Identification of Growth Related Quantitative Trait Loci within the Abalone *Haliotis midae*, Using Comparative Microsatellite Bulked Segregant Analysis

by

Ruhan Slabbert

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Supervisor: Dr. Rouvay Roodt-Wilding
Faculty of Agrisciences
Department of Genetics

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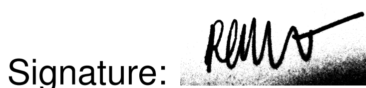
DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Signature: 

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ABSTRACT

The South African abalone, *Haliotis midae*, is a commercially valuable mollusc and is mostly exported to the Far East. Genetics research on *H. midae* has increased substantially since a genetic improvement programme was introduced in 2006 by collaboration between Stellenbosch University, government and industry partners. The development of molecular markers, QTL-mapping, gene-expression and genome manipulations are the main focuses of the research currently being conducted. The end goal is to create high quality and fast growing animals for the industry. The present study focused on the development of microsatellite markers and the detection of quantitative trait loci (QTL) affecting growth traits (shell length, shell width, wet weight) in this species. A combination of three methods, namely selective genotyping and bulked segregant analysis (pooling analysis), single marker regression and interval mapping were used to identify putative QTL in two full-sib families from two different farmed locations. Additional methods and protocols were developed that can assist the industry in other molecular research aspects. A total of 125 microsatellite loci were characterised. A total of 82 of these loci were isolated using second generation sequencing, a first for any abalone species. A preliminary, low-density framework linkage map was constructed containing 50 loci that mapped to 18 linkage groups. The observed genome length was 148.72cM with coverage of ±47%. QTL analyses revealed two putative QTL for shell width and wet weight, with 17% and 15% variance explained, that mapped on one linkage group in the first family and three putative QTL, for shell length, shell width and wet weight, with 33%, 28.5% and 31.5% variance explained, that mapped on one linkage group in the second family. Additional methods and protocols developed include an automated high-throughput DNA isolation protocol, a real-time PCR assay for *H. midae* x *H. spadicea* hybrid verification, a triploid verification microsatellite assay and a pre- and post-PCR multiplex setup and optimisation protocol. Future studies focussing on QTL and marker assisted selection (MAS) should verify the QTL found in this study and also utilise additional family structures and determine QTL-marker phase within the commercial populations.

OPSOMMING

Die Suid-Afrikaanse perlemoen, *Haliotis midae*, is 'n kommersieel waardevolle weekdier en word hoofsaaklik na die Verre-Ooste uitgevoer. Genetiese navorsing op *H. midae* het aansienlik toegeneem sedert 'n genetiese verbeteringsprogram in 2006 deur samewerking tussen die Universiteit van Stellenbosch, die regering en industrievennote ingebring is. Die ontwikkeling van molekulêre merkers, KEL-kartering, geen-uitdrukking en genoom manipulasies is die hooffokusse van die navorsing wat tans uitgevoer word. Die einddoel is om hoë kwaliteit en snelgroeiende diere vir die industrie te skep. Die huidige studie het op die ontwikkeling van mikrosatelliet merkers en die opsporing van groeiverwante (skulplengte, -breedte en nat gewig) kwantitatiewe eienskap lokusse (KEL) in hierdie spesie gefokus. 'n Kombinasie van drie metodes, naamlik selektiewe genotipering en versamelde segregaat analise (samevoegingsanalise), enkel merker regressie en intervalkartering is gebruik om waarskynlike KEL in twee vol-sibbe families van twee verskillende produksiegebiede te identifiseer. Aanvullende metodes en protokolle is ontwikkel wat die industrie in ander molekulêre navorsingsaspekte kan ondersteun. 'n Totaal van 125 mikrosatelliet lokusse is beskryf. 'n Totaal van 82 van hierdie lokusse is deur die gebruik van derde generasie volgordebepaling geïsoleer, 'n eerste vir enige perlemoen spesie. 'n Voorlopige, laedigtheid raamwerkkoppelingskaart is saamgestel met 50 lokusse wat op 18 koppelingsgroepe gekarteer is. Die waarneembare genoomlengte was 148.72cM met 'n dekking van ±47%. KEL-analises het twee waarskynlike KEL vir skulpbreedte en nat gewig blootgelê wat 17% en 15% variasie verduidelik en is op een koppelingsgroep in die eerste familie gekarteer asook drie waarskynlike KEL, vir skulplengte, -breedte en nat gewig wat 33%, 28.5% en 31.5% variasie verduidelik en is op een koppelingsgroep in die tweede familie gekarteer. Aanvullende metodes en protokolle wat ontwikkel is, sluit 'n geoutomatiseerde hoë-deurgang DNS-isolasieprotokol, 'n intydse PKR-proef vir *H. midae* x *H. spadicea* hibried verifikasie, 'n triploïed verifikasie mikrosatellietproef en veelsoortige pre- en post-PKR opstelling en optimaliseringsprotokol in. Toekomstige studies wat fokus op KEL en merker ondersteunde seleksie (MOS) behoort die KEL wat in hierdie studie gevind is te verifieer en ook bykomende familie strukture te benut om KEL-merker fases binne die kommersiële populasie te bepaal.

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The following researchers contributed to this section: Nicola Ruivo and Nicol van den Berg as part of their M.Sc. thesis and Darrell Lizamore as part of his B.Sc. (Hons) study. Data analyses, interpretation and manuscript preparation was performed by myself (Ruhan Slabbert). The markers designed by myself are shown in Appendix A, Table A.1.

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Chapter 5, Section 5.5:

All practical and data analysis was performed by myself (Ruhan Slabbert).

LIST OF ABBREVIATIONS

3’	3-prime
5’	5-prime
[]	concentration
℃	degrees Celsius
>	greater than
<	less than
±	more or less
%	percentage
μg	microgram
μg/ml	micrograms per millilitre
μl	microlitres
μM	micromolar
A	adenine
AA	acrylamide
Acc. Nr.	GenBank accession number
AFLP	amplified fragment length polymorphism
ANOVA	analysis of variance
APS	ammonium persulfate
ATP	adenosine triphosphate
bp	basepairs
BLAST	basic local alignment search tool
Blastn	nucleotide-nucleotide BLAST
BSA	bovine serum albumin

cDNA	complimentary DNA
CH_3COOH	acetic acid
CTAB	N-cetyl-N, N, N-trimethyl ammonium bromide
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	di-thio-threitol
EDTA	ethylene diamine tetra-acetate
e.g.	for example
EST	expressed sequence tag
et al.	and company
F	forward
F1	filial generation one
FAM	carboxyfluorescein
FIASCO	Fast Isolation by AFLP of Sequences Containing Repeats
G	guanine
g	gram
GLM	general linear model
g/ml	grams per millilitre
H_o	observed heterozygosity
H_e	expected heterozygosity
H_L	height of large allele
H_S	height of small allele
HCl	hydrochloric acid

Hm and *Hmid*	*Haliotis midae* (locus abbreviation)
HRMC	high resolution melt-curve analysis
H-W	Hardy-Weinberg
Kb	kilobases
KCl	potassium chloride
KEGG	Kyoto Encyclopedia of Genes and Genomes
kg	kilogram
LB	Luria Bertani medium
LOD	natural logarithm of the likelihood ratio
M	molar
MAS	marker assisted selection
Mb	megabases
mM	millimolar
mg	milligram
$MgCl_2$	magnesium chloride
ml	millilitre
mm	millimetres
mg/ml	milligrams per millilitre
mM/L	millimolar per litre
mtDNA	mitochondrial deoxyribonucleic acid
n	number of individuals
n_a	number of alleles
N	indicate any one of A, T, C or G
N/A	not applicable

NaCl	sodium chloride
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
ND5	NADH dehydrogenase subunit 5
ng	nanogram
ng/μl	nanograms per microlitre
NS	non-significant
P	probability
PAA	poly-acrylamide
PAGE	poly-acrylamide gel electrophresis
PCR	polymerase chain reaction
pers. comm.	personal communication
pH	concentration of hydrogen ions in a solution is expressed conventionally as its pH
PIC	polymorphic information content
pmol	picomol
QTL	quantitative trait loci / locus
R	reverse
RAPD	random amplified polymorphic DNA
rcf	relative centrifugal force
RFLP	restriction fragment length polymorphism
rfu	relative fluorescent units
RSA	Republic of South Africa
SDS	sodium dodecyl sulfate
SNP	single nucleotide polymorphism

SNX	*Stu*I, *Nhe*I, *Xmn*I
spp.	group of species
SSC	standard saline citrate
SSR	simple sequence repeat
STR	simple tandem repeat
T	thymine
T_a	annealing temperature
Taq	*Thermus aquaticus* DNA polymerase
TBE	Tris, boric acid, EDTA
TD-PCR	touch-down PCR
TE	Tris, EDTA
Temed	N, N, N', N'-tetramethyl-ethylenediamine
TEN	Tris, EDTA, NaCl
Tris	2-amino-2-(hydroxymethyl)-1, 3-proanediol
U	unit
USD	United States Dollar (currency)
UV	ultra-violet
v/v	volume to volume
w/v	weight to volume
ZAR	South African Rand (currency)

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CHAPTER 1

INTRODUCTION

1.1) Overview of Taxonomy, Biology and Ecology

Haliotis midae (Phylum: Mollusca; Class: Gastropoda; Family: Haliotidae) is one of five endemic abalone species found along the coastal waters of South Africa. This animal can reach shell lengths of up to 230mm (Hecht, 1994) and reach an age of more than 30 years. These abalones are dioecious. Research on *H. midae* found that 100% maturity is reached at 7.2 years of age (Tarr, 1995) and even as early as three years of age on the warmer East coast or under cultured conditions (Wood, 1993). Males are identified by a cream coloured gonad while females possess a green coloured gonad. They are broadcast mass spawners and fertilization is external (Tarr, 1987). The fertilized eggs develop into trochophore larvae, then into non-feeding planktonic veliger larvae and finally settle after about 5 days. The larvae usually settle on shallow coral, while juveniles shelter and feed between sea urchins (e.g.: *Parechinus angulosus*; Mayfield and Branch, 2000) or beneath boulders or in crevices, while older animals will move to a deeper habitat as their size increases and tend to stay in the same position for extended periods of time (Tarr, 1987, 1995). Most adult abalone will reside in the kelp beds at a depth of less than 10 metres (Tarr, 1987).

Larvae are preyed on by filter-feeders and small planktonic predators, while juveniles are targeted by animals such as whelks, crabs and reef fish. Adult abalones have no natural enemies other than humans (Tarr, 1987). Juvenile abalone feed on small algae and diatoms found on rock surfaces. Adult abalone feed on drifting seaweed (*Ecklonia maxima* and *Plocamium* spp.) by trapping it using the foot and rasping it to pieces with the radula (tongue-like structure).

Haliotis midae show great variability in growth rate. Tarr (1995) observed that the growth increment of similar sized animals vary from 9mm to 33mm over a three year period for animals sized 68mm at the beginning of the study. The study of Tarr (1995) suggests that temperature does not significantly influence growth rate, while studies such

as McShane *et al.* (1988) done on *H. rubra*, demonstrated that food availability may be an important factor influencing growth.

1.2) Overview of Global and Local Abalone Aquaculture

1.2.1) Global Aquaculture

Many *Haliotis* spp. are currently produced under cultured conditions. Excluding *H. midae*, these include *H. rufescens* (e.g.: Searcy-Bernal *et al.*, 2009), *H. fulgens*, *H. corrugata* (e.g.: Leighton, 1989), *H. asinina* (e.g.: Encena, 2009; Jarayabhand *et al.*, 2009), *H. diversicolor supertexta* (e.g.: Jarayabhand *et al.*, 2009), *H. tuberculata* (e.g.: Huchette and Clavier, 2004), *H. iris* (e.g.: Henriques *et al.*, 1989), *H. discus discus* (e.g.: Wang, 2004), *H. discus hannai* (e.g.: Xu, 2004), *H. rubra* (e.g.: Liu *et al.*, 2009) and *H. laevigata* (e.g.: Reaburn and Edwards, 2003), while *H. varia* (Najmudeen and Victor, 2004) aquaculture is being developed. The total worldwide fisheries landings and cultured production of abalone was an estimated 45000 metric tonnes in 2008 (www.fishtech.com; accessed 05/08/2010) for all species. Current prices for abalone species are being pushed down by factors such as the economic down-turn, increased production outputs and the lack of local markets other than the Asian-Pacific region (Gordon and Cook, 2009).

Each species is cultured under different environmental and management conditions. General hatchery practices include a series of events. The following description is adapted from the FAO Training Manual on Artificial Breeding of Abalone (*Haliotis discus hannai*) in Korea (Fisheries and Aquaculture Department, 1990). Mature broodstock are usually collected from the wild, but established farms select animals produced on site for breeding as well. Both wild and hatchery produced broodstock need to be conditioned for future spawning to ensure gamete quality and quantity. For this, animals will be kept under a constant water temperature, optimal water flow-rate and oxygen levels and adequate, high quality feed should be supplied. Various method for spawning induction are used and includes 1) exposure to ultraviolet light, 2) exposure to air for a period of time after which filtered seawater is added, 3) thermal shock by increasing and then decreasing water temperature and 4) chemical induction. Eggs are fertilised in special containers using optimal sperm vs. egg concentrations. Free-swimming larvae are closely monitored after hatching and are settled after a few days on diatom covered plates. Some studies have

already shown that settlement is dependent on the presence of specific diatom species on the plate (Kawamura and Takami, 1995; Kawamura *et al.*, 1995; Gordon *et al.*, 2006). The larvae are grown to a certain size before they are moved to different grow-out systems, tanks and raceways. The size of the abalones is monitored and they are size graded regularly to ensure optimal growth rates and quality. When the abalones reach export weight they are quality graded and sent for either live-export preparation or processed for canning or other product types. Abalone farms are usually land-based flow-through systems. A newer direction in abalone culture research is recirculation and integrated systems. Integrated systems use more than one aquaculture species, for example abalone, fish and seaweed (Figure 1.1). These systems diversify product output and allow for a better use of resources. In combination with recirculation systems, an integrated system will increase the sustainability and yield, reduce water usage and ensure optimal water quality (Neori *et al.*, 2000; Schuenhoff *et al.*, 2003).

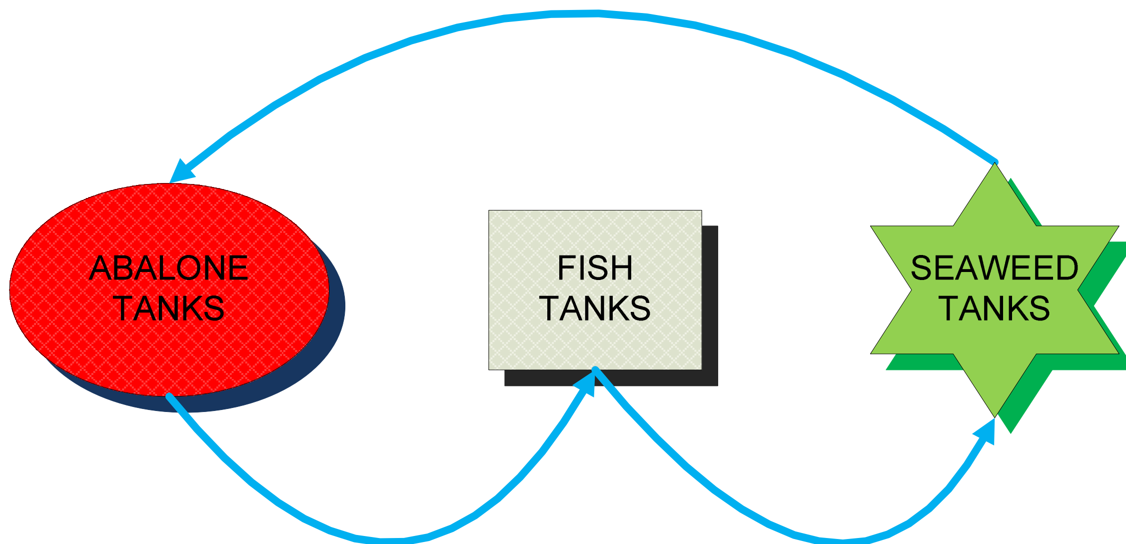


Figure 1.1: A simple diagramme representing an integrated recirculating system. The blue arrows indicate the direction of the waterflow through the system.

1.2.2) Local Aquaculture

The abalone industry is the most valuable aquaculture sector in South Africa. Only *Haliotis midae* is cultivated and exported. *Haliotis midae* is the largest South African abalone species and is relatively abundant, non-cryptic and easily accessible in comparison to the other species. This makes it an ideal target for recreational, subsistence and commercial harvesting activities. Harvesting activities on *H. midae* commenced in 1949 in the Gansbaai area. The first restrictions on abalone catches were set in 1968 (Tarr, 1992) and were kept more or less the same until 1997 when it was lowered (Cook, 1998). Illegal harvesting of abalone poses a serious problem for sustainable abalone harvesting, since most poached abalones are undersized (Hauck and Sweijd, 1999). These individuals are most likely immature and are therefore removed from any reproductive activities. The high number of recruits removed from this reproductive cycle could eventually cause the collapse of natural populations (e.g.: *H. sorenseni*; Tegner *et al.*, 1996). In addition to harvesting, an ecological factor has also contributed to a decrease in abalone populations: an increased rock lobster (*Jasus lalandii*) populations preying on juvenile abalone as well as on sea urchins (*Parechinus angulosus*) which play an important role in the settlement and protection of *H. midae* larvae (Tarr *et al.*, 1996; Day and Branch, 2002). The decline in abalone populations eventually led to the closure of the abalone fishery on 31 January 2008. The fishery was reopened in June 2010 for commercial diving (Essop, 2010).

A logical step in the evolution of a sustainable abalone aquaculture industry in South Africa was to culture this product within a controlled environment; increasing yield and availability. Countries such as China have been culturing abalone for decades. Even though knowledge obtained from these countries can be universally applied, it is important to consider that the aspects of abalone culturing differ according to the economical, political and environmental status within a country at a specific time (Fleming and Hone, 1996) as well as the biology of the target species. Farming of *H. midae* became an economic reality after 1981, when Genade *et al.* (1985, 1988) spawned *H. midae* in captivity. A programme to establish commercial abalone farming was initiated in 1990 as a joint effort by industry and academic institutions (Cook and Britz, 1991), while technology transfer also played a part in the development process (Sales and Britz, 2001). By 2009 some 15 licensed commercial farms had been established with an estimated total live

mass production of 870 tonnes in 2008 fetching around USD 38 per kg (pers. comm.: Wayne Barnes, Abalone Farmers Association of South Africa).

1.3) Overview of General and Molecular Research on Abalone Aquaculture

1.3.1) General Research on Abalone

The sustainability, development and profitability of the abalone industry are dependent on the type and quality of research outputs. Much time, effort and resources go into such research endeavours. Such projects cover a legion of topics. These include handling, production and management issues (e.g.: White *et al.*, 1996; Britz *et al.*, 1997; Reddy-Lopata *et al.*, 2006; Vosloo and Vosloo, 2006; Yearsley, 2007; Robertson-Andersson *et al.*, 2009), diseases (e.g.: Haaker *et al.*, 1992; Nicolas *et al.*, 2002; Bower, 2003; Lleonart *et al.*, 2003; Xu, 2004; Balseiro *et al.*, 2006; Cai *et al.*, 2006a, b, c; Hooper *et al.*, 2007; Cai *et al.*, 2008; Cheng *et al.*, 2008; Ying *et al.*, 2008), nutrition (e.g.: Britz, 1994; Gomez-Gil *et al.*, 2000; Macey and Coyne, 2005; Naidoo *et al.*, 2006; Troell *et al.*, 2006; Smit *et al.*, 2007), hybridisation (e.g.: Hoshikawa *et al.*, 1998; Ibarra *et al.*, 2005; Cai *et al.*, 2009; Carr and Appleyard, 2009; Lafarga-de la Cruz *et al.*, 2009; Luo *et al.*, 2009); reproduction (e.g.: Encena *et al.*, 1998; Park *et al.*, 2006; Fukazawa *et al.*, 2007; Roux *et al.*, 2008) and various genetic disciplines. Genetic improvement will be discussed in the following section.

1.3.2) Genetics Research on Aqua- and Mariculture Species

Genetic techniques and technologies are becoming an integrated part of modern aqua- and mariculture. These techniques and technologies can be applied to many aquaculture related issues such as stock identification, the monitoring of founding stocks, assisting with environmental impact and recovery studies and assisting with breeding programmes (Magoulas, 1998). The success of these genetic applications rests on molecular genetic markers. Many different markers exist and one, namely microsatellite markers, will be covered in Chapter 2. Table 1.1 gives a summary of a few types of molecular markers used in aquaculture.

Table 1.1: Different marker systems used in aquaculture genetics.

Marker type	Abbreviation	Description	Reference
Allozymes	N/A	Co-dominant, single locus markers based on polypeptides.	May *et al.* (1980)
Amplified fragment length polymorphism	AFLP	Dominant, multi-locus markers generated by restriction digestion and selective PCR.	Vos *et al.* (1995)
Expressed sequence tags based markers (e.g.: Microsatellites or SNPs)	EST	Markers generated from coding DNA (cDNA) and used to study gene expression and diversity.	Liu and Cordes (2004)
Microsatellites	SSR / STR	Co-dominant, multi-allelic markers consisting of repeat units 1-6bp in length and genotyped by PCR	Liu and Cordes (2004)
Minisatellites	N/A	Multi-locus, multi-allelic markers consisting of repeat units of >10bp and generated by PCR.	Liu and Cordes (2004)
Mitochondrial DNA	mtDNA	Single-locus, multi-allelic markers situated on the mitochondria and generated by PCR or RFLP.	Liu and Cordes (2004)
Random amplified polymorphic DNA	RAPD	Dominant, multi-locus, bi-allelic markers generated using short oligonucleotides and PCR.	Welsh and McClelland (1990) Williams *et al.* (1990)
Restriction fragment length polymorphism	RFLP	Co-dominant, bi-allelic markers generated by restriction enzyme	Liu and Cordes (2004)

		digestion.	
Single nucleotide polymorphism	SNP	Co-dominant, bi-allelic markers based on single base differences within a PCR generated fragment.	Liu and Cordes (2004)

Genetic improvement programmes rely on high quality genetic material within commercial populations (Frankham, 1995; Hill, 2000). When starting out with a genetic improvement programme for any aquaculture species, the acquisition of a base population that contains adequate genetic diversity is of the utmost importance (Borrell *et al.*, 2007). High genetic diversity will ensure long-term genetic response to selection (Hayes *et al.*, 2006). This diversity, which translates into phenotypic diversity, will allow the measuring and mapping of economically important traits (commercial breeding). The base populations will not only be important for maintaining an industry, but in some instances it is vital for protecting and rebuilding the natural finfish and shellfish stocks (supportive breeding). They serve as large genetic banks which contain all or most of the available genetic diversity found in the wild. The level of genetic diversity within commercial or supportive breeding stocks is dependent on large effective population sizes (N_e; number of individuals that contributes different alleles to the next generation). Effective population size influences the levels of heterozygosity, affects genetic drift, increase the chances of lethal allelism and influences linkage disequilibrium (Pollak, 1983; Nei and Tajima, 1987; Frankham, 1995; Falconer and Mackay, 1996). A small N_e can lead to inbreeding depression which could lower reproductive success and survival traits (Falconer and Mackay, 1996) of aquaculture species. By applying genetic markers, Hayes *et al.* (2006) maintained diversity in commercial stocks by using AFLPs to select broodstock based on individual heterozygosity levels or contributions to the overall diversity, while Ditlecadet *et al.* (2006) and Borrell *et al.* (2007) selected Arctic charr and sea bream broodstocks with microsatellites based on individual relatedness. A practical example of how molecular technology can be applied for selecting broodstock is by Doyle and Herbinger (1995) who used a within-family selection protocol based on DNA-fingerprints. First, the animals were ranked based on trait superiority. The highest rank individual was then chosen for breeding, followed by the next unrelated individual, followed by the next individual

unrelated to the previous two. This was repeated until the maximum number of broodstock that is required was reached.

The effectiveness and impact of supportive breeding programmes can be monitored by using markers such as microsatellites (Koljonen *et al.*, 2002; Blanchet *et al.*, 2008). The same goes for monitoring the effect of domestication via intentional and unintentional selection and adaptation to culture conditions on the genetic composition and life stages of a population being domesticated. Most aquaculture stocks are still in their early domestication stages (Mignon-Grasteau *et al.*, 2005) making this an ideal situation for molecular genetics to play a significant role alongside other disciplines. By selecting superior breeders based on phenotypes only, breeders can skew the genetic structure of the hatchery populations. Low genetic diversity could lead to the collapse of hatchery stocks (Bentsen and Olesen, 2002)!

The loss of genetic diversity within farms and hatcheries are well documented (e.g.: Hauser *et al.*, 2002; Sekino *et al.*, 2002; Lewis *et al.*, 2006; Thai *et al.*, 2007; Lind *et al.*, 2009). There are numerous explanations for these observations which range from biological factors compounded by human activity to only human activity. An example of a biological aspect is the differential contributions of broodstock individuals to their offspring, which is a natural phenomenon during spawning (Hedgecock, 1994; Arnason, 2000), due to various environmental or physical reasons. When differential contributions are seen within a small broodstock population the loss of genetic material can be quite large. Molecular markers can be applied to reconstruct pedigrees and quantify the contributions of broodstock individuals (e.g.: Garant *et al.*, 2001). An example of a hatchery (human) activity that can lower genetic diversity is culling (Taris *et al.*, 2006) where animals are permanently removed from the genetic pool on a farm.

It is clear from the abovementioned examples that molecular technology is already an essential part of the domestication process of many aquaculture species. By quantifying the levels of genetic diversity within hatcheries and broodstocks, effective management strategies can be developed to maintain genetic diversity and therefore lay the foundations for successful improvement strategies.

When a good base population is established and the most important mechanisms that influence both the biology and the genetics of a species have been identified, aquaculturists can move towards the molecular and technical aspects of the genetic

improvement programme. The basic components of a molecular breeding programme consists of the construction of a molecular linkage map, identification and locating of quantitative trait loci (QTL), followed by marker assisted selection (MAS).

Genetic linkage maps are statistical and graphical representations of the positions of different molecular markers within a segregating population. Marker-based linkage maps are the scaffold for any molecular genetics based improvement and selection programme. These allow for the analyses of important economic traits (Lander and Botstein, 1989) and marker assisted selection (Cho *et al.*, 1994). A number of important aquaculture species already has linkage maps consisting of variable numbers of different marker types. A summary is shown in Table 1.2.

Table 1.2: Some aquaculture species (excluding abalone) with linkage maps and the marker type and number mapped on these.

Species	Reference
Finfish	
Arctic char (*Salvelinus alpines*)	Woram *et al.*, 2004
Atlantic salmon (*Salmo salar*)	Gilbey *et al.*, 2004 Moen *et al.*, 2004b
Bighead carp (*Aristichthys nobilis*)	Liao *et al.*, 2007
Brown trout (*Salmo trutta*)	Gharbi *et al.*, 2006
Channel catfish (*Ictalurus punctatus*)	Waldbieser *et al.*, 2001 Liu *et al.*, 2003
Common carp (*Cyprinus carpio*)	Sun and Liang, 2004
European sea bass (*Dicentrarchus labrax*)	Chistiakov *et al.*, 2005
Japanese flounder (*Paralichthys olivaceus*)	Coimbra *et al.*, 2003
Nile tilapia (*Oreochromis niloticus*)	Kocher *et al.* 1998 McConnell *et al.*, 2000 Lee *et al.*, 2005
Rainbow trout (*Oncorhynchus mykiss*)	Young *et al.*, 1998; Sakamoto *et al.*, 2000

	Nichols *et al.*, 2003
Silver carp (*Hypophthalmichthys molitrix*)	Liao *et al.*, 2007
Turbot (*Scophthalmus maximus*)	Bouza *et al.*, 2007
Walking catfish (*Clarias macrocephalus*)	Poompuang and Na-Nakorn, 2004
Yellowtail (*Seriola lalandi*; *S. quinqueradiata*)	Ohara *et al.*, 2005
Shellfish	
Black tiger shrimp (*Penaeus monodon*)	Wilson *et al.*, 2002
Blue mussel (*Mytilus edulis*)	Lallias *et al.*, 2007
Eastern oyster (*Crassostrea virginica*)	Yu and Guo, 2003
Japanese scallop (*Patinopecten yessoensis*)	Xu *et al.*, 2008
Kuruma prawn (*Penaeus japonicus*)	Li, Y. *et al.*, 2003
Marine shrimp (*Penaeus chinensis*)	Li, Z. *et al.*, 2006
Pacific oyster (*Crassostrea gigas*)	Li and Guo, 2004 Hubert and Hedgecock, 2004
Sea urchin (*Strongylocentrotus* spp.)	Zhou *et al.*, 2006
White shrimp (*Penaeus vannamei*)	Pérez *et al.*, 2004
Zhikong scallop (*Chlamys farreri*)	Wang *et al.*, 2004 Li, L. *et al.*, 2005

Following map construction, important economical traits are measured and the effect, number and position of loci affecting these traits are determined (Lander and Botstein, 1989). Such loci are called quantitative trait loci (QTL) and are also under the influence of environmental factors. Some examples of QTL studies are given in Table 1.3. The traits targeted by such studies are mostly of economic importance or production-related (references in Table 1.3). The strategy used for detecting QTL in aquaculture species depends on the biology and farming methodology of the species which are usually highly fecund, making breeding often difficult to control (Gjedrem *et al.*, 2005), influencing the family structure that can be used in QTL-mapping.

Table 1.3: A few examples of QTL found in various aquaculture species (excluding abalone).

Species	Trait	Reference
	Finfish	
Atlantic salmon (*Salmo salar*)	Infectious salmon anemia resistance Flesh colour	Moen *et al.*, 2007 Baranski et al., 2010
Asian seabass (*Lates calcarifer*)	Growth related traits	Wang *et al.*, 2006
Coho salmon *Oncorhynchus kisutch*	Spawning time	Araneda *et al.*, 2009
European sea bass (*Dicentrarchus labrax*)	Morphometric traits	Chatziplis *et al.*, 2007
Rainbow trout (*Oncorhynchus mykiss*)	Spawning time Upper thermal tolerance Embryonic development rate IHN-resistance Pyloric caeca number Cortisol levels Early maturation	Sakamoto *et al.*, 1999; Leder *et al.*, 2006 Danzmann *et al.*, 1999; Perry *et al.*, 2001 Robison *et al.*, 2001 Khoo *et al.*, 2004 Zimmerman *et al.*, 2005 Drew *et al.*, 2007 Haidle *et al.*, 2008
Tilapia (*Oreochromis* spp.)	Cold tolerance and fish size Immunity, stress response, blood parameters and size Cold tolerance	Cnaani *et al.*, 2003 Cnaani *et al.*, 2004 Moen *et al.*, 2004a
	Shellfish	
Bay scallop (*Argopecten irradians*)	Size-related traits	Qin *et al.*, 2007
Eastern oyster (*Crassostrea virginica*)	Disease resistance	Yu and Guo, 2006
European flat oyster (*Ostrea edulis*)	Disease resistance	Lallias *et al.*, 2009

Kuruma prawn (*Marsupenaeus japonicus*)	Growth related traits	Lyons *et al.*, 2007

Massault *et al.* (2008) divided QTL-mapping designs into three groups: 1) hierarchical design, 2) the mass-spawning design and 3) the large full-sib family design.

Hierarchical design: This design is ideal for species where family structure and origin can be controlled in full. Full-sib families of variable sizes are available as well as multi-generation information. Atlantic salmon and trout are examples of species fitting this design.

Mass-spawning design: This design can be used for species where the creation of single full-sib or half-sib families is difficult or impossible. Mass-spawning species spawn in groups causing the mapping population to consist of a number of different families. These families need to be identified first using molecular pedigree reconstruction. An issue with the families is the unequal number of offspring in each because of unequal parentage contribution (e.g.: Brown *et al.*, 2006). Only the largest families are selected for the mapping studies. Sea bream is a perfect example of where this design can be applied.

Large full-sib family design: This design is used where artificial spawning facilitates the use of large full-sib families. Large families are ideal for selective genotyping, reducing cost and increasing experimental power (Lebowitz *et al.*, 1987). Species suited for this design include molluscs such as oysters and abalone.

All three above mentioned designs are linkage based methods requiring family structures, unlike linkage disequilibrium methods which are also based on association studies. The main drawback of these three designs is therefore that the identified QTL-trait linkage is only specific for the family being studied, and not necessarily to the entire population.

The close association between a marker and a QTL as well as knowledge on the performance of the QTL in different environments (Danzmann *et al.*, 1999) can be used for marker-assisted selection (MAS; Hallerman and Beckman, 1988; Ferguson, 1994). Individuals with the desired trait are selected based on their genotype. This genotype must

be linked to the trait or traits to be selected for. MAS is particularly useful for traits where phenotypes are hard to measure, such as disease resistance (Fuji *et al.*, 2007; Moen *et al.*, 2009). The main restraint of most MAS-programmes in aquaculture is however the lack of high-resolution linkage maps (Sonesson, 2003). MAS not only has the potential to increase the genetic gain for a trait, but also to decrease the negative aspects of selection such as inbreeding (Sonesson, 2007).

1.3.3) Genetics Research: *Haliotis* spp.

A number of abalone species are cultured worldwide. Numerous molecular markers have been designed for these *Haliotis* species including microsatellites (e.g.: Evans *et al.*, 2000; Selvamani *et al.*, 2000; An and Han, 2006; Baranski *et al.*, 2006b; Sekino *et al.*, 2006b; Zhan *et al.*, 2009), SNPs (Qi *et al.*, 2008, 2010) and mitochondrial markers (Klinbunga *et al.*, 2003) to name a few. These markers were then used to investigate different aspects of various research programmes.

Molecular parentage assignments were performed in abalone as part of marker characterisation (Li, Q. *et al.*, 2003; Sekino *et al.*, 2006), to study the contributions of individual broodstock animals to the F1 population (Selvamani *et al.*, 2001), or as part of a breeding programme (Hara and Sekino, 2007b). Levels of genetic variation have been studied extensively within wild and hatchery abalone stocks. Studies such as those by Smith and Conroy (1992), Mgaya *et al.* (1995), Evans *et al.* (2004a), Li, Q. *et al.* (2004), Hara and Sekino (2007a), and Li, Q. *et al.* (2007) detected a loss of genetic diversity within different hatchery populations. Such results suggest that the various hatchery activities and management strategies can have a negative impact on a population without proper knowledge and genetic regulatory processes.

Molecular linkage maps have also been generated for a few species of abalone. Baranski *et al.* (2006a) mapped 122 microsatellite loci to 18 linkage groups in *H. rubra*. The linkage map of Liu *et al.* (2006), for *H. discus hannai*, consisted of microsatellite, AFLP and RAPD markers and mapped 119 loci to 22 linkage groups in the female and 94 loci to 19 linkage groups in the male. The microsatellite marker based map of Sekino and Hara (2007b) for the same species had 160 markers placed on 19 linkage groups for the female and 18 linkage groups with 167 markers for the male. Shi *et al.* (2010) constructed

a map for *H. diversicolor* with 90 AFLP markers on 17 linkage groups in the female and 94 AFLP markers on 18 linkage groups in the male map. Quantitative trait loci for growth related traits have already been identified for two abalone species: *H. discus hannai* (Liu *et al*., 2007) and *H. rubra* (Baranski *et al*., 2008). Twenty-eight significant QTL were identified for nine characteristics by Liu *et al.* (2007) – shell length (4), shell width (4), total weight (2), shell weight (1), soft part weight (4), muscle weight (3), gonad and digestive gland weight (4), mantle weight (4) and gill weight (2). Baranski *et al.* (2008) identified 10 QTL segregating in a male and five in a female animal, all associated with growth rate.

Genomic research is also becoming more popular for *Haliotis*. A cDNA library was constructed for *H. asinina* by Jackson and Degnan (2006) with the aim of studying growth, development and reproduction processes. A number of genes has been cloned and characterised in addition to their transcriptional profile (Ekanayake *et al*., 2008; De Zoysa *et al*., 2009, 2010; Nikapitiya *et al*., 2009; Zhou and Cai, 2010). These studies investigated the gene expression profiles under various physiological and immunological stresses with the ultimate aim of elucidating complex molecular mechanisms to assist genetic breeding programmes.

Another area of research includes the production of triploid abalones (Arai *et al*., 1986; Stepto and Cook, 1998; Yang *et al.,* 1998; Norris and Preston, 2003; Li, Y. *et al.,* 2007a; Okumura *et al.,* 2007) and hybrids (Brown, 1995; Hoshikawa *et al*., 1998; Cai *et al*., 2009) between species. Triploid induction is one strategy for increasing production output by creating animals with greater meat to shell ratios, while hybridisation has the potential to tap into the hybrid vigour of the species in the hope of increasing growth rates, size and other commercial traits (Elliott, 2000).

1.3.4) Genetics Research: *Haliotis midae*

The most studied area for commercially grown *H. midae* is genetic population structure and diversity. Studies to date have reported different outcomes. Evans *et al.* (2004a) detected a loss in genetic diversity between natural and commercial populations, while Lambrechts (2002) and Slabbert *et al.* (2009a) detected no such loss. However, Slabbert *et al.* (2009a) did find a loss of diversity in a single spawning group within a particular farm due to differential broodstock contributions. These three studies indicate

that levels of diversity may differ between and within farms and is influenced by a range of different parameters. The mechanisms and causes for the loss or retention of genetic diversity within the commercial environment could aid in the management of important *H. midae* genetic resources and should be the focus of future studies. A number of molecular pedigree analyses have also been done to identify and select broodstock individuals that either does not contribute to the offspring at all (Slabbert *et al.* 2009a) or only contribute to faster growing offspring (Ruivo, 2007; Van den Berg and Roodt-Wilding, 2010).

Genetics research on *H. midae* has increased substantially since a genetic improvement programme was introduced in 2006 by a collaboration effort between Stellenbosch University, government and industry partners (Brink *et al.*, 2009b; Slabbert *et al.*, 2009b). The importance and application of genetics in abalone breeding schemes is reviewed by Elliott (2000) and Roodt-Wilding and Slabbert (2006). Current molecular genetic tools for *H. midae* include 215 microsatellite markers (Bester *et al.*, 2004; Rhode, 2010; Chapter 2), 38 SNPs (Bester *et al.*, 2008; Rhode *et al.*, 2008; Rhode, 2010) as well as a number of AFLP markers (Badenhorst, 2008). A number of DNA analyses methods have so far been optimised in the course of the improvement programme, including DNA extraction protocols (Badenhorst and Roodt-Wilding, 2007).

Preliminary gene-expression studies in cultured *H. midae* looked at the differential expression of some 39 genes in animals exposed to varying levels of oxygen and temperature regimes (Vosloo *et al.*, 2009). A study by Macey and Coyne (2005) investigated gene expression during the immune response of *H. midae* during probiotic treatment. This treatment was shown to have an immunostimulating effect on abalones. Two genes, cytochrome *b* and cytochrome *c* oxidase III, were found to be upregulated during these treatments, indicating the role of these genes during the immune response of *H. midae* (Van Rensburg and Coyne, 2009). Studies such as these will increase the understanding of abalone response and differential adaptation to environmental stress and help researchers understand biological pathways within these fascinating animals.

