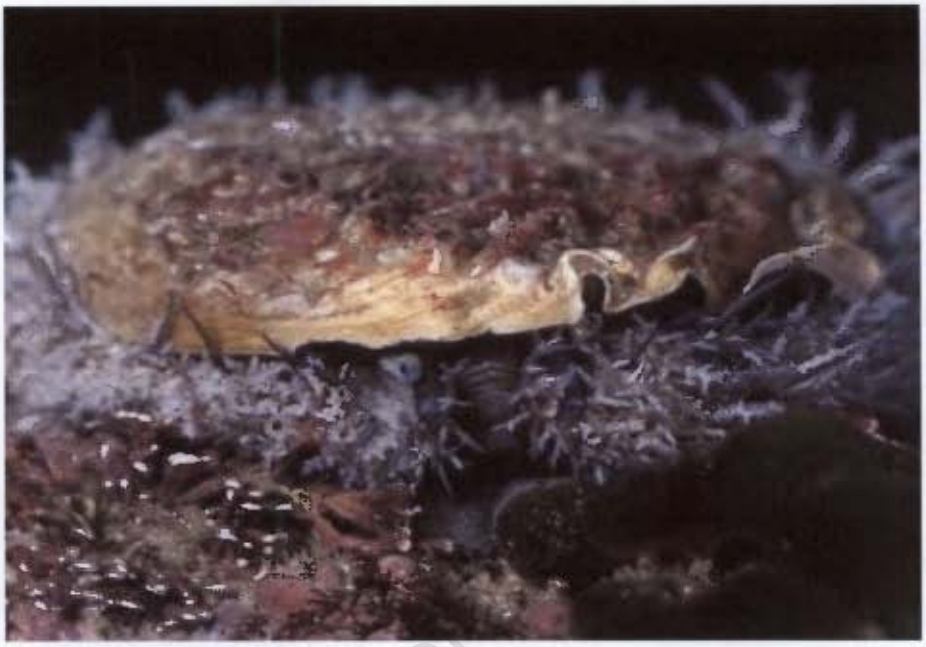
Characterisation of the Population Genetics of Farm-Bred *Haliotis midae* using microsatellite DNA markers



by

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This thesis is dedicated to my grandma,

Roseline Esme Green

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Acknowledgements

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Abstract

The abalone *Haliotis midae* is a gastropod mollusc which is of commercial importance in South Africa due to its high export value. During this study the genetic structure of farmed *Haliotis midae* was investigated in order to determine whether microsatellite DNA analysis could be used to separate farmed abalone into phenotypically different groups with respect to growth rate. Microsatellites display high levels of variability and this makes them suitable for a wide variety of applications in aquaculture, particularly where genetic differentiation between population groups may be limited. This study investigates the genetic composition of 120 individuals from 2 spawning events that had been classed as either fast or slow growing by the farm managers. Three highly polymorphic microsatellite loci were selected and used to determine the extent of the genetic diversity which exists amongst the 120 individuals tested from the Jacobsbaai abalone farm. Additionally, these microsatellite loci were used to determine whether abalone classed as either fast or slow growing could be differentiated into specific genetic population groups. Neighbour-joining trees constructed using genetic distance data obtained for all three loci demonstrated that a distinct separation between the fast and slow growing abalone was evident. It was also found that there has been a loss of genetic diversity on the Jacobsbaai abalone farm in terms of heterozygosity. This has been attributed to the high levels of inbreeding as evidenced by the high F_{is} values. All population groups were found to deviate significantly from Hardy-Weinberg equilibrium and this

is most likely due to the occurrence of non-random mating on the Jacobsbaai abalone farm. This study has successfully demonstrated that a combination of microsatellite loci with high allelic diversity can potentially be employed as a tool for distinguishing between fast and slow growing farmed abalone. This study needs to be validated by testing larger sample sizes and the use of additional farms.

Chapter 1

Introduction

1.1 *Haliotis midae*

Abalone are marine herbivorous gastropods, that belong to the family *Haliotidae* [8]. In their natural habitat, they are slow feeding nocturnal herbivores, with the adults grazing predominantly on seaweeds and the juveniles on microalgae and diatoms found on the surfaces where they settle [22]. These animals are highly prized for their large adductor (foot) muscle [22].

Haliotidae (Gastropoda) includes more than 100 species of abalone distributed in temperate and tropical waters of all continents [1,5]. About 10 % of these are subjected to commercial exploitation in Australia, Japan, South Africa, North America and Southeast Asia [1,6,8]. There are six abalone species (*H. midae, H. parvum, H. spadicea, H. queketti, H. speciosa, H. pustulata*) that are endemic to South Africa (Fig. 1) [23]. *Haliotis midae* (Fig. 2), known locally as 'perlemoen', is the only species that is harvested commercially [6].

Commercial fisheries now exist for over 30 species worldwide, and the products are sold in live, frozen, canned or dried form. The main markets reside in China, Japan, Hong Kong (and South-East Asia), the USA, Mexico, Korea and Europe [24]. The annual commercial quota for *Haliotis midae* stands at 650 metric tons [7]. It has been estimated that the amount of abalone exploited by recreational divers is the same as

the annual commercial quota. Abalone losses due to poaching has been difficult to quantify but available estimates have shown that in just one area in the Western Cape province at least 40 tons of *H. midae*, valued at US $1 million to local producers, is poached annually [7].

Fig. 1: Distribution map of the six abalone species that are endemic to South Africa [65].

Fig. 2: Image showing the commercially important South African abalone *Haliotis midae* (Photograph courtesy of Bronwyn Arendze-Bailey)

1.2 Abalone natural stock populations

The worldwide popularity of abalone has led to the decline of this species (Fig. 3) in recent years through commercial overfishing, excessive poaching and large recreational catches [22, 6]. As such local extinctions has been forecasted to take place in the next five years [6]. The high demand, combined with the recent decline in abalone fisheries, has increased the pressure on natural stocks, which need to be maintained if the fishery industry is to remain viable [8]. This has lead to the growth of

the abalone aquaculture industry and genetic improvement of abalone stocks has been identified as an important factor if future market demands are to be met [3]. Thus, there is a need for fisheries management to understand the genetic structure of abalone populations and to identify stocks of commercially important *Haliotis midae* [8].

There are two areas in which genetics is especially important in aquaculture development:

1.) the genetic improvement of important production traits
2.) genetic implications of the intentional relocation of organisms for aquaculture or restocking programmes [21]

During this study I will be investigating the first area. N. G. Elliot [22] defines genetic improvement as 'a gain in the cultured production of an abalone species through the exploitation or manipulation of the genetic variation present within the particular species' [22]. He goes on to state that in general there are four 'inputs' to a culturing system that a manager can alter to improve production gain. These are farm size, management practices (including husbandry, tank design etc.), nutrition and genetics. The first three factors are associated with the environment in which the animals live and are the areas usually targeted first with any culture venture. Genetics however is associated with the biological potential of the species to exploit the environment. The aim of any genetic improvement program therefore is to increase the production gain by exploiting the inherent biological potential of the cultured population [22].

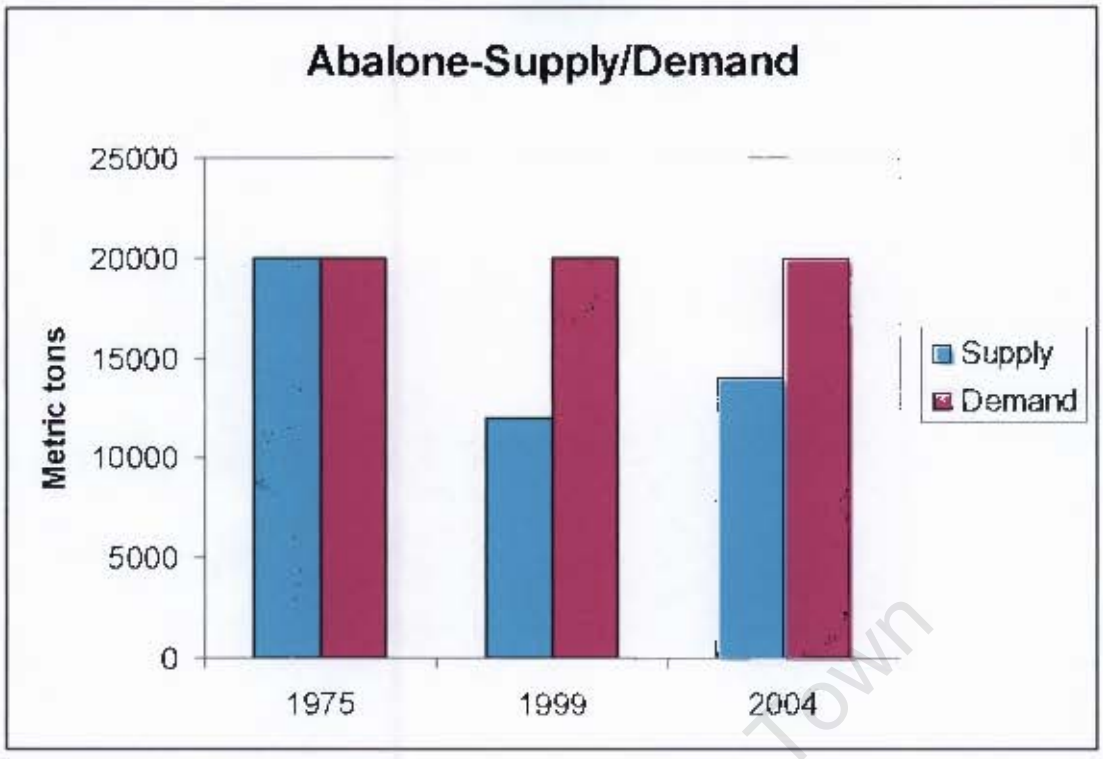


Fig. 3: Bar graph showing the worldwide supply and demand of abalone over the last 29 years in cultured *H. supertexta* [64]

1.3 Abalone Aquaculture

Abalone are attractive aquaculture species as in most instances, they already have a recognized status and attract a high price in the international market place. They are a sought after seafood delicacy, especially in the Far East where they are usually prepared for special or ceremonial occasions. Cultured abalone fill a different market niche to the commercial fishery products as they are generally harvested at a smaller size, 50-80 mm [22].

Aquaculture of the South African abalone *Haliotis midae* developed through the 1990s, in parallel with the emergence of the culture of other commercially important species in Asian countries, the USA, Australia, New Zealand and Chile. The South African abalone fishery industry has existed since 1949 [23]. The first attempts to cultivate *Haliotis midae* were made in 1981 [23]. A research and development iniative to establish commercial abalone farming was initiated by the University of Cape Town, the Council for Scientific and Industrial Research and Rhodes University in partnership with three fishing companies. To date, there are about 12 abalone farms that have since been established, ranging in distribution from Port Nolloth on the Atlantic coast to East London on the Indian Ocean. Of these, approximately ten farms are presently exporting abalone on a regular basis to the Far East. It has been estimated that approximately US $12 million has been invested in the industry and the projected production of the existing shore–based farms is 500-800 tons [23]. At present abalone can fetch as much as US$ 38 per kg on the overseas market.

1.4 Genetic improvement techniques

The process of domestication has resulted in major production gains and efficiency increases in modern agriculture [25]. Genetic improvement programs in terrestrial species have been shown to benefit greatly from knowledge of pedigree and individual performance [10]. In the aquaculture industry, however, selection programs have been implemented in only a few species. In fact many of the species exploited for commercial aquaculture are essentially still 'wild' [26]. Genetic improvement of farmed aquaculture species, therefore, has the ability to deliver cumulative and

sustained improvements in production efficiency, product quality and financial profitability of aquaculture enterprises and industries. The potentials of these gains have long been recognized as a significant impetus for domestication and controlled breeding for a range of aquaculture species. Gains in profit resulting from genetic improvement have been realized in terrestrial domesticated livestock species, agricultural, horticultural and ornamental plants, forest trees and some aquaculture species, notably salmonids [20].

Selective breeding programs have been instituted for a number of aquaculture species. The main task when carrying out such programs is to start from appropriate broodstocks, both regarding management of genetic resources and knowledge of parentage relationships among these individuals [3]. Simple mass selection programs have been commonly applied to fish and it has been shown that maximum genetic progress at any selection intensity is reached with a combination of within and between family selection [27]. In a study conducted by Jones *et al* on redclaw crayfish *Cherax quadricarinatus* a size-selected breeding program was set up [90]. They were able to show in their study that improvement in economically viable traits in this species could be achieved as they were able to obtain a 9.5 % gain in growth rate with two selected generations. Selective breeding programs have been shown to be successful in fish species with a 10-15 % increase in growth rate being reported [26]. These include Atlantic salmon [26], rainbow trout [91] and channel catfish [92]. Thus selective breeding programs in aquaculture have been shown to be successful. There are a number of associated benefits with selective breeding programs. Selective breeding programs will ensure higher production rates and decreased production costs because of increased growth rates, shorter turnover rates and improved food

conversion efficiency. Domesticated animals will become better adapted to captivity resulting in a decrease in stress and disease mortality. Additionally use of land and food resources will be optimized because of higher growth rates and higher retention of protein and energy. The consequent increase in disease resistance will result in a decrease in the use of chemotherapeutic agents and so contribute to better animal welfare and environmental management. Lastly the increase in flesh quality of the aquaculture species would result in greater consumption by the consumer [26]. Selective breeding programs based on phenotypic traits such as growth rate hold lots of potential and implementation of this in *Haliotis midae* would greatly benefit the South African aquaculture industry.

Despite the obvious advantages associated with selected breeding programs, there are, however, a number of potential problems associated with selective breeding strategies. It has been found that aquaculture practises may inadvertently decrease the genetic variability present in farmed stocks by breeding related individuals or by the use of small numbers of broodstock [11, 29]. It has been reported that hatchery-produced juveniles tend to have reduced genetic variability, measured as the number of alleles (as determined by microsatellite analysis) and heterozygosity, when compared to natural populations because of the small effective number of parents used in producing subsequent generations [11, 28, 29, 30]. As such there is a potential for genetic deterioration of the broodstock if a small effective number of breeding animals is kept within the hatchery [31, 32]. Some selective breeding programs breed from a small number of 'superior' families that may be related. Other farms operate a mass selection approach with high selection intensities.

One of the main problems associated with selective breeding strategies is inbreeding. Inbreeding will result in a decrease in genetic variability which will limit the potential for future gain from artificial selection. As more intensive breeding practices are put in place care must be taken to avoid loss of genetic variation and accumulation of inbreeding within farmed stocks [9]. Pedigree records need to be maintained to minimize the probability of selecting related individuals as parents which could increase inbreeding [9].

