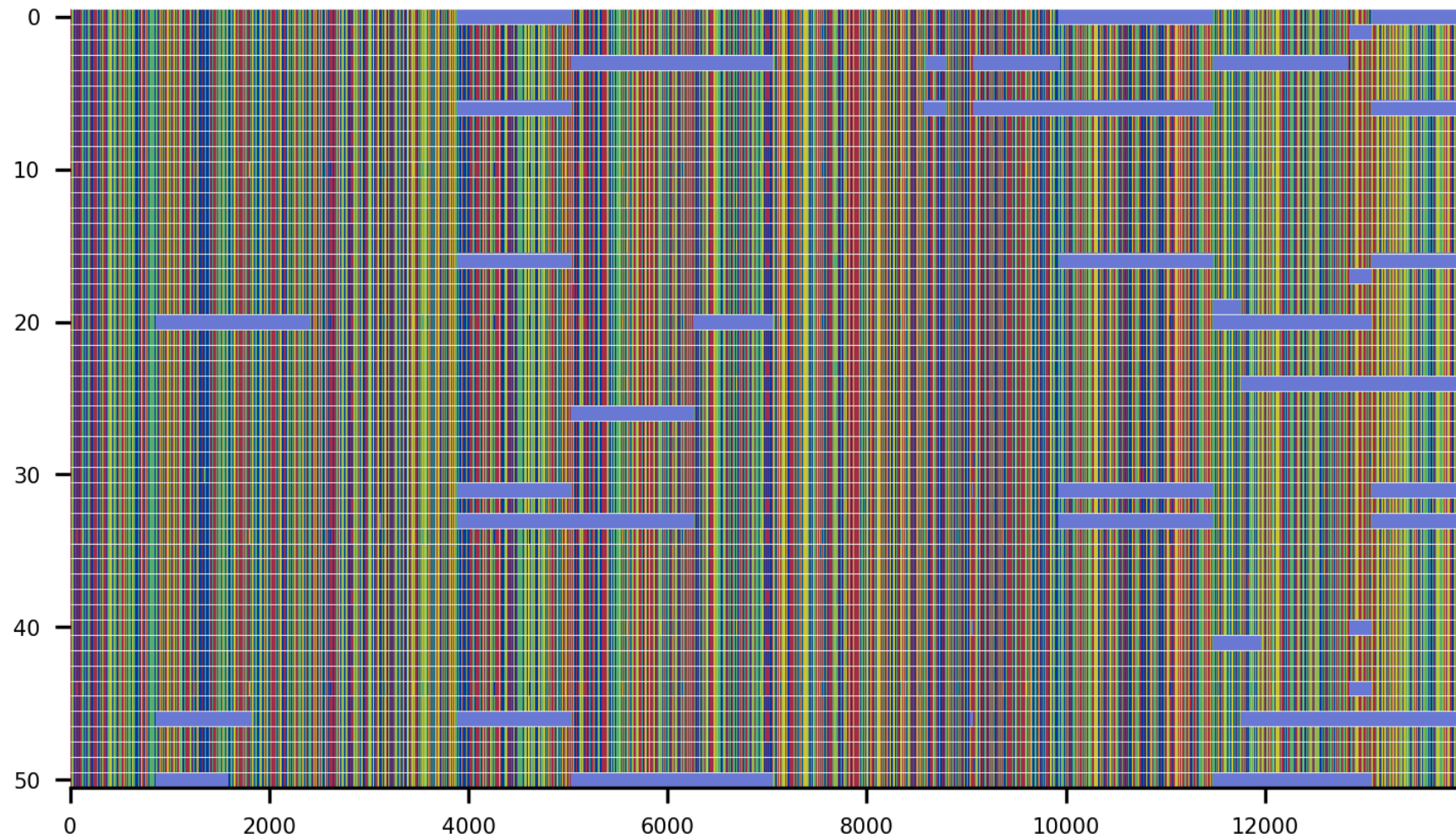


Figure 4.15: Schematic representation of the alignment of 51 partially complete mitogenomes using sequences generated using the ezRAD protocol. Different colours represent different nucleotides (yellow is guanine, red is thymine, green is adenine, and dark blue is cytosine), and the horizontal bars of continuous light blue are the segments that are missing from that assembly.

A phylogenetic tree identified two major groups within sea urchin populations, one older lineage, consisting of four haplotypes, and a younger clade, featuring all the remaining individuals. The most recent common ancestor of these two sister groups dated back to 1.64 mya (95% confidence interval, 1.46 – 1.81). Similarly, within the younger clade, an additional two nested sister groups were identified, which consisted of the majority of the individuals in this study. The divergence between these two groups was more recent and dated back to 0.49 (95% confidence interval, 0.42 – 0.57) mya. The remaining splits occurred within the past 100 000 years (Figure 4.16).

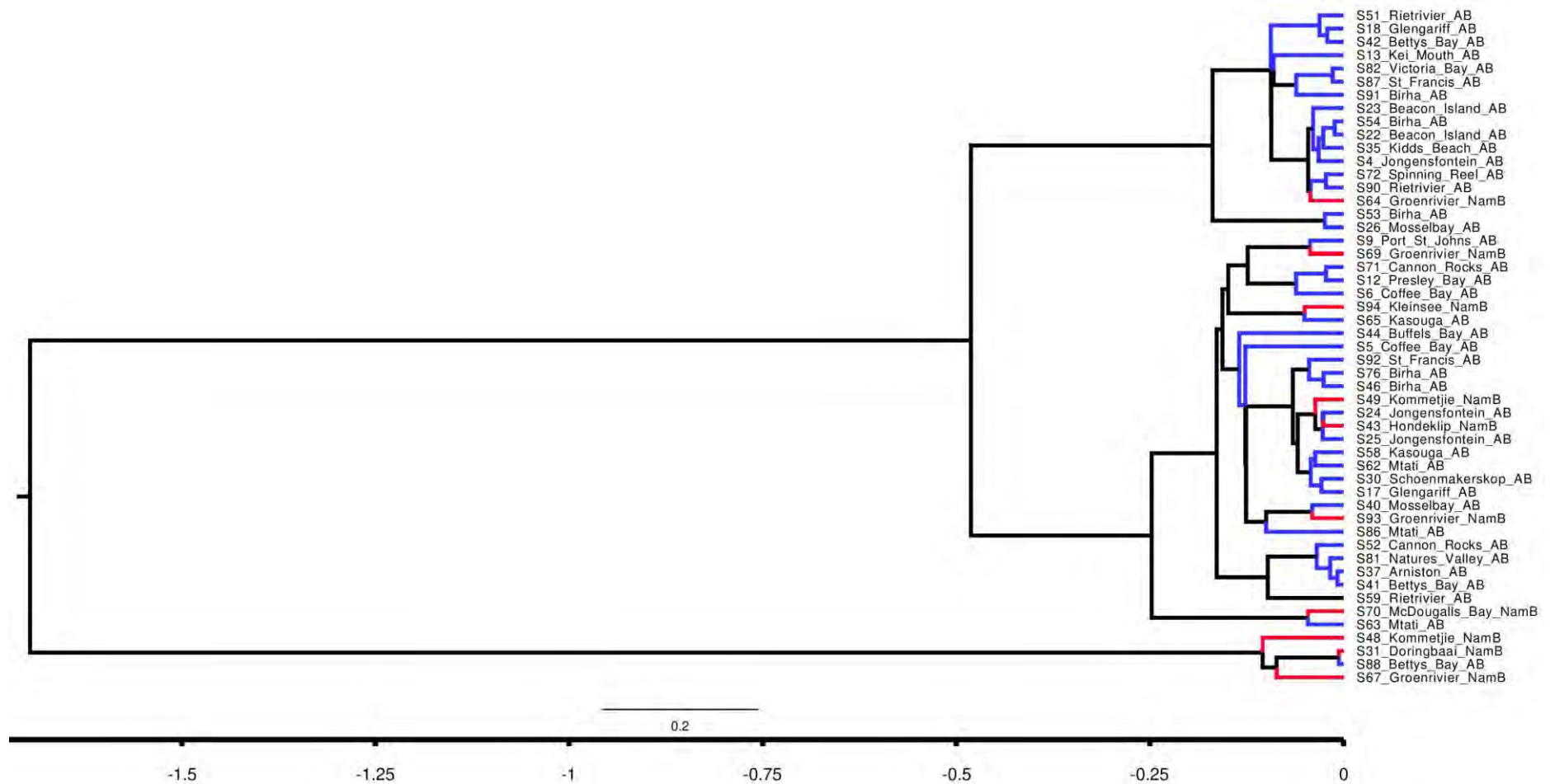


Figure 4.16: Phylogenetic tree representing individuals from different populations of the Cape sea urchin, *Parechinus angulosus* along the South African coastline. The red and blue colours show branches leading to individuals from the east and west coasts, respectively. The x-axis is time to most recent common ancestor in millions of years.

Among the competing phylogeographic models, a diffusion model in the discrete space with asymmetrical migration rates between populations had the highest posterior support. This was reasonable given that it is largely accepted that migration in the marine environment usually follows gradients (e.g., temperature, salinity) and a symmetric mode is less likely to take place. The reconstruction of the dispersal history of *P. angulosus* identified the most likely root point of colonisation on the south west coast at Kommetjie. Then, multiple waves of colonisation, first towards the east coast (Figure 4.17), and more recently towards the west coast (Figure 4.18) took place and colonised South African coastlines.



Figure 4.17: Initial eastward expansion of the Cape sea urchin, *Parechinus angulosus*, from the root point at Kommetjie ($n = 30$, 1 cm = 50 000 cm). The green line indicates the dispersion of the species.

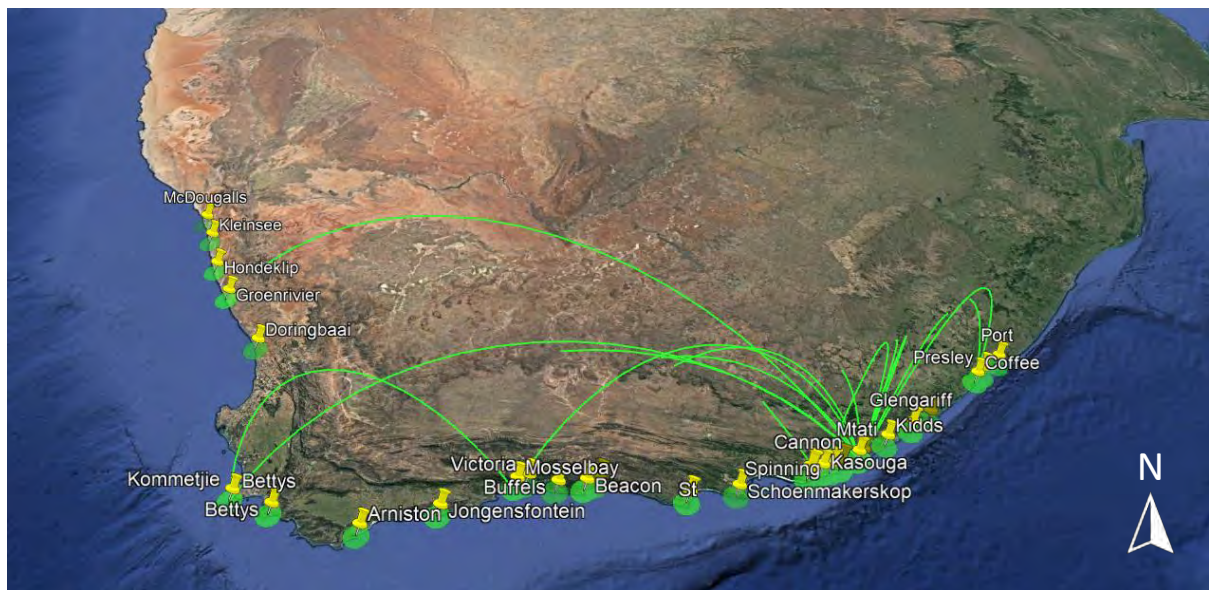


Figure 4.18: Secondary expansion of the distribution range of *Parechinus angulosus* westwards from the east coast sites colonised in the initial wave of eastward expansion ($n = 30$, 1 cm = 50 000 cm). The green line indicates the dispersion of the species.

4.3.3 De novo assembly of ezRAD sequences

The de novo assembly of unmapped sequences resulted in 1 435 632 contigs with a total length of 831 594 379 bp. The N50, which is a genome assembly statistic that shows 50% of the assembled contigs are shorter than this particular value, was 1 116 bp. After applying the same filtering step as explained for the reference-based method, a total of 2 791 SNPs were identified. The average percentage of missing data per individual was 19%, and each variable site across the population was covered by an average of 34.52 reads. Similar to the reference-based assembly, no correlation between sequencing depth and a decline in heterozygosity was observed.

The level of heterozygosity varied from 0.16 in Presley Bay to 0.03 in Kleinsee, and population differentiation indices G_{st} and G'_{st} were estimated at 0.18 and 0.21, respectively. The Fixation index showed that the majority of loci have a differentiation less than 0.15. As in the reference-based assembly, the estimated Tajima's D was negative, pointing to an excess of rare alleles. The results of the DAPC analysis show no clear clustering pattern within the data based on the population of origin, and the LEA cross-entropy method that performed reasonably well in the reference-based dataset performed poorly in the de novo method in assigning individuals into

a conclusive number of clusters. The OutFLANK and pcadapt analyses identified a total of 14 outlier loci, and the two outliers were common between the different methods. Similarly, GWDA identified three loci that are putatively under selection in populations from the east and west coasts of South Africa.

No genotype-phenotype analysis was performed on the de novo dataset since as explained in the methodology section, this analysis is sensitive to the correct identification of underlying population structure that is integrated into the model as a latent variable, and since LEA package could not conclusively identify the hidden population structure, results from this analysis would be unreliable.

4.4 Discussion

In this chapter, the genome-wide variation, and the presence of fine-scale local adaptation and phylogeography of *P. angulosus* were investigated along the South African distribution of this echinoderm. The observed spatial patterns of genome-wide variation in populations of *P. angulosus* across the South African coastline showed the presence of a concealed population differentiation. The estimates for two commonly used indices of population differentiation, F_{st} and G_{st} , exceeded the median reported for marine organisms (Waples 1998), and this points to the presence of adaptive divergence in *P. angulosus*. These results are consistent with those estimated from mitochondrial DNA by Muller (2011) and Mertens *et al.* (2018). Furthermore, these estimates are significantly higher than population differentiation indices reported from four decapod crustaceans in the same geographic region (Emami-Khoyi *et al.* 2021). Further, allele frequencies of several genotyped loci differed significantly from genome-wide background rates of differentiation between bioregions. Loci that show high levels of genetic differentiation represent candidate regions in the genomes that could be subject to natural selection (Beaumont and Nichols 1996). High levels of genetic differentiation often indicate that gene flow does not affect these loci to the same extent that it does affect background neutral loci across the genome, which could point to the development of specific genetic adaptations to the local environmental conditions (Kawecki and Ebert 2004). Furthermore, the identification of these outlier loci revealed the presence of nuanced fine-scale local adaptation to the local environmental conditions present at the individual rocky shore sites along the eastern and western coasts of South Africa. Similar findings have been reported in earlier genome-wide studies of marine organisms in other regions that illustrated that the presence of

a subset of loci that significantly deviate from the background genomic rate is common-place, and several instances have been reported in red abalone (De Wit and Palumbi *et al.* 2013), Atlantic herring (Corander *et al.* 2013), pink salmon (Johnston *et al.* 2014), and sea scallops (Van Wyngaarden *et al.* 2017).

In the absence of a reference genome for *P. angulosus*, assigning functional significance to outlier SNPs was based on the homology to annotated genes in the surrogate reference genome (*Lytechinus variegatus*) used in this study. Among the identified outlier loci, however, several were within uncharacterised proteins reported from *L. variegatus*. Interestingly, among the remaining loci, one was within a gene that is involved in transcriptome initiation, another in rRNA binding protein 26-like, and another was within the mitochondrial genome. This is consistent with earlier studies in *P. angulosus* using mitochondrial DNA since both studies reported comparatively high differentiation in the markers placed on mitochondrial DNA (Muller 2011, Mertens *et al.* 2018).

The level of genetic variation among populations varied more than three-fold between the coasts. Consequently, evolutionary potential within different populations is expected to differ significantly. This is important because the concealed genetic variation within populations serves as raw material for natural selection to act upon and select the individuals with higher fitness (Charlesworth and Charlesworth 1978, Charlesworth *et al.* 1982, Charlesworth *et al.* 2009, Charlesworth and Charlesworth 2010, Charlesworth 2017, Charlesworth and Charlesworth 2017, Charlesworth *et al.* 2017). Populations with higher genetic variation are expected to be more resilient in the face of changing environmental conditions population (Charlesworth and Charlesworth 2010). Estimates for Tajima's D in *P. angulosus* were consistently negative, pointing to the presence of rare alleles. The excessive segregation of rare alleles within a population is typical of a selective sweep (i.e., replacement of all variants with a variant of higher fitness), or population expansion following a population bottleneck (Eckshtain-Levi and Weisberg 2018). The magnitudes of the estimates in this study were, however, not statistically significant to infer the cause of the observed patterns. As a rule of thumb, values larger than +2 or smaller than -2 are considered statistically significant (Tajima 1989), which was not the case here.

Finally, I reconstruction of the phylogeography of *P. angulosus* along the South African coastline places the root of the current populations in the south-western Cape bioregion (within

the west coast). From this location, an old, distinct lineage diverged more than 1.5 mya. In parallel, another branch gives rise to a clade that included the majority of the individuals' genotypes in this study. The geographic pattern of dispersal illustrates that localities situated on the east coast were originally colonised (Figure 4.17), followed by settlement in localities situated on the west coast. (Figure 4.18).

Among the multiple findings yielded by this genetic study, the discovery of genetic structuring and putative adaptive divergence suggests the possibility of local adaptation by sea urchin populations. This finding contributes significantly to the growing body of evidence that challenges the traditional view of high gene flow and large effective population size (n_e), preventing the emergence of distinct spatial genetic patterns across seascapes. Studies on marine vertebrates and invertebrates have showed that the presence of a population structure is more prevalent than originally thought (Hauser *et al.* 2008, Sanford *et al.* 2010). While it is expected that local adaptations are more prevalent in organisms with a direct development (i.e., where gravid males and females exchange genetic material in a predetermined location), as opposed to broadcast spawning, the latter review of Sanford *et al.* (2010) reports that distinct population structure in species with planktonic dispersal constitutes approximately 66% of the reviewed study cases. The idea of a well-mixed population spanning oceanic basins stems from theoretical studies that show the migration of only a few individuals that can successfully establish in a new habitat and contribute to future generations is enough to prevent significant population structure as the primary source of genetic variation is recombination, and not mutation rate which, would otherwise require the movement of a large number of individuals (Waples 1998). Development of local adaptations is the product of natural selection and gene flow between populations. The apparent lack of impediments to the movement of marine organisms and the life history of many of these species that involve a planktonic larval stage similar to the life history of *P. angulosus* suggests that, compared to terrestrial ecosystems, the occurrence of local adaptations among marine organisms should not be widespread. This mainly stems from the concept that in marine ecosystems, the balance between gene flow and natural selection is in favour of the homogenising effect of gene flow, eroding any genome-wide footprints indicative of local adaptations (Puebla 2009).

This emerging paradigm that underlines the presence of subtle local adaptation in coastal adaptation unsurprisingly coincides with recent unprecedented advancements in the affordability and availability of high throughput sequencing, which makes the detection of even

the subtlest signals of adaptation across an organism's genome possible. Conventional genetic approaches, often reliant on single genes or a combination of few genetic markers, generally lack the robustness and statistical power required to decipher these subtle changes in allele frequency and to detect cryptic population structure (Hemmer-Hansen *et al.* 2014). The investigation of the source of genetic variation in marine organisms driving local adaptation is being intensively researched (Nielsen *et al.* 2009, Bierne *et al.* 2016). Determining whether the underlying genetic variation stems from existing variation segregating within populations, or through the occurrence of new mutations within marine populations is a question that still persists. Furthermore, understanding the evolutionary mechanisms that maintain population structure despite significant gene flow, and the covariance of allelic effects in different loci continues to be a challenge with many possible solutions. Recent findings from various genome-wide association studies, quantitative genetics investigations, and experimental biology point to the multi-genic origin of fine-scale local adaptations (see Dylan 2020 for a comprehensive review). Local adaptations of polygenic origin can arise in marine organisms under three different scenarios: A) significant changes in allele frequency across multiple loci with small to moderate effects that promote superior fitness under different marine conditions (Barton 1999); B) subtle changes in allele frequencies within the functional genome components possessing adaptive significance; C) through a presence of supergenes or genomic islands of divergence (Kirkpatrick 2010, Schwander *et al.* 2014). Consequently, the polygenic nature of local adaptations in marine organisms makes it possible to create or maintain subtle genetic structure, despite significant levels of gene flow, since the diminishing of allelic effects across multiple genes mitigates the impact of migration in swamping the genomic signal of local adaptations (Yeaman 2015, Wellenreuther and Hansson 2016). While the polygenic origin cannot be fully inferred from the current results, the changes in allele frequency of outlier loci of *P. angulosus* were mainly subtle to moderate hinting to some, yet limited, local adaptation. In population two ($n = 3$, Kommetjie), however, the changes in allele frequency were extreme, with complete fixation of the alternative alleles. These patterns indicate the potential relevance of this site for local adaptation.

After the formation of population structure, its long-term persistence and development of local adaptation also depends on the species dispersal capability (Endler 1977). Accurately estimating dispersal in marine species indeed poses significant challenges. For example, neighbourhood sizes of planktonic larval dispersal could range from 10 – 100 km for invertebrates and 50 – 200 km for fish (Palumbi 2004). When a species' dispersal is smaller

than the extent of the selective marine gradient that promotes the formation of population structure, fine-scale local adaptation can take place. If the scale of dispersion is, however, greater than the potential of the species for dispersal, the formation of local adaptation is expected to be more complex, and other processes, such as balanced polymorphisms, could play an important role, which typically involves recurring selection acting on each generation of migrants in the new habitat for which they are maladapted (Sanford and Kelly 2011).

Recent evidence suggests that, when local adaptation occurs, marine populations can develop complex genomic mechanisms in the form of chromosomal rearrangement, or the development of cold spots of recombination to minimise the loss of these fitness-related footprints in the genome (Dyan 2020). For example, a study of genomic divergence in Atlantic cod (Berg *et al.* 2016), Atlantic herring (Martinez Barrio *et al.* 2016) and stickleback (Marques *et al.* 2016, Roesti *et al.* 2015) show that adaptive loci that infer fitness-related benefits in these species are tightly linked by genomic inversion. For such underlying genomic mechanisms to act in favour of maintaining population structure in marine populations, it is not always necessary for the focal populations to show highly significant population structure, since evidence for similar patterns has been reported in species with less pronounced and cryptic population structure, such as Atlantic cod (Barney *et al.* 2017, Barth *et al.* 2017, Sodeland *et al.* 2016) and the seaweed fly (Wellenreuther *et al.* 2017).

It is noteworthy that different demographic scenarios can also create genomic signatures that are similar to those left behind by local adaptations, while the underlying mechanisms are completely different, and this can result in a high false positive rate in the identification of loci under selection (Dyan 2020). For instance, balanced polymorphism explained in a previous paragraph, which involves the generational competitive exclusion of dispersed individuals by local residents that have developed fine-scale local adaptations to their environment, can create obvious population structure, while the underpinning mechanism may be to some extent different (Grosberg and Cunningham 2001, Schmidt and Rand 2001). For example, in a study on the acorn barnacle, Schmidt and Rand (2001) found that if individuals were sampled soon after planktonic larval stages, the genetic diversity across high and low intertidal populations was homogenous. The differential survival rate of individuals exposed to post-settlement conditions, however, resulted in obvious changes in allelic frequencies and the formation of population structure, as is expected from species experiencing selection pressure. While this phenomenon has been labelled as a ‘phenotype-environment mismatch’ by Marshall *et al.*

(2010), others suggested that such dynamics can be described as post-settlement or post-recruitment balanced polymorphisms between individuals (Grosberg and Cunningham 2001, Schmidt and Rand 2001).

Phenotypic plasticity, which occurs when a particular genotype produces different phenotypes under contrasting environmental conditions (Holloway 2022), can similarly create patterns that mimic that of local adaptations. While there is no doubt that the expression of phenotypic plasticity is to some extent modulated by genetic factors (Zai *et al.* 2013), the underlying mechanisms are different, since the development of phenotypic plasticity does not necessarily point to the presence of high genetic variation within populations (Mata and Zas 2023). Distinction between local adaptations, balanced polymorphisms, and phenotypic plasticity is important since each point to a different underlying adaptive mechanism that could influence the overall species' resiliency in the face of future ecological challenges (Osovitz and Hofmann 2005, Kuo and Sanford 2009). In phenotypic plasticity and balance polymorphisms, the species has the complete genomic repertoire to express adaptive responses, however, local adaptations mean that some distinct populations developed a specific genetic variation that can make them resilient against environmental conditions. As such, the conservation status of these populations needs to be closely evaluated since these very same populations may represent adaptive responses that are unique and can be used to replenish genetically depauperated populations that are not able to respond optimally to the challenges they are facing (Sanford and Kelly 2011).

4.5 Conclusion

The information gathered through this study can be used to better predict the distribution and interaction of ecological communities in the face of projected scenarios of climate change, since the underlying statistical methods for widely used projection methods assume that the entire population of a species is homogenous, and the presence of the fine-scale local adaptation in populations is largely ignored. In this study, evidence from the measurement of morphometric characters (Chapter 3), genome-wide heterozygosity, and population differentiation shows that *P. angulosus* population is heterogeneous in both the morphological and genomic makeups. Evidence was found of cryptic speciation, adaptive divergence, and fine-scale local adaptation across rocky shore sites. The genetic variation found among populations points to potential differences in evolutionary potential, further supported by the

association found between outlier loci and functional genes. A design such as the one used in the current study can help identify subtle genetic relations with morphometric traits and the environment (temperature; Figure 4.13). This multidisciplinary approach therefore helped in identifying those intra- and inter-population variations in adaptive responses to current and future climatic challenges, information that can help to incorporate in marine ecosystem management plans and identify both resilient and vulnerable populations.

A better understanding of the underpinning processes involved in local adaptations in marine organisms is critical in the establishment of marine conservation areas and spatial management of marine populations with subtle population structure. While the major focus of these ecosystem management plans is on the representativity and connectivity of the designated areas, the role of local adaptation in these populations in these management plans is largely ignored. This is critical since the unimpeded movement of an individual into a habitat where they are maladapted, will only exacerbate the competition for common ecological resources with local residents, and if these individuals are to contribute to the local gene pool, there is a significant risk that the adaptive mechanisms that local populations developed over time would get swamped with sub-optimal genetic variants that immigrants bring into new habitats. The history of aquaculture and game management is full of instances where translocation resulted in the collapse of sink populations (Conover 1998, Yanick *et al.* 2003). The identification of resilient populations could, however, provide marine ecosystem managers with suitable candidates that can serve as a source to replenish diversity in genetically vulnerable and less resilient populations.

Chapter five: An ecological study of the gut microbiota of the Cape sea urchin, *Parechinus angulosus*

5.1 Introduction

5.1.1 Bacteria in the environment

Bacteria have been found in every conceivable ecosystem on earth, including terrestrial (Bergey and Holt 1923), aquatic (Zobell 1942, MacLeod 1965), extreme (Zeikus 1979), and even deep subsurface (Sinclair and Ghiorse 1989) environments, as well as on/in the bodies of almost all organisms (Baines 1956, Fuller and Brooker 1974, Chivers and Hladik 1980, Wilkinson and Garrone 1980). Their ubiquity underscores the foundational role that bacteria play in the functioning of all ecosystems, where they perform the vital, yet still largely unexplored ecological processes of nutrient cycling (Likens and Bormann, Rosswall 1976, Reichle 1977), energy flow (Odum 1968, McIntyre *et al.* 1970), and decomposition (Gray and Baxby 1968, Boylen and Brock 1973). These interconnected processes are crucial for maintaining the healthy functioning of ecosystems (Tláskal *et al.* 2021).

A wide diversity of bacterial taxa is found in the marine environment, including *Proteobacteria* (Gonzalez and Moran 1997), *Cyanobacteria* (Burja *et al.* 2001), *Bacteroidetes* (Fernandez-Gomez *et al.* 2013), *Actinobacteria* (Bull *et al.* 2005), and *Firmicutes* (Moreno-Letelier *et al.* 2011). The composition of bacterial communities in marine environments depends on a number of factors (Fuhrman *et al.* 2015) broadly characterised by space and time (Van der Gucht *et al.* 2007, Gilbert *et al.* 2012). Variations in latitude and longitude (McBee and McBee 1956, Sieburth 1971, Kriss *et al.* 1960, Kriss 1976), and seasonal and diurnal changes (Miyamoto *et al.* 1962, Yetinson and Shilo 1979) result in differences in temperature (McEwen 1912, Coker 1918, Orton 1920), sunlight availability (Klugh 1929, Lee 1974) and ocean current patterns (Goldsbrough 1933, Stommel 1953, Wyrski 1974), contributing to the formation of distinct microbial community structures within the world's oceans. Physico-chemical factors such as temperature (Sunagawa *et al.* 2015, Xie *et al.* 2022), salinity (Doherty *et al.* 2017), nutrient availability (Bannon *et al.* 2022), and pH (Jin and Kirk 2018), alter the structure of microbial communities as all affect the growth rates and metabolic activity of bacteria (Gilbert and Kana 2016). Likewise, factors, such as dissolved organic matter (Biddanda *et al.* 2001), water turbidity (Zamxaka *et al.* 2004), hydrodynamic processes (Saito *et al.* 2019, Li *et al.* 2015, Liu *et al.* 2021, Amano *et al.* 2022), and water depth and stratification (Peoples *et al.* 2018, Milke *et al.* 2022), affect growth rate and metabolism of microbial communities (Thomen *et al.* 2017). In addition, water turbidity (Quang *et al.* 2017) has a pronounced impact on light penetration

(Cózar *et al.* 2019), particularly affecting those species of bacteria that are photo-dependent (DeVilbiss 2023). One of the least explored factors responsible for shaping the diversity of bacterial community structure is the distance of the immediate marine environment from land masses (Scicchitano *et al.* 2022). Proximity, or lack thereof, to land affects nutrient input into the ecosystem, as well as sedimentation likelihood or occurrence, and the frequency, if any, of anthropogenic activities and their effects (Paulsen *et al.* 2017, Doane *et al.* 2020). Substrate type and habitat similarly affect microbial community composition through architectural complexity and the availability of surfaces for bacteria to colonise (Bruhn *et al.* 2021), as well as the creation of unique niches, physical and ecological, for bacteria to occupy (Fahimipour and Gross 2020, Miao *et al.* 2021). In sedimentous environments, sediment type also affects bacteria settlement due to differences in oxygen availability (Qiao *et al.* 2018).