A form of genetic manipulation, namely triploidy, is thought to increase growth rates and improve product quality (Elliott, 2002). Triploidy has been successfully induced in *H. midae* using either cytochalasin B (Stepto and Cook, 1998) or through hydrostatic pressure (De Beer, 2004). An ongoing growth trial however showed no significant weight, length or yield gain after 48 months between diploid and triploid abalone, but did find that

gonad development was significantly less in triploid animals (Brink *et al.*, 2009a) which may suggest that these animals are sterile.

1.4) Dissertation Layout

1.4.1) Aims

The two main aims of this dissertation are the development of molecular genetic markers, namely microsatellite loci, and the detection of growth related quantitative trait loci (QTL) in *Haliotis midae*. Three traits are targeted for QTL-detection, namely shell length, shell width and wet weight. A combination of three strategies, namely selective genotyping and bulked segregant analysis (pooling analysis), single marker regression and interval mapping is used to identify putative QTL. A minor aim during this study is to develop additional methods and protocols that can be used to assist the industry in other molecular breeding aspects and to assist further research on QTL-detection and marker assisted selection (MAS).

The layout of each chapter is as follows:

1.4.2) Chapter 2

The isolation and characterisation of microsatellite loci in *Haliotis midae* are described in four sections. The general protocol called the Fast Isolation by AFLP of Sequences Containing repeats (FIASCO) is given in the first section (Section 2.1). The following two sections (Sections 2.2 & 2.3) describe the construction of repeat enriched libraries screened using colony PCR. The last section (Section 2.4) covers a high-throughput method of marker development using pyrosequencing. The construction of a preliminary framework linkage map is also outlined in Section 2.4.

1.4.3) Chapter 3

Bulked segregant analysis and selective genotyping using microsatellite loci are applied in *Haliotis midae* in a preliminary scan for growth related QTL. Three phenotypes are measured, namely shell length, shell width and wet weight. The top and bottom 10% phenotypic extremes of two full-sib families from two different hatcheries are used in the construction of DNA pools. These pools are genotyped and the peak fluorescent intensities for each locus are compared between them. Peak fluorescence is representative of allele frequencies. Loci showing significant difference between top and bottom pools in peak fluorescence are further used in single marker regression. This is used to confirm the accuracy of the pooled analysis. Single marker regression is followed by interval mapping to map the position of the QTL as well as calculate the effect of any QTL.

1.4.4) Chapter 4

Various studies and methodologies supporting Chapters 2 and 3 are discussed. Section 4.1 covers the non-destructive juvenile sampling by taking epipodial tentacles. DNA quality and animal survival are evaluated. Section 4.2 gives a short description of a questionnaire based assessment of the traits chosen by hatchery managers, which are seen as being important for production and profit. The final section (Section 4.3) discusses the setup and optimisation of PCR multiplex protocols and post-PCR family specific multiplexes. Microsatellite markers are divided into multiplexes according to allele sizes and fluorescent dye after which PCR conditions are optimised. With family-specific multiplexes a post-PCR multiplex is set up by dividing markers according to the allele sizes observed after the genotyping of the parental samples within a family.

1.4.5) Chapter 5

The optimisation and design of a number of protocols intended to facilitate molecular studies in *H. midae* are described. Section 5.1 compares different kit-based silica membrane and home-brew extraction protocols for their use in purifying high quality genomic DNA from abalone tissue. DNA quantity and quality are evaluated. Section 5.2

describes a high-throughput, automated DNA extraction protocol using a commercial kit and an automated robotic platform. Section 5.3 is linked to the previous section; where the same silica plate used by the commercial kit is depurinated for re-use. The quality of the DNA purified by the regenerated plate and the possibility of cross-contamination is studied. Section 5.4 modifies an existing protocol for species identification between *H. midae* and *H. spadicea* by developing a real-time PCR assay. This protocol is applied to identify the first experimental hybrid individuals of these two species. In Section 5.5 a microsatellite assay for the identification of triploid individuals is developed and optimised for fast throughput.

1.4.6) Chapter 6

In this concluding chapter the utility of the various aspects of this study is discussed. Challenges encountered are elaborated on along with recommendations on how to overcome or avoid these in future studies. Future work is also touched upon.

CHAPTER 2

MICROSATELLITE LOCI ISOLATION

SECTION 2.1

The Fast Isolation by AFLP of Sequences Containing Repeats (Zane *et al.*, 2002)

2.1.1) Introduction

Microsatellites are simple sequence repeats found in 1kb or less tracts within the genomes of higher organisms. Microsatellites are defined as containing repeat units of 1-6bp (Goldstein and Pollock, 1997) or even 2-8bp (Armour *et al.*, 1999). Microsatellites are more diverse in vertebrates than in invertebrates while cold-blooded species have longer repeat numbers.

When isolating microsatellite loci most researchers choose loci with 10 or more repeat units in the belief that these will have high levels of size polymorphism (Schlötterer, 1998). Tri- or tetranucleotide repeat loci are preferred for analysis, because dinucleotide repeat loci usually show stronger stutter bands, complicating analyses. Amplification conditions for newly designed loci should be optimised and standardised, since similar conditions will facilitate multiplexing and make data collection efforts more time-efficient (Chambers and McAvoy, 2000).

The method used in this study for microsatellite library construction and isolation was the FIASCO-method (Zane *et al.,* 2002). This section briefly describes the protocol as applied in the laboratory. The results shown are those generated during the isolation process and are basically identical between different experiments. The sections following this one will report on polymorphic microsatellite loci developed during this study.

2.1.2) Materials and Methods

2.1.2.1) Step 1: Samples and DNA Extractions

DNA from wild and farmed abalones was used as templates for the construction of the repeat-containing genomic libraries. The individuals were from the Saldanha Bay population and Roman Bay Sea Farm. All DNA extractions were performed from muscle tissue using the CTAB-method (section 5.1.2.1.1) from Saghai-Maroof *et al.* (1984).

2.1.2.2) Step 2: Restriction and Ligation

A *Mse*I-restriction enzyme digestion and the ligation of the *Mse*I AFLP-adaptor (5'-TAC TCA GGA CTC AT-3' / 5'-GAC GAT GAG TCC TGA G-3') were done simultaneously in a 25μl reaction [250ng Genomic DNA, 1x One-Phor-All Buffer, 5mM DTT (di-thio-threitol), 50μg/ml BSA (Bovine Serum Albumin), 1μM *Mse*I-adaptor, 200μM ATP, 2.5U *Mse*I, 1.0U T4-Ligase (all reagents from New England Biosystems)]. The reaction was incubated at 37℃ in a waterbath for 3 hours. A 1:10 dilution was made of the reaction.

2.1.2.3) Step 3: 1st AFLP Amplification

An AFLP-amplification was done using AFLP-adaptor specific primers (5'-GAT GAG TCC TGA GTA A**N**-3' referred to as *Mse*I-N). A 20μl reaction volume (1x $MgCl_2$ free buffer, 1.5mM $MgCl_2$, 200μM of each dNTP, 120ng *Mse*I-N, 0.4U Go*Taq* DNA polymerase, 5μl ligation reaction) was used to amplify the fragments. The PCR-programme was set for 94℃ for 30 seconds, 53℃ for 60 seconds and 72℃ for 60 seconds. Different reactions using different cycle numbers were performed and compared to each other. The number of cycles varied from 16 to 26. The results were analysed on an agarose gel (2% w/v agarose; 1x TBE [1.08% w/v Tris, 0.54% w/v boric acid, 0.058% w/v EDTA]; ethidium bromide) and visualised while being exposed to UV-light. The reaction showing the best distribution of DNA fragments was used for the hybridisation step.

2.1.2.4) Step 4: Hybridisation

The DNA was hybridised with biotinylated $(AC)_{12}$, $(GATC)_6$, $(CAA)_8$ and $(GTGC)_6$ probes. This was performed in a 100μl reaction containing 250-500ng of DNA, 50-80pmol of a probe, 4.2x SSC (standard saline citrate) and 0.07% v/v SDS (sodium dodecyl sulphate). Four hybridisations were performed, each with a different probe. The DNA was denatured at 95℃, 3 minutes and hybridised at 25℃, 15 minutes. The hybridisation reaction was diluted by adding 300μl TEN_{100} [10mM Tris-HCl (pH 8), 1mM EDTA (pH 8), 100mM NaCl (pH 8)].

2.1.2.5) Step 5: Selective Capturing of Hybridised DNA

A total of 1mg (100μl) streptavidin coated beads were washed four times with an equal volume of TEN_{100} and resuspended in 40μl of the same buffer. A volume of 10μl of an unrelated PCR-product was added to the beads to minimize non-specific binding of genomic DNA. Forty microlitres of the beads were then added to 400μl of the hybridisation reaction and incubated at room temperature for 30 minutes while it was slowly shaken on a Vortex Genie 2 (*Scientific Industries*).

The beads-probe-DNA complex was separated using a magnetic field and the hybridisation buffer was discarded. Non-specific DNA was removed by three non-stringency washes (NS) and three stringency (S) washes. The supernatant of the third wash was saved each time and stored at -20℃. The non-stringency washes were performed by adding 400μl TEN_{1000} [10mM Tris-HCl (pH 8), 1mM EDTA (pH 8), 1M NaCl, pH 8] to the beads, gently mixing and leaving the mixture at room temperature for 5 minutes after which the beads were magnetically removed and the aqueous phase discarded. The stringency washes were performed by adding 400μl of 0.2x SSC, 0.1% v/v SDS to the beads, gentle mixing and leaving the mixture at room temperature for 5 minutes after which the beads were magnetically removed and the aqueous phase discarded.

DNA was separated from the beads-probe complex by two denaturation steps. The first denaturation step was performed by adding 50μl TE [10mM Tris-HCl (pH 8), 1mM EDTA (pH 8), pH 8] to the beads and incubating the mixture for 5 minutes at 95℃. The

supernatant (D1) was removed and quickly stored at -20℃. The second denaturation step was performed by adding 15µl 0.15M NaOH to the beads. The supernatant (D2) was neutralised by adding 1µl 0.1667M CH_3COOH (acetic acid) and then stored at -20℃.

DNA was precipitated by adding 1x volume isopropanol and 0.15M Sodium Acetate to the Eppendorf tubes containing the supernatants of the four different washes. The tubes were left overnight at -20℃. The DNA was pelleted by centrifugation at 16.1rcf for 30 minutes. The pellet was dried and resuspended in 50µl ddH_2O.

2.1.2.6) Step 6: 2nd AFLP Amplification

Two microlitres of the DNA obtained from the washes and denaturation steps were amplified using the same conditions as used during the 1st AFLP amplification (section 2.1.2.3), the only difference being the use of 30 cycles with the 2nd amplification. The products were analysed on an agarose gel (2% w/v agarose, 1x TBE; ethidium bromide) and visualised while being exposed to UV-light.

Steps 7-9 were left out for Section 2.4 and were replaced by second-generation sequencing, in this case pyrosequencing.

2.1.2.7) Step 7: Cloning

The Qiagen p-Drive vector (*Qiagen*) was used to clone the desired PCR-product. Luria Bertani (LB) medium (1% w/v bacto-tryptone, 0.5% w/v bacto-yeast, 1% w/v NaCl, 1.5% bacterial agar, pH 7.0) containing 100µg/ml ampicillin (*Roche Applied Science*), 80µg/ml X-gal and 5µM IPTG, was prepared and poured into Petri dishes.

DNA from the first denaturing step (D1; section 2.1.2.5) was amplified as described for the 2nd AFLP amplification (section 2.1.2.6) and purified using SigmaSpin Post-Reaction Purification Columns (*Sigma-Aldrich*). A transformation reaction containing 4µl purified PCR-product, 1µl pDrive cloning vector and 5µl ligation master mix (2x) was incubated at 4℃ for 30 minutes, after which 2µl of the reaction was added to competent cells and mixed gently. The competent cells were incubated on ice for 5 minutes, heat-

shocked at 42℃ for 30 seconds and immediately put on ice again for 2 minutes. A volume of 250µl SOC-medium (2% bacto-tryptone, 0.5% yeast extract, 0.05% NaCl; g/ml; 10ml 250mM KCl, 18ml 20% sterile glucose, 5ml 2M sterile $MgCl_2$, pH 7) at room temperature was added to the transformed cells and between 50µl and 100µl was plated out and incubated overnight at 37℃.

2.1.2.8) Step 8: Screening of Clones

Single colonies were selected from the plates and a colony-PCR was performed. The M13 forward (5'-GGTTTTCCCAGTCACGAC-3') and reverse (5'-GGAAACAGCTATGACCATG-3') vector-specific primers were used to screen the colonies in a 20µl PCR-reaction {2x $MgCl_2$ free buffer, 1.5mM $MgCl_2$, 200µM of each dNTP, 5pmol M13 forward and reverse primer, 0.2µl Go*Taq* polymerase (*Promega*), DNA from picked colony}. The PCR-programme was set for 94℃ for 10 minutes; 25 cycles of 94℃ for 60 seconds, 55℃ for 60 seconds, 72℃ for 60 seconds followed by 72℃ at 10 minutes. The PCR-products were analysed on an agarose gel (2% w/v agarose, 1x TBE, ethidium bromide) along with a 100bp size marker and visualised while being exposed to UV-light.

2.1.2.9) Step 9: Sequencing of Clones

The clones were sequenced in a 10µl sequencing-reaction containing 1.3µl BigDye v3 terminator (*Applied Biosystems*), 2.7µl 5x BigDye sequencing buffer (*Applied Biosystems*), 1.1pmol primer (M13F) and 20ng DNA. Clones expected to contain TG-rich regions were sequenced with the M13R primer in order to prevent too much sequence drop out when long TG-tracts are encountered. The sequencing reactions were analysed with the 3730xl DNA Analyzer (*Applied Biosystems*).

2.1.2.10) Step 10: Designing of Microsatellite Primers

The software used for designing the primer sets is given in Sections 2.2, 2.3 and 2.4.

2.1.2.11) Step 11: Amplification with Unlabelled Primers

The unlabelled primer pairs were used during this step. The PCR conditions are described for each primer pair in Sections 2.2, 2.3 and 2.4. Note that the PCR conditions were identical for both unlabelled and labelled primer pairs.

2.1.2.12) Step 12: Poly-acrylamide Gel Electrophoresis (PAGE)

PCR reactions for polymorphism testing were performed using eight different individuals from a Black Rock wild population (Aletta Bester-Van der Merwe, Stellenbosch University, Department of Genetics, Aquaculture Division) using the PCR conditions optimised for the specific marker being tested. Reactions were analysed using a 100bp size standard, by agarose gel electrophoresis (2% w/v agarose, 1x TBE, ethidium bromide) and visualised while being exposed to UV-light. The reactions were then analysed using a 100bp size marker on the PAA-gels (12% v/v acrylamide:bisacrylamide mix (Table 2.1.1) on the Cleaver Scientific Ltd. Gel system. The PAA-gels were visualised by ethidium bromide staining for 10 minutes and UV-light exposure.

Table 2.1.1: The recipe for the 12% v/v poly-acrylamide gel mix.

	STOCK	**[FINAL]**
Acrylamide/Bisacrylamide Mix	49:1 (40%)	12% (v/v)
TBE	5x	1x
Ammoniumpersulfate (APS)	1mg/ml	0.8% (v/v)
Temed		0.16% (v/v)

2.1.2.13) Step 13: Labelling of Primers

Only one primer per pair was labelled of the primer pairs that were judged to be polymorphic after PAGE. Three general criteria were taken into account when a primer for labelling was selected: 1) the longest primer in the set is chosen; or 2) the primer closest to the repeat is chosen; or 3) the primer containing the most cytosine and guanine at the 3'-end is chosen. Primers were labelled with the fluorescent labels VIC, NED, FAM and PET (*Applied Biosystems*).

2.1.2.14) Step 14: Characterisation of Labelled Primers

This step is described in Sections 2.2, 2.3 and 2.4

2.1.3) Results and Discussion

2.1.3.1) Step 3: 1st AFLP Amplification

Figure 2.1.1 shows the results of the PCR reactions performed at 18, 20, 22, 24 and 26 cycles. The optimal number of PCR cycles was found to be 18 and that PCR product was used further. The products were mostly above 200bp in size and smears were seen. The PCR product with the most evenly spread smear was used in the steps to follow. This would be to ensure that the fragment sizes are represented equally in the rest of the protocol.

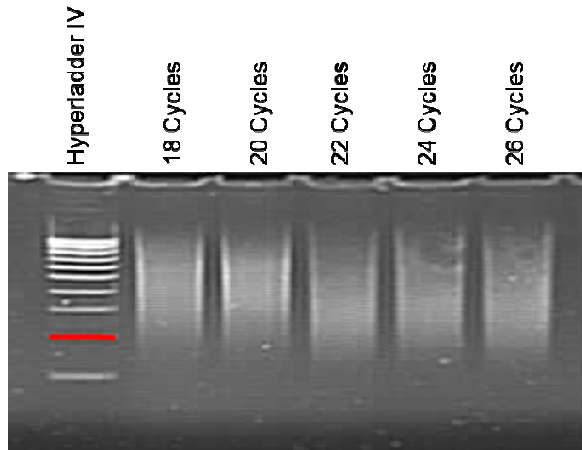


Figure 2.1.1: Agarose gel showing the results of a 1st AFLP amplification (18 to 26 cycles). The red line indicates the 200bp band.

2.1.3.2) Step 6: 2nd AFLP Amplification

The PCR results of the non-stringency (NS) wash step, stringency (S) wash steps, first denaturation (D1) and second denaturation (D2) is shown in Figure 2.1.2. The smears were above 200bp as expected, since the same size range was seen after the 1st AFLP amplification. D1 was used in further steps.

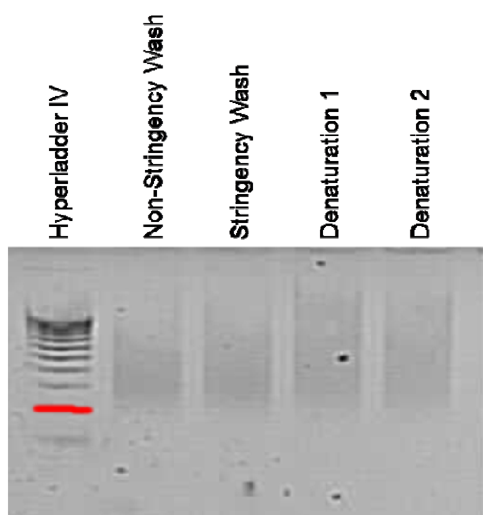


Figure 2.1.2: Agarose gel showing the results of a 2nd AFLP after the washing steps. The red line indicates the 200bp band.

2.1.3.3) Step 8: Screening of Clones

Figure 2.1.3 shows the results of a colony screening. All clones above 250bp were sequenced. The PCR product size of vector DNA without inserts is 250bp and anything above that served as a positive indication that an insert was present.

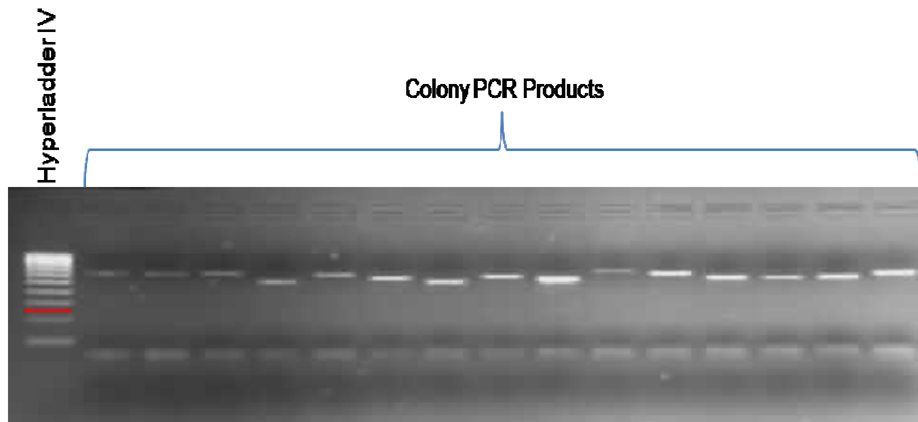


Figure 2.1.3: Agarose gel showing the results of a colony PCR. The red line indicates the 250bp position.

2.1.3.4) Step 11 and 12: Primer Optimisation and PAGE

All primer pairs that amplified the expected fragment size were analysed with PAGE to test levels of polymorphism. Figure 2.1.4 shows the results of *Hm*LCS1T optimisation and Figure 2.1.5 shows polymorphism testing of the same marker.

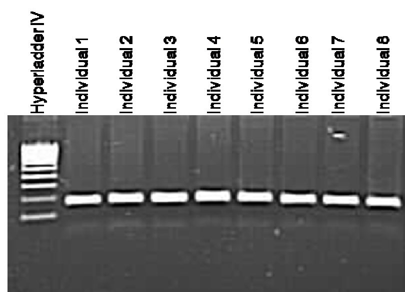


Figure 2.1.4: Agarose gel results for *Hm*LCS1T.

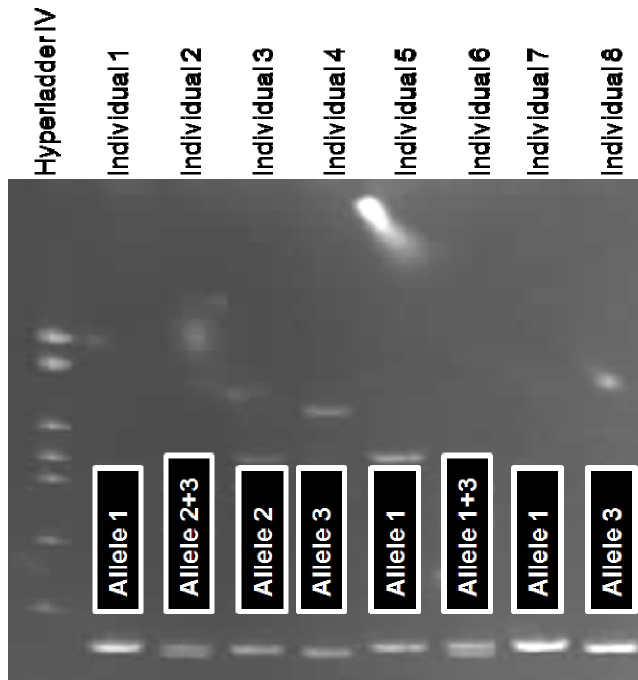


Figure 2.1.5: Poly-acrylamide gel electrophoresis results for *Hm*LCS1T, showing 3 alleles.

2.1.3.5) Step 14: Characterisation of Labelled Primers

See Sections 2.2, 2.3 and 2.4 for results and discussions.

SECTION 2.2

Isolation and Characterisation of 63 Microsatellite Loci for the Abalone, *Haliotis midae*

Published as: Slabbert, R., Ruivo, N.R., Van den Berg, N.C., Lizamore, D.L., Roodt-Wilding, R., 2008. Isolation and characterisation of 63 microsatellite loci for the abalone, *Haliotis midae*. Journal of the World Aquaculture Society 39:429-435. (Appendix C)

2.2.1) Introduction

Haliotis midae is an important species within the fisheries industry of South Africa, with an output of almost 750 tonnes in 2005 (Loubser, 2005). *Haliotis midae* has been cultured in captive conditions since 1981 (Genade *et al.*, 1985, 1988), but the first real effort to establish commercial abalone farms was made in 1990 by industrial and academic institutions (Sales and Britz, 2001). The species is as yet undomesticated and few breeding programmes have been undertaken. Genetic characterisation could play a large role in further development of the resource. Currently, molecular work done on *H. midae* has been limited to population structure studies and some parentage analyses. To assist the industry in genetic improvement programmes to enhance their stocks, more molecular markers are required. These will be used to construct the first linkage map for this species, identify QTL and perform accurate parentage assignments. To date, 11 polymorphic microsatellite loci have been reported for *H. midae* (Bester *et al.*, 2004). Here we report the characterisation of 63 microsatellite loci that will facilitate future molecular studies and breeding programmes.

2.2.2) Materials and Methods

2.2.2.1) DNA Extractions

Genomic DNA was isolated from mantle tissue following a standard CTAB extraction method (Saghai Maroof *et al.*, 1984). Tissue was homogenized in 500µl of CTAB lyses buffer containing 0.5mg/ml Proteinase K (*Sigma-Aldrich*) and incubated at 60°C in a

waterbath. Following chloroform: isoamyl alcohol (24:1) extractions, the supernatant were precipitated with ⅔ volume of 100% cold isopropanol. DNA was washed with 200μl of 70% ethanol, redissolved in 100μl of distilled water and stored at -20℃.

2.2.2.2) Microsatellite Enrichment

In this study, microsatellite repeat sequences were isolated using an enrichment technique (FIASCO) (Zane *et al.*, 2002). Enriched partial genomic libraries were constructed using DNA from four individuals. For this, 250ng DNA was simultaneously digested with *Mse*I and ligated to *Mse*I AFLP adaptors. DNA was selectively amplified using a mixture of four adaptor specific primers (*Mse*I-N) and hybridized independently with a biotinylated $(AC)_{12}$ and a $(GATC)_6$ probe. Repeat-containing fragments were recovered by streptavidin magnetic particles and cloned into a Qiagen p-Drive vector (*Qiagen*) in order to produce a highly enriched microsatellite library.

Clones were sequenced using a 3730xl DNA Analyzer (*Applied Biosystems*) to verify the presence of repeat motifs. Primer sets were designed using Oligo version 4.1 (Rychlik and Rhoads, 1989). All primer information was submitted to GenBank (Table 2.2.1).

2.2.2.3) Genotyping

A total of 32 individuals from Black Rock, on the East coast of South Africa, were genotyped to test the level of polymorphism of the markers. For each primer pair, one of the primers was labelled with FAM, NED, VIC or PET (*Applied Biosystems*) dyes. All polymerase chain reactions (PCRs) were conducted in a Geneamp 2700 thermo cycler (*Applied Biosystems*) in 10μl reactions containing 20ng DNA, 0.2μM of each primer, 200μM deoxyribonucleotide triphosphates (dNTP's), 0.1U of Go*Taq* polymerase (*Promega*), 1x Go*Taq* Flexi Buffer (*Promega*) and 2mM $MgCl_2$. Various PCR programmes were used. Programme 1: initial denaturing step at 94℃ for 5 minutes followed by 25 cycles of 30 seconds at 94℃, 30 seconds at 55℃, and 30 seconds at 72℃, and a final extension of 7 minutes at 72℃. Programme 2: initial denaturing step at 94℃ for 5 minutes followed by 35 cycles of 45 seconds at 94℃, 45 seconds at 55-65℃, and 45 seconds at 72℃, and a final extension for 7 minutes at 72℃. Programme 3: initial denaturing step at

94°C for 5 minutes followed by 30 cycles of 30 seconds at 94°C, 1 minute at 55-65°C, and 1 minute at 72°C, and a final extension for 10 minutes at 72°C. Programme 4: initial denaturing step at 94°C for 5 minutes followed by 25 cycles of 45 seconds at 94°C, 45 seconds at 55°C, and 45 seconds at 72°C, and a final extension for 10 minutes at 72°C. Programme 5 (touch-down): an initial denaturing step of 94°C for 5 minutes followed by 2 cycles of 30 seconds at 94°C, 30 seconds at 65°C and 30 seconds at 72°C. Thereafter the annealing temperature was lowered by 1°C in consecutive cycles, until an annealing temperature of 55°C was reached and maintained for 30 cycles for 60 seconds at 94°C, 60 seconds at 55°C and 60 seconds at 72°C, with a final extension for 7 minutes at 72°C. PCR products were separated on a 3730xl DNA Analyzer (*Applied Biosystems*) and analysed using GeneMapper version 4 (*Applied Biosystems*).

2.2.2.4) Statistical Analyses

Observed and expected heterozygosities, null allele frequencies and the probability of Hardy-Weinberg equilibrium were calculated using CERVUS version 3.0.3 (Kalinowski *et al*., 2007), GENEPOP version 4 (Raymond and Rousset, 1995) and MICRO-CHECKER version 2.2.3 (Van Oosterhout *et al*., 2004). Corrections of the significance levels for multiple tests were performed following the Bonferroni procedure (Rice, 1989).

2.2.3) Results and Discussion

A total of 983 recombinant clones were sequenced of which 47% contained repeat motifs. A total of 192 primer pairs were designed and 63 were found to amplify polymorphic loci. The PCR primer sequences, optimal annealing temperature, repeat motif, and allele size ranges are shown in Table 2.2.1. Loci showed an average of 13.1 (range 2-27) alleles per locus and mean observed and expected heterozygosities of 0.573 (range 0-0.955) and 0.775 (range 0.082-0.958), respectively. A total of 25 loci did not conform to Hardy-Weinberg expectations (Table 2.2.1). This could be caused by null alleles (24 of these loci exhibited null alleles) but can also be attributed to other factors such as non-random sampling (not sampled over a large geographical area), sample size or the evolutionary history of a population. Null alleles, caused by homozygote excess,

were detected in a total of 33 loci (Table 2.2.1). The high levels of polymorphism and heterozygosity are comparable to previous isolation studies in *H. midae* (Bester *et al.*, 2004; H_o = 0.14 to 0.93), *H. rubra* (Baranski *et al.*, 2006b; H_o = 0.00 to 1.00) and *H. discus hannai* (An and Han, 2006; H_o = 0.17 to 1.00). The newly isolated loci will provide an invaluable tool for population and kinship studies. The reported markers are currently being applied in parentage as well as QTL studies. The new markers should also be very useful in future mapping studies and molecular breeding programmes for *H. midae*.

Table 2.2.1: Primer sequences and characteristics of 63 *Haliotis midae* microsatellite loci.

Locus	Repeat Sequence	Primer Sequence (5'-3')	n	Size range (bp)	T_a (P^a)	H_o	H_e	n_a	P	Null	Accession number
*Hm*AD102T	$(ACTC)_{15}$	**F** ACATTGGGGTTCTCAATCA **R** TAACGGGACAATGAATAAACTA	28	199-263	TD (5)	0.32	0.93	16	***	Y	DQ785747
*Hm*DL34aR	$(ATC)_{13}$	**F** TGCAAGTCCAGAGTATGTGG **R** TGTGCTTGAGAGAGATGGTG	25	230-320	60°C (4)	0.56	0.94	20	***	Y	EF054860
*Hm*DL34bT	$(CAGA)_{16}$	**F** CATCACCATCTCTCTCAA **R** TAAATCACATAATCATGAACCTG	32	104-166	TD (5)	0.84	0.94	16	***	N	EF054860
*Hm*DL50M	$(TGTC)_{11}(GGTC)_6$	**F** TTATTGCGCCATACAGTTCG **R** TCAGGCAGACAGACATACCG	25	202-280	60°C (4)	0.80	0.93	18	0.022	Y	EF054861
*Hm*DL110T	$(TCAC)_{23}$	**F** TCCTAAAAGCTGCATAACACCA **R** TGAAGGGGATAAAACCAGGA	32	138-278	55°C (4)	0.75	0.92	17	0.027	Y	EF054864
*Hm*DL123D	$(CT)_{20}$	**F** ATCCCCTAAATCTGCGTCAA **R** CGCCTGTAAAATGCACAAGA	27	266-374	60°C (4)	0.22	0.95	20	***	Y	EF054865
*Hm*DL131M	$(AC)_{14}(TC)_8$	**F** GTGACAGAAGGTGGAAGTGGA **R** TTACGACAAGCATGGGTTACTG	22	195-277	55°C (4)	0.50	0.73	10	0.027	Y	EF054867
*Hm*DL151T	$(CTGA)_{14}$	**F** AATAAGCCAAAACACGGAGCA	30	156-234	55°C (4)	0.53	0.54	8	0.675	N	EF054868

		R GGGTTCGATTCCCCACAA									
*Hm*DL207M	$(CT)_7(TG)(CT)_5$ $(TT)(CT)_6(CA)_6$	**F** CATACACACGCATTCACATACA **R** CATCAACAGGTCCAAGGAAG	25	211-283	55°C (4)	0.80	0.90	15	0.279	N	EF054869
*Hm*DL214T	$(TGAG)_{15}$	**F** CCTGCATCCATTTAGCTCTGAT **R** GTTGTGCTGGATTGGGATGT	32	199-249	60°C (4)	0.28	0.31	5	0.126	N	EF054871
*Hm*G16D	$(GT)_{13}$	**F** ATATTGCTGAATGAGGGGTA **R** CACCACCACTACCACCATAC	32	285-321	TD (5)	0.94	0.88	9	0.066	N	DQ785744
*Hm*G46D	$(TG)_{17}$	**F** GATGAGTCCTGAGTAAGTAAATAAT **R** ATCCGTGTACACACTCACTG	30	221-237	TD (5)	0.60	0.60	6	0.755	N	DQ785745
*Hm*G53T	$(CACT)_{31}$	**F** TGCTGTTGAAGTCTTTGTCC **R** TATCAGTCCCGCATCTATTG	25	104-200	TD (5)	0.32	0.96	20	***	Y	DQ785746
*Hm*IF33M	$(GATA)_3(GACA)_{30}$	**F** ATGGATAGCTAGCGAGATATAGA **R** TAGTGATTTTACGGAAACGG	30	104-310	TD (5)	0.23	0.85	17	***	Y	DQ785769
*Hm*LCS1T	$(CGTG)_6$	**F** TTGAAAAACACAGGAAATGC **R** AGTAAAGGTTGTTTCGTGAAAG	27	154-174	TD (5)	0.67	0.63	9	0.895	N	DQ825701
*Hm*LCS5M	$(GCTC)_4(ACTC)_3$	**F** AACTCAATCCCATCTATGGC **R** CTTTGACCACTAGGCTACCC	24	472-482	TD (5)	0.00	0.08	2	0.006	N	DQ825705

*Hm*LCS7M	$(GT)_7(GCGT)_6(GT)_7$	**F** ATGATGCTATTCAGCTCTCG **R** ATGATGAAAGTGGCGTAAAA	29	196-270	TD (5)	0.69	0.89	16	***	Y	DQ825707
*Hm*LCS9M	$(GC)_2(GT)_2(GCGTGT)_2$ $(GCGT)_2(GC)$	**F** TTGGCATAGGATGGACTTGT **R** GATGCGGCCACAGGC	30	281-299	TD (5)	0.27	0.51	4	0.001	Y	DQ993214
*Hm*LCS18M	$(CAC)_2(GAC)(TAC)_3$ $(CAC)_4(TAC)_3(CACGAC)$ $(TAC)_{12}(CAC)_3(TAC)_4$ $(AAC)(TAC)(CAC)_2(TAC)_4$ $(CAC)_2(TAC)_4(CAC)$	**F** CGGTGATAACGATAGTTGGT **R** GGTAGTTGCAGTAATGGTATTC	26	144-266	55℃ (1)	0.81	0.91	19	0.083	N	DQ993217
*Hm*LCS37M	$(GA)_{13}(CA)(GA)_8(CA)(GA)_4$ $(CAGA)_9(CA)_5$	**F** ACCTTGAGGTCCTGTCAGTC **R** AAGTATTCCAGAAACGCTTCT	21	194-376	55℃ (1)	0.76	0.96	23	***	Y	DQ993229
*Hm*LCS47M	$(AC)_6(AG)(AC)_{15}$	**F** CAAAACAAAAAAACAACAAC **R** ATCACGTATTGATTGATTCTAT	32	172-208	TD (5)	0.78	0.85	13	0.044	N	DQ993228
*Hm*LCS48M	$(CT)_{14}(CA)_9$	**F** ATGTGTGAGCACGTGTTTCT **R** AGTCACAAGCTACATCGAATCT	21	334-352	TD (5)	0.52	0.55	9	0.466	N	DQ993227
*Hm*LCS55T	$(GTGA)_5$	**F** ATTGTTGATAATGGCATTGG **R** TTCATTACACGTCTAAATCCAA	32	268-286	TD (5)	0.25	0.43	7	0.006	Y	DQ993226
*Hm*LCS63T	$(CACT)_4$	**F** AGTCTTCCTCCAGTTCTCCA **R** AGCAAACATACGTGACTTGG	26	208-230	TD (5)	0.04	0.54	4	***	Y	DQ993223

*Hm*LCS67M	$(GAGT)_3(GT)_5(GC)_4$	**F** ATGGCGGAGGATATAATGAT **R** GAAGCCTATTTCTGGTGTCC	31	258-296	TD (5)	0.45	0.73	8	***	Y	DQ993222
*Hm*LCS72M	$(TG)_5(CGTG)_2$	**F** TGTGACAGGAAAGCCTAAAG **R** GTGATAGAGGGAGAAAGTATGG	28	261-281	TD (5)	0.25	0.76	7	***	Y	DQ993220
*Hm*LCS73T	$(GAGT)_{10}$	**F** CCATGGCTCAGAATATTGAA **R** CATGTTGGAGATCTGGTTTG	26	151-245	TD (5)	0.31	0.76	15	***	Y	DQ993219
*Hm*NR20M	$(TCC)_5(TAC)_7$	**F** CTACAACAAACGCCGATG **R** TGCAGTAATAGGGGTACCAG	27	187-289	60℃ (2)	0.85	0.85	11	0.716	N	EF063097
*Hm*NR54H	$(TTAGGG)_4$	**F** TAACACTAAGTCCCTCACCC **R** CATTCTACATTCGACATTCG	32	329-407	60℃ (2)	0.78	0.76	10	0.217	N	EF063103
*Hm*NR106D	$(TG)_{15}$	**F** TCCTTGGCCAGAATAACC **R** TATATGGTCTGCATCGCTG	31	329-389	60℃ (2)	0.81	0.88	16	0.076	N	DQ825709
*Hm*NR120T	$(TGAG)_{23}$	**F** TTGAGCATGAGTCGTTGAGC **R** ACCTGCTCTTTAGCTCAGATGG	29	235-347	62℃ (2)	0.90	0.94	24	0.197	N	EF121745
*Hm*NR136D	$(CA)_{11}$	**F** GAGTAATATGGGCACCTCG **R** GTTTGGAATGTCTGATTGGA	32	211-309	60℃ (2)	0.75	0.80	20	0.156	N	DQ825710
*Hm*NR185D	$(GT)_{13}$	**F** TAGAGTTCATGTGTGTACGTGTGC	31	132-160	65℃ (2)	0.61	0.88	11	0.010	Y	EF121750