A major consequence of inbreeding is inbreeding depression. Inbreeding depression arises during artificial selection practices because of a small population size [58]. This results in a steady increase in the inbreeding coefficient in the selected population with a consequent reduction in reproduction and survival (reproductive fitness) [58, 59]. One of the major consequences of inbreeding depression is that on average birth rates are reduced and death rates increase [60]. Traits that are closely related to fitness show the greatest inbreeding depression [58].

Inbreeding, however, can be managed through a structured breeding program that allows a high degree of relationship to exist between some individuals but prevents them mating. This results in a slower accumulation of the amount of inbreeding within populations. This issue has been examined in nucleus breeding structures in terrestrial livestock where inbreeding can be high [62]. Inbreeding is of particular concern where inbreeding depression is high for commercially important traits or where the genetic load is so high as to significantly reduce viability [20].

Properly designed breeding programs can make appropriate use of additive and non-additive genetic variation for traits of economic importance and so minimize the negative effects of inbreeding [10]. For most aquaculture species the analytical tools to carry out genetic improvement techniques are available. They have not been implemented successfully though because of the difficulty of measurement and/or lack of domestication or controlled breeding [20]. Novel genetic technologies involving the use of DNA-based tools are under development for a range of aquaculture species. These gene marker technologies can be used for identification and monitoring of lines, families and individuals, monitoring and control of inbreeding, diagnosis of simply inherited traits and genetic improvement for favourable genes and gene combinations [20].

1.5 Microsatellite DNA markers

Microsatellite markers are short stretches (tens to hundreds of base pairs) of DNA composed of one to eight nucleotide repeats arrayed in tandem [9,15]. These microsatellite DNA loci are flanked by unique sequence DNA. Primers for polymerase chain reactions (PCR) can be designed from the flanking DNA sequence, ensuring specific amplification of a single locus. Microsatellites display polymorphism by means of having variable numbers of tandem repeat motifs resulting in size variation which can then be visualized by PCR with pairs of locus-specific flanking primers, followed by electrophoresis of the amplification product [16]. Moreover with the current molecular methods it is feasible to score

microsatellite length polymorphisms in large numbers of individuals for genetic analyses within and between populations. The relatively short length of the array facilitates size fractionation and precise scoring of alleles on polyacrylamide sequencing gels [4]. Some microsatellite loci have very high numbers of alleles per locus (>20), making them very useful for applications such as parent-offspring identification in mixed populations, while others have lower numbers of alleles and may be more suited for population genetics and phylogenetic studies [13, 33, 34, 35].

Microsatellites have proved useful in distinguishing populations [35]. They generally appear to be non-coding and selectively neutral. They are frequently polymorphic as a result of allelic variation in the number of repeats in the tandem array. Such characteristics offer great potential for their use as genetic markers [36]. Previous work has shown that some microsatellite loci are conserved within related taxa so that primers developed to amplify markers in one species may amplify the homologous markers in related species as well [37,39,40].

Microsatellites have become the markers of choice for a wide range of applications in genetic mapping and genome analysis [41]. Microsatellites represent ideal molecular markers because they have multiple alleles that are highly polymorphic among individuals. Additionally microsatellite loci are highly abundant and dispersed evenly through eukaryotic genomes. They are also inherited in a co-dominant fashion and are fast and easy to assay. Moreover they can serve as sequence-tagged sites for anchoring in genetic and physical maps [16,41]. They therefore present the most widely applicable DNA technology [9].

Microsatellite markers show higher levels of genetic variability than allozyme markers. This variability makes for suitability in a variety of applications in fisheries and aquaculture, particularly where genetic differentiation between populations may be limited. Potential applications in aquaculture include monitoring changes in genetic variation, as a consequence of different breeding strategies, parentage assignment, and estimation of relatedness between potential breeding pairs [9]. In addition these molecular markers can be used to identify lines or strains, define stock diversity, monitor inbreeding, diagnose simply inherited traits and even improve stocks through marker assisted selection [2, 42, 43, 44].

Microsatellite loci have been isolated from a number of temperate abalone species [45, 46, 47]. Their ability to readily genotype individuals in culture circumvents problems associated with traditional selective breeding programs, in which individual family lines have to be cultured separately. The rearing together of offspring from multiple parents allows for a better scrutiny of the genetic effects underlying a targeted trait by reducing the confounding effects of the environment [1]. Determination of the genetic structure of abalone populations will allow farmers to manage and conserve their diversity.

There are a number of advantages associated with microsatellite DNA markers. Firstly, sampling is non-lethal and only a few samples are required when screening an individual. Secondly, due to their ability to reveal extensive allelic variation and high levels of variability these markers frequently detect differences even among closely related populations. Hence, microsatellite polymorphisms have become the favoured

tool in aquaculture stock management, fish population analysis and biodiversity conservation [33, 36].

1.6 Breeding objectives for *Haliotis midae*

Breeding objectives for *Haliotis midae* include the improvement of growth and survival. The traits in the breeding objective can usually be defined but they cannot always be measured. Thus, traits are used as selection criteria that are known to have some genetic relationship with traits in the objective. Growth rates and feed conversion efficiency are both examples of traits that form part of the breeding objective for many aquaculture species. Growth rates are usually the targeted traits as they can be measured easily and are therefore a good selection criterion. Traits that are chosen as selection criteria are those that will be used to make the genetic change towards the breeding objective. They therefore need to be heritable, inexpensive to measure and, most importantly, genetically correlated with the traits in the breeding objective [20]. Some aquaculture species already have published parameters for key traits such as growth [48,49,50]. However for most aquaculture species studies are limited and there have been no parameters published [20].

In most genetic improvement programs the first trait of interest is growth, with the aim of producing faster growing individuals and thus reducing the production time and cost for a market size individual. Growth is an important factor in abalone aquaculture as many people describe the slow growth rate as a hindrance to their aquaculture potential. Genetic changes in the red abalone *Haliotis rufescens*

(Swainson) have been reported to provide 50 % to 100 % increases in growth rates [51,52].

Genetics can potentially do more than just increase growth rates. It can assist in the provision of abalone strains that are better suited to particular grow-out environments, are disease resistant, have higher survival rates, have better food conversion ratios, have desirable market traits (e.g. colour, texture, taste, tenderness), return a better meat yield, have a higher fecundity with age, have a higher or lower age at maturity, are sterile, are single sex only and can be used to enhance pearl production [22].

One of the first steps in a genetic improvement programme is for the industry to decide what are the main characters of economic importance that need to be targeted [22]. Although (as mentioned previously) selective breeding programmes are not commonly used in aquaculture they have proven to be successful when instituted in many areas of primary production. The application of such technology for genetic improvement in aquaculture production, apart from Atlantic salmon, has been slow and there are very few reports of research in this area for abalone [22, 63]. However, in studies conducted by Hara [66] and Kobayashi *et al* [67, 68] abalone growth has been assessed in culture and it has been suggested that there are genetic factors involved in growth and that selective breeding strategies could improve this trait. Third generation mass selection for faster growth in *H. discus hannai* produced a 21 % increase in daily growth rate in animals of shell size 20-30 mm and a 65 % increase in those from 30-70 mm, compared with the growth of control commercial animals [22].

In Iceland, a large research programme on the red abalone *Haliotis rufescens* was started in 1996 to study genetic variation in survival, growth rate, meat yields and age at maturity [63]. They have produced 100 families using a hierarchical mating system of one male to 2-6 females. In order to understand the importance of these results heritabilities have to be defined. The heritability of a trait is defined as the proportion of the total (phenotypic) variation that is explained by the genetic variation and heritability values give an indication of how a trait will respond to natural or artificial selection. Results to date have returned heritabilities (genetic proportion of observed variation) of 0.11 for survival at four months of age, 0.08 for shell length at eight months (~13 mm) and a value of 0.30 for shell length after 17 months growth (~36 mm). These results suggest that commercially important traits could be improved through selection in abalone [63].

These preliminary studies indicate that selective breeding programs may be a good way of increasing outputs on abalone farms. This increase in outputs will result in gains for farmers and an increase in revenue.

1.7 The use of microsatellite DNA markers in *Haliotis spp.*

Research to date on the use of microsatellite DNA markers in *Haliotis spp.* has focussed mainly on the determination of the loss of genetic variation between hatchery and wild stocks, and parentage determination studies. Microsatellite DNA markers have been developed for a number of *Haliotis* species. These include *Haliotis midae* [6], *Haliotis rubra* [47], *Haliotis discus discus* [55], *Haliotis kamtschatkana* [56], *Haliotis rufescens* [46], *and Haliotis asinina* [57].

Selvamani *et al.* [1] conducted a parentage assignment study in *Haliotis asinina*. Microsatellite DNA markers were used to genotype individual abalone larvae. Previously they had isolated 11 microsatellite loci from *Haliotis asinina* and five of these were used during this study. They devised a system that allowed mass screening of individual larvae which enabled them to readily analyse at least 10 loci from a single larva. The obtained microsatellite data was used to assign each larva to its parents. This allowed the relative contribution of each of the broodstock to the next generation to be determined. From this study it is evident that highly polymorphic microsatellite loci are effective markers for use in parentage assignment studies [1].

In a similar study Li *et al.* [54] investigated the inheritance of microsatellite DNA markers in the Pacific abalone *Haliotis discus hannai*. Here they reported on the mode of inheritance of seven microsatellite loci. These seven microsatellite markers were used to verify the mode of the mating system for this abalone. Additionally, during their study, they achieved a 60 % success rate in the amplification of *Haliotis discus discus* markers in *Haliotis discus hannai*. This showed that microsatellites developed for one species can be used successfully in a closely related subspecies [54].

In a study conducted by Evans *et al.* [53] the loss of genetic variation at microsatellite loci in hatchery produced abalone in Australia, and South Africa was investigated. *Haliotis rubra*, an abalone species endemic to Australia and *Haliotis midae*, an abalone species endemic to South Africa, were used in the study. During this study a comparison of genetic variability within and between samples of cultured and wild *Haliotis midae* at three microsatellite loci was made. Five microsatellite loci were

used to compare the level of genetic variability which exists between cultured and wild stocks of *Haliotis rubra*. It was found that genetic differences between wild and hatchery stocks do exist. This was evidenced by the observed loss of rare alleles across most loci in all the hatchery stocks when compared to the wild stocks (35-62 %). This however was not accompanied by a decline in overall heterozygosity and it is suggested that this could be an indication of a short term bottleneck. This study demonstrates how highly polymorphic microsatellite DNA markers can be used to compare levels of genetic variability between wild and hatchery-reared stocks of commercially important abalone species [53].

In a more recent study conducted by Guitierrez-Gonzalez *et al.* [18] a genetic evaluation of the stock enhancement programs of the blue abalone *Haliotis fulgens* in Baja California, Mexico was conducted. The first part of their study was centred on estimating the genetic composition and diversity of hatchery-reared progeny by using microsatellite DNA markers. The next stage involved assessing the efficiency of these genetic tags in determining the hatchery origin of released organisms. The results generated during this study showed that the genetic diversity within the stock enhancement programs at two Baja California hatcheries was not compromised. The authors however caution that care should be taken to avoid a decrease in the genetic variability in hatchery populations [18].

1.8 Research objectives of this study

This study has two objectives. The first objective will be to determine the nature of the population diversity that exists within an abalone farm in order to gain

information which will help in the formulation of an "on-farm" breeding strategy. This breeding strategy will seek to maintain and possibly maximise genetic biodiversity of the farmed abalone. These abalone could then be used to restock the natural resource. The second goal will be to improve abalone mariculture by developing a directed breeding program that will allow informed crossing of broodstock to produce progeny that possess desirable traits such as increased growth rates and disease resistance.

There are two key questions that will be dealt with:

1.) What is the extent of the genetic diversity of farm-bred abalone?
2.) Is there a correlation between genetic populations and desirable traits?

The desirable trait that will be tested during this study will be growth rate. This study will seek to ascertain if it is possible to distinguish between fast and slow growing animals at the genetic level and if so, to what extent. This will be investigated using microsatellite DNA marker technology.

This study has been divided into three parts:

1.) The identification of *Haliotis midae* microsatellite loci that could be used to address the aims of this study.
2.) The optimised PCR conditions for amplification of each microsatellite locus will be determined.

3.) Genotype individuals using microsatellites with a high degree of length polymorphism. Statistical analysis of microsatellite data will be used to investigate the level of genetic diversity between phenotypically fast and slow growing individuals. The information gained will be used to determine whether abalone can be grouped according to commercially important phenotypic traits, with the eventual goal of assessing whether post-larval animals can be identified as potentially fast or slow growing animals.

Population genetic analysis software will be used to determine the extent of the genetic variation within and between fast and slow-growing animals. Outlined below are the genetic variability measures and tests that will be employed to achieve this objective.

1.8.1 *Genetic variability measures*

The level of genetic variation within and between the phenotypic groups will be evaluated during this study. Various genetic variability measures will be assessed. Allelic diversity which has been shown to be an effective measure of genetic diversity will be calculated for each phenotypic group at each locus. This will be done by determining the mean number of alleles per locus. The observed heterozygosity (H_0) and expected heterozygosity (H_e) values which are important indicators of the level of diversity will be calculated. These values will show the extent of the genetic variation which exists between the fast and slow growing animals.

1.8.2 *Hardy-Weinberg equilibrium*

In a large randomly breeding population, allelic frequencies will remain the same from generation to generation. This phenomenon is known as Hardy-Weinberg equilibrium. There are a few conditions which need to be met in order for Hardy-Weinberg equilibrium to be attained. In addition to being large and breeding randomly there needs to be no mutation, no migration and no selection [58,59,60].

Although in principle, the Hardy-Weinberg equilibrium is very simple it plays an important role in conservation and evolutionary genetics. It provides the basis for detecting deviations from random mating, testing for selection, modelling the effects of inbreeding and selection, and estimating the allele frequencies of loci showing dominance [59]. The four groups of abalone investigated during this study will be subjected to a Hardy-Weinberg equilibrium test to determine whether the population deviates or conforms to Hardy-Weinberg equilibrium. Deviations from Hardy-Weinberg equilibrium **(Equation 1)** were determined using chi-squared analysis [61] **(Equation 2)**.

$$p^2 + 2pq + q^2 = 1 \qquad \textbf{Equation 1}$$

$$X^2 = \Sigma(obs - exp)^2 / exp \qquad \textbf{Equation 2}$$

1.8.3 *Estimates of Population Differentiation*

Wright's F-statistics are widely used to characterize population genetic structure. These statistics allow the partitioning of genetic diversity within and among

populations. The F_{st} value, referred to as the fixation index which is used as a measure of genetic differentiation, will be determined during this study. The F_{st} value is used to determine the effect of population sub-division on inbreeding. It is the probability that two alleles drawn randomly from a population fragment are identical by descent [78].