5.1.2 Importance of bacteria

Marine bacteria perform the same ecosystem functions as their terrestrial counterparts throughout the marine environments, from the coast (Purdy *et al.* 2002, Dang *et al.* 2008) to pelagic ecosystems (Schut *et al.* 1997, Long and Azam 2001, Welsh and Castadelli 2004). Nutrient cycling in the marine environment is facilitated by bacterial decomposition of organic matter (Nixon 1981, Flanagan and Cleve 1983, Banta *et al.* 2004, Chen 2009) resulting in the release into the surrounding water column of nitrogenous compounds like ammonia (Mackin and Swider 1989), phosphorus compounds like phosphate (Ingall and Cappellen 1990), carbon compounds like carbon dioxide (Middleburg *et al.* 1993), and trace elements like iron, manganese and zinc (Gerringa 1990, Rysgaard *et al.* 2001, John and Conway 2014). The bioavailability of these compounds in turn boosts primary production (Howarth 1988, Buitenhuis *et al.* 2012, Egan and Gardiner 2016). Bacteria, however, also play a direct role in the primary production of organic matter (Sorokin *et al.* 1995, Zhou *et al.* 2014, Patel *et al.* 2018) through photosynthesis (Thornton 2012, Flombaum *et al.* 2013, Teto *et al.* 2020). Photosynthetic bacteria, such as *Cyanobacteria* (e.g., *Prochlorococcus* species, Teto *et al.* 2019), are foundational to marine food webs and contribute to oxygen production (Berman-Frank *et al.* 2003) and carbon cycling (Biller *et al.* 2015). This cycle supports the microbial loop (Hermann *et al.* 2000), where energy and nutrients are transferred to higher trophic levels (Srisomwong *et al.* 2019) through the consumption of bacteria by planktonic organisms, which are in turn consumed by larger organisms (Meyer 1994, Pomeroy *et al.* 2007).

In addition to nutrient cycling and primary productivity, bacteria also contribute to the health of ecosystems by regulating the pathogenic establishment on/in macrobiota, which, if left unchecked, could destabilise community structure (Van Elsas *et al.* 2012). This is achieved by outcompeting pathogens for the available resources of space (Hornef 2015), nutrients (Kamada *et al.* 2012), carbon sources (Franz *et al.* 2007), oxygen (Litvak *et al.* 2019), amino acids (Olive and Sasseti 2016) and access to food sources (Rohmer *et al.* 2011), depending on species. Bacteria further contribute to optimal marine ecosystem functioning through indirect water purification when decomposing detritus (Ducklow 1983) as well as through methane regulation (Kruger *et al.* 2005), the latter of which is a common emission from waterbodies (Rehder *et al.* 1998, Sauter *et al.* 2006). These additional services are intrinsically linked to nutrient cycling, in the first instance through removal of organic pollutants (Espinosa-Ortiz *et al.* 2022), contaminants (Dell'Anno *et al.* 2020), and excess nutrients (Shi *et al.* 2012) in the water medium, and in the second instance through the consumption of methane by methanotrophic bacteria (Yamamoto 1980). Bacteria may also contribute to stabilisation of coastal substrates, like rocks and sediments, by forming biofilms on these structures, thereby contributing to nutrient retention and erosion prevention (Decho 2000).

5.1.3 Bacterial symbioses

The recent hologenome theory aims to explain the microbial variation seen in organism-associated bacteria and posits that the combined genetic information of the host and its microbiota can determine the integrated fitness of eukaryotic organisms (Zilber-Rosenberg and Rosenberg 2008). This theory builds on the traditional evolutionary principle describing the highly dynamic, yet obligate, synergistic evolution of hosts and their symbiotic microbes when subjected to evolutionary processes (Rosenberg *et al.* 2009). As a result, both hosts and host-associated microbiota can be considered as a single unit, termed a holobiont (Rosenberg *et al.* 2009, Bosch and McFall-Ngai 2011). In ecology, the holobiont approach underscores the crucial and intricate interplay between organisms and their microbiota throughout evolution, a process also known as phyllosymbiosis (Kohl 2020, Perez-Lamarque *et al.* 2022). As a result, the co-evolution between hosts and their microbiota results in similar microbial compositions across similar hosts, reflecting phylogenetic relatedness between hosts (Ochman *et al.* 2010).

When considering symbiotic microbial communities, the structure and activities of the bacterial communities are affected by the physiology and diet of the host species (Pierce and Ward 2019).

Microbial community structure is shaped by the feeding behaviour of the host organism as the hosts ingest different types of edible material and therefore require different commensal bacterial species to aid in digestion (Muegge *et al.* 2011, Schwab *et al.* 2011, Lau *et al.* 2018, Zhu *et al.* 2018, Xy *et al.* 2022). The impacts of ontogeny and life stages of an organism on microbial communities are significant (Trabal *et al.* 2012, Trabal *et al.* 2014). Maternal transition of microbes often seeds the gut with the necessary dominant bacteria to most effectively break down food items commonly ingested by a species (Sato *et al.* 2019), though as the organism grows, new microbes are acquired from the environment resulting in a shift in the microbiome diversity due to the changes in nutrient requirements, feeding habits and interaction with other organisms (Sullam *et al.* 2012, Li 2016). In instances where the host becomes infected by a pathogen, the host organism's natural immune system response also affects the balance of beneficial and harmful bacteria in the host (Chang *et al.* 2020). Alongside this, an over-proliferation of non-harmful bacteria within a community can cause an imbalance in the community structure of the given ecosystem or environment due to a drastic change in species abundance, which can result in disease or serious ecosystem services disruption (Clemente *et al.* 2012). The organism-to-organism interactions between microbes likewise can affect the community structure through competition between bacterial species for resources (Cordero and Datta 2016).

While bacteria can be found in many organs, bacterial diversity and abundance is highest in the gut of an organism (Yang *et al.* 2021). This is true for many invertebrates too, including the those in the Phylum Echinodermata, which are similarly aided by bacteria in the digestion of food items (Bosch 1992, Ward-Rainey *et al.* 1996, Thorsen 1999), though their feeding methods and habits are markedly different. Sea urchins can be classified as non-obligate herbivores (Vadas 1977), graze-feeding on plant material (Yang *et al.* 2021), but also on small organisms (Byrnes and Reed 2013) or non-vegetative detritus that is hypothesised to be of animal origin (Kelly *et al.* 2012). This variable diet results in a diverse gut bacterial community, including the major groups of *Gammaproteobacteria* (Hakim *et al.* 2015), *Bacteroidetes* and *Alphaproteobacteria* (Weigel 2020), *Vibrio* (Sawabe *et al.* 1995), and *Planctomycetes* (Rodriguez-Barreras *et al.* 2021). These are variously involved in the degradation of complex carbohydrates like cellulose, proteins, and lipids, but also are involved in nitrogen cycling (Liu *et al.* 2020, Zheng *et al.* 2020).

Bacteria have symbiotic relationships with a variety of hosts, which involve varying degrees of dependence and cooperation (Gerardo 2015). For example, bacteria engage in mutualistic symbiosis with corals (Ritchie 2011) by providing the corals with essential nutrients in the form of amino acids and vitamins (Lema *et al.* 2012, Hazraty-Kari *et al.* 2022), which is reciprocated by the corals with a supply of organic material and protection for the bacteria (Gairuso *et al.* 1999, Furla *et al.* 2000, Fitt and Cook 2001). The dominant bacterial group engaging in this mutualism is known as *Endozoicomonas* (Shiu *et al.* 2020). Another example of mutualistic symbiosis is the relationship that exists between bacteria and coastal zone vegetation (Donnelly and Herbert 1998, Nielsen *et al.* 2001). Bacteria found on the roots of *Zostera*, *Posidonia* and mangroves, contribute to varying degrees of nutrient cycling, nitrogen fixation and disease suppression (Flores-Mireles *et al.* 2007, Jensen *et al.* 2007, Garcias-Bonet *et al.* 2012), and in turn receive refuge and nutrients to metabolise. Similarly, intertidal macroalgae host bacteria, but on their fronds and in the form of biofilms that aid in nutrient uptake and protection against pathogens while accessing nutrients and being provided with a living substrate (Tornblom and Sondergaard 1999, Haglund *et al.* 2002). Bacteria can be found on almost all body surfaces of almost marine organisms (Hakim *et al.* 2016), whether on the exoskeleton (Becker 1996, Hultgren and Stachowicz 2011) or the soft-bodied variants of organisms whose bodies are supported by an endo-or hydroskeleton (Szewzyk *et al.* 1991, Dobretsov and Qian 2004, Kittelmann and Harder 2005). Here, the bacterial epibionts play a role in disease control, which is achieved by outcompeting pathogenic microbiota for resources (Nadell *et al.* 2009), but also through bacterial production of anti-microbial compounds (Wannamaker 1980) which inhibit the establishment and/or proliferation of harmful microbiota (Hibbing *et al.* 2010).

Bacteria can establish in the gills of clams (Vetter 1985) and mussels (Prieur *et al.* 1990); in the tissues, skeleton, and polyps and tentacles of corals (Wegley *et al.* 2007, Olson *et al.* 2009, Kimes *et al.* 2010); in the mesohyl matrix, spongin, spicules, choanocyte chambers, and canals and chambers of sponges (Hentschel *et al.* 2006); and the tentacles and oral disc of anemones (Palinscar *et al.* 1989). Other invertebrate species for which the host-symbiont relationship is reasonably well described are sea urchins. Sea urchins maintain symbiotic relationships with diverse bacterial communities primarily residing in their digestive tract (Pol *et al.* 2018, Yu *et al.* 2019). These symbionts, including *Vibrio*, *Pseudomonas*, *Flavobacterium*, and *Aeromonas* (Unkles 1977) and various other bacterial species, play essential roles in aiding digestion, nutrient acquisition, and overall host health (Schlosser *et al.* 2005, Arafa *et al.* 2012). The bacteria contribute to the breakdown of complex organic matter, assisting in the digestion of

algae or other food sources consumed by sea urchins (Pol *et al.* 2018). Additionally, some bacterial species may facilitate nitrogen fixation (Haditomo *et al.* 2021), provide essential nutrients (Hammer *et al.* 2019), and/or aid in the detoxification process (Da Silva *et al.* 2006). This mutualistic association ensures a symbiotic balance, where both the sea urchin and its bacterial symbionts benefit, contributing to the overall fitness and well-being of the sea urchin host (Hakim *et al.* 2015).

5.1.4 Rationale and aims

A research gap was identified in the knowledge of South African sea urchin gut microbiota. The research objectives for this study were to reconstruct the diversity of the gut microbiome in the Cape sea urchin, *Parechinus angulosus*, and to investigate the potential for spatial variation of the gut microbiome diversity in individuals collected from the east and west coasts of South Africa. Potential variation in gut microbial community structure in sea urchins in response to the distinct environmental conditions prevalent on the east and west coasts of South Africa provides a unique opportunity to define those mechanisms that modulate bacterial diversity along the entire South African coastline.

5.1.5 Hypotheses and research questions

To assess whether there is variation in the alpha and beta diversity of the gut microbiome of *Parechinus angulosus* individuals collected from the east and west coasts of South Africa, the study aimed to test two hypotheses: A) the existence of a core group of dominant taxa in individuals across all populations along the South African distribution range of *P. angulosus*, and B) the potential variations in gut bacterial community composition among populations of *P. angulosus* as associated with distinct environmental conditions on the east and west coasts of South Africa. These hypotheses were investigated using sequencing methods targeting the 16S rRNA gene. Such information was necessary to ascertain whether the survey of gut microbial community structure could serve as a reliable bioindicator of ecological resiliency in sea urchins in South Africa.

5.2 Materials and methods

5.2.1 Collection & Processing

During the Austral summer of 2019-2020, (October to January) six adult individuals of *Parechinus angulosus*, 40-60 mm spine-to-spine diameter, were collected by hand at 43 sites along the South African coastline, from McDougall's Bay on the west coast (-29.29037; 16.87785) to Port St John's on the east coast (-31.65125; 29.51822, Chapter 2; Figure 2.1). Collected specimens were placed in sterile jars and completely submerged in 99% ethanol. Upon return to the laboratory, specimens were stored in a freezer at -27°C at the South African Institute for Aquatic Biodiversity (SAIAB), Makhanda, South Africa.

Intestinal tissue adjacent to the anus was dissected and removed from each of 222 sea urchins and placed into Eppendorf tubes containing absolute sterile ethanol after which samples were once again kept at -27°C.

5.2.2 Genomic DNA extraction and preparation of metabarcoding libraries

Although the initial study design intended for the tissue of six individuals to be used per site, due to challenges during the optimisation of the protocol, tissue from only three samples out of six could be extracted for each site. Using the modified protocol of DNA extraction by 'salting out' (Sunnucks and Hales 1996), with the addition of 20 ng/μL RNase subsequent to the pelleting step, genomic DNA was extracted from the intestinal tissues of three individuals from each of the 37 sites.

Following quantification by Nanodrop spectrophotometry, amplification of the bacterial 16S rRNA gene was done as described in Walters *et al.* (2015). The locus-specific and specially designed primer set used in this study targeted the V4 and V5 hypervariable regions of the bacterial 16S rRNA gene and included the forward primer 16Sa-F (3'-GTAAGGTTTCYTCGCGT-5') and reverse primer 16Sa-R (3'-CAGCAGCCGCGGTAA-5'). Suitable Illumina adaptor sequences were added on to the locus-specific primer sequences to enable multiplexing during the subsequent sequence analysis. Each PCR reaction was composed of the specifically designed primer set, template DNA, KAPA High Fidelity Hotstart Kit (Roche) which contains 2.5 mM MgCl₂ (1X), 0.3 mM of each dNTP (1X) and 0.5 U of KAPA HiFi HotStart DNA Polymerase per 25 μL, and PCR molecular grade water to a final volume of 25 μL. Thermocycling parameters began with a five minute denaturation step at

98°C, followed by 25 cycles of denaturation for 15 seconds at 98°C, annealing for 30 seconds at 55°C, and extension for 30 seconds at 72°C, with a final extension step of 1 minute at 72°C. Amplification of the correct gene fragment was verified by inspecting five µL of the resultant PCR product on a 1% -agarose gel subjected to electrophoresis and viewed on a Uvitec Cambridge FireReader. PCR products were purified using Mag-Bind® Total Pure NGS SPRI beads (Omega Bio-Tek Inc), at a ratio of 1:1.2 sample to SPRI bead solution according to the manufacturer's instruction manual. Product concentrations were determined using the Invitrogen™ Qubit™ High Sensitivity Assay (Thermo Fisher Scientific), also according to manufacturer's instructions.

Individual sample amplicons were then indexed using the Nextera XT V2 adaptors (Illumina) according to the manufacturer's instructions. The resultant indexed amplicon libraries were then purified using ProNex® Size-Selective Purification System (Promega). In turn, the purified amplicon libraries were quantified using the Qubit dsDNA HS assay on a Qubit 3 Fluorometer (Invitrogen, ThermoFisher Scientific) and then pooled in equimolar concentrations. Sequence analysis was conducted on the MiSeq platform (Illumina) at the Aquatic Genomics Research Platform (AGRP-SAIAB) using a Nextera V3 600 cycle sequencing kit (Illumina) and loading 10pM of the pooled libraries with a 10% PhiX spike.

5.2.3 Computational analysis

Analysis of the microbiome diversity in the Cape sea urchin, *Parechinus angulosus* was conducted using both Mothur v1.44.3 (Schloss *et al.* 2009) and Qiime2 v.2023.9 pipelines. Similar trends were observed resulting from both methods of data analysis. The output from the Qiime analysis is presented in this chapter with the results from the Mothur analysis detailed in Appendix 2.

To note, in this subsection, the same terminology was used as that of the Qiime2 developer for clustered sequences, who uses the term 'sequence feature' as an alternative to 'OTU' (operational taxonomic unit) or 'ASV' (amplicon sequence variant).

Prior to data analysis, the quality of demultiplexed sequences was visually inspected in the Qiime2 package. The first step in dataset curation is denoising, which refers to the removal of low-quality sequences, adapter contamination, potential chimeric sequences, and potential

PCR-induced errors. Qiime2 offers two options to denoise data, namely DADA2 and Deblur. For this study, Deblur (Bokulich *et al.* 2013) was selected as it is a method that differentiates between true biological sequences and the presence of sequencing errors, based on sequence-associated error profiles. The Deblur pipeline cannot, however, handle paired-end sequences such as those generated in this study. As a workaround for this limitation, VSEARCH (Rognes *et al.* 2016) was used to merge forward and reverse reads prior to using Deblur. To assign taxonomic rank to denoised sequences, the Qiime2 built-in q2-feature-classifier trained on the SILVA 99% OTU full-length database (Bokulich *et al.* 2018, Robeson *et al.* 2020) was used. Following taxonomic rank assignment, all putative sequences that originated from the mitochondrion, chloroplast, and Eukaryota, as well as unassigned taxa, were removed from the analysis. In addition, all sequencing features that formed with less than 25 sequences, which is the lowest quantile of sequence abundance in the data, were removed.

Qiime2 plug-ins for the assessment of α - and β -diversity indices (<https://forum.qiime2.org/t/alpha-and-beta-diversity-explanations-and-commands/2282>) often require a phylogenetic tree to be constructed. To this end, the denoised sequences were first aligned using MAFFT-aligner (Katoh and Kuma 2002, Katoh *et al.* 2005), and subsequently a phylogenetic tree was reconstructed using Fast-Tree (Morgan *et al.* 2009). The resulting tree was rooted using a mid-point rooting approach as explained in the programme's manual (<https://docs.qiime2.org/2023.9/>). To take into account differences in sequencing depth, which are known to negatively affect the estimation of some biodiversity indices, the sequencing depth was subsampled into an equal number of sequences using the Qiime pipeline. The sequencing depth across all samples was therefore visually inspected and interactively selected such that the maximum number of sequencing features in the highest number of samples could be retained. Ultimately, it was decided to use two populations for this study, the east coast which encompasses all populations to the east of and including Arniston, and the west coast which encompasses all sites to the west of and including Betty's Bay (Figure 2.1). This follows the literature in which the east and west coasts are well-described as distinct from each other, as opposed to using three bioregions as identified in other studies (Bustamante and Branch 1996), the identification of which relies on many factors besides the prevailing Agulhas and Benguela currents.

Two primary measures of diversity, evenness (α -diversity, Pielou's 1966) and weighted-UniFrac (β -diversity, Lozupone and Knight 2005) were selected since these two indices are

among the most informative biodiversity indices in comparative microbiome studies (Sato *et al.* 2020). In particular, weighted-UniFrac was chosen as it is not sensitive to the controversial topic of rarefaction (Hong *et al.* 2022). The statistical significance of variation in α -diversity among populations was tested using a Kruskal-Wallis test (Kruskal and Wallis 1952). Similarly, the statistical significance of the weighted-UniFrac β -diversity index between individuals from the east and west coasts of South Africa was tested using a PERMANOVA test (999 unique permutations).

To identify putative sequencing features that are differentially abundant between the east and west coasts of South Africa, the Gneiss pipeline (Morton *et al.* 2017) was implemented in Qiime2 v.2018.11. Gneiss is based on the concept called ‘balances’. Balances are a statistical framework to address one of the major limitations of measuring bacterial abundances as proportions. This problem arises when the proportional abundance of a single taxa changes between two locations or conditions, meaning that the remaining taxa also need to change in proportion since the proportion needs to sum to 1 (100%). This results in a high number of false positives that are in fact noise that results from reporting abundance as a proportion rather than true differentially abundant taxa. Balances are linear combinations of log-transformed proportions of features, in this case bacterial taxa, in a clustering tree. The clustering tree can be generated using correlation clustering, gradient clustering, and even a phylogenetic tree. As recommended in the Qiime2 manual, the balances were created using correlation-clustering, which is an unsupervised method based on Ward’s hierarchical clustering (Ward 1963). In this method, a co-occurrence tree is created that shows how frequently two microbial taxa co-occur in sequences sampled. Then, a multivariate response linear regression model was reconstructed to investigate the correlation between different balances and different coasts. If a correlation was found to be statistically significant ($p < 0.05$), the bacterial taxa in that particular balance were reported and graphically visualised.

The graphical representation of the balance concept illustrated in Figure 5.1, shows, on the left panel, a correlation clustering tree, with each terminal node representing different taxa indicated by the different colours. The corresponding node annotations indicate the log-transformed relative abundances of each taxon. On the right, an analogous tree is presented, with node annotations depicting different conditions or geographical locations. While the real change only happened in the abundance of one specific taxon (purple), without considering the balance concept, it could be wrongly inferred that all taxa have changed in terms of relative

abundance. When using the concept of balances (log-ratio abundances), however, only balances associated with the purple taxon will show statistically significant changes, and others such as b_3 remains unchanged, because it exclusively assesses the ratio between the red and green taxa that are not affected by changes in the abundance of purple taxa. By considering balances on clustering trees, as opposed to taxon proportions, some of the confounding factors that typically arise due to unequal numbers of sequences between conditions or geographical locations, can be reduced or eliminated by focussing on the differentially abundant taxa within a specific balance.

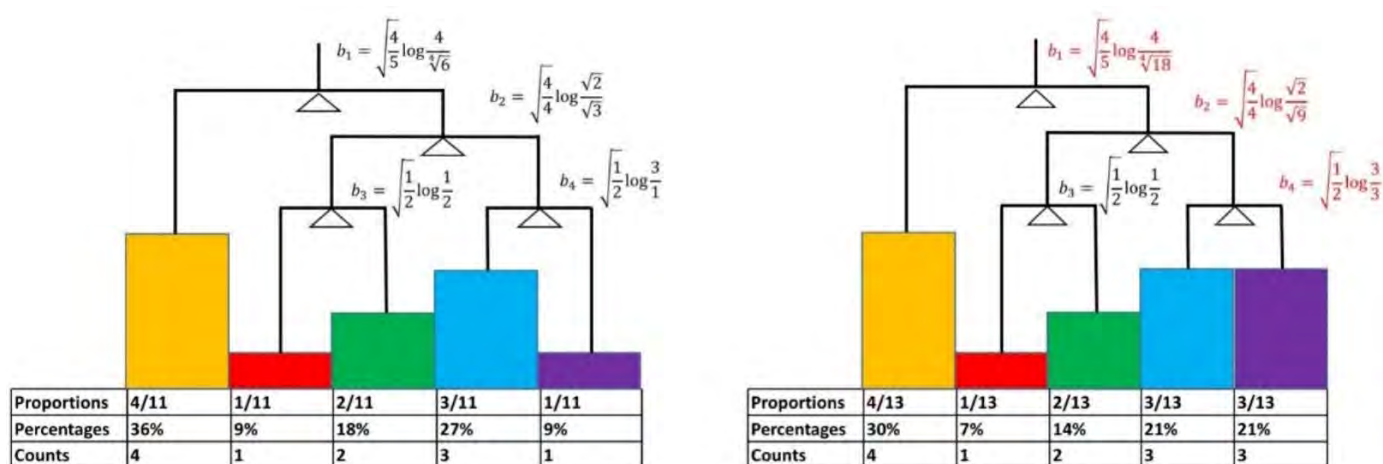


Figure 5.1: Example of correlation clustering tree constructed using example data to describe the concept of balances. The values on different nodes are proportional to the abundance of bacterial taxa in all branches descending from that node. Different colours show presence of different bacterial taxa. The log-ratio highlighted in red are balances that are different between two biogeographical regions (Source: ‘Differential abundance analysis with gneiss’ in ‘Qiime2docs’ 2018, <https://docs.qiime2.org/2018.8/tutorials/gneiss/>).

The method selected to construct a balance tree is correlation clustering which is the appropriate option if there is no prior information about how to cluster the bacteria. This was done using the correlation-clustering command, after which balances were calculated using the following equation:

$$b_i = \sqrt{\frac{rs}{r+s}} \log \frac{g(x_r)}{g(x_s)}$$

where i represents the i^{th} internal node in the tree, $g(x)$ represents the geometric mean within set x , and x_r represents the set of taxa abundances in the numerator of the balance, x_s represents the set of taxa abundances in the denominator of the balance, and r and s represent the number of taxa within x_r and x_s respectively.

Using balances it is not possible to infer absolute changes of bacteria in a sample, but balances can reduce or limit the number of possible scenarios, and in this case taxa abundances in both the numerator and denominator of y_0 decreased, but taxa abundances in the y_0 denominator increased more as compared to the y_0 numerator.

In addition to balance analysis, an analysis of the composition of the microbiome with bias correction (ANCOM) was performed in the Qiime2. While the implementation of this method and the theoretical base behind it is comparatively more straightforward, one major limitation of this method is that the number of differentially abundant sequencing features should be less than 25% of the total number of features, which requires that this limitation be considered when interpreting the results.

Lastly, the functional content of the gut bacterial community of *P. angulosus* in terms of the Kyoto Encyclopaedia of Genes and Genomes (KEGG; Kanehisa and Goto 2000, Kanehisa 2019, Kanehisa *et al.* 2023) pathways were investigated using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUST2, Douglas *et al.* 2020), and the major bacterial phyla identified in this step were summarised using the KEGG database.

5.3 Results

The sequencing run resulted in a total of 6 687 795 sequences. On average, each sample yielded 69 664 quality filtered sequences. After merging sequences in VSEARCH, a total of 4 725 850 merged sequences with a median length of 463 bp were generated (Table 5.1).

Table 5.1: Summary statistics of the data set containing the merged forward and reverse sequences of gut bacteria from the sea urchin *Parechinus angulosus*.

Merged sequences	
Minimum	11
Median	49648.5
Mean	49227.604
Maximum	99625
Total	4725850

After denoising, the quality filtered sequences collapse into 1 842 unique sequencing features, with a median frequency of 19 369 (Table 5.2).

Table 5.2: Summary statistics of sequences collapsed to 1842 unique sequencing features.

Frequency	
Minimum frequency	194.0
1st quartile	14 866.0
Median frequency	19 369.0
3rd quartile	24 371.5
Maximum frequency	35 705.0
Mean frequency	19 551.547

The Qiime2 taxonomy classifier successfully assigned a taxonomic rank to all 1 842 sequencing features, with an average confidence level of 0.93. In total, bacterial taxa belonging to 22 unique phyla, 43 unique classes, 116 unique orders, 184 unique families, and 255 known unique genera were identified (Figure 5.2).

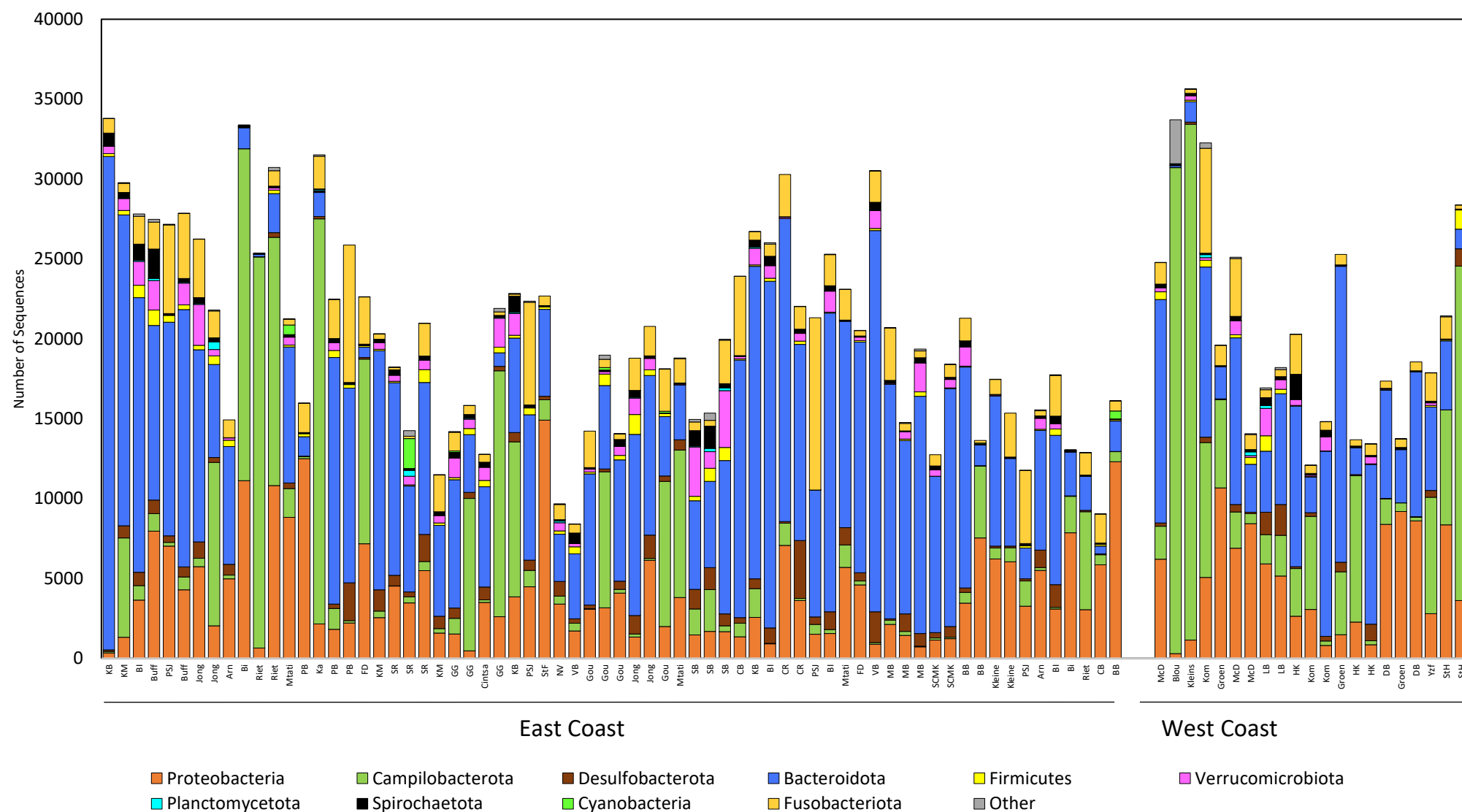


Figure 5.2: Bacterial diversity reported at phylum level from the gut microbiota of the Cape sea urchin, *Parechinus angulosus*, from individuals collected from the east and west coasts of South Africa. Abbreviations on the x-axis represent population (sampling site) names which can be viewed in Chapter 2; Figure 2.1.

Alpha rarefaction curves confirm that sampling at the selected sequencing depth (13 842) was appropriate to capture diversity within populations from different coasts since the rarefaction plots indicate that additional sequences beyond this value would not identify significant numbers of new sequencing features (Figures 5.3).

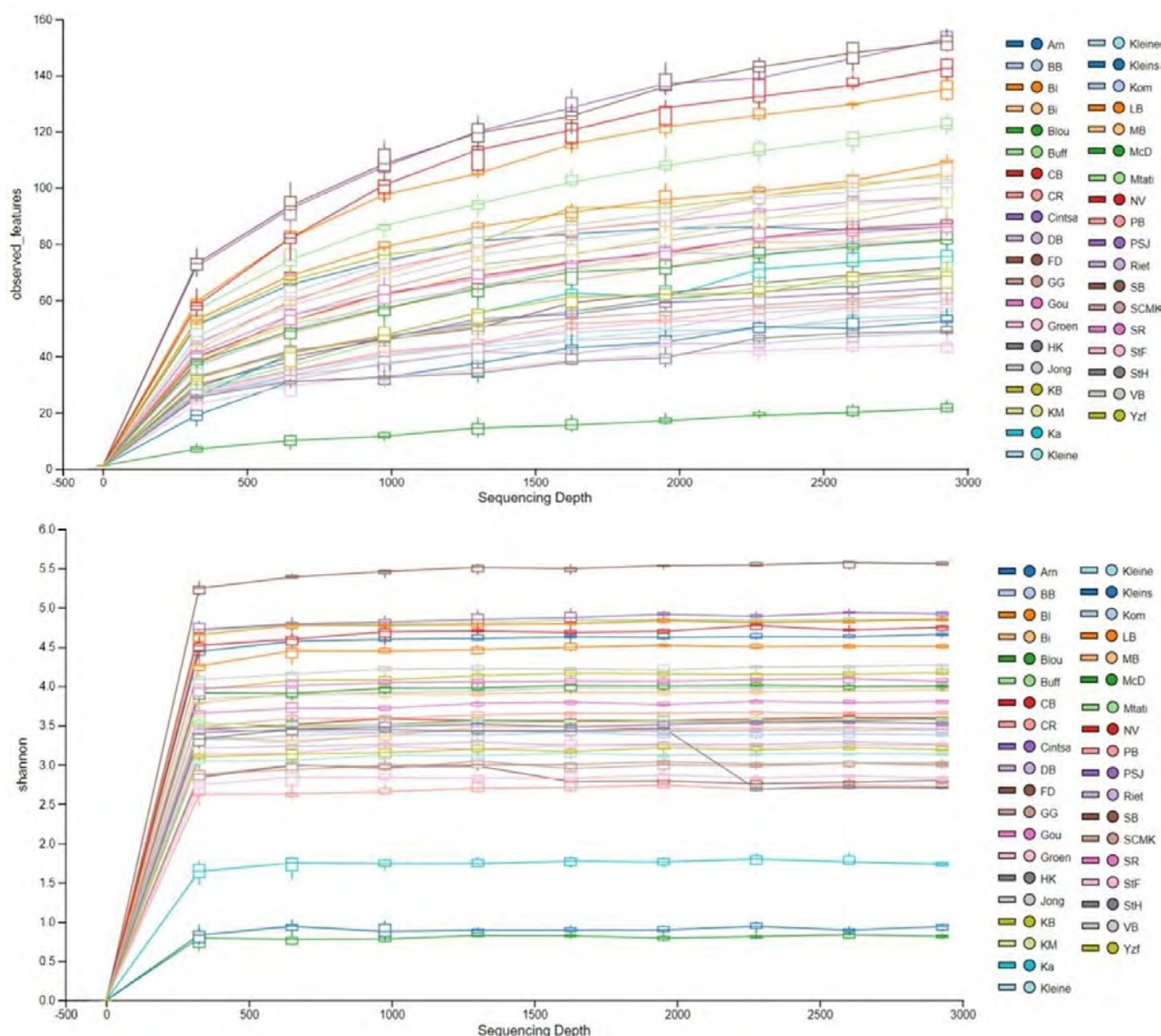


Figure 5.3: Rarefaction curves of Observed sequence features (top) and the Shannon diversity (bottom) indices of the gut bacterial taxa from 101 individuals of *Parechinus angulosus* collected from 37 sites along the South African coastline. For better visualisation, the sequencing depth up to only 3000 was plotted.