		R TACCTGTAACGCGCTTGCT									
*Hm*NR191T	(GAGT)$_6$	**F** CCACATGGGTACAAAGTCC **R** TTAGTTTTACGCCGCACTC	31	241-497	62°C (2)	0.81	0.86	16	0.136	N	EF121752
*Hm*NR180D	(GT)$_{24}$	**F** ACAAGGAGGCGTGAAATCTGC **R** GCATTGTTACCCCCTACAAAGACC	31	269-297	65°C (2)	0.52	0.91	12	***	Y	EF121748
*Hm*NR224T	(CATA)$_{18}$	**F** TGTCCATAGCAGCCCCTTAC **R** ACATCTTGTTGCCGTTGTTG	22	444-540	65°C (2)	0.95	0.95	20	0.529	N	EF512269
*Hm*NR258R	(CAA)$_{11}$	**F** GCATCGCCTGATTTGATTC **R** CAGAAGGGTGGGTTGTAGTATG	32	239-257	62°C (2)	0.72	0.76	6	0.627	N	EF512272
*Hm*NR281P	(CTCAA)$_{24}$	**F** AACCTTCAGTAACCCATGC **R** TGAATAGGCACCATAAAGGG	28	225-375	60°C (2)	0.71	0.92	21	0.026	Y	EF512274
*Hm*NR289P	(GTTGT)$_5$	**F** GCAAGACAGACATCCAAGAC **R** TACAAATCCCGACACAAGAG	32	301-316	65°C (2)	0.25	0.26	4	0.487	N	EF512275
*Hm*NS6T	(ACGC)$_6$	**F** TGAGAGACATTTGAAGCATTTA **R** AACACTCACGTACGCATACAC	32	186-230	55°C (3)	0.59	0.78	9	0.031	Y	EF367117
*Hm*NST7T	(CACT)$_{26}$	**F** CACATGGGTACAATGTGTGAAG **R** GGTAGCACTGTTTCTCACGA	32	228-328	60°C (3)	0.94	0.95	21	0.565	N	EF455618

*Hm*NS14R	(TTG)$_5$	**F** GCTCTGGTGTATGTTGTGTCA **R** TTGATCAAGTTGCACATGAAT	31	252-261	60℃ (3)	0.03	0.21	4	***	Y	EF367115
*Hm*NS19L	(AACACCC)$_9$	**F** ACAACAACAAAGGTGGTCAA **R** CAATGAATAGCTATGGGTCG	32	178-252	55℃ (3)	0.56	0.95	25	***	Y	EF033330
*Hm*NS28D	(CA)$_{16}$	**F** CAGTCAATTTTCATCGCATT **R** AGGTCGTTTTTCTCCTTCAG	32	123-185	55℃ (3)	0.91	0.94	19	0.450	N	EF033332
*Hm*NS31D	(GT)$_4$(CT)(GT)$_8$	**F** CTCGGGTTCAGTTACCTACA **R** CTTGCTGACTTCGATCACAC	32	238-288	55℃ (3)	0.34	0.80	14	***	Y	EF033333
*Hm*NSa34D	(AC)$_7$	**F** CATTCCACGCTGAAGAAATC **R** TGAGATGAGCGTGAAAATGT	31	185-189	50℃ (3)	0.10	0.36	3	***	Y	EF367118
*Hm*NS38T	(TCAC)$_{10}$	**F** CTGAGACCCAAAGTTTTCTTTA **R** ATCTATGTTCAGGGTGTCAGTG	31	402-474	55℃ (3)	0.74	0.83	11	0.596	N	EF367113
*Hm*NS56D	(CA)$_{20}$	**F** TTCGGCAAGTGAATGTCTAG **R** CCGAGTTTGGAATGTCTGAT	31	211-253	55℃ (3)	0.84	0.85	16	0.528	N	EF455619
*Hm*NS58D	(GTT)$_8$	**F** TGCCACTCAAATGTTCCTTA **R** CTATTTCAGGTGTCCCCAGT	32	233-272	60℃ (3)	0.78	0.86	10	0.034	N	EF367119
*Hm*NS100T	(GAGT)$_{16}$	**F** CAGTTTTTGTTAGGGATTTCAT	32	232-454	60℃ (3)	0.53	0.92	14	***	Y	EF367114

		R GAAAAAGACTGTTGATGGGG									
*Hm*RS27T	$(TCAC)_{30}$	**F** TACCGGTATAAACCGAACAC **R** GTTCAGCAAGAAATCAGTCG	29	224-428	TD (5)	0.41	0.93	23	***	Y	DQ785751
*Hm*RS36T	$(CTCA)_{7}$	**F** TCAACTCACTCAACCAACCA **R** TAGTCTATGTTGCGGTCTGC	25	348-366	TD (5)	0.52	0.67	6	0.398	N	DQ785753
*Hm*RS37D	$(AC)_{15}$	**F** AACTTTCAGGACGAAAGGG **R** ATATGTTAGATGTGCGGCAA	29	335-353	TD (5)	0.55	0.69	7	0.001	N	DQ785754
*Hm*RS38M	$(GT)_{14}(GA)_{9}$	**F** ATCAAGATATCTCCCAAGGG **R** CACACATACACACAAACACACA	31	229-269	TD (5)	0.58	0.89	13	***	Y	DQ785755
*Hm*RS54D	$(AC)_{13}$	**F** TTTGTGAAATAGCATGGAGC **R** TGTAAATAATCGAGCCTGGA	31	224-236	TD (5)	0.8387	0.5928	4	0.022	N	DQ785774
*Hm*RS61H	$(GAGATA)_{3}$	**F** GGTTTACTCAGGGTTTAGGG **R** AAATTTTGGGGAGTTTACAAC	32	507-549	TD (5)	0.91	0.86	12	0.017	N	DQ785776
*Hm*RS62D	$(GT)_{12}$	**F** ATCCACTTTGACTTGTTTATTTG **R** GTGTGTACTGATGTTCTGCCA	26	262-300	TD (5)	0.42	0.86	14	***	Y	DQ785777
*Hm*RS80M	$(GAGT)_{17}(GA)_{3}(GAGT)$	**F** AATGGTTCTTTTGATCCCTT **R** TCATTATAACATCTGGCCTTG	31	178-240	TD (5)	0.71	0.92	18	0.005	Y	DQ785756

*Hm*RS83M	(GTTT)$_2$(GT)$_{34}$(TTTG)$_6$	**F** TGACTCTCAGTTTCACATCCA **R** ATATGTCACATATCACAAATGCA	31	192-362	TD (5)	0.65	0.96	27	***	Y	DQ785757
*Hm*RS88M	(GT)$_{10}$(GCGT)$_2$(GT) (GCGT)(GT)$_2$	**F** TCAGAATATTGCACCCAAAC **R** CATGAACCATCAATACTGCC	30	311-349	TD (5)	0.80	0.87	12	0.282	N	DQ785758
*Hm*RS90M	(GT)(GTGC)$_2$(GT)$_4$(GTG C) (GT)(GTGC)	**F** ATTTGATACCTTGTCTCGCTT **R** TGAGATCGAAAATCCCACTAT	31	434-458	TD (5)	0.23	0.54	4	***	Y	DQ785759
*Hm*RS117M	(GAGT)$_{33}$(GCGT)$_3$	**F** GAGCACACGAATACCAAGAG **R** AATTCAACCCCTCCTCACT	30	171-307	TD (5)	0.43	0.91	22	***	Y	DQ785765
*Hm*RS129D	(GT)$_{15}$	**F** TTGAATCTGACTGAACTGGG **R** TATAAGCCACATTCTGAGGAA	29	251-295	TD (5)	0.52	0.91	16	***	Y	DQ785766

n = sample size; *Ta* = optimal annealing temperature; TD = touchdown polymerase chain reaction; P[a] Polymerase chain reaction programme; H_o = observed heterozygosity; H_e = expected heterozygosity; n_a = number of alleles; Y = null alleles present; N = null alleles not present.

*** $P < 0.0001$ (for Hardy-Weinberg Equilibrium).

SECTION 2.3

Isolation and Segregation of 44 Microsatellite Loci in the South African Abalone *Haliotis midae* L.

Published as: **Slabbert, R., Hepple, J., Venter, A., Nel, S., Swart, L., Van den Berg, N.C., Roodt-Wilding, R., 2010. Isolation and segregation of 44 microsatellite loci in the South African abalone *Haliotis midae* L. Animal Genetics 41:332-333. (Appendix C)**

2.3.1) Introduction

Haliotis midae is an important commercial aquaculture resource in South Africa and as a result the focus of the industry is shifting towards a genetic improvement programme. Molecular markers are an important component of such a programme and can be used for various applications including pedigree analysis, linkage mapping, QTL mapping and marker assisted selection (Baranski *et al.*, 2006a; Hayes *et al.*, 2007; Slabbert *et al.*, 2009a). Microsatellite loci were isolated from *H. midae* genomic DNA using either the FIASCO method (Zane *et al.*, 2002) or the SNX Unilinker method (Hamilton *et al.*, 1999).

2.3.2) Materials and Methods

The FIASCO method was modified to accommodate additional restriction enzymes, namely *Mse*I, *Eco*RI and *Msp*I (*New England Biolabs*). New adaptor and primer sequences were designed for *Eco*RI and *Msp*I (Table 2.3.1). *Alu*I and *Rsa*I were used for the SNX method. The DNA libraries were enriched using an AC-rich probe and cloned using the Qiagen PCR Cloning kit. Sequencing was performed on a 3730xl DNA analyser (*Applied Biosystems*). Sequences were edited using Sequence Scanner version 1 (*Applied Biosystems*). Primers were designed for repeat containing sequences using BatchPrimer version 1 (You *et al.*, 2008) and labeled using fluorescent dyes.

DNA from 32 individuals from each of two full-sib families (Family 7B and Family 42A) was extracted and used for PCR amplification. PCR reactions were carried out in a final volume of 10μl containing 20ng DNA, 0.2μM of each primer, 200μM deoxyribonucleotide triphosphates (dNTPs), 0.1U of Go*Taq* polymerase (*Promega*), 1x Go*Taq* Clear Flexi Buffer (*Promega*) and 2mM $MgCl_2$. PCR programmes are as follows: P1) an initial denaturing step of 94℃ for 3 minutes followed by 37 cycles of 40 seconds at 93℃, 40 seconds at variable temperatures and 45 seconds at 72℃ with a final extension for 10 minutes at 72℃; P2) an initial denaturing step of 94℃ for 5 minutes followed by 10 cycles of 30 seconds at 94℃, 30 seconds at 65℃ and 30 seconds at 72℃. The annealing temperature was lowered by 1℃ in consecutive cycles, until an annealing temperature of 55℃ was reached. This was then followed by 30 cycles of 30 seconds at 94℃, 30 seconds at 55℃ and 30 seconds at 72℃, with a final extension for 7 minutes at 72℃; P3) an initial denaturing step of 94℃ for 5 minutes followed by 25 cycles of 30 seconds at 94℃, 60 seconds at variable temperatures and 60 seconds at 72℃, with a final extension for 10 minutes at 72℃. Genotyping was performed using a 3730xl DNA Analyser (*Applied Biosystems*) and GeneMapper version 4 (*Applied Biosystems*). The Mendelian segregation patterns (1:1:1:1, 1:2:1, 1:1) of the selected markers were examined in these families using the chi-square test.

Table 2.3.1: Primer and adaptor sequences of *Eco*RI and *Msp*I restriction enzymes which were used for the FIASCO method.

Adaptor / Primer Name	**Sequence (5'-3')**
*Eco*RI-1 primer	GACTGCGTACCAATTCA
*Eco*RI-2 primer	GACTGCGTACCAATTCT
*Eco*RI-3 primer	GACTGCGTACCAATTCC
*Eco*RI-4 primer	GACTGCGTACCAATTCG
*Eco*RI-Adaptor 1	CTCGTAGACTGCGTACC
*Eco*RI-Adaptor 2	AATTGGTACGCAGTCTAC

*Msp*I-1 primer	GATGAGTCCTGAGGCGGA
*Msp*I-2 primer	GATGAGTCCTGAGGCGGT
*Msp*I-3 primer	GATGAGTCCTGAGGCGGC
*Msp*I-4 primer	GATGAGTCCTGAGGCGGG
*Msp*I-Adaptor 1	GACGATGAGTCCTGAG
*Msp*I-Adaptor 2	CGCTCAGGACTCAT

2.3.3) Results and Discussion

A total of 978 recombinant clones were sequenced, 49% of these contained repeat motifs. A total of 222 primer pairs were designed of which 44 were found to be polymorphic (Table 2.3.2).

Among a total of 88 marker-family combinations (44 x 2), 38 of these were informative (at least one parent must be heterozygous for a locus), 18 combinations were monomorphic, eight combinations had three or more alleles and 24 combinations could not be reliably scored or amplified (Table 2.3.2). Of the 38 informative marker-family combinations 10 did not conform to expected Mendelian segregation patterns ($P < 0.05$). Of these 10 combinations, three (*Hmid*0006M for Family 42A and *Hmid*2044T, *Hm*LCS147T for Family 7B) could be explained by the presence of null alleles. *Hmid*0006M and *Hm*LCS147T conformed to Mendelian segregation after we corrected for the null alleles: Due to the polymorphic nature of the markers, the null alleles for *Hmid*0006M and *Hm*LCS147T were easily identified. The offspring were then reassigned as being heterozygotes for the null allele after which a second segregation test was performed. These markers then conformed to the expected segregation patterns (1:1:1:1; $P > 0.05$; Table 2.3.2). For example: if one parent is heterozygous A_1/A_2 and the other homozygous A_3/A_3, the offspring should contain the genotypes A_1/A_3 and A_2/A_3, with an expected segregation ratio of 1:1. However, if the second parent possesses a null allele and therefore only appears to be homozygous, the offspring should contain two heterozygote types: A_1/null, A_2/null, A_1/A_3 and A_2/A_3 with an expected segregation pattern of 1:1:1:1. The

null allele for *Hmid*2044T could not be identified because heterozygous genotypes could not be distinguished from the homozygotes. The distortion of the other combinations (*Hmid*0053D, *Hmid*0310D, *Hmid*4018D, *Hm*LCS71T and *Hm*NSp31M for Family 42A and *Hmid*0310D and *Hmid*0065M for Family 7B) could possibly be explained by PCR errors or scoring difficulties (Jones and Ardren, 2003). These markers should be used with caution.

Table 2.3.2: Marker information, PCR conditions, segregation analyses and accession numbers of 44 novel microsatellite loci for *Haliotis midae*. Informative parent combinations are shown by a cross between two genotypes, while non-informative combinations will be monomorphic, duplicated or have non-reliable genotypic data.

Locus Name	Repeat Sequence (5'-3')	Primer Sequence (5'-3')	PCR Programme T_a	Family 42A			Family 7B			GenBank Accession Number
				Parents (male x female)	Expected Segregation	*P*-value[1]	Parents (male x female)	Expected Segregation	*P*-value[1]	
*Hmid*0006M	(ACAT)$_n$(AC)$_n$(ACAT)$_n$	**F** ATGAATTAGTGAACGCCTT **R** TTTGTTAGATATTCGCCAT	P2 Touch-down	312/312[2] x 273/281 312/U[3] x 273/281	1:1[2] 1:1:1:1[3]	***[2] 0.771[3]	Non-reliable Genotypes			GQ927108
*Hmid*0007C	(AC)$_n$...(ACGC)$_n$	**F** AAATTATTCGGCTAAATGC **R** AGGCCACACACCCATTTCC	P2 Touch-down	Non-reliable Genotypes			359/359 x 349/359	1:1	0.080	GQ927109
*Hmid*0053D	(GT)$_n$	**F** AAACTGACCATCGCACTT **R** AGACACATGCTCACGCAC	P2 Touch-down	096/096 x 102/108	1:1	***	102/106 x 094/096	1:1:1:1	0.372	GQ927110
*Hmid*0065C	(CT)$_n$...(AC)$_n$...(AC)$_n$...(AC)$_n$	**F** TATGAGCACCAATCAACGC **R** TTTCCTGTCTCTTCCGCAC	P2 Touch-down	246/253 x 242/253	1:1:1:1	0.054	234/251 x 209/234	1:1:1:1	***	GQ927111
*Hmid*0136T	(ACTC)$_n$	**F** GATATCGTTATAAGCGGTG **R** ATATCGGGTAGCATTGTCA	P2 Touch-down	085/089 x 085/085	1:1	0.160	Monomorphic			GQ927112
*Hmid*0154C	(GAT)$_n$...(GAT)$_n$...(GAT)$_n$	**F** GTAACAGTGTTTAGCACCA **R** CATGTTTTGTATCATAAGG	P2 Touch-down	243/247 x 227/243	1:1:1:1	0.068	214/229 x 214/243	1:1:1:1	0.289	GQ927113

*Hmid*0166R	(GAT)$_n$	**F** GCATGTGTTTATGACTGATGGC **R** ACACTCAAGCGACTTTGGC	P2 Touch-down	Duplicated Locus			Duplicated Locus			GQ927114
*Hmid*0221T	(ACAG)$_n$	**F** GGTAGATCAACTGTCGCAGG **R** ATATTATCAAATCTGTTGCC	P2 Touch-down	156/168 x 156/172	1:1:1:1	0.321	164/172 x 156/168	1:1:1:1	0.741	GQ927115
*Hmid*0310D	(GT)$_n$	**F** GATATGTTGAAATGGGGTT **R** AACAAACACAGACACTCAG	P2 Touch-down	111/114 x 118/124	1:1:1:1	***	110/118 x 105/116	1:1:1:1	***	GQ927119
*Hmid*0315M	(GCGT)$_n$(GT)$_n$	**F** CGGTACTATGGTGTTCGCTT **R** AAAAACTGTTAGGGAGGCAA	P2 Touch-down	113/128 x 122/122	1:1	0.263	113/117 x 113/128	1:1:1:1	0.431	GQ927120
*Hmid*0321C	(GT)$_n$...(GT)$_n$	**F** AATATGTGGAGCACGACGC **R** AGGGCTACCCCACTGGTC	P2 Touch-down	147/155 x 155/155	1:1	1.00	Monomorphic			GQ927121
*Hmid*0553D	(GT)$_n$	**F** ACTAAGTTTCATAACGAACGCAC **R** TGTTCAGGACACACCCCAC	P2 Touch-down	Non-reliable Genotypes			160/160 x 160/162	1:1	0.576	GQ927122
*Hmid*0558D	(GT)$_n$	**F** TGGAGCGTGTGAGTGAGAG **R** GATTTGGGCGCATGGACAG	P2 Touch-down	111/117 x 111/113	1:1:1:1	0.626	113/117 x 107/111	1:1:1:1	0.654	GQ927123
*Hmid*0561D	(CG)$_n$	**F** ACAACATGCACACAGACACG **R** ACATGTGCACGCCTTTTG	P2 Touch-down	Monomorphic			Monomorphic			GQ927116
*Hmid*0563M	(AG)$_n$(ACAT)(AG)$_n$(ACAGAG)$_n$(ACAG)$_n$(ACTG)(ACAG)$_n$	**F** AGCGTGTGTGTGTTGTGTG **R** TGTCCGTGGTTCGCATAAAG	P2 Touch-down	Monomorphic			221/225 x 202/221	1:1:1:1	0.080	GQ927117
*Hmid*0610D	(GT)$_n$	**F** AGCAGTAAAGTCCTAGGGTGTG **R** ACTTTTACATCTAGACACAGGGC	P2 Touch-down	121/121 x 106/125	1:1	0.077	119/123 x 119/119	1:1	0.479	GQ927118
*Hmid*2009AD	(TG)$_n$	**F** TGTAGTAGACGGTGCAAGGAT **R** GGTGCCAACTGTTCACAATA	P2 Touch-down	Monomorphic			Monomorphic			GQ927144

*Hmid*2015M	(TG)$_n$(TGTC)$_n$	**F** GCCTGTGTCGGTCTATCTGT **R** ATCGGAGACTCAACATTTGC	P2 Touch-down	Non-reliable Genotypes			Non-reliable Genotypes			GQ927124
*Hmid*2032C	(GA)$_n$...(GA)$_n$...(GA)$_n$...(GA)$_n$...(AG)$_n$...(GT)$_n$...(GT)$_n$...(GA)$_n$...(GA)$_n$...(GA)$_n$...(GAGG)$_n$	**F** CAGTGTGTTTGTGTGTTGCTC **R** CACCTGTTGTTGCTGCTCT	P2 Touch-down	Non-reliable Genotypes			Non-reliable Genotypes			GQ927125
*Hmid*4T	(GAGT)$_n$	**F** AATTTAGACGAGTGGCTTGTG **R** CACATGGGTACATTGTGTGAG	P2 Touch-down	Monomorphic			158/171 x 171/171	1:1	***	GQ927126
*Hmid*2047BD	(GT)$_n$	**F** GTACGTTCAACACACCAGTCA **R** ACCTTCTACGCATTGAACATC	P2 Touch-down	Monomorphic			Monomorphic			GQ927127
*Hmid*4009C	(TG)$_n$...(TG)$_n$...(TG)$_n$...(TG)$_n$...(TG)$_n$...(TG)$_n$	**F** GTGTGCGTCGAATACTTTCA **R** GTATTCGAAGCACACCAACA	P1 53°C	Monomorphic			Monomorphic			GQ927128
*Hmid*4010D	(AC)$_n$	**F** TCCTGATCATGAAAGCAAAAC **R** CTGAACATGGTGAGCAACTG	P2 Touch-down	Non reliable genotypes			189/203 x 199/201	1:1:1:1	0.741	GQ927131
*Hmid*4018D	(GT)$_n$	**F** ACATATCCACGCTCGTAAGC **R** CTACACCTGCACCTACACCA	P2 Touch-down	212/225 x 200/225	1:1:1:1	***	Monomorphic			GQ927129
*Hmid*4019D	(GT)$_n$	**F** TCAGCACAATGCTACGTCAT **R** CCCACCACACAGACATACAC	P2 Touch-down	Monomorphic			Monomorphic			GQ927132
*Hmid*4022C	(TG)$_n$...(TG)$_n$...(TG)$_n$...(TG)$_n$...(TG)$_n$...(TG)$_n$...(GT)$_n$...(TGA)$_n$	**F** TGTGAATTATCATGGGCATCT **R** CCCTCCTGTAACTTGTCCTG	P2 Touch-down	Monomorphic			Monomorphic			GQ927130
*Hm*NSS1H	(GGGTTA)$_n$	**F** TAA TTC CAG CAG CTG AAA AA **R** AAC AAC GAC CCT AAA CCA TC	P3 56°C	Non-reliable Genotypes			Duplicated Locus			EF033331
*Hm*NS18M	(ACCA)(AGG)$_n$(ACC)$_n$AG(ACC)$_n$AG(AAC)	**F** AATTGTCTCCTTTGTTCTTCTTT **R** TATTTGTGACTTTAGGTGAGGAC	P3 60°C	Duplicated Locus			200/208 x 179/179	1:1	0.367	EF367120
*Hm*LCS152M	(CAA)$_n$(CCA)$_n$(CTA)$_n$(CCA)$_n$(CTA)$_n$(CCA)(CTA)$_n$	**F** TGATAAATGTCACTTGGGATCA **R** ACACGTGCAAACTGGTCTTC	P2 Touch-down	144/149 x 149/149	1:1	0.114	Duplicated Locus			GQ927139

*Hm*LCS34M	(TCAC)$_n$(TCAA)(TCAC)(TCAA)$_n$	**F** TAAAGTTGGCGTAAAATCTCA **R** AACCAAGATGTAGCCAGGAT	P1 58°C	Non-reliable Genotypes			Non-reliable Genotypes			DQ993230
*Hm*R16T	(CAGT)$_n$	**F** TGGGTACAATATGTGAAGCC **R** GCAAATTCAAAATGCTCAAA	P2 Touch-down	Non-reliable Genotypes			Non-reliable Genotypes			GQ927138
*Hm*LCS71T	(GGAT)$_n$	**F** GTCACTGTATGTCTCGCACAC **R** ATTTTGACGTTACTGCACGTA	P2 Touch-down	186/231 x 186/186	1:1	***	Duplicated Locus			DQ993221
*Hm*LCS388M	(GCGT)$_n$(GTT)$_n$(GT)$_n$	**F** GACAACCGGGATTCAAAC **R** ACTTCTTCAGGCAATATTACTAAG	P2 Touch-down	Non-reliable Genotypes			144/149 x 144/144	1:1	0.317	GQ927140
*Hm*NSp31M	(CAA)$_n$(CAG)$_n$(CAA)$_n$	**F** CTCGGGTTCAGTTACCTACA **R** CAAGTCAGGGTGGTCGTCTTTCC	P3 60°C	288/307 x 288/291	1:1:1:1	***	Monomorphic			EU126856
*Hm*NSp42C	(AGT)$_n$...(AGT)$_n$...(AGT)$_n$...(TGT)$_n$	**F** CCTGAATTTATAGTAGTA **R** CTCACTCAAACACACCCAAA	P3 60°C	286/311 x 286/323	1:1:1:1	0.411	293/305 x 281/305	1:1:1:1	0.431	EU126858
*Hm*LCS58M	(TG)$_n$(CG)(TG)$_n$(CGTG)(TGCG)$_n$(TG)$_n$	**F** GACAATTGAGAACATGTTTTTG **R** AACACTGATATGGTCAGTCCA	P1 58°C	Duplicated Locus			Non-reliable Genotypes			DQ993225
*Hm*LCS383T	(GTGA)$_n$	**F** TAAACTGCAAAATACCCACC **R** TCTTTACATCAAAGCTCCGT	P2 Touch-down	Non-reliable Genotypes			402/405 x 409/412	1:1:1:1	0.289	GQ927141
*Hm*S104T	(GAGT)$_n$	**F** GTCGTCAGAAGCAGTTTGAA **R** AAATCAATTTCTAGTGCCCC	P1 51°C	Non-reliable Genotypes			Non-reliable Genotypes			GQ927137
*Hm*2H6FT	(CACT)$_n$	**F** GTACTCATGCTCAACTGCGT **R** TGTCAAGTTACCGATCAAGG	P2 Touch-down	Non-reliable Genotypes			Non-reliable Genotypes			GQ927136
*Hm*3A11FM	(CA)$_n$(CG)(CA)$_n$	**F** TGCTCGTCCTATAGCAATGT **R** ATAGAGCAACGTGCATTCAC	P2 Touch-down	Non-reliable Genotypes			Non-reliable Genotypes			GQ927135
*Hm*LCS147T	(GAGT)$_n$	**F** CTTCTGTCATCCACAAGAGC **R** GCTGGAATATTGTTGAAAGC	P2 Touch-down	Non-reliable Genotypes			187/187[2] x 110/126	1:1[2]	***[2]	GQ927134

							187/U[3] x 110/126	1:1:1:1[3]	0.154[3]	
*Hm*NS32M	$(CAA)_n(GTC)(GTT)_n(GTC)(GTT)_n$	**F** TTCCGGATAAAGTAAATCGTC **R** ACACTATAGAATACAGCGGCC	P3 52°C	Non-reliable Genotypes			Duplicated Locus			GQ927142
*Hm*LCS175M	$(GT)_n(GA)_n$	**F** TTCACACTCTACAGGGTTGG **R** AACACATTCAAAACCCACC	P2 Touch-down	278/278 x 276/280	1:1	0.479	247/282 x 280/280	1:1	0.576	GQ927133
*Hm*NS21M	$(CT)_n(C)_n(CT)_n(T)_n(CT)_n$	**F** ACAAACTCACGCACACATTC **R** AGGTTGAAGAATGAGTGCGT	P2 Touch-down	235/240 x 215/227	1:1:1:1	0.355	221/229 x 217/221	1:1:1:1	0.089	GQ927143

[1] Departure from Mendelian segregation, *** $P < 0.05$.

[2] Genotype, segregation ratio and *P*-value without correction for null allele.

[3] Genotype, segregation ratio and *P*-value with correction for null allele.

<u>SECTION 2.4</u>

Microsatellite Marker Development in the Abalone *Haliotis midae* Using Pyrosequencing (454): Characterisation, *In Silico* Analyses and Linkage Mapping.

2.4.1) Introduction

There are five abalone species found in the waters of South Africa, of which only one, *Haliotis midae*, is cultivated and exported. The *H. midae* (abalone) industry is the most lucrative aquaculture sector in South Africa, with 15 active farms producing some 870 tonnes in 2008 fetching around USD 38 per kg (pers. comm.: Wayne Barnes, Abalone Farmers Association of South Africa). Commercial abalone farming of *H. midae* was initiated in 1990 (Cook and Britz, 1991) and 15 years later a genetics research programme was introduced by a collaboration effort between academic institutions, industry and government (Brink *et al.*, 2009b; Slabbert *et al.*, 2009b). The overall aim of the programme is the genetic characterisation and enhancement of *H. midae* within its natural and commercial settings. To achieve this, various molecular genetic markers are needed to facilitate different aspects of the programme such as genetic diversity studies, pedigree reconstructions, linkage mapping and QTL discovery.

Microsatellite markers are an extremely popular marker in modern molecular disciplines as a search of the ISI Web of Knowledge shows (10048 publications since 2006; accessed 05/08/2010). It is also a widely used marker in abalone genetic studies, including population structure studies, genetic diversity studies, parentage studies, linkage mapping and QTL mapping (Baranski *et al.*, 2006a; Gutiérrez-Gonzales *et al.*, 2007; Li, Q. *et al.*, 2007; Liu *et al.*, 2007; Slabbert *et al.*, 2009a; Shi *et al.*, 2010). Microsatellite loci have already been isolated for various abalone species, including *H. rubra* (Evans *et al.*, 2000; Baranski *et al.*, 2006b), *H. asinina* (Selvamani *et al.*, 2000), *H. corrugata* (Diaz-Viloria *et al.*, 2008) and *H. discus hannai* (An and Han, 2006; Sekino *et al.*, 2006; Li and Akihiro, 2007). It is also the most popular marker used thus far in the *H. midae* genetics research programme with 133

(excluding current Section 2.4) microsatellites (Bester *et al.*, 2004; Slabbert *et al.*, 2008; Rhode, 2010; Slabbert *et al.*, 2010).

The development of microsatellite markers is, unfortunately, very labour-intensive and costly. Genomic libraries need to be constructed and enriched for microsatellite repeats, clones screened and then sequenced (Zane *et al.*, 2002). Even more advanced isolation protocols such as the Fast Isolation by AFLP of Sequences Containing Repeats (FIASCO; Zane *et al.*, 2002) and the SNX-Unilinker method (Hamilton *et al.*, 1999) are still not suited for high-throughput development of markers, because of labour-intensive cloning and screening steps. The advent of new generation sequencing technologies such as sequencing-by-synthesis (Ronaghi *et al.*, 1998) and ligation-mediated sequencing (Shendure *et al.*, 2005) could therefore provide an alternative to generating large data sets with minimal effort and a decrease in costs.

Pyrosequencing is based on the real-time acquisition of DNA synthesis data via bioluminescence and is driven by four enzymes, Klenow DNA Polymerase I, ATP sulfurylase, Luciferase and Apyrase (Ronaghi *et al.*, 1998; Ahmadian *et al.*, 2006). This technology has already been used in mutation detection studies (Garcia *et al.*, 2000), SNP genotyping (Ahmadian *et al.*, 2000), bacterial genotyping (Monstein *et al.*, 2001), viral genotyping (Gharizadeh *et al.*, 2001) and EST sequencing (Agaton *et al.*, 2002). Pyrosequencing was recently employed to develop microsatellite markers for a fungus (*Fusarium circinatum*), an insect (*Sirex noctilio*), a wasp (*Deladenus siricidicola*), various moa species and the duck, *Hymenolaimus malacorhynchos* (Abdelkrim *et al.*, 2009; Allentoft *et al.*, 2009; Santana *et al.*, 2009). These studies showed that pyrosequencing is a good platform for the automation of certain analytical steps within a standard marker development protocol making it a more time efficient strategy.

In this study, pyrosequencing was used to generate data from a repeat enriched genomic library and compared to conventional methods of data collection used in the laboratory. Primers for microsatellite loci were designed and characterised further. The data generated by pyrosequencing was also checked against BLAST databases to distinguish type 1 and type 2 markers. The newly developed markers were then mapped onto a preliminary microsatellite linkage map.

2.4.2) Materials and Methods

2.4.2.1) Sample Collection and DNA Extractions

Haliotis midae samples were collected from the wild at Saldanha Bay (West coast of RSA) and DNA was extracted from 16 individuals. Samples from a full-sib family were collected on Roman Bay Sea Farm (Gansbaai, RSA). DNA was extracted from the sire (M342) and the dam (F617) and 32 (O01 to O32) random offspring aged 3 months. Genomic DNA from both sampling sites was isolated from muscle tissue using a CTAB-protocol (Saghai-Maroof *et al.*, 1984).

2.4.2.2) Genomic Library Construction

The Fast Isolation by AFLP of Sequences Containing Repeats (FIASCO, Zane *et al.*, 2002) method was used to construct a repeat enriched genomic library. A total of 250ng of *H. midae* DNA was digested with *Mse*I (*New England Biolabs*) and ligated to *Mse*I-adaptors. This sample was then selectively amplified and separately enriched with biotinylated $(AC)_{12}$, $(GATC)_6$, $(CAA)_8$ and $(GTGC)_6$ probes and recovered by streptavidin magnetic particles. These enriched particles were again selectively amplified using an *Mse*I-specific primer mix.

2.4.2.3) Pyrosequencing and Primer Design

A final amount of 5μg PCR product was sequenced using the Roche 454 GS-FLX system at Inqaba Biotech (Pretoria, South Africa). Samples were prepared and analysed at Inqaba Biotech according to the protocol of the manufacturer. Single reads were obtained and contigs constructed using the software Newbler version 1.1.03.24. All the contigs were trimmed of any adaptor sequences using the Find and Replace module of Microsoft Word. These contigs were then analysed for length, GC-content and repeat motifs using the online software programme BatchPrimer3 version 1 (You *et al.*, 2008). The same software package was used to design primers for repeat containing contigs where adequate flanking regions were available. The settings for BatchPrimer3 are shown in Table 2.4.1.

Table 2.4.1: The parameters for BatchPrimer3 v 1 for designing microsatellite primer pairs.

Parameter	**Value**
Primer length	16-25 (21 as optimal)
GC-content	30-70% (50% as optimal)
Annealing temperature	50-70 (57 as optimal)
Product size	90-350 (120 as optimal)
Other parameters	Default

To avoid primer redundancy, all contigs for which primers were designed were screened against a local BLAST database (created with BioEdit version 5.0.9; Hall, 1999) which contains all the microsatellite sequences generated thus far for *H. midae*.

2.4.2.4) Genotyping

All polymerase chain reactions (PCR) were conducted in a Geneamp 2700 thermo cycler (*Applied Biosystems*) in 10µl reaction volumes containing 20ng DNA, 0.2µM of each primer, 200µM of each dNTP, 0.1U of 2G Fast*Taq* polymerase (*KAPA Biosystems*), 1x Buffer B (*KAPA Biosystems*) and 2mM $MgCl_2$. A fast touch-down PCR programme was used: an initial activation and denaturing step of 95℃ for 2 minutes followed by 10 cycles of 1 second at 94℃ and 5 seconds at 65℃ and 30 cycles of 1 second at 94℃ and 5 seconds at 55℃. A final elongation step is performed at 72℃ for 10 seconds. PCR products were separated on a 3730xl DNA Analyzer (*Applied Biosystems*) and scored using GeneMapper version 4 (*Applied Biosystems*).

2.4.2.5) Statistical Analyses, Linkage Mapping and Bioinformatics

Sixteen Saldanha Bay samples were used to characterise the microsatellite loci. The number of alleles, observed and expected heterozygosities and polymorphic information content (PIC) were calculated using CERVUS version 3.0.3 (Kalinowski *et al.*, 2007). Deviation from Hardy-Weinberg equilibrium and F_{is}, as a measure of heterozygote deficiency or excess (Weir and Cockerham, 1984), were calculated using Genepop version 4 (Rousset, 2008). Sequential Bonferroni correction was performed for multiple tests (Rice, 1989).

Linkage mapping was performed using two parents and a subset of 32 offspring. The parents were first screened with previously developed (Bester *et al.*, 2004; Slabbert *et al.*, 2008; Slabbert *et al.*, 2010) and the newly isolated markers. Polymorphic markers were arranged into multiplexes based on allele size and fluorescent label type (Appendix B, Table B.2). Genotypes were then assessed for the offspring. Linkage analysis was performed with JoinMap version 4.0 (Van Ooijen, 2006) using regression mapping at an independence LOD threshold of 4.0 and the CP population model. Map distances were calculated using the Kosambi map function. The linkage map was drawn using MapChart version 2.2 (Voorrips, 2002). The expected genome size was estimated based on the constructed map. Two methods were followed: 1) GE_1, 2s was added to the observed length of each linkage group, where s is the average distance between markers (Fishman *et al.*, 2001); 2) GE_2, the observed length of each linkage group was multiplied by $(m + 1) / (m – 1)$, where m is the number of markers in each group (Chakravarti *et al.*, 1991). The estimated length is then equal to the sum of the revised length of the linkage groups. The map coverage was calculated as total observed length as a percentage of GE_1 and GE_2.

Bioinformatic analyses were performed to classify microsatellite loci as either type 1 or type 2 and also to identify similarities with microsatellite loci isolated from other *Haliotis* spp. The software package, dCAS version 1.4.1 (Guo *et al.*, 2009) was used to search all the constructed contigs against the KEGG (Kyoto Encyclopedia of Genes and Genomes) nucleotide database and a customized database containing all available *Haliotis* spp. nucleotide data downloaded from the National Center for Biotechnology Information (NCBI; accessed 20/11/2009). For the BLAST search, the

threshold of minimum identity percentage and minimum length of match was set to 90% and 30 nucleotides respectively. All BLAST hits with E-value ≤ 10^{-9} were regarded as significant.

2.4.3) Results

2.4.3.1) Pyrosequencing and Primer Design

A total number of 11271 single sequence reads was generated covering 1.82 Mb and assembled into 1067 contigs (Table 2.4.2). One-hundred and forty-one dinucleotide, 22 trinucleotide, 264 tetranucleotide, 20 pentanucleotide and 14 hexanucleotide repeats were detected within 297 contigs (Table 2.4.3). Primer pairs were successfully designed for 185 repeat-containing contigs using BatchPrimer3. Of these primer pairs 27 were discarded as some individual primers were situated within repeat tracts, which may cause difficulties in downstream applications such as PCR amplification and size calling of alleles. Another three sequences were discarded due to similarity to previously isolated loci for which primers already existed. A total of 155 primer pairs were chosen for further analysis.

Table 2.4.2: Pyrosequencing and primer design data for various species using the FIASCO-method for library construction.

Species	Method	Sequences generated (Mb)	Contigs and singletons (≥ 100bp)	Microsatellite containing sequences	Amplifiable sequences with repeats	Polymorphic primers
S. noctilio[1]	454	1.47	1840	463 (25%)[a]	336 (18%)[a]	--
D. siricidicola[2]	454	1.22	1040	421 (40%)[a]	296 (28%)[a]	--
H. midae[3]	454	1.82	1067	297 (28%)[a]	185 (17%)[a]	82 (8%)
H. midae[4]	Traditional	--	250	112 (45%)[a]	20 (8%)[a]	11 (4%)
H. midae[5]	Traditional	--	983	462 (47%)[a]	192 (20%)[a]	63 (6%)
H. midae[6]	Traditional	--	978	479 (49%)[a]	222 (23%)[a]	44 (4%)

a: Percentages are calculated out of total of contigs and singletons.

1 and 2: Santana *et al.*, 2009

3: This Section 2.4

4: Bester *et al.*, 2004

5: Slabbert *et al.*, 2008

6: Slabbert *et al.*, 2010

Table 2.4.3: Microsatellite repeat motifs found in contig sequences.