Genetic distance measures will be calculated using the allele frequency data generated using the program GenAlEx [73]. The program Mega version 3.1 [88] will be used to plot neighbour-joining trees which will further assess the level of genetic variability which exists between the fast and the slow growing individuals within each spawning group.

Chapter 2

Materials and methods

2.1 Sampling techniques

Blood samples were obtained from farm-bred *Haliotis midae* in culture at Jacobsbaai abalone farm, South Africa. One to two millilitres (dependent on the size of the abalone) of blood was extracted from the pedal sinus of individual abalone from different grow-out tanks using a 2 ml syringe and 21 G (0.80 x 40 mm) needle (Promex). On the first abalone farm visit 100 individual animals were sampled and on the second abalone farm visit 120 individual animals were sampled (Table 1 and 2). The blood samples were stored on ice and transported back to the laboratory for DNA extraction. Blood samples spotted onto the Whatman FTA cards did not require storage on ice.

At Jacobsbaai abalone farm a colour coded classification system is used to distinguish between batches of abalone which are fast growing and those which are slow growing. Each abalone is graded individually by the farm managers on the basis of size and the growth data which is generated is used to classify the individual abalone as either fast or slow growing (Data Confidential). Thus it is known which tanks contain animals which are fast growing and which tanks contain animals that are slow growing. We were given this information and selected four tanks to sample. In each tank fast and slow growing animals were randomly selected for sampling.

The pink and white colour codes are used to identify fast growing individuals and the green and blue colour codes are used to identify the slow growing individuals (Table 1 and 2). The blood samples collected on the 17th November 2004 were used to carry out preliminary investigations and therefore only ten individuals were sampled from each tank (Table 1). After selection of the three loci to be used for genotyping, additional blood samples were collected on the 17th February 2005. This time only four tanks were sampled. Thirty individual abalone were sampled from each tank, so that in total, 120 individual abalone were sampled. Sixty of these had been classified as slow growing individuals and 60 of these had been classified as fast growing individuals by the farm managers based on growth data generated from the spawning date of each batch of animals (Table 2).

Table 1: *Haliotis midae* blood samples collected at Jacobsbaai abalone farm (17th November 2004)

Tank	Spawn Date	Growth phenotype	Samples
91D	5\00	Green[2]	10
78G	5\00	Pink[1]	10
79G	4\00	Blue[2]	10
93A	4\00	Pink[1]	10
29E	1\01	Green[2]	10
34F	1\01	Pink[1]	10
90G	4\01	Green[2]	10
96B	4\01	White[1]	10
88H	5\01	Green[2]	10
106A	5\01	Pink[1]	10

[1] fast grower, [2] slow grower

Table 2: *Haliotis midae* blood samples collected at Jacobsbaai abalone farm (17th February 2005)

Group	Tank	Spawn date	Colour code	No. of samples
1	29E	1\01	Green[1]	1 to 30
2	34F	1\01	Pink[2]	31 to 60
3	82E	9\00	Pink[2]	61 to 90
4	82E	9\00	Blue[1]	91 to 121*

[1]slow grower [2] fast grower, * Note: sample 120 was lost and so An additional sample (121) was taken but the number of animals sampled remained constant at 30.

2.2 DNA extraction

2.2.1 DNA extraction protocol I

DNA was isolated from individual blood samples using a modification of the DNA extraction procedure developed by Ausabel *et al* [69, 76]. The individual blood samples were centrifuged at 12 000 g for 5 minutes to pellet the haemocytes. The supernatant was discarded. One millilitre of ice-cold 1x phosphate buffered saline, pH 7.4, was added to the pelleted haemocytes. The haemocytes were re-suspended and re-centrifuged at 12 000 rpm for 5 minutes. The supernatant was again discarded. Haemocytes were re-suspended in 567 µl 0.5 M Tris-EDTA buffer, pH 7.6, 30 µl, 10 % SDS and 3 µl proteinase K (20 mg/ml). Samples were mixed thoroughly and

incubated at 37°C for 1 hour. One hundred microlitres of 5 M NaCl and 80 µl of CTAB/NaCl was added. A 10 minute incubation step at 65°C followed. The DNA was extracted with an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged for 5 minutes at 12 000 rpm. The aqueous phase was transferred to an eppendorf tube and the DNA precipitated with 0.6 volumes of isopropanol. The precipitate was washed with 70 % ethanol for 5 minutes at 12 000 rpm. The supernatant was discarded. The DNA pellet was air-dried and subsequently re-suspended in 50 µl Tris-EDTA buffer and 1µl RNase A (10 mg/ml).

2.2.2 DNA extraction protocol II

In addition to DNA extraction protocol I a new DNA isolation procedure was investigated. This DNA extraction protocol involved the use of Whatman FTA technology. In this protocol Whatman FTA cards are used to collect, archive, transport and isolate nucleic acids at room temperature. Each card contains four circles on which individual samples can be spotted (Fig. 4). One hundred microlitres of each individual blood sample was spotted onto each circle on the FTA card. Each FTA card was then allowed to air-dry for 1 hour. The cards were sealed in a foil pouch and transported back to the laboratory and stored in the dark at room temperature until such time as a polymerase chain reaction (PCR) reaction was to be conducted. A 1.2 mm Harris micro punch (Fig. 5) was used to punch the cards so as to acquire a 1.2 mm disk bearing abalone blood. Cross contamination was prevented by cleaning the punch between samples with ethanol. Each disk was washed thrice with FTA purification reagent (Whatman Biosciences). This reagent removes contaminants and the DNA immobilized on the washed disk can then be amplified by PCR. Each

disk was allowed to air-dry for 1 hour after washing, before the PCR was set up. Each sample disk was added to a PCR mix and the microsatellite sequences were amplified.

Fig. 4: The image above shows the Whatman FTA classic card that was used to collect and store blood samples [77].

Fig. 5: The Harris micro punch that was used to cut out a sample disk from the FTA card [77].

These cards are effective for this type of research because each card contains a chemical matrix which is able to lyse the cells, inactivate proteins and immobilize the genomic nucleic acids. The immobilized nucleic acids are trapped within the fibres of the card. After drying, the nucleic acids are protected from enzymatic, microbial, oxidative and free-radical degradation. The samples can be stored for years at room temperature before analysis.

2.3 Selection of microsatellite loci and primer design

The first stage of the project involved the screening of genetic databases for loci in *Haliotis* species which contained microsatellite repeats. The search yielded ten loci that included a variety of microsatellite repeats. The PCR primer sequences for two of these loci had been published, while specific primers were designed and tested for the other eight loci. The program DNAman version 4.13 [82] was used to design primers specific to each locus.

2.4 PCR amplification of microsatellite loci

PCR optimisation studies were carried out on the ten microsatellite loci. During these optimisation studies varying concentrations of $MgCl_2$, *H. midae* genomic DNA template and a range of annealing temperatures were investigated. Three microsatellite markers, namely CmrHr 2.29 [53], HmD11 and HmD61 [6], were optimised for use in *H. midae* using an Applied Biosystems 2720 thermal cycler. Amplification conditions were modified from Evans *et al.* [53] and consisted of an initial denaturation step at 94°C for 3 min, an annealing step at 53°C for 30 secs and an extension step at 72°C for 1 min. This was followed by 30 cycles of denaturation at 94°C for 30 secs, annealing at 53°C for 30 secs and extension at 72°C for 10 min. Cycling was followed by a 10 min extension step at 72°C. Reactions were performed in 10 µl volumes consisting of approximately 24 ng of genomic template DNA, 200 µM of each dNTP, 23.4 picomoles of each oligonucleotide primer, 2.5 mM $MgCl_2$

and 0.75 units of Taq DNA polymerase. The reactions were made up to volume with distilled water.

2.5 Radioactive labelling of microsatellites

A radioactive labelling protocol by Vos *et al* [72] was used. Forward primers were radioactively labelled at the 5' end with γ^{32}P ATP (18 μCi/μl). Labelling reactions were set up in a total volume of 25 μl with 1x T4 polynucleotide kinase buffer, 3 units of T4 polynucleotide kinase, 5 μl of γ^{32}P ATP (18 μCi/μl), 100 ng of forward primer (100 ng/μl) and nuclease free water made up to volume. The labelling reaction was incubated at 37 ^{O}C for 30 minutes. The reaction was stopped at 65 ^{O}C for 5 minutes. Unincorporated nucleotides were removed by adding an equal volume of 4 M ammonium acetate. The reaction mix was mixed well, centrifuged briefly and 10 volumes of 100 % ethanol was added. The reaction mix was mixed, centrifuged briefly and incubated at 20 ^{O}C for 30 minutes. The reaction mix was centrifuged for 15 minutes and the supernatant was carefully removed. The pellet was resuspended in 25 μl of nuclease-free water. The counts of the labelled primer was determined by adding 1 μl of labelled primer to 2 μl of scintillation fluid. The labelling reaction was deemed to be successful if the counts were above 10^5 dpm/μl. PCR reactions were set up in 10 μl volumes with 200 μm dNTP, 60-100 ng of DNA template, 2.5 mM $MgCl_2$, 1x Taq buffer, 0..25 units of Taq DNA Polymerase and equal concentrations of forward and reverse primers. The primer concentrations used to amplify the different loci varied, ranging from 53 pmol/μl to 62 pmol/μl. Amplification conditions were modified from Evans *et al* [53] and consisted of an initial denaturation

step at 94°C for 3 min, an annealing step at 53°C for 30 sec and an extension step at 72°C for 1 min. This was followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 53°C for 30 sec and extension at 72°C for 10 min. Cycling was followed by a 10 min extension step at 72°C.

2.6 Gel Electrophoresis

2.6.1 Vertical polyacrylamide gel electrophoresis

The method employed by Vos *et al* [72] was adapted as follows. Gels were prepared in 1x Tris-Borate EDTA (TBE) from a stock acrylamide solution containing 40 % acrylamide/1.5 % bisacrylamide to generate a 6 % polyacrylamide gel. Two hundred microlitres of 10 % ammonium persulphate (APS) and 50 µl N,N,N',N'-tetramethylenediamine (TEMED) was added to the gel mix. The gel was mixed and dispensed between two glass plates (45 cm x 30 cm) using a 50 ml syringe. The gel was allowed to polymerize for 3 hours. Two microlitres of tracking dye was added to the PCR samples. The tracking dye was made up in a volume of 10 ml and consisted of 9.6 ml of formamide, 500 µl of 0.2M EDTA and 100 µl of dye (50 mg xylene cyanol, 50 mg bromophenol blue, made up to 1 ml with distilled water). The gel was run at 30 milliamps for about 3 hours. Following completion of the gel run the gel was removed from the gel apparatus. The gel was picked up by using a Whatman 3 MM sheet of paper with the same dimensions as the gel and covered with cling wrap. The Whatman sheet was marked clearly so that the orientation of the gel was known. The gel was dried for 30 minutes on a gel drier with the gel facing up. The

PCR products prepared by the radioactive-labelling protocol were then visualised by autoradiography. This involved the exposure of each gel to X-ray film overnight (approximately 15 hours). If this exposure time was too long, then the gel was exposed to a new sheet of X-ray film for a shorter time period (approximately 5 hours). Each X-ray film was developed manually using AGFA developer and fixer solutions. The developer solution was made up of 2 litres of distilled water and 500 ml of developing solution (G118). The fixer solution (G334i Part A and B) was made up of 1.4 litres of distilled water, 500 ml of fixer solution A and 100 ml of fixer solution B. During the development process the X-ray film was submerged in the developing solution for a few minutes until the bands became visible. The X-ray was then rinsed in distilled water after which it was submerged in the fixer solution for three minutes. The X-ray film was rinsed again and left to air dry.

2.6.2 Horizontal polyacrylamide gel electrophoresis

Gels were prepared in 1x Tris-Borate EDTA (TBE) from a stock acrylamide solution containing 40 % acrylamide/1.5 % bisacrylamide to generate a 6 % polyacrylamide gel. Four millilitres of 10 % ammonium persulphate (APS) (less than one week old) and 340 µl N,N,N',N'-tetramethylenediamine (TEMED) were added to initiate polymerization. The gel was swirled to mix in the TEMED and poured within 10 sec of the addition of TEMED. Filtering or degassing the solution before pouring was not necessary. The dimensions of the gel was 19.7 cm x 15.8 cm. The gel was allowed to polymerize overnight at room temperature, and the comb was then removed. Ten microlitres of each PCR reaction was added to 2 µl of bromophenol blue tracking dye.

The running voltage was maintained at 150 V. Gels were run in the absence of ethidium bromide.

The Ethidium bromide (EtBr) staining protocol used to visualise the microsatellites was developed by Bellomy and Record [70]. The gel was stained in 0.5 μg/ml EtBr for one hour and then destained in distilled water for 3 hours. A ϕX174 DNA/Hinf I dephosphorylated marker (Promega) was used as the standard. The concentration of the marker was 50 ng/μl and 2 μl of marker was loaded onto each gel. DNA fragments were visualised on a 254 nm wavelength transilluminator. Band sizes were determined using the DNA FRAG computer program [71].

2.7 Population genetics data analysis

The Jacobsbaai abalone farm operates a rotational mating system with their broodstock. Each broodstock abalone tank contains between 6 and 9 animals per tank. A single spawning event involves the use of 8 tanks of which 4 contain males and 4 contain females. The tanks are rotated so that every 8 weeks the same broodstock pool of animals is used. There are currently 277 broodstock animals in use on the Jacobsbaai abalone farm. One hundred and fifty one of these have originated from the wild and 126 have originated from the farm. In this study we were working with individuals which arose from two separate spawning events. It was thus decided to first analyse the 120 sampled individuals as a whole and then each spawning batch was analysed separately as the information obtained could be different due to the fact that different broodstock parents were used.

Arlequin version 3 [93] was employed to calculate the mean allele number, allele frequencies, observed and expected heterozygosity (H_o and H_e) values for all three loci. The inbreeding estimate of Weir and Cockerham [75], F_{is} was calculated using GENEPOP version 1.2 [94] and departure from Hardy-Weinberg equilibrium was calculated using the Arlequin program. Deviations from Hardy-Weinberg equilibrium (Equation 1) were determined using chi-squared analysis [74] (Equation 2).

Equation 1 $\mathbf{P^2 + 2pq + q^2 = 1}$

Equation 2 $\boldsymbol{X^2 = \Sigma(obs - exp)^2 / exp}$

F_{st} [75] and R_{st} [95] values were calculated using the Arlequin program. It was used to calculate pairwise group comparison values for Wright's fixation index [75] and R_{st} [95]. R_{st} is an analogue of F_{st} that utilises a stepwise mutation model, which is thought to better describe the mutational process of microsatellites. These values were tested for significant departure from zero using a random permutations procedure (10 000 permutations).