Subsampling to an equal sequencing depth of 13 842 resulted in 58.13% (1 079 676) of sequencing features to be retained in 82.11% ($n = 78$) of samples. The α -diversity index (Pielou's evenness) showed some level of variation among populations and among individuals from within the east and west coasts of South Africa (Figure 5.4). The results from the Kruskal-Wallis test on the evenness measures of α -diversity among different populations were, however, not statistically significant ($p = 0.3$) and those estimated from individuals originating from within the east or west coasts of South Africa were also not-significant ($p = 0.05$).

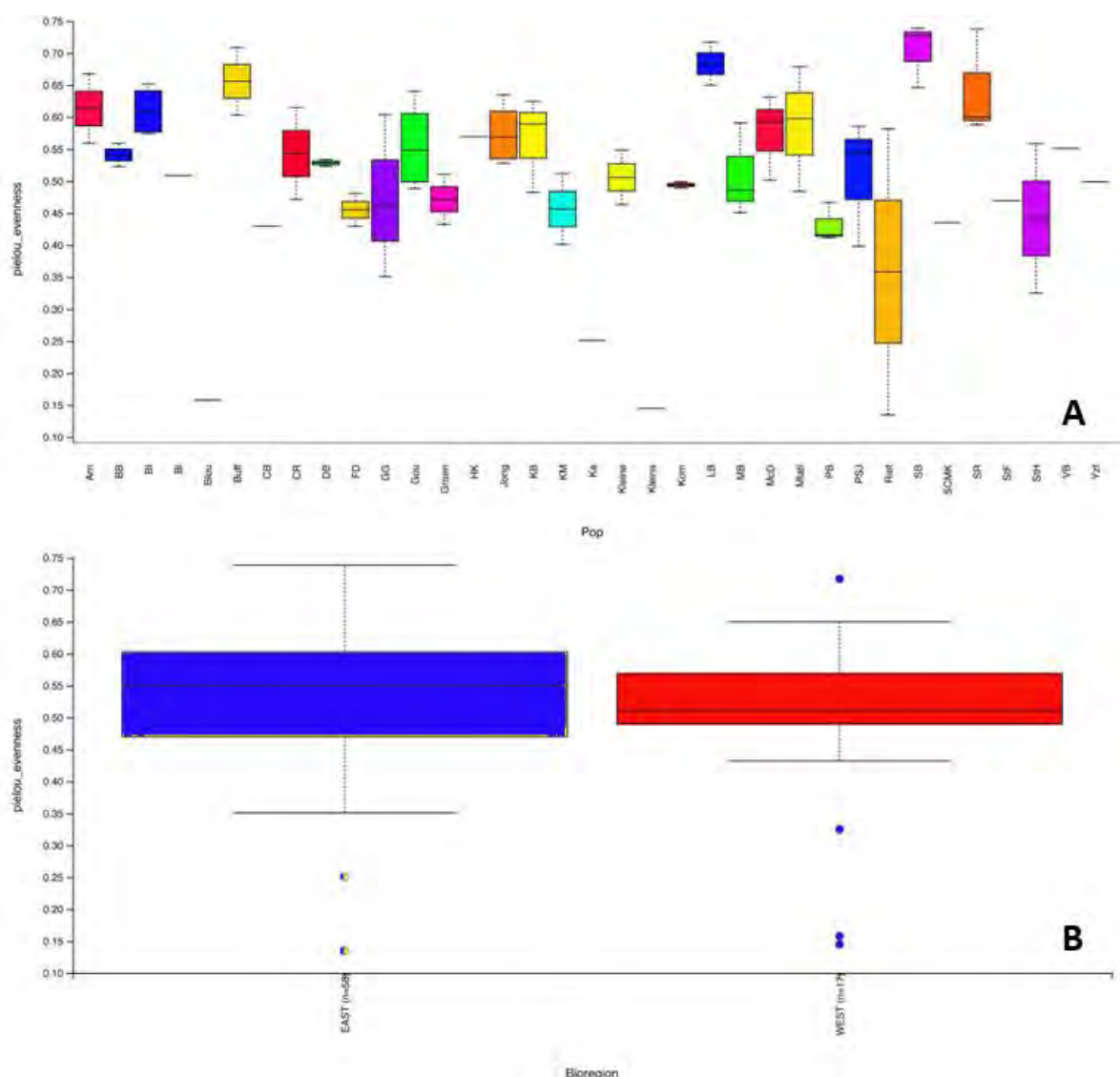


Figure 5.4: Boxplots of the α -diversity measure of evenness among populations (top) and between coasts (bottom). The horizontal line inside the box represents the median value, and the box spans from the 25th percentile to the 75th percentile, while the vertical lines extend to the 5th and 95th percentile.

The weighted and unweighted-UniFrac indices and the Bray-Curtis index of biodiversity show variation among populations from the different coasts (Figure 5.5).

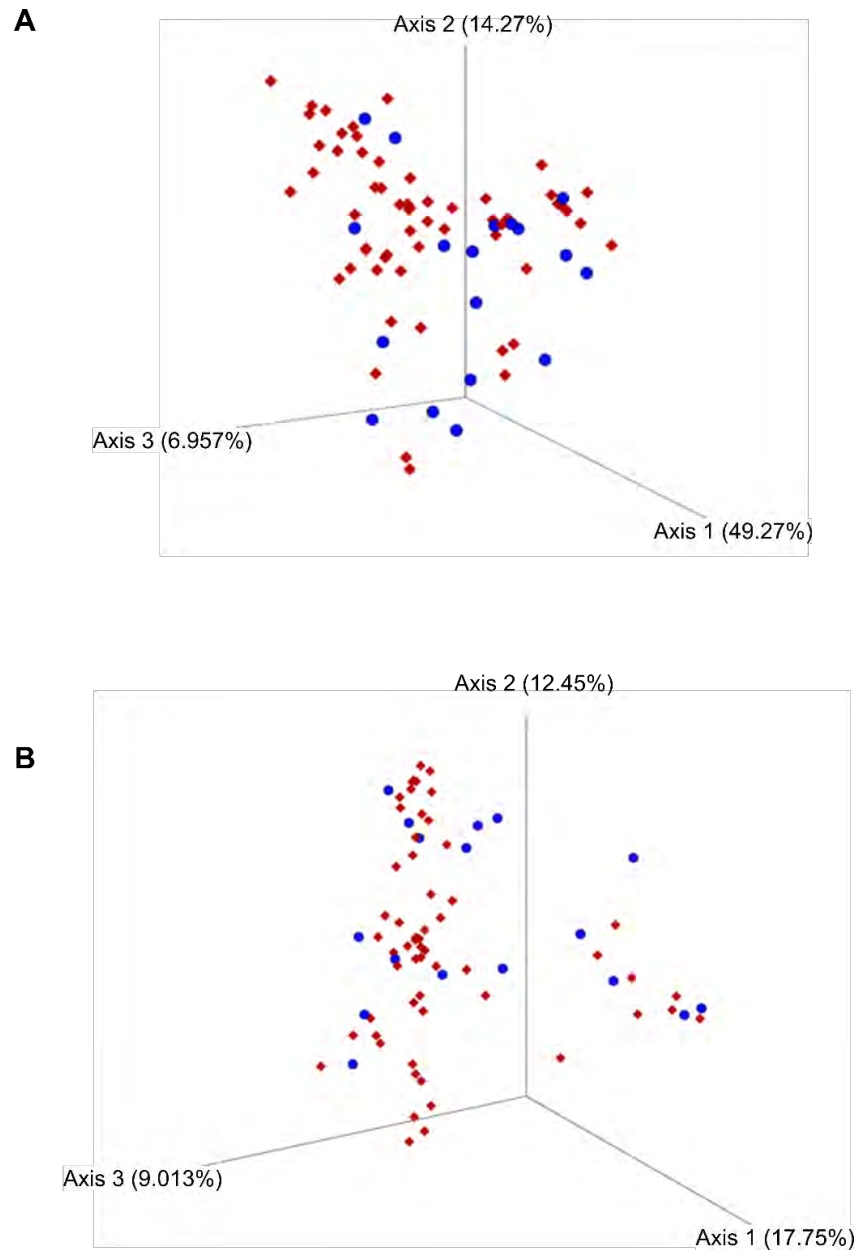


Figure 5.5: Principal coordinate analysis ordination plot of weighted-UniFrac (A) and unweighted UniFrac (B) distances generated on gut bacterial sequences of the sea urchin *Parechinus angulosus* collected from the east (red diamonds) and west (blue circles) coasts of South Africa.

A PERMANOVA test on weighted and unweighted-UniFrac indices of β -diversity showed that the compositional microbiome diversity of gut tissue in individuals from the east and west coasts of South Africa was significantly different, though pseudo-F value suggests that the differences in the gut microbiota community structure between the two coasts are subtle (Table 5.3, Table A1.2 in Appendix 1). The pseudo-F value is influenced by the small number of samples on the west coast as compared to the east coast.

Table 5.3: Results of PERMANOVA on the weighted (the abundance of bacteria) and unweighted-UniFrac (presence or absence of bacteria) diversity indices generated on gut bacterial sequence feature data of the Cape sea urchin, *Parechinus angulosus* collected from the east and west coasts of South Africa.

	Weighted-UniFrac	Unweighted-UniFrac
Sample size	75	75
Permutations	999	999
pseudo-F	3.718	2.896
p-value	0.015	0.002
q-value	0.015	0.002

Differential abundance analysis of identified taxa from individuals collected on the east and west coasts using balance regression in Gneiss identified two particular balances (y0 and y2). These were worth investigating as both had a higher number of bacterial taxa in the numerator compared to the taxa abundance in the denominator. Changes in the abundances of taxa in the selected balances could reflect the influence of biotic and abiotic factors that vary between the east and west coasts in shaping microbial diversity.

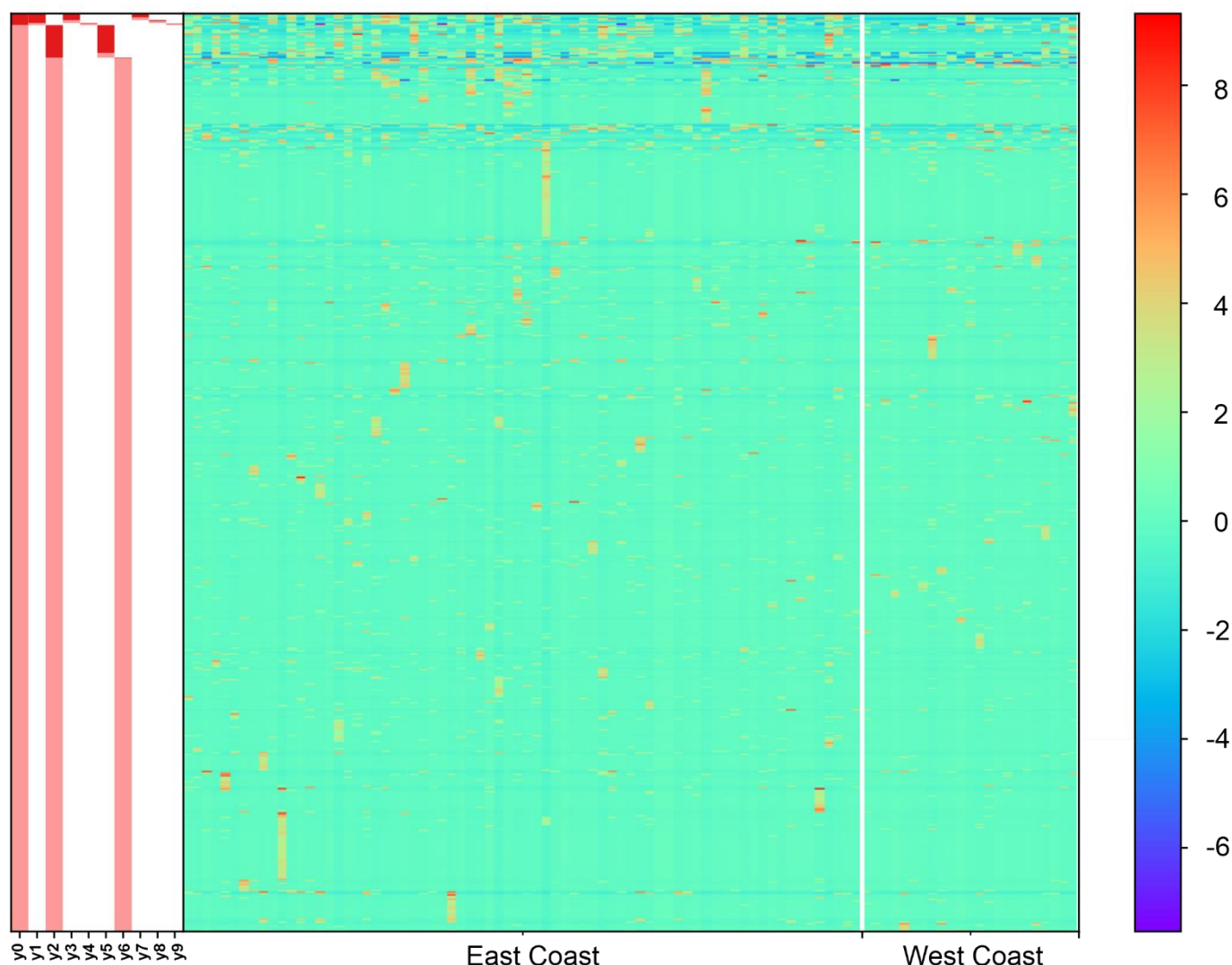


Figure 5.6: Heatmap showing the differential abundances (log-scale abundance ratio) of gut bacterial taxa of individuals of *Parechinus angulosus* collected from the east and west coasts of South Africa. In the bar plot on the far left, the bright red bars represent a combination of taxa in the denominator of the balance, while the paler red bars indicate the subset of taxa found in the numerator. In the heat map on the right, different colours show the log-scale abundance ratio of different taxa.

In the simplicial linear regression (summarised in Table 5.4, for full table please see Appendix 1: Table A1.3), the p -value for both intercepts and the coefficients for balance y_0 was statistically significant ($p = 0.003$) between coasts, and for the balance y_2 , this value was non-significant ($p = 0.06$). The simplicial linear regression explained more than 3% of compositional variation in the data ($R^2 = 0.0312$), and the ten-fold cross-validation show that the model error was more than the predicted error in all cross-validation, therefore, overfitting

was not of concern here. This level of statistical power in explaining microbiome is expected since the diversity of host-associated microbiota is influenced by the confounding effects of a variety of biotic and abiotic factors (e.g., genetics, diet, age, gender, physiological condition, health status) that are difficult to take into account in wild populations. To contextualise, the same method applied to the diversity of gut microbiota in humans had an R^2 -value of approximately 10% (<https://docs.qiime2.org/2018.11/tutorials/gneiss/>).

Given all of these factors, it was decided to investigate the proportion of different bacterial taxa that form these balances in more detail. A closer look at these balances (Figure 5.7) shows that the numerator consists of sequencing features identified to their lowest taxonomic ranks as that of the genera *Bacteroidia*, *Vibrio*, *Bacteroidetes*, *Colwellia*, *Desulfotalea*, *Labilibacter*, and two strains of uncultured bacteria. The bacteria in the denominator were identified to their respective lowest taxonomic ranks of *Bacteroidetes*, *Bacteroidales*, *Bacteroidia*, *Sphingobacteriales*, and a strain of uncultured bacteria.

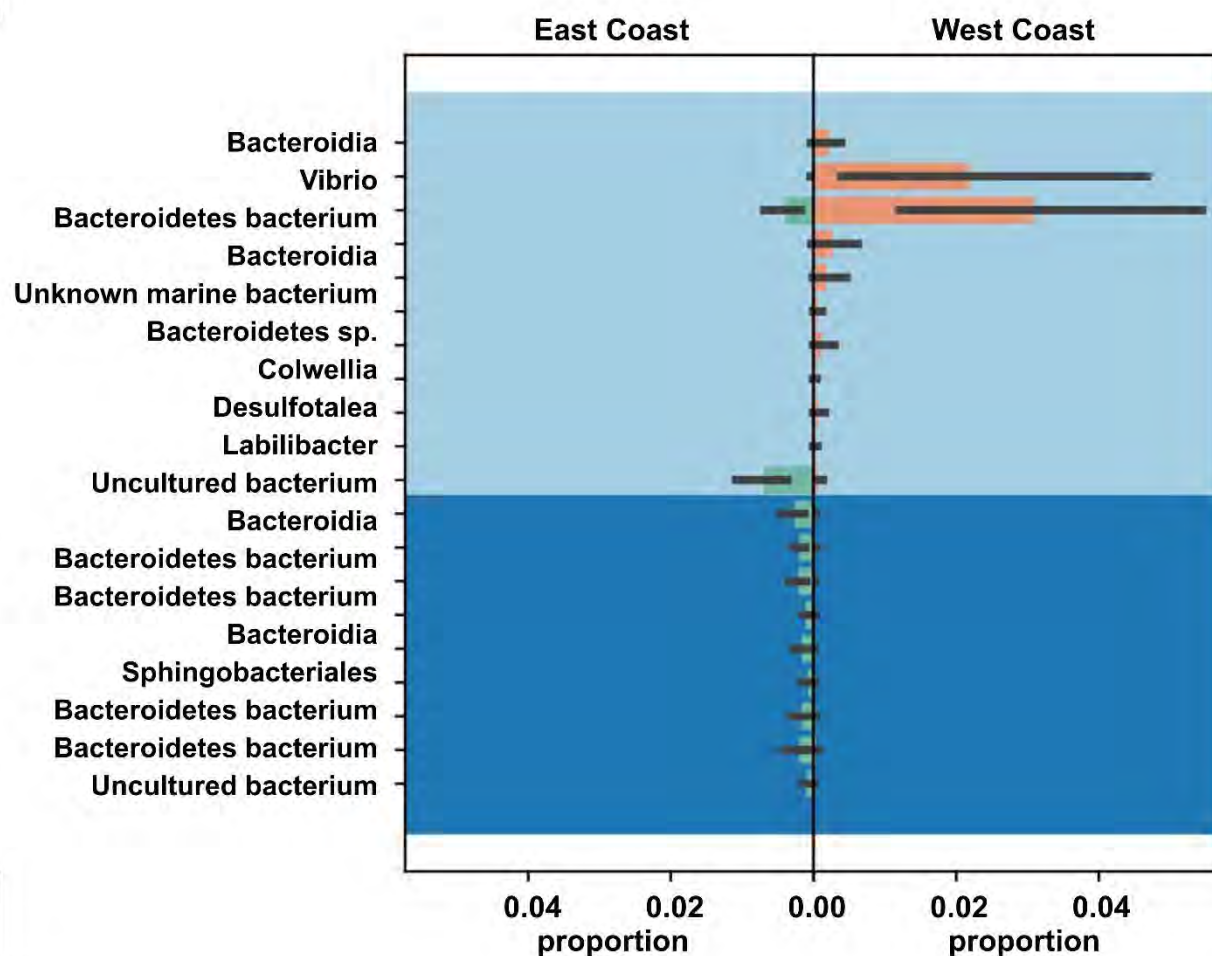


Figure 5.7: Proportion plot of the y_0 balance analysis from Gneiss showing differentially abundant bacterial groups between the east and west coasts of South Africa. Bacteria in the light-blue section of the graph are the bacteria found in the numerator of the balance, and those in the dark-blue section are bacteria found in the denominator of the balance.

The analysis of compositions of microbiomes with bias correction (ANCOM) identified a sequencing feature being assigned to a taxonomic rank of *Bacteroidales* ($W = 356$), and another unidentified member of genus *Psychromonas* ($W = 334$) as bacterial taxa that significantly change in abundance between the east and west coasts of South Africa. As mentioned in the methods section, however, the performance of the ANCOM is unpredictable when the number of differentially abundant sequencing features exceeds 25% and this result needs to be interpreted in terms of both type I and type II errors.

Finally, the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUST2) pipeline was used to predict the metagenomic functional content of the microbiome from the marker genes. The functional content of the gut microbiome in *P. angulosus* was then classified against the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database to understand the molecular interaction, reaction, and relation networks for metabolism (e.g., carbohydrate metabolism), genetic information processing (e.g., transcription), environmental information processing (e.g., membrane transport), cellular processes (e.g., cell growth and death), and organismal systems (e.g., the immune system). A summary of the main biochemical pathways and their functions show that the majority of the taxa are involved in carbohydrate metabolism, followed by amino acid and energy metabolism (Table 5.5, for an expanded version of this table please see Appendix 1: Tables A1.4 – A1.8). It is important to note that some of these pathways have only been investigated in certain species, and so these results are homologous, that is to say, based on the similarity of the sequence to a ‘reference’ sequence for which the biochemical pathway has been mapped, performing similar if not identical functions.

Table 5.4: Summary table of the functional content of gut bacterial taxa of individuals of the Cape sea urchin, *Parechinus angulosus* collected from the east and west coasts of South Africa, reported as a number of biochemical pathways.

Biochemical pathways	Number of linked KEGG annotations
Global and overview maps (core biochemical functions)	4 523
Carbohydrate metabolism	940
Amino acid metabolism	643
Energy metabolism	494
Membrane transport	488
Signal transduction	457
Metabolism of cofactors and vitamins	366
Xenobiotics biodegradation and metabolism	315
Cellular community – prokaryotes	278
Glycan biosynthesis and metabolism	251
Lipid metabolism	188
Nucleotide metabolism	184
Metabolism of other amino acids	146
Metabolism of terpenoids and polyketides	145
Biosynthesis of other secondary metabolites	128

Biochemical pathways	Number of linked KEGG annotations
Replication and repair	106
Translation	98
Cell motility	77
Folding, sorting and degradation	66
Cell growth and death	48
Endocrine system	47
Transport and catabolism	46
Environmental adaptation	23
Digestive system	17
Nervous system	16
Aging	15
Immune system	14
Transcription	13
Cellular community – eukaryotes	4
Circulatory system	4
Excretory system	4
Information processing in viruses	2
Signalling molecules and interaction	2
Development and regeneration	2
Sensory system	1

5.4 Discussion

Following the resurgence of microbial genomics (Haditomo *et al.* 2021), assisted by the recognised bacteria's critical role in aiding their host organism's survival, adaptations to various environmental stressors (Zeng *et al.* 2023) and the recently discovered role of microbiome in the development of resistance to non-infectious diseases (Pol *et al.* 2018), the focus of the current research was on the microbiome diversity in the Cape sea urchin, *Parechinus angulosus*, a widely distributed sea urchin in southern Africa. Despite this growing interest in microbiome research, there is a noticeable gap in studies that investigate microbiome diversity in marine organisms, particularly with respect to gut microbiota in marine invertebrates. In this study, metabarcoding of the bacterial 16S rRNA gene was used to assess the composition of the gut microbiome of *P. angulosus* sampled from the east and west coasts of South Africa.

Owing to the diverse and contrasting prevalent oceanic currents on the east and west coasts of South Africa, the Benguela and the Agulhas Currents, respectively, and the pronounced variability in the environmental conditions of both coasts, it was expected that gut microbial

communities would differ. The reconstruction of microbial diversity found that the gut microbiota from individuals sampled from multiple populations around the South African coastline differed significantly from one another, and so a core community of microbial taxa at a lower taxonomic ranking that occurs in all samples along the east and west coasts could not be identified, confirming the hypothesis of regional and intra-regional differences. Nevertheless, when the bacterial diversity indices were calculated, the α -diversity (Pielou's evenness) index showed moderate variation among populations and between coasts, although this variation did not differ statistically. Due to the borderline lack of significance ($p = 0.06$), the trends observed in the variation of α -diversity, however, may have yielded significant results should additional sample sites be added. Furthermore, the β -diversity analyses (both weighted and unweighted-UniFrac) were significantly different between the east and west coasts of South Africa, underlining, however, a subtle variation.

Four predominant phyla were identified from the gut bacteria of *Parechinus angulosus* – *Campylobacterota*, *Bacteroidota*, *Fusobacteriota*, and *Proteobacteria*. These microbial taxa are ubiquitous and have been reported in a variety of environments as well as in the digestive tracts of different terrestrial and marine organisms (Emami-Khoyi *et al.* 2019, Emami-Khoyi *et al.* 2020, Rodriguez-Barreras *et al.* 2023). These bacterial taxa encompass a vast diversity of microorganisms whose functions in the associated host vary from pathogenic to highly beneficial. For instance, some members of the *Campylobacterales* order are implicated in diseases such as *Campylobacter* infection, raising questions about the potential impacts this group of bacteria could have on the sea urchins health while their functionality already seems to be under physiological pressure owing to changing environmental factors (Van der Walt *et al.* 2021). Similar to the current study, Hakim *et al.* (2016) found *Campylobacterales* to be the predominant order of bacteria observed in the gut tissues of *Lytechinus variegatus*, with 93% of the representative sequences being assigned to *Campylobacteriaceae*. The same pattern was also found in a study by Brothers *et al.* (2018) in the same urchin species where individuals were held in captivity for the duration of a feeding experiment. Haditomo *et al.* (2021) concluded that an increase in *Campylobacteriaceae* may lead to these families being considered 'fluctuated communities', a phenomenon that Gonzalez and Descamps-Julien (2004) posit as a response to fluctuations of environmental factors. It is also known that in the sea urchins *Mesocentrotus nudus* and *Strongylocentrotus intermedius*, increases in *Campylobacteriaceae* have been noted in specimens reared in facilities for the duration of the studies (Haditomo *et al.* 2021), a finding supported by Hakim *et al.* (2016) who worked on lab-cultured specimens.

In contrast, members of *Bacteroidia* play a critical role in supplying energy to the host through the fermentation of indigestible polysaccharides, further demonstrating the cosmopolitan importance of the symbiotic relationships between these microorganisms and their sea urchin hosts (Simsek *et al.* 2013).

When family-level taxa were considered, 184 unique families were identified. The most dominant of these families were *Prolixibacteraceae*, *Arcobacteraceae*, *Vibrionaceae*, *Fusobacteriaceae*, *Marinifilaceae*, and *Cryomorphaceae*. A search of available literature about the potential functions of these families shows that *Prolixibacteraceae* is a family that is notable for having at least 11 genera that are responsible for the oxidation of metallic elements (such as iron) and nitrate-reducing bacterial genera (Lino *et al.* 2015). *Arcobacteraceae* is a family that is widely distributed in aquatic environments and is comprised of at least seven genera. Many of these have been considered emerging enteropathogens in humans (Ho *et al.* 2006, Snelling *et al.* 2006), though their role in the bacterial community of sea urchins remains undetermined. The family *Vibrionaceae* is also abundant in the world's oceans, but compared to *Arcobacteraceae* it is well reported, consisting of eight genera that are involved in nitrogen cycling, but also in the generation of microbial films which ease the settlement and establishment of other bacterial species (Palit and Nair 2014, Takemura *et al.* 2014). The family of *Fusobacteriaceae* consists of five genera and, while species within these genera are largely pathogenic, members of this family ferment carbohydrates, and amino acids and peptides to produce acids of varying pH (Stanley and Whitman 2010). *Marinifilaceae* is a family consisting of three genera and though its presence has been reported in the gut, its function has only been reported as one of mineralisation of organic matter in the deep ocean (Li *et al.* 2022). Finally, *Cryomorphaceae* is a family containing at least nine genera that appear to be involved in the carbon cycle to some extent (Bowman 2014). Further research is required to determine the specific role of each of these families in shaping the gut microbiota of *P. angulosus* in the regions of focus, but most of these families share the important roles of nutrient cycling, which is likely to be of fundamental functional significance towards the health of the sea urchin gut.

The predicted functional content of sea urchins' gut microbial community in this study revealed involvement in some of the core biochemical pathways that are critical for the survival of their host. An analysis of KEGG pathways revealed that the identified bacterial taxa were mainly involved in carbohydrate metabolism (n = 940), energy metabolism (n = 494), and lipid metabolism (n = 188). The fact that a very high proportion of taxa are involved in carbohydrate

metabolism is not surprising considering that sea urchins are non-obligate herbivores, feeding primarily on plant material, but also on detritus if present, which most often takes the form of faecal matter from, or body tissues of other organisms (Vadas 1977). This would also explain the relatively high proportion of lipid metabolising taxa, as animal tissues have a higher lipid content compared to plant material (Post *et al.* 2007), aside from the fact that the two metabolic pathways are linked by various biochemical processes (Oliver *et al.* 2001). Energy metabolism comprises the subcategories of oxidative phosphorylation, carbon fixation, methane metabolism, nitrogen metabolism, and sulphur metabolism (Su *et al.* 2016, Wei *et al.* 2019, Liu *et al.* 2022). Oxidative phosphorylation refers to the process where energy is harnessed for the production of adenosine triphosphate (ATP), which is used in many essential cellular processes of bacteria (Green and Zande 1982), whereas carbon fixation describes the process of converting inorganic carbon into carbohydrates which can be metabolised by the urchin (Evans *et al.* 1966, Fuchs 1985). Bacterial taxa that engage in methane metabolism use methane as a carbon source (Hanson and Hanson 1996) which could theoretically be used to produce carbohydrates similar to the taxa that perform carbon fixation. Nitrogen metabolism by contrast refers to the synthesis and conversion of amino acids through the reduction of nitrate (Gale 1947), which are readily absorbed without the need for prior digestion as in the case of proteins to amino acid products. Finally, sulphur metabolism is the process of the absorption of inorganic sulphur for inclusion into amino acids and other secondary metabolites (Postgate 1984). It is clear that all the taxa engaging in the biochemical pathways described in the subcategories of energy metabolism in one form or another, releases energy from the ingesta for assimilation of these compounds into the gut of *P. angulosus*.