Repeat Types	Total
Dinucleotide repeats	141
Trinucleotide repeats	22
Tetranucleotide repeats	264
Pentanucleotide repeats	20
Hexanucleotide repeats	14
Total microsatellite repeats	461
Total individual contigs containing repeats	297

2.4.3.2) Statistical Analyses, Linkage Mapping and Bioinformatics

Screening of a wild population revealed a total of 82 polymorphic microsatellite markers (Table 2.4.4). Ten of these loci had more than three alleles indicating a duplication event within the same locus or possibly between loci. The number of observed alleles for the non-duplicated alleles ranged from two to 21, expected heterozygosity ranged from 0.063 to 0.968, observed heterozygosity ranged from 0.000 to 1.000 and PIC content from 0.059 to 0.934. Seventeen of the 72 potential microsatellite markers did not conform to Hardy-Weinberg equilibrium after sequential Bonferroni correction ($P < 0.05$).

The loci that were used in the construction of the linkage map and Mendelian segregation results are shown in Table 2.4.5. The preliminary linkage map is shown in Figure 2.4.1. A total of 50 loci were successfully mapped onto 18 linkage groups, while 28 loci remained unlinked. Of the latter, seven were excluded due to deviation from expected Mendelian segregation ratios ($P < 0.01$; Table 2.4.5). The newly isolated microsatellite loci (indicated by * in Figure 2.4.1) accounted for 18 of the 50 mapped loci and were spread over 12 linkage groups. Total observed genome length was 148.72cM, while the estimated genome lengths were 316.06cM for GE_1 and 321.42cM for GE_2 (Table 2.4.6). The map coverage was 47.06% for GE_1 and 46.27% for GE_2 (Table 2.4.6).

The repeat-containing contigs showed no significant identities in either database (E-value $\geq 10^{-9}$). Two non-repeat containing contigs did however show similarities. Contig688 showed between 80% and 86% identity ($E = 2 \times 10^{-43}$ to 10^{-27}) to a *Haliotis* spp. NADH dehydrogenase subunit 5 (ND5) gene and Contig923 had an 83% identity ($E = 3 \times 10^{-26}$) to *H. diversicolor* cytidine deaminase. Locus *Hmid*PS1.868T (Contig868) showed similarity ($E = 10^{-13}$) with *H. d. hannai* microsatellite locus Awb003 and *Hmid*PS1.1007C (Contig1007) showed similarity ($E = 2 \times 10^{-11}$) with *H. d. hannai* microsatellite locus Awb068. *Hmid*PS1.868T and Awb003 [$(TGAG)_n$] and *Hmid*PS1.1007C and Awb068 [$(ACTC_n)$] have identical repeat tracts, but share only one flanking sequence. Eighteen non-polymorphic, repeat-containing contigs showed similarity to the flanking regions of microsatellite loci isolated for other *Haliotis* spp (Table 2.4.7).

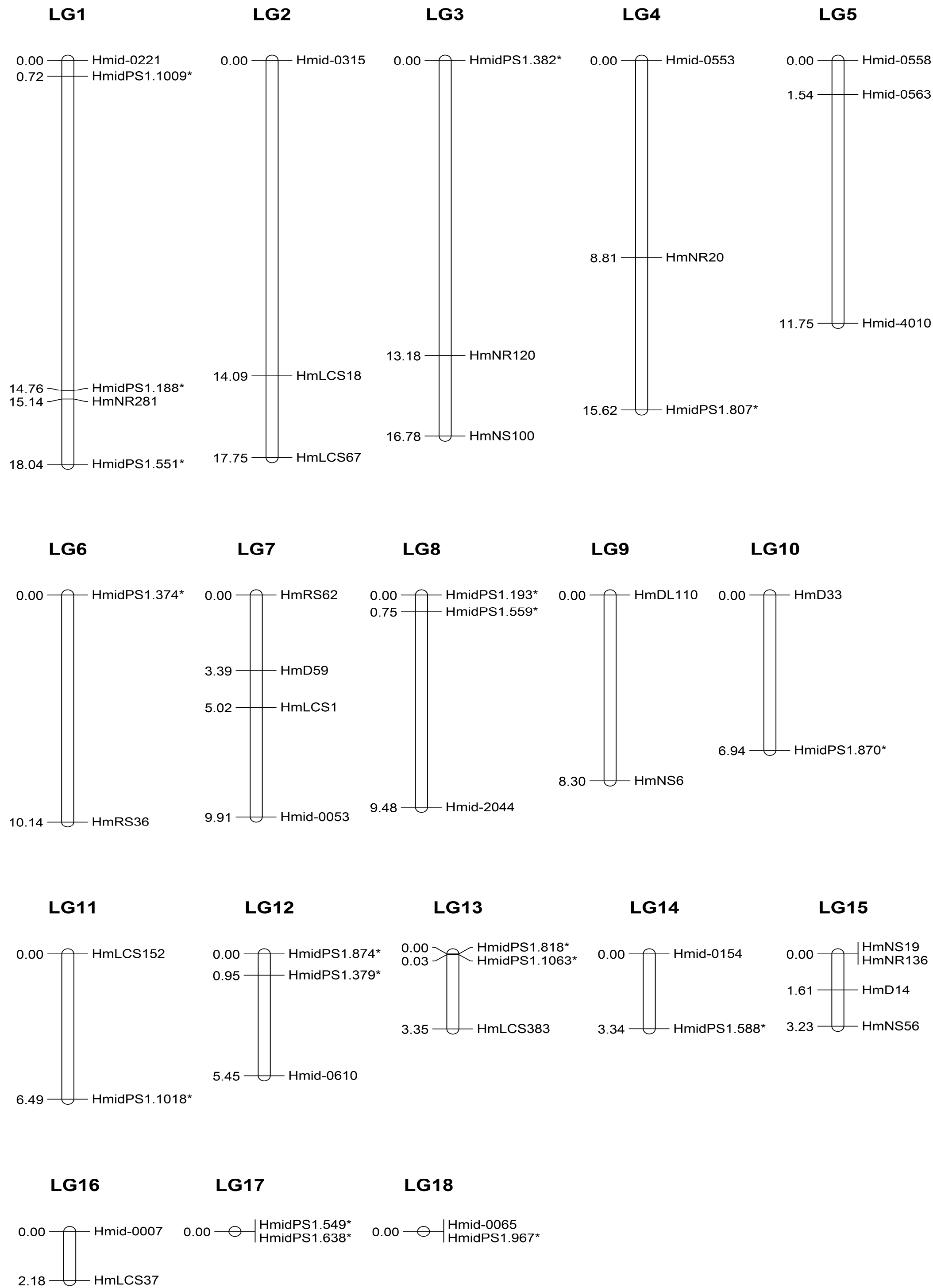


Figure 2.4.1: Preliminary genetic linkage map of *Haliotis midae*. All microsatellite loci marked with * indicate markers isolated in this study. Distances are given in cM.

Table 2.4.4: Eighty-two polymorphic microsatellite loci isolated using pyrosequencing for *Haliotis midae*.

Locus Name	Repeat Tract	Primer Sequence (forward & reverse; 5'-3')	PCR Programme	n_a[a]	H_o[b]	H_E[c]	H-W (P)[d]	PIC[e]	Accession Number
*Hmid*PS1.38T	$(CCAA)_n$	**F** TCACATCCTCCTATCATCCAC **R** GTTGGAAGATGTAATCGTTGG	TD-PCR	6	0.500	0.518	0.372	0.478	GU256656
*Hmid*PS1.42C	$(ATCC)_n...(ATCC)_n$	**F** CATTTTCCCATGTATCCAAAC **R** GGATTTGGATGGAAAAATCTC	TD-PCR	3	0.714	0.532	0.394	0.450	GU256657
*Hmid*PS1.95H	$(CACACG)_n$	**F** ATTCAGACTGGTACGATTTCC **R** ACCTATGTGTATGTGAGTGTGTG	TD-PCR	2	0.125	0.121	1.000	0.110	GU256658
*Hmid*PS1.124D	$(AC)_n$	**F** ATTTTATTGGGGGAAAGAATG **R** ATGGGTGAGTGACTCTGTGTT	TD-PCR	2	0.375	0.315	1.000	0.258	GU256659
*Hmid*PS1.138D	$(CA)_n$	**F** AAACACATACAGGCACTCACA **R** AGACAGGGTGTAACATTCTATTCA	TD-PCR	8	0.375	0.760	0.000***	0.706	GU256660
*Hmid*PS1.147M	$(TG)_n(GA)_n$	**F** ATGCGTTGCGTACGTGT **R** TTTTCCTCTGTCTCTCATCTCTC	TD-PCR	3	0.063	0.179	0.035	0.166	GU256661
*Hmid*PS1.150C	$(CA)_n...(CA)_n...(CACT)_n$ $(CA)_n...(CA)_n$	**F** AAACGCTCATGCTCACATACT **R** AGCCTAACAAACACTTTGCTG	TD-PCR	21	0.750	0.968	0.000***	0.934	GU256662
*Hmid*PS1.155D	$(CA)_n$	**F** GGACCAACAGACAATTGAAAC **R** AGGATCTGTCACCTACAGACG	TD-PCR	2	0.063	0.063	-	0.059	GU256663
*Hmid*PS1.156D	$(GT)_n$	**F** ACGTAAGGCAGATTGATTTTG	TD-PCR	6	0.111	0.739	0.000***	0.669	GU256664

		R CATACACACATACACACATACG							
*Hmid*PS1.160T	$(CACT)_n$	**F** AAAGTTTTGGTCACAATACCG **R** CTTTGTGTGAGTGCGTGAGT	TD-PCR	4	DUP				GU256665
*Hmid*PS1.171C	$(CACCA)_n$...$(CACAC)_n$	**F** TATTCAACGACTGACCATTCC **R** TTGGTGTGTGGGTGTGC	TD-PCR	2	0.125	0.121	1.000	0.110	GU256666
*Hmid*PS1.179R	$(CAA)_n$	**F** CAGCAAACAAGTATCAACAGC **R** AATCTTGTGTTCTGTTTTGG	TD-PCR	6	DUP				GU256667
*Hmid*PS1.188C	$(GTGC)_n$...$(GT)_n$	**F** GGACTGACCATTTGATAATGTG **R** CTGCAAAGAATACTATTTGAGGAA	TD-PCR	4	0.188	0.236	0.192	0.220	GU256668
*Hmid*PS1.193C	$(ACTC)_n$...$(TCAC)_n$... $(CACT)_n$...$(CACT)_n$... $(TCAC)_n$	**F** CTCAATATATCCCCACGAGAA **R** TTGAGTCGGATATTGTCACAG	TD-PCR	9	0.500	0.845	0.011	0.800	GU256669
*Hmid*PS1.195T	$(ACGC)_n$	**F** GCTTTGAACCCGTTATTTGTT **R** GGATGTTGACCGATTATTCAT	TD-PCR	2	0.313	0.272	1.000	0.229	GU256670
*Hmid*PS1.197C	$(TG)_n$...$(TG)_n$	**F** GTCTGTCAGGTGTGTGTGAGA **R** CGAACACAAGATCTCTATCTCTCT	TD-PCR	2	0.000	0.121	0.032	0.110	GU256671
*Hmid*PS1.206C	$(GACT)_n$...$(GTGA)_n$... $(AGTG)_n$	**F** ATCCAGGCTGATTGTGAGA **R** TGTGACAGATGAGTTTGACAAC	TD-PCR	6	0.375	0.601	0.017	0.517	GU256672
*Hmid*PS1.207C	$(AGTG)_n$...$(GTGA)_n$	**F** TTGATTGATCGATTGATTGAG **R** GTCACCATGTGTCACAGAGAA	TD-PCR	2	0.250	0.226	1.000	0.195	GU256673

*Hmid*PS1.208D	(AC)$_n$	**F** CTATTTCGCACACGCTGAT **R** GTGTGTGCGTGCTTGC	TD-PCR	4	0.143	0.550	0.001***	0.483	GU256674
*Hmid*PS1.222T	(AGTG)$_n$	**F** GAAATGCGATCTCTGATTGAG **R** AGCGTTACACTCATTCACTCAC	TD-PCR	3	0.125	0.123	1.000	0.116	GU256675
*Hmid*PS1.227T	(ATGT)$_n$	**F** TAACACGTACACTCCAGTCC **R** ACATGCTTGACTGTGACTCTCT	TD-PCR	4	0.250	0.603	0.002	0.496	GU256676
*Hmid*PS1.228T	(ACTC)$_n$	**F** TAACAAGTCAGCCACTCAACA **R** GTGTGAGTGAGGTGAGGAGAC	TD-PCR	4	0.500	0.575	0.697	0.459	GU256677
*Hmid*PS1.247M	(GA)$_n$(GAAT)$_n$	**F** ATCAGAGTGTGTGCATCAGTG **R** CTGACATACACACAGACATCCA	TD-PCR	4	0.875	0.665	0.345	0.573	GU256678
*Hmid*PS1.305T	(GCAC)$_n$	**F** CTCGAGTTTCAACCATTGAGT **R** GGGTGGGTGTTACGAGTG	TD-PCR	5	0.714	0.762	0.307	0.688	GU256679
*Hmid*PS1.332D	(AC)$_n$	**F** TGAAACACTCACACATCGACT **R** TGGTTCATGCATAAATGTTGT	TD-PCR	18	0.563	0.950	0.000***	0.915	GU256680
*Hmid*PS1.353T	(CACG)$_n$	**F** CGGAATAGAAGACGAGCAAT **R** ATTTGACAGAAGGTGGTGGT	TD-PCR	3	0.188	0.365	0.048	0.309	GU256681
*Hmid*PS1.355R	(TCA)$_n$	**F** GCCCATGTGCGAAGTT **R** ATGTTTTGAGGAGGGAGTTTC	TD-PCR	7	0.375	0.716	0.005	0.663	GU256682
*Hmid*PS1.370C	(CAACC)$_n$...(CACT)$_n$	**F** ACAACCAAACTCAACCCAAC **R** TCATGTGACACGAGTGTGTG	TD-PCR	4	0.600	0.561	1.000	0.454	GU256683

*Hmid*PS1.374T	$(GAGT)_n$	**F** TGACAAGTTTGGATTTGTTTTC **R** TAGCTGGAATATTGCTGAGTG	TD-PCR	4	0.375	0.333	1.000	0.299	GU256684
*Hmid*PS1.375C	$(GTGA)_n$...$(GTGA)_n$	**F** GGAGTGAACGAGTGAAGAAGT **R** ACAACTCACCTGTCTTGTTTGT	TD-PCR	2	0.182	0.485	0.059	0.356	GU256685
*Hmid*PS1.379T	$(GTGC)_n$	**F** TACTGTCTCTCTCGACGGTTC **R** GCAAACACAATAAAACACCAA	TD-PCR	8	0.625	0.696	0.145	0.648	GU256686
*Hmid*PS1.382D	$(TG)_n$	**F** TGGAATAACTGTCTATTTCGTCA **R** TCAGACAGAAAGACACACACG	TD-PCR	7	0.385	0.812	0.002	0.749	GU256687
*Hmid*PS1.398P	$(AGGTG)_n$	**F** ACAAGCCTCTAAAATGCCTCT **R** TGCACGGTAAACTCAATCT	TD-PCR	4	0.125	0.579	0.000***	0.482	GU256688
*Hmid*PS1.405T	$(CAAC)_n$	**F** CCTGCCACTCACTCAACTATT **R** GGATAAGTGATTGGATGGGATA	TD-PCR	7	DUP				GU256689
*Hmid*PS1.433H	$(CACACG)_n$	**F** AGTCCTGACCAGAACAAACAG **R** GAGGTGAAAAGGATTTTGATG	TD-PCR	5	DUP				GU256690
*Hmid*PS1.457T	$(GAGT)_n$	**F** TGGATGAGTGAATGAGAATGA **R** ACACGATGACAAACAATGTGA	TD-PCR	8	0.308	0.665	0.007	0.620	GU256691
*Hmid*PS1.469R	$(ATC)_n$	**F** TTGGTCAGCCATGTAGTCATA **R** ATGATGGTGGGGATGATG	TD-PCR	2	1.000	0.516	0.000***	0.375	GU256692
*Hmid*PS1.484C	$(GAGT)_n$...$(GTGA)_n$... $(GTGA)_n$	**F** ATATCTGACGTCTACCCCACA **R** CCCATCCTGTGAAGAACATAC	TD-PCR	4	0.250	0.288	0.283	0.267	GU256693

*Hmid*PS1.487T	$(TGAG)_n$	**F** ACGTACACGGACTGACAATTT **R** ACTTGAAGAAGCGTAAAACCA	TD-PCR	6	0.357	0.638	0.001***	0.575	GU256694
*Hmid*PS1.521T	$(GAGT)_n$	**F** ATCTGTGTGCACTCAATCTGT **R** ACCCTACCACCCATTTAACTT	TD-PCR	4	0.333	0.303	1.000	0.276	GU256695
*Hmid*PS1.549D	$(TG)_n$	**F** GTTGTGTGGGAGCGTATGTAT **R** TACACCCCACATATACACCAA	TD-PCR	10	0.813	0.885	0.225	0.842	GU256696
*Hmid*PS1.551C	$(TATG)_n$...($TGTA)_n$	**F** GCTCCACCAAAATTGATATGT **R** ACATACACATAGGTACACACACA	TD-PCR	4	0.688	0.595	0.905	0.506	GU256697
*Hmid*PS1.559M	$(CA)_n(G)(TCAC)_n$	**F** TAAGGATCATACACACTCGT **R** CTTCAGTCGAAATGTGTATAACG	TD-PCR	2	0.000	0.121	0.032	0.110	GU256698
*Hmid*PS1.561C	$(TTGC)_n$...$(TGTT)_n$	**F** AGGGTAGAACAATTTCCTGCT **R** ATGGTCACTTGGGTTTGTCTA	TD-PCR	3	0.063	0.284	0.003	0.257	GU256699
*Hmid*PS1.588C	$(CACT)_n$...($TCAC)_n$...($CACT)_n$	**F** GGAATATTGCTAAATGGTGGA **R** TGAGTGAGTAATTGGGTAGGTG	TD-PCR	5	0.250	0.716	0.000***	0.641	GU256700
*Hmid*PS1.629C	$(TTGT)_n$...($TGGG)_n$...($TAGG)_n$	**F** TGCATTTTGTAGTGTGTTTGC **R** CCCTCCCTACCTACCTACCTA	TD-PCR	6	DUP				GU256701
*Hmid*PS1.635D	$(AC)_n$	**F** CCAACAGTTTTCTGAATGTGA **R** TGAAGTAGATGATGGGTGCAT	TD-PCR	2	0.063	0.063	-	0.059	GU256702
*Hmid*PS1.638T	$(GTGA)_n$	**F** CAAGATCTAAAAATGGCCTCA **R** CTTCACACTCACAAACTCAGC	TD-PCR	3	0.375	0.331	1.000	0.294	GU256703

*Hmid*PS1.692T	(ATAC)$_n$	**F** TAAGACTGAGGGCGCTTTT **R** TGGTGTGGATATGTGAAAATG	TD-PCR	2	0.063	0.063	-	0.059	GU256704
*Hmid*PS1.711T	(GTGA)$_n$	**F** TAAACTGCTGTCACCAAGGA **R** TACCCACACAACAGACTACCC	TD-PCR	5	DUP				GU256705
*Hmid*PS1.728D	(CA)$_n$	**F** ACTTCACACATGAATGCACAC **R** GTTGTTTTACAGACCGTCGAG	TD-PCR	2	0.125	0.121	1.000	0.110	GU256706
*Hmid*PS1.768T	(ACTC)$_n$	**F** TAAAGCGGCGTAAAACTGA **R** AATAGCCTGTCAGGTCATCG	TD-PCR	2	0.000	0.444	0.000***	0.337	GU256707
*Hmid*PS1.805T	(CACG)$_n$	**F** AGAGGTTTGACATGACTTCCA **R** ATGCGTGTTTGTATATGTG	TD-PCR	4	0.200	0.598	0.002	0.511	GU256708
*Hmid*PS1.807T	(GAGT)$_n$	**F** TGTTTGAATAACCACCCTCTT **R** CATTAGCTAACCCACAAATCC	TD-PCR	4	0.438	0.760	0.006	0.688	GU256709
*Hmid*PS1.811C	(TTGT)$_n$...(TG)$_n$	**F** ATTGAAATAAATGCGCTTCAG **R** CAAACACATAGATAGACGCACTT	TD-PCR	11	0.462	0.837	0.004	0.789	GU256710
*Hmid*PS1.818C	(ATGG)$_n$...(TGGA)$_n$...(AC)$_n$	**F** AATGTAGGGTTGCTTCAAATG **R** GAGTGTGTGGGTGTCTCTTTC	TD-PCR	9	0.625	0.738	0.165	0.696	GU256711
*Hmid*PS1.831M	(CACC)$_n$(CACT)$_n$(CACC)$_n$(CACT)$_n$(CAC)(CACT)$_n$	**F** CTCACTCACTCCCTCATTCAC **R** CCTGTACTGGTTAACAATATTTGAG	TD-PCR	17	DUP				GU256712
*Hmid*PS1.840D	(AC)$_n$	**F** CATACAGAACACTGCGGAAC **R** AACCACTTAGTGTGTGCGATT	TD-PCR	2	0.063	0.417	0.002	0.323	GU256713

*Hmid*PS1.844M	(GAGT)$_n$(GTGA)$_n$	**F** ACATATGCGCCTTTGTGTTAT **R** CAGGTAACTCACTCACTCACG	TD-PCR	7	DUP				GU256714
*Hmid*PS1.859T	(CTCA)$_n$	**F** AAGACCGTACACTCTCACTCG **R** TGGTGAGATATACAGGGTGAAA	TD-PCR	6	0.250	0.435	0.002	0.404	GU256715
*Hmid*PS1.860D	(GT)$_n$	**F** AGTAGGTGGACCTCTCTCCAT **R** ACAGAATCTACACGCACACAC	TD-PCR	3	0.438	0.365	1.000	0.309	GU256716
*Hmid*PS1.868T	(TGAG)$_n$	**F** TGTAGGGATGAGAACGAAAAG **R** GGCGTAAAACCATACTCACTC	TD-PCR	3	0.875	0.534	0.007	0.412	GU256717
*Hmid*PS1.870C	(CACACG)$_n$...(AC)$_n$	**F** ACAACAACACACACGCACA **R** GTGCCAAAACATATTTCAAAAC	TD-PCR	15	0.875	0.938	0.493	0.901	GU256718
*Hmid*PS1.873T	(TCAC)$_n$	**F** AACAGTGTCAATAAAGCTGGAA **R** TGGACCAGAGTGATAATAGTGTG	TD-PCR	3	0.467	0.393	1.000	0.342	GU256719
*Hmid*PS1.874C	(CACG)$_n$...(AC)$_n$	**F** AACGAAGGACAGTAAAAACAACT **R** CAGCTAGACTGAGTGTGACCA	TD-PCR	14	0.625	0.927	0.000***	0.890	GU256720
*Hmid*PS1.890M	(CACT)$_n$(CT)$_n$	**F** TTCTCATTTCACACACACAGG **R** CCTTTTCACTTCATAGCGTGT	TD-PCR	14	DUP				GU256721
*Hmid*PS1.906T	(CCAC)$_n$	**F** CACTCACTCACTAACCCACCT **R** ATAAACCCTGAAACCCTGAAA	TD-PCR	3	0.231	0.218	1.000	0.198	GU256722
*Hmid*PS1.952D	(TG)$_n$	**F** TGAGTCCTGAGTAACTGCAAA **R** TGGATTGAACAACTTTCAACA	TD-PCR	8	0.357	0.690	0.000***	0.638	GU256723

*Hmid*PS1.961T	(GTAG)$_n$	**F** AAACTAGAAAGGAGGCACGTT **R** ATACTACACGCGCACACATAC	TD-PCR	2	0.125	0.444	0.007	0.337	GU256724
*Hmid*PS1.967M	(TGTC)$_n$(TG)$_n$	**F** ATATGCACACCGAGTGAAATC **R** CTAACATGACCAGCGATTGTT	TD-PCR	6	0.563	0.798	0.021	0.738	GU256725
*Hmid*PS1.972T	(TCAC)$_n$	**F** CCCACTCACTCACATATCCAC **R** GCATGGAAAAACAAAATGTCT	TD-PCR	5	0.688	0.593	0.446	0.492	GU256726
*Hmid*PS1.981T	(CTCA)$_n$	**F** CTGGAATATTGCTAAAAGTGG **R** TTAGAGACTGAAGACGGGATG	TD-PCR	9	0.600	0.869	0.057	0.821	GU256727
*Hmid*PS1.982M	(TGTA)$_n$(TG)$_n$	**F** TCCTGAGTAATCGTACTCTGTGT **R** CCAACAATGTAACCACAGGAT	TD-PCR	3	0.214	0.415	0.019	0.359	GU256728
*Hmid*PS1.1007C	(ACTC)$_n$...(TCAA)$_n$	**F** ATATTGCCGATGTGACGTTAT **R** TGATTGATTGTAGTGATTGAGTTG	TD-PCR	5	0.800	0.651	0.001***	0.566	GU256729
*Hmid*PS1.1009H	(GTGGGT)$_n$	**F** TGTAAGAAGTGGACCAGCAGT **R** TGCTCCACAAAACTGAGTACA	TD-PCR	9	DUP				GU256730
*Hmid*PS1.1012R	(CAT)$_n$	**F** CCCACACACATGAGAAATGT **R** CAAGTATGTGTGGGTGGTGTT	TD-PCR	9	0.733	0.885	0.166	0.839	GU256731
*Hmid*PS1.1018T	(TTGT)$_n$	**F** CTGCGTCCTTGTGTGTGT **R** TGACGAGAGGGTCAGATTAGA	TD-PCR	6	0.500	0.694	0.034	0.630	GU256732
*Hmid*PS1.1026M	(GTGA)$_n$(GTGC)$_n$	**F** GTGTGCGTGAGTGAGTGAGT **R** CACCAAATTACATCCCATACC	TD-PCR	6	0.250	0.679	0.000***	0.610	GU256733

*Hmid*PS1.1038T	$(GTGA)_n$	**F** TATGTGCATGTGGGGTTATG **R** AACAAACACATGGATACACCA	TD-PCR	5	0.688	0.653	1.000	0.562	GU256734
*Hmid*PS1.1058C	$(TGAG)_n$...$(AGTG)_n$...$(AGTG)_n$	**F** GTAATTGGATCAAAGATGC **R** AAATGACAGCTCTCAGATTGC	TD-PCR	11	0.600	0.903	0.000***	0.860	GU256735
*Hmid*PS1.1063C	$(TC)_n$...$(CGTG)_n$	**F** AAAGGTTTGTGGAATGTGTGT **R** TACCACACACCCTCAAGTATG	TD-PCR	11	0.533	0.903	0.001***	0.860	GU256736
*Hmid*PS1.1066M	$(GT)_n(TG)_n(TGTT)_n$	**F** AATCCAACAAAGGAAATACCC **R** CACACCAAACAAACAAACAAA	TD-PCR	6	0.188	0.516	0.000***	0.474	GU256737

a: n_a = number of observed alleles.

b: H_o = Observed heterozygosity.

c: H_E = Expected heterozygosity.

d: *** $P < 0.05$ = significant departure from Hardy-Weinberg equilibrium after sequential Bonferroni correction.

e: PIC = polymorphic information content.TD-PCR = Touch-down PCR.

DUP = Duplication of locus.

Table 2.4.5: Informative microsatellite loci used to construct a preliminary linkage map for *Haliotis midae*.

Locus	Segregation model (Male x Female)[a]	Mendelian segregation[b]	Accession number	Reference
*Hm*D14	ef x eg	NS	AY303333	1
*Hm*D33	ef x eg	NS	AY303334	1
*Hm*D55	ab x cd	NS	AY303337	1
*Hm*D59	ab x cd	NS	AY303338	1
*Hm*AD102T	ef x eg	NS	DQ785747	2
*Hm*DL34aR	ab x cd	NS	EF054860	2
*Hm*DL110T	ef x eg	NS	EF054864	2
*Hm*LCS1T	ef x eg	NS	DQ825701	2
*Hm*LCS18M	ef x eg	NS	DQ993217	2
*Hm*LCS37M	ab x cd	NS	DQ993229	2
*Hm*LCS67M	lm x ll	NS	DQ993222	2
*Hm*NR20M	ef x eg	NS	EF063097	2
*Hm*NR120T	ab x cd	NS	EF121745	2
*Hm*NR136D	ef x eg	NS	DQ825710	2
*Hm*NR224T	lm x ll	NS	EF512269	2
*Hm*NR281P	ab x cd	NS	EF512274	2
*Hm*NS6T	hk x hk	NS	EF367117	2
*Hm*NS19L	ab x cd	NS	EF033330	2
*Hm*NS56D	ef x eg	NS	EF455619	2
*Hm*NS100T	ab x cd	NS	EF367114	2
*Hm*RS27T	ab x cd	NS	DQ785751	2

*Hm*RS36T	ef x eg	NS	DQ785753	2
*Hm*RS62D	ab x cd	NS	DQ785777	2
*Hm*RS129D	ab x cd	NS	DQ785766	2
*Hm*NS32M	lm x ll	NS	GQ927142	3
*Hm*LCS152M	ab x cd	NS	GQ927139	3
*Hm*LCS383T	lm x ll	NS	GQ927141	3
*Hmid*0007C	ab x cd	NS	GQ927109	3
*Hmid*0053D	ef x eg	NS	GQ927110	3
*Hmid*0065M	ab x cd	NS	GQ927111	3
*Hmid*0154M	ef x eg	NS	GQ927113	3
*Hmid*0221T	ab x cd	NS	GQ927115	3
*Hmid*0310D	ab x cd	NS	GQ927119	3
*Hmid*0315M	ab x cd	NS	GQ927120	3
*Hmid*0553D	nn x np	NS	GQ927122	3
*Hmid*0558D	ef x eg	NS	GQ927123	3
*Hmid*0563M	lm x ll	NS	GQ927117	3
*Hmid*0610D	ef x eg	NS	GQ927118	3
*Hmid*2044T	ab x cd	NS	GQ927126	3
*Hmid*4010D	ab x cd	NS	GQ927131	3
*Hmid*PS1.38T	nn x np	NS	GU256656	4
*Hmid*PS1.147M	lm x ll	NS	GU256661	4
*Hmid*PS1.150C	ef x eg	***	GU256662	4
*Hmid*PS1.188C	lm x ll	NS	GU256668	4
*Hmid*PS1.193C	ef x eg	NS	GU256669	4
*Hmid*PS1.206C	nn x np	***	GU256672	4

*Hmid*PS1.247M	lm x ll	NS	GU256678	4
*Hmid*PS1.332D	ab x cd	NS	GU256680	4
*Hmid*PS1.374T	nn x np	NS	GU256684	4
*Hmid*PS1.379T	nn x np	NS	GU256686	4
*Hmid*PS1.382D	ab x cd	NS	GU256687	4
*Hmid*PS1.405T	lm x ll	***	GU256689	4
*Hmid*PS1.457T	lm x ll	NS	GU256691	4
*Hmid*PS1.484C	nn x np	NS	GU256693	4
*Hmid*PS1.487T	nn x np	NS	GU256694	4
*Hmid*PS1.549D	ef x eg	NS	GU256696	4
*Hmid*PS1.551C	lm x ll	NS	GU256697	4
*Hmid*PS1.559M	nn x np	NS	GU256698	4
*Hmid*PS1.588C	nn x np	NS	GU256700	4
*Hmid*PS1.638T	lm x ll	NS	GU256703	4
*Hmid*PS1.805T	hk x hk	***	GU256708	4
*Hmid*PS1.807T	nn x np	NS	GU256709	4
*Hmid*PS1.818C	ab x cd	NS	GU256711	4
*Hmid*PS1.859T	nn x np	NS	GU256715	4
*Hmid*PS1.860D	lm x ll	***	GU256716	4
*Hmid*PS1.870C	ab x cd	NS	GU256718	4
*Hmid*PS1.873T	hk x hk	***	GU256719	4
*Hmid*PS1.874C	ab x cd	NS	GU256720	4
*Hmid*PS1.961T	nn x np	NS	GU256724	4
*Hmid*PS1.967M	nn x np	NS	GU256725	4
*Hmid*PS1.981T	ef x eg	NS	GU256727	4

*Hmid*PS1.1009H	lm x ll	NS	GU256730	4
*Hmid*PS1.1012R	ef x eg	NS	GU256731	4
*Hmid*PS1.1018T	ef x eg	NS	GU256732	4
*Hmid*PS1.1026M	ef x eg	***	GU256733	4
*Hmid*PS1.1038T	nn x np	NS	GU256734	4
*Hmid*PS1.1058C	ef x eg	NS	GU256735	4
*Hmid*PS1.1063C	ab x cd	NS	GU256736	4

a: Coding according to JoinMap version 4: <abxcd> = locus heterozygous in both parents with 4 alleles; <efxeg> = locus heterozygous in both parents with three alleles; <hkxhk> = locus heterozygous in both parents with two alleles; <lmxll> = locus heterozygous in the first parent; <nnxnp> = locus heterozygous in the second parent.

b: *** $P < 0.01$ = significant departure from Mendelian segregation ratio.

NS = non-significant.

1: Bester *et al*., 2004.

2: Slabbert *et al*., 2008.

3: Slabbert *et al*., 2010.

4: This Section 2.4.

Table 2.4.6: Average genome coverage and number of markers per linkage group and observed and estimated length per linkage group.

		Map length (cM, Kosambi)		
Linkage group (LG)	**Nr. of markers**	**Observed**	**GE_1**	**GE_2**
LG1	5	18.04	27.34	27.06
LG2	3	17.75	27.04	35.49
LG3	3	16.78	26.07	33.55
LG4	3	15.62	24.91	31.23

LG5	3	11.75	21.04	23.49
LG6	2	10.14	19.43	30.41
LG7	4	9.91	19.20	16.51
LG8	3	9.48	18.77	18.96
LG9	2	8.30	17.59	24.89
LG10	2	6.94	16.24	20.82
LG11	2	6.49	15.78	19.46
LG12	3	5.45	14.75	10.91
LG13	3	3.35	12.64	6.69
LG14	2	3.34	10.01	12.63
LG15	4	3.23	12.52	5.38
LG16	2	2.18	11.47	6.53
LG17	2	0.00	9.30	0.00
LG18	2	0.00	9.30	0.00
Total	50	148.72	316.06	321.42
Genome coverage (%)			47.06	46.27

Observed = Map length observed in cM; GE_1 = Map length estimated according to Fishman *et al.* (2001); 2) GE_2 = Map length estimated using according to Chakravarti *et al.* (1991).

Table 2.4.7: Contigs showing significant similarity to microsatellite loci from other *Haliotis* spp.

Contig	Microsatellite Locus of Other *Haliotis* spp.	E-value
Contig 486	*H. discus hannai* Afa047	$E = 4 \times 10^{-13}$
Contig 386	*H. discus hannai* Ahdh644	$E = 2 \times 10^{-20}$
Contig 440	*H. discus hannai* Eab638	$E = 10^{-13}$
Contig 871	*H. discus hannai* Eab638	$E = 3 \times 10^{-13}$

Contig 880	*H. discus hannai* Hdh-k282	$E = 3 \times 10^{-17}$
Contig 993	*H. discus hannai* Hdh-k282	$E = 10^{-13}$
Contig 761	*H. discus hannai* Hd715	$E = 2 \times 10^{-21}$
Contig 531	*H. discus hannai* Hd731	$E = 4 \times 10^{-10}$
Contig 572	*H. rubra* Hrub1	$E = 4 \times 10^{-14}$
Contig 1036	*H. rubra* Hrub1	$E = 2 \times 10^{-17}$
Contig 782	*H. rubra* Hrub1	$E = 10^{-13}$
Contig 910	*H. rubra* Hrub1	$E = 2 \times 10^{-11}$
Contig 633	*H. rubra* Hrub9	$E = 8 \times 10^{-26}$
Contig 576	*H. rubra* Hrub9	$E = 10^{-25}$
Contig 530	*H. rubra* Hrub9	$E = 8 \times 10^{-12}$
Contig 383	*H. rubra* Hrub11	$E = 4 \times 10^{-16}$
Contig 175	*H. rubra* Hrub13	$E = 10^{-15}$

2.4.4) Discussion

A total of 82 microsatellite markers were developed for *H. midae* using the FIASCO-method and 454-pyrosequencing. The results in terms of data generated (Table 2.4.2) is comparable to those for other species that also used similar strategies. When the results are compared to previous isolation attempts in *H. midae* (Bester *et al.*, 2004; Slabbert *et al.*, 2008; Slabbert *et al.*, 2010) fewer microsatellite containing sequences were generated (28% *vs.* 45-49% of all contigs and singletons generated; Table 2.4.2), but a higher number of polymorphic markers were eventually obtained (8% vs. 4-6% of all contigs and singletons generated; Table 2.4.2). A major advantage of this study was that all the pyrosequencing data was generated in a single event in contrast to multiple cloning reactions for previous publications. Pyrosequencing therefore entailed far less laboratory work by eliminating library construction and screening of clones through direct sequencing of the genomic library.

The usefulness of the newly designed microsatellite markers for future applications such as population structure analysis, parentage assignments and linkage mapping were assessed by calculating various parameters. Deviations from Hardy-Weinberg equilibrium (Table 2.4.4) were mostly a result of heterozygote deficiency caused by either the presence of null alleles (O'Connell and Wright,

1997), and allele drop-out or scoring errors (Jones and Ardren, 2003). Two observations (*Hmid*PS1.469R and *Hmid*PS1.1007C) were explained by heterozygote excess caused by either allele scoring errors or selection against homozygotes or more likely by the small sample used for analyses. These loci should be used with caution although loci such as *Hmid*PS1.469R and *Hmid*PS1.1007C could be interesting candidates for studying selection processes in the life history of *H. midae*. The high PIC-values obtained for many of the loci makes them good candidates for parentage assignments and linkage mapping, since the chances of it being informative in both parent and offspring are good. Amplification of more than the expected two alleles has previously been observed in *H. midae* (Slabbert *et al.*, 2010) and *H. rubra* (Evans *et al.*, 2001; Baranski, 2006). The exact mechanisms responsible for this occurrence are still unclear, but could be explained by genome duplication, polyploidy, aneuploidy or conserved microsatellite repeat tracts and flanking regions found in mobile elements (Hubert *et al.*, 2000; Rodzen and May, 2002; David *et al.*, 2003; Meglécz *et al.*, 2004). A recent study by Rhode (2010) found that 21% of all known *H. midae* microsatellite loci are associated with characterised transposable elements, which play a role in locus duplication. This high association could therefore be the most likely explanation for duplications seen in abalone microsatellites. An example where duplication was found within the current study is locus *Hm*LCS152 (Section 2.3), which is associated with a retrotransposon (pers. comm.: Clint Rhode, Molecular Aquatic Research Group).

A search of *Haliotis* spp. and KEGG databases found no significant identities between any of the polymorphic loci and genes. Only two non-repeat containing contigs showed similarities to genes, namely NADH dehydrogenase subunit 5 (ND5) and cytidine deaminase. The similarities found for the non-polymorphic loci isolated in this study to other published microsatellite loci are further confirmation for microsatellite conservation between abalone species (Table 2.4.7; Evans *et al.*, 2001, 2004a; Sekino and Hara, 2007a; Rhode, 2010). Unfortunately the monomorphic nature of the loci won't allow comparative genome analysis using linkage mapping, but second generation sequencing would be very useful for such future studies (e.g.: Green *et al.*, 2010). The more in depth characterisation of these {and other such loci identified by Rhode (2010)} will allow studies into the evolutionary dynamics of microsatellites and their functions (Liu and Ely, 2009).