Linkage disequilibrium between all pairs of loci was assessed using GENEPOP version 1.2 [94]. The significance of departure from equilibrium levels was determined using a Markov chain procedure with dememorization of 1000, 1000 batches and 10 000 iterations.

Genetic distances were calculated using the genetic distance co-dominant genotypic option in the GenAlEx program [73]. A pairwise individual by individual genetic distance matrix representing the differences between all alleles was generated for each

individual locus and the three loci combined [17]. Neighbour-joining trees [14] were constructed from the genetic distance data for each spawning group using Mega version 3.1 [88] to determine whether the fast and slow growing individuals within each spawning group could be differentiated from each other.

Chapter 3

Results

3.1 Identification of microsatellite loci

The first stage of this study involved the identification of microsatellite loci that could be used to differentiate between fast and slow growing individuals. The National Centre for Biotechnology Information (NCBI) site was screened for microsatellite loci that were found to occur in *Haliotis* species. Ten loci were selected which included a variety of microsatellite repeats (Table 3). Four of these loci had been isolated previously from *Haliotis rubra* and the remaining 6 loci had been isolated from *Haliotis midae*. The Polymerase Chain Reaction (PCR) primer sequences for two of these loci (CmrHr 2.30 and CmrHr 2.36) were known. Specific primer sequences were designed and tested for the other eight loci employed in this study.

3.2 Optimisation of PCR conditions

The second stage of this study involved optimisation of the PCR reaction conditions for the ten selected loci. Genomic DNA used as a template for these PCR reactions was isolated from blood samples taken from individual abalone using a standard genomic DNA isolation procedure. A number of different parameters were investigated. The effect of changes in the magnesium chloride concentration was

examined by carrying out magnesium titration experiments. A magnesium chloride concentration of 2.5 mM was found to be optimum for amplification of each locus.

Table 3: Summary of the ten microsatellite loci found to occur in *Haliotis* species. The PCR primer sequences are shown as well as the expected size of the PCR product

Species	Locus	GenBank accession number	Primer Sequence (5'-3') (F-Forward, R-Reverse)	Reference	Published expected size (bp)
Haliotis rubra	CmrHr 2.23	AF302832	F- TGGAAGCTTTTCAAACATTGG R-TACAATGGGATTAAGAAGC	Evans *et al.* [53]	243,253*
Haliotis rubra	CmrHr 2.29	AF302834	F-TGATTGGTGTGTGAGGTGAAA R-CCGATGCCCTTATCATCACT	Evans *et al.* [53]	426-468*
Haliotis rubra	CmrHr 2.30	AF194959	F-TTGGCAGTGATGGAAACTTG R-TTCCAAACTGACACAGACGC	Evans *et al.* [47]	284-328*
Haliotis rubra	CmrHr 2.36	AF194960	F-CACCCTTTGGCATGAAAGAT R-ACCAACAGGGGCAGATACAG	Evans *et al.* [47]	83-121*
Haliotis midae	HmD11	AY303341	F-CTGCATTTGACGTCTCCC R-ACAGGGAATACAGGGTCG	Bester *et al.* [6]	-
Haliotis midae	HmD33	AY303339	F-TGTGGATGTGTCGATTTCC R-AAAGCCCATTTCTGGTGG	Bester *et al.* [6]	-
Haliotis midae	HmD44	AY303334	F-TATTTGAAGGCACGGTGG R-AGGTAGCCCAGTGTATATTGC	Bester *et al.* [6]	-
Haliotis midae	HmD61	AY303338	F-TCGTACAACTCGAGCAATG R-CAGCAACCCTTACAGCCT	Bester *et al.* [6]	-
Haliotis midae	$HmSp_3$	AY303343	F-CTCGCGTGTGTAATTATGG R-TTATTTACGGGTTGATCAGG	Bester *et al.* [6]	-
Haliotis midae	$HmSp_6$	AY303345	F-AGGTTTGGTTGCACGACG R-TGTGATGCCCATTTCTGG	Bester *et al.* [6]	-

* These are published expected ranges; - indicates that at the commencement of this study there were no expected size ranges published for these loci.

DNA titration experiments showed that a DNA concentration between 40 ng and 60 ng was adequate to yield sufficient PCR product. The optimal primer concentrations were found to differ slightly for each primer set.

For the third stage of this study, samples of between 10 and 20 abalone were screened to determine the level of polymorphism at specific loci using the optimised PCR conditions determined during stage two. The preceding experiments were performed by separating the PCR products by electrophoresis through a 2 % TBE agarose gel and staining with ethidium bromide (EtBr) in order to visualise the PCR products on a 254 nm wavelength UV-transilluminator. However, a more sensitive electrophoresis method was required to detect base pair differences in allele sizes between individuals in order to determine the level of polymorphism of each locus. A radioactive labelling protocol in which ^{32}P-labelled primers were used was investigated. PCR products generated using ^{32}P-labelled primers were characterised following separation by denaturing polyacrylamide gel electrophoresis and subsequent autoradiography. Although this is the method of choice for detection of microsatellites, the technique proved to be fraught with technical problems and was very time-consuming, especially when large numbers of samples needed to be screened. Thus, horizontal polyacrylamide gel electrophoresis was adopted for screening microsatellites [72]. It is a simple technique which is as sensitive as traditional methods, but is less finicky and results are available within one day.

Of the ten selected loci, only four produced results with alleles that could be reliably scored. These were loci CmrHr 2.29, HmD11, HmD61 and CmrHr 2.30. The expected size ranges as determined during this study for these loci were as follows: alleles

detected at the CmrHr 2.29 locus had a size range of 340-580 base pairs (bp), alleles detected at the CmrHr 2.30 locus had a size range of 141-155 bp, alleles detected at the HmD11 locus had a size range of 239-359 bp and alleles detected at the HmD61 locus had a size range of 487-561 bp. Although the CmrHr 2.30 locus was initially identified as a potential microsatellite marker it was not employed in this study as the results obtained were not reproducible. This, combined with the fact that the difference in the allele size range determined during this study for CmrHr 2.30 in *Haliotis midae* differed markedly from the size range reported in *Haliotis rubra* (Table 3), led to the rejection of this locus. Thus, three loci, CmrHr 2.29, HmD11 and HmD61 were selected for further use in this study as they exhibited high levels of polymorphism with 12, 8 and 10 alleles being detected, respectively, at each locus.

3.3 Population Genetic Analysis

3.3.1 Genetic variability

3.3.1.1 Allelic variation

As mentioned in the methods the Jacobsbaai abalone farm operates a rotational mating system with their broodstock. In this study we were working with individuals which arose from two separate spawning events. It was thus decided to first analyse the 120 sampled individuals as a whole and then each spawning batch was analysed separately as the information obtained could be different due to the fact that different broodstock parents were used. The two spawning events will be referred to as spawning event one (SPG 1) and spawning event two (SPG 2). Four abalone groups

were selected for this study. Groups one and two originated from spawning event one and groups three and four were from spawning event two. Animals were selected from each spawning event on the basis of growth rate. Groups one and four comprised the slow growing individuals and groups two and three comprised the fast growing individuals. The groups were based on size measurements generated during grading of the animals by the farm managers (Table 2). Thirty individuals were sampled from each group.

Allelic diversity (i.e. the number of alleles displayed per locus) is a useful measure of genetic variability within a group, provided comparisons are of samples of similar size [9]. The genetic diversity indicators for analysis of all 120 sampled individuals are displayed in Table 4. The results generated from analysis of these samples were used as an indication of the extent of the genetic diversity which is present on the Jacobsbaai abalone farm. It can be seen that high levels of polymorphism were detected at each locus. Thirty one, fifty nine and twenty seven alleles were detected at the HmD61, CmrHr 2.29 and HmD11 loci respectively.

Table 4: Allelic variation, Observed heterozygosity (H_0), Expected heterozygosity (H_e) and F_{is} values for the 120 samples taken on the Jacobsbaai Abalone Farm (JAF).

		No. of samples	No. of alleles	Observed heterozygosity (H_0)	Expected heterozygosity (H_e)	F_{is}
JAF samples	**HmD61**	120	31	0.17*	0.95	0.82
	CmrHr 2.29	120	59	0.44*	0.97	0.55
	HmD11	120	27	0.36*	0.93	0.61

* signifinificant deviation from Hardy-Weinberg equilibrium

The observed heterozygosity levels (H_0) ranged from 0.17 at the HmD61 locus to 0.44 at the CmrHr 2.29 locus. Significant deviation ($P<0.001$) from Hardy-Weinberg equilibrium was detected at each locus. The inbreeding/non-random mating coefficient F_{is} measures the reduction in heterozygosity within each population. F_{is} values ranged from 0.55 at the CmrHr 2.29 locus to 0.82 at the HmD61 locus.

The genetic data for each individual spawning group was compared in Table 5. It was decided that the genetic diversity estimates across all three loci for the two spawning groups would be compared as this provides a more accurate measure of the genetic diversity which is present within each spawning group. Both SPG 1 and SPG 2 had a mean allele number of 19. The H_0 values were low with a mean value of 0.27 and 0.37 being determined for SPG 1 and SPG 2 respectively. The fixation index (F) is calculated for each locus to determine the level of inbreeding which exists within each spawning group. It differs from the F_{is} values as the values displayed in Table 5 are averaged for the two groups generated from each spawning event. The levels of

inbreeding were high for each group with SPG 1 having a value of 0.72 and SPG 2 having a value of 0.74.

Table 5: Allelic variation, Observed heterozygosity(H_0), Expected heterozygosity (H_e) and F coefficient values for each spawning group on the Jacobsbaai abalone farm.

Group	No. of samples	Mean no. of Alleles	Mean observed heterozygosity (H_0)	Mean expected Heterozygosity (H_e)	Fixation index (F)
SPG 1	60	19	0.27	0.92	0.72
SPG 2	60	19	0.37	0.87	0.74

Note: SPG 1-Spawning group 1, SPG 2-Spawning group 2.

Each phenotypic group was analysed separately to determine whether a distinction could be drawn between those individuals which are slow growing and those which are fast. The mean number of alleles, observed (H_0) and expected heterozygosity (H_e) values for each phenotypic group are displayed in Table 6. It has already been determined that all three loci are polymorphic (Table 4) for the four study groups and thus genetic diversity estimates across all three loci were determined.

Table 6: Allelic variation, Observed heterozygosity (H_o) and Expected heterozygosity (H_e) at three loci for 4 abalone groups

Group	No. of samples	Mean no. of alleles	Mean observed heterozygosity (Ho)	Mean Expected heterozygosity (He)	F_{is}
Group 1 (slow)[a]	30	10	0.24*	0.84	0.73
Group 2 (fast)[a]	30	10	0.32*	0.90	0.70
Group 3 (fast)	30	15	0.37*	0.87	0.58
Group 4 (slow)	30	12	0.38*	0.82	0.58

[a] slow and fast refers to the growth rate of the abalone group
* significant differentiation from Hardy-Weinberg equilibrium $P< 0.05$

A mean allele number of 10 was determined for both group one and group two across the three loci. Group three and four had mean allele numbers of 15 and 12 respectively. The mean observed heterozygosity values were low for all four groups. Groups one and four which comprised slow growing individuals had values of 0.24 and 0.38 respectively. Groups two and three which comprised fast growing individuals had mean observed heterozygosity values of 0.32 and 0.37. The F_{is} value is an inbreeding coefficient which measures the reduction of heterozygosity within a population due to inbreeding. All the values were positive indicating that there is a deficit of heterozygotes in the four groups. The highest value was observed in group one (0.73) with a similar value of 0.70 being determined for group two. The F_{is} values for group three and four were the same with a value of 0.58 being determined. It can be seen that highest levels of inbreeding was observed in group one and two which originated from the same spawning group.

The Hardy-Weinberg equilibrium principle is an important concept in population genetics studies. Hardy –Weinberg equilibrium is attained when the allele frequencies

remain constant from one generation to the next. It is this constancy of the allele frequencies which serves to preserve the genetic variation within natural populations [78].

In this study the four abalone groups were found to deviate significantly from Hardy-Weinberg equilibrium across all the loci. This deviation is most likely due to the deficiency of heterozygotes within the groups as already evidenced by the low heterozygosity values.

3.3.1.2 Allele frequency

Daniel Hartl in his book 'A primer of population genetics' states that genetic variation can be quantified using the concept of allele frequency [58]. Allele frequency is defined as the proportion of all alleles of a gene that are of a specified type [78].

The allele frequency distributions at each microsatellite locus for each spawning batch are depicted in Appendix one. The allele frequency data for each spawning event was analysed separately. One dominant allele was detected at the HmD61 locus for the slow (group one) growing abalone group. Allele 523 occurred at a frequency of 0.265 in group one. Three dominant alleles were detected at the HmD61 locus for the fast (group two) growing abalone group. Alleles 509, 515 and 523 were all found to occur at a frequency of 0.174. Alleles 513 and 523 are shared by both the fast and the slow growing abalone groups. Allele 513 occurred at a higher frequency in group two and allele 525 occurred at a higher frequency in group one. Alleles 517, 527, 528, 531,

535, 537, 545, 553 and 541 were detected only in group one, while alleles 509, 515, 519, 521, 539, 547, 551 and 557 were detected only in group two.

Rare alleles are those that occur at frequencies of less than 5 % and are useful in parentage assignment studies. Two rare alleles (alleles 517 and 561) were detected in group one and three rare alleles (allele 539, 547 and 557) were detected in group two.

Group one (slow growers) has three dominant alleles at the CmrHr 2.29 locus and group two (fast growers) has one dominant allele. In group two, allele 500 occurred at a frequency of 0.167, while alleles 468, 510 and 540 occurred at a frequency of 0.094 in group one. Alleles 460, 468 and 476 are shared by both groups. Alleles 460 and 476 were found to occur at a higher frequency in the slow growing abalone group and allele 468 occurred at a slightly higher frequency in group two. Alleles 339, 472, 478, 484, 488, 493, 497, 498, 504, 510, 520, 532, 540, 546 and 572 were detected only in group one, while alleles 446, 452, 472, 480, 486, 496, 500, 506, 512, 514, 516, 522, 523 and 528 were detected only in group two.

Seven rare alleles (339, 488, 497, 498, 504, 546 and 572) were detected in group one and seven rare alleles (alleles 460, 468, 480, 486, 496, 516 and 522) were detected in group two.