The involvement of gut microbiota in essential biochemical pathways reflects recent trends in microbiome research, necessitating a reassessment of an organism's overall fitness (Evariste *et al.* 2019) and its long-term evolutionary potential to effectively respond to future challenges (Voolstra and Ziegler 2020). Fitness and evolutionary potential encompass the host's genome along with the diversity and functional content of its host-associated microbiome (Henry *et al.* 2019, 2021). Recent research highlights the critical role of a functionally diverse host-associated microbial community to serve as a reservoir of biochemical pathways (Thapa 2019). These can act as an evolutionary buffer to perform critical physiological processes in the host as it responds to current and future stressors (O'Brien *et al.* 2020). This backup of biochemical pathways is particularly important when species are exposed to extreme and unpredictable environmental pressures, such as extreme climatic events (Li *et al.* 2023) or the emergence of

new diseases (Yang *et al.* 2022), since natural selection is a slow-moving process (Haldane 1957), and the generation of adaptive genetic variants takes a long time (Bailey *et al.* 2021). During environmental pressure, therefore, the redundant biochemical pathways in the host-associated microbiome can serve as a temporary buffer zone to protect species from extinction (Fontaine and Kohl 2023). As such, drastic shifts in the host's core microbiome can point to the presence of an underlying stressor (or set of stressors), preceding observable negative impacts on the broader community structure and ecosystem function (Ribas *et al.* 2023). This makes the microbiome a suitable bioindicator of the health of coastal ecosystems (Astudillo-Garcia *et al.* 2019). For microbiome diversity to serve as an optimal bioindicator, however, several criteria need to be satisfied (Roitman *et al.* 2018). These criteria and the potential of the sea urchin microbiome as a candidate bioindicator are detailed in the general discussion (Chapter 6).

Describing microbiome diversity and its functional content in non-model organisms, similar to the investigation into the gut microbial diversity of *Parechinus angulosus* in this study, has several limitations. One major challenge lies in the variability of microbial communities within individuals and populations. It is known that several factors, such as the host's genetic diversity, physiological conditions, and exposure to differing environmental variables prior to sample collection, can introduce considerable noise into the data, complicating the interpretation of results. While in experimental setups there are some possibilities to control for these confounding factors, such options are not always available for field conditions and so these results need to be conservatively interpreted as a snapshot of microbial diversity. Though the repeated sampling of the same individuals can partly mitigate these limitations, in most cases, a combination of logistic and financial challenges makes repeated sampling impossible. As such, the spatially broad cross-sectional nature of many studies may not capture the true dynamic of temporal changes in microbial communities, which can be influenced by factors like seasonality, diet variations, and host age and health status. When feasible, longitudinal studies, which refer to the tracking of the same individuals over time, would provide valuable insights into the temporal dynamics of microbial communities and their responses to environmental changes, though this adds to the complexity of resampling motile organisms. Some of the sampling challenges include the fact that temporally multiple collections are often sacrificed over spatial coverage. Additionally, the reliance on DNA-based sequencing may provide insights into microbial taxonomy but may not fully capture functional aspects of the functional content of microbiome diversity. These methods are highly dependent on the

presence of a complete genetic and functional trait reference database, which is typically scarce for under-studied, non-model species. In the absence of such databases, the results of such studies need to be interpreted within these limitations. Interdisciplinary and collaborative studies that gather experts from different fields such as experimental and clinical microbiology, microbial and community ecology, and computational biology, may aid in developing more holistic frameworks that explain the interplay between microorganisms, their environments, and their hosts. Moreover, the application of advanced statistical methods, machine learning algorithms, and network analyses will undoubtedly contribute to a more complete understanding of the complex relationships between hosts and their microbial ecosystems.

5.5 Conclusion

In this metabarcoding analysis of gut microbial diversity of the Cape sea urchin, *Parechinus angulosus*, a significant, yet subtle variation in the gut bacterial abundances among individuals collected from the east coast and those collected from the west coast was observed. These findings nevertheless add to the growing body of evidence that emphasises the pivotal roles that regional environmental factors and host physiological status play in shaping the microbial composition of the gut, and further underscores the intricate interplay between local environmental conditions and other factors such as dietary preferences in creating gut microbial diversity in *P. angulosus*. Insights gained through this study can contribute to the growing understanding of the complex dynamics that modulate interactions between host and bacteria in marine ecosystems, with potential uses in ecological and environmental studies.

Chapter six: General discussion

This study investigated the morphology (Chapter 3), genomic diversity, presence of population structure, fine-scale local adaptation (Chapter 4), and gut microbial diversity (Chapter 5) of the Cape sea urchin, *Parechinus angulosus* along the South African coastline, exposed to the characteristic contrasting oceanic regimes present on the South African coast (Filander 2014). Such multidisciplinary approaches have long been due, given that marine organisms have developed a diverse array of behavioural (Candolin and Rahman 2023), morphological (Leeuwis and Gamperl 2022), physiological (Bal *et al.* 2021), genomic (Alter *et al.* 2021), and epigenomic adaptations (Beal *et al.* 2020) to minimise the negative impacts on species' life histories and long-term survival induced by the ever-increasing anthropogenic pressures on world's oceans. Understanding the underpinning multiple dynamics of marine ecosystems has never been more important to address the resiliency of population to climate change and help minimise and mitigate the risks that otherwise lead to either functional (Solan *et al.* 2004, Pimiento *et al.* 2020) or numerical extinction (Bakker *et al.* 2021).

To assess the resiliency of marine populations, gradual changes in the functional diversity of marine ecosystems need to be investigated (Gladstone-Gallagher *et al.* 2019, Biggs *et al.* 2020, Rincon-Diaz *et al.* 2021). The long-term monitoring of marine ecosystems is important because deterioration in the health of marine ecosystems can be gradual and typically remains unnoticed for decades (Conley *et al.* 2007, Diez *et al.* 2009). As a result, when the ecological signature of degradation in marine ecosystems is detected, it is often too late to address the root cause of the problem and implement effective restorative strategies (Sinclair *et al.* 1995, Connors 2014, Moser *et al.* 2020). Thus, the development of a suite of methodologies that can identify changes in the biological, chemical, and physical attributes that are associated with the proper functioning of marine ecosystems, is crucial for timely and effective management plans. This thesis partly filled this gap by targeting disciplines that could highlight signs of past and recent adaptive divergence and resiliency. Conventional methods of monitoring marine ecosystems rely on the application of several biological (Sukhotin and Berger 2013), biochemical (Dahlhoff 2004), and physical properties (Rombouts *et al.* 2013) of systems that can reflect the health and resiliency of marine habitats. More recently, the usefulness and applicability of using some species that are collectively known as bioindicator species has drawn significant interest among researchers investigating ecological resilience (Bayouli *et al.* 2021). A bioindicator species can reflect the degradation of the health of marine systems through measurable physiological, morphological, or genomic changes in their communities (Holt and Miller 2011, Helmholz *et al.* 2016, Manickavasagam *et al.* 2019).

Quantitative analysis of shape and size has been widely used as an informative bioindicator to monitor the health of ecosystems (Kunakh *et al.* 2022) since the changes in shapes and sizes of body parts in an organism can reflect the influence of environmental factors on an individual's fitness. In line with the findings of others, this study (Chapter 3) indicated that changes in *P. angulosus*' morphometrics to some extent reflect the adaptive responses of this species to the contrasting environmental conditions they are exposed to across the South African coastline. These driving environmental factors are likely temperature, habitat (substrate type), and current speed. Harnessing this information can provide valuable information on an individual's fitness (Pecorino *et al.* 2012, Zhao *et al.* 2018). Specifically, understanding how these environmental variables shape the morphology of sea urchin populations contributes to our knowledge of local adaptation and ecological responses to environmental gradients.

A combination of eight of the most important morphometric traits of the test and spine measurements in *P. angulosus* indicated differences in individuals from the east and west coasts of South Africa (Chapter 3). When these measurements were used to calculate the three indices of globosity, volumetry, and density, similar patterns of variation were found, with consistently higher values on the west coast for all three indices. A supervised machine learning algorithm (K-Nearest Neighbour – KNN) to a large extent further distinguished between individuals from the east and west coasts based on spine morphometric measurements (length and width for large and small spines). By assessing the performance of these methods in distinguishing between east and west coast populations, the study provides insights into the feasibility and limitations of using morphometric data for population classification. While the Bayesian analysis performed on spine morphometrics performed poorly in the classification of populations, this was primarily due to significant intra-population variation. The morphometric analysis almost uniformly indicates that *P. angulosus* morphology of test and spines could be considered useful bioindicators to quantify adaptive divergence and identify resiliency to change, demonstrating the power of morphometric traits in characterising and comparing sea urchin populations. While the overall, yet not consistent, higher morphometric values on the west than on the east coast is interesting, it cannot fully indicate which morphotype may be morphologically at risk or advantage as further analyses would be required to isolate the physiological trends linked to these different forms. In conclusion, this study demonstrates that individuals from the east and west coasts of South Africa exhibit variation in aspects of their phenotype (the test and Aristotle's lantern), which indicates that the drastically different

ecological conditions found on each of the coasts have led to phenotypic differences in the South African *P. angulosus* population.

The formation of genetic structure in marine organisms is the product of natural selection (Nicolaus and Edelaar 2018), random genetic drift, and gene flow (Chiu *et al.* 2023). The population of *P. angulosus* showed a moderate level of genomic differentiation between populations on the eastern and western coasts of South Africa (Chapter 4), confirming previous findings on other marine invertebrates along the South African coastline (Mertens *et al.* 2018, Emami-Khoyi *et al.* 2021). This pattern was, however, only obvious when the sea urchin genome was scanned using thousands of high-quality SNPs (generated independently from a combination of de novo and reference-based approaches) dispersed across the entirety of the genome. The presence of multiple outlier loci that significantly deviate from the background rate of genomic differentiation; other loci that illustrated significant association between genotypes; and their characteristic environmental and morphological variations typical of the east and west coasts of South Africa, provide evidence for the presence of adaptive differentiation and cryptic genetic structure among *P. angulosus* populations. It is noteworthy that assigning functional significance to outlier loci or the loci identified in the genotype-phenotype association studies in non-model marine organisms like *P. angulosus* needs high-quality annotated reference genomes for adequate assignment (Hakim *et al.* 2019). In the absence of such a reference template, functional significance assigned to these putative variant sites needs to be interpreted within these limitations (Ishizuka *et al.* 2023). While some of these loci could be directly linked to the underpinning molecular mechanisms involved in local adaptation or are in linkage to those loci involved in the process, several other evolutionary forces can leave behind genome-wide footprints that are practically indiscernible from those left behind by natural selection (Lotterhos and Whitlock 2015, Dylan 2020; Chapter 4). By investigating genome-wide variation, this study provides valuable insights into the spatial distribution of genetic diversity and adaptive potential within the species. In summary, genome-wide variation analysis revealed cryptic population structure and the presence of adaptive divergence between *P. angulosus* individuals collected from the east and west coasts. The identification of outlier loci associated with local adaptation suggests that individuals of the South African population of *P. angulosus* have evolved genetic adaptations to the prevailing environmental conditions of their respective habitats.

The reconstruction of the phylogenetic history of *P. angulosus* places the most likely geographic location of the root in the south-western Cape bioregion which is nested in the west coast (Chapter 4). From the root location, the individuals first disperse eastwards until comparatively recently, after which individuals disperse westwards of the root point. A deep divergence between four haplotypes identified from Betty's Bay (east coast), Groenrivier (west coast), Doringbaai (west coast), and Kommetjie (south-western Cape bioregion, nested in the west coast) and the rest of the individuals' mitogenome could point to the significance of this location in the evolutionary history of sea urchins since these locations may harbour a rare, ancient mitogenomic lineage of evolutionary significance that merit further investigation.

Interestingly, genome-wide scans of three individuals from Kommetjie, a key site in the south-western Cape bioregion (nested in the west coast) as located in the border zone between the sea urchin populations from what has been defined as the east and west coasts, shows that these individuals are distinct since their genomic makeup does not contain a genomic signature that is shared between individuals from both the east and west coasts (Chapter 4). This result substantiates earlier findings of the presence of unique mitogenomic lineages around this location and merits further investigation by including more samples compared to what has been used in this study. Surprisingly, the Kommetjie population, which harbours one of the oldest mitogenomic haplotypes, shows the largest shift in allele frequency between current and predicted scenarios of climatic change, which makes this population an interesting subject for future studies that focus on investigating the resiliency of sea urchin populations. This conclusion is, however, based on the assumption that outlier loci detected are positively related to the individual's fitness.

Modelling changes in allele frequency in the population of *Parechinus angulosus* in response to extreme scenarios of future climatic change also shows that different populations are likely to respond to these changes differently (Chapter 4). The average change in the allele frequency of a subset of selected loci varied between 0.006 and -0.003. While these numbers in absolute terms might not look significant, studies on fine-scale local adaptation and adaptive divergence in marine and terrestrial organisms show that the major contributor to the development of local adaptations is in fact the integrated impact of small to moderate changes in allele frequencies (Hornoy *et al.* 2015, Oleksiak and Rajora 2020). Establishing a direct link between changes in allele frequency and the impact it would have on an animal's overall fitness, in the absence of an annotated reference genome and its functional identity, is a challenging task (Ballouz *et al.*

2019, Svensson and Connalón 2019). It is, however, reasonable to assume that significant changes in allele frequency can have unpredicted impacts on population resiliency against environmental pressures (Drury 2020). Changes such as an increase in the frequency of a beneficial allele that can boost the fitness of the population; an increase in the frequency of alleles that do not infer any fitness-related benefits; or increase in alleles that have negative impacts under new environmental conditions, can disrupt the evolutionary potential of a population in the face of climatic challenges (Whitlock 2000, Orr 2003, Kassen and Battallion 2006, Martin and Lenormand 2008). Until a reference genome with complete annotation for *P. angulosus* becomes available, such results merely point to the heterogeneous responses of current populations to future climatic scenarios.

An analysis of the gut microbial diversity and its functional content in *P. angulosus* showed significant variation in the compositional community diversity and the biochemical functions they perform (Chapter 5). The majority of the host-associated gut microbiota in *P. angulosus* was found to be involved in carbohydrate, amino acid, and energy metabolism, which is in line with the findings in other similar organisms (Hakim *et al.* 2016). These differences in bacterial taxa and metabolic pathways hint at regional and intra-regional variations in the gut microbiome. Modelling the variation in gut microbiota among populations of sea urchins that are exposed to markedly different ocean regimes on the east and west coasts of South Africa using different methods, such as balance regression, and ANCOM analyses consistently reported some taxa as being differentially abundant between the east and west coasts of South Africa. A direct link between differentially abundant taxa and the oceanic features on the east and west coasts of South Africa needs to be interpreted with caution since controlling for confounding effects of host physiology, health status, age, sex, and prior exposure to different environmental stressors under remote field conditions remains logistically challenging (Wunsch 2007, Ewert and Sibthorp 2009).

The intricate relationship between hosts and host-associated microbiomes opens a new avenue for its application as a bioindicator to monitor the health of coastal ecosystems (Ribas *et al.* 2023). Studies show that when performed under stringent protocols, the diversity of host-associated microbiomes can detect perturbation in ecosystem functioning far before traditional physical and biochemical bioindicators (Burns *et al.* 2017, Harrison *et al.* 2017, Glasl *et al.* 2018). Before using *P. angulosus* microbiome as an emerging bioindicator, additional analyses need to be performed to justify its application. The recommended criteria for microbial

diversity to be assigned as an informative bioindicator vary significantly depending on the habitat in which the animals are found (Rasmussen *et al.* 2021, Stefunkova *et al.* 2021). There are, however, certain universal requirements that need to be satisfied prior to this designation. These criteria are specificity, monotonicity, variability, and practicality (Roitman *et al.* 2018). Briefly, specificity refers to an organism's potential to uniquely respond to the presence or absence of environmental stressors. Monotonicity points to the extent of an organism's response, both in terms of intensity and duration to the presence of stressors. Variability means that the response of the community to the presence or absence of environmental stressors needs to be consistent. Lastly is practicality, which means feasibility, cost-effectiveness, and ease of gathering and analysing collected data. It is also noteworthy that an informative bioindicator should be ecologically sound and various studies validate such suitability of microbiome in both terrestrial and marine ecosystems (Nugues *et al.* 2004). Significant changes in microbial diversity in response to increased abiotic stressors such as pCO₂ levels in the water column have been reported as being used as an early alarm system for acidification in aquatic ecosystems (Liu *et al.* 2010). More studies are needed to investigate the response of sea urchin gut microbiota to evaluate how well compositional variation of gut microbial communities satisfies the criteria, as such information cannot be inferred from the current study. For this purpose, the survey of gut microbial diversity and its function can serve as a first step towards this goal. A multidisciplinary bioindicator that harnesses the combined power of changes in host genomic diversity, modifications to the host-associated microbiome, and the measurement of morphological characters to detect early signs of disturbance in marine ecosystems will provide researchers with a novel set of interdisciplinary methodologies to address some of the most devastating impacts of changes in the earth's climatic patterns.

Upon conclusion of this study, it is worth critically assessing whether the results of this study may have been different had the sampling range been extended to encompass the whole of *P. angulosus*' distribution range. While sampling on the east coast began at the eastern-most limit of the species, rendering further collection from warmer waters not relevant, opportunities for extending sampling northward, and technically westward, exist along the west coast. Despite temperature fluctuations on the west coast of Africa due to El Nino events, Angola's warmer waters present interesting prospects for individuals of *P. angulosus* that are potentially better adapted to warm environments similar to that which is found on the east coast of South Africa. Although extending sampling into Namibia and Angola may enhance our understanding of regional patterns, it is unlikely to alter the fundamental conclusions regarding local adaptation,

genetic divergence, and microbial diversity in *P. angulosus* populations. This stems from the considerable ecological and genetic differentiation already observed within the South African distribution range of this species, coupled with the fact that the eastern-most distribution limit corresponds to the suspected upper thermal limits for this species. Consequently, any additional data garnered by sampling north of South Africa's border on the west coast, would likely mirror the ecological conditions already represented by populations sampled in this study. Therefore, the inclusion of such data is unlikely to yield meaningful contributions.

The development of more sensitive biochemical assays and DNA sequencing technologies, combined with high-precision devices to measure various biotic and abiotic factors in marine ecosystems enable scientists to develop a new generation of multidisciplinary monitoring tools that combine the power of conventional methods with recent developments in computational techniques (Creer *et al.* 2016, Hering *et al.* 2018, Pieretti and Danovaro 2020, Laini *et al.* 2022). These new multidisciplinary bioindicators are comparatively less labour-intensive, often faster than traditional methods to assess ecosystems, and easier to implement under challenging marine conditions (Deiner *et al.* 2017). Since the amount of data produced by these new generations of biomonitoring techniques is significantly larger than the widely used conventional techniques, comparable bioinformatic expertise and computational resources are needed to interpret these results (Fernandez *et al.* 2014, Grolinger *et al.* 2016, Attwood *et al.* 2019). An integration of these multidisciplinary approaches must closely be considered for further ecological applications that aim to manage resources at risks due to the multiple effects of climate change.

In recent decades, there has been a rising momentum in global conservation and restoration efforts. These initiatives, which encompass restoration programmes, establishment of marine protected areas (MPAs), and promotion of blue economy strategies, point to developing sustainable frameworks for the reconciliation of economic benefits and preservation of biodiversity in marine ecosystems (Jeffery *et al.* 2022). The establishment, implementation, monitoring, and management of MPAs require that the two major core criteria of connectivity and representability of marine ecosystems be met (Lee *et al.* 2022). It also requires a thorough evaluation of the suitability of marine habitats for sustainable blue economy development to be considered prior to the selection of oceanic habitats for MPA establishment (Cziesielski *et al.* 2021). This necessitates the integration of the novel set of biological, biochemical, and physical bioindicators, coupled with emerging multi-omic approaches such as genomics,

microbiomics, transcriptomics, epigenomics, lipidomics, and metabolomics which collectively allow ecosystem managers to identify the most suitable habitats for this purpose. Based on this, the current research uniquely proposes multidisciplinary scientific model to be incorporated in coastal ecosystem management and development plans to protect the vulnerable coastal ecosystems subjected to increasing environmental pressures.

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Appendix 1 (Supplementary material for Chapter 4 and 5)

Table A1.1: Codon changes in the genome of the Cape sea urchin, *Parechinus angulosus* collected from sites along the South African coastline.

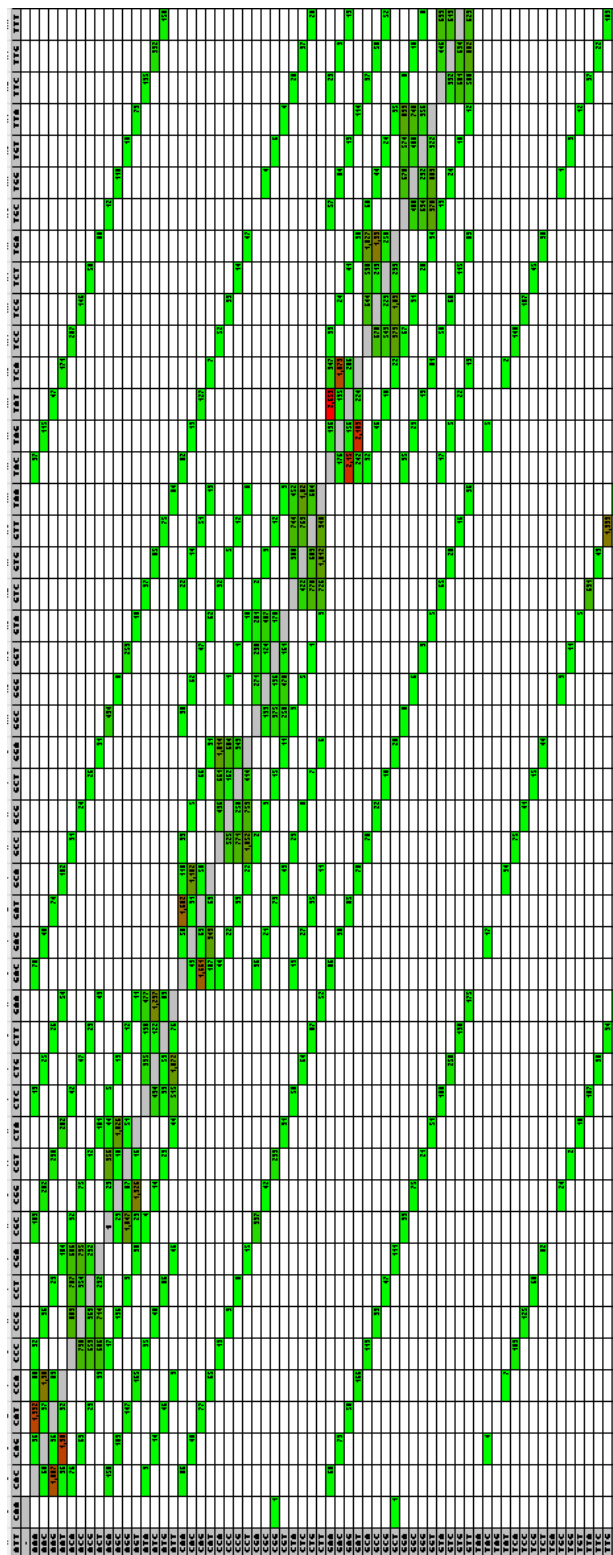


Table A1.2.: PERMANOVA results on unweighted and weighted UniFrac indices of β -diversity.

	Group 1	Group 2	Sample size	Permutations	pseudo-F	p- value	q- value
Unweighted	EAST	WEST	75	999	2.8963745006307753	0.002	0.002
Weighted	EAST	WEST	75	999	3.718319509970393	0.015	0.015

Table A1.3: The results of simplicial linear regression for all balances.

Balance	Intercept	p-value
y0	5.751768196094561e-25	0.0033183478455725487
y1	2.2382533062398775e-28	0.006592066437058289
y2	2.8571918172107904e-15	0.05702050919853683
y3	0.06769530356948876	0.06769530356948876
y4	7.469876262119233e-22	0.18976853545468025
y5	5.364136400990007e-20	0.574300264088986
y6	7.206696945720889e-11	0.007323961691976339
y7	1.227037527620842e-06	0.6426677768107484
y8	0.031537753072578244	0.6830102732445564
y9	0.676827784855083	0.09930391728335432
y10	4.4351171138061006e-07	0.8628281898621292
y11	0.9874433874092801	0.9874433874092801
y12	1.7680377058195485e-07	0.40982979377219364
y13	0.0023380749118722304	0.12218144368429976
y14	0.0007547544176995356	0.02404962586902745
y15	2.1397054050340614e-07	0.0658922813150238
y16	0.0009295239098025988	0.602968601394267
y17	2.79753428408818e-19	0.7669344267727484
y18	0.20946469937424803	0.2921542903936084
y19	8.019366837204641e-05	0.08912459883075113
y20	5.7005555637637696e-12	0.004327225446048862
y21	0.13593126992099852	0.9079241424779975

Balance	Intercept	p-value
y22	0.012667127882039964	0.4806502933686835
y23	5.270647819953781e-29	7.92804206212488e-06
y24	0.6124511624138087	0.6124511624138087
y25	1.562525646140398e-09	0.14559320547613436
y26	4.438488302072555e-08	0.052170084127765935
y27	0.00012110518768136075	0.8737862446060845
y28	0.03357698886420826	0.310798388566635
y29	4.345074389568783e-07	0.431453692438691
y30	0.0008819654075746325	0.0008819654075746325
y31	0.404101274872369	0.34205711582545373
y32	0.0011730530732291463	0.040357492585578844
y33	3.226183560541057e-11	0.24332817080493804
y34	0.5813651509173632	0.8415820316009514
y35	4.013185449009965e-10	0.7914055484602516
y36	0.42612519476077915	0.2820577317650872
y37	0.00011205457345478987	0.09880313670008803
y38	5.036387633726392e-06	0.019819528611779092
y39	3.7700492096109695e-07	9.075323359275348e-10
y40	0.4110439131690925	0.4110439131690925
y41	0.6145658809050596	0.6145658809050596
y42	0.30624051168646765	0.726672135588257
y43	0.159433994843368	0.159433994843368
y44	0.0006043360020235342	0.3025933554757548
y45	0.7192871106568154	0.03143140504816445
y46	0.0077759740342638935	0.4732519235736514
y47	4.301940073759802e-07	0.19608107075990522
y48	0.23906230957369815	0.04144972910736345
y49	0.4923720565463531	0.020013592978390154
y50	0.4525597062970055	0.402751796046137
y51	0.026841207065792832	0.9269154296066368
y52	0.28012640110212833	0.6399812843217644

Balance	Intercept	p-value
y53	1.7836024285918643e-13	0.024116419815484718
y54	0.00014790312591239006	0.07754031594964289
y55	0.000186347229779001	0.09382869589644602
y56	0.2965799545691761	0.9326015296278514
y57	0.03269747550031587	0.9835286900042006
y58	0.7591073927840764	0.30746984531647453
y59	0.09474045492748115	0.13728703070331383
y60	0.08146820443257669	0.9820094236139868
y61	0.171098863261807	0.4057817115977207
y62	0.17577015884718686	0.2301981581366749
y63	7.76006167533726e-05	0.4940067447400597
y64	0.0009891427096689741	0.25657185497652285
y65	9.783245381429701e-07	0.7317089849365453
y66	6.619759987553872e-06	0.054368967277105974
y67	0.3518980089935221	0.7638985092841967
y68	0.010636464694020107	0.3285449330145621
y69	0.014018739135494415	0.6859098692513752
y70	0.04421056534099913	0.20355284585201652
y71	0.0031274836425828644	0.2058988191211306
y72	0.8860371167866996	0.8860371167866996
y73	0.5649592156016578	0.5649592156016578
y74	0.4666442352551697	0.9441221356440156
y75	1.188208195428966e-16	0.976292086902695
y76	0.028458952384804205	0.028458952384804205
y77	0.0003742695761711305	0.0026746094336201807
y78	0.08612757910024853	0.2759823245113043
y79	0.0007625552418647194	0.2618389562089247
y80	0.0044909816825517215	0.7054100462439887
y81	0.06462282898968674	0.6281968757007126
y82	0.009893644887468186	0.22709849622568626
y83	0.0005325332672358056	0.0016133929058725594

Balance	Intercept	p-value
y84	0.013404379964969215	0.04038129531322094
y85	0.026994183463772498	0.37306100301411693
y86	3.424669056817899e-49	0.7045035801631433
y87	0.13242304451738712	0.0655204028876726
y88	4.999690138809274e-16	0.0007716324561954593
y89	0.8905371001833735	0.29816021089788036
y90	0.1229330517648836	0.25184319907891006
y91	0.011377332752415422	0.27540649885148716
y92	0.16187334039853657	0.30663082083282395
y93	0.4641215951572355	0.4641215951572355
y94	0.1122507983663446	0.8837952909452671
y95	0.0005196594432412892	0.061088178994836075
y96	0.0774278529993181	0.10627704453631764
y97	2.2746857196529353e-05	0.2734951975660727
y98	5.491940463853338e-10	0.05342171488569561
y99	3.2384870997818535e-12	0.0705197251283412
y100	0.812082056954463	0.812082056954463
y101	0.0003986244293860191	0.031989088245701534
y102	0.6514997269829221	0.6514997269829221
y103	0.32122264891845864	0.9242003249189652
y104	0.8137499862804158	0.8137499862804158
y105	3.6813375775280256e-06	0.022646135162977744
y106	0.4049563144218048	0.3854638401064736
y107	0.019447007501382094	0.3727101584679837
y108	0.001747856614216638	0.4470967324771764
y109	0.0004477422229899936	0.8645751408947417
y110	5.002885044766868e-06	0.23488623971970085
y111	0.07033070907091359	0.43241925802791015
y112	0.8143245642628777	0.8143245642628777
y113	0.0002010408421341995	0.06509565815202001
y114	0.9288241018618402	0.9288241018618402

Balance	Intercept	p-value
y115	0.921152806299035	0.921152806299035
y116	0.019661861761304498	0.4698982956676121
y117	0.09460971501196441	0.32521661232164933
y118	0.0009128199269532792	0.12777141508525564
y119	0.18294508127283943	0.36635951401598
y120	0.5825540973683285	0.5825540973683285
y121	0.3381382600534487	0.3381382600534487
y122	2.885647976546704e-07	0.05204732773391101
y123	2.0242094042215926e-09	0.1259506157658446
y124	0.06817056795270209	0.9460840959190571
y125	7.13350677496982e-05	0.011615357564494033
y126	0.00010672924014926607	0.9633875112864257
y127	0.3417296356100791	0.3417296356100791
y128	0.3902960098343745	0.3902960098343745
y129	0.8126803845339988	0.8126803845339988
y130	0.9131394024633124	0.9131394024633124
y131	0.6786866236731166	0.6786866236731166
y132	0.8710234039672172	0.42365311023736535
y133	0.11303187955869091	0.3445204755388259
y134	0.4414320053666062	0.5455781328650255
y135	0.001999897803766997	0.7450355412801197
y136	0.20077034198134264	0.717321656602819
y137	0.0017152332291011387	0.6464186172644865
y138	0.8508147641290236	0.9743309420342269
y139	0.5795126157130734	0.5795126157130734
y140	0.11501440788418897	0.45699429104528944
y141	0.11822299404997783	0.9621760244238535
y142	1.620167395676283e-08	0.6020462855569615
y143	0.8673628347200166	0.8673628347200166
y144	0.9910302696983758	0.9910302696983758
y145	0.8117384080056635	0.8117384080056635