The application of the markers for mapping and their distribution within the genome was assessed by constructing a preliminary linkage map for *H. midae*. The application of the newly developed markers for linkage mapping was proven since eight new linkage groups were constructed after their addition. The estimated genome length (316.06 and 321.42cM) was much smaller than for *H. rubra* (male = 940.5cM; female = 1586.2cM; Baranski *et al.*, 2006a), *H. discus hannai* (male = 866.7 and 931.4cM; female = 1126.7 and 1186.7cM; Sekino and Hara, 2007b) and *H. diversicolor* (male = 1896.5cM and female = 1875.2cM; Shi *et al.*, 2010). This is to be expected for the current linkage map since only 50 markers and 32 individuals were used for its construction and therefore the recombination rate between markers is most probably underestimated. Many linkage groups contained only two or three markers and the potential exist that these could either be expanded or incorporated into other linkage groups. The large number of unlinked loci may be due to the small mapping population size resulting in many linkages staying undetected (Coimbra *et al.*, 2003). A more comprehensive linkage map, using additional markers and larger sample sizes is in progress, for which the current map serves as a reference.

The amount of data generated by the combination of a microsatellite enrichment strategy (FIASCO) and pyrosequencing (454) were accurate and adequate enough for developing and characterising 82 polymorphic microsatellite markers. Pyrosequencing provides sequence information on all available DNA fragments present within the enriched library in contrast to traditional cloning where technical, time and budget constraints causes the loss of much information. The characterisation of the newly designed markers showed that many of them would be useful for parentage and population studies. Linkage mapping showed that the markers are distributed throughout the genome. The additional markers will also contribute to the construction of a more detailed linkage map. *In silico* analysis of the 454-data also identified the potential targets for further evolutionary and mechanistic studies regarding flanking regions and repeat tracts.

CHAPTER 3

Genome Scan for QTL Affecting Size in *Haliotis midae* Using Selective DNA Pooling and Microsatellite Loci

3.1) Introduction

Haliotis midae is a valuable commercial export product for the South African aquaculture industry fetching around USD 38 per kg (pers. comm. Mr. Wayne Barnes, Abalone Farmers Association). This species became the subject of a genetic improvement programme in 2006 (Brink *et al.,* 2009b; Slabbert *et al.,* 2009b).

Growth rate is key to a profitable abalone industry (Elliott, 2000). It was found that heritabilities for growth rate for two abalone species (*H. rufescens* and *H. asinina*) are high, but only at two years of age and older (Jonasson *et al.,* 1999; Lucas *et al.,* 2006). This makes it difficult to select animals when they are still young. The best strategy would then be marker assisted selection (MAS), which uses data from QTL studies. Hayes *et al*. (2007) simulated a MAS programme and found that the genetic gains for growth can be as high as 15% within a single generation.

A number of QTL have been identified in aquatic invertebrate and mollusc species. These include oysters (Yu and Guo, 2006; Lallias *et al.,* 2009), scallop (Qin *et al.,* 2007) and shrimp (Lyons *et al.,* 2007). QTL affecting growth rate have also been mapped for the abalone species *Haliotis discus hannai* (Liu *et al.,* 2007) and *H. rubra* (Baranski *et al*., 2008). Sufficient molecular tools and full- and half-sib families (spawned from December 2006) have now become available for *H. midae* to justify the construction of a linkage map and QTL analysis. To date, 215 microsatellite markers (Bester *et al.,* 2004; Rhode, 2010; this dissertation) have been developed for this species, allowing a QTL mapping study.

The high fecundity of abalone makes it an ideal species for a large full-sib family design (Massault *et al*., 2008). This design is used where artificial spawning facilitates the use of large full-sib families. Large families are ideal for selective genotyping, which is based on the differences in allele frequencies between the

upper and lower tails of the phenotypic distributions. To reduce the costs of genotyping such large families, a combination of bulked segregant analysis (Michelmore *et al.,* 1991), selective DNA pooling (Darvasi and Soller, 1994) and tail analysis (Hillel *et al.,* 1990) can be used for QTL detection. This design was evaluated by Baranski *et al*. (2008) in abalone. Selective DNA pooling reduces the amount and costs of genotyping required for QTL-mapping and is sensitive enough to detect small differences between the different phenotypic pools (Darvasi and Soller, 1994; Breen *et al.,* 1999). DNA pooling has already been used for successful QTL identification in cattle, chicken and abalone (Lipkin *et al.,* 1998, 2002; Fisher and Spelman, 2004; Cobanoglu *et al.,* 2005; Baranski *et al*., 2008). It has also been utilised in applications such as detecting signatures of selective sweeps (Thomas *et al.,* 2007), population studies (Hillel *et al.,* 2003) and allele frequency determination (Schwarz *et al.,* 2004; Skalski *et al.,* 2006).

For verification of the pooling analysis results it can be followed up using single marker analysis by applying ANOVA to test for differences in locus means between traits (Kearsey, 1998). Such an approach requires no linkage map since no positional information is given after this analysis. Single marker regression can in turn be followed up by interval mapping (Lander and Botstein, 1989). Interval mapping uses the information of adjacent loci to map the position of a QTL (Kearsey, 1998).

In this study a genome-wide search of two full-sib families was conducted to identify QTL for shell length, shell width and wet weight. Pooling analysis was conducted in an initial screening using microsatellite loci. The significant markers identified in this way were genotyped in the individual animals in addition to a number of non-significant or linked loci to verify the accuracy of the pooling analysis. A linkage map was constructed to identify any linkage groups containing segregating QTL. Finally these linkage groups were used for interval mapping to identify the position and effect of the QTL.

3.2) Materials and Methods

3.2.1) Sampling and Phenotyping

Two full-sib families were chosen from the 73 full- and half-sib families subscribed to a genetic improvement programme for *Haliotis midae*. Family 7B was located on the Roman Bay Hatchery (Gansbaai, RSA) and Family 42A was situated on HIK Abalone Hatchery (Hermanus, RSA). These locations are approximately 50km apart and have different management and production protocols. The environmental conditions for each family therefore differed.

The four parental animals from Family 42A and Family 7B were sampled by taking two epipodial tentacles per individual and were stored in 100% ethanol until the extraction step. All available offspring was also sampled for each family. A total of 931 individuals, aged 19 months (spawned 7 January 2007) from Family 42A and 424 individuals aged 20 months (spawned 7 December 2006) from Family 7B were tagged using bee-tags (*Swienty*, Norway; Figure 3.1). Two to three epipodial tentacle samples were taken of each animal according to Slabbert and Roodt-Wilding (2006; Figure 3.2) and stored in 100% ethanol. Phenotypic measurements of total wet weight (g), shell length (mm) and shell width (mm) were recorded using a balance and calliper. Both shell length and width were measured at the widest parts of the shell (Figure 3.3). Approximately 10% of the individuals at the phenotypic tails were selected for DNA extraction and pool construction.

Figure 3.1: An abalone tagged with a bee-tag (green).

Figure 3.2: Sampling equipment used for collection of epipodial tentacles.

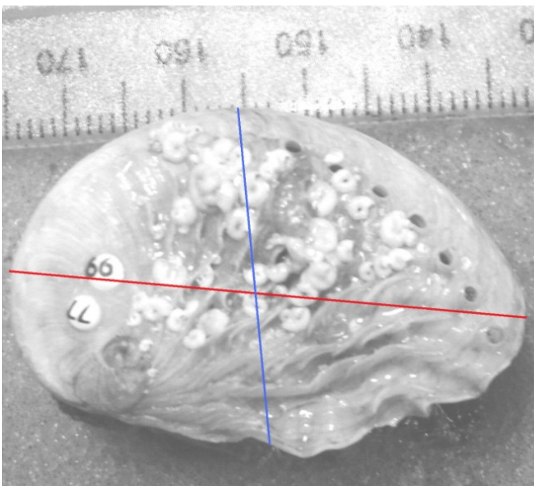


Figure 3.3: The measuring of shell length, indicated in red, and shell width, indicated in blue.

3.2.2) DNA Extractions and Pool Construction

DNA was purified from 202 individuals (sum of ~10% from the upper and ~10% of the bottom phenotypic tail) from Family 42A and 82 individuals (sum of ~10% from the upper and ~10% of the bottom phenotypic tail) from Family 7B. The NucleoSpin Plant II kit (*Macherey-Nagel*) was used for performing the DNA extractions. Extractions were done on the Genesis RMP200 (*Tecan*) robotic platform. Two to three epipodial tentacles were added to 400μl of Buffer PL2 as well as 2μl of 10mg/ml proteinase K (*Sigma-Aldrich*) and incubated overnight at 60℃. A volume of

100μl Buffer PL3 was then added and the mixture was incubated on ice for 5 minutes and centrifuged at 6000g for 30 minutes. A total of 400μl supernatant was mixed with 450μl binding buffer PC and transferred to a silica membrane plate. The mixture was sucked through the membrane by a vacuum system (-0.4 bar). The bound DNA was washed to remove proteins and salts with 400μl buffer PW1 and twice with 700μl buffer PW2. A vacuum step followed each washing step (-0.4 bar). The bound DNA was eluted twice with 100μl volumes of elution buffer preheated to 70°C. DNA quantification was performed using absorbance at 260nm with the Infinite F200 NanoQuant (*Tecan*).

To ensure that each individual is represented by an equal amount of DNA in each pool, all samples were standardised to a concentration of 5ng/μl. Two pools were created in duplicate for each phenotypic tail (Top and Bottom) for each family; therefore eight DNA pools were made. Each pool contained an equal number of individuals. The layout of these pools is shown in Table 3.1.

Table 3.1: The names and number of individuals of each DNA pool.

Family	Pools	Number of Individuals
42A	Top A	51
	Top B	50
	Bottom A	51
	Bottom B	50
7B	Top A	20
	Top B	20
	Bottom A	21
	Bottom B	21

3.2.3) Parental and Pools Microsatellite Genotyping

Both parental and pooled samples were genotyped using the markers described in Sections 2.2 to 2.4 (163 in total; 26 loci developed by Alida Venter in Section 2.3 was excluded, because it was not yet characterised at the time of the pooling analyses) as well as 6 additional markers described by Bester *et al.* (2004). The parental genotyping was performed by Dr. P.Z. Wang and J. Hepple. The PCR

conditions of these markers were discussed in Sections 2.2 to 2.4 and in Bester *et al.* (2004). These markers were multiplexed before the automated electrophoresis analyses into 36 different multiplexes (Appendix B, Table B.1). The final DNA concentration per reaction was approximately 10ng. Genotyping was performed on a 3730xl DNA Analyzer (*Applied Biosystems*) and genotyping and peak heights were recorded with GeneMapper version 4 (*Applied Biosystems*). According to Breen *et al.* (1999) either peak heights or peak area can be used when collecting fluorescent intensity data during pooling analyses, since the results of both are similar. In the current study peak heights between 50 and 30 000 fluorescent units (RFU) were scored.

3.2.4) Pooling Analysis

The ratio of the parental alleles was calculated as the height of the small allele (H_S) to the height of the large allele (H_L; see Figure 3.4): H_S/H_L and converted to log ratios using Microsoft Excel 2007. The null hypothesis to be tested was $(H_S/H_L)_{top} = (H_S/H_L)_{bottom}$, hence $(H_S/H_L)_{top} / (H_S/H_L)_{bottom} = 1.0$. A general linear model (GLM), loosely based on Baranski *et al.* (2008), was used to test this null hypothesis using the following independant variables: Pool (the DNA pool of the tails), Gender (sire or dam which contributed the allele pair), Marker (microsatellite locus) and all the relevant interactions. The GLM was fitted with Pool, Parent and Marker as the main effects. All possible interactions up to the 3rd level were included in the model. Each family was analysed separately. Analysis was performed using SAS version 9 (SAS Institute Inc., 2004). If the null hypothesis was rejected, therefore indicating that there were differences present between the top and bottom tails, a t-test was subsequently performed to test for individual parent-marker significance. Bonferroni correction for multiple tests was applied for the pooled data (Rice, 1989).

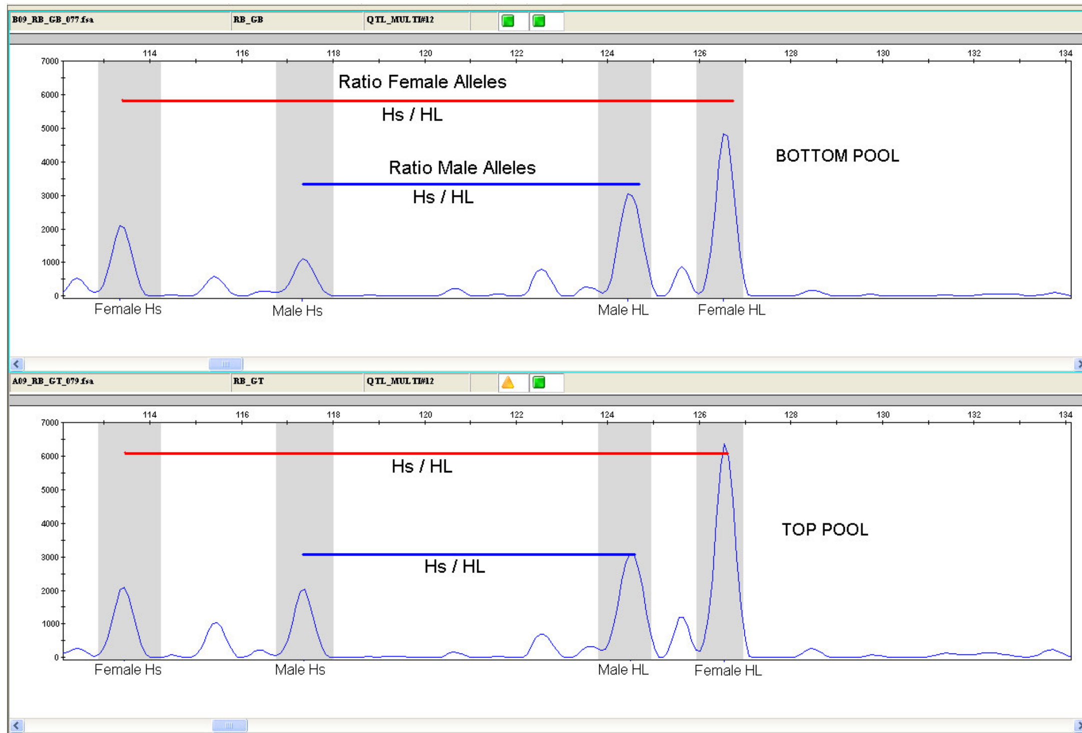


Figure 3.4: An electropherogramme showing the parental alleles for Family 7B, locus *Hm*D59. The ratios for female (red line) and male alleles (blue line) are calculated by dividing the height of the smaller allele (H_S) by the height of the larger allele (H_L) and converting it to logratios.

3.2.5) Individual Genotyping and Single Marker Regression

Single marker regression was performed in order to assess the accuracy of the pooling analyses. Loci showing significant differences in the pooling analyses were individually typed in both families (42A and 7B). Additional loci, showing no significant differences or that were on the same linkage group as the loci identified after pooled analysis, were also individually typed in each family. These markers were chosen from the linkage map in Section 2.4 and the preliminary linkage map that became available early 2010, constructed by Dr. P.Z. Wang and J. Hepple (unpublished) so that it covers different linkage groups. To save costs the loci were then chosen so they could be multiplexed in a single genotyping run (Appendix B, Table B.3). A protocol for family specific multiplex setups is described in Section 4.3. The PCR's were performed separately and then pooled before genotyping. The amplification conditions of the markers are discussed in Sections 2.2 to 2.4.

Genotyping was performed on a 3730xl DNA Analyzer (*Applied Biosystems*) and genotyping with GeneMapper version 4 (*Applied Biosystems*). Single marker regression, which does not require a linkage map, was carried out in MapQTL version 5.0 (Van Ooijen, 2004) by nonparametric means using the Kruskal-Wallis test.

3.2.6) Linkage Mapping and Interval Mapping

A linkage map was created for each family using the genotype data obtained in in section 3.2.5. JoinMap version 4.0 (Van Ooijen, 2006) was used applying regression mapping at an independence LOD threshold of 3.0 and the CP population model. Map distances were calculated using the Kosambi map function. The linkage map was drawn using MapChart version 2.2 (Voorrips, 2002). Linkage groups were named according to origin (Family 42A or 7B) and number (arranged from longest to shortest map distances). These maps were constructed for interval mapping since the map constructed in Section 2.4 was assembled using a different family and the linkage maps constructed in another project (Wang and Hepple; unpublished) were still preliminary and some genotype and phenotypic data were unavailable for that data set. The complete linkage groups from this chapter, Section 2.4 and the preliminary map of Wang and Hepple was compared using MapChart version 2.2 (Voorrips, 2002). Linkage groups from Section 2.4 will be preceded by the suffix 2.4 and the preliminary map of Wang and Hepple by PZJH.

Only complete linkage groups were used for interval mapping and any loci showing segregation distortion ($P < 0.01$) was excluded from this analysis. Interval mapping was performed with a maximum likelihood approach (Titterington *et al.*, 1985; McLachlan and Basford, 1988) in MapQTL version 5 (Van Ooijen, 2004) using the default analysis parameters and the CP population model. A LOD score of > 3 was taken as indicating a significantly segregating QTL. The percentage of variance explained by the QTL was also calculated using this software. Three traits, namely wet weight, shell width and shell length were analysed.

3.3) Results

3.3.1) Phenotyping

The phenotypic averages of the combined top and combined bottom pools per family as well as the percentage difference between the phenotypic values are shown in Table 3.2. The differences between the phenotypic extremes are 25% and more for each trait.

Table 3.2: Phenotypic averages for length, width and weight per family.

Trait	Phenotype	Family 42A	Family 7B
Length (mm)	Top	42.86 (±6.98)	45.00 (±3.89)
	Bottom	31.33 (±2.69)	29.58 (±3.65)
% Difference		26%	34%
Weight (g)	Top	14.27 (±6.92)	16.18 (±6.56)
	Bottom	5.46 (±0.66)	5.31 (±1.04)
% Difference		61%	67%
Width (mm)	Top	26.96 (±2.42)	28.35 (±1.97)
	Bottom	20.06 (±0.78)	19.27 (±2.17)
% Difference		25%	32%

Family 42A: 202 individuals; Family 7B: 82 individuals.

3.3.2) Pooling Analysis

Only informative loci were analysed. A locus was regarded as informative when it amplified successfully, had no null alleles and was heterozygous in at least one parent. A total of 58 of 163 (36%) microsatellite loci were informative in Family 7B, while 93 of 163 (57%) loci were informative for Family 42A. A total of 118 parent-marker combinations could be analysed in Family 7B and 142 in Family 42A. The null hypothesis of no difference between the tail pools for all three traits combined was rejected by the general linear model with $P < 0.001$ (Table 3.3).

Table 3.3: General linear model results per family for marker interactions.

Source of Variation	Sum of Squares	Mean Square	F-Value	*P*-value
Family 42A				
Parent.Marker	21.38	0.63	18.98	***
Pool.Parent.Marker	7.41	0.22	6.58	***
Family 7B				
Parent.Marker	14.30	0.65	24.80	***
Pool.Parent.Marker	1.99	0.09	3.45	***

*** = Significant for $P < 0.001$.

A total of five parent-marker combinations in Family 42A and two in Family 7B deviated from $P = 1$ (Table 3.4). Not all combinations were significant, but the *P*-values were still small enough to warrant further investigation. In Family 42A loci *Hm*D59, *Hm*LCS1, *Hm*RS62, *Hm*D44 and *Hm*LCS147 were candidates for linkage to all three size related (length, width and weight) QTL, while locus *Hmid*PS1.818 was identified in Family 7B. These markers were then used for individual genotyping.

Table 3.4: Results for the individual t-tests of loci with $P \leq 1$.

Family	**Locus**	**Parent**	***P*-value**
42A	*Hm*D59	Male	0.294
	*Hm*LCS1	Male	**
	*Hm*RS62	Male	***
	*Hm*D44	Female	***
	*Hm*LCS147	Female	***
7B	*Hmid*PS1.818	Male	0.277
	*Hmid*PS1.818	Female	0.071

** = Significant for $P < 0.05$.

*** = Significant for $P < 0.01$.

3.3.3) Individual Genotyping and Single Marker Regression

A total of 18 loci were typed in Family 42A using the individuals from the pooled analyses. In Family 7B, a total of 14 loci were typed using the individuals used in the pooled analyses. These loci are shown in Table 3.5. The linkage groups indicated in Table 3.5 are based on the sex averaged maps of Wang and Hepple that became available in 2010.

Table 3.5: Loci used for individual genotyping shown per family. The linkage group number is based on the 2010 preliminary map of Wang and Hepple.

Family	Locus	Linkage Group
42A	*Hm*LCS147[#]	1
	*Hm*NR281[##]	1
	*Hmid*PS1.551[##]	1
	*Hmid*PS1.1066	2
	*Hmid*PS1.972[##]	3
	*Hmid*PS1.433[##]	3
	*Hm*2H6FT[##]	3
	*Hm*RS62[#]	3
	*Hm*D59[#]	3
	*Hm*LCS1[#]	3
	*Hm*D55	6
	*Hm*RS27	9
	*Hmid*PS1.818	10
	*Hmid*PS1.1063	10
	*Hmid*PS1.247	10
	*Hm*NR20	12
	*Hmid*PS1.860	17
	*Hm*D44	Unlinked
7B	*Hmid*PS1.1009	1

	*Hmid*PS1.551	1
	*Hm*D59	2
	*Hm*RS62	2
	*Hmid*PS1.818#	5
	*Hmid*PS1.1063##	5
	*Hm*LCS383##	5
	*Hmid*PS1.179##	5
	*Hmid*PS1.860	6
	*Hmid*PS1.981	8
	*Hmid*PS1.1012	11
	*Hmid*PS1.1058	12
	*Hm*NR120	15
	*Hm*D55	18

= Significant locus identified after pooled analysis.

= Locus on the same linkage group as significant locus identified after pooled analysis.

Locus *Hm*D44 (Family 42A) and locus *Hmid*PS1.1009 (Family 7B) failed to amplify at all and was discarded. Locus *Hm*2H6FT (Family 42A) was discarded after more than half the individuals failed to amplify. The individual genotyping revealed that some of the individuals within the experimental populations were not part of the families under investigation. Within Family 42A a total of 44 individuals (22%) from both top and bottom phenotypic pools were discarded from further analyses. A total of 13 individuals (16%) from the top and bottom phenotypic pools of Family 7B were discarded due to either missing genotype data or because they were part of a different family. The numbers of the phenotypic extremes were readjusted so that an equal number of individuals represent the top and bottom groups (Table 3.6). The

phenotypic averages of the reassembled extremes are shown in Table 3.7 and were comparable to those of the original population (Table 3.2).

Table 3.6: The number of individuals of the original and the reconstructed phenotypic extremes.

Family	Pools	Original Phenotypic Extremes: Number of Individuals	Reconstructed Phenotypic Extremes: Number of Individuals
42A	Top	101	61
	Bottom	101	61
7B	Top	40	37
	Bottom	42	36

Table 3.7: Phenotypic averages for length, width and weight per family after reconstruction of the extremes.

Trait	Phenotype	Family 42A	Family 7B
Length (mm)	Top	42.64 (±7.03)	44.90 (±2.36)
	Bottom	31.51 (±2.72)	29.60 (±3.88)
% Difference		26%	34%
Weight (g)	Top	13.70 (±4.99)	15.86 (±3.49)
	Bottom	5.64 (±0.74)	5.30 (±1.15)
% Difference		58%	67%
Width (mm)	Top	26.65 (±1.96)	28.18 (±1.41)
	Bottom	20.26 (±0.76)	19.20 (±2.35)
% Difference		24%	32%

The single marker regression analysis is shown in Table 3.8. This analysis confirmed the findings of the pooled analysis for loci *Hm*RS62, *Hm*D59 and *Hm*LCS1 in Family 42A and for locus *Hmid*PS1.818 in Family 7B. Locus *Hm*LCS147, which

showed in Family 42A highly significant ($P < 0.001$) association with a potential QTL did not show any significance after single marker regression analysis. This could be explained by the segregation distortion seen for this marker (see below) due to genotyping difficulty within the female. We were unable to amplify the female alleles in the individual offspring, even though the alleles were present in the pools. A possible reason can be allele drop out caused by the change in PCR conditions during pooled sample and individual sample amplification. The higher copy numbers of different individuals in a pooled sample could have increased the chances of amplifying these alleles. Additional associations were found in Family 42A where loci *Hmid*PS1.433, *Hmid*PS1.247, *Hmid*PS1.1063 and *Hm*RS27 showed significance for length, width and weight (Table 3.8). In Family 7B locus *Hmid*PS1.551 was significant for all three traits (Table 3.8). Interestingly this locus showed no significance in Family 42A for any trait, indicating a possible false result or that the QTL are not segregating on that chromosome in this family. Locus *Hmid*PS1.1063 stands out in both families, making that linkage group an excellent candidate for more detailed QTL mapping (underlined and bolded in Table 3.8).

Table 3.8: Results of Kruskal-Wallis single marker regression analysis as performed in MapQTL version 5.

Family	**Locus**	**Linkage Group**	**Significance**		
			Length	**Width**	**Weight**
42A	*Hm*LCS147#	1	NS	NS	NS
	*Hm*NR281##	1	NS	NS	NS
	*Hmid*PS1.551##	1	NS	NS	NS
	*Hmid*PS1.1066	2	NS	NS	NS
	*Hmid*PS1.972##	3	NS	NS	NS
	*Hmid*PS1.433##	3	$P < 0.0001$	$P < 0.0001$	$P < 0005$
	*Hm*RS62#	3	$P < 0.01$	$P < 0.05$	$P < 0.1$

	*Hm*D59[#]	3	*P* < 0.001	*P* < 0.005	*P* < 0.01
	*Hm*LCS1[#]	3	*P* < 0.01	*P* < 0.05	*P* < 0.1
	*Hm*D55	6	NS	NS	NS
	*Hm*RS27	9	*P* < 0.001	*P* < 0.005	*P* < 0.001
	*Hmid*PS1.818	10	NS	NS	NS
	_Hmid_PS1.1063	10	*P* < 0.1	*P* < 0.01	*P* < 0.05
	*Hmid*PS1.247	10	*P* < 0.05	*P* < 0.005	*P* < 0.05
	*Hm*NR20	12	NS	NS	NS
	*Hmid*PS1.860	17	NS	NS	NS
7B	*Hmid*PS1.551	1	*P* < 0.01	*P* < 0.05	*P* < 0.01
	*Hm*D59	2	NS	NS	NS
	*Hm*RS62	2	NS	NS	NS
	*Hmid*PS1.818[#]	5	*P* < 0.0005	*P* < 0.005	*P* < 0.0005
	_Hmid_PS1.1063[##]	5	*P* < 0.0005	*P* < 0.0005	*P* < 0.0005
	*Hm*LCS383[##]	5	*P* < 0.005	*P* < 0.05	*P* < 0.01
	*Hmid*PS1.179[##]	5	*P* < 0.01	*P* < 0.005	*P* < 0.005
	*Hmid*PS1.860	6	NS	NS	NS
	*Hmid*PS1.981	8	NS	NS	NS
	*Hmid*PS1.1012	11	NS	NS	NS
	*Hmid*PS1.1058	12	NS	NS	NS
	*Hm*NR120	15	NS	NS	NS
	*Hm*D55	18	NS	NS	NS

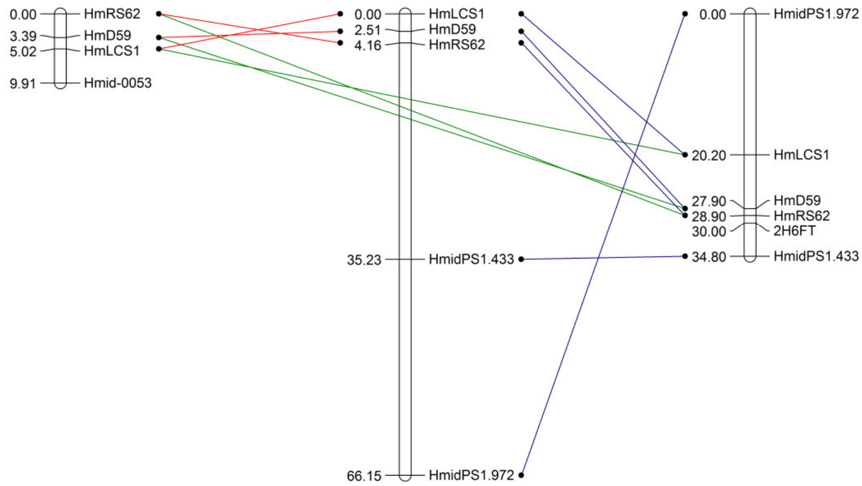
= Significant locus identified after pooled analysis.

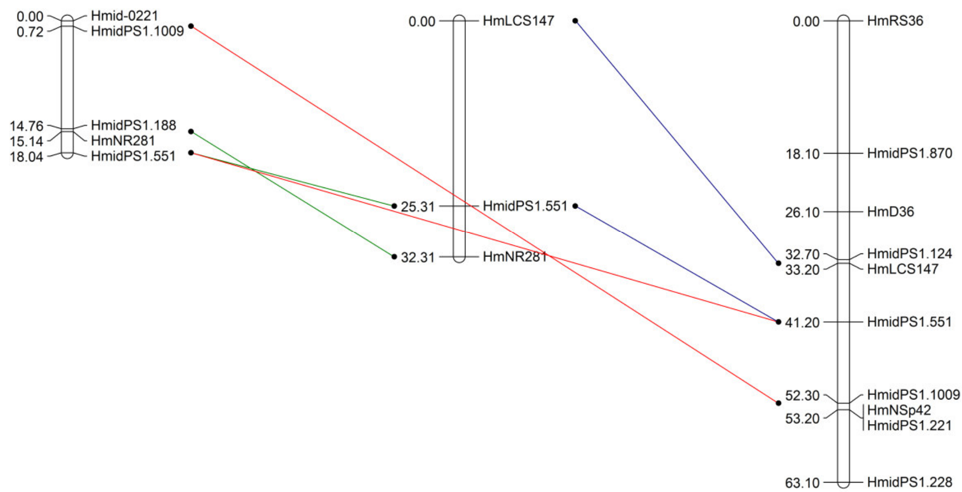
= Locus on the same linkage group as significant locus identified after pooled analysis.

NS = Non-significant.

3.3.4) Linkage Mapping

Linkage mapping of the experimental families were performed for the loci given in Table 3.8. Three linkage groups were created for Family 42A, namely 42A_LG1, 42A_LG2 and 42A_LG3. Two groups were constructed for Family 7B, namely 7B_LG1 and 7B_LG2. Locus *Hmid*PS1.972 was forced onto 42A_LG1 by lowering the LOD threshold to 1.0. Loci *Hm*LCS147 and *Hmid*PS1.433 showed significant segregation distortion ($P < 0.0001$) and hence were removed from this analysis. Locus *Hm*NR281 was not mapped on the Wang and Hepple linkage map. Linkage groups assigned with 2.4 and PZJH are from Section 2.4 and the preliminary linkage maps respectively. The linkage groups for Family 42A are shown in Figure 3.5. Linkage group 42A_LG1 was similar to linkage group 7 of the Section 2.4 map and to linkage group 3 from the PZJH preliminary map for Family 42A. Linkage group 42A_LG2 was similar to linkage group 1 of the Section 2.4 map and to linkage group 1 from the PZJH preliminary map for Family 42A. Linkage group 42A_LG3 was similar to linkage group 13 of the Section 2.4 map and to linkage group 10 from the PZJH preliminary map for Family 42A.


2.4_LG7
42A_LG1
PZJH_42A_LG3
0.00 HmRS62
3.39 HmD59
5.02 HmLCS1
9.91 Hmid-0053
0.00 HmLCS1
2.51 HmD59
4.16 HmRS62
35.23 HmidPS1.433
66.15 HmidPS1.972
0.00 HmidPS1.972
20.20 HmLCS1
27.90 HmD59
28.90 HmRS62
30.00 2H6FT
34.80 HmidPS1.433


2.4_LG1
42A_LG2
PZJH_42A_LG1
0.00 Hmid-0221
0.72 HmidPS1.1009
14.76 HmidPS1.188
15.14 HmNR281
18.04 HmidPS1.551
0.00 HmLCS147
25.31 HmidPS1.551
32.31 HmNR281
0.00 HmRS36
18.10 HmidPS1.870
26.10 HmD36
32.70 HmidPS1.124
33.20 HmLCS147
41.20 HmidPS1.551
52.30 HmidPS1.1009
53.20 HmNSp42
HmidPS1.221
63.10 HmidPS1.228

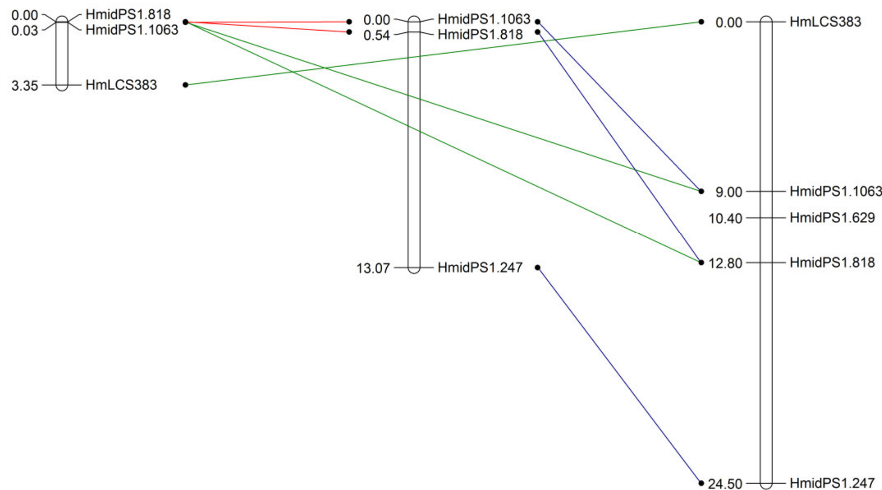


Figure 3.5: Linkage groups comparison for the QTL sample map of Family 42A between the map of Section 2.4 (2.4_LGx), the QTL samples in this chapter (42A_LG1-3) and the map of Wang and Hepple (PZJH_42A_LGx).

The linkage groups for Family 7B are shown in Figure 3.6. Loci *Hmid*PS1.860 and *Hmid*PS1.1012 showed significant segregation distortion ($P < 0.0001$) and hence were removed from this analysis. Locus *Hmid*PS1.179 did not map on 7B_LG1 as expected, but still showed weak linkage to that group. Linkage group 7B_LG1 was similar to linkage group 13 of the Section 2.4 map and to linkage group 5 from the PZJH preliminary map for Family 7B. Linkage group 42A_LG1 was similar to linkage group 7 of the Section 2.4 map and to linkage group 2 from the PZJH preliminary map for Family 7B.

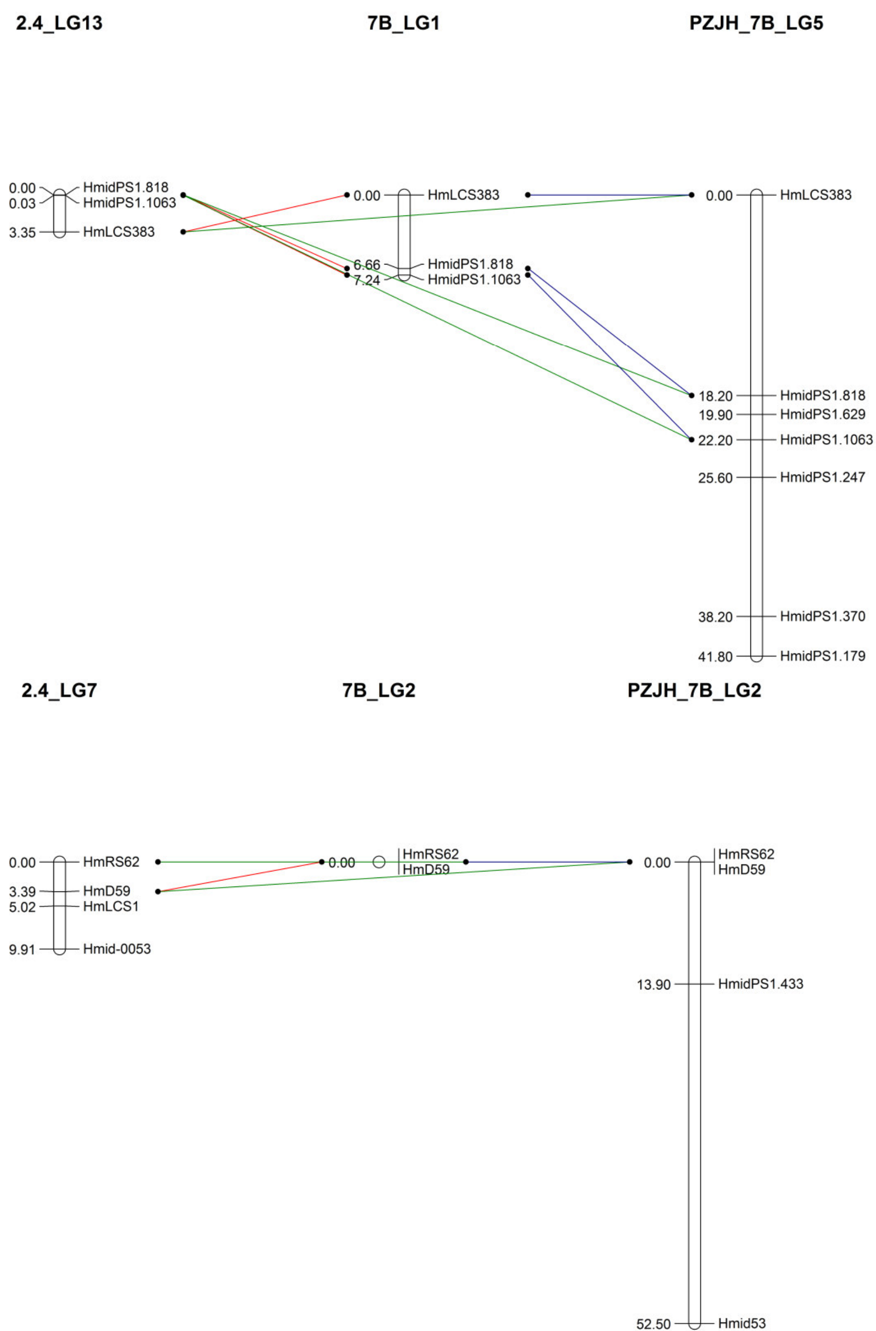


Figure 3.6: Linkage groups comparison for the QTL sample map of Family 7B between the map of Section 2.4 (2.4_LGx), the QTL samples in this chapter (7B_LG1-2) and the map of Wang and Hepple (PZJH_7B_LGx).

3.3.5) Interval Mapping

Linkage groups 42A_LG1-3 and 7B_LG1-2 were subsequently used for interval mapping.