Two dominant alleles were detected at the HmD11 locus in animals derived from spawning event one. Group one (slow growers) and group two (fast growers) possessed one dominant allele each. Allele 259 in group one had an allele frequency of 0.464, while in group two, allele 267 occurred at a frequency of 0.219. Alleles 255

263 are shared by both the slow and fast growing abalone groups. Both alleles occurred at a greater frequency in group one. Alleles 251 and 259 were detected only in slow growing abalone group one, while alleles 267, 271, 275, 279 and 283 were detected only in fast growing abalone group two.

One rare allele (allele 251) was found in group one and no rare alleles were detected in group two.

Two dominant alleles were found at the HmD61 locus for the individuals generated from spawning event two. In group three (fast growers), allele 499 occurred at a frequency of 0.205, while allele 491 found in group four (slow growers) occurred at a frequency of 0.313. Alleles 491, 515, 525 and 541 are shared by both the slow and the fast growing abalone groups. All these alleles were found to occur at a higher frequency in the slow growing abalone group. Alleles 487, 499, 509, 517, 519, 533, 535, 547 and 553 were detected only in group three, while alleles 495, 521 and 531 were found only in group four.

Nine rare alleles (alleles 491, 515, 519, 525, 529, 535, 541, 547 and 553) were detected in group three and one rare allele (allele 531) was detected in group four.

Two dominant alleles were found at the CmrHr 2.29 locus for groups three (fast growers) and four (slow growers). Allele 522 occurred in group three at a frequency of 0.115, while allele 516 occurred at frequency of 0.238 in group four. Alleles 470, 504, 512 and 522 are shared by both groups. Alleles 470 and 504 occur at a higher frequency in group four and alleles 512 and 522 occur at a higher frequency in group

three. Alleles 454, 464, 472, 474, 478, 482, 486, 490, 492, 510, 526, 528, 530, 532, 536, 540, 552, 564, 576 and 580 were detected only in group three. Alleles 440, 456, 462, 496, 498, 508 and 516 were detected only in group four.

Sixteen rare alleles (alleles 470, 472, 474, 478, 482, 486, 490, 492, 504, 512, 526, 530 532, 552, 576 and 580) were detected in group three and three rare alleles (496, 508 and 512) were detected in group four.

Two dominant alleles were detected at the HmD11 locus for groups three (fast growers) and four (slow growers). Allele 259 in group three occurred at a frequency of 0.421 and at a frequency of 0.400 in group four. Alleles 251, 255, 259 and 263 are shared by both groups. Allele 251 and 259 was found at a higher frequency in group three. Alleles 255 and 263 occurred at a much greater frequency in group four. Alleles 239, 252 and 256 are found only in group three, while alleles 243, 247 and 359 are found only in group four.

Four rare alleles (alleles 252, 256, 263 and 267) were detected in group three and four rare alleles (alleles 243, 247, 251 and 359) were detected in group four.

Private alleles were observed in all four groups. Private alleles are important because they play an important role in parentage assignment studies. The percentage of private alleles was similar across all four study groups. Group three had the highest percentage of private alleles (42 %) with a mean total of 6 private alleles being detected across the three loci. Groups one and two which originated from the same spawning event had the same amount of private alleles (37 %) and group four had the lowest number of private alleles (32 %).

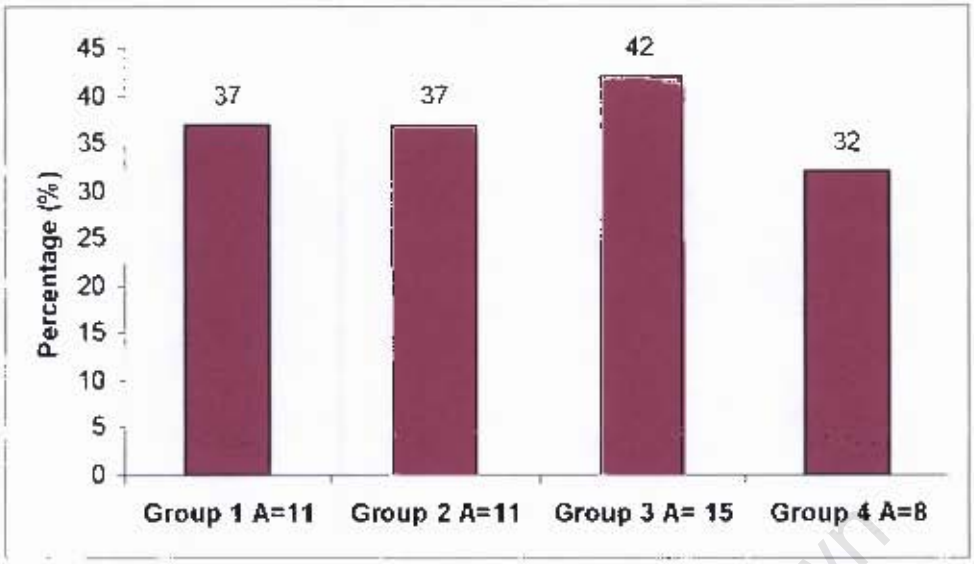


Fig 6: Mean values for private alleles detected at the three loci for the four abalone groups

3.3.2 Population differentiation

3.3.2.1 F_{st} comparisons and R_{st} comparisons

Wright has devised a system for determining the level of genetic differentiation based on F_{st} values [78]. Values in the range of 0 to 0.05 are considered to indicate little genetic differentiation. Values that occur in the 0.05 to 0.15 range are considered to indicate moderate genetic variation. Values in the 0.15 to 0.25 range are considered to indicate great genetic differentiation and F_{st} values of greater than 0.25 are considered to indicate very great genetic differentiation. A pairwise comparison of the four abalone groups using allele frequency data showed low F_{st} values for all population pairs (Table 7). Using Wright's system there appears to be a low to moderate amount

of genetic differentiation present between the four groups. The highest F_{st} value across the three loci was 0.091 calculated when comparing group two (fast growers) with group four (slow growers). The low F_{st} values generated for the four abalone groups shows that little variation exists between groups and that most of the variation exists within groups [78]. F_{st} significance values which are determined by bootstrapping over samples for each locus and jackknifing over all loci show that all pairwise group comparisons with the exception of groups one and three were found to be significantly different to each other.

Table 7: Pairwise group genetic differentiation F_{st} values for the four abalone groups (F_{st} (via Frequency) values below diagonal).

	Group 1[a]	Group 2[b]	Group 3[b]	Group 4[a]
Group 1[a]	0.00000			
Group 2[b]	0.078*	0.00000		
Group 3[b]	0.027	0.049*	0.00000	
Group 4[a]	0.082*	0.091*	0.071*	0.00000

[a] slow growers, [b] fast growers

Table 8: Pairwise group genetic differentiation R_{st} values for the four abalone groups

(Sum of squared difference estimates below diagonal)

	Group 1[a]	Group 2[b]	Group 3[b]	Group 4[a]
Group 1[a]	0.000			
Group 2[b]	-0.001	0.000		
Group 3[b]	0.108*	0.076	0.000	
Group 4[a]	0.162*	0.090*	0.076*	0.000

[a] slow growers, [b] fast growers

R_{st} is an analogue of F_{st} but it utilises a step-wise mutation model which is thought to better describe the mutational process of microsatellites and thus R_{st} values are thought to be a more accurate measure of population differentiation. The results determined here are different to those obtained using the F_{st} measurements. The R_{st} values for pairwise comparisons between groups one and three, groups one and four, groups two and four and groups three and four were determined to be significantly different to 0 which means that these groups can be genetically differentiated from each other. A non-significant value has been determined for groups two and three and this indicates that these cannot be genetically differentiated from each other. The negative value determined for group one and two indicates that the allelic size variation within the groups, were greater than between the two groups.

Linkage disequilibrium was assessed for the three loci and no significant departure from equilibrium levels was detected ($P< 0.05$) indicating that the three loci are not linked to each other.

3.3.2.2 Neighbour-joining trees

Genetic distance trees are commonly used in microsatellite studies to illustrate the differences which exist between different population groups. For the purposes of this study, genetic distances between the individual abalone were determined (Appendix one) and these genetic distances were used to determine whether a distinction can be drawn between the fast and the slow growing individuals.

Two neighbour-joining trees based on genetic distance data for all three loci were plotted for each spawning group. The genetic distance tree constructed for spawning event one is displayed in Fig. 7.

The genetic distances were combined for all three loci and a neighbour-joining tree constructed showing the relationship between individuals from SPG 1 (Fig. 7). It can be seen that when all the loci are included that there is a clear distinction between the fast and the slow growing individuals. There are two branches X and Y. Two clusters originate at node X, A and B and cluster C originates at node Y. Cluster A comprises slow growing individuals only and Cluster C comprises slow growing individuals with a single fast growing individual. Cluster B consists of a mixture of fast and slow growing individuals grouped in a number of sub-clusters.

The neighbour-joining tree plotted in Fig. 8 was generated from the individuals obtained from spawning event two. It combines the genetic distances calculated for each individual from SPG 2 at all three loci. Clustering between the fast and slow

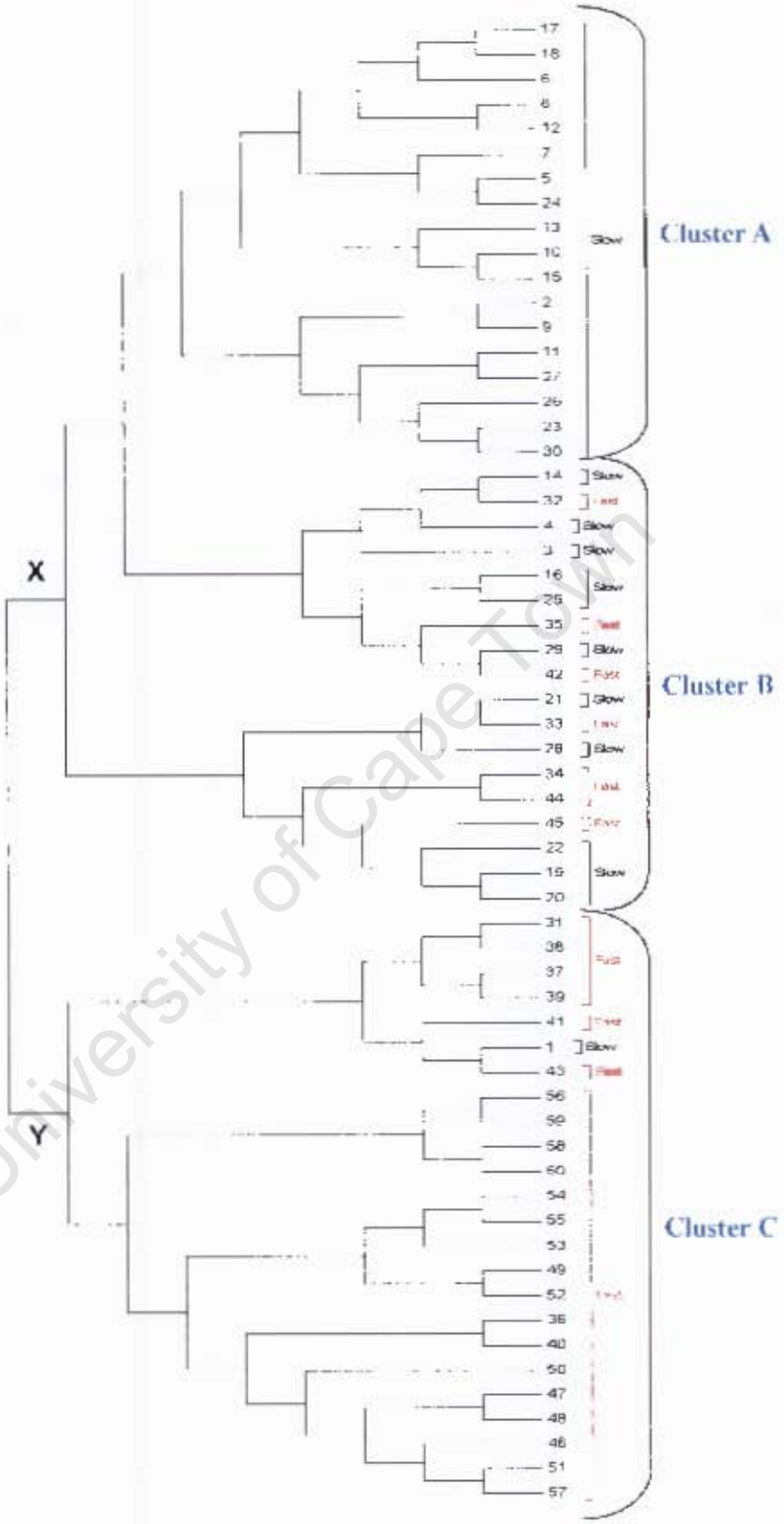


Fig 7 Neighbour-joining genetic distance tree for the individuals from SPG 1 across all three loci.