Balance	Intercept	p-value
y146	0.9407334516994913	0.9407334516994913
y147	0.5739248178424201	0.5739248178424201
y148	0.8580191235294559	0.8580191235294559
y149	0.9173874552736471	0.9173874552736471
y150	0.021343056493571298	0.20302260326077948
y151	0.006167874159282902	0.18773646982921588
y152	0.3216694779071673	0.3216694779071673
y153	0.055611770557508676	0.5520949786596221
y154	0.007880370931272924	0.3126924008457877
y155	0.5025798470634979	0.5025798470634979
y156	0.003964585698240705	0.6765140300583532
y157	0.06080488170302898	0.26150405973840896
y158	0.028953225679707283	0.9116341794865419
y159	1.2926748022858942e-05	0.0445229967777724
y160	0.4283714757534752	0.7371800118201088
y161	0.7860001298075254	0.6833886742071893
y162	0.6044661841762822	0.05945918595766179
y163	0.003001235420018911	0.15268699986531573
y164	0.1145212112023893	0.1145212112023893
y165	0.4388189315393418	0.8545137318699463
y166	0.921002074246881	0.921002074246881
y167	0.1202594278958721	0.351544603848733
y168	0.30330317890331787	0.47870173493920953
y169	0.9950261518841282	0.9950261518841282
y170	0.1258940411101001	0.1258940411101001
y171	0.5965818303307908	0.5965818303307908
y172	0.5500094449706355	0.5500094449706355
y173	0.06892758204515778	0.1034235245639426
y174	0.642120618406431	0.642120618406431
y175	0.48008632276415897	0.5936658872694551
y176	0.017818095230525778	0.31404596714909533

Balance	Intercept	p-value
y177	0.07586314486602248	0.30290773060459736
y178	0.008211993115385313	0.1367329204410846
y179	0.03419774660771368	0.03419774660771368
y180	0.027398592066826002	0.38073828146602107
y181	0.7087989217116064	0.7087989217116064
y182	0.8906019896268931	0.8906019896268931
y183	0.673445083640724	0.673445083640724
y184	0.07425223365652203	0.07425223365652203
y185	0.9047702267274307	0.9047702267274307
y186	0.20260766914156764	0.31889748260100725
y187	0.050080467287960104	0.10367151663900791
y188	0.017378541339602052	0.3013344646385969
y189	5.569788523420373e-05	0.028825741300138513
y190	0.9886852562518911	0.9886852562518911
y191	0.017964594988240466	0.6448077453827757
y192	0.25227465722262565	0.25227465722262565
y193	0.0071657411205544455	0.4392205961369049
y194	0.002935856764194751	9.012010019295212e-05
y195	0.5521989476001479	0.5911414718230337
y196	0.06587781695992725	0.20788368002239424
y197	0.39672917810798786	0.5254289044434606
y198	0.1084842714850463	0.3399536177100517
y199	0.0004673521220590517	0.3688946561460439
y200	0.014563841785014724	0.16053155316427448
y201	0.3285704331197222	0.3285704331197222
y202	0.9372664446783047	0.9372664446783047
y203	0.09781499678293494	0.09781499678293494
y204	0.005765265039073233	0.3541812739378454
y205	0.0013356071579680843	0.48622616388841433
y206	0.028537610013331566	0.028537610013331566
y207	0.0014775792072945191	0.4424657881808528

Balance	Intercept	p-value
y208	0.1647073302322285	0.1647073302322285
y209	0.7772271429520453	0.7772271429520453
y210	0.5263341089925818	0.5809751322670945
y211	0.11780420323301226	0.3491892467027349
y212	0.1316480282021223	0.36209028480610417
y213	0.14360148623147292	0.3228826941378097
y214	0.5514987835961931	0.5514987835961931
y215	0.6321904282856967	0.6321904282856967
y216	0.7854557006802887	0.7854557006802887
y217	0.8157521130768397	0.5160966651628054
y218	0.037103642582060704	0.06407607625349075
y219	0.06361167519375346	0.7770910612435781
y220	0.21094143779506233	0.004635813192517505
y221	0.08210601792234537	0.6642316174609076
y222	0.905176736564259	0.905176736564259
y223	0.7817243769507576	0.7817243769507576
y224	0.25516568003862616	0.4512956832678169
y225	0.9901946615400994	0.9901946615400994
y226	0.6492163386416403	0.6492163386416403
y227	0.47147553592377084	0.5584936238764998
y228	0.04947991562111688	0.21215574939898188
y229	0.6609778629663148	0.6609778629663148
y230	0.0025440915333594712	0.48601977706362387
y231	0.6046726618359484	0.006421463853772923
y232	0.1430193642430591	0.9195440632486818
y233	0.5989046721137625	0.5989046721137625
y234	0.4643149118332688	0.5554570796415756
y235	0.7558186428833313	0.7558186428833313
y236	0.6239772970270798	0.6239772970270798
y237	0.2446185002928039	0.44488973260407394
y238	0.6118774371650205	0.6118774371650205

Balance	Intercept	p-value
y239	0.180356126883648	0.07827089583438977
y240	0.023698158632287247	0.5257874384238665
y241	0.0037209365432030288	0.0529583801775746
y242	0.08708596266319552	0.00033654219300383286
y243	0.3097957568199521	0.08350828655727192
y244	0.2099977955954475	0.8866828137996589
y245	0.0002873212400498712	0.055972486084266276
y246	0.5056083261408653	0.5726362431229381
y247	0.09462472497948658	0.3252332787348803
y248	0.7782337652686778	0.7782337652686778
y249	0.870075810918449	0.40493426540387906
y250	0.5667962297205628	0.5967689584985153
y251	0.620740519380838	0.2660809569421514
y252	0.13533452252039158	0.13533452252039158
y253	0.31193216904495763	0.014754193264867203
y254	0.00022496407344072268	0.31172364526406265
y255	6.171050379641836e-06	0.9074323503849887
y256	0.008960035855858898	0.48317179543411004
y257	0.002859252337358769	0.0483054002265541
y258	0.37790011317509975	0.021584458157889144
y259	0.9228400730219386	0.9228400730219386
y260	0.00212440187860128	0.8987588794375014
y261	0.5056083261407862	0.5726362431229307
y262	0.4194329306042018	0.5358115158836869
y263	0.17129327886109194	0.3948783749249939
y264	0.8173650825824603	0.8173650825824603
y265	0.28580776005371034	0.28580776005371034
y266	0.49973380650653626	0.5702396248748105
y267	0.3223246756299316	0.3223246756299316
y268	0.779665615289113	0.011240168108291333
y269	0.20062968302575457	0.7743934456083231

Balance	Intercept	p-value
y270	0.005876920200263714	0.7108864548101006
y271	0.004288088799770792	0.0513976619766503
y272	0.6696873250045254	0.6696873250045254
y273	0.7840299237565033	0.7840299237565033
y274	0.15440049632984898	0.15440049632984898
y275	0.10166156559873135	0.3328675340390911
y276	0.7065016377055511	0.7065016377055511
y277	0.11587608133633219	0.055159113517602325
y278	0.01864339459267082	0.48259031287210896
y279	0.00021745316421397311	0.10255241761754426
y280	0.035115648155375837	0.5104265918768303
y281	0.5056083261408433	0.5726362431229362
y282	0.38194566916425565	0.518488293363639
y283	0.31004137482394883	0.34552635568605283
y284	0.9541344818988138	0.9541344818988138
y285	0.6873013278845899	0.6873013278845899
y286	0.4837229256825376	0.4837229256825376
y287	0.33968723121469024	0.30577470516494065
y288	0.537557928976563	0.06867206646099296
y289	0.70903081855821	0.0004770004688830844
y290	0.01980433776472718	0.13269881588466204
y291	0.011429252526438652	0.3625415099757232
y292	0.005261011865425709	0.6641939513113725
y293	0.02136599554063589	0.20309054569509738
y294	0.0012551416640366024	0.12683606203009135
y295	0.0018702947929699753	0.11983566493731455
y296	0.556946618635239	0.08762024536586357
y297	0.0022156670835619305	0.26896941158692245
y298	0.005523719426818301	0.7929788086343232
y299	0.15104455480931023	0.15104455480931023
y300	0.5720587066291561	0.9051926457446022

Balance	Intercept	p-value
y301	0.00030934895763624703	0.8509903894711626
y302	0.5056083261408645	0.5726362431229381
y303	0.4884157253078213	0.5655792114570124
y304	0.8001348946342887	0.18464778731637949
y305	0.9631596930670379	0.9631596930670379
y306	0.0035692854276845915	0.5824113966920901
y307	0.5403709189901781	0.979332694749867
y308	0.7808881319911358	0.9016195106004422
y309	0.6486767761160925	0.7253215596403878
y310	0.7040447433594524	0.7040447433594524
y311	0.699148937065922	0.699148937065922
y312	0.012425323672043138	0.33977476336724133
y313	0.16470593319252488	0.38978481957038014
y314	0.0004279814473241176	0.21141635882286672
y315	0.007514577195391203	0.3622472899292062
y316	0.7108114174458662	0.0005651680988667326
y317	0.0038294632988164923	0.33802325561274704
y318	0.9652825529415994	0.9652825529415994
y319	0.1920579806307956	0.1920579806307956
y320	0.02805005343760891	0.3108803442519134
y321	0.3729247678009988	0.5467295884951329
y322	0.013325631538471642	0.7690243540269717
y323	0.836461209267864	0.003222606432170557
y324	0.5056083261406522	0.5726362431229126
y325	0.9945873249869678	0.9945873249869678
y326	0.8503865972233526	0.07860259253852853
y327	0.3351679897416191	0.7370934830718237
y328	0.5475768741735598	0.7756944677657753
y329	0.0029085575487927473	0.9351685057101032
y330	3.688836590715687e-05	0.4313004543827358
y331	0.0006383055036871483	0.6928369892171908

Balance	Intercept	p-value
y332	0.4375467492136006	0.4375467492136006
y333	0.004236001902940218	0.20171019959714032
y334	0.36454633111713797	0.36454633111713797
y335	0.010799461397407477	0.5691822327747881
y336	0.6637552237875427	0.6637552237875427
y337	0.001487000005349927	0.41514915354900883
y338	0.00034114409004263707	0.25692795620001363
y339	0.00036579225004816467	0.28370839546986815
y340	0.38990957943407867	0.38990957943407867
y341	0.5019679359473427	0.23269717815085178
y342	0.908350284851055	0.42691566483636967
y343	0.2546015688773394	0.5395368612383737
y344	0.44001256322320403	0.44001256322320403
y345	0.4488027635259335	0.4488027635259335
y346	0.06895103707333798	0.6265770703301641
y347	0.03838105709991625	0.3127762549352804
y348	0.9786289355277209	0.9786289355277209
y349	0.0009389929759767255	0.7337239412014294
y350	0.019278767400424935	0.8702228486690342
y351	0.5056083261408744	0.5726362431229571
y352	0.5056083261408651	0.5726362431229381
y353	0.9998846428126094	0.9998846428126094
y354	0.43327506013975636	0.9683161899935915
y355	0.20640965663410255	0.6756554770212035
y356	0.763834497200044	0.763834497200044
y357	0.029213638436217562	0.7530068208884582
y358	0.44805780620848146	0.7871591339269687
y359	0.07700504537289242	0.37422199371956133
y360	0.00014550101342469557	0.05921045931548994
y361	0.6343514386165285	0.3032828015301852
y362	0.596479250634056	0.825118109854742

Balance	Intercept	p-value
y363	0.306105293217377	0.9726242801031453
y364	0.09986598212663414	0.6706951671353525
y365	0.014018536776200399	0.35680946872021924
y366	0.9747736594968335	0.9747736594968335
y367	0.05317231646147212	0.3587494752470247
y368	0.00015012552284390947	0.09976669838779864
y369	0.0014449048350975648	0.22993352248763546
y370	0.05476460718734815	0.05476460718734815
y371	0.6765636980398235	0.2722162454137213
y372	0.32308168880904065	0.6368531565804378
y373	0.0302990626110913	0.02505499711007718
y374	0.828047546292983	0.828047546292983
y375	0.01442466414941518	0.13179350836699336
y376	0.6649556277843072	0.6649556277843072
y377	0.7265055894430523	0.793073135758029
y378	0.6843586136696607	0.699993351534969
y379	0.6345887177898056	0.6683430389237175
y380	0.021906234788944528	0.773085966646188
y381	0.04170812221260638	0.5068768550085907
y382	0.28142628651738355	0.8686876359987578
y383	0.7957304345396237	0.7957304345396237
y384	0.0540642933045509	0.7330198127939989
y385	0.1270555702392895	0.4681769308465732
y386	0.003185450356671324	0.0007105181679472784
y387	0.5056083261402932	0.5726362431228829
y388	0.5056083261406479	0.5726362431229178
y389	0.9158951614523869	0.2340433043082552
y390	0.056534131751665335	0.19018561454208113
y391	0.9516256176447339	0.9516256176447339
y392	0.059317655919563214	0.6750292388985049
y393	0.06491454659298185	0.4919925766398552

Balance	Intercept	p-value
y394	0.11801240674519022	0.3493901804876829
y395	0.48990897963617375	0.31277344208877955
y396	0.023207827986027076	0.33344762317785726
y397	0.09038206689064983	0.6809156373167835
y398	0.11235176210596669	0.16309730549112322
y399	0.03424571765612825	0.3233142075711265
y400	0.03820337119591377	0.6790796871964293
y401	0.754224742576433	0.754224742576433
y402	0.04394085921212199	0.3674587289551583
y403	0.029302857100318002	0.7556559139728706
y404	0.00033547820922535503	0.6875617755410763
y405	0.652310366474308	0.662452747128101
y406	0.0014142602866540814	0.5007265252097362
y407	0.44640627326745796	0.44640627326745796
y408	0.02814300703538441	0.1493920728640113
y409	0.883064725160024	0.883064725160024
y410	0.8200570795536108	0.32310647370609175
y411	0.7527713121922334	0.0013727566434810047
y412	0.008189492434892653	0.9111133280102958
y413	0.9731913344696415	0.9731913344696415
y414	0.0001168052057153777	0.09467627749127192
y415	0.8852564881939619	0.8852564881939619
y416	0.7707623427625332	0.9498818224720122
y417	0.6565901710441753	0.6565901710441753
y418	0.6556582589253481	0.6564296395043546
y419	0.08380517433702173	0.18796750751971136
y420	0.8964187107390285	0.8964187107390285
y421	0.934990549297305	0.934990549297305
y422	0.039802700586748785	0.12634700246541108
y423	0.3493497354469564	0.810488893565146
y424	0.7597757242254155	0.15147527803740934

Balance	Intercept	p-value
y425	0.15829174270151455	0.9897229982677007
y426	0.7479497025300117	0.8317923108840157
y427	0.011283077160266487	0.02909425515086498
y428	0.14786764993255166	0.14786764993255166
y429	0.1052438543873916	0.4680948078188224
y430	0.5412191215770541	0.5412191215770541
y431	0.5056083261408639	0.5726362431229375
y432	0.2321458276636655	0.2321458276636655
y433	0.10823364252917457	0.7433741723449093
y434	0.5012451076602089	0.14949243870849466
y435	0.0011858643750497719	0.05696511212359721
y436	0.5137683244803849	0.827845455539162
y437	0.12030544308163824	0.4520442849854036
y438	0.505863476428366	0.675757164526416
y439	0.27666043976926874	0.799456656344835
y440	0.28427068049889487	0.4212729165075746
y441	0.012767515861487763	0.6997280183169785
y442	0.9926990561685504	0.9926990561685504
y443	0.29479228349568026	0.47405295912972467
y444	0.646802386617219	0.7261912015129535
y445	0.2528692723660919	0.2528692723660919
y446	0.01563143206497181	0.1842934487300816
y447	0.06116268198496712	0.33179923797871513
y448	0.6011231493268264	0.7259717181556796
y449	0.8780273942567414	0.8780273942567414
y450	0.0015352022668365082	0.9976597466737591
y451	0.8840667893417926	0.22056033150322946
y452	0.12019638441997699	0.21614353311171913
y453	0.7519068706453886	0.8995394712741613
y454	0.23926416870956235	0.9213464531950637
y455	0.18045454205901434	0.18045454205901434

Balance	Intercept	p-value
y456	0.839418418600243	0.06837896429021911
y457	0.8849956203626275	0.15898533427930267
y458	0.5657974852995473	0.11572892772227585
y459	0.5969195630683048	0.1332501225897271
y460	0.6504175167651822	0.5846016583490856
y461	0.5717870331543953	0.5717870331543953
y462	0.1944875849471455	0.1944875849471455
y463	0.1864584861722611	0.28027699844156684
y464	0.7862351470887861	0.7862351470887861
y465	0.544210057684084	0.7168362973326212
y466	0.8920713640619455	0.8920713640619455
y467	0.08415033035789157	0.8720464983541377
y468	0.6430696417776193	0.6430696417776193
y469	0.784407553080811	0.784407553080811
y470	0.46850205184207416	0.46850205184207416
y471	0.9346883506462338	0.21113709391219956
y472	0.7794818781588623	0.7794818781588623
y473	0.2107194261918398	0.1731222125671078
y474	0.6635779422823123	0.0985002068619226
y475	0.9620514349302145	0.06192470434566254
y476	0.12413542801105903	0.9803684809572895
y477	0.8658737011045876	0.21361278850144172
y478	0.376962699749933	0.7414429201106978
y479	0.6125656408927145	0.011637271562424261
y480	0.3000480343686475	0.9835182834043341
y481	0.8308871181038393	0.008359258574329625
y482	4.778438793398427e-05	0.9381877326858274
y483	0.15171662839689945	0.4751409314302376
y484	0.7036339996092903	0.7036339996092903
y485	0.09570142986666015	0.5467989624295262
y486	0.49571480188749106	0.49571480188749106

Balance	Intercept	p-value
y487	0.9061380560212915	0.00429793828622004
y488	0.3820007899785155	0.3820007899785155
y489	0.20196331745626753	0.3632943360751647
y490	0.40535775629225246	0.15782971587270728
y491	0.6687038577240432	0.32120876632097195
y492	0.5149555146083079	0.5343463631754906
y493	0.883153275586296	0.883153275586296
y494	0.10104853009540228	0.8331246618934108
y495	0.6615726433566039	0.6615726433566039
y496	0.8325266572983816	0.31854073447408937
y497	0.9265511453121278	0.9265511453121278
y498	0.36790326215664726	0.36790326215664726
y499	0.05573571526280455	0.21405709437638293
y500	0.6625834569968292	0.6625834569968292
y501	0.9158352626863694	0.9158352626863694
y502	0.5765719900690998	0.5765719900690998
y503	0.8424729470693626	0.9081305391068413
y504	0.999999999999807	0.15028938150266877
y505	0.7600690423800835	0.10420918879761798
y506	0.7866513197677951	0.7866513197677951
y507	0.40140749821625965	0.01561700208699518
y508	0.023670963564329274	0.5751640138466829
y509	0.56697673974933	0.56697673974933
y510	0.5851046686736677	0.5851046686736677
y511	0.8208689412466075	0.061901878150326085
y512	0.999999999999929	0.02399888189823305
y513	0.4160979982160171	0.4160979982160171
y514	0.7046154661120182	0.6425777261090532
y515	0.5835138137301046	0.008787510025757167
y516	0.18028122379394854	0.9419758679614914
y517	0.0035227221448016263	0.2377594606184398

Balance	Intercept	p-value
y518	0.11168889018442281	0.27343428278218046
y519	0.4235165622458261	0.4235165622458261
y520	0.5340302402424804	0.6076858475891029
y521	0.07714841471232647	0.020601430323154646
y522	0.947374636929706	0.947374636929706
y523	0.9545730079315913	0.9545730079315913
y524	0.11183635741471493	0.14932650172831083
y525	0.19506583480323392	0.3827623336060022
y526	0.4403322846947644	0.9631823121848795
y527	0.9660721007277798	0.16339009765968107
y528	0.8190698053135403	0.17703462673887058
y529	0.9962090192215167	0.701223312879875
y530	0.5056083261408462	0.5726362431229365
y531	0.16187152397864518	0.3076913404952152
y532	0.790480532095858	0.5257033845286193
y533	0.904219994504473	0.1461647154880883
y534	0.4987726520902971	0.17187814050647338
y535	0.22275834495804864	0.7797828588744596
y536	0.6952734585679228	0.26318135498718326
y537	0.18710575682337574	0.29526472780726837
y538	0.060948944117434886	0.2824439162476462
y539	0.8792920914355586	0.0028274052439431915
y540	0.4144451550804077	0.4144451550804077
y541	0.14881251599932765	0.5654150280410721
y542	0.9457872663648172	0.9457872663648172
y543	0.7608834200676139	0.04127459891697935
y544	0.1269724933154912	0.1269724933154912
y545	0.6891324778373713	0.6891324778373713
y546	0.4629189289572489	0.5548621357968693
y547	0.5020591806446388	0.5020591806446388
y548	0.27570240614608327	0.46333136963637833

Balance	Intercept	p-value
y549	0.9953022638063682	0.9953022638063682
y550	0.3618261128170365	0.5414674625807638
y551	0.16320584460874935	0.8224709737025147
y552	0.29496468912270013	0.29496468912270013
y553	0.48307650569897254	0.5633605140956227
y554	0.19805350188253734	0.41441478508831364
y555	0.9883838573257921	0.9883838573257921
y556	0.9766868260572976	0.8510230918336483
y557	0.13221301868923313	0.20655997272812224
y558	0.881919502844842	0.881919502844842
y559	0.2167748066681051	0.4271392738683104
y560	0.5082248756465269	0.5736989165498759
y561	0.45193356004722873	0.45193356004722873
y562	0.4186613103280715	0.4186613103280715
y563	0.33536831353950547	0.884687060027677
y564	0.9999999999999998	0.15028938150267004
y565	0.8984310063227596	0.013687994492828521
y566	0.6452995214332303	0.6452995214332303
y567	0.7413369683956277	0.3727636184586519
y568	0.9858424631018563	0.9858424631018563
y569	0.3246361638283053	0.3246361638283053
y570	0.5388321140039688	0.5388321140039688
y571	0.7211715036896897	0.7211715036896897
y572	0.11507793122784193	0.9540107876460969
y573	0.6142604994726204	0.6142604994726204
y574	0.42528170299872287	0.6737460639748528
y575	0.2517585881593996	0.7546990310155357
y576	0.1428430597675228	0.13479524459975997
y577	0.7933495403918714	0.7933495403918714
y578	0.45414037133567053	0.013301165389428837
y579	0.030116927787555786	0.3597760889448123

Balance	Intercept	p-value
y580	0.18234628973750133	0.970361410001045
y581	0.9324133926215584	0.9324133926215584
y582	0.0983296211381179	0.0983296211381179
y583	0.005643399227002789	0.6539271593534141
y584	0.0013250149883289094	0.124213227471743
y585	0.7866192186182932	0.7866192186182932
y586	0.0005922409641034099	0.3556408815819949
y587	0.5759767842240848	0.5759767842240848
y588	0.8840441021750811	0.8840441021750811
y589	0.8155282175963836	0.39960569567943494
y590	0.25666574697961847	0.45219385892045316
y591	0.701179405049969	0.023581751956255583
y592	0.7567561664530474	0.7567561664530474
y593	0.16172128155394064	0.7987610078012078
y594	0.5827401883028225	0.8459638922487686
y595	0.7317668565935511	0.7317668565935511
y596	0.3973362462218376	0.26275173250891726
y597	0.29148511806210803	0.1132639388752918
y598	0.33429472410632666	0.495017111918862
y599	0.05912605453206624	0.4724981904293145
y600	0.9208256869139677	0.9208256869139677
y601	0.9216001228319317	0.29385513180133843
y602	0.27912640389788496	0.27912640389788496
y603	0.6390209045169007	0.6390209045169007
y604	0.633297244213344	0.633297244213344
y605	0.5992441570800265	0.9815490691360306
y606	0.07092549395008547	0.3427895850483601
y607	0.8946449124528357	0.8946449124528357
y608	0.4127454370052953	0.4127454370052953
y609	0.772600041114159	0.07672118530434699
y610	0.9744597390358534	0.2907055050776481

Balance	Intercept	p-value
y611	0.6295904453949881	0.6295904453949881
y612	0.7689391584430475	0.7689391584430475
y613	0.6339333501439001	0.7592866180541895
y614	0.6317283063426125	0.6317283063426125
y615	0.5565931145243312	0.5565931145243312
y616	0.5405934086308457	0.4513756197558612
y617	0.6576716558779692	0.6576716558779692
y618	0.5104701938228057	0.574608492457086
y619	0.06473496871678402	0.2879228311738814
y620	0.9218139569592259	0.9218139569592259
y621	0.9333824105111301	0.9333824105111301
y622	0.09243652274128998	0.322785475908524
y623	0.2427838933135933	0.3191336388056283
y624	0.15861181825297574	0.38495903462091274
y625	0.6765855321254739	0.6765855321254739
y626	0.645862239407757	0.645862239407757
y627	0.9999999999999689	0.1502893815026683
y628	0.895141994090729	0.191247835664041
y629	0.26037983731094505	0.4544043456655379
y630	0.5088304289495789	0.5088304289495789
y631	0.6964473575438795	0.6964473575438795
y632	0.5174807390814335	0.08193479474759055
y633	0.999999999999956	0.04638753952419156
y634	0.844129021391663	0.26323082236711365
y635	0.3830919052603893	0.5190317830225308
y636	0.1634247492022752	0.3403458669424402
y637	0.25173514345339004	0.761988256525886
y638	0.034907471514624804	0.2367981785678934
y639	0.4341487736320099	0.5423750979532432
y640	0.15045726165130516	0.15045726165130516
y641	0.1377236482716882	0.8060730829589332

Balance	Intercept	p-value
y642	0.6459663630834378	0.0063708582343612215
y643	0.8124345892656861	0.007354276602733335
y644	0.05128421270471117	0.6292943823765065
y645	0.8301852838715673	0.9512759247244039
y646	0.07565009041080284	0.6642813851153728
y647	0.0014464571322844182	0.10764578484453591
y648	0.7120644225617039	0.7120644225617039
y649	0.029972126502801653	0.19811470095416095
y650	0.2526346571154971	0.44202571953318137
y651	0.04530205020204887	0.3642202199542337
y652	0.24900801616559287	0.73517855197834
y653	0.0010312822187329353	0.05958882099974924
y654	0.5580672808342528	0.5580672808342528
y655	0.00018496519538122396	0.5058941082970445
y656	0.6999445446435668	0.6999445446435668
y657	0.690450408038337	0.690450408038337
y658	0.30148590297846145	0.4777155742049892
y659	0.8531660207065441	0.19075096775104577
y660	0.37953892927639205	0.5173441308100957
y661	0.3679358067665014	0.5117695885810689
y662	0.3616214687120101	0.9520848295415911
y663	0.47574116247625353	0.1630003151511617
y664	0.05269470069296332	0.2622645408300093
y665	0.6987295538422305	0.6987295538422305
y666	0.695096265273833	0.695096265273833
y667	0.5175011759839641	0.7837310935017254
y668	0.08766727636028625	0.2463465054381362
y669	0.5851394391218717	0.7272749782530543
y670	0.7689457064986066	0.7689457064986066
y671	0.9939681727223881	0.9939681727223881
y672	0.6929609062952784	0.12079463979544695

Balance	Intercept	p-value
y673	0.6917725402583895	0.07174543905588626
y674	0.0676714974502878	0.09114111294297847
y675	0.7359439315340318	0.6929272734233987
y676	0.3889690239002992	0.2013610119876387
y677	0.17233810358776216	0.6101343747676913
y678	0.9999999999998429	0.1502893815026629
y679	0.34823442921357617	0.7404524218536117
y680	0.5494608057302852	0.5494608057302852
y681	0.8128408427137932	0.8128408427137932
y682	0.8206037449233866	0.8206037449233866
y683	0.08563747094317736	0.08563747094317736
y684	0.7252932864258845	0.7252932864258845
y685	0.5220633820128806	0.861437350288024
y686	0.5884704899687515	0.60498760143951
y687	0.10253534033867145	0.3337916442968497
y688	0.6550172160246899	0.6550172160246899
y689	0.6086128910112849	0.6086128910112849
y690	0.9170466933283197	0.9170466933283197
y691	0.7692576643483581	0.7692576643483581
y692	0.5830186139774034	0.021935267189762464
y693	0.9847193795977035	0.9847193795977035
y694	0.5241108622188425	0.5800890812344688
y695	0.25002273109006207	0.4481922038258688
y696	0.21247005974962688	0.8505337942814435
y697	0.16120919546677218	0.16120919546677218
y698	0.5004072752505181	0.5004072752505181
y699	0.7323279110027935	0.25993984562647554
y700	0.4992101722883207	0.5700252678112621
y701	0.030465194658738513	0.2268959459639757
y702	0.08479687306980495	0.08479687306980495
y703	0.0834759786169999	0.31235374837244223

Balance	Intercept	p-value
y704	0.4375009254846899	0.5438529618802241
y705	0.11716032057236195	0.5878775614859048
y706	0.7874745532335472	0.7874745532335472
y707	0.3473530162820077	0.5016295247082482
y708	0.4189984858972201	0.7946025788348412
y709	0.7861767978529501	0.7861767978529501
y710	0.01648439471012224	0.4800915731521863
y711	0.5752671838197069	0.6000000945126125
y712	0.9477458134828664	0.9477458134828664
y713	0.0036819887169934327	0.34427147369223465
y714	0.16779957494544837	0.36005781388256086
y715	0.3273485846338607	0.43360038718933935
y716	0.030583962396421468	0.0702332382704875
y717	0.7033837946018293	0.005242071565033792
y718	0.28827044830855114	0.28827044830855114
y719	0.04538920728973723	0.24287350558019205
y720	0.3548191273779645	0.9366813852602659
y721	0.06155492674123455	0.3614971725337346
y722	0.8963827514626018	0.8963827514626018
y723	0.2857920117362695	0.3986759540947623
y724	0.005879569474610282	0.15688366728590075
y725	0.3459212207623516	0.45906888895602715
y726	0.3452545885937909	0.5005766678404688
y727	0.505608326140941	0.572636243122945
y728	0.7252553435092741	0.3372826070714782
y729	0.34253692972419986	0.8617627312235193
y730	0.5056083261408988	0.5726362431229483
y731	0.16284804863831534	0.3781754879973922
y732	0.6086636954636802	0.6125054881536494
y733	0.9667555403266375	0.1918560913064191
y734	0.9341002118352384	0.20645587115733965

Balance	Intercept	p-value
y735	0.4297087310987673	0.4297087310987673
y736	0.8617504455144636	0.8617504455144636
y737	0.10035333360098199	0.3314744084109289
y738	0.38593013853711744	0.15193606225490153
y739	0.2357082319605505	0.2357082319605505
y740	0.02118408287496736	0.4980282922324264
y741	0.38542269329966283	0.13406220464849233
y742	0.5352704339361506	0.5352704339361506
y743	0.6987423934462814	0.004558450877145205
y744	0.9803495879809085	0.8006254224549738
y745	0.9999999999999982	0.15028938150266943
y746	0.9999999999999998	0.15028938150267004
y747	0.8849775574607783	0.027210007671998555
y748	0.9876663841725563	0.9876663841725563
y749	0.04421538130954503	0.2551060220449587
y750	0.3394234728427855	0.3394234728427855
y751	0.9167009224576755	0.9167009224576755
y752	0.31200826247694596	0.483378516215316
y753	0.3015212209096999	0.4777347727973883
y754	0.8971835993626303	0.8971835993626303
y755	0.6574773601187207	0.6574773601187207
y756	0.7055752870686838	0.7055752870686838
y757	0.32261135252606987	0.32261135252606987
y758	0.8647822705296413	0.8647822705296413
y759	0.7661613997148093	0.7661613997148093
y760	0.06446374693289332	0.2408172323689181
y761	0.7894930759510307	0.7894930759510307
y762	0.3978580130078858	0.5259528574318204
y763	0.8309711027125393	0.8309711027125393
y764	0.9369974287613118	0.1899941384603134
y765	0.9617456894364123	0.9617456894364123