3.3.5.1) Family 42A

The results of the interval mapping for Family 42A are shown in Figure 3.7 for shell length, Figure 3.8 for shell width and Figure 3.9 for wet weight. Loci *Hmid*PS1.433 and *Hm*LCS147 were excluded from this analysis due to segregation distortion which could lead to incorrect results. Two significant (LOD > 3.0) putative QTL were mapped on 42A_LG3 for the traits shell width and wet weight explaining 17% and 15% of the phenotypic variance, respectively. The QTL for these traits mapped to the same position, suggesting that the same gene (s) or chromosomal segment is involved in the control of these traits. This result correlates with the single marker regression (see section 3.3.3). The other significant associations identified by single marker regression for 42A_LG1 were not observed after interval mapping analysis.

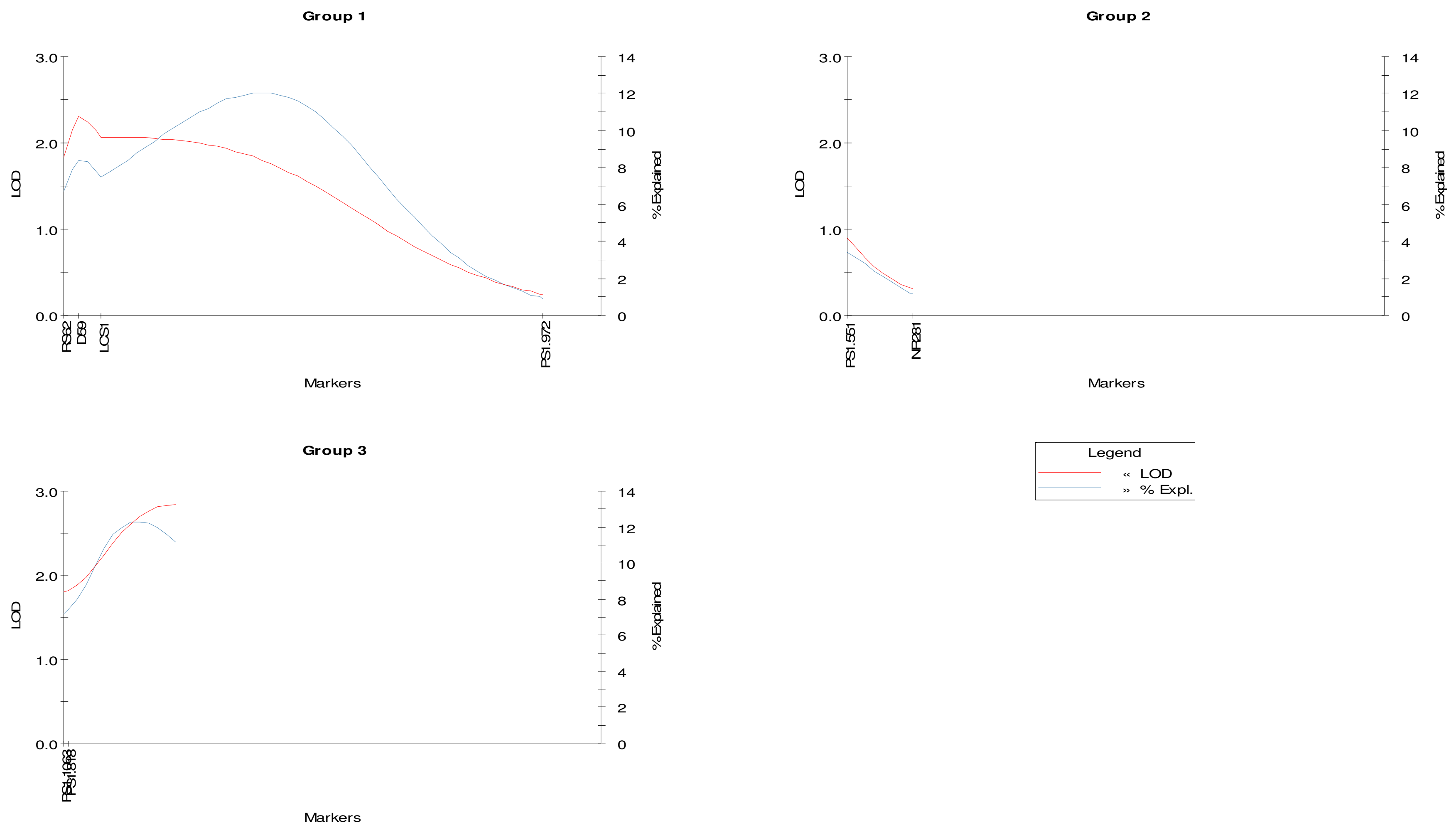


Figure 3.7: Interval mapping results for Family 42A for shell length. Group 1 = 42A_LG1; Group 2 = 42A_LG2; Group 3 = 42A_LG3.

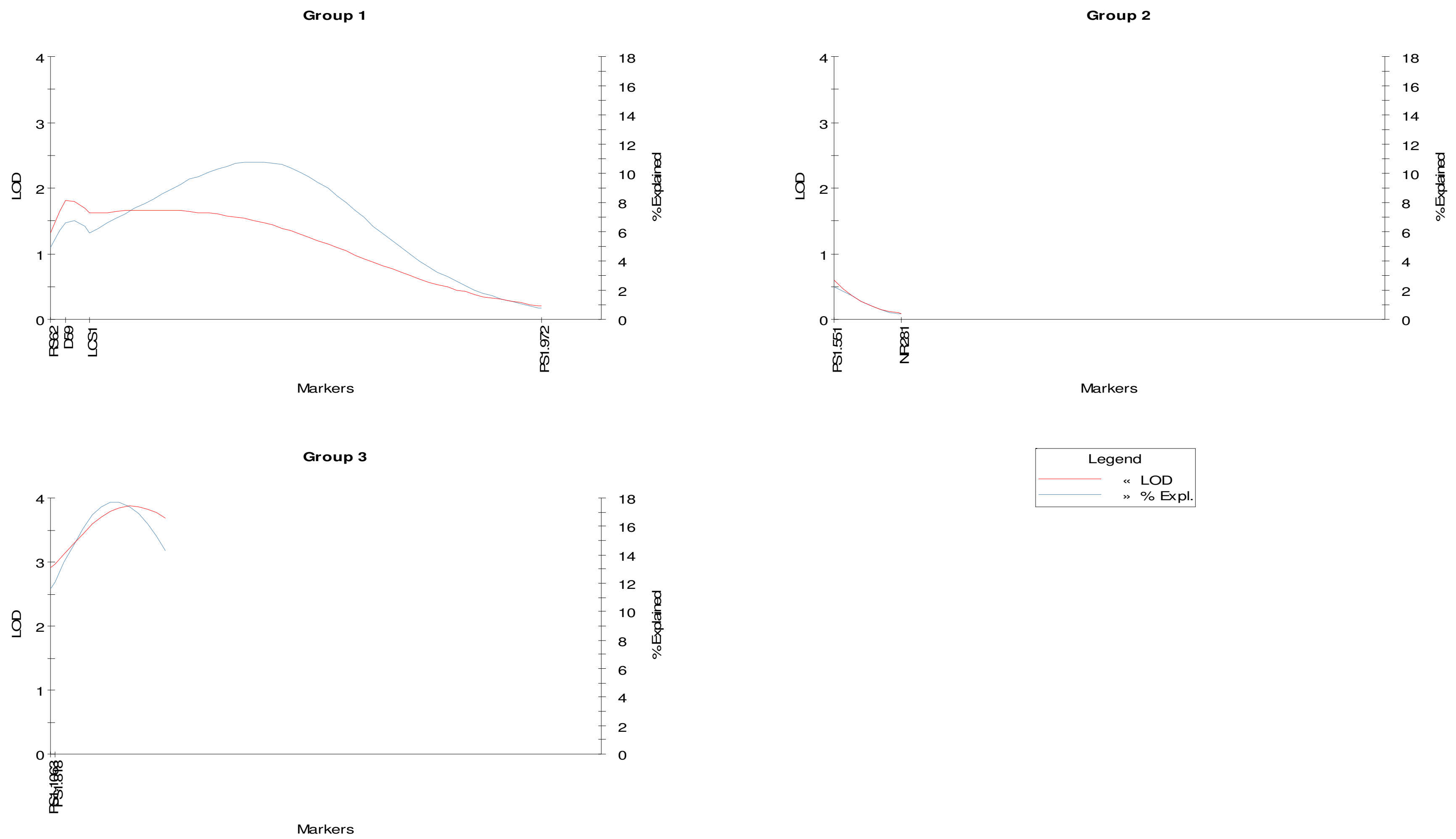


Figure 3.8: Interval mapping results for Family 42A for shell width. Group 1 = 42A_LG1; Group 2 = 42A_LG2; Group 3 = 42A_LG3.

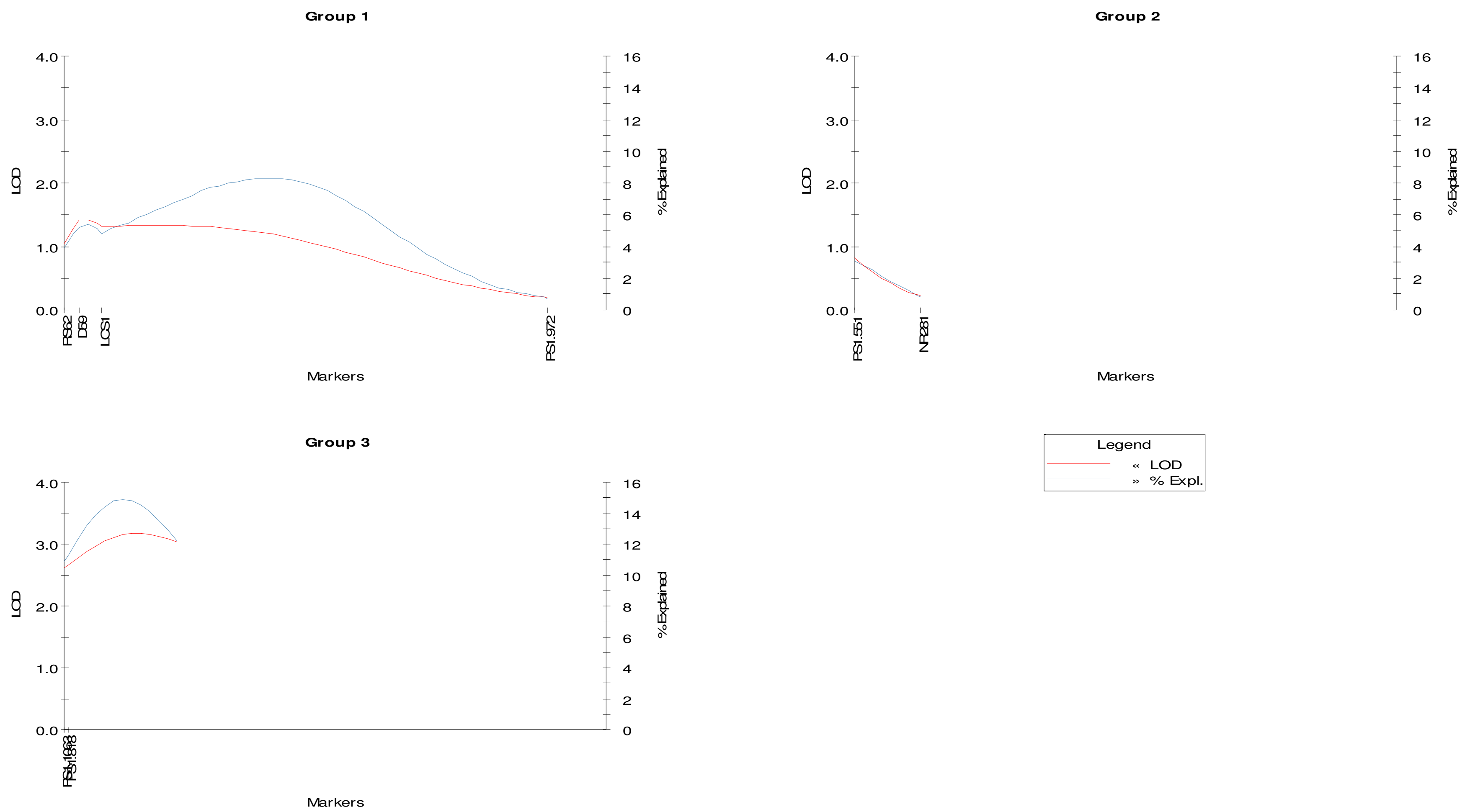


Figure 3.9: Interval mapping results for Family 42A for wet weight. Group 1 = 42A_LG1; Group 2 = 42A_LG2; Group 3 = 42A_LG3.

3.3.5.2) Family 7B

The results of the interval mapping for Family 7B are shown in Figure 3.10 for shell length, Figure 3.11 for shell width and Figure 3.12 for wet weight. Only 7B_LG1 was used for interval mapping since 7B_LG2 had no recombination data. Three significant (LOD > 3.0) putative QTL were mapped on 7B_LG1 for shell length, shell width and wet weight, explaining 33%, 28.5% and 31.5% of the phenotypic variance, respectively. The QTL for these traits mapped to the same position, suggesting that the same gene (s) or chromosomal segment is involved in the control of these traits. These results are consistent with the single marker regression analysis (see section 3.3.3). If *Hmid*PS1.179 could have been mapped a more accurate position for the QTL could have been obtained.

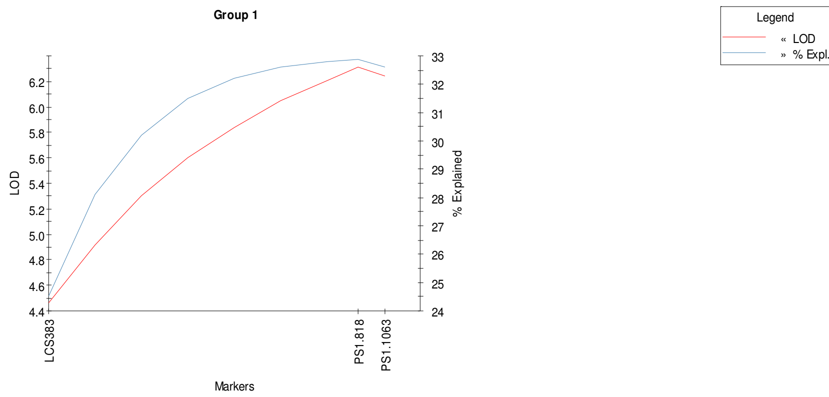


Figure 3.10: Interval mapping results for Family 7B for shell length. Group 1 = 7B_LG1.

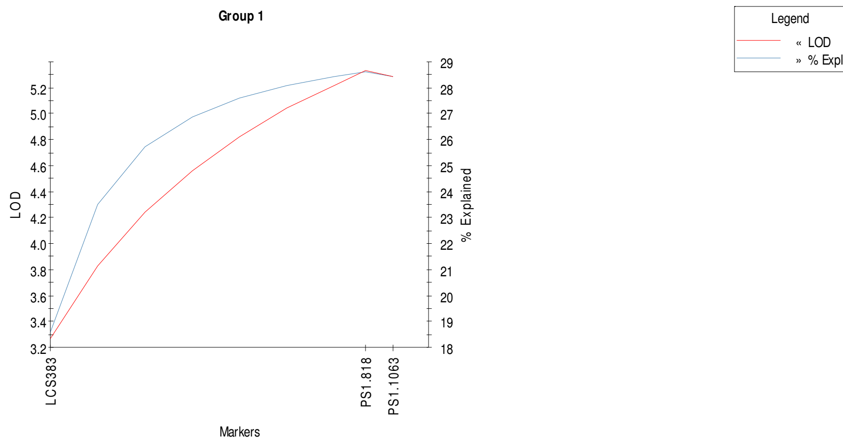


Figure 3.11: Interval mapping results for Family 7B for shell width. Group 1 = 7B_LG1.

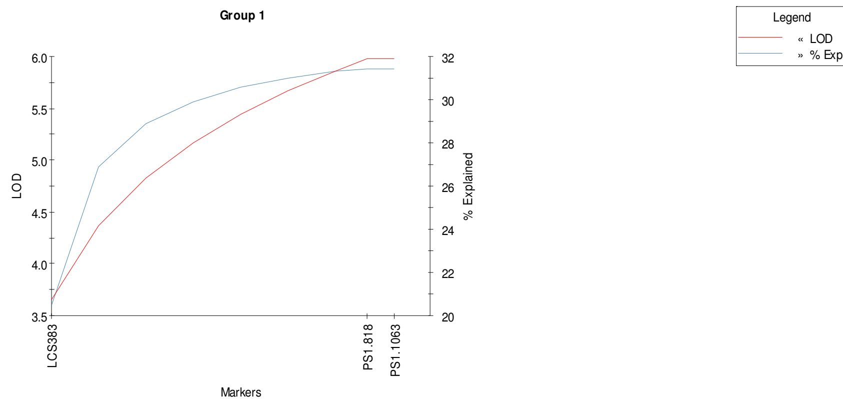


Figure 3.12: Interval mapping results for Family 7B for wet weight. Group 1 = 7B_LG1.

3.4) Discussion

A combination of strategies, namely selective genotyping and bulked segregant analysis (pooling analysis), and individual genotyping with single marker regression and interval mapping, was applied in order to identify putative QTL for three traits, namely shell length, shell width and wet weight. These size related traits are important to the industry as is seen in Section 4.2 where size and growth related traits scored highly during a survey conducted to pinpoint desirable traits as identified by hatchery managers.

The pooling analysis identified six markers possibly linked to putative QTL for growth related traits, five in Family 42A and one in Family 7B. Two markers from Family 42A were eliminated after single marker regression analysis, but four (three in Family 42A and one in Family 7B) were confirmed while six (two from Family 42A and four from Family 7B) additional loci were added. Interval mapping analysis identified two putative QTL in Family 42A for shell width and wet weight. Three putative QTL, for shell length, shell width and wet weight, was identified in Family 7B. The loci associated with the QTL in Family 7B were identified by all three strategies (pooled analysis, single marker regression and interval mapping), while only single marker regression and interval mapping identified the QTL in Family 42A. Two reasons can be given for the differences between the pooled data and the individual genotyping strategies: 1) The pooling results for Family 42A were influenced by the large number of individuals that was not part of the family. A total of 40% of the individuals was eventually removed from any further analyses after individual genotyping, while the number removed from Family 7B was negligible. It is clear that any of the non-sibling abalones carrying identical sized alleles for any of the loci under investigation would severely skew the results and lessen the power of detecting any differences in fluorescent units between the phenotypic extremes. 2) Interval mapping is more sensitive when looking at phenotypic differences within a pool and also utilises the information gained from flanking loci, adding to the sensitivity of QTL detection (Mariasegaram *et al.*, 2007).

A number QTL for size related traits have been mapped in various aquaculture species. In Asian sea bass, Wang *et al.* (2006) mapped the QTL for length and weight to the same position, explaining 28.8% and 59.7% of the phenotypic variance,

respectively. Two additional QTL for weight was mapped to two other chromosomal regions and explained 6.4 and 8.8% of the variance. Lyons *et al.* (2007) mapped length and weight in Kuruma prawn to the same position. The region explained 16% of the phenotypic variance. In Bay scallop shell length, shell width and total weight was also mapped to a single chromosomal position, explaining 11.5% of the variance (Qin *et al.*, 2007). An initial selective pooling screen for QTL in three full-sib families of the abalone, *Haliotis rubra*, by Baranski *et al.* (2008) found QTL for growth rate on nine linkage groups which explained between 16-47% of the observed variance. Liu *et al.* (2007) identified QTL in a single family of another abalone, *H. discus hannai*. Four QTL for shell length and four for shell width were found on four different linkage groups. The QTL for length and width mapped at the same position on each of the four groups. The amount of variance explained for length ranged from 8-13% and for width 8-18%. Two QTL on two different linkage groups were found for total weight. The positions of these QTL did not correspond to those of length and width. A total of 12% and 12.7% of the observed variance were explained by these two QTL. In the current study a QTL was found for each of the three traits that were examined. Shell length, shell width and wet weight mapped to the same chromosomal position in both *H. midae* families. In Family 7B the variance explained for these three traits were 33%, 28.5% and 31.5% and in Family 42A, 17% and 15% of the variance were explained for width and weight, respectively. These size related traits also mapped to the same linkage groups within most of the species discussed above, with the exception of *H. discus hannai*. The amount of variance explained by the QTL found in the current study is also comparable to that found in the previously mentioned studies. Six additional QTL (two for each of the three traits), identified by single marker regression, remain unmapped since the loci associated with the QTL were the only loci genotyped for each linkage group involved. This study found less significant QTL than the only other two previous studies on abalone species (Liu *et al.*, 2007; Baranski *et al.*, 2008). The main reasons for this could be the admixture of one family, lowering detection power as discussed above and the small number of markers that were informative in the other family, therefore covering less of the genome and lowering detection power.

The linkage groups containing locus *Hm*RS27 in Family 42A and locus *Hmid*PS1.551 in Family 7B should be typed and the possibility of a segregating QTL

must be studied using interval mapping. These two loci could not be mapped onto a linkage group (see section 3.3.4). The chromosomal region containing the mapped putitative QTL (associated with loci *Hmid*PS1.818 and *Hmid*PS1.1063) is shared between the two families and in future this region should be studied in other families to see if it is segregating over the entire population or only within the families studied (e.g.: Wang *et al.*, 2007). This verification of the QTL, their effects and phases is essential before the onset of marker assisted selection (MAS) in *H. midae* (Wang *et al.*, 2007).

CHAPTER 4

SUPPORTING METHODS AND STUDIES

SECTION 4.1

Non-Destructive Sampling of Juvenile Abalone using Epipodial Tentacles and Mucus: Method and Application

Published as: Slabbert, R., Roodt-Wilding, R., 2006. Non-destructive sampling of juvenile abalone using epipodial tentacles and mucus: method and application. African Journal of Marine Science 28:719-721. (Appendix C)

4.1.1) Introduction

Deoxyribonucleic acid (DNA) quality is important for applications such as polymerase chain reactions (PCR), endonuclease restriction enzyme digests, Southern blot analysis, genomic library construction and DNA sequencing. The first step in these molecular genetic studies is DNA extraction. For abalone, the quality of DNA obtained is dependent on the biological material used for the extraction. The quality of DNA can be tested by agarose gel electrophoresis. Degraded DNA is characterised as a smear whereas intact DNA is usually viewed as a single band (Sarsfield *et al*., 2000).

Non-destructive sampling is desirable for, among other things, endangered species (e.g. Wasko *et al*., 2003) or for organisms that are needed for future studies, such as those in breeding programmes (Chaline *et al*., 2004). Withler *et al*. (2001) reported non-destructive sampling of epipodial tentacles in adult abalone *Haliotis kamtschatkana* and Maynard *et al.* (2004) reported the sampling of such tentacles from *H. laevigata* exceeding 100mm in length. Li, Q. *et al*. (2004) sampled the haemolymph of wild *H. discus hannai* and Elliott *et al*. (2002) used mucus as a sampling media for *Haliotis* sp. Sweijd *et al*. (1998) extracted DNA from meat from canned *Haliotis* sp. for forensic analysis. However, such studies on juvenile abalone have been mostly done on dead specimens (e.g. Evans *et al*., 2004a; Li, Q. *et al*.,

2004; Gutierrez-Gonzales and Perez-Enriquez, 2005) by dissecting the animal for muscle or gill tissue.

This study reports on the extraction of DNA from epipodial tentacles and mucus from live juvenile abalone *H. midae*. The DNA quality and application of these samples for PCR and genotype analysis were also determined.

4.1.2) Material and Methods

Epipodial tentacles and mucus from 12 juvenile abalones (7-15 months old; 19-35.8mm in length and weighing 1.08-7.11g) were collected from the Irvin and Johnson abalone hatchery at Danger Point (Gansbaai, South Africa). The animals were placed upside down in a Petri-dish containing water such that their tentacles would extrude. The tentacles were cut using dissection scissors, and were stored in 99.9% ethanol (*Merck*) until DNA extractions. Mucus samples were taken by putting the animal on sterile Whatman no1 filter paper and allowing the animal to deposit mucus. The mucus was allowed to dry and the filter paper was sealed and stored at 4℃. Each animal was placed in a separate tank to assess survival after sampling.

4.1.2.1) DNA Extractions

DNA extractions were performed according to the CTAB-method described by Saghai Maroof *et al*. (1984), using 300µl of extraction buffer per epipodial tentacle sample (one tentacle) and 500µl per mucus sample (1cm^2 piece of mucus-containing filter paper; Figure 4.1.1).

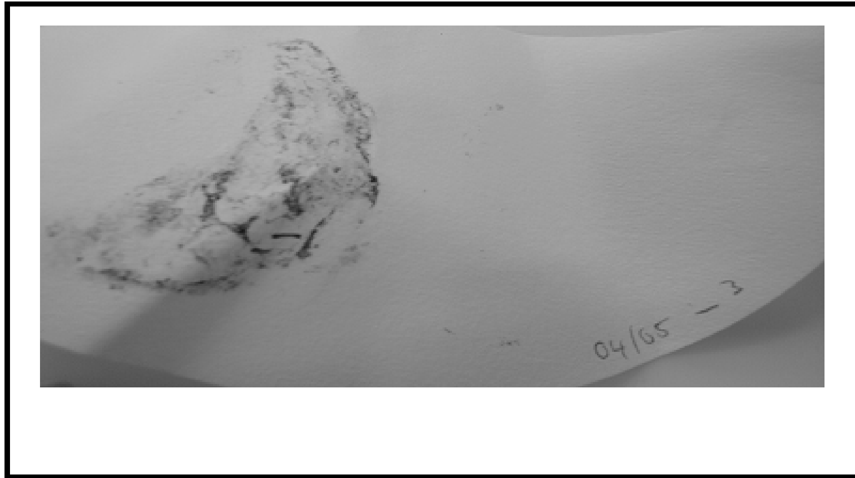


Figure 4.1.1: An example of a mucus-containing filter paper.

DNA quality was tested using agarose (0.7%) gel electrophoresis. Microsatellite DNA markers were used to assess the applicability of the DNA with regards to PCR and genotyping. Undiluted DNA was used for PCR reactions. Two highly polymorphic DNA markers, *Hm*D55 and *Hm*D59 (Bester *et al.*, 2004), were used for genotyping. A 10μl multiplex PCR reaction (1x Flexi Buffer [*Promega*], 0.8mM $MgCl_2$, 0.5mM of each dNTP, 1pmol of each primer, 0.25U Go*Taq*® [*Promega*]) was performed using a touch-down protocol. An initial denaturising step of 94℃ for 5 minutes was followed by 2 cycles of 94℃ for 30 seconds, 65℃ for 30 seconds and 72℃ for 30 seconds. Thereafter, the annealing temperature was lowered by 1℃ in each two consecutive cycles, until an annealing temperature of 55℃ was reached and maintained for 30 cycles of 94℃ for 60 seconds, 55℃ for 60 seconds and 72℃ for 60 seconds, with a final elongation step at 72℃ for 7 minutes.

4.1.3) Results and Discussion

Non-destructive methods of sampling juvenile abalone were successful. All 12 juvenile abalone survived for the two-month study. DNA extraction from epipodial tissue and mucus was also successful. The agarose gel electrophoresis results for DNA quality are shown in Figure 4.1.2. All DNA samples are shown as single bands, indicating good-quality DNA. Some protein impurities can be seen in the mucus samples of animals 11 and 12. Mucus samples extractions tended to have more

impurities than epipodial samples. The DNA was also successfully used in PCR analysis by means of polymorphic microsatellite markers. The PCR products for both sample types within an individual abalone sample amplified identical alleles (Figure 4.1.3). The DNA obtained can therefore be successfully used in PCR and genotyping analysis. Unlike the method used in the Elliott *et al.* (2002) study, the mucus samples in this study were dried before DNA extraction and the DNA was not diluted before PCR reactions.

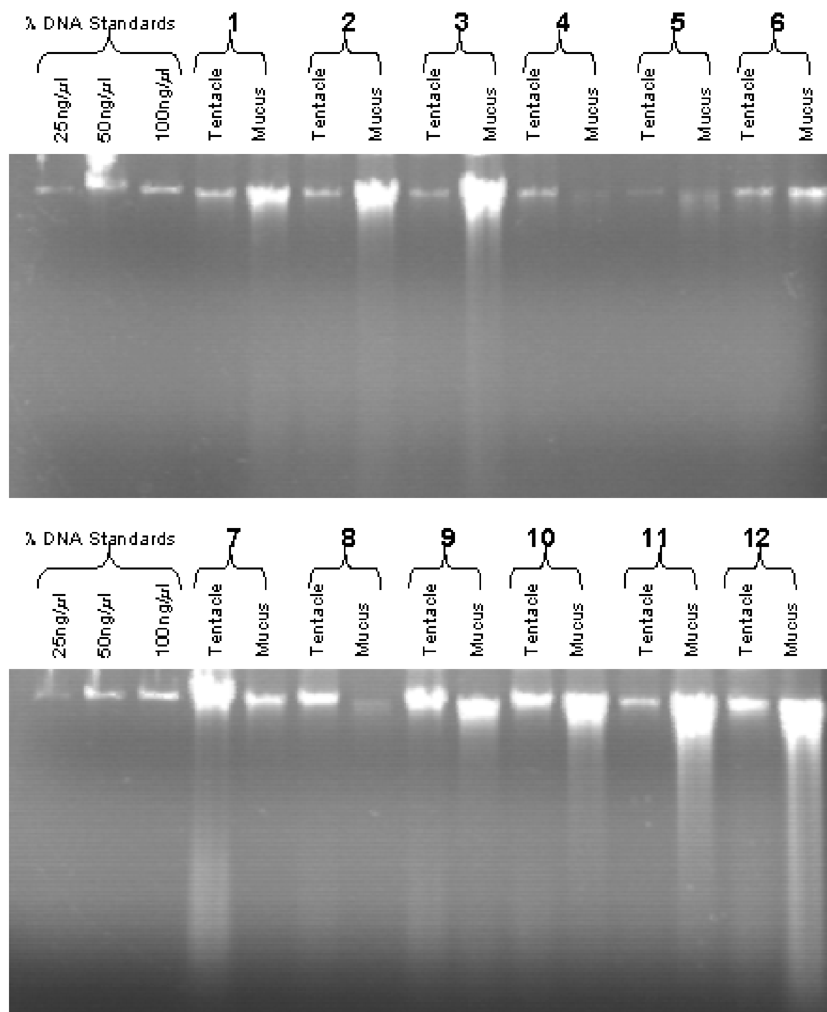


Figure 4.1.2: Results of agarose gel electrophoresis (0.7%), showing the DNA concentrations and quality of tentacles and mucus samples from the 12 juvenile abalones studied.

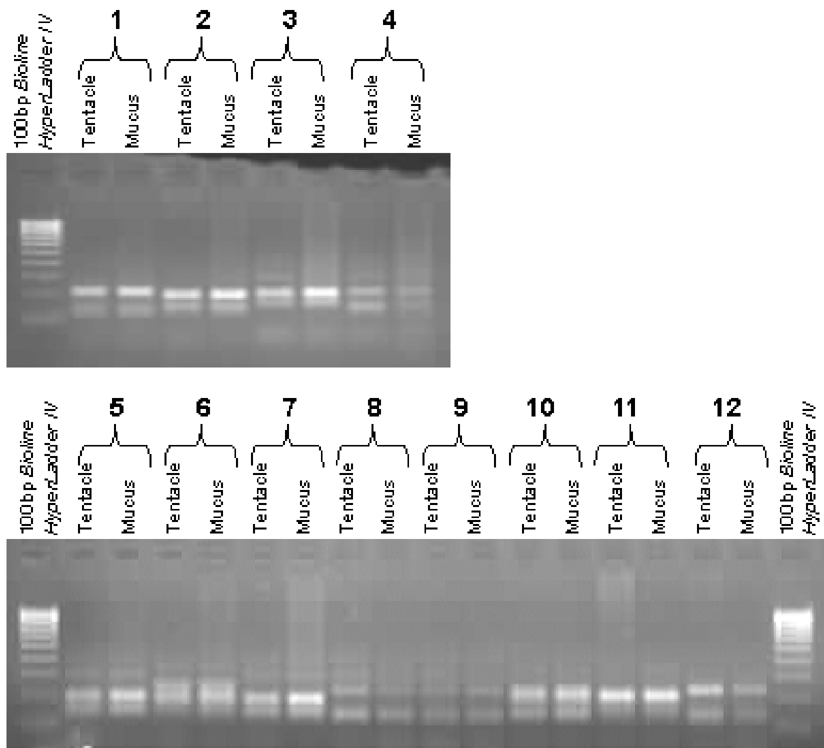


Figure 4.1.3: Results of PCR reactions for tentacles and mucus samples from the 12 juvenile abalones studied, using *Hm*D55 and *Hm*D59.

Non-destructive sampling methods are cost effective for commercial abalone farms, especially for older animals because these are not harmed or killed. This method is also useful for research purposes, because a large number of commercial animals can be sampled for genetic population or parentage studies. Further, non-destructive sampling methods could be used effectively for identifying genetically superior abalone at an early age, after the development of technologies to enable marker assisted selection, for use in future selective breeding programmes. This sampling method will also have application in broodstock and offspring monitoring with the ultimate goal of re-introduction of abalone into the wild. This will become an important alternative in view of the rapid decline in natural abalone stocks in South Africa (Tarr *et al.*, 1996). Monitoring will enable selection of a highly heterozygous reseeding stock, resulting in offspring with increased heterosis, thus, increasing the fitness of the offspring or masking the effects of deleterious alleles (Ingvarsson and Whitlock, 2000; Whitlock *et al.*, 2000). However, the introduction of foreign alleles may also lead to outbreeding depression. In view of the significant genetic differentiation between West and East coast populations of wild *H. midae* (Evans *et*

al., 2004b), it is important to select reseeding stock that is not too divergent from the local population, so limiting the number of foreign alleles that are introduced. Non-lethal sampling can also be used in transgenic studies on abalone. The current method of collecting and processing mucus samples can be applied in forensic science, where mucus is found on clothes or equipment of alleged poachers. This would ensure reliable molecular identification of *H. midae* trace elements (Sweijd *et al*., 1998).

In summary, non-destructive sampling protocols allow for the collection of ample tissue to isolate DNA and perform molecular studies of abalone, which are beneficial to both abalone farmers and researchers alike.

SECTION 4.2

A Questionnare Based Evaluation of Economically Important Traits in *Haliotis midae*

4.2.1) Introduction

A great variety of economically important phenotypic traits exist for any commercial aquaculture species. These traits are targeted by investigators during QTL-mapping experiments (e.g.: Wang *et al.*, 2006; Chatziplis *et al.*, 2007; Liu *et al.*, 2007; Lyons *et al.*, 2007; Qin *et al.*, 2007; Baranski *et al.*, 2008, Haidle *et al.*, 2008; Lallias *et al.*, 2009). The information obtained during QTL-studies can be used for marker assisted selection (MAS) (e.g.: Dekkers, 2004) where molecular markers are used to select individual animals carrying one or more economically valuable phenotype. Information on which traits to select for could be a useful resource, since the investigator can focus on relevant phenotypes.

In order to assess which traits are seen as important by the abalone industry a questionnaire was created covering twelve traits. These traits were taken from literature (Wang *et al.*, 2006; Chatziplis *et al.*, 2007; Liu *et al.*, 2007; Lyons *et al.*, 2007; Qin *et al.*, 2007; Baranski *et al.*, 2008, Haidle *et al.*, 2008; Lallias *et al.*, 2009) and from discussions with a few abalone hatchery managers. The phenotypic measurements for the twelve traits could be taken in three ways: 1) by killing the animals (destructive-analysis), 2) by continuous data collection throughout the production cycle (temporal-analysis) or 3) measuring once at a specific point in the production cycle (point-analysis).

4.2.2) Materials and Methods

4.2.2.1) Questionnaire

The twelve traits chosen for the questionnaire and the measurement type of each trait are shown in Table 4.2.1. The questionnaire was given to four abalone hatchery managers. A ranking scale from 1 to 12 was given for each individual trait and a participant had to rank them according to importance, 1 being least important and 12 the most important. It was also possible to rank different traits as being of equal importance.

Table 4.2.1: Economically important traits as given in the questionnaire.

Number	Trait	Type
1	Disease resistance	Temporal- or destructive-analysis
2	Early growth rate	Temporal-analysis
3	Flesh colour	Destructive-analysis
4	Food conversion efficiency	Temporal-analysis
5	Larval survival / quality	Point-analysis
6	Meat to shell ratio	Destructive-analysis
7	Reproductive traits	Temporal-, destructive- or point-analysis
8	Shell colour	Point-analysis
9	Shell shape	Temporal- or point-analysis
10	Size at harvest	Point-analysis
11	Size uniformity	Point-analysis
12	Taste	Destructive-analysis

4.2.2.2) Data Analysis

The traits were ranked per questionnaire according to the score given by each representative. Each trait was then given a ranked score out of a total of 12. The score of each trait from each questionnaire was then added to give a total out of a maximum of 48 points. The percentage of marks for each trait were then calculated from this total and plotted from least to most important on a histogramme using Microsoft Excel 2007. Traits with more than 50% of the points were arbitrarily taken

as being of higher priority for QTL-mappping and MAS in the South African abalone industry.

4.2.3) Results

The results of the ranked traits are shown in Figure 4.2.1. Early growth rate, size at harvest, meat to shell ratio, reproductive traits, disease resistance and larval survival/quality scored above 50%.

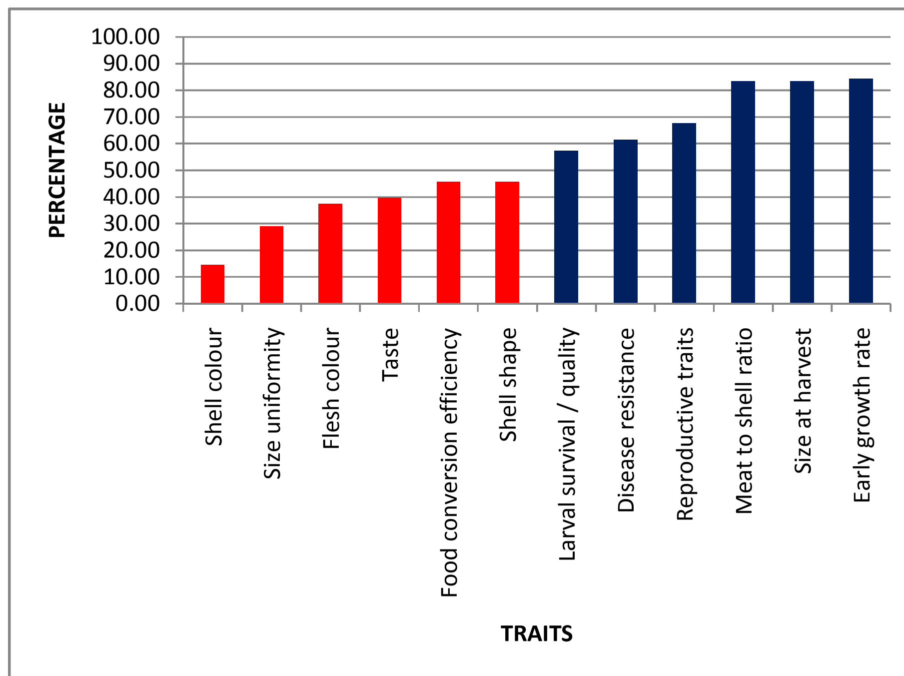


Figure 4.2.1: Histogramme of ranked economically important traits. Red indicates traits scoring below 50% and blue those scoring above 50%.