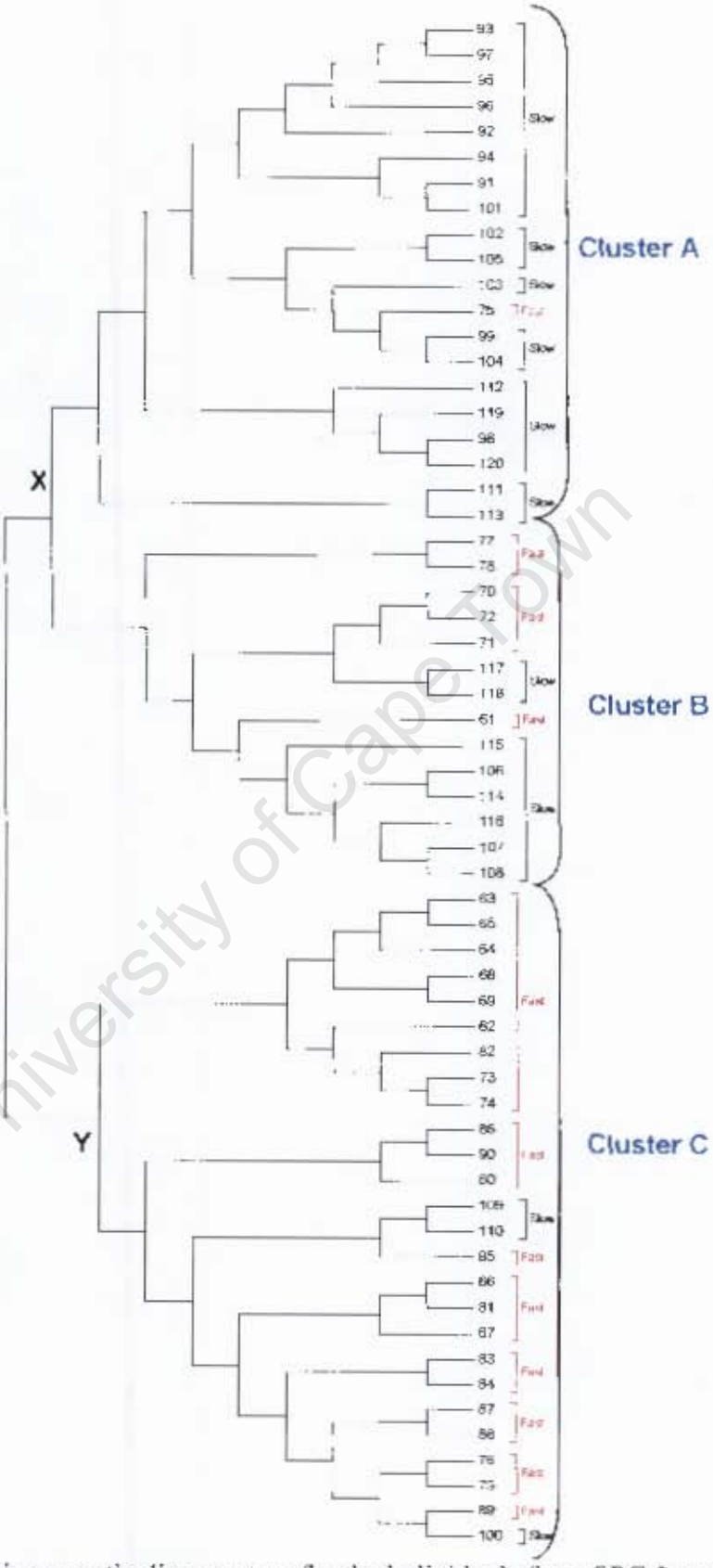


Fig 8. Neighbour-joining genetic distance tree for the individuals from SPG 2 across all three loci

growing individuals is more apparent. There are two major branch points X and Y. Clusters A and B originate at node X and Cluster C originates at node Y. Cluster A is mostly comprised of slow growing individuals with a single fast growing individual. Cluster B comprises 6 fast growing individuals and 8 slow growing individuals and cluster C branching off from node Y includes mostly fast growing individuals (14 fast growing individuals and 3 slow growing individuals). Although the division is not as clear as it was for spawning group one, differentiation between the fast and slow growing abalone from SPG 2 is indeed evident.

Chapter 4

Discussion

'A universal attribute of natural populations is that organisms within the population differ in phenotype with respect to many traits [78].' The aim of population genetics and therefore this study is to deal with this phenotypic diversity which is a consequence of differences which exist at the genetic level.

In Chapter 1 the objectives of this study were outlined. The first objective was to quantify the extent of the genetic diversity which exists on the Jacobsbaai abalone farm with respect to allelic diversity. This was approached in two parts. Firstly, the genetic diversity which exists between all 120 sampled individuals was investigated. Genetic data obtained would provide an indication of the extent of the genetic diversity which is present on the Jacobsbaai abalone farm. Secondly, the genetic diversity which exists within each spawning group was investigated to determine whether one spawning group is more genetically diverse than the other. The second objective was to determine whether a correlation could be drawn between genetic groups and desirable traits. Here, the different phenotypic groups were compared in order to determine whether the fast and slow growing individuals (phenotypic trait) could be differentiated from each other at the genetic level. The findings of this study, therefore, is of commercial importance as the ability to differentiate between animals which are fast and slow growing with the aid of microsatellite loci would allow for

selection of the fast growing animals, which in turn would increase turnover rates and revenues on abalone farms.

4.1 Genetic diversity on Jacobsbaai abalone farm

4.1.1 Allelic variation

The extent of the genetic diversity which exists on the Jacobsbaai abalone farm can be determined by looking at genetic diversity indicators. These include allele number, observed (H_o) and expected heterozygosity (H_e). These factors, together with the inbreeding coefficient (F) and Hardy-Weinberg equilibrium testing, were thus employed to determine the extent of the genetic diversity which is present between individuals on the Jacobsbaai abalone farm.

The three loci HmD11, HmD61 and CmrHr 2.29 were all polymorphic for the 120 individuals sampled (Table 4). A large number of alleles were detected amongst the 120 individuals at the three loci. Thirty one, fifty nine and twenty seven alleles were identified at the HmD61, CmrHr 2.29 and HmD11 loci respectively. The observed heterozygosity values were low with only 17.4 % of the individuals being heterozygous at the HmD61 locus, 43.9 % of the individuals at the CmrHr 2.29 locus and 34.9 % of the individuals at the HmD11 locus. The inbreeding coefficient F_{is} (which is a measure of inbreeding between individuals with respect to the whole population) at each locus is high with 81.6 %, 54.9 % and 61.2 % inbreeding/non-random mating being detected at the HmD61, CmrHr 2.29 and HmD11 loci

respectively. CmrHr 2.29, the most heterozygous locus, included the highest number of alleles and reflected the lowest percentage of inbreeding.

These results show that there has been a loss of heterozygosity on the Jacobsbaai abalone farm despite the high levels of polymorphism detected at each locus. Therefore the next step in this study was to determine whether each spawning group exhibited this trait or whether one spawning group was more genetically diverse than the other. This is important because, as mentioned previously, between 48 and 72 broodstock animals are used for each spawning event. Therefore a more diverse group could have been generated from a larger-sized pool of broodstock parents.

The allelic data for spawning group one (SPG 1) and spawning group two (SPG 2) are displayed in Table 5. Each spawning group consisted of 60 individuals and high levels of polymorphism were detected with a mean allele number of 19 being determined for each spawning group. It can be seen that the heterozygosity levels are low in both spawning groups and this concurs with what was found for all 120 individuals. Both SPG 1 and SPG 2 have high levels of inbreeding as evidenced by the high inbreeding values. It is interesting to note that despite SPG 2 having an increased amount of heterozygotes the level of inbreeding is much the same as in SPG 1.

It can be clearly seen that overall, heterozygosity levels in our study are low, indicating that there is an obvious heterozygote depression on the Jacobsbaai abalone farm. This is to be expected as this is a closed farm population where broodstock numbers are small and migration is kept to a minimum. Heterozygote depression is a common occurrence in inbred groups and is expected to appear at multiple, if not all,

loci [83]. It is therefore important to determine whether this homozygote excess is indeed due to the occurrence of inbreeding between the broodstock animals as this has been shown to decrease genetic diversity. Consequently Hardy-Weinberg equilibrium testing was undertaken to confirm this.

Hardy-Weinberg equilibrium is a common concept used in population genetic studies. A population is said to be in Hardy-Weinberg equilibrium when allele frequencies remain constant from one generation to the next. This constancy of the allele frequencies implies that when a population is large with no migration or mutation and natural selection does not affect the alleles under consideration, then the mechanism of Mendelian inheritance will keep the allele frequencies constant and this will serve to preserve genetic variation [58].

In this study the Hardy-Weinberg equilibrium, together with F coefficient estimates, provided little evidence of random mating occurring within the four groups. Significant deviation from Hardy-Weinberg equilibrium was detected in all four groups across the three loci due to a deficit of heterozygotes [2]. This would suggest that genetic variation is not being maintained on the farm. This result was not unexpected as there are a number of requirements that need to be fulfilled before Hardy-Weinberg equilibrium can be achieved. The population needs to be free of selection, mutation, random mating needs to occur and genotypic proportions should be constant from generation to generation [58, 59, 78].

There could be a number of reasons for this significant deviation from Hardy-Weinberg equilibrium. The deviation from Hardy-Weinberg equilibrium observed in

this study is most likely due to the occurrence of inbreeding which could have occurred due to poor broodstock management and the utilization of a small broodstock pool. Indeed, inbreeding can occur at the Jacobsbaai abalone farm as 126 of the broodstock animals have originated from the farm itself.

While the deviation from Hardy-Weinberg equilibrium has been attributed to the occurrence of inbreeding on the Jacobsbaai abalone farm, it would be useful to carry out testing with a larger sample size [2]. A larger sample size reflects genotype frequencies more accurately and minimizes bias and sampling variance effects on variability and genetic distance estimates [84]. It should be noted that an increased sample size might not change results but instead might serve to confirm what has already been found.

4.1.2 Population differentiation

4.1.2.1 F_{st} and R_{st} comparisons

The inbreeding coefficient F is used to quantify the amount of inbreeding present within a population. The F coefficient is a probability value which ranges from 0 to 1 in individuals [60]. Assumptions about inbreeding and/or non-random mating in a group can be made if the majority of the loci give consistent results across the group [81]. During this study the F_{st} values were found to be similar across the four abalone groups, indicating that despite the inbreeding that is occurring amongst broodstock animals on the Jacobsbaai abalone farm, there is a low to moderate level of genetic differentiation (according to Wright's classification scale) across the three

loci tested. The F_{st} values also show that all the groups can be significantly differentiated from each other with the exception of groups one and three. This is unexpected as these groups have originated from separate spawning and thus one would expect that they could be genetically differentiated from each other. Thus Rst testing was undertaken as R_{st} values have been shown to be a more accurate indication genetic differentiation when the use of microsatellite data is employed. The estimates here are different to those determined for the F_{st} values and these results have determined that groups one and two cannot be genetically differentiated from each other and groups two and three cannot be differentiated from each other. The fact that group one and two cannot be differentiated from each other is not surprising as they have originated from the same spawning event. However, these results have to be interpreted with caution as there is no indication that groups three and four are similar and they have originated from the same spawning event too. Therefore no conclusions can be drawn about whether it is possible to differentiate between those individuals which are fast growing and those which are slow growing and differentiation cannot be made on the basis of spawning groups either. Therefore this data just indicates that genetic differentiation does exist on the Jacoobsbaai abalone farm and thus it was possible to proceed with the study.

It has been shown that when measures are taken to avoid inbreeding as part of a breeding program, genetic variation need not be reduced despite a small effective population size [89]. This may be achieved by obtaining pedigree information on all individuals in the breeding program and using this information to arrange matings that will ensure only minimum increases in non-random mating in every generation [9]. It has been shown previously that although breeding methods inevitably influence the

genetic variability of any farmed population, a rotational mating selection method has helped maintain the stock's genetic diversity by minimizing non-random mating [2]. Institution of these practices on the Jacobsbaai abalone farm would serve to decrease the level of inbreeding which is occurring which would in turn increase the amount of heterozygotes and contribute to making the various abalone groups more genetically variable.

In conclusion, the first objective of my study was successfully completed and the genetic diversity of the animals sampled from the Jacobsbaai abalone farm has been quantified. It was observed that there has been a loss of heterozygosity on the Jacobsbaai abalone farm despite the high allelic diversity observed as evidenced by the low observed heterozygosity values. Additionally, the mean observed heterozygosity values determined for the individuals generated from spawning event one show that these individuals are 14 % less heterozygous than the individuals generated from spawning event two. This loss of heterozygosity has been attributed to inbreeding which is occurring between the broodstock at the Jacobsbaai abalone farm as evidenced by the Hardy-Weinberg testing and the F coefficient values.

4.2 Genetic population groups and phenotypic traits

The second objective of this study was to determine whether a correlation could be drawn between genetic population groups and desirable traits. As mentioned previously, the desirable trait investigated during this study was growth rate, and as such, we wanted to determine whether the four abalone groups could be genetically differentiated from each other with respect to growth rate.

4.2.1 Allelic variation

It has been shown previously in a study conducted by Norris *et al* [9] on hatchery salmon and farmed rainbow trout that allelic diversity is a more sensitive measure of differences in genetic variation between wild and farmed populations than overall heterozygosity. In this study the three loci, HmD11, HmD61 and CmrHr 2.29 were all polymorphic for the four abalone groups. The mean allele number ranged from 10 in groups one and two to 15 in group three for the three loci tested.

The level of allelic variation between the fast and slow growing abalone groups which were composed of individuals generated from spawning event one were compared in order to determine how different these phenotypic groups are. Group one and two had had an identical mean allele number of 10. The H_0 values for both groups were low with group two (fast growers) having a slightly higher value. As both group possess the same number of alleles it is interesting that the fast growing abalone group has a higher level of heterozygosity.

In summary, it is clear that there is no obvious difference between the fast and slow growing groups generated from spawning event one with respect to allelic diversity. A difference in H_0 was observed with the fast growing abalone group having a higher value than the slow growing abalone group. However, it can not be stated conclusively that a more heterozygous group is able to attain a faster growth rate as there is not enough evidence to confirm this at this stage.

The level of allelic diversity which exists between the fast and slow growing abalone groups which comprised individuals generated from spawning event two will now be discussed.

The mean allele number of 15 was higher for the two groups generated from spawning event two than for the individuals from spawning event one. Group three (fast growers) had an allele number of 15 which was higher than the 12 alleles possessed by group four (slow growers). The H_0 values were almost identical for group three and group four.

It can be seen that the results obtained for groups three and four do not allow one to distinguish between the fast and slow growing abalone groups on the basis of allelic diversity at the HmD11 and HmD61 loci. However, the fast growing abalone group possesses twice as many alleles at the CmrHr 2.29 locus as the slow growing abalone group.

In terms of allelic diversity therefore, the fast and slow growing abalone from either spawning group cannot be differentiated from each other. This does not mean that the levels of allelic diversity are low. On the contrary, all four abalone groups possessed a high number of alleles at the three loci tested. However the data obtained does not allow one to draw any conclusions about whether the fast and slow growing abalone groups can be differentiated from each other.

In order to determine whether the values obtained in this study are similar to those obtained in other studies of a similar kind, our results were compared to those

obtained in a study conducted by Bester et al [6]. This is the only study to date that has characterized genetic information for the HmD11 and HmD61 loci, two of the loci that were used during this study. In their study, they successfully isolated and characterized 11 microsatellite loci in the South African abalone *Haliotis midae*. Allele numbers of 31 and 27 for the HmD61 and HmD11 loci, respectively are substantially larger than the value of 11 and 5 calculated for the HmD61 and HmD11 loci in the Bester *et al*. study[6]. This, however, was not unexpected as a much larger sampling group was used in our study.

The mean observed heterozygosity value of 0.36, determined for locus HmD11 in our study, is similar to the H_0 value of 0.32 reported by Bester *et al* [6]. However, the mean observed heterozygosity value for locus HmD61 deviated from the value obtained by Bester *et al*, as the mean H_0 value of 0.17 obtained during this study was markedly lower than their value of 0.61.

There are two possible reasons for the differences observed between the two studies. During this study 120 individual abalone samples were analysed as opposed to the 28 samples analysed by Bester *et al*. [6]. Therefore, the larger sampling size might account for the differences in allele number and heterozygosity. Secondly, the abalone investigated in the Bester *et al*. [6] study originated from a different farm to the abalone characterized in this study and consequently, the genetic information obtained could be different due to the use of different broodstock animals on the two farms.