Balance	Intercept	p-value
y766	0.8978414707691591	0.8978414707691591
y767	0.24931629088260474	0.44776294329800936
y768	0.845635241827145	0.845635241827145
y769	0.21229215421807252	0.21229215421807252
y770	0.8713990008139131	0.8713990008139131
y771	0.43452933198174054	0.5425431906987901
y772	0.7607127310439802	0.7607127310439802
y773	0.5452270585637755	0.5452270585637755
y774	0.22054226458449677	0.429618440964184
y775	0.6009618818284835	0.9481268886851666
y776	0.16693636893230784	0.373191949249154
y777	0.5627947617360824	0.5952339239453575
y778	0.5621112163936484	0.5621112163936484
y779	0.007406897586869362	0.15831624756635315
y780	0.10143317749435801	0.859025672290685
y781	0.6303364230903874	0.6303364230903874
y782	0.5705673633084795	0.3580304035579133
y783	0.42164194803440813	0.7815511308014664
y784	0.06780246625998376	0.16828777595641117
y785	0.13228077968194973	0.2328735098586295
y786	0.37264953082145236	0.37264953082145236
y787	0.9463187647514615	0.9463187647514615
y788	0.0377327092807446	0.9643783915736837
y789	0.6323566964186916	0.6384840435352219
y790	0.07355004654737086	0.07355004654737086
y791	0.5188222498658457	0.5188222498658457
y792	0.5056083261407984	0.572636243122892
y793	0.9357131650708774	0.9357131650708774
y794	0.5056083261406343	0.5726362431229166
y795	0.846125312441339	0.846125312441339
y796	0.22715841016878696	0.7024017375798766

Balance	Intercept	p-value
y797	0.5056083261407612	0.5726362431229279
y798	0.5810139173236545	0.6021780940562422
y799	0.8085638533345968	0.2590860788300405
y800	0.5056789511420773	0.5578455729318339
y801	0.21405075856439715	0.42533043347767974
y802	0.2570142259603721	0.4524020657597674
y803	0.683847794206799	0.06781920715525555
y804	0.0533326681213621	0.389318997953623
y805	0.8641099118400419	0.8641099118400419
y806	0.2561002174086579	0.4518556119169531
y807	0.1460871416260759	0.3746661382690589
y808	0.15385954399334945	0.18459853760753991
y809	0.9258307201005083	0.9258307201005083
y810	0.041098350943519935	0.24928706212357798
y811	5.870854748771356e-07	0.01946753129063754
y812	0.9999999999999993	0.15028938150267046
y813	0.999999999998508	0.15028938150267687
y814	0.3576758085887174	0.251123161664295
y815	0.3456890932727665	0.5007949748275728
y816	0.9700476455627358	0.9700476455627358
y817	0.7037840089032299	0.7037840089032299
y818	0.7568564962817814	0.7568564962817814
y819	0.930405571600474	0.930405571600474
y820	0.7861226129377344	0.7861226129377344
y821	0.5056083261408644	0.572636243122938
y822	0.5382150600320393	0.5856769441533773
y823	0.9024567411653905	0.19676708209347396
y824	0.23712657631576167	0.3716494172250844
y825	0.6730741082812515	0.6730741082812515
y826	0.5343860882029465	0.8564558751728257
y827	0.7368210540410769	0.7368210540410769

Balance	Intercept	p-value
y828	0.8751150574493409	0.8751150574493409
y829	0.3451635720035377	0.5005309186856334
y830	0.7429250065520999	0.7429250065520999
y831	0.7552237477174069	0.7552237477174069
y832	0.8144483003057847	0.8144483003057847
y833	0.7024551787224911	0.7024551787224911
y834	0.7177904019222129	0.7177904019222129
y835	0.5484902676511003	0.5484902676511003
y836	0.001990451405171345	0.08726013577593639
y837	0.6516777351554155	0.6516777351554155
y838	0.042268363288698825	0.2515044157414077
y839	0.057655694121655075	0.197563458487925
y840	0.1083299797284688	0.29261169959015965
y841	0.5425039077206918	0.9927845584514969
y842	0.014711363207292804	0.10158924442441215
y843	0.10386368851360932	0.10386368851360932
y844	0.015590363823717153	0.5480717749291854
y845	0.9431724059788666	0.9431724059788666
y846	0.7582910073376659	0.550597010108517
y847	0.45405993753436846	0.45405993753436846
y848	0.14257747352680653	0.3716836981886579
y849	0.7267597082251662	0.7267597082251662
y850	0.8900403948823208	0.11187437264546561
y851	0.7012335239793273	0.7012335239793273
y852	0.622381654058627	0.7976849034563718
y853	0.3119768190562089	0.3119768190562089
y854	0.11027286579950973	0.34236263458329663
y855	0.8263003293693939	0.8263003293693939
y856	0.12103700319498373	0.3980632547889946
y857	0.0005729619064547441	0.1345289483700115
y858	0.9727069760120848	0.9727069760120848

Balance	Intercept	p-value
y859	0.1978158515611876	0.1978158515611876
y860	0.782164618003466	0.2824257842963586
y861	0.9372574788535257	0.9372574788535257
y862	0.5902569209008154	0.5902569209008154
y863	7.195709004274193e-07	0.8123187346361572
y864	0.5056083261408668	0.5726362431229387
y865	0.5056083261409251	0.5726362431229444
y866	0.7327467740291522	0.7327467740291522
y867	0.5877559856717964	0.5877559856717964
y868	0.6282805738831083	0.6282805738831083
y869	0.5056083261408647	0.5726362431229384
y870	0.6000024930409413	0.6000024930409413
y871	0.9999999999998421	0.15028938150266274
y872	0.4037101393127105	0.20681204702082498
y873	0.6310759514018238	0.6310759514018238
y874	0.9524607553778426	0.09095721362533157
y875	0.24499089238472563	0.44511868796479837
y876	0.8994503716427685	0.2508332767708806
y877	0.7684848440610764	0.7684848440610764
y878	0.6752553242245072	0.6752553242245072
y879	2.1002750177231554e-09	0.014410918664446945
y880	0.9999999999997035	0.15028938150265625
y881	0.9999999999993754	0.15028938150264123
y882	0.7635560893741651	0.5310384273049554
y883	0.505608326140864	0.5726362431229375
y884	0.7347524810952224	0.11921634734530367
y885	0.6425376690439031	0.6425376690439031
y886	0.5056083261400094	0.5726362431228578
y887	0.505608326141219	0.5726362431229712
y888	0.5056083261408653	0.5726362431229384
y889	0.2180184677706379	0.6109898695164168

Balance	Intercept	p-value
y890	0.3457894881633192	0.3457894881633192
y891	0.6191466051451104	0.6191466051451104
y892	0.4204054326308603	0.4204054326308603
y893	0.7501577531328162	0.7501577531328162
y894	0.7000197138687803	0.7000197138687803
y895	0.10840260833770171	0.33987050630607396
y896	0.10855901226129332	0.34002964909534217
y897	0.6374413326533158	0.6374413326533158
y898	0.5056083261408848	0.5726362431229397
y899	0.6591348300414661	0.6591348300414661
y900	0.9316846433258943	0.9316846433258943
y901	0.4763801792480469	0.4763801792480469
y902	0.053255945153538375	0.242594331140339
y903	0.7817583062899521	0.7817583062899521
y904	0.003735633600850737	0.07614837261773283
y905	0.5682165385178122	0.5682165385178122
y906	0.7711213167137483	0.7711213167137483
y907	0.334288445993706	0.334288445993706
y908	0.0044995654655080285	0.23880946908391762
y909	0.7972188615367656	0.7972188615367656
y910	0.8350034650065823	0.1101754332043797
y911	0.30058728347451186	0.23859645996513762
y912	0.09534507919262555	0.8107643217296716
y913	0.2594930579315399	0.7618507732025155
y914	0.5865437591308739	0.5584845211537813
y915	0.6324884075008483	0.6324884075008483
y916	0.6862017337587603	0.6862017337587603
y917	0.9983375539630802	0.9983375539630802
y918	0.09705007555513986	0.7188207711617678
y919	0.15596959867686738	0.29070205988247905
y920	0.6516638953969123	0.9239087222487954

Balance	Intercept	p-value
y921	0.2548874468324074	0.02455902495876767
y922	0.20181847248449938	0.753168066213933
y923	0.39724426610198316	0.9302000962770811
y924	0.05524740699544809	0.22992887660407232
y925	0.772698877394161	0.772698877394161
y926	0.0036044201688566753	0.10140506449235154
y927	0.8236015058355124	0.8236015058355124
y928	0.18957657664464514	0.18957657664464514
y929	0.9997148572985122	0.9997148572985122
y930	0.34784864482851113	0.4168968593240747
y931	1.980333365690767e-08	0.6236509359049036
y932	0.5056083261408642	0.5726362431229379
y933	0.3256587258320084	0.49056202990595954
y934	0.9472536734383483	0.9472536734383483
y935	0.5056083261407777	0.5726362431229299
y936	0.5050595873848003	0.956469625931331
y937	0.5702900942888655	0.5981046646944641
y938	0.5056083261408645	0.5726362431229381
y939	0.5056083261409772	0.5726362431229486
y940	0.7800234850832641	0.7800234850832641
y941	0.7189749059143481	0.13335878348624686
y942	0.999999999999901	0.15028938150266552
y943	0.3639693896710967	0.25113692515971314
y944	0.5056083261408661	0.5726362431229387
y945	0.4046931149622559	0.22829924048465108
y946	0.6392667722145018	0.6392667722145018
y947	0.5949184150227641	0.6074023246117538
y948	0.5500559324445842	0.5815165604792298
y949	0.9999999999999727	0.037622893233597715
y950	0.3331133981717007	0.3331133981717007
y951	0.5056083261409495	0.5726362431229464

Balance	Intercept	p-value
y952	0.6576065646219589	0.6576065646219589
y953	0.50560832614089	0.5726362431229393
y954	0.5056083261409884	0.5726362431229498
y955	0.9999999999999091	0.15028938150266527
y956	0.8924441571036696	0.06195509216706871
y957	0.8394558654624211	0.7194912876959876
y958	0.24414311640897726	0.24414311640897726
y959	0.26810260716465645	0.45894144761039923
y960	0.8924167871080528	0.8924167871080528
y961	0.5056083261408422	0.5726362431229358
y962	0.47404395099578667	0.5595766796217337
y963	0.4670217217121958	0.4670217217121958
y964	0.5056083261408675	0.5726362431229394
y965	0.9166707773660727	0.9166707773660727
y966	0.17243743117583965	0.28996617435874783
y967	0.2356042332143757	0.3929273117705392
y968	0.8484672200829602	0.8484672200829602
y969	0.1579973111284054	0.38446607647707665
y970	0.3512623267735404	0.3512623267735404
y971	0.2874795661339237	0.9314035288423513
y972	0.28133989575763463	0.4665418353809059
y973	0.8677340233549203	0.8677340233549203
y974	0.3630614724063337	0.17823350236037824
y975	0.578459618763373	0.6805607506698066
y976	0.4312102566407103	0.9386979733917128
y977	0.6127014787206391	0.6127014787206391
y978	0.6336546793340396	0.1306589010391876
y979	0.033393658779419834	0.2470927898601891
y980	0.25820295097903995	0.8291584295304995
y981	0.03334020480339677	0.03334020480339677
y982	0.6992465748030321	0.6992465748030321

Balance	Intercept	p-value
y983	0.7034412447266981	0.7034412447266981
y984	0.3012841360221194	0.4776058698463237
y985	0.2204266776634074	0.4295427616269897
y986	0.3520542595565279	0.36180509779771086
y987	0.22700705193203446	0.35995545206739177
y988	0.16873696738024507	0.8265706789573168
y989	0.15865860435627363	0.17945323898206292
y990	0.2464020920189029	0.378385866418832
y991	0.29003978441857237	0.29003978441857237
y992	0.08191133461187074	0.6469163038197846
y993	0.7773474276086991	0.7773474276086991
y994	0.09122546404816351	0.37335912128028526
y995	0.8458326108421135	0.8458326108421135
y996	0.8030170043099372	0.8030170043099372
y997	0.8356012980907563	0.8356012980907563
y998	0.057145627311566936	0.23299770304622042
y999	0.009734935552062488	0.43841457301912945
y1000	0.18562480706833157	0.3157238372291835
y1001	1.4535662863503475e-10	0.22516033183893494
y1002	5.836495923935886e-07	0.8280882599556565
y1003	7.668044096965856e-07	0.8340427651470332
y1004	0.8140070139107031	0.8140070139107031
y1005	0.8037324985994754	0.8037324985994754
y1006	0.5056083261407537	0.5726362431229283
y1007	0.6606422837679211	0.6606422837679211
y1008	2.2247846126564183e-06	0.851023829363596
y1009	0.5056083261406268	0.5726362431229155
y1010	0.505608326140608	0.5726362431229133
y1011	0.8653219546436639	0.6298750677721848
y1012	0.9999999999999976	0.15028938150266924
y1013	0.4144099914171777	0.19804664229330776

Balance	Intercept	p-value
y1014	0.0003942121619356673	0.5278320469774582
y1015	0.5056083261409353	0.5726362431229519
y1016	0.6119627430711885	0.6119627430711885
y1017	0.5041208908933824	0.5041208908933824
y1018	0.2427789005997812	0.17776461784803277
y1019	0.21095420748879576	0.4232572173853373
y1020	1.6079398820906645e-08	0.01601256044375985
y1021	0.9999999999999706	0.06896365272309551
y1022	0.5056083261408644	0.5726362431229377
y1023	0.5056083261408035	0.5726362431229324
y1024	0.9999999999999998	0.15028938150266985
y1025	0.9999999999999987	0.15028938150267004
y1026	0.5493314151675548	0.5493314151675548
y1027	0.7840834153669383	0.1382251970570269
y1028	0.21899898726334663	0.21899898726334663
y1029	0.6174497058820196	0.6174497058820196
y1030	0.5056083261410804	0.5726362431229581
y1031	0.2990875908663197	0.4764087821028937
y1032	0.40812758374828295	0.40812758374828295
y1033	0.5056083261404725	0.5726362431229011
y1034	0.9017167245696653	0.38982252571283976
y1035	0.40529622870037585	0.40529622870037585
y1036	0.3569482920462814	0.5063980638183476
y1037	0.20818410881650287	0.20818410881650287
y1038	0.08493942548669796	0.17431406265162347
y1039	0.13367837309809455	0.3639092562316991
y1040	0.31587140419132637	0.31587140419132637
y1041	0.21349494033632402	0.3914597671053138
y1042	0.5718122541520089	0.5718122541520089
y1043	0.5129629259569531	0.11767223790903654
y1044	0.20522223495013797	0.20522223495013797

Balance	Intercept	p-value
y1045	0.45269476662704045	0.45269476662704045
y1046	0.766425787252417	0.31204883487414514
y1047	0.5263030776904554	0.8682611513314014
y1048	0.32850645585858107	0.32850645585858107
y1049	0.06537689063224568	0.06537689063224568
y1050	0.026634202766345934	0.8162365249957555
y1051	0.4119328647344077	0.27440988290543533
y1052	0.4788081616082856	0.5615772738429758
y1053	0.841197544886182	0.841197544886182
y1054	0.9932430299410986	0.9047369609893996
y1055	0.6080603193495273	0.6080603193495273
y1056	0.7441311995154896	0.7441311995154896
y1057	0.7133428146855763	0.7133428146855763
y1058	0.6315747639201282	0.665976310584276
y1059	0.7797795946574295	0.7797795946574295
y1060	0.3641241042051605	0.3641241042051605
y1061	0.7183483370651284	0.7183483370651284
y1062	0.11221979552281704	0.34371363075627237
y1063	0.2185846775677872	0.4283334800539762
y1064	0.17223055266992815	0.35245985119188705
y1065	0.7183827181392315	0.7183827181392315
y1066	0.7007523523491932	0.7007523523491932
y1067	0.6554808781451382	0.6554808781451382
y1068	0.9739195554609623	0.9739195554609623
y1069	0.3793481783957264	0.5172532729772178
y1070	0.011879731568951103	0.16928155392695396
y1071	0.07834646319392216	0.39925267525031405
y1072	0.24431881905082906	0.3537873375306311
y1073	0.5053605779895027	0.5053605779895027
y1074	5.497774251569174e-09	0.5277255875547125
y1075	0.5056083261407279	0.5726362431229255

Balance	Intercept	p-value
y1076	0.5056083261409491	0.5726362431229461
y1077	0.4536743726140995	0.5382942398551911
y1078	0.5056083261404816	0.5726362431229015
y1079	0.2468893226483438	0.41481646779884396
y1080	0.999999999990709	0.15028938150271187
y1081	0.999999999995616	0.15028938150264978
y1082	0.33639266028445736	0.13581002700016934
y1083	0.5056083261408055	0.5726362431229326
y1084	0.5596530709296116	0.5596530709296116
y1085	0.8450576123559674	0.8450576123559674
y1086	0.5056083261411034	0.572636243122961
y1087	5.3147875569383345e-05	0.02125905896804251
y1088	0.999999999991314	0.1502893815027153
y1089	0.99999999999146	0.15028938150271517
y1090	0.999999999998062	0.15028938150268006
y1091	0.5056913843272832	0.5889525042649234
y1092	0.5010210678632193	0.5010210678632193
y1093	0.999999999999765	0.1502893815026715
y1094	0.7854793425814148	0.7854793425814148
y1095	0.7838606117753047	0.1563336817401196
y1096	0.9313149751172347	0.9313149751172347
y1097	0.5220914235706295	0.5792825267911688
y1098	0.5056083261409495	0.5726362431229466
y1099	0.6300252112610198	0.6300252112610198
y1100	0.40077942263617894	0.40077942263617894
y1101	0.763346782603199	0.763346782603199
y1102	0.4861622641024538	0.4861622641024538
y1103	0.6368917491465709	0.6368917491465709
y1104	0.2124713275776081	0.49104541838145976
y1105	0.5437106524630155	0.5437106524630155
y1106	0.19757106872040733	0.19757106872040733

Balance	Intercept	p-value
y1107	0.029128296869925997	0.07101150686774352
y1108	0.252167972223902	0.9311197464881336
y1109	0.31579467738449996	0.31579467738449996
y1110	0.2709936947370316	0.36979570467440714
y1111	0.9790008371445801	0.0519936822221135
y1112	0.25717327383060945	0.4148830992925244
y1113	0.8654024237365885	0.8654024237365885
y1114	0.6496962725804276	0.4333350321165589
y1115	0.551579522602071	0.31144792474874006
y1116	0.1443699118412718	0.37890305484620146
y1117	0.9738143572303136	0.4151846433363742
y1118	0.23094688965707558	0.4363351125614032
y1119	0.3987260351072413	0.5263551867889871
y1120	0.8702444674703866	0.8702444674703866
y1121	0.20548752583478375	0.36284440569522725
y1122	0.4654609320469141	0.22779464808364133
y1123	0.1448641448649692	0.3038522159076816
y1124	0.7844553047943317	0.7844553047943317
y1125	0.1046838666753536	0.22331169993339464
y1126	0.8095255211681942	0.8095255211681942
y1127	0.24405159319711514	0.44454078431627286
y1128	0.2238021022430137	0.3691224422989108
y1129	0.3087588035175009	0.047752366520704866
y1130	0.3214467019057308	0.3214467019057308
y1131	0.09882792249759186	0.3298352768922467
y1132	0.7880322860244009	0.7880322860244009
y1133	0.5846679329033202	0.6035571725745035
y1134	0.9445649609099671	0.9445649609099671
y1135	0.7452705447356611	0.7452705447356611
y1136	0.45088638653805213	0.45088638653805213
y1137	0.7993868654596521	0.30142298904662534

Balance	Intercept	p-value
y1138	0.9792669884836055	0.14639528300759386
y1139	0.32387735978137117	0.8291430828723189
y1140	0.505608326141409	0.5726362431229888
y1141	0.5056083261414795	0.5726362431229955
y1142	0.6783098340835241	0.1203730427012204
y1143	0.7088001895718963	0.11722991632500618
y1144	1.739746855795494e-07	0.01844550764446592
y1145	0.9999999999996012	0.15028938150265184
y1146	0.3316852585529774	0.9654069423519845
y1147	0.5056083261408166	0.5726362431229337
y1148	0.5214638859353717	0.5214638859353717
y1149	0.3998432109471026	0.5735438196244109
y1150	0.999999999999998	0.15028938150266957
y1151	0.9999999999998914	0.1502893815026646
y1152	0.5056083261409335	0.572636243122945
y1153	0.6101692906826688	0.1309980621600333
y1154	0.999999999999951	0.15028938150267188
y1155	0.999999999999998	0.15028938150267035
y1156	0.5596169540828929	0.594010805206552
y1157	0.6817212929395225	0.6817212929395225
y1158	0.8928885410354395	0.8928885410354395
y1159	0.5056083261410707	0.5726362431229581
y1160	0.6265370267606132	0.6265370267606132
y1161	0.25094080350521236	0.9179434595643057
y1162	0.47000812006008785	0.47000812006008785
y1163	0.5056083261409239	0.5726362431229438
y1164	0.5410917245654977	0.14256557038093845
y1165	0.4195348625094698	0.5358574148513735
y1166	0.73480110816683	0.73480110816683
y1167	0.3927600595293571	0.3927600595293571
y1168	0.31754078476920683	0.31754078476920683

Balance	Intercept	p-value
y1169	0.6020627068565372	0.6020627068565372
y1170	0.9999999999999187	0.1502893815026659
y1171	0.5691922777389136	0.7412595009825278
y1172	0.09815785789697722	0.3291101438866104
y1173	0.17763594771217478	0.39966964704972896
y1174	0.03071070647545006	0.19244626041953503
y1175	0.30508975560728635	0.4230169152699613
y1176	0.16043324085597044	0.19733891210207746
y1177	0.018355397477273296	0.9868435129456637
y1178	0.9530137656015205	0.9530137656015205
y1179	0.5074665059350314	0.6521918948678853
y1180	0.9445828500672431	0.019135981203843636
y1181	0.28771558525599905	0.02675686040154707
y1182	0.028748477339351958	0.028748477339351958
y1183	0.5056083261407425	0.5726362431229264
y1184	0.7159070000200756	0.7159070000200756
y1185	0.7139007401137629	0.7139007401137629
y1186	0.32130118900802207	0.4882881728325099
y1187	0.4207453853754146	0.43989315504461446
y1188	0.623604309806199	0.623604309806199
y1189	0.4035499943688978	0.9912746324085338
y1190	0.3390450313056575	0.4974394406925573
y1191	0.17412917746373935	0.3001003233516637
y1192	0.42149306128355407	0.5367379742442933
y1193	0.6610524924637949	0.5396901704016549
y1194	0.7693080254202775	0.7693080254202775
y1195	0.6657766736652251	0.6657766736652251
y1196	0.42848759505880807	0.5398648830659696
y1197	0.7222161069684883	0.7222161069684883
y1198	0.8163260999767987	0.8163260999767987
y1199	0.5702233773884168	0.598079198664127

Balance	Intercept	p-value
y1200	0.8401683677260903	0.8401683677260903
y1201	0.875212397344187	0.875212397344187
y1202	0.6639107954054988	0.12190376820052903
y1203	0.5056083261409091	0.5726362431229425
y1204	1.9909099764453952e-09	0.1918259163179302
y1205	0.00014322939535310174	0.9677834565979031
y1206	0.6969473069559861	0.6969473069559861
y1207	0.7985394377263308	0.7985394377263308
y1208	0.5056083261405188	0.5726362431229071
y1209	0.5056083261410722	0.572636243122957
y1210	0.3223317110244661	0.8341657915315973
y1211	0.7832135653933658	0.7832135653933658
y1212	0.19775551197925725	0.8419719713117794
y1213	1.2014963295455186e-06	0.01616774441378166
y1214	0.999999999998943	0.15028938150267554
y1215	0.999999999997857	0.1502893815026813
y1216	0.5056083261408627	0.5726362431229368
y1217	0.50560832614099	0.5726362431229505
y1218	0.999999999996421	0.15028938150265206
y1219	0.999999999997649	0.1502893815026581
y1220	0.32255469581243373	0.488944097932866
y1221	0.8186435258899091	0.8186435258899091
y1222	2.740313344095519e-07	0.7966989964212357
y1223	0.2433621580943239	0.4441157623480374
y1224	0.8173477443157106	0.09523662057392883
y1225	0.9001985558728129	0.4606764874247533
y1226	0.5056083261409158	0.572636243122943
y1227	0.5731491163581107	0.3273432817468727
y1228	0.5307341529478291	0.9137612607787211
y1229	0.999999999998774	0.15028938150266452
y1230	0.40597845096066604	0.4252376684342801

Balance	Intercept	p-value
y1231	0.8570665558440187	0.8570665558440187
y1232	0.12384450574790745	0.22870321733563417
y1233	0.7017512298850466	0.7017512298850466
y1234	0.4854813490918576	0.4854813490918576
y1235	0.6275150142232159	0.724287517156452
y1236	0.43116290873381113	0.43116290873381113
y1237	0.16414065586138157	0.16414065586138157
y1238	0.4961150112351581	0.4961150112351581
y1239	0.999999999999717	0.1502893815026711
y1240	0.7213139296550276	0.7213139296550276
y1241	0.7544399479031576	0.8859931696436515
y1242	0.08083435297913999	0.32099627194852653
y1243	0.006200831583156026	0.31278009613000873
y1244	0.5056083261405471	0.5726362431229091
y1245	0.3912035006810244	0.3912035006810244
y1246	0.09800644727257861	0.32894585152202094
y1247	0.5876325947902309	0.5876325947902309
y1248	0.8521327491175493	0.8521327491175493
y1249	0.6709059016186671	0.021191949254446167
y1250	0.8257360361927225	0.8257360361927225
y1251	0.8963296995260466	0.8963296995260466
y1252	0.699040790335406	0.699040790335406
y1253	0.9018023121645554	0.9018023121645554
y1254	0.6774827979940063	0.6774827979940063
y1255	0.6677793919280273	0.6677793919280273
y1256	0.725009878803348	0.725009878803348
y1257	0.879616594022964	0.879616594022964
y1258	0.8595680452735202	0.8595680452735202
y1259	0.19990474831147662	0.41570480363429807
y1260	0.9591193367556177	0.9591193367556177
y1261	0.9271625365005737	0.9271625365005737

Balance	Intercept	p-value
y1262	0.35539980877719096	0.4685840141871225
y1263	0.5056083261406246	0.5726362431229153
y1264	3.55141997930582e-05	0.47670461640098105
y1265	0.6257453351159794	0.6257453351159794
y1266	0.5686180958626174	0.5974659909654543
y1267	0.5056083261404863	0.5726362431229045
y1268	0.003950726547279988	0.9277082285333079
y1269	0.1495665371946691	0.3775789854332311
y1270	0.1829846608314573	0.3672291259663185
y1271	1.1071111866105634e-09	0.010260682643515063
y1272	0.9999999999999999	0.1502893815026702
y1273	0.9999999999999984	0.1502893815026702
y1274	0.9999999999996204	0.1502893815026502
y1275	0.5056083261408651	0.5726362431229377
y1276	0.5056083261408644	0.5726362431229385
y1277	0.5056083261409122	0.5726362431229425
y1278	0.505608326140768	0.5726362431229293
y1279	0.505608326140912	0.5726362431229426
y1280	0.5857352084039246	0.6039591382292122
y1281	0.4892798619072652	0.5704663415857338
y1282	0.1775531317101779	0.39960777288365334
y1283	0.7946763366281306	0.7946763366281306
y1284	1.1168298458324422e-06	0.8366544165250577
y1285	0.5056083261408655	0.5726362431229384
y1286	0.19198816911480115	0.19198816911480115
y1287	0.2130671633171154	0.42467387489985575
y1288	0.7253244702707162	0.38965933861311475
y1289	0.8505534436950893	0.8505534436950893
y1290	0.6460746246361924	0.6460746246361924
y1291	0.7664380155197744	0.7664380155197744
y1292	0.03492808218568617	0.6110421382116347

Balance	Intercept	p-value
y1293	0.658893346300486	0.658893346300486
y1294	0.3504288459755894	0.8474664543950255
y1295	0.3102469094980832	0.3102469094980832
y1296	0.3806870661623887	0.3806870661623887
y1297	0.7465085513937862	0.7465085513937862
y1298	0.7015117566970146	0.6081766691586946
y1299	0.999999999999121	0.15028938150266605
y1300	0.999999999999998	0.15028938150266974
y1301	0.3201404514196813	0.13576757366110878
y1302	0.2285646716875192	0.2285646716875192
y1303	0.6928225246731843	0.12519655624287718
y1304	0.007202874211372351	0.08766168916291422
y1305	0.5182373571045875	0.5182373571045875
y1306	0.27799005134545574	0.27799005134545574
y1307	0.9660439574057887	0.9660439574057887
y1308	0.46008508472879883	0.46008508472879883
y1309	0.5056083261408657	0.5726362431229387
y1310	0.8596683235854107	0.8596683235854107
y1311	0.9465328435310035	0.9465328435310035
y1312	0.8388918127656344	0.8388918127656344
y1313	0.8105130929698824	0.8105130929698824
y1314	0.6562594228811878	0.6562594228811878
y1315	0.7123789397808592	0.7123789397808592
y1316	0.9393990751480561	0.9393990751480561
y1317	0.4989148044993447	0.569904301648751
y1318	0.27610190117672484	0.46356014339828167
y1319	0.9028352009345414	0.9028352009345414
y1320	0.4037318835336887	0.4819874096533311
y1321	4.447967079942807e-05	0.9278173839702827
y1322	1.3225746658928512e-06	0.8233462934997345
y1323	0.4571017113652851	0.5523723455071703

Balance	Intercept	p-value
y1324	0.5056083261408201	0.5726362431229339
y1325	0.0026942811420286074	0.9388331377213077
y1326	0.17057905795629924	0.6005038877370217
y1327	1.1099873714258494e-09	0.010262335009918452
y1328	3.445676470105388e-08	0.012522981309047288
y1329	0.999999999997239	0.15028938150265533
y1330	0.999999999999999	0.15028938150267004
y1331	0.5056083261413223	0.5726362431229824
y1332	0.505608326142123	0.5726362431230598
y1333	1.027398683244581e-05	0.8791789975885544
y1334	0.5056083261408657	0.5726362431229385
y1335	0.5056083261408647	0.572636243122937
y1336	0.5056083261408015	0.572636243122933
y1337	0.5056083261407113	0.5726362431229239
y1338	0.5056083261408638	0.5726362431229371
y1339	0.8112931133308334	0.8112931133308334
y1340	0.1308899894964886	0.33552666195116876
y1341	0.8354431365044669	0.8354431365044669
y1342	0.5056083261410619	0.5726362431229571
y1343	0.5056083261407208	0.5726362431229245
y1344	0.4023406163723673	0.4023406163723673
y1345	0.6708612436474664	0.12607513788227576
y1346	0.6833849401894554	0.6833849401894554
y1347	0.9277523268130438	0.9277523268130438
y1348	0.6179097100403548	0.6179097100403548
y1349	0.914023465423887	0.5547482378619626
y1350	0.9999999999999631	0.15028938150266796
y1351	0.05354033930977988	0.924385339769223
y1352	0.7324853417854427	0.7324853417854427
y1353	0.18371462980209788	0.21527329078023902
y1354	0.9521583848572133	0.9521583848572133