4.2.4) Discussion

The aim of the questionnaire was to find out which traits are seen by abalone farm hatchery managers as important for selection and therefore QTL-mapping. The questionnaire covered 12 traits, but is by no means exhaustive.

Growth related traits have been studied in abalone aquaculture (Capinpin and Corre, 1996; Kawahara *et al.*, 1997; Jonasson *et al.*, 1999; Lucas *et al.*, 2006; Kube *et al.*, 2007). The size of abalone at harvest is important for the economic viability of the international abalone farming industry (Viana, 2002). A trait such as early growth ensures that animals reach a larger size much quicker and could therefore spend less time within the production cycle, making a farm more profitable, since less money is spent feeding the animals (pers. comm.: Stephen Ashlin, Roman Bay Hatchery and Louise Jansen, HIK Abalone Farm). Also, both a larger size at harvest and a higher meat to shell ratio increases the total weight of meat that can be sold by a farm. It should be noted that size at harvest can be linked to early growth rate. By keeping a consistently faster growing animal for the same time within the production cycle as a slower grower, a farmer will generate more wet weight and therefore more income. Larval survival and quality and overall disease resistance will contribute to healthier animals as well as increased outputs (e.g.: Roberts *et al.*, 2001; Jackson *et al.*, 2005; Macey and Coyne, 2005). Reproductive traits, including sperm- and egg quality, also scored high, since these are essential in creating a high quality commercial stock (e.g.: Fukazawa *et al.*, 2005).

The above mentioned traits can be prioritised, where possible, within a genetic improvement programme by calculating heritabilities, followed by QTL-mapping and MAS. Traits that are difficult to measure and select for, such as meat to shell ratio, disease resistance and some reproductive traits, are ideal targets for QTL-mapping (e.g.: Moen *et al.*, 2009). The data collected during this study can now be used to plan the study of multiple trait QTL-mapping and MAS-programmes.

SECTION 4.3

Multiplex Setup Protocol: Pre-PCR and Family Specific Multiplexing

4.3.1) Introduction

Multiplex PCR was first described by Chamberlain *et al.* (1988). A multiplex PCR amplifies two or more loci in the same reaction, saving costs and increasing data generation per assay. Multiplex PCR became more attractive as a technique for data collection when fluorescent labels (Ziegle *et al.*, 1992; Mansfield *et al.*, 1994) and capillary electrophoresis (Heller, 2001) became available. These techniques enabled researchers to analyse more than one marker per single capillary based on non-overlapping amplicon size and label wavelength (Hayden *et al.*, 2008). Markers that can be multiplexed include mitochondrial markers, microsatellite loci and SNPs. Applications of multiplex PCR include species identification (Hill *et al.*, 2001; Marshall *et al.*, 2007), broodstock management (Porta *et al.*, 2006), population studies (Lerceteau-Köhler and Weiss, 2006; Apostolidis *et al.*, 2007; Li, Y. *et al.*, 2007b), parentage assignment (Johnson *et al.*, 2007) and SNP genotyping (Sanchez *et al.*, 2006).

Some techniques to facilitate multiplex PCR have been developed. One such technique is the multiplex-ready PCR described by Hayden *et al.* (2008). This method combines a M13-tailed primer method (Oetting *et al.*, 1995) and multiplex PCR. M13-tailed primer methods comprise of three primers: a locus-specific forward primer with a 5'-end extension identical to a M13-primer, its respective reverse primer and a fluorescently labelled M13-primer. The labelled M13-primer will therefore add fluorescence to the amplification product during the PCR reaction, eliminating the need to label each primer set individually. Markers with different sized amplicons are multiplexed in a single PCR reaction, labelled and analysed. Multiplex-ready PCR has several advantages: 1) uniform amplification facilitates the use of a standard protocol for marker analysis; 2) allele pull-up is reduced; 3) a marker can be labelled with a fluorophore of choice making marker separation more

flexible, since overlapping markers can be labelled differently and pooled; 4) costs of labelling each marker individually are eliminated, requiring only four fluorescently labelled primers.

The PCR multiplex-ready method is however impractical if more than one locus with overlapping allele sizes are to be multiplexed in a single PCR reaction. Individually fluorescently labelled markers are therefore more suitable for PCR multiplexing. Multiplex PCR conditions however need to be optimised and can be quite time consuming and labour intensive. Optimisation is required to overcome some technical difficulties such as primer-primer interactions and non-specific amplification (Elnifro *et al.*, 2000).

Multiplex PCR has become more practical and less time consuming by the development of multiplex-specific PCR kits. One such kit is the Qiagen Multiplex PCR Kit and it is the first commercially available kit of this kind. The kit consists of a master mix containing HotStart*Taq* DNA Polymerase and a unique Qiagen Multiplex PCR Buffer with a factor MP, which facilitates primer binding. This kit has found application in previous abalone studies such as for linkage mapping (Baranski *et al.*, 2006a).

Here the methodology involved in setting up and optimising multiplex PCR reactions using the Qiagen Multiplex PCR Kit is described. The practical theory behind family specific multiplexing will also be discussed.

4.3.2) Materials and Methods

4.3.2.1) Theoretical Multiplex Set-up

Firstly, markers were sorted into multiplexes according to label colour and amplicon size using Microsoft Excel 2007 (Figure 4.3.1). A minimum of four markers can be multiplexed, because four fluorescent labels are available, namely FAM, ROX, VIC and NED (*Applied Biosystems*). An additional marker labelled with the same label as another one can be added if the amplification products do not overlap (e.g.: Figure 4.3.1, *Hm*LCS47 and *Hm*LCS48).

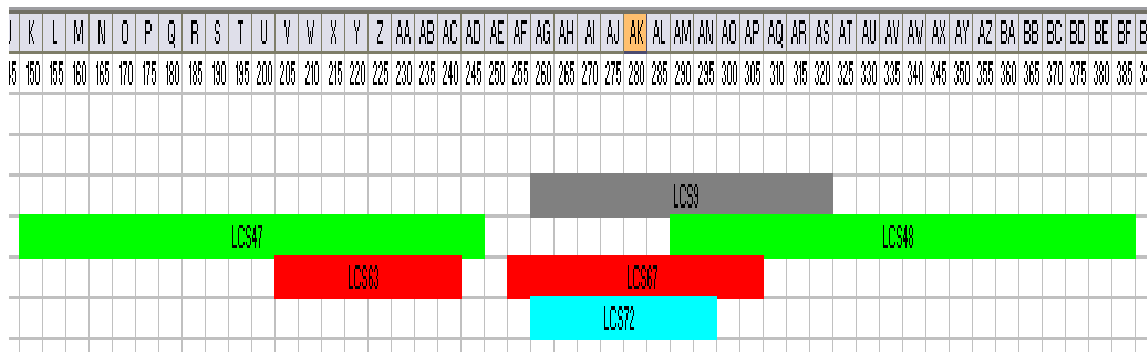


Figure 4.3.1: A Microsoft Excel sheet showing the initial multiplexing of markers. LCS9 = NED, LCS47 and LCS48 = VIC, LCS63 and LCS67 = PET and LCS72 = FAM.

<u>4.3.2.2) PCR Multiplexing Optimisation</u>

DNA from a single abalone from Roman Bay (Gansbaai, RSA) was used to set up the multiplex protocol for the following loci: *Hm*LCS9, *Hm*LCS47, *Hm*LCS48, *Hm*LCS63, *Hm*LCS67 and *Hm*LCS72. This multiplex combination is used as an example for describing the protocol setup. Each locus was amplified individually using the optimal PCR conditions as described in Section 2.2. These reactions served as positive controls and would be compared to the multiplex reaction. The Qiagen Multiplex PCR kit (*Qiagen*) was used to perform the multiplex reactions. Multiplex reactions were set up in 10μl volumes. A final [DNA] of 20ng was used per single PCR and multiplex reaction. The PCR reaction mix for the Qiagen Multiplex PCR kit is shown in Table 4.3.1.

Table 4.3.1: Multiplex PCR conditions for the Qiagen Multiplex Kit.

Component (Stock concentration)	Volume	Final concentration
Qiagen Multiplex PCR Master Mix[a] (2x)	5μl	1x
Primer Mix (2μM each primer)[b]	2μl	0.2μM each primer
RNase-free water	Variable	-
DNA	Variable	20ng
Total volume	10μl	

a: Contains HotStart*Taq* DNA Polymerase, Multiplex PCR Buffer, dNTP Mix and 6mM $MgCl_2$.

b: The primer mix consisted of a mixture of all primer pairs.

The annealing temperature for the multiplex reaction was optimised using a *Px*2 Thermal Cycler (*Thermo Electron Corporation*) to perform a gradient PCR. Annealing temperatures of 57℃, 60℃ and 63℃ were tested. The PCR cycling programme for the Qiagen Multiplex PCR was as follows: an initial activation step to activate the HotStart*Taq* DNA Polymerase was performed at 95℃ for 15 minutes followed by 35 cycles of 30 seconds at 94℃, 90 seconds at 57℃, 60℃ and 63℃ and 60 seconds at 72℃ with a final extension step at 60℃ for 30 minutes. Amplification success was analysed on an agarose gel (2%, 1x TBE, ethidium bromide) along with a 100bp size marker (HyperLadder IV, *Bioline*) and visualised while being exposed to UV-light. All PCR reactions were then analysed along with the LIZZ600 (*Applied Biosystems*) size standard on a 3730xl DNA Analyzer (*Applied Biosystems*) and genotypes were scored with GeneMapper version 4 (*Applied Biosystems*).

The multiplex reactions were compared to the control reactions (amplification using only one primer set) according to allele size and peak heights. If a marker failed to amplify the correct allele size within any of the three multiplex reactions it was discarded from the multiplex. If a marker amplified the correct allele size within two or more multiplex reactions, the peak heights were compared between these reactions. The better reaction is considered to be the one with higher peak fluorescence.

4.3.2.3) Family-Specific Multiplexing

When performing marker analysis within a full-sib family, multiplexes can be set up for loci with similar fluorescently labelled tags according to individual allele sizes instead of the entire amplicon range. The data for the one multiplex that was collected after genotyping the dam and sire of Family 42A (Chapter 3) is used as an example. The family-specific multiplex was constructed using Microsoft Excel 2007. The loci used are shown in Table 4.3.2. The loci can be amplified individually and multiplexed for electrophoresis after the PCR or the PCR can be optimised in the same manner as described above.

Table 4.3.2: Loci used for one of the Family 42A specific multiplexes and their corresponding fluorescent labels.

Locus	**Fluorescent Label**
*Hmid*PS1.818	VIC
*Hmid*PS1.860	VIC
*Hm*D55	VIC
*Hmid*PS1.1063	PET
*Hmid*PS1.1066	PET
*Hm*NR20	FAM
*Hmid*PS1.247	FAM
*Hmid*PS1.433	NED
*Hmid*PS1.972	NED
*Hm*RS27	NED

4.3.3.) Results and Discussion

4.3.3.1) PCR Multiplexing Optimisation

The results of the single PCR and multiplex PCR are shown in Figure 4.3.2. Amplification was achieved for the individual loci as well as for two annealing temperatures tested for the multiplex reactions. The multiplex reaction performed at 63°C failed to amplify anything.

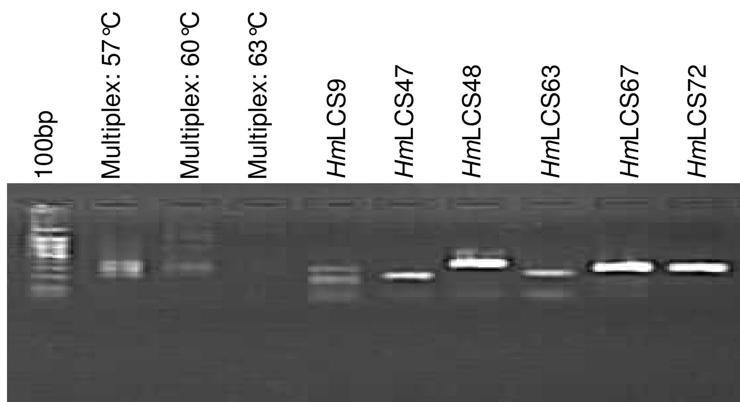


Figure 4.3.2: Multiplex and control reactions results.

The genotyping results are shown in Figure 4.3.3. The multiplex reaction performed at 57°C, successfully amplified the correct alleles for each locus (Figure 4.3.3: a1, b1, c1, d1, e1, f1). The 60°C multiplex reaction failed to amplify anything for locus *Hm*LCS47 (Figure 4.3.3: b2) while amplifying the incorrect allele size for *Hm*LCS9 (Figure 4.3.3: a2). The allele size and peak height data are shown in Table 4.3.3. In the four loci where both multiplex reactions amplified the correct allele size, the 57°C reaction showed better amplification when peak heights were compared; e.g.: for *Hm*LCS72 – 57°C:60°C = 5.9:1. The optimal annealing temperature for this multiplex reaction was therefore 57°C.

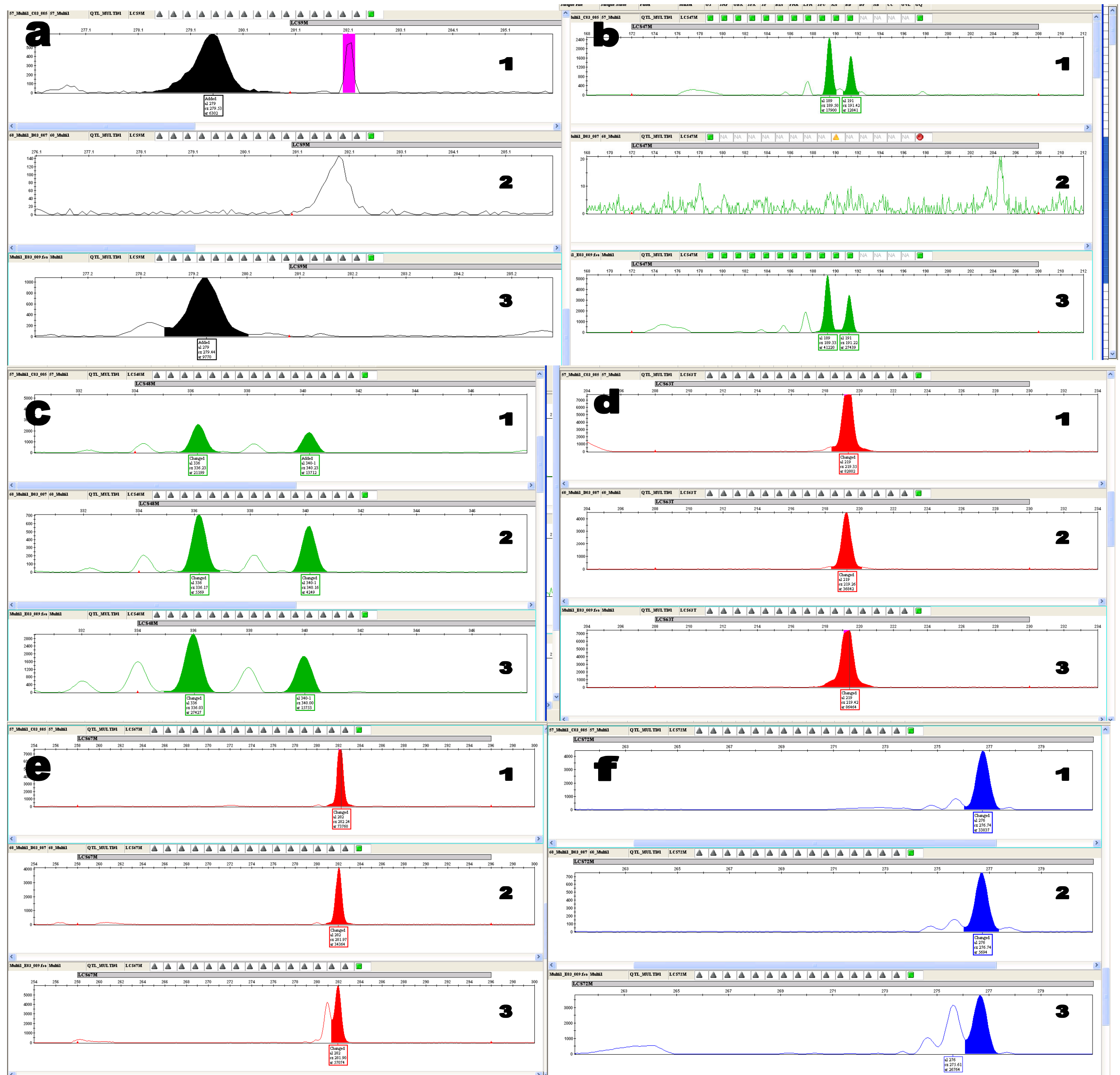


Figure 4.3.3: Genotyping results for Multi#1. a) *Hm*LCS9M; b) *Hm*LCS47M; c) *Hm*LCS48M; d) *Hm*LCS63T; e) *Hm*LCS67M; f) *Hm*LCS72M. 1) Multiplex at 57℃; 2) Multiplex at 60℃; 3) Control reaction.

Table 4.3.3: Allele size and peak height data from Multi#1.

Reaction	Locus	Allele 1	Allele 2	Height 1	Height 2
Multiplex: 57℃	*Hm*LCS9M	279	-	650	-
Multiplex: 60℃	*Hm*LCS9M	282	-	140	-
*Hm*LCS9M	*Hm*LCS9M	279	-	1082	-
Multiplex: 57℃	*Hm*LCS47M	189	191	2487	1674
Multiplex: 60℃	*Hm*LCS47M	-	-	-	-
*Hm*LCS47M	*Hm*LCS47M	189	191	5345	3447
Multiplex: 57℃	*Hm*LCS48M	336	340	2601	1863
Multiplex: 60℃	*Hm*LCS48M	336	340	711	569
*Hm*LCS48M	*Hm*LCS48M	336	340	3019	1875
Multiplex: 57℃	*Hm*LCS63T	219	-	7736	-
Multiplex: 60℃	*Hm*LCS63T	219	-	4497	-
*Hm*LCS63T	*Hm*LCS63T	219	-	7434	-
Multiplex: 57℃	*Hm*LCS67M	282	-	7625	-
Multiplex: 60℃	*Hm*LCS67M	282	-	4082	-
*Hm*LCS67M	*Hm*LCS67M	282	-	5966	-
Multiplex: 57℃	*Hm*LCS72M	276	-	4444	-
Multiplex: 60℃	*Hm*LCS72M	276	-	748	-
*Hm*LCS72M	*Hm*LCS72M	276	-	3166	-

4.3.3.2) Family-Specific Multiplexing

All ten loci could be multiplexed post-PCR for this family. The PCR reactions of all the family-specific multiplexes were not optimised during this study. Only post-PCR multiplexing was done. The combined parental allele sizes for each locus are shown in Table 4.3.4 and the electropherogrammes showing the positions relative to each other is shown in Figure 4.3.4-4.3.7. Family specific-multiplexing has the potential for greater cost saving, but will require some optimisation.

Table 4.3.4: Loci used for one of the Family 42A specific multiplexes and their combined parental allele sizes (bp).

Locus	Allele 1	Allele 2	Allele 3	Allele 4
*Hmid*PS1.818	155	157		
*Hmid*PS1.860	77	85		
*Hm*D55	182	190	202	208
*Hmid*PS1.1063	114	120	124	134
*Hmid*PS1.1066	97	99	101	
*Hm*NR20	221	232	245	321
*Hmid*PS1.247	112	114	118	
*Hmid*PS1.433	117	129	134	
*Hmid*PS1.972	120	124	132	
*Hm*RS27	268	307	319	

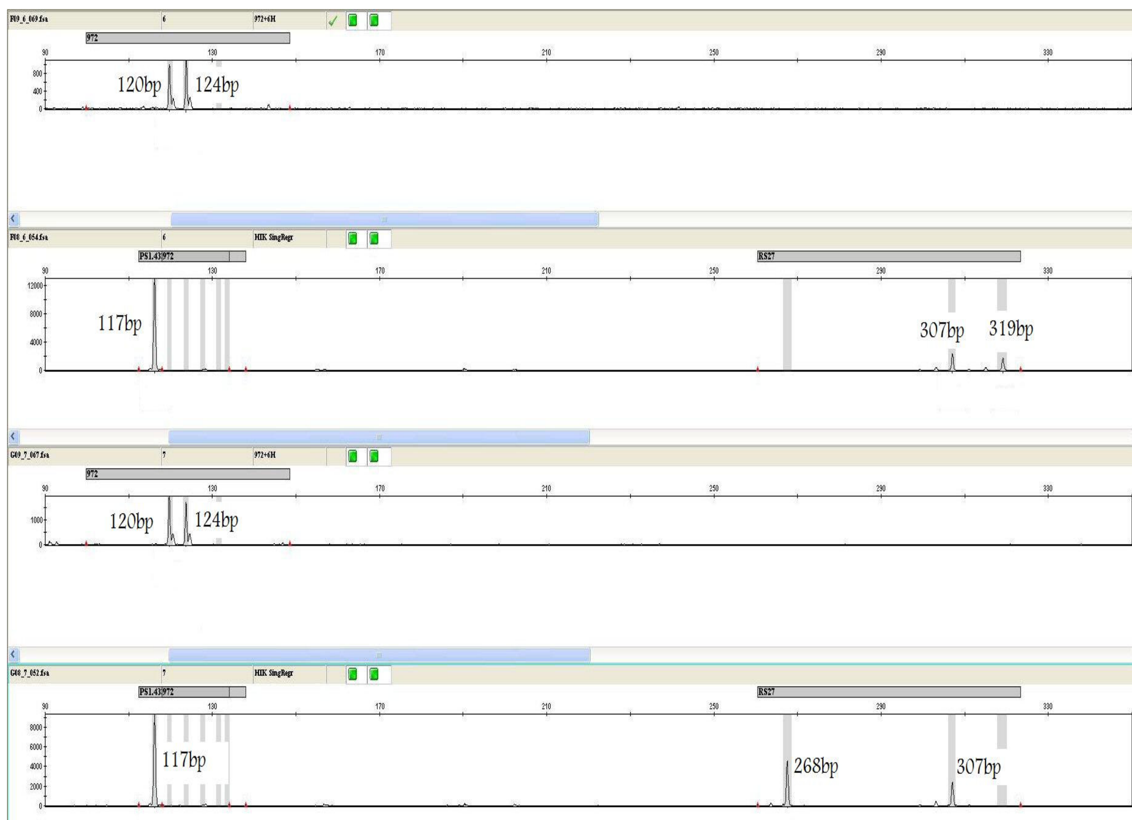


Figure 4.3.4: Alleles of the NED labelled loci.

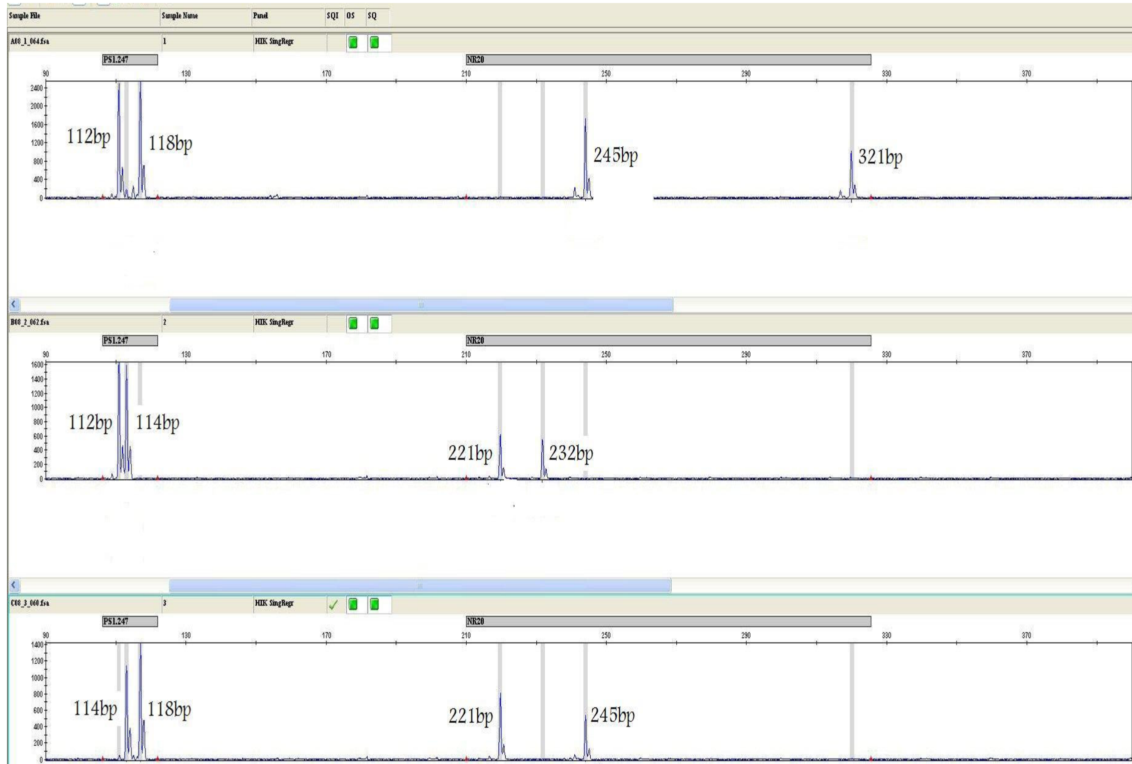


Figure 4.3.5: Alleles of the FAM labelled loci.

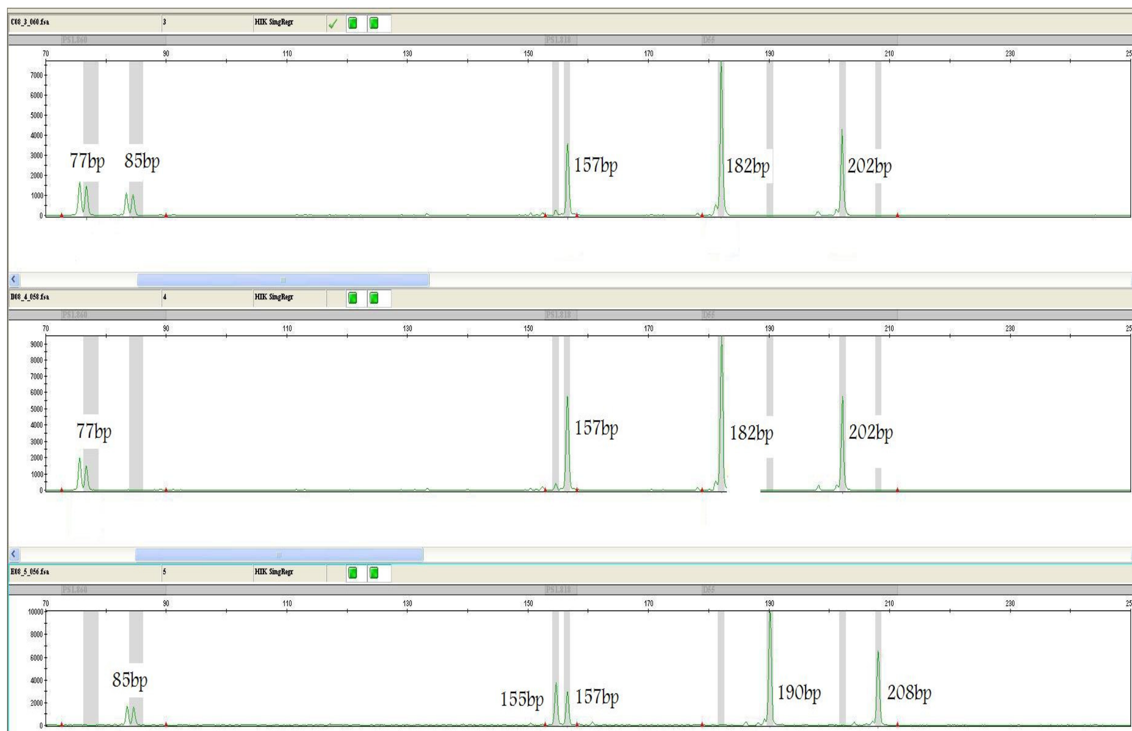


Figure 4.3.6: Alleles of the VIC labelled loci.

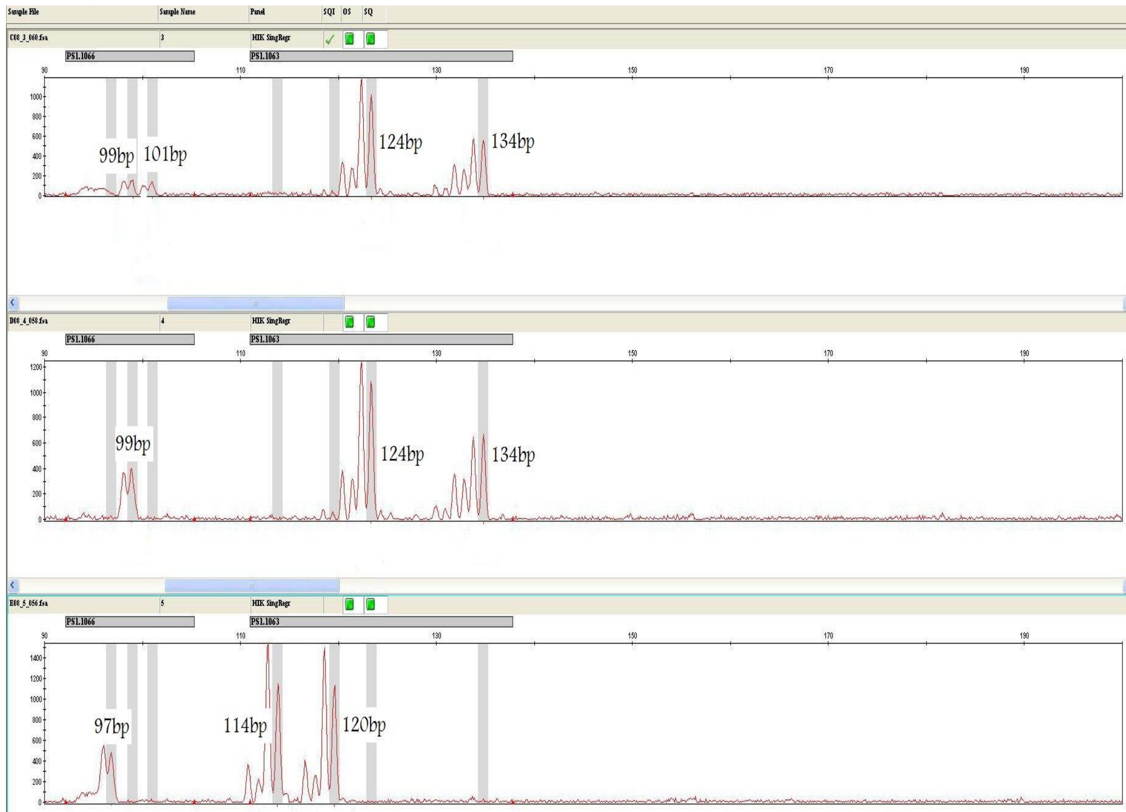


Figure 4.3.7: Alleles of the PET labelled loci.

For a layout of all the multiplexes refer to Appendix B. All the multiplexes which were designed during the course of this study are shown in Appendix B, Table B.1. The family specific multiplexes are shown in Appendix B, Table B.2.

CHAPTER 5

PROTOCOL DEVELOPMENT

SECTION 5.1

DNA Extraction Method Comparison for *Haliotis midae*

5.1.1) Introduction

DNA yield and quality are essential for any downstream application such as restriction digestions, polymerase chain reactions (PCR), real-time PCR procedures and genotyping to name a few. The DNA extraction protocol followed can have a significant influence on the extracted DNA obtained. DNA extractions employ either organic solvents, silica membrane or gel based methods or magnetic bead methodology. Variables influencing the extraction include the type and composition of the extraction buffer; whether home-brewed or kit-based. Studies by Shan *et al.* (2008; activated sludge), Waldschmidt *et al.* (1997; bees), Cao *et al.* (2003; paraffin embedded tissues and buccal cells), Di Pinto *et al.* (2007; food products), Cler *et al.* (2006; clinical samples) and Chapela *et al.* (2007; canned tuna), found that different buffers gave variable qualities and quantities of purified DNA.

The objective of this study was to compare home-brew methods and commercial kits for their application in purifying high-quality genomic DNA from *Haliotis midae* tissue.

5.1.2) Materials and Methods

5.1.2.1) Extraction Protocols

Four different extraction protocols were used for DNA isolation: Protocol A used a home-brewed CTAB buffer, protocol B a home-brewed SDS buffer, protocol C a CTAB kit-based buffer and protocol D a SDS kit-based buffer.

5.1.2.1.1) Protocol A: home-brewed CTAB buffer

This protocol is based on Saghai-Maroof *et al.* (1984). Buffer A consisted of 2% (v/v) CTAB, 1.4M/L NaCl, 20mM/L EDTA (pH 8.0), 100mM/L Tris-HCl (pH 8.0) and 0.2% (v/v) 2-mercapto-ethanol. Approximately 0.2g of tissue was added to 500μl Buffer A and 2μl 10mg/ml proteinase K (*Sigma-Aldrich*), homogenised using the TissueLyser (*Qiagen*) and incubated overnight at 60°C. After incubation an equal volume of chloroform:isoamylalcohol (24:1) was mixed with the sample, centrifuged at maximum for 5 minutes and the supernatant was transferred to a clean 1.5ml tube. This step was repeated. A volume of 200μl 100% isopropanol was added to precipitate the DNA and incubated at -20°C for at least 1 hour. The sample was centrifuged at maximum speed (16.1rcf) for 20 minutes and the supernatant was removed by decanting. Two-hundred microlitres of 70% ethanol was then added to wash out any excess salts from the DNA pellet. It was centrifuged again at maximum speed (16.1rcf), the supernatant removed and the pellet dried and finally resuspended in 100μl distilled water.

5.1.2.1.2) Protocol B: home-brewed SDS buffer

Buffer B consisted of 0.5% (v/v) SDS, 0.5M/L NaCl, 20mM/L EDTA (pH 8.0) 100mM/L Tris-HCl (pH 8.0) and 0.2% (v/v) 2-mercapto-ethanol. Approximately 0.2g of tissue was added to 500μl Buffer B and 2μl 10mg/ml proteinase K (*Sigma-Aldrich*), homogenised using the TissueLyser (*Qiagen*) and incubated overnight at 60°C. After incubation, 170μl of a 5M stock of potassium acetate was mixed with the sample, incubated on ice for 5 minutes, centrifuged at maximum speed for 10 minutes and the supernatant transferred to a clean 1.5ml tube. An equal volume of 100% isopropanol was added to precipitate the DNA and incubated at -20°C for at least 1 hour. The sample was centrifuged at maximum speed for 20 minutes and the supernatant was removed by decanting. Two-hundred microlitres of 70% ethanol was then added to wash out any access salts from the DNA pellet. It was centrifuged again at maximum speed, the supernatant was removed and the pellet dried and finally resuspended in 100μl distilled water.

5.1.2.1.3) Protocol C: kit-based CTAB buffer

Buffer PL1 (Buffer C), which is CTAB-based, of the NucleoSpin PlantII kit (*Macherey-Nagel*) was used. Approximately 0.2g of tissue was added to 500µl Buffer C and 2µl of 10mg/ml proteinase K (*Sigma-Aldrich*), homogenised using the TissueLyser (*Qiagen*) and incubated overnight at 60℃. After incubation 10µl of RNase (supplied with the kit) was added to half of the samples and incubated for an additional hour at 65℃. The sample was then centrifuged at 6.0rcf for 20 minutes. The rest of the protocol was performed on a robotic platform, a Genesis RMP200 (*Tecan*). A total of 400µl supernatant was mixed with 450µl binding buffer PC and transferred to a silica membrane plate. The mixture was pulled through the membrane by a vacuum system. The bound DNA was washed to remove proteins and salts with 400µl buffer PW1 and twice with 700µl buffer PW2. The bound DNA was eluted twice with 100µl volumes of elution buffer preheated to 70℃.

5.1.2.1.4) Protocol D: kit based SDS buffer

Buffer PL2 (Buffer D), which is SDS-based, of the NucleoSpin PlantII kit (*Macherey-Nagel*) was used. Approximately 0.2g of tissue was added to 400µl Buffer D and 2µl 10mg/ml of proteinase K (*Sigma-Aldrich*), homogenised using the TissueLyser (*Qiagen*) and incubated overnight at 60℃. After incubation 10µl of RNase (supplied with the kit) was added to half of the samples and incubated for an additional hour at 65℃. A volume of 100µl Buffer PL3 (potassium acetate) was added to the sample and incubated on ice for 5 minutes. The sample was then centrifuged at 6.0rcf for 20 minutes. The rest of the protocol was performed on a robotic platform, a Genesis RMP200 (*Tecan*). A total of 400µl supernatant was mixed with 450µl binding buffer PC and transferred to a silica membrane plate. The mixture was pulled through the membrane by a vacuum system. The bound DNA was washed to remove proteins and salts with 400µl buffer PW1 and twice with 700µl buffer PW2. The bound DNA was eluted twice with 100µl volumes of elution buffer preheated to 70℃.

5.1.2.2) DNA Yield and Quality Evaluation

DNA yield and quality was measured spectrophotometrically at 260nm with a NanoDrop ND-1000 and the purity estimated using the A_{260}/A_{230} and A_{260}/A_{280} ratios. These ratios measure humic acid contaminants and protein based impurities respectively (Wilfinger *et al.*, 1997; Sambrook and Russell, 2001). The recommended A_{260}/A_{230} ratio is ≈ 1.8 and the A_{260}/A_{280} ratio is > 2.0.

5.1.2.3) Tissue Collection and Sample Nomenclature

Mantle tissue was collected from *Haliotis midae* individuals on the HIK abalone farm, Hermanus, RSA. The samples were stored in 100% ethanol at 4°C until DNA extractions could be performed.

The samples were named according to buffer and individual. Buffer C and D samples have additional appendices namely woR (without RNase) and wR (with RNase).

5.1.3) Results

The results of the NanoDrop readings are shown in graph format in Figures 5.1.1-5.1.3. Figure 5.1.1 shows the yield for each buffer. Buffer B, C and D showed the highest yields of DNA. Buffer A gave the least amounts of DNA. Buffer D also gave slightly higher yields compared to Buffer A and C. The variation in total DNA between the samples could be due to insufficient digestion of cells by proteinase K, releasing less nucleic acids for purification. The samples digested in Buffer C and D that contained no RNase yielded more DNA, but this could only indicate the presence of an excess of RNA and cause the overestimation of DNA concentration.

Figure 5.1.2 shows the graph of 260/230 ratios. All buffers gave comparable ratios, all between 1.8 and 2.2. The levels of humic acid contaminants were therefore low throughout. Figure 5.1.3 shows the 260/280 ratios. Only Buffer D gave consistent ratios of 2.0 and higher. Buffer A had extremely low ratios, suggesting that protein impurities were not sufficiently removed using this protocol.