The CmrHr 2.29 locus was isolated and characterized from the Australian abalone, *Haliotis rubra* [47]. In a subsequent study, Evans *et al* [53] investigated the loss of

genetic variation at microsatellite loci in hatchery produced abalone. Testing was performed on both the Australian abalone *Haliotis rubra* and the South African abalone *Haliotis midae*. Testing was carried out on four farms producing *H. midae*. A mean observed heterozygosity value of 0.64 was calculated for this locus by Evans *et al* [55] compared to the value of 0.44 determined in our study. The discrepancy between the data obtained in the two studies could be due to Evans *et al* [55] investigating the genetic diversity which exists between abalone groups from 4 different farms while we investigated the genetic diversity which is present between abalone groups on one abalone farm.

4.2.2 Allele frequency

Allele frequency distributions have been shown to be useful in population genetic studies as they can be used to draw distinctions between different population groups. Norris *et al.* [9] conducted a study in which the microsatellite genetic variation between and within farmed and wild Atlantic salmon (*Salmo salar*) populations was investigated. They showed that wild and farmed Atlantic salmon could be differentiated from each other by using allele frequency distributions. A common trend that was found was that alleles that occurred at high frequencies in the wild populations were not detected in the farmed populations. In another study Gutierrez-Gonzalez *et al* [18] showed that allele frequency distributions could be used to determine whether differences existed between the offspring and the broodstock of the blue abalone *Haliotis fulgens*. They showed that alleles that occurred at low frequencies in the broodstock were lost to subsequent generations and therefore not found in the offspring. Therefore, in this study it was hoped that allele frequency data

could be used to draw distinctions between the fast and slow growing abalone groups. It was hoped that specific alleles would be found in the fast growing groups or that these alleles, if found in the slow growing abalone groups, would occur at a much lower frequency in the latter group. In other words the fast growing abalone groups were expected to possess a greater pool of alleles. A larger number of alleles would increase genetic variability which has been shown to directly improve the fitness of an individual [86, 87]. Thus, alleles found at high frequencies in the fast growing groups may be associated with the faster growth rate.

The allele frequency data for each spawning group was analysed and no meaningful deductions could be made which would allow one to differentiate between the fast and slow growing abalone. Groups one (slow growers) and two (fast growers) were generated from spawning event one and groups three (fast growers) and four (slow growers) were generated from spawning event two. Eight alleles which were unique to group one (slow growers) and Eight alleles unique to group two (fast growers) were identified at the HmD61 locus. Groups one and two shared two alleles at the HmD61 locus. Allele 513 was found at a higher frequency in group two and allele 523 was found at a much higher frequency in group one.

At the HmD11 locus both the shared alleles occurred at a greater frequency in group one (slow growers). Two unique alleles were identified in group one (slow growers) and five unique alleles were identified in group two (fast growers) at this locus.

Three alleles at the CmrHr 2.29 locus were shared by groups one and two. Alleles 460 and 468 occurred at a higher frequency in group one and allele 476 occurred at about

the same frequency in both groups Fifteen alleles which were unique to group one (slow growers) at this locus were identified and 12 alleles which were unique to group two were identified.

The results were equally variable for groups three and four which were generated from spawning event two. Nine alleles unique to group three (fast growers) and three alleles unique to group four (slow growers) were identified at the HmD61 locus. Four alleles were shared between groups three and four. They all occurred at a much higher frequency in group four (slow growers).

There were four shared alleles and they all occurred at a higher frequency in group four (slow growers) with the exception of allele 522 which occurred at a higher frequency in group three (fast growers). Nineteen unique alleles were identified in group three (fast growers) and seven unique alleles were identified in group four (slow growers) at this locus.

Threee unique alleles were identified in group three (fast growers) and three unique alleles were identified in group four (slow growers) at the HmD11 locus. Group three had higher frequency values for alleles 251, 259 and 263 at the HmD11 locus. It should also be noted that the increased frequency of allele 263 was markedly higher in group four.

While there are some clear differences in the allele frequency data for the fast and slow growing abalone groups, no definitive trend was observed and thus no significant conclusions can be drawn between fast and slow growing abalone within

each spawning batch. For the two groups that were generated from spawning event one the majority of the alleles that were shared at the HmD61, CmrHr 2.29 and HmD11 loci were found to occur at a higher frequency in the slow growing abalone group. Thus it could be postulated that these alleles could be somehow linked to genes which are involved in the determination of the growth rate within abalone and therefore abalone which possess these alleles have a slower growth rate. However, this has not been confirmed and has to be interpreted with caution until such time as this can be proved conclusively.

If we now look at groups three and four we can see that at the HmD61 locus the shared alleles occurred at higher frequencies in the slow growing abalone group. At the CmrHr 2.29 the results loci were similar to those obtained for spawning event one with majority of the alleles occurring at a higher frequency in the slow growing abalone group. There was an even spread of allele frequencies at the HmD11 locus for each group.

The allele frequency data obtained for the four groups therefore does not allow one to distinguish between fast and slow growing abalone within each spawning group. It should however be noted that very few of the alleles were shared between the fast and slow growing individuals of each spawning event despite the fact that both groups originated from the same pool of broodstock parents. In fact a large proportion of the allele pool for each group was unique to the group at the CmrHr 2.29 and HmD61 loci. At the HmD11 locus a large proportion of the total number of alleles were shared by both the fast and the slow growing abalone groups. The difference in alleles between the fast and slow growing abalone groups could be due to the fact that during

each spawning event on the Jacobsbaai abalone farm between 48 and 72 broodstock animals are used. Thus, the difference in the genetic composition of each group is most likely due to the fact that the individuals originated from different parents. The only way, therefore, to determine whether there is a relationship between phenotype and allele frequency would be to conduct more testing with more abalone groups. Genotyping of the broodstock would also need to be conducted to determine parentage of the individual animals.

Interestingly, a large number of rare and private alleles were found in both the slow growing and fast growing abalone groups. As mentioned previously, rare alleles are alleles that occur at a frequency of less than 5 % and contribute little to heterozygosity [9,12, 80]. During this study, groups three and four, which were spawned from the same broodstock animals, had more rare alleles than groups one and two that were spawned from a different group of broodstock animals. Thus, it would be easier to assign the individuals in groups three and four to their respective parents.

Private alleles are also important as they are regarded as being effective genetic markers for parentage determination, and have been shown to be effective for monitoring genetic diversity in hatchery populations [2]. The percentage of private alleles was similar between the fast and slow growing abalone at the three loci. Group one and two which were generated from the same spawning event possessed the same percentage of private alleles (37 %). For the individuals from spawning event two group four (slow growers) possessed less private alleles than group three (fast growers). Although parentage determination was not the objective of this study, the

high percentage of private and rare alleles at the three loci would make this an excellent choice for use in such studies

4.2.3 Neighbour-joining trees

Since it was not possible to distinguish between fast and slow growing abalone on the basis of allele frequency, the next step involved an investigation of the use of genetic distances between fast and slow growing individuals as a means of differentiation. Most microsatellite studies conducted with aquacultured species have focused on investigating the extent of genetic variation which exists between wild and cultured species. Melon Barroso *et al* [85], Norris *et al* [9], Alam and Islam [12] and Kohlmann *et al* [19] used neighbour joining genetic distance trees to illustrate the genetic diversity which exists between wild and farmed populations of *Brycon opalinus*, *Catla catla*, Atlantic salmon (*Salmo Salar*) and the common carp (*Cyprinus carpio* L.) respectively. In this study the objective was different as the genetic differences which exist between phenotypically distinct abalone groups was investigated and as such I am unable to compare the results obtained here with those obtained in other studies as I have not found any studies which have the same objective as this study.

Neighbour-joining trees plotted from genetic distances between individuals from spawning event one are displayed in Fig 7. The tree generated from genetic distances between individuals at all three loci shows that there is a clear distinction between the fast and slow growing individuals belonging to this spawning group (Fig. 7). Furthermore, it is evident that genetic diversity exists amongst the members of the fast and slow growing abalone groups as evidenced by the large amount of sub clusters.

The division between the fast and slow growing can be seen but there is still a mixture of fast and slow growing individuals in cluster B.

If we now look at the genetic distance tree in Fig. 8 which was plotted for the individuals generated from spawning event two, it can be that the pattern is much the same as for SPG 1. For the animals of SPG 2 there appears to be a larger amount of genetic diversity between the slow growing individuals as the fast growing individuals are more closely related by genetic distance. For both spawning group one and two it can be seen that there is a distinction between the fast and slow growing abalone. However, both spawning groups possess a cluster (Cluster B) which consists of a mixture of slow and fast growing individuals with no distinct separation being evident. The indistinct grouping of some animals from SPG 1 and SPG 2 with respect to phenotype is most likely due to the fact that different broodstock parents are used in each spawning event. As mentioned earlier, when animals are spawned on the Jacobsbaai abalone farm, between 48 and 72 broodstock animals are used at a time and this would account for the extensive sub-clustering that is evident in the genetic distance trees. The use of a variety of broodstock parents may also result in individuals with an intermediate growth rate which could not be grouped distinctly with regard to phenotype. Alternatively, the animals that appear to have been clustered incorrectly with respect to phenotype may in fact have been incorrectly identified as fast or slow growing abalone by the farm managers during grading.

In summary, it can be seen that fast and slow growing abalone groups generated from SPG 1 and SPG 2 can be differentiated from each other on the basis of genetic distance. This is most accurate when a combination of loci are employed as the

discriminating power of single loci is insufficient. The reason for the indistinct grouping of some of the individuals is not obvious and it would be necessary to identify the broodstock parents in order to solve this problem.

The clustering observed in the genetic distance trees demonstrates that there is some differentiation among the fast and the slow growing individuals. This is important finding as the next step in this study would be to genotype these individuals in order to determine who the broodstock parents are. Determination of this would allow one to perform crosses with broodstock parents that are known to generate fast growing progeny. The resulting larvae can then be analysed to determine if their microsatellite profiles are similar to those determined in this study for those individuals which are fast growing and thus selection for broodstock parents which give rise to fast growing progeny can be instituted.

4.3 Conclusion

In conclusion, this study has shown that a combination of three highly polymorphic microsatellite DNA markers can be employed to distinguish between fast and slow growing individuals on the Jacobsbaai abalone farm. In future studies additional microsatellite loci should be investigated in order to identify additional markers that could assist in differentiating between phenotypically different *H. midae*. Most microsatellite studies use between five and six loci and it has been recommended that a sampling group of about 50 individuals be used. At the commencement of this study Bester *et al* [6] had not yet published their results. From their study it is clear that loci HmSP1, HmD14 and HmD59 would be good choices as they have allele numbers

of 16, 15 and 21 respectively [6]. In addition, the observed heterozygosity values obtained for these loci are all higher than 0.45. Secondly, the abalone sampled should all originate from the same spawning event so as minimize the variables which might exist within the study.

It was also found that there has been a loss of genetic diversity in terms of heterozygosity as the number of heterozygotes in each group is low. However, Norris *et al* [9] have shown that allelic diversity is a more sensitive measure of differences between wild and farmed populations than heterozygosity. Thus, in terms of allelic diversity, therefore, this study found that the four abalone groups displayed high levels of polymorphism but this variability cannot be used to distinguish between the fast and slow growing abalone.

To date there have not been many studies with similar objectives as our study. Most microsatellite genetic studies involve investigation of the loss of genetic variation on aquaculture farms in hatchery bred stocks. In the introduction of this study I have mentioned the various ways in which microsatellites have been used in aquaculture. Only one phenotypic based study employing the use of microsatellites was found. In a study conducted by Calvo *et al* [96] they were able to successfully distinguish between groups of sheep with different colour phenotypes using microsatellite analysis and random fragment length polymorphisms (RFLPs). However, the loci selected for use in this study were known to be associated with a gene that influences melanin production.

This has been a preliminary study and future studies need to be conducted to confirm the results obtained here. This study also demonstrates the high levels of polymorphism detectable with microsatellite loci within farmed stocks of *Haliotis midae*. In an aquaculture situation this technology has the potential to be of great use in detecting levels of genetic variation and would enable farmers to breed with broodstock which have been shown to produce offspring which are able to grow faster. This in turn would benefit the farmer and strengthen the commercial viability of abalone farming. It is also essential to monitor any change in the genetic structure of the hatchery population with respect to a base population or wild populations in a closed farm setting [9,12]. This is important because a loss of genetic variation is considered to be the loss of genetic potential for stock improvement and adaptation to environmental changes [12].