Balance	Intercept	p-value
y1355	4.591165282177447e-05	0.027820599154282397
y1356	0.9999999999996216	0.15028938150265397
y1357	0.9999999999997857	0.15028938150267898
y1358	0.08307021336239218	0.42060686436174
y1359	0.9736412269306434	0.9736412269306434
y1360	0.9270783176558306	0.5849759408706965
y1361	0.4772770158735111	0.4772770158735111
y1362	0.830349717213963	0.03338624768001707
y1363	0.5253560752447707	0.8013673993757049
y1364	0.5056083261408983	0.5726362431229413
y1365	0.8952908413158884	0.17645810552908064
y1366	0.9390081045943156	0.9390081045943156
y1367	0.9970489581321968	0.1960608911355203
y1368	0.5056083261413948	0.572636243122989
y1369	0.5056083261412673	0.5726362431229762
y1370	0.6408990071976	0.6408990071976
y1371	0.47496585077536774	0.5599646521980735
y1372	0.9214151371818098	0.9214151371818098
y1373	0.9295379114012035	0.9295379114012035
y1374	0.764023698067503	0.764023698067503
y1375	0.5056083261409035	0.5726362431229415
y1376	0.3325730947838264	0.4941343123444427
y1377	0.719783926264391	0.11910156217522226
y1378	0.50560832614196	0.572636243123039
y1379	0.6413470552808243	0.6413470552808243
y1380	0.5056083261408639	0.5726362431229381
y1381	0.5056083261411872	0.5726362431229673
y1382	0.5772731337988272	0.5772731337988272
y1383	0.6677237080496572	0.6677237080496572
y1384	0.6436672390018692	0.38532155608780516
y1385	3.966269650384481e-07	0.014991052290140632

Balance	Intercept	p-value
y1386	0.3267526188672789	0.38577250997456136
y1387	0.5056083261416003	0.5726362431230088
y1388	0.505608326140861	0.5726362431229349
y1389	0.5056083261408636	0.5726362431229377
y1390	0.00027426684194916713	0.9653602558636061
y1391	0.5056083261408668	0.572636243122939
y1392	7.71015869221455e-07	0.8357596605525202
y1393	0.6420734269709913	0.6420734269709913
y1394	0.6223499759858862	0.6223499759858862
y1395	0.4877324852734596	0.4877324852734596
y1396	0.5056083261407223	0.5726362431229242
y1397	0.7551861463009811	0.7551861463009811
y1398	0.522435425690345	0.7758844693650874
y1399	0.9999999999999633	0.15028938150266843
y1400	0.6197402030440693	0.6197402030440693
y1401	0.6747872727192907	0.6747872727192907
y1402	0.8391668347807643	0.44044393262128695
y1403	0.24643265729445987	0.5233088577283292
y1404	0.3268178448858876	0.27510155697560296
y1405	0.2673968339838275	0.3354805365984388
y1406	0.2724652719075272	0.2724652719075272
y1407	0.5565598789588397	0.5565598789588397
y1408	0.12459970664626788	0.35563503575119226
y1409	0.5056083261409883	0.5726362431229498
y1410	0.8388788495620485	0.8388788495620485
y1411	0.5059487300846457	0.6059568679260929
y1412	0.56487487974585	0.56487487974585
y1413	5.638618051003647e-07	0.836757180192762
y1414	1.5637073098599723e-05	0.9050679839688135
y1415	0.40980665234035984	0.5314486084062875
y1416	0.6276643277240536	0.6276643277240536

Balance	Intercept	p-value
y1417	0.339827989454504	0.49783681829663473
y1418	6.339256356995236e-08	0.8007600905548948
y1419	0.5056083261412315	0.5726362431229775
y1420	0.5056083261406199	0.5726362431229142
y1421	0.5056083261408663	0.5726362431229387
y1422	0.5056083261411085	0.572636243122961
y1423	0.505608326140773	0.5726362431229298
y1424	0.5056083261406543	0.572636243122919
y1425	0.7482063898278423	0.37479377744221454
y1426	0.23852519130211308	0.4411134014795144
y1427	0.9319576190199036	0.1851755483240796
y1428	3.061599355573642e-08	0.012659733045801739
y1429	6.210917494045195e-06	0.8707762067551224
y1430	0.6875483052266915	0.6408322947892
y1431	0.0007956965964059437	0.9945550288281548
y1432	9.491588693492914e-05	0.9375655390620146
y1433	0.5056083261415821	0.5726362431230073
y1434	0.5056083261407394	0.5726362431229259
y1435	0.9448922752939469	0.9448922752939469
y1436	0.6466593818217756	0.6466593818217756
y1437	0.9315270811434019	0.08694471457617754
y1438	0.505608326140981	0.5726362431229499
y1439	0.7151785989033934	0.7151785989033934
y1440	0.999999999999172	0.5580531809697467
y1441	0.2170966944720912	0.2170966944720912
y1442	0.9258750654321598	0.8401260346335304
y1443	0.33202755267703477	0.33202755267703477
y1444	0.20644918826418499	0.20644918826418499
y1445	0.08631163639198888	0.48696425718874525
y1446	0.7977829536471899	0.7977829536471899
y1447	0.8286844543445115	0.6734887525671436

Balance	Intercept	p-value
y1448	0.2956968826124359	0.2956968826124359
y1449	0.5056083261408539	0.5726362431229384
y1450	0.4348223319893191	0.18389784051520208
y1451	0.5699467817599457	0.5979736048217268
y1452	0.13551349857124706	0.3376918315599624
y1453	2.6483661942410945e-07	0.8137788212454038
y1454	0.5056083261409501	0.5726362431229461
y1455	0.5056083261408645	0.5726362431229384
y1456	1.5648795083657822e-08	0.7509937683891035
y1457	1.2678896234076337e-06	0.858701529651037
y1458	0.4050269787374089	0.16857675361154015
y1459	0.5056083261406992	0.5726362431229235
y1460	0.5056083261408713	0.5726362431229428
y1461	0.5056083261413084	0.5726362431229799
y1462	0.5056083261404628	0.5726362431229004
y1463	0.5056083261414119	0.5726362431229898
y1464	5.181976907813584e-06	0.865729382527008
y1465	0.5056083261408636	0.5726362431229379
y1466	0.5056083261412058	0.572636243122969
y1467	0.5056083261409628	0.5726362431229466
y1468	0.5918266941921433	0.6626406654922026
y1469	7.97088558974806e-06	0.8766514394306524
y1470	0.5056083261405571	0.5726362431229086
y1471	0.26279277588265865	0.45583041273726077
y1472	0.7179841260012074	0.11646204594474499
y1473	0.2852283834058665	0.2852283834058665
y1474	0.5056083261408647	0.5726362431229381
y1475	0.5056083261410456	0.5726362431229564
y1476	0.999999999999972	0.15028938150266888
y1477	0.9731773312510876	0.1453215483968257
y1478	0.7764341642142458	0.7764341642142458

Balance	Intercept	p-value
y1479	0.6279259162232167	0.6279259162232167
y1480	0.37745606611410876	0.23356556827595837
y1481	0.25418583540878315	0.25418583540878315
y1482	0.7511260680975267	0.7511260680975267
y1483	0.5601872443962992	0.5601872443962992
y1484	0.632806884499306	0.632806884499306
y1485	0.7301545399267884	0.7301545399267884
y1486	0.24900833093976416	0.24900833093976416
y1487	0.7144388921708931	0.7144388921708931
y1488	0.5056083261407933	0.5726362431229313
y1489	0.5056083261408639	0.5726362431229381
y1490	0.5056083261409151	0.572636243122943
y1491	9.456664991926181e-07	0.8452921372456415
y1492	3.117844053503637e-07	0.806523388138244
y1493	9.824185917350248e-07	0.8531711674700578
y1494	0.505608326140609	0.5726362431229148
y1495	0.5056083261415006	0.5726362431229971
y1496	0.5056083261403164	0.5726362431228893
y1497	0.5056083261410951	0.5726362431229597
y1498	2.510835156261667e-06	0.8617079701716752
y1499	2.9382656874462026e-06	0.8579712759548255
y1500	1.618934955105491e-05	0.9067483274957178
y1501	1.0485279134078955e-05	0.8907463161699114
y1502	0.5056083261412255	0.5726362431229721
y1503	0.5056083261416946	0.5726362431230161
y1504	0.9297061153980724	0.13772767705605807
y1505	0.6086926558310739	0.6086926558310739
y1506	0.5056083261407837	0.5726362431229305
y1507	0.6501869645657705	0.6501869645657705
y1508	0.999999999999145	0.1502893815026743
y1509	1.252379203326149e-05	0.8858873004653859

Balance	Intercept	p-value
y1510	4.213036904056897e-05	0.9155869363062019
y1511	0.5056083261401902	0.5726362431228735
y1512	0.5056083261411319	0.5726362431229648
y1513	0.9999999999994973	0.15028938150269253
y1514	0.9999999999998912	0.15028938150266474
y1515	0.5056083261406045	0.5726362431229128
y1516	0.999999999999913	0.999999999999913
y1517	0.8155103118455409	0.2156951290049056
y1518	0.3244196917920521	0.3244196917920521
y1519	0.999999999999875	0.150289381502669
y1520	0.835349219153027	0.09766768168014847
y1521	0.447893411531886	0.447893411531886
y1522	0.6551764717900231	0.6551764717900231
y1523	0.658111299410543	0.658111299410543
y1524	0.5056083261414337	0.5726362431229933
y1525	0.292662316849209	0.292662316849209
y1526	0.4245308267926766	0.4245308267926766
y1527	0.5056083261411449	0.5726362431229648
y1528	0.5056083261406416	0.5726362431229166
y1529	0.5056083261406847	0.5726362431229214
y1530	1.431155684345184e-07	0.7893702280663928
y1531	9.401146966386082e-07	0.8734204035598082
y1532	8.649496528023753e-07	0.822044359744077
y1533	6.68078552559484e-05	0.9489102517663834
y1534	3.7001206684603616e-05	0.9376775357781473
y1535	9.907245007930099e-06	0.9037551081799018
y1536	0.14405201875309798	0.4670898286927876
y1537	7.467999555409903e-06	0.8937361998836685
y1538	2.2632852281035703e-05	0.9135054430030386
y1539	2.1225053996815623e-06	0.8503662636161247
y1540	9.436576018330343e-06	0.9016382122962194

Balance	Intercept	p-value
y1541	1.6613159508746802e-05	0.9073595647447757
y1542	2.831146028547985e-06	0.848506669378642
y1543	6.651201634892566e-06	0.8883902415365976
y1544	3.978017924334836e-05	0.9283088831751303
y1545	0.5206659810194558	0.5787122143922633
y1546	0.5538627111509374	0.5539474178171078
y1547	0.5056083261405699	0.57263624312291
y1548	0.505608326141063	0.5726362431229575
y1549	0.5056083261409506	0.5726362431229453
y1550	0.999999999999187	0.15028938150267415
y1551	4.327771127041741e-05	0.9162583442116659
y1552	2.3821170455212576e-06	0.8469881972722928
y1553	7.546464514291691e-06	0.8920927704866891
y1554	3.557308881265487e-06	0.021526815129419848
y1555	0.999999999992342	0.1502893815027049
y1556	0.999999999997649	0.15028938150265891
y1557	0.999999999999182	0.15028938150266655
y1558	0.20727903446812615	0.20727903446812615
y1559	0.28609206647042823	0.28609206647042823
y1560	0.36707631901892707	0.7826725580862803
y1561	0.6564172562304471	0.3362008900592165
y1562	0.2807410249127246	0.0512875246548735
y1563	1.1170674617924421e-05	0.8790967531781204
y1564	0.29642858831564506	0.29642858831564506
y1565	0.9668079447122342	0.35689890986124795
y1566	0.5056083261408154	0.5726362431229335
y1567	0.9048068792180775	0.1352817405060213
y1568	3.5749773816753043e-06	0.8621477905011664
y1569	0.5056083261405557	0.5726362431229073
y1570	0.5056083261400318	0.5726362431228582
y1571	0.5056083261408655	0.5726362431229384

Balance	Intercept	p-value
y1572	0.00010390999498377187	0.9436095628477001
y1573	0.5056083261418058	0.5726362431230283
y1574	3.872477601795376e-07	0.8321177310809711
y1575	6.263806745855304e-07	0.8291989681775802
y1576	3.7215099199638006e-06	0.8903679492246889
y1577	4.615228075438385e-05	0.9399398291784006
y1578	3.0072541143055824e-05	0.9273175027900975
y1579	9.627014994489033e-06	0.8914375115827948
y1580	1.9875775714083427e-05	0.9261481114128842
y1581	3.4377266706144395e-06	0.8806397378884625
y1582	1.1573078244557776e-05	0.8976632014940562
y1583	3.4418146200626676e-06	0.8709549734249055
y1584	2.0829368033395063e-05	0.9127300821114426
y1585	0.5056083261407395	0.5726362431229268
y1586	0.6424224928871379	0.6424224928871379
y1587	6.6968900763881675e-06	0.8727007264759244
y1588	0.5056083261406995	0.5726362431229225
y1589	6.099429117550383e-07	0.8111680763438286
y1590	0.5056083261413074	0.5726362431229793
y1591	0.999999999991775	0.15028938150262847
y1592	0.999999999994749	0.15028938150264362
y1593	4.318700271072715e-06	0.8466744223956155
y1594	6.65203078825732e-07	0.01954157321808018
y1595	0.999999999997653	0.15028938150268062
y1596	0.44785640221328105	0.44785640221328105
y1597	0.9508139872484814	0.5562526840366818
y1598	0.29661241763472346	0.29661241763472346
y1599	0.5329114026507633	0.14695417795460597
y1600	0.6875833927693805	0.6875833927693805
y1601	0.6478291956719118	0.6478291956719118
y1602	0.99999999999863	0.15028938150267443

Balance	Intercept	p-value
y1603	0.28184580288516986	0.28184580288516986
y1604	0.5056083261415705	0.5726362431230028
y1605	0.5056083261408651	0.5726362431229381
y1606	0.5056083261408657	0.5726362431229384
y1607	1.7413336654322271e-06	0.8502859274358543
y1608	1.2827498336354906e-05	0.8920267524065989
y1609	7.853886921745699e-05	0.9364883969118147
y1610	1.2492606377696343e-05	0.8913966888661048
y1611	1.616939442324793e-05	0.8975629449792724
y1612	6.004894159101792e-05	0.9330719072445457
y1613	3.164519482224588e-07	0.8384868191599235
y1614	3.2525748150779785e-07	0.8127451843985608
y1615	5.2373773892217796e-06	0.8504258080198369
y1616	2.726349065148237e-05	0.9223384528152225
y1617	4.377233215150159e-06	0.8671456008400625
y1618	1.949114298232068e-05	0.9194560926804057
y1619	4.04626000010577e-06	0.8798013768003675
y1620	0.13570416318240888	0.4644850224062247
y1621	0.5056083261409153	0.5726362431229444
y1622	0.5056083261408361	0.5726362431229359
y1623	0.33064960698134255	0.44831960631176604
y1624	1.320384752257133e-06	0.8386721045870176
y1625	0.5056083261415497	0.5726362431230047
y1626	0.5056083261411575	0.5726362431229669
y1627	3.2920868490949275e-08	0.013941396271313026
y1628	5.63913362897652e-09	0.013302070531807791
y1629	1.6847333352466596e-06	0.83909260529642
y1630	0.9999999999998305	0.1502893815026629
y1631	1.0	1.0
y1632	0.5012169097027414	0.14005413885178095
y1633	0.2981520294102476	0.2963437844196591

Balance	Intercept	p-value
y1634	0.6717560623452221	0.6717560623452221
y1635	0.8828431961190797	0.8828431961190797
y1636	0.09956154509659479	0.07128996856262107
y1637	0.48099912495373653	0.48099912495373653
y1638	0.964259475742797	0.964259475742797
y1639	0.9999999999998664	0.15028938150266313
y1640	0.5056083261403845	0.5726362431228943
y1641	0.5056083261409908	0.5726362431229498
y1642	0.5056083261413897	0.5726362431229866
y1643	1.5697701561064294e-05	0.8968528194043778
y1644	2.606358004862553e-05	0.9091062824204993
y1645	1.0924916085530691e-07	0.7805392814986695
y1646	1.0758612566330582e-06	0.8333054708614793
y1647	2.886001544812558e-06	0.8612118445404708
y1648	2.18517903104879e-05	0.9221405505892086
y1649	0.19811451739541458	0.6308710185883124
y1650	2.134388112368517e-06	0.8692277858884773
y1651	4.40359845313833e-06	0.8743197964175192
y1652	0.5056083261408605	0.5726362431229371
y1653	0.5056083261408642	0.5726362431229379
y1654	0.5056083261408642	0.5726362431229379
y1655	0.5056083261409311	0.5726362431229445
y1656	0.8141638180653561	0.22370742868142354
y1657	2.1937760142014093e-06	0.8549557715078512
y1658	1.4257546046495737e-05	0.8984436190839719
y1659	1.0364310034677555e-05	0.8907104798305903
y1660	2.425654188491829e-06	0.8507232292138369
y1661	1.8889581603530545e-09	0.011761451911286386
y1662	4.374719564574995e-08	0.019275448433283607
y1663	0.999999999999978	0.1502893815026706
y1664	0.2010004916738488	0.2010004916738488

Balance	Intercept	p-value
y1665	0.2822845561546956	0.2822845561546956
y1666	0.5056083261408902	0.5726362431229407
y1667	0.43986251366798623	0.43986251366798623
y1668	0.596029158062976	0.3708865201409443
y1669	0.7760640308955712	0.7760640308955712
y1670	0.1128850682254525	0.1128850682254525
y1671	0.5188496444994446	0.7966613076427967
y1672	0.8654340492836666	0.8654340492836666
y1673	0.9999999999999989	0.15028938150266952
y1674	0.9999999999999302	0.15028938150267315
y1675	1.058298302323549e-07	0.8030472163054806
y1676	0.13345495520592732	0.46381499727581355
y1677	1.0492034183519258e-06	0.8551263104872706
y1678	3.9978803569604614e-06	0.8630906511617366
y1679	1.0331254506125047e-05	0.8939596363441029
y1680	9.607750370521601e-06	0.8995381726073881
y1681	0.5056083261419297	0.5726362431230325
y1682	6.4743905299240275e-06	0.9144910551396506
y1683	0.5056083261405299	0.5726362431229083
y1684	0.0008905771121305426	0.9685934001929121
y1685	0.000877151958806036	0.9689900911005139
y1686	0.5056083261402454	0.5726362431228829
y1687	0.5056083261399935	0.5726362431228604
y1688	0.9186504139167968	0.13702901375130583
y1689	1.6611758769213735e-06	0.8487085551880394
y1690	7.839899547954995e-06	0.8810072377525133
y1691	5.734609996922689e-08	0.01936107957827714
y1692	5.7480470063400456e-08	0.018999206617752958
y1693	0.8670731624102742	0.8670731624102742
y1694	0.5056083261408957	0.5726362431229415
y1695	0.9813686218569291	0.9813686218569291

Balance	Intercept	p-value
y1696	0.9999999999999998	0.15028938150266974
y1697	0.9999999999997565	0.15028938150268267
y1698	0.9999999999999993	0.15028938150266954
y1699	2.577555927461135e-07	0.8324244671628787
y1700	2.770149526013575e-07	0.8239788634151313
y1701	8.528240300117417e-07	0.8564538288398523
y1702	1.8275299530101527e-06	0.8622839634783928
y1703	9.967059794997753e-06	0.898386210112273
y1704	1.335274279557933e-05	0.9106410428089773
y1705	2.5209882425912403e-05	0.937395024729047
y1706	0.5056083261403472	0.572636243122892
y1707	0.5056083261404763	0.5726362431228994
y1708	1.715984428282844e-06	0.8494354675263094
y1709	2.181169742649211e-08	0.018645929432343177
y1710	3.442574327864041e-08	0.019055442862186224
y1711	0.5056083261407408	0.5726362431229262
y1712	0.3108536608998179	0.48276261583777913
y1713	0.559261170185745	0.5938736409241623
y1714	4.978549908189825e-07	0.016689235441764164
y1715	0.9999999999996421	0.1502893815026521
y1716	1.3559356309794553e-08	0.013180969304732277
y1717	0.9999999999997039	0.1502893815026845
y1718	1.89838630804852e-09	0.011334165523635073
y1719	0.9999999999993895	0.1502893815027007
y1720	2.9767305630157455e-07	0.8356795265306398
y1721	4.489278833478759e-06	0.8841345308143714
y1722	3.993887061665772e-06	0.8651072610151572
y1723	6.476411082158402e-06	0.8940487361511414
y1724	8.355419762571067e-06	0.9200953432306579
y1725	0.5056083261410389	0.5726362431229524
y1726	0.5056083261411288	0.5726362431229625

Balance	Intercept	p-value
y1727	6.014775103723369e-08	0.014643300139131834
y1728	9.556735176344227e-10	0.011611117880099543
y1729	5.171983597033235e-09	0.012650748645807983
y1730	3.1106179633848965e-07	0.8277497034997059
y1731	0.13729028791877954	0.4649667818859392
y1732	2.321713338642522e-06	0.8654626972764808
y1733	1.6857418095723342e-09	0.7262092038172033
y1734	1.0442438561300387e-08	0.7681856615681166
y1735	0.5056083261408688	0.5726362431229393
y1736	0.5056083261414039	0.5726362431229908
y1737	6.828023874435244e-09	0.012858685670443298
y1738	8.847603633469484e-07	0.8488652713796289
y1739	0.4549860675658709	0.7818509849643374
y1740	6.000558181762477e-07	0.8712722001637991
y1741	0.765393616870953	0.765393616870953
y1742	1.8968267628174853e-06	0.8902171292516936
y1743	1.7426430080836352e-06	0.8696469453468768
y1744	5.634165628236684e-07	0.8393266616596062
y1745	1.0888646980351367e-06	0.851234482218445
y1746	2.045788093465402e-06	0.8606758760196976
y1747	1.117564643567503e-07	0.8054286616807218
y1748	1.1347854863392812e-07	0.832037902713729
y1749	1.188790885842076e-06	0.8756844537825432
y1750	6.589943687110364e-07	0.8609902991789194
y1751	1.9631682452541802e-07	0.8560576368018079
y1752	1.9868550432775457e-06	0.8676505812934967
y1753	2.7147569650550507e-06	0.8721805988391602
y1754	1.4208008776116433e-07	0.8364443084912804
y1755	7.52928503659779e-07	0.9038372252357426
y1756	3.6576123346076974e-06	0.9020814378123203
y1757	4.3147899181914845e-07	0.8571490463683845

Balance	Intercept	p-value
y1758	4.133301070017185e-08	0.8007173935599493
y1759	1.5439818680172852e-06	0.8621199012448306
y1760	9.558189826472305e-08	0.8377488855053117
y1761	8.742976403287812e-10	0.42970917445703616
y1762	1.5071643704269123e-06	0.8524780849093397
y1763	2.3307408780634083e-08	0.8033432546797983
y1764	0.04370672373790019	0.6491351863002977
y1765	0.8166844719297507	0.8166844719297507
y1766	0.11940491522088194	0.537193490152583
y1767	9.850411442724111e-07	0.8862944896489586
y1768	5.967273508595674e-07	0.8810972322991244
y1769	2.3203500881904635e-07	0.8324151548113492
y1770	1.601296058544822e-07	0.8182235812217512
y1771	1.7003734350740148e-07	0.8229564476577278
y1772	1.7050835491393714e-07	0.8255290595011131
y1773	4.15025414329435e-08	0.8192393486725651
y1774	4.4793424238879844e-07	0.8579171580227117
y1775	3.967766111432103e-07	0.8554305675130104
y1776	1.0275932567039993e-08	0.798207753666484
y1777	2.0035301137746833e-07	0.8418104092579662
y1778	0.12799691958589682	0.551181501656518
y1779	1.7527411427889108e-07	0.8256229230210868
y1780	6.99350606183762e-08	0.7872692813245146
y1781	1.7158602608235656e-07	0.812331529358887

Table A1.4: The functional content of gut bacterial taxa of individuals of the Cape sea urchin, *Parechinus angulosus* collected from the east and west coasts of South Africa, reported as a number of biochemical pathways.

Biochemical pathways	Number of linked KEGG annotations
Global and overview maps	4523
01100 Metabolic pathways	2108
01110 Biosynthesis of secondary metabolites	698

Biochemical pathways	Number of linked KEGG annotations
01120 Microbial metabolism in diverse environments	692
01200 Carbon metabolism	232
01210 2-Oxocarboxylic acid metabolism	66
01212 Fatty acid metabolism	41
01230 Biosynthesis of amino acids	188
01232 Nucleotide metabolism	76
01250 Biosynthesis of nucleotide sugars	96
01240 Biosynthesis of cofactors	252
01220 Degradation of aromatic compounds	74
Carbohydrate metabolism	940
00010 Glycolysis / Gluconeogenesis	78
00020 Citrate cycle	48
00030 Pentose phosphate pathway	58
00040 Pentose and glucuronate interconversions	49
00051 Fructose and mannose metabolism	79
00052 Galactose metabolism	48
00053 Ascorbate and aldarate metabolism	29
00500 Starch and sucrose metabolism	77
00520 Amino sugar and nucleotide sugar metabolism	107
00620 Pyruvate metabolism	94
00630 Glyoxylate and dicarboxylate metabolism	78
00640 Propanoate metabolism	68
00650 Butanoate metabolism	86
00660 C5-Branched dibasic acid metabolism	21
00562 Inositol phosphate metabolism	20
Energy metabolism	494
00190 Oxidative phosphorylation	99
00195 Photosynthesis	55
00196 Photosynthesis - antenna proteins	24
00710 Carbon fixation in photosynthetic organisms	26
00720 Carbon fixation pathways in prokaryotes	71
00680 Methane metabolism	110
00910 Nitrogen metabolism	42
00920 Sulfur metabolism	67
Lipid metabolism	188
00061 Fatty acid biosynthesis	26
00062 Fatty acid elongation	2
00071 Fatty acid degradation	30
00100 Steroid biosynthesis	6
00120 Primary bile acid biosynthesis	3
00121 Secondary bile acid biosynthesis	4

Biochemical pathways	Number of linked KEGG annotations
00140 Steroid hormone biosynthesis	4
00561 Glycerolipid metabolism	32
00564 Glycerophospholipid metabolism	45
00565 Ether lipid metabolism	3
00600 Sphingolipid metabolism	16
00590 Arachidonic acid metabolism	3
00591 Linoleic acid metabolism	2
00592 alpha-Linolenic acid metabolism	4
01040 Biosynthesis of unsaturated fatty acids	8
Nucleotide metabolism	184
00230 Purine metabolism	113
00240 Pyrimidine metabolism	71
Amino acid metabolism	643
00250 Alanine, aspartate and glutamate metabolism	48
00260 Glycine, serine and threonine metabolism	79
00270 Cysteine and methionine metabolism	76
00280 Valine, leucine and isoleucine degradation	48
00290 Valine, leucine and isoleucine biosynthesis	16
00300 Lysine biosynthesis	32
00310 Lysine degradation	32
00220 Arginine biosynthesis	40
00330 Arginine and proline metabolism	67
00340 Histidine metabolism	34
00350 Tyrosine metabolism	44
00360 Phenylalanine metabolism	45
00380 Tryptophan metabolism	32
00400 Phenylalanine, tyrosine and tryptophan biosynthesis	50
Metabolism of other amino acids	146
00410 beta-Alanine metabolism	27
00430 Taurine and hypotaurine metabolism	20
00440 Phosphonate and phosphinate metabolism	14
00450 Selenocompound metabolism	23
00460 Cyanoamino acid metabolism	13
00470 D-Amino acid metabolism	28
00480 Glutathione metabolism	21
Glycan biosynthesis and metabolism	251
00510 N-Glycan biosynthesis	5
00513 Various types of N-glycan biosynthesis	4
00531 Glycosaminoglycan degradation	11
00563 Glycosylphosphatidylinositol GPI-anch	1

Biochemical pathways	Number of linked KEGG annotations
00603 Glycosphingolipid biosynthesis - globo and isoglobo series	3
00604 Glycosphingolipid biosynthesis - ganglio series	1
00511 Other glycan degradation	14
00540 Lipopolysaccharide biosynthesis	47
00542 O-Antigen repeat unit biosynthesis	2
00541 O-Antigen nucleotide sugar biosynthesis	47
00550 Peptidoglycan biosynthesis	39
00552 Teichoic acid biosynthesis	16
00571 Lipoarabinomannan LAM biosynthesis	12
00572 Arabinogalactan biosynthesis - Mycobacterium	11
00543 Exopolysaccharide biosynthesis	38
Metabolism of cofactors and vitamins	366
00730 Thiamine metabolism	21
00740 Riboflavin metabolism	23
00750 Vitamin B6 metabolism	11
00760 Nicotinate and nicotinamide metabolism	41
00770 Pantothenate and CoA biosynthesis	30
00780 Biotin metabolism	18
00785 Lipoic acid metabolism	22
00790 Folate biosynthesis	47
00670 One carbon pool by folate	23
00830 Retinol metabolism	5
00860 Porphyrin metabolism	85
00130 Ubiquinone and other terpenoid-quinone biosynthesis	40
Metabolism of terpenoids and polyketides	145
00900 Terpenoid backbone biosynthesis	29
00909 Sesquiterpenoid and triterpenoid biosynthesis	6
00906 Carotenoid biosynthesis	24
00905 Brassinosteroid biosynthesis	1
00981 Insect hormone biosynthesis	1
00908 Zeatin biosynthesis	2
00903 Limonene degradation	4
00907 Pinene, camphor and geraniol degradation	13
01052 Type I polyketide structures	1
00522 Biosynthesis of 12-, 14- and 16-membered macrolides; Including: Tylosin biosynthesis, Mycinamicin biosynthesis, Erythromycin biosynthesis, Oleandomycin biosynthesis, Pikromycin/methymycin biosynthesis, Avermectin biosynthesis	1

Biochemical pathways	Number of linked KEGG annotations
01051 Biosynthesis of ansamycins	3
01056 Biosynthesis of type II polyketide backbone	5
01057 Biosynthesis of type II polyketide products	6
00253 Tetracycline biosynthesis	5
00523 Polyketide sugar unit biosynthesis	9
01054 Nonribosomal peptide structures	5
01053 Biosynthesis of siderophore group nonribosomal peptides	25
01055 Biosynthesis of vancomycin group antibiotics	5
Biosynthesis of other secondary metabolites	128
00940 Phenylpropanoid biosynthesis	4
00945 Stilbenoid, diarylheptanoid and gingerol biosynthesis	1
00941 Flavonoid biosynthesis	1
00944 Flavone and flavonol biosynthesis	1
00943 Isoflavonoid biosynthesis	1
00946 Degradation of flavonoids	3
00901 Indole alkaloid biosynthesis	1
00950 Isoquinoline alkaloid biosynthesis	11
00960 Tropane, piperidine and pyridine alkaloid biosynthesis	13
00996 Biosynthesis of various alkaloids; Including: Cucurbitacin biosynthesis, Solanine and tomatine biosynthesis, Ephedrine biosynthesis, Capsaicin biosynthesis, Acridone alkaloid biosynthesis	1
00232 Caffeine metabolism	2
00965 Betalain biosynthesis	3
00966 Glucosinolate biosynthesis	2
00311 Penicillin and cephalosporin biosynthesis	6
00332 Carbapenem biosynthesis	3
00261 Monobactam biosynthesis	22
00521 Streptomycin biosynthesis	13
00524 Neomycin, kanamycin and gentamicin biosynthesis	2
00525 Acarbose and validamycin biosynthesis	2
00401 Novobiocin biosynthesis	8
00404 Staurosporine biosynthesis	2
00405 Phenazine biosynthesis	6
00333 Prodigiosin biosynthesis	3
00998 Biosynthesis of various antibiotics; Including: Kanosamine biosynthesis, Aurachin biosynthesis,	8

Biochemical pathways	Number of linked KEGG annotations
Bacilysin biosynthesis, Puromycin biosynthesis, Dapdiamides biosynthesis, Fosfomycin biosynthesis, Cremeomycin biosynthesis, Fumagillin biosynthesis, Pentalenolactone biosynthesis, Terpentecin biosynthesis, Roseoflavin biosynthesis, Cycloserine biosynthesis	
00999 Biosynthesis of various plant secondary metabolites; Including: Crocin biosynthesis, Ginsenoside biosynthesis, Saponin adjuvant biosynthesis, Cannabidiol biosynthesis, Mugineic acid biosynthesis, Pentagalloylglucose biosynthesis, Benzoxazinoid biosynthesis, Gramine biosynthesis, Coumarin biosynthesis, Furanocoumarin biosynthesis, Hordatine biosynthesis, Podophyllotoxin biosynthesis	6
00997 Biosynthesis of various other secondary metabolites; Including: Ditryptophenaline biosynthesis, Fumiquinazoline D biosynthesis, Paerucumarin biosynthesis, Staphyloferrin B biosynthesis, Cyclooctatin biosynthesis, Lovastatin biosynthesis, Grixazone biosynthesis, Staphyloferrin A biosynthesis, Ethynylserine biosynthesis, Aerobactin biosynthesis	3
Xenobiotics biodegradation and metabolism	315
00362 Benzoate degradation	66
00627 Aminobenzoate degradation	36
00364 Fluorobenzoate degradation	10
00625 Chloroalkane and chloroalkene degradation	17
00361 Chlorocyclohexane and chlorobenzene degradation	20
00623 Toluene degradation	14
00622 Xylene degradation	18
00633 Nitrotoluene degradation	14
00642 Ethylbenzene degradation	4
00643 Styrene degradation	15
00791 Atrazine degradation	10
00930 Caprolactam degradation	13
00363 Bisphenol degradation	2
00621 Dioxin degradation	10
00626 Naphthalene degradation	6
00624 Polycyclic aromatic hydrocarbon degradation	10
00365 Furfural degradation	7
00984 Steroid degradation	14
00980 Metabolism of xenobiotics by cytochrome P450	6
00982 Drug metabolism - cytochrome P450	6

Biochemical pathways	Number of linked KEGG annotations
00983 Drug metabolism - other enzymes	17

Table A1.5: The functional content of gut bacterial taxa of individuals of the Cape sea urchin, *Parechinus angulosus* collected from the east and west coasts of South Africa, reported as a number of genetic information processing pathways.