Appendix 1

Allele frequency Distributions

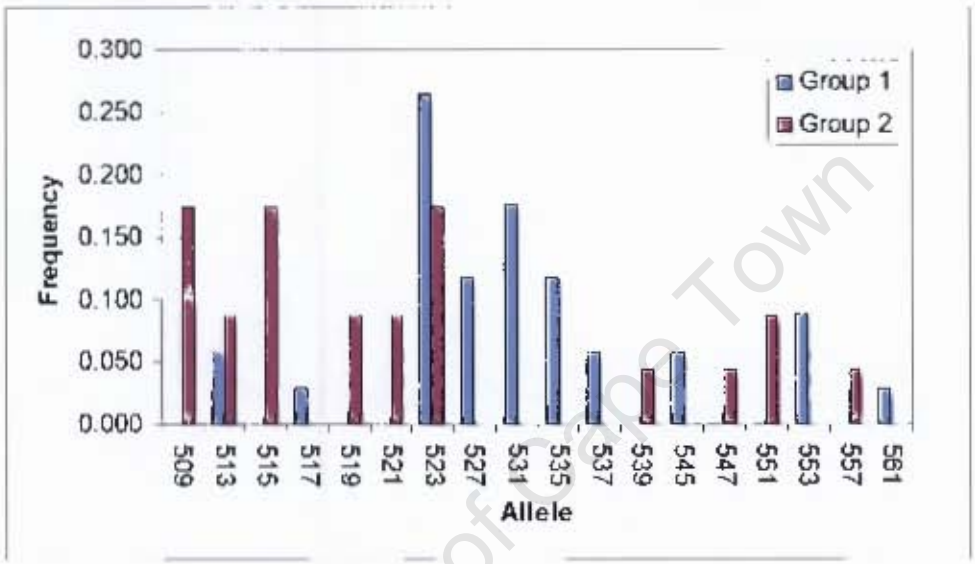
Private Allele Distributions

Genetic Distance Matrices

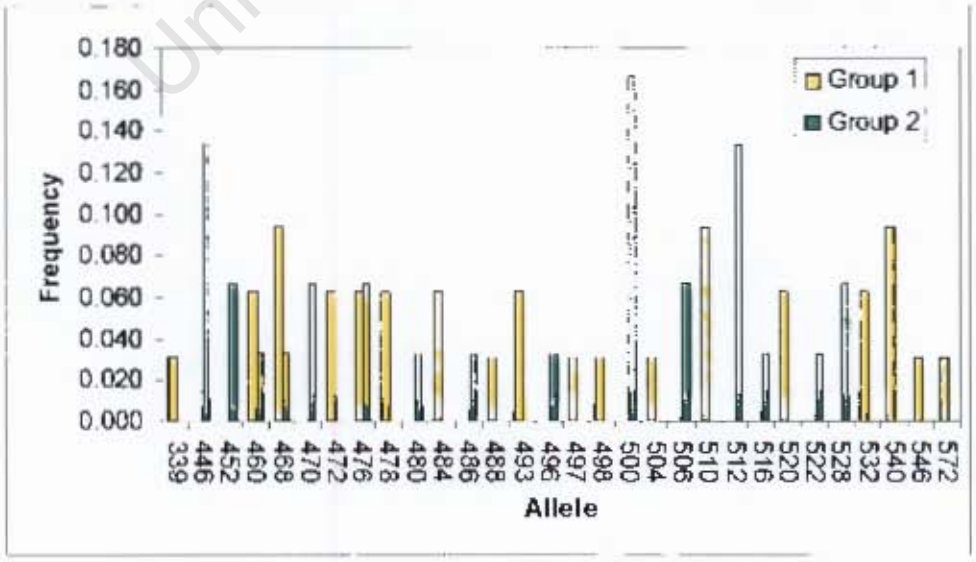
Allele Frequency Distributions

Spawning group one

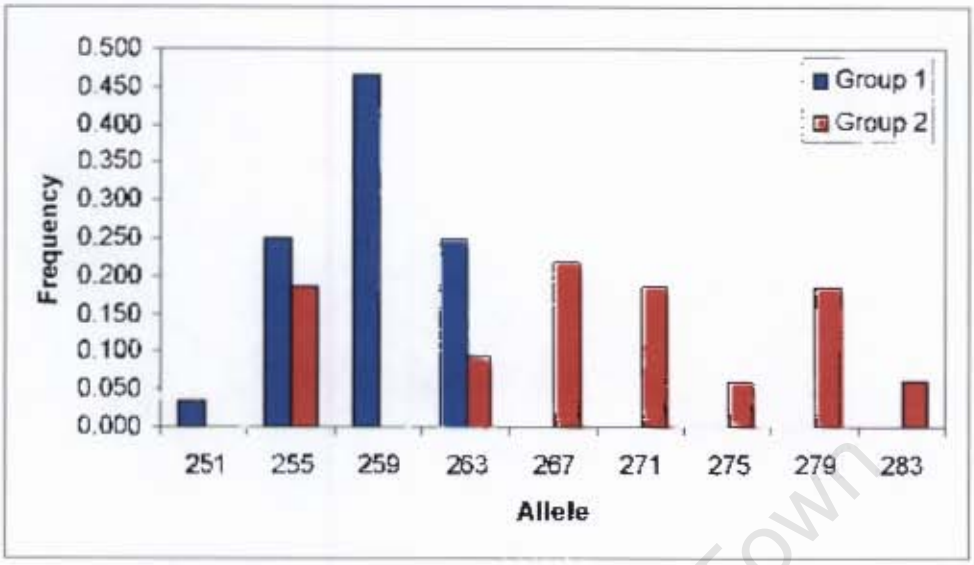
Locus HmD61



Locus CmrHr 2.29

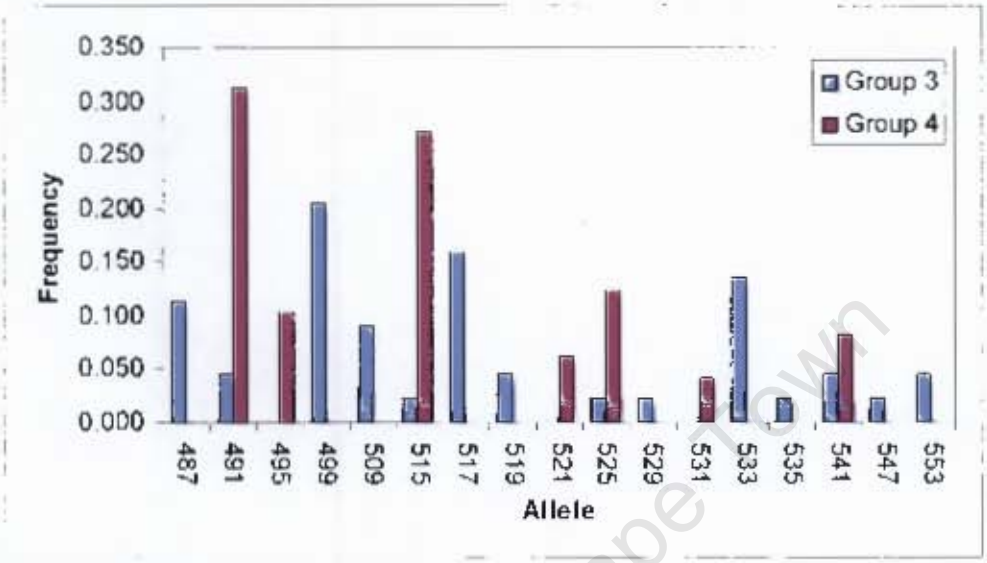


Locus HmD11

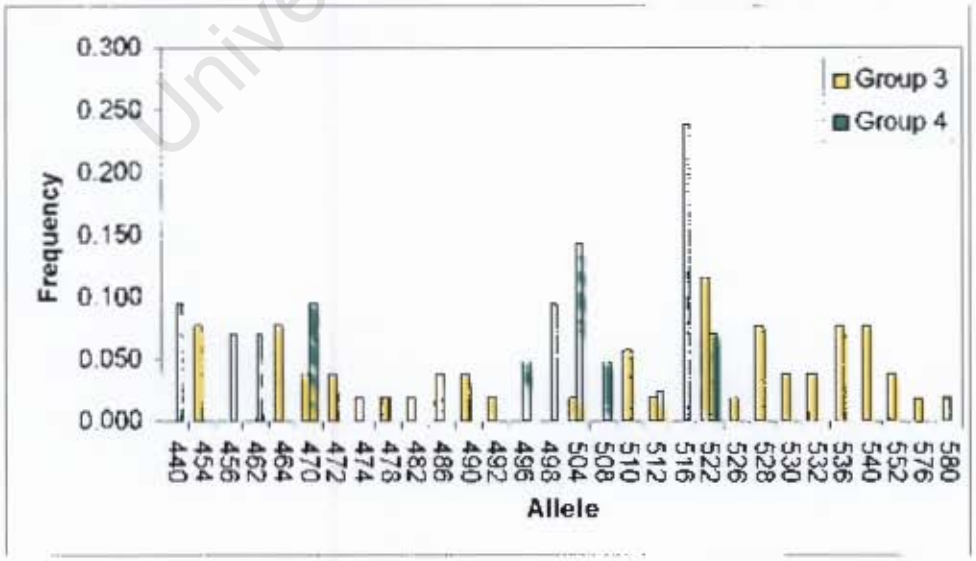


Spawning event two

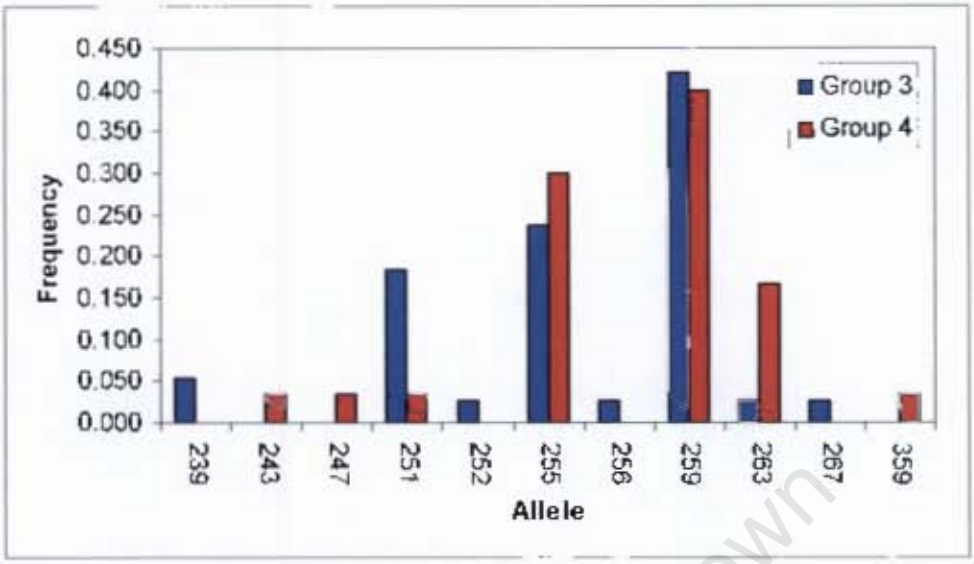
Locus HmD61



Locus CmrHr 2.29

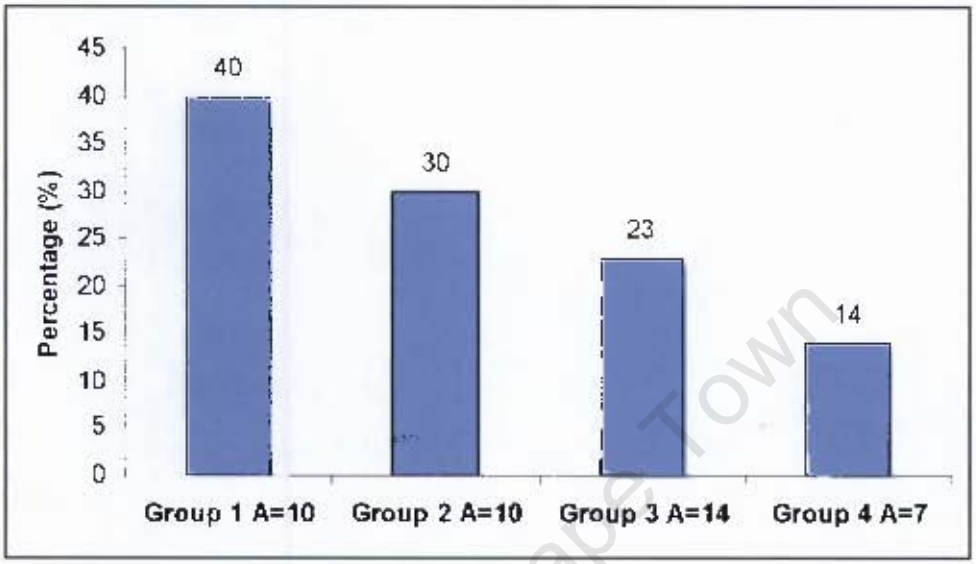


Locus HmD11



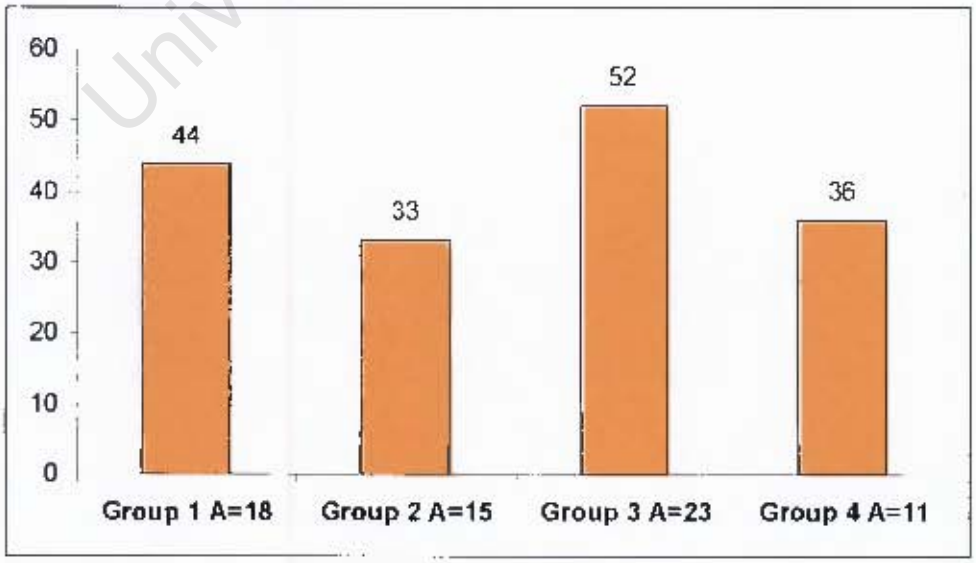
Private Allele Data

Locus HmD61



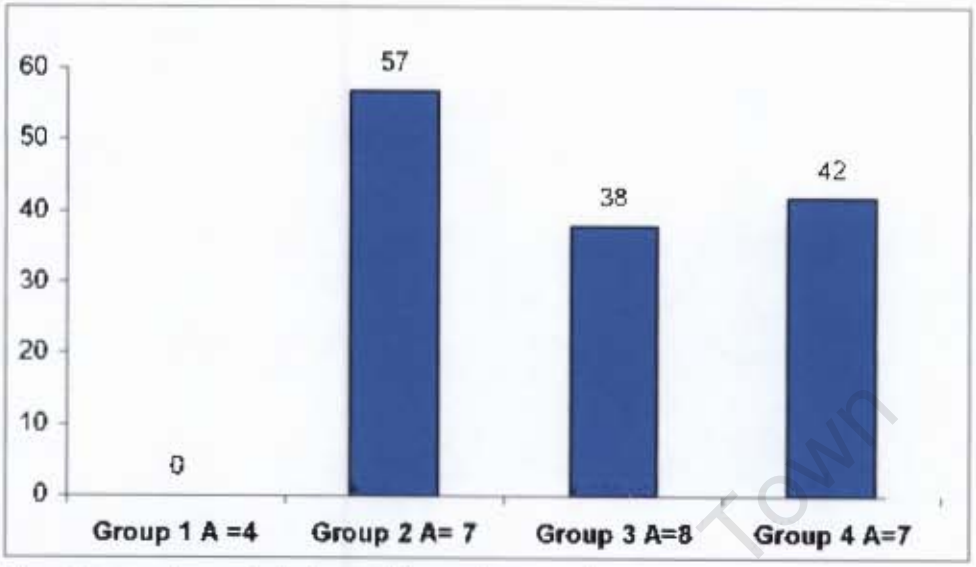
(A = the number of alleles within each group).

Locus CmrHr 2.29



(A = the number of alleles within each group).

Locus HmD11



(A = the number of alleles within each group).

Fig 1: Genetic distance matrix generated for all three loci for the four abalone groups used during this study

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