Genetic Information Processing	Number of linked KEGG annotations
Transcription	13
03020 RNA polymerase	7
03022 Basal transcription factors	2
03040 Spliceosome	4
Translation	98
03010 Ribosome	55
00970 Aminoacyl-tRNA biosynthesis	35
03013 Nucleocytoplasmic transport	1
03015 mRNA surveillance pathway	2
03008 Ribosome biogenesis in eukaryotes	5
Folding, sorting and degradation	66
03060 Protein export	20
04141 Protein processing in endoplasmic reticulum	5
04122 Sulfur relay system	18
03050 Proteasome	3
03018 RNA degradation	20
Replication and repair	106
03030 DNA replication	21
03410 Base excision repair	19
03420 Nucleotide excision repair	10
03430 Mismatch repair	25
03440 Homologous recombination	29
03450 Non-homologous end-joining	2
Information processing in viruses	2
03250 Viral life cycle - HIV-1	1
03266 Virion - Herpesvirus	1

Table A1.6: The functional content of gut bacterial taxa of individuals of the Cape sea urchin, *Parechinus angulosus*, collected from the east and west coasts of South Africa, reported as a number of environmental information processing pathways.

Environmental Information Processing	Number of linked KEGG annotations
Membrane transport	488

02010 ABC transporters	365
02060 Phosphotransferase system PTS	56
03070 Bacterial secretion system	67
Signal transduction	457
02020 Two-component system	408
04013 MAPK signaling pathway - fly	1
04016 MAPK signaling pathway - plant	3
04011 MAPK signaling pathway - yeast	2
04330 Notch signaling pathway	1
04370 VEGF signaling pathway	1
04371 Apelin signaling pathway	1
04066 HIF-1 signaling pathway	10
04068 FoxO signaling pathway	4
04020 Calcium signaling pathway	3
04070 Phosphatidylinositol signaling system	5
04072 Phospholipase D signaling pathway	2
04071 Sphingolipid signaling pathway	3
04024 cAMP signaling pathway	1
04022 cGMP-PKG signaling pathway	2
04151 PI3K-Akt signaling pathway	3
04152 AMPK signaling pathway	7
Signaling molecules and interaction	2
04512 ECM-receptor interaction	1
04514 Cell adhesion molecules	1

Table A1.7: The functional content of gut bacterial taxa of individuals of the Cape sea urchin, *Parechinus angulosus*, collected from the east and west coasts of South Africa, reported as a number of cellular processing pathways.

Cellular Processes	Number of linked KEGG annotations
Transport and catabolism	46
04144 Endocytosis	2
04145 Phagosome	1
04142 Lysosome	17
04146 Peroxisome	19
04138 Autophagy - yeast	1
04137 Mitophagy - animal	2
04148 Efferocytosis	4
Cell growth and death	48
04112 Cell cycle - Caulobacter	30
04113 Meiosis - yeast	2
04210 Apoptosis	4
04214 Apoptosis - fly	2

04215 Apoptosis - multiple species	1
04216 Ferroptosis	3
04217 Necroptosis	5
04115 p53 signaling pathway	1
Cellular community - eukaryotes	4
04530 Tight junction	1
04540 Gap junction	2
04550 Signaling pathways regulating pluripotency of stem cells	1
Cellular community - prokaryotes	278
02024 Quorum sensing	130
05111 Biofilm formation - <i>Vibrio cholerae</i>	59
02025 Biofilm formation - <i>Pseudomonas aeruginosa</i>	46
02026 Biofilm formation - <i>Escherichia coli</i>	43
Cell motility	77
02030 Bacterial chemotaxis	26
02040 Flagellar assembly	50
04814 Motor proteins	1

Table A1.8: The functional content of gut bacterial taxa of individuals of the Cape sea urchin, *Parechinus angulosus*, collected from the east and west coasts of South Africa, reported as a number of organismal system processing pathways.

Organismal Systems	Number of linked KEGG annotations
Immune system	14
04611 Platelet activation	1
04613 Neutrophil extracellular trap formation	1
04624 Toll and Imd signaling pathway	1
04621 NOD-like receptor signaling pathway	5
04622 RIG-I-like receptor signaling pathway	1
04623 Cytosolic DNA-sensing pathway	1
04612 Antigen processing and presentation	1
04659 Th17 cell differentiation	1
04657 IL-17 signaling pathway	1
04666 Fc gamma R-mediated phagocytosis	1
Endocrine system	47
04910 Insulin signaling pathway	7
04922 Glucagon signaling pathway	12
04920 Adipocytokine signaling pathway	2
03320 PPAR signaling pathway	10
04915 Estrogen signaling pathway	1
04914 Progesterone-mediated oocyte maturation	1
04917 Prolactin signaling pathway	2

Organismal Systems	Number of linked KEGG annotations
04921 Oxytocin signaling pathway	1
04918 Thyroid hormone synthesis	2
04919 Thyroid hormone signaling pathway	2
04928 Parathyroid hormone synthesis, secretion and action	1
04916 Melanogenesis	1
04924 Renin secretion	2
04614 Renin-angiotensin system	3
Circulatory system	4
04260 Cardiac muscle contraction	3
04270 Vascular smooth muscle contraction	1
Digestive system	17
04970 Salivary secretion	2
04972 Pancreatic secretion	2
04976 Bile secretion	2
04973 Carbohydrate digestion and absorption	2
04974 Protein digestion and absorption	4
04975 Fat digestion and absorption	1
04978 Mineral absorption	4
Excretory system	4
04964 Proximal tubule bicarbonate reclamation	4
Nervous system	16
04724 Glutamatergic synapse	2
04727 GABAergic synapse	4
04725 Cholinergic synapse	1
04728 Dopaminergic synapse	4
04726 Serotonergic synapse	2
04730 Long-term depression	1
04723 Retrograde endocannabinoid signaling	2
Sensory system	1
04744 Phototransduction	1
Development and regeneration	2
04361 Axon regeneration	2
Aging	15
04211 Longevity regulating pathway	2
04212 Longevity regulating pathway - worm	8
04213 Longevity regulating pathway - multiple species	5
Environmental adaptation	23
04713 Circadian entrainment	1
04714 Thermogenesis	10
04626 Plant-pathogen interaction	12

Appendix 2 - Mothur (Supplementary material for Chapter 5)

A2.1 Processing

The data were first denoised by the removal of low-quality reads and sequences that had not successfully been demultiplexed. Reads outside of the desired 350-550bp range, reads containing any ambiguous nucleotides, as well as all chimeric sequences [identified using the VSEARCH (Schloss 2009) algorithm] were removed. Then a taxonomic rank was assigned to further curate the resulting dataset using the Naive Bayesian classifier (Wang *et al.* 2007) with the SILVA full length reference database (v1.44.3) (Quast *et al.*, 2013). All sequences that presented a taxonomic rank of chloroplasts, mitochondria, Archaea, Eukaryota, and sequences classified as 'unknown' were removed from the dataset. Operational Taxonomic Units (OTUs) were constructed using an uncorrected pairwise distance of 0.03 against the SILVA (v1.44.3) (Quast *et al.*, 2013) database.

Before diversity analysis, sequences were subsampled to a sequencing depth of 10 900 in order to normalise the data to allow direct comparison between samples.

To determine if the data set met the assumption of homogeneity of variance, a recommended procedure for some downstream statistical tests, the permutational multivariate analysis of dispersion (PERMDISP) function was applied in Primer v7 (Clarke and Gorley, 2015). Principal Coordinate Analysis (PCO) ordination plots were generated based on Bray-Curtis dissimilarity (Bray and Curtis 1975). Similarly, the same package was also used to perform the permutational analysis of variance (PERMANOVA) tests to investigate the possible effect of site nested within region/coast on gut bacterial composition, with site given as a random factor, and region set as a fixed factor. Finally, a similarity percentage (SIMPER) analysis was used to determine which bacterial phyla contributed the most towards the dissimilarity observed between sites and regions, based on the results of the pairwise PERMANOVA. A series of heatmaps were generated to visualise the abundance of amplicon sequence variants between sites within the eastern and western coasts of South Africa, which aim to cluster closely related sites based on the abundance of particular phyla.

A.2.2 Results

After removing taxonomic ranks reported as unclassified and unidentified bacteria, a total of 18 bacterial phyla were identified. These phyla were reported as individuals belonging to *Acidobacteriota*, *Actinobacteriota*, *Bacteroidota*, *Bdellovibrionota*, *Campylobacterota*, *Chloroflexi*, *Cyanobacteria*, *Dadabacteria*, *Deinococcota*, *Dependentiae*, *Desulfobacterota*, *Fibrobacterota*, *Firmicutes*, *Fusobacteriota*, *Patescibacteria*, *Planctomycetota*, *Proteobacteria*, and *Verrucomicrobiota*. Of these, *Bacteroidota*, *Campylobacterota*, *Fusobacteriota*, and *Proteobacteria* were found to be the most dominant phyla (Figure A2.1).

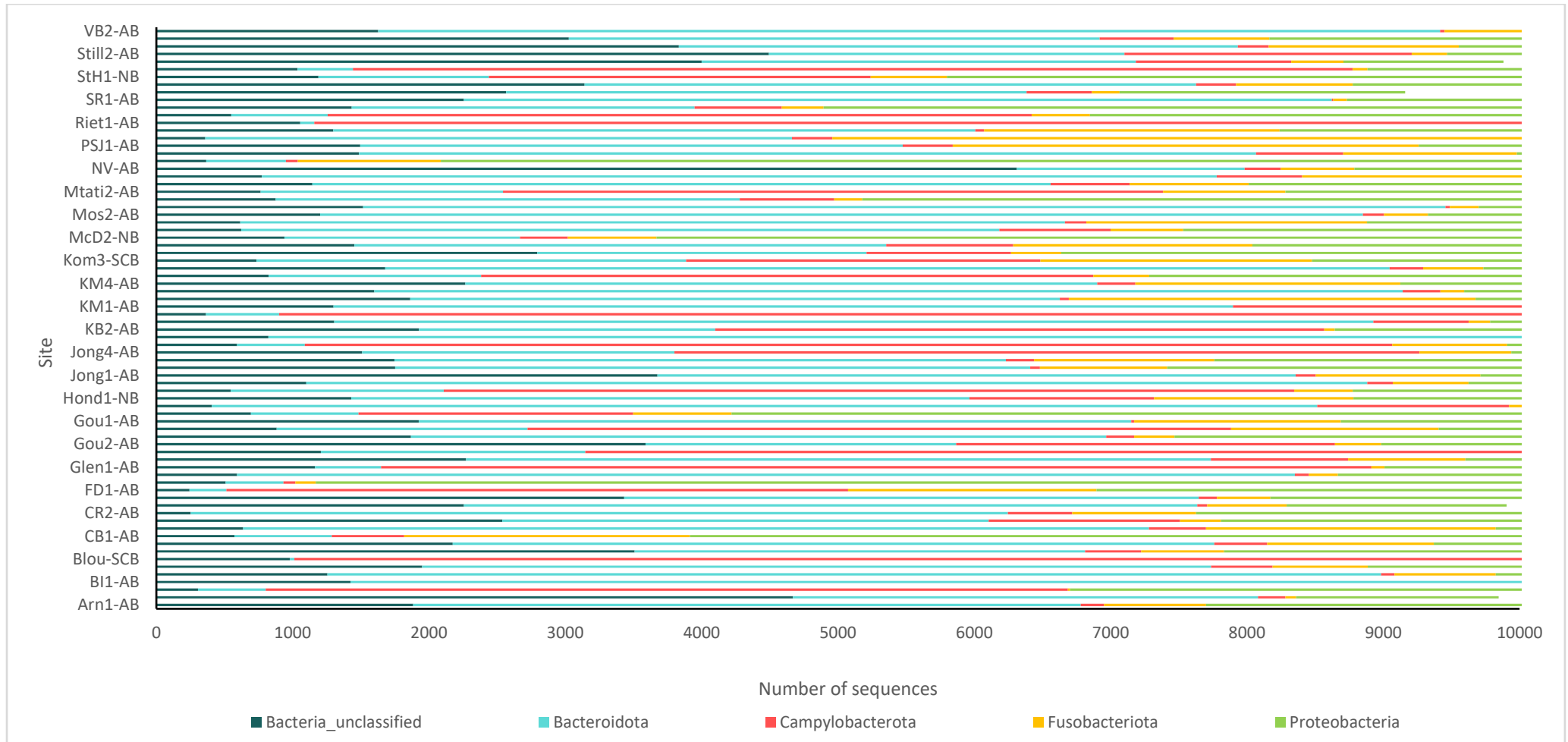


Figure A2.1: Number of sequences per phylum per site for the four most dominant phyla, *Bacteroidota*, *Campylobacterota*, *Fusobacteriota*, *Proteobacteria*, and a class consisting of all the remaining less dominant, and unknown/unclassified phyla found in the gut microbial community of *Parechinus angulosus* individuals collected from 32 sites on the South African coastline.

The results from the PERMDISP test for homogeneity of variances in the nested design of population within coast at the phylum level were not significant ($p = 0.1$ and $p = 0.444$ respectively), indicating that the assumption of homogeneity of variances was not violated. Principal component analysis ordination plots of population and coast showed that the first two axes explained 79% of the variance observed in the data set (Figures A2.2 and A2.3, respectively).

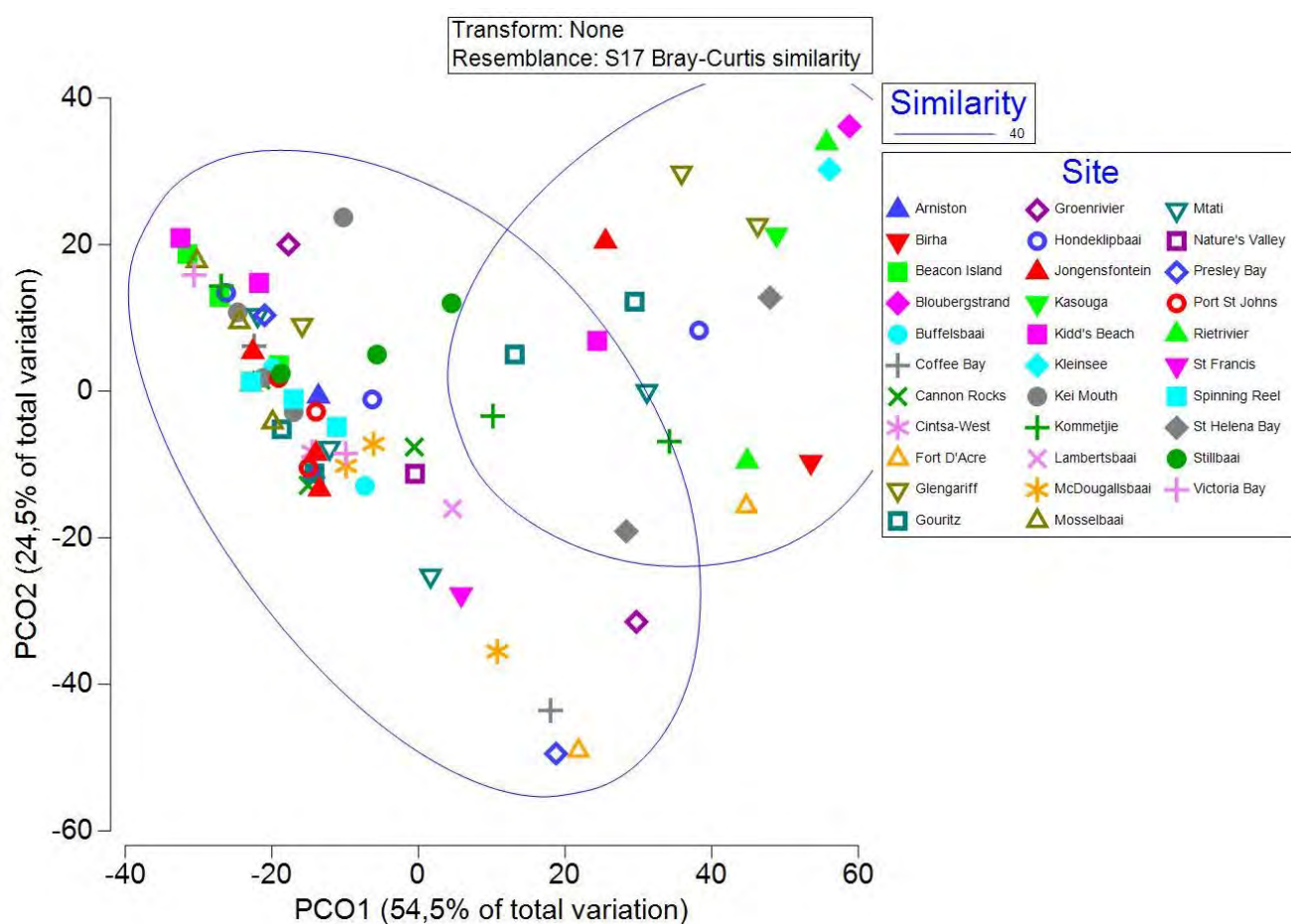


Figure A2.2 A principal component analysis ordination plot of populations (i.e., sites) from which individuals of *Parechinus angulosus* were collected for analysis of their gut microbiota, showing clusters generated at 40% similarity. Each site is represented using a combination of different colours and symbols.

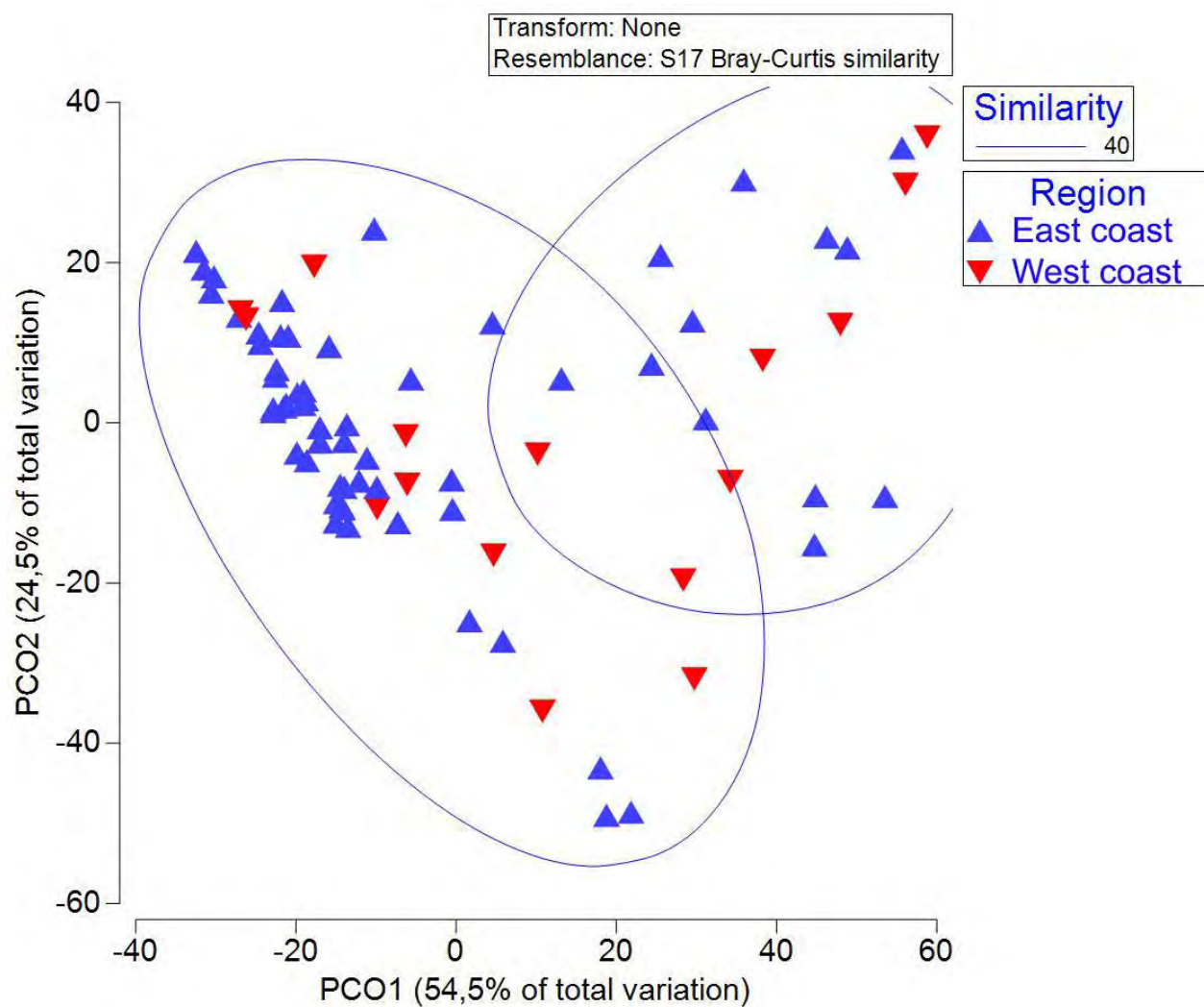


Figure A2.3: A principal component analysis ordination plot constructed using the same gut bacterial data of *Parechinus angulosus* individuals as in Figure A2.3 above, but of the coast factor level, clustering together individuals from the east and west coasts based on 40% similarity. Blue triangles represent individuals from the east coast, red triangles individuals from the west coast.

The results of the PERMANOVA revealed that differences between coasts and between populations within coast were statistically significant (Table A2.1).

Table A2.1: PERMANOVA main test results examining the effect of coast, or populations within coast, on bacterial phyla from the gut of *Parechinus angulosus* individuals collected at sites along the South African coastline [Degrees of Freedom, Df: Sum of Squares, SS: Mean Squares; Pseudo F-Ratio; P-value (permutation); Monte Carlo P-values].

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms
Coast	1	5223,1	5223,1	3,5266	0,018	998
Site(coast)	30	51933	1731,1	2,0234	0,001	996
Residual	44	37643	855,52			
Total	75	94090				

Thereafter, a pairwise PERMANOVA test was performed to determine which sites differed from one another within the east and west coasts respectively, followed by a SIMPER analysis to determine which bacterial phyla contributed the most towards the significant dissimilarity observed. Only certain sites within the east coast were found to differ significantly from one another, of which Rietrivier was consistently found to be the most dissimilar to all other sites, and none were found to differ significantly within the west coast (Table A2.2). SIMPER analysis showed that *Campylobacterota* and *Bacteroidota* contributed the most towards the observed dissimilarity between Rietrivier and the other sites from which it differed significantly. *Campylobacterota* alone contributed as much as 43.18% towards the dissimilarity between Rietrivier and Spinning Reel.

Table A2.2: Results of the PERMANOVA pairwise and SIMPER analyses showing the percentage contribution of each morphometric trait collected from individuals of *Parechinus angulosus* between pairs of populations within the east coast.

Groups	t	P(permanova)	Unique perms	Species	Percentage contribution	Cumulative percentage
Rietrivier, Spinning Reel	3,5849	0,085	10	Campylobacterota	43,18	43,18
				Bacteroidota	27,7	70,88
Rietrivier, Stillbaai	3,3957	0,097	10	Campylobacterota	39,9	39,9
				bacteria_unclassified	21,65	61,55
				Bacteroidota	18,95	80,5
Rietrivier, Port St Johns	3,6689	0,099	10	Campylobacterota	41,89	41,89
				Bacteroidota	23,46	65,35
				Fusobacteriota	20,11	85,47
Rietrivier, Mosselbaai	3,7442	0,113	10	Campylobacterota	41,4	41,4
				Bacteroidota	39,5	80,91
Rietrivier, Beacon Island	3,9574	0,097	10	Campylobacterota	40,88	40,88
				Bacteroidota	40,31	81,18
Rietrivier, Cannon Rocks	2,9693	0,107	10	Campylobacterota	44,02	44,02
				Bacteroidota	30,52	74,54
Rietrivier, Jongensfontein	2,4735	0,063	15	Campylobacterota	42,44	42,44
				Bacteroidota	26,28	68,72
				Proteobacteria	12,7	81,41
Rietrivier, Kei Mouth	3,4633	0,079	15	Campylobacterota	40,7	40,7
				Bacteroidota	34,05	74,75
Birha, Beacon Island	5,0321	0,236	4	Bacteroidota	38,21	38,21
				Campylobacterota	31,67	69,88

				Proteobacteria	18,11	87,99
Birha, Kei Mouth	3,3031	0,207	5	Bacteroidota	32,7	32,7
				Campylobacterota	31,46	64,15
				Proteobacteria	18,28	82,43
Birha, Mosselbaai	4,1539	0,23	4	Bacteroidota	38,75	38,75
				Campylobacterota	33,29	72,04
Birha, Port St Johns	4,0246	0,282	4	Campylobacterota	34,35	34,35
				Bacteroidota	23,34	57,69
				Fusobacteriota	21,76	79,45
Birha, Spinning Reel	3,9895	0,246	4	Campylobacterota	35,86	35,86
				Bacteroidota	28,01	63,86
				bacteria_unclassified	14,98	78,84
Birha, Stillbaai	4,2049	0,259	4	Campylobacterota	29,77	29,77
				bacteria_unclassified	23,95	53,72
				Proteobacteria	19,99	73,7
Beacon Island, Kasouga	4,6652	0,24	4	Campylobacterota	46,62	46,62
				Bacteroidota	41,13	87,75
Beacon Island, Port St Johns	2,7562	0,107	10	Bacteroidota	37,55	37,55
				Fusobacteriota	37,15	74,7
Beacon Island, St Francis	3,1167	0,252	4	Proteobacteria	45,29	45,29
				Bacteroidota	43,14	88,43
Beacon Island, Stillbaai	3,3784	0,102	10	Bacteroidota	44,32	44,32
				bacteria_unclassified	27,91	72,23
Cannon Rocks, Kasouga	2,9793	0,256	4	Campylobacterota	47,2	47,2
				Bacteroidota	28,88	76,08
Kasouga, Kei Mouth	3,1103	0,201	5	Campylobacterota	46,76	46,76
				Bacteroidota	34,62	81,38

Kasouga, Mosselbaai	4,0323	0,265	4	Campylobacterota	46,63	46,63
				Bacteroidota	39,84	86,47
Kasouga, Port St Johns	3,8709	0,234	4	Campylobacterota	48,76	48,76
				Bacteroidota	24,17	72,93
Kasouga, Spinning Reel	4,1832	0,254	4	Campylobacterota	47,02	47,02
				Bacteroidota	26,76	73,78
Kasouga, Stillbaai	3,838	0,27	4	Campylobacterota	46,84	46,84
				bacteria_unclassified	24,16	71
Kei Mouth, Nature's Valley	2,2406	0,191	5	bacteria_unclassified	39,98	39,98
				Bacteroidota	36,99	76,97
Kei Mouth, Stillbaai	2,3179	0,027	35	Bacteroidota	31,87	31,87
				bacteria_unclassified	28,97	60,84
				Fusobacteriota	13,84	74,68
Mosselbaai, Port St Johns	2,3134	0,103	10	Bacteroidota	38,66	38,66
				Fusobacteriota	36,57	75,23
Mosselbaai, Stillbaai	3,3586	0,1	10	Bacteroidota	39,17	39,17
				bacteria_unclassified	30	69,17
				Campylobacterota	10,43	79,6
Mtati, Stillbaai	2,0383	0,028	35	bacteria_unclassified	31,68	31,68
				Proteobacteria	20,14	51,82
				Bacteroidota	19,47	71,29
Port St Johns, Spinning Reel	2,3876	0,106	10	Fusobacteriota	42,93	42,93
				bacteria_unclassified	21,49	64,42
				Bacteroidota	12,75	77,17
Port St Johns, Stillbaai	3,2618	0,1	10	bacteria_unclassified	32,39	32,39
				Fusobacteriota	30,83	63,22
				Bacteroidota	11,23	74,44

Spinning Reel, Stillbaai	2,1513	0,107	10	Bacteroidota	25,02	25,02
				bacteria_unclassified	21,99	47,01
				Proteobacteria	14,78	61,8
				Campylobacterota	14,64	76,43

A heatmap was generated to investigate clustering between populations within either the east or west coasts based on phylum (Figure A2.5)

Figure A2.4: A clustered heatmap displaying the frequency of the four dominant gut microbial phyla in the sea urchin *Parechinus angulosus*, revealing clusters of populations irrespective of their east or west coast locations.

There was an obvious clustering of sites when the abundances of the *Bacteroidota*, *Campylobacterota* and *Proteobacteria* phyla (Table A2.3) are considered in isolation. This demonstrates that the clusters observed include populations from both the east and west coast, suggesting that factors other than biogeographic region and distance between populations are responsible for the clustering observed.

Table A2.3: Clusters with the highest number of sequence features of sites from both the east (pink) and west (blue) coasts per bacterial phylum according to Figure A2.5.

Phylum		
Bacteroidota	Campylobacterota	Proteobacteria
Spinning Reel1	Mtati2	McDougall's Bay2
Fort D'Acre3	Kommetjie1	Coffee Bay1
Mosselbaai2	Kidd's Beach2	Presley Bay1
Kei Mouth3	Jongensfontein4	Fort D'Acre2
Kidd's Beach3	Gouritz4	St Helena1
Hondeklip3	Hondeklip2	Groenrivier1
Beacon Island 2	Rietrivier2	St Francis
Victoria Bay2	Birha	Mtati1
Mosselbaai3	Fort D'Acre1	
Kommetjie2	St Helena2	
Kei Mouth1	Glengariff1	
Groenrivier2	Kasouga	
Mtati4	Glengariff3	
Coffee Bay2	Rietrivier1	
Presley Bay2	Blouberg	
Mosselbaai1	Kleinsee	
Kidd's Beach1		
Beacon Island1		