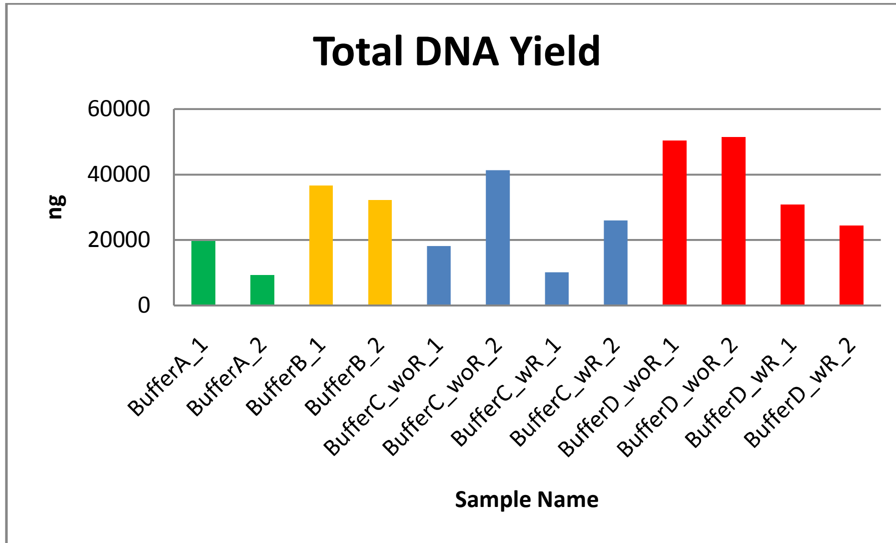


Figure 5.1.1: A graphical representation of the yields for each buffer type.

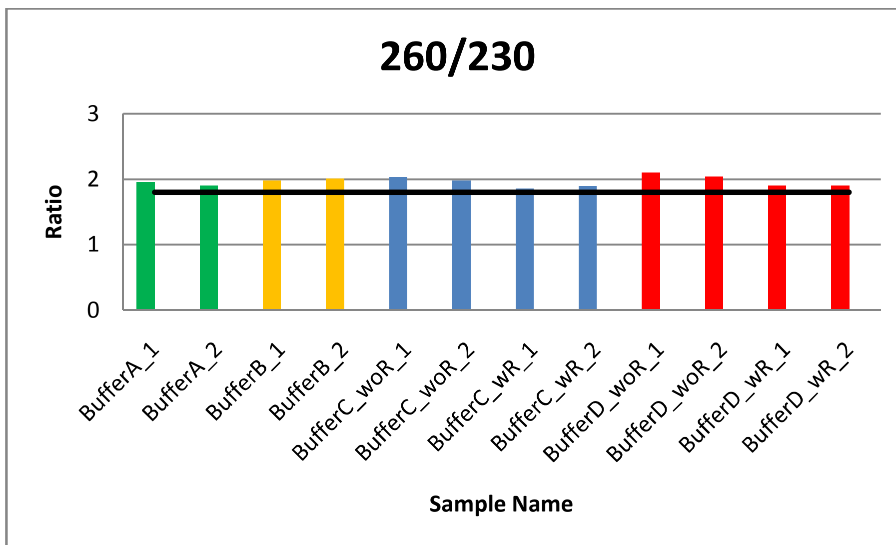


Figure 5.1.2: A graphical representation of the 260/230 ratios for each buffer type. The black line indicates the desirable 1.8 ratio.

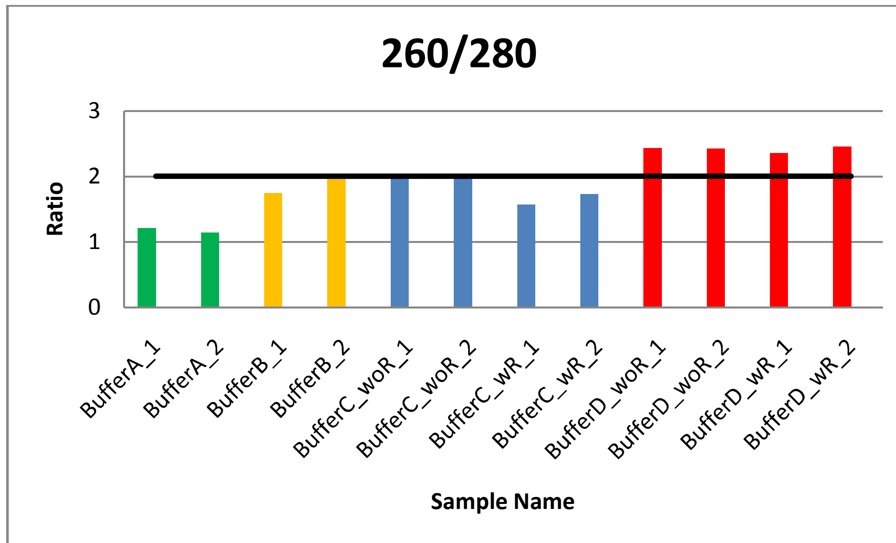


Figure 5.1.3: A graphical representation of the 260/280 ratios for each buffer type. The black line indicates the minimum ratio of 2.0.

5.1.4) Discussion

Four different DNA extraction buffers were tested for their application in purifying DNA from abalone tissue. The buffers were either CTAB- or SDS-based. One CTAB and one SDS buffer formed part of a commercially available extraction kit, while the remaining two were home-brewed. The results of this study found that the SDS-based buffers, Buffer B and D, were more effective in isolating large amounts of high quality DNA in *H. midae*.

Both Buffer B and D have their advantages and disadvantages. The greatest advantage of both is their suitability to high-throughput extractions using robotic platforms. A disadvantage of Buffer B would be the possibility of inconsistency in DNA purities as far as protein based impurities and salt removal is concerned. This is less of an issue with Buffer D that comes with a silica column based kit. Silica columns are preferred when extracting pure and high-quality DNA (Siddappa *et al.*, 2007; Tagliavia *et al.*, 2009) as proteins and salts are removed much more effectively. The greatest disadvantage of Buffer D is the costs involved per sample due to the silica columns, which could increase the price 3 to 4 times compared to the home-brew methods. It must be mentioned that an automated protocol for the

home-brew protocols will also be more expensive if not done by hand, since the consumables used by robotic platforms are pricey.

The buffers most suited for abalone extractions are SDS-based. When quality is more important than costs, a silica column extraction method is recommended above others, but when money is a limiting factor a home-brew SDS buffer and protocol would suffice.

SECTION 5.2

High-throughput DNA Extraction Protocol for *Haliotis midae*

5.2.1) Introduction

One of the bottlenecks in molecular breeding programmes is the large number of DNA extractions that is required for down-stream analyses such as linkage and QTL-mapping (Mace *et al.*, 2003). Automated, high-throughput extraction procedures has the potential to decrease the time spent per sample and also lessen any handling errors that goes with manual protocols (Zilberman *et al.*, 2006). Silica based commercial kits are well suited to automation and also yield high-quality DNA (Elphinstone *et al.*, 2003).

Here we present a high-throughput DNA extraction protocol for the abalone *Haliotis midae*. The protocol uses a robotic platform and a silica-based commercial kit.

5.2.2) Materials and Methods

5.2.2.1) Sample Preparation

The NucleoSpin Plant II kit (*Macherey-Nagel*) was used for performing the DNA extractions. Extractions were done on the Genesis RMP200 (*Tecan*; Figure 5.2.1) using a script written by Carel van Heerden (DNA Sequencing Facility, Stellenbosch University, RSA). A new script was created to add 400µl of Buffer PL2 to each of the 96 wells of a round-well block (*Macherey-Nagel*) after 2µl of 10mg/ml proteinase K (*Sigma-Aldrich*) was added. Two epipodial tentacles which were stored in 100% ethanol were added to each well, sealed with a mat and incubated overnight at 60℃. The same script was then used to add 100µl Buffer PL3 to each well. The block was incubated on ice for 5 minutes and centrifuged at 6.0rcf for 30 minutes. A total of 400µl supernatant was mixed with 450µl binding buffer PC and transferred to a silica

membrane plate. The mixture was pulled through the membrane by a vacuum system (-0.6 bar). The bound DNA was washed to remove proteins and salts with 400μl buffer PW1 and twice with 700μl buffer PW2. A vacuum step followed each step (-0.6 bar). The bound DNA was eluted twice with 100μl volumes of elution buffer preheated to 70℃. DNA quantification was performed using the Infinite F200 NanoQuant (*Tecan*).

Figure 5.2.1: The eight syringe Genesis RMP200 system.

5.2.3) Results and Discussion

This protocol was used to extract DNA from almost 4500 abalone epipodial tentacle and muscle tissue samples. The pre-digestion sample preparation took about 60 minutes or less per 96 tissue samples. The overnight digestion step can be lessened to 1 hour, but there is the risk of getting lower DNA yields. The automated steps were completed in 40 minutes per 96 samples. The quantification of these 96 samples was completed within 10 minutes using the Infinite F200 NanoQuant (*Tecan*) and a 16-well optical plate. Table 5.2.1 gives a comparison between the high-throughput and the standard laboratory extraction protocol in terms of consumables, handling of samples and time spent per 96 samples.

Table 5.2.1: A comparison between the high-throughput and the standard laboratory extraction protocols in terms of consumables, handling of samples and time spent per 96 samples.

Parameters	Laboratory Extraction (CTAB or SDS Buffers)	High-throughput Extraction (Silica Column-based)
Tubes Used or 96-Round-Well Blocks Used	288 0	96 1
Tips Used	198	145
Times Sample is Handled by User	5	1
Time for [DNA] Readings	60 minutes (NanoDrop ND-1000)	10 minutes (Infinite F200 NanoQuant)
Time from Tissue to DNA (excluding overnight digestion)	± 1 to 2 Days	± 2 hours
Cost per Extraction as Charged by the Sequencing Facility, Stellenbosch University for 2010.	ZAR 25.00	ZAR 15.00

The automated high-throughput protocol reduced the number of tubes by at least 66% and the number of tips by at least 77%. The chance of human-error is reduced since the samples are only handled once during pre-digestion sample preparation. The overall time spent in purifying DNA was cut from days to just hours. The amount of samples from which DNA could be extracted increased. Three groups of 96 samples could be extracted within a normal working day. The use of the robotic platform also enables a researcher to load samples and then continue to perform other duties while the robotic platform is in operation. Even though silica columns are more expensive (ZAR 25.00) compared to some home-brewed methods (ZAR 10.00-15.00) the increase in volume and quality of DNA and decrease in time spent on extractions should make up for this cost difference.

SECTION 5.3

Depurination and Regeneration of 96-well Commercial Silica DNA Extraction Plates

5.3.1) Introduction

Silica columns are used for extracting pure and high-quality DNA (Siddappa *et al*., 2007; Tagliavia *et al*., 2009). Such columns are also perfect for automated platforms and high-throughput protocols. Their main disadvantages are high costs and that they can only be used once, because trace amounts of bound DNA remain in the silica after elution steps. The columns can be re-used by removing the DNA using strong acids and alkalines. Siddappa *et al*. (2007) used hydrochloric acid (HCl) to denature and hydrolyse any DNA molecules into very small fragments of below 36bp in length. Tagliavia *et al*. (2009) described a protocol using Triton-X in combination with an alkaline (NaOH) wash, followed by an acid wash (HCl) and a final NaOH wash. Both methods showed efficient removal of bound DNA without the risk of any contamination after reuse.

In this study the regeneration and reuse of a 96-well commercial plate using sodium hydroxide (NaOH) and hydrochloric acid (HCl) is described.

5.3.2) Materials and Methods

5.3.2.1) First DNA Extraction (Pre-Regeneration)

The Nucleospin Plant II extraction kit (*Macherey-Nagel*) was used for DNA extraction. Tissue from a single abalone (*Haliotis midae*) was used for the extraction. Sixteen tissue samples of variable sizes, but no larger than $5mm^3$ was added to 400μl of lysis buffer PL2 (*Macherey-Nagel*) and 2.5μl of 10mg/ml proteinase K (*Sigma-Aldrich*) and digested at 60°C overnight. A volume of 100μl of buffer PL3 was added to each sample, mixed, incubated on ice for 5 minutes and centrifuged at 6.0rcf for 20 minutes. The Tecan Genesis 2000 robotic platform was used to

complete the remaining steps which were performed according to the recommendations of the manufacturer. The samples were loaded in the last 16 wells of the 96-well plate (Figure 5.3.1). DNA was quantified using a Nanodrop ND 1000.

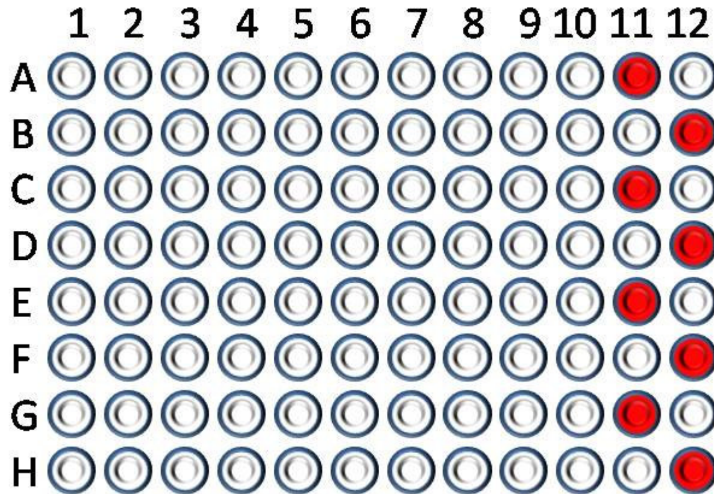


Figure 5.3.1: A diagramme of a 96-well plate. The last two columns, 11 and 12, were used for DNA extractions pre- and post-regeneration. The red wells indicate those that contained tissue during the second (post-regeneration) DNA extraction.

5.3.2.2) Column Regeneration

The plate was rinsed in ddH_2O to remove traces of the wash buffers. The entire plate was then submerged in 1M NaOH and left for 10 hours. The plate was removed and rinsed with ddH_2O to remove excess NaOH. The wells were then washed three times with ddH_2O by filling the entire well (approximately 1.5ml) and applying a vacuum (-0.6 bar) until the contents passed through the membrane. The plate was then submerged in 1M HCl for 10 hours. It was once again rinsed and washed using ddH_2O as described above. Before re-use the membranes were equilibrated by washing them twice with buffer PW2 and drying it for 15 minutes by vacuum (-0.6 bar).

5.3.2.3) Second DNA Extraction (Post-Regeneration)

A different *H. midae* individual was used for the second extraction. Eight tissue samples of variable sizes, but no larger than 5 mm^3, were used. An extraction reaction without tissue was put into every second well (Figure 5.3.1). The same protocol was followed as for the first extraction. The same 16 wells on the regenerated plate were used in the extraction. DNA was quantified using a Nanodrop ND1000.

5.3.2.4) Microsatellite and Real-time-PCR Analyses

The *H. midae* specific microsatellite locus *Hm*RS129 (Acc. Nr.: DQ785766) was amplified using fluorescent labelled primers (VIC) and real-time PCR and was carried out on the Rotor-Gene 6000 with the SensiMix CybrGreen reaction kit (*Quantace*) and 0.1 optical PCR tubes in 10µl volumes. All reactions from the run were prepared from the same master mix. Each reaction contained 1µl template from the undiluted extraction, 1x SensiMix (*Quantace*), CybrGreen and 5µM of each primer. PCR conditions were 95℃ for 10 minutes, 10x (30 seconds at 95℃, 30 seconds at 65℃ dropping 1℃ each cycle, 30 seconds at 72℃), 30x (30 seconds at 95℃, 30 seconds at 55℃, 30 seconds at 72℃). Melt analyses were performed from 67℃ to 95℃ rising by 1℃ every 5 minutes. Data was acquired on the green channel. Data analyses were performed using the Melt module of the Rotor-Gene 6000 Series Software Version 1.7. Fluorescent microsatellite electrophoresis was performed on the 3730xl DNA Analyzer (*Applied Biosystems*) and genotyped using Genemapper Version 4 (*Applied Biosystems*).

5.3.3) Results

5.3.3.1) DNA Quantification

The results of the NanoDrop readings are shown in Table 5.3.1. No DNA was detected in the wells that contained no tissue for the extraction after regeneration.

Table 5.3.1: NanoDrop results of post-regeneration DNA extractions.

Sample Name	[DNA] (ng/µl)	260/280 Ratio	260/230 Ratio
Row A Column 11 (Tissue)	237.68	1.91	2.46
Row B Column 11	--	--	--
Row C Column 11 (Tissue)	225.67	1.90	2.43
Row D Column 11	--	--	--
Row E Column 11 (Tissue)	208.67	1.90	2.39
Row F Column 11	--	--	--
Row G Column 11 (Tissue)	237.58	1.92	2.41
Row H Column 11	--	--	--
Row A Column 12	--	--	--
Row B Column 12 (Tissue)	265.72	1.92	2.43
Row C Column 12	--	--	--
Row D Column 12 (Tissue)	353.50	1.91	2.32
Row E Column 12	--	--	--
Row F Column 12 (Tissue)	217.81	1.94	2.41
Row G Column 12	--	--	--
Row H Column 12 (Tissue)	294.43	1.90	2.40

5.3.3.2) Real-time PCR and Microsatellite Genotyping

The results of the melt analysis are shown in Figure 5.3.2. The figure shows the melt profile of the eight wells that contained tissue samples post-regeneration in red, while no amplification was observed for the wells that contained no tissue (blue). The green line indicates the melt profile of an individual from the pre-regeneration extraction. The genotype results are shown in Table 5.3.2. The genotype of the pre-regeneration individual was 276/282 and that of the post-regeneration individual 263/284. The alleles of the original individual were not detected in any of the post-regeneration samples.

Table 5.3.2: Genotyping results for pre- and post-regeneration extractions.

Sample Name	**Allele1**	**Allele2**
Pre-Regeneration Sample Genotype	276	282
Post-Regeneration Genotypes		
Row A Column 11 (Tissue)	263	284
Row B Column 11	0	0
Row C Column 11 (Tissue)	263	284
Row D Column 11	0	0
Row E Column 11 (Tissue)	263	284
Row F Column 11	0	0
Row G Column 11 (Tissue)	263	284
Row H Column 11	0	0
Row A Column 12	0	0
Row B Column 12 (Tissue)	263	284
Row C Column 12	0	0
Row D Column 12 (Tissue)	263	284

Row E Column 12	0	0
Row F Column 12 (Tissue)	263	284
Row G Column 12	0	0
Row H Column 12 (Tissue)	263	284

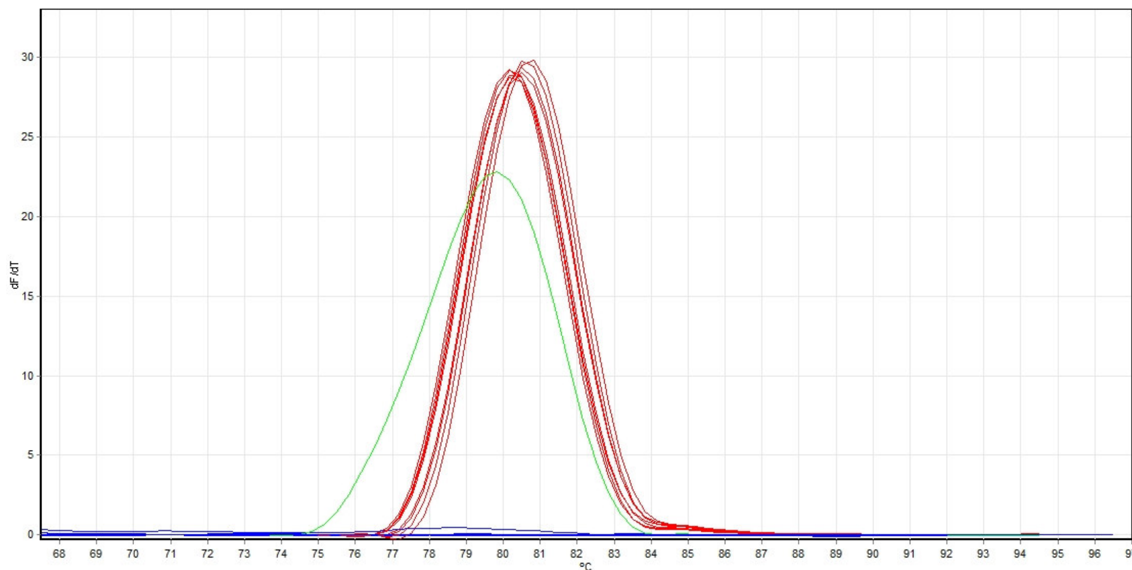


Figure 5.3.2: The melt-analysis of the post-regeneration PCR (in red = with tissue and blue = without tissue) and a pre-regeneration sample (in green).

<u>5.3.4) Discussion</u>

A protocol allowing the re-use of commercial 96-well silica plates was set up following Siddappa *et al*. (2007) and Tagliavia *et al*. (2009) by washing the plates with both sodium hydroxide (NaOH) and hydrochloric acid (HCl). DNA quantification analysis, real-time PCR and microsatellite genotyping showed that all the trace DNA was successfully removed after the various washing steps. The quality and DNA yield remained high after plate regeneration extractions. The regeneration of these plates can save almost 40% in consumable cost after one regeneration step.

SECTION 5.4

Hybrid Discrimination using High-Resolution Melt Curve Analysis in *Haliotis midae* x *Haliotis spadicea*

5.4.1) Introduction

The abalone industry in South Africa is very lucrative, fetching around USD 38 per kg in 2008 (pers. comm.: Wayne Barnes, Abalone Farmers Association of South Africa). Recently industry became interested in creating an interspecific hybrid between the cultured species, *Haliotis midae* and another naturally abundant species, *H. spadicea*. Hybrid abalones are of great interest to breeders and could potentially combine desirable traits into a single organism (Elliott, 2002). In oysters the cross between *Crassostrea gigas* and *C. rivularis* combines the disease resistance and hardiness of *C. gigas* with the physiological tolerances of *C. rivularis* (Allen and Gaffney, 1993). Hybridisation in the genus *Haliotis* thus far primarily focused on increasing growth rates (Ibarra *et al*., 2005).

A number of hybrids exist for abalone, for example *H. rubra* x *H laevigata* (Brown, 1995), *H. kamtschatkana* x *H. discus hannai* (Hoshikawa *et al*., 1998), *H. fulgens* x *H. rufescens* (Ibarra *et al*., 2005) and *H. diversicolor* x *H. discus discus* (Cai *et al*., 2009). Hybrids are either defined by their phenotypic characteristics (Owen *et al*., 1971) or by genetic techniques such as allozyme and microsatellite analyses (Brown, 1995; Ibarra *et al*., 2005). Another way for discriminating hybrids would be the introgression of nuclear genes by identifying species-specific differences in them. Sweijd *et al*. (1998) developed a PCR-RFLP method using a nuclear lysin gene to discriminate between *H. midae* and *H. spadicea*.

In this study a modified protocol was developed using high resolution melt-curve analysis (HRMC) for hybrid identification to be used in future. HRMC has successfully been used previsouly to identify fish species or identify fish mitochondrial haplotypes (Wattanabe *et al*., 2004; Dalmasso *et al*., 2007; Haynes *et al*., 2009).

5.4.2) Materials and Methods

5.4.2.1) Sample Preparation

Tissue samples were taken from a *H. midae* female and a *H. spadicea* male. Two-day old larvae produced from a cross between these animals were also sampled before they were destroyed (Roux *et al.*, in prep.). Genomic DNA was isolated following a standard CTAB extraction method (Saghai Maroof *et al.*, 1984). DNA was quantified using the NanoDrop ND1000.

5.4.2.2) Real-time PCR

Primers specific to abalone lysin were used to amplify a 120bp product. The DNA used for analysis consisted of one *H. midae* and one *H. spadicea* sample, a mixture of DNA from these two individuals (hybrid control) and the larvae sample of the interspecific cross. Real-time PCR and HRMC analysis was performed using a Rotorgene 6000 PCR machine. PCR was carried out in a final volume of 25μl and conditions were as follows: 1x SensiMix HRM (*Quantace*), 1μl EvaGreen dye, 5μM of each primer and 40ng of DNA template. Cycling conditions were initial denaturation and activation at 95℃ for 10 minutes followed by 50 cycles of 95℃ for 10 seconds, 53℃ for 20 seconds and 72℃ for 30 seconds. HRMC analysis was done using the Rotorgene 6000 Series Software version 1.7 after the temperature was raised from 70℃ to 90℃ at 0.1℃ increments and a hold of 2 seconds for each step. PCR products were sequenced afterwards as verification of the HRMC results, using the 3730xl DNA Analyzer (*Applied Biosystems*).

5.4.3) Results and Discussion

The result of the HRMC analysis is shown in Figure 5.4.1. *Haliotis midae*, *H. spadicea* and the hybrid control genotypes were clearly distinguishable. The larvae sample was classified as hybrid based on these results. The sequencing results confirmed this conclusion showing the single basepair difference between *H. midae* and *H. spadicea* as well as the presence of both genotypes in the hybrid larvae sample (Table 5.4.1).

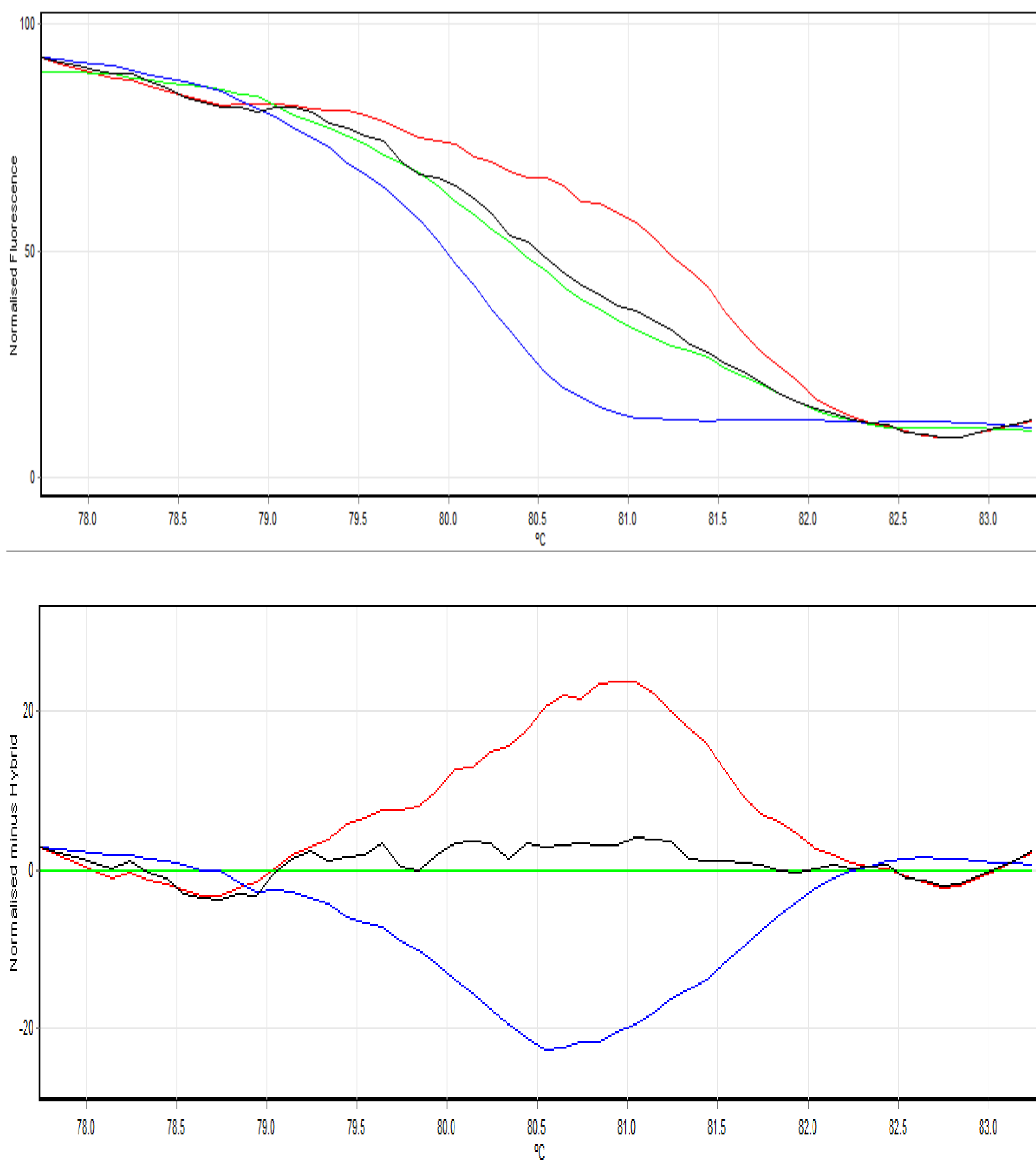


Figure 5.4.1: The normalised (top) and difference graph (bottom) of the high-resolution melt analysis of the sperm lysin SNP for *Haliotis midae* (red), *H. spadicea* (blue), the hybrid control (green) and the *H. midae* / *H. spadicea* hybrid (black).

Table 5.4.1: The sequences of *Haliotis midae*, *H. spadicea* and their hybrid for a section of the abalone sperm lysin gene.

Species	**Sperm lysin sequence**
Haliotis midae	CTGGATGGAGCCAATGACGTCATCCCAGC**G**CGGGGTGGCCAGATTTTTCAC
Haliotis spadicea	CTGGATGGAGCCAATGACGTCATCCCAGC**A**CGGGGTGGCCAGATTTTTCAC
Interspecific hybrid larvae	CTGGATGGAGCCAATGACGTCATCCCAGC**R**CGGGGTGGCCAGATTTTTCAC

The high resolution melt-analysis presented here will rapidly and accurately verify the presence of hybrid larvae in future breeding experiments in the South African abalone industry or help in searching for evidence of hybridisation occurring in nature. Even though this protocol was optimised for the two species it can easily be applied in other abalone species using different nuclear or mitochondrial regions (Supernault *et al.*, 2010).

SECTION 5.5

A Microsatellite Panel for Triploid Verification in the Abalone, *Haliotis midae*

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5.5.1) Introduction

There are five abalone species found in the waters of South Africa, of which only one, *Haliotis midae*, is cultivated and exported with a current farm production of 870 tonnes in 2008 fetching around USD 38 per kg (pers. comm.: Wayne Barnes, Abalone Farmers Association of South Africa). One strategy for increasing production output is triploid induction (Elliott, 2002). Triploid abalone can be produced using various methods including pressure shock (e.g.: Arai *et al*., 1986), thermal shock (e.g.: Yang *et al.,* 1998), 6-dimethylaminopurine treatment (e.g.: Norris and Preston, 2003), caffeine treatment (e.g.: Okumura *et al.,* 2007) and cytochalasin B treatment (e.g.: Li, Y. *et al.,* 2007a). Triploidy was successfully induced in *H. midae* using cytochalasin B (Stepto and Cook, 1998) and hydrostatic pressure (De Beer, 2004).

It is important to verify the ploidy level of the experimental population after induction. Various methods exist for such verification including flow cytometry, karyotyping, nucleolar organizer region analysis and particle size analysis (Harrell *et al.,* 1995). The use of flow cytometry has thus far been the preferred direct method for triploid verification in abalone (Stepto and Cook, 1998; Norris and Preston, 2003; De Beer, 2004; Liu *et al.,* 2004; Li, Y. *et al.,* 2007a; Okumura *et al.,* 2007), because hundreds of samples can be analysed at a time and the relative percentage of triploid induction can also be analysed for an experimental population (Harrell *et al.,* 1995). Another direct method of triploidy verification is the use of polymorphic DNA markers (Magoulas, 1998) such as microsatellites. Triploidy in molluscs are induced by suppressing the formation of either the first or second polar body resulting in an

extra set of chromosomes from the female (Gérard *et al.,* 1999). The usefulness of the molecular markers therefore relies on the heterozygosity of a locus within the female and on the allele inherited from a male having a different size than those two female alleles. A large number of loci need to be tested to identify the combination of markers that can reliably identify triploid individuals. This became practical for *H. midae* after the development of 200 microsatellite markers (Bester *et al.,* 2004; Slabbert *et al.,* 2008, 2010).

In this study we tested 30 polymorphic microsatellite loci for their application in ploidy verification. The development of an accurate protocol for triploidy verification for *H. midae* using appropriate polymorphic microsatellite markers are described followed by its application in identifying diploid and induced triploid individuals within an experimental population.

5.5.2) Materials and Methods

5.5.2.1) Triploidy Induction and Sample Preparation

Abalones were spawned on I&J Abalone and triploidy was induced according to De Beer (2004). Tentacles were taken from 8 triploid and 8 diploid abalones to be used as control samples for the initial genotyping. DNA extractions were performed using the CTAB extraction protocol of Saghai-Maroof *et al.* (1984). A further 42 ploidy-unknown, three triploid and three diploid juvenile abalones were sampled by taking two tentacles. All tissue samples were stored in 100% ethanol for transportation and kept at room temperature until extraction (5.5.2.4).

5.5.2.2) Initial Genotyping

The 16 control samples were genotyped with 30 microsatellite loci shown in Table 5.5.1. PCR amplification was performed according to the conditions described in Bester *et al.* (2004) and Slabbert *et al.* (2008, 2010). Fluorescent analysis was performed with a 3730xl DNA Analyzer (*Applied Biosystems*) and genotyping with GeneMapper version 4 (*Applied Biosystems*). An individual was classified as triploid if three alleles were detected for a locus. Markers for the triploidy verification

multiplex were chosen based on amplification success, unambiguous identification between ploidy levels and the number of correct triploid individuals assigned (cut-off = 3 out of 8 control samples). Any loci showing 4 or more alleles were discarded from further analyses.

Table 5.5.1: Microsatellite loci tested for suitability for triploidy verification.

Locus	n_a	H_o	H_e	**Acc. Number**	**Group**[d]
*Hm*AD102T[b]	16	0.32	0.93	DQ785747	15
*Hm*D55[a]	9	0.68	0.80	AY303337	Ungrp
*Hm*D59[a]	15	0.78	0.84	AY303338	5
*Hm*LCS7M[b]	16	0.69	0.89	DQ825707	Ungrp
*Hm*LCS47M[b]	13	0.78	0.85	DQ993228	Ungrp
*Hm*LCS48M[b]	9	0.52	0.55	DQ993227	Ungrp
*Hm*LCS63T[b]	4	0.04	0.54	DQ993223	Ungrp
*Hm*LCS67M[b]	8	0.45	0.73	DQ993222	Ungrp
*Hm*NR20M[b]	11	0.85	0.85	EF063097	6
*Hm*NR106D[b]	16	0.81	0.88	DQ825709	Ungrp
*Hm*NR120T[b]	24	0.90	0.94	EF121745	4
*Hm*NR224T[b]	20	0.95	0.95	EF512269	Ungrp
*Hm*NR258R[b]	6	0.72	0.76	EF512272	Ungrp
*Hm*NR281P[b]	21	0.71	0.92	EF512274	1
*Hm*NS19L[b]	25	0.56	0.95	EF033330	2
*Hm*NS58D[b]	10	0.78	0.86	EF367119	Ungrp
*Hmid*PS1.305T[c]	5	0.71	0.76	GU256679	Ungrp
*Hmid*PS1.332D[c]	18	0.56	0.95	GU256680	14

*Hmid*PS1.549D[c]	10	0.81	0.89	GU256696	8
*Hmid*PS1.811C[c]	11	0.46	0.84	GU256710	Ungrp
<u>*Hmid*PS1.818C</u>[c]	11	0.63	0.74	GU256711	8
<u>*Hmid*PS1.870D</u>[c]	15	0.88	0.94	GU256718	1
*Hmid*PS1.874C[c]	14	0.63	0.93	GU256720	7
*Hm*PS1.1058C[c]	11	0.60	0.90	GU256735	13
*Hm*PS1.1063C[c]	11	0.53	0.90	GU256736	3
*Hm*RS27T[b]	23	0.41	0.93	DQ785751	13
*Hm*RS36T[b]	6	0.52	0.67	DQ785753	1
*Hm*RS37D[b]	7	0.55	0.69	DQ785754	Ungrp
*Hm*RS80M[b]	18	0.71	0.92	DQ785756	Ungrp
*Hm*RS83M[b]	27	0.65	0.96	DQ785757	Ungrp

a: Bester *et al.,* 2004.

b: Slabbert *et al.,* 2008.

c: Section 2.4.

Ungrp = Not assigned to a linkage group (based on linkage map in Section 2.4).

Underlined loci indicate those used for triploid verification in this study.

5.5.2.3) Statistical Analysis

Population estimates such as number of alleles and observed and expected heterozygosities (Table 5.5.1) were taken from Bester *et al.* (2004) and Slabbert *et al.* (2008, 2010). A one-way ANOVA (Statistica 8) was performed to ascertain if markers for ploidy verification should be chosen based on number of observed alleles, observed heterozygosity or expected heterozygosity. Loci that correctly identified (3 alleles) 3 out of 8 triploid controls were compared to loci that did not identify (2 or less alleles) any triploid controls. Each parameter was tested separately.

5.5.2.4) Multiplex Set-up and Experimental Triploidy Verification Protocol

The triploid verification multiplex reaction was optimised using the Qiagen Multiplex Kit (*Qiagen*). PCR reactions were performed using a 2720 Thermal Cycler (*Applied Biosystems*) in a final volume of 10µl and contained 20ng of genomic DNA, 1x Qiagen Multiplex PCR Master Mix and 2µl of the primer mix (4µM each of *Hm*D59 and *Hm*NR106D and 2µM each of *Hm*NR20M, *Hm*NR120T, *Hm*NS19L, *Hmid*PS1.305T, *Hmid*PS1.818C and *Hmid*PS1.870D). The PCR programme was as follows: an initial activation step was performed at 95℃ for 15 minutes followed by 35 cycles of 30 seconds at 94℃, 90 seconds at 57℃ and 60 seconds at 72℃ with a final extension step at 60℃ for 30 minutes. Fluorescent genotyping was done using a 3730xl DNA Analyzer (*Applied Biosystems*).

This optimised protocol was then applied to identify 42 ploidy-unknown samples and three triploid and three diploid control samples to verify their ploidy status. DNA was extracted from tentacle tissue using a faster method than used in section 5.5.2.1, namely the KAPA Quick Extract kit (*KAPA Biosystems*). Extractions were performed using a 2720 Thermal Cycler (*Applied Biosystems*) in a final volume of 100µl. The reaction contained 1x KAPA Quick Extract Buffer, 2U KAPA Quick Extract Enzyme (*KAPA Biosystems*) and a 1mm^2 piece of epipodial tentacle. The PCR programme was as follows: an incubation step was performed at 60℃ for 15 minutes followed by a heat-inactivation step at 95℃ for 5 minutes. The multiplex PCR reaction was then performed followed by fluorescent genotyping. An individual was classified as triploid if three alleles were detected for at least two loci. Samples that had any loci showing four or more alleles should be discarded from further analyses as this could suggest the possibility of DNA contamination from other sources.

5.5.3) Results

5.5.3.1) Initial Genotyping

Nine loci met the minimum criteria set for triploid verification in this study. *Hm*D59, *Hm*LCS7T, *Hm*NR20M, *Hm*NR106D, *Hm*NR120T, *Hm*NS19L, *Hmid*PS1.305T, *Hmid*PS1.818C and *Hmid*PS1.870D identified all diploid individuals correctly and identified at least 3 out of 8 triploid individuals correctly (Table 5.5.2). The remaining 21 loci identified all diploid individuals correctly, but only 2 or less triploid individuals. Loci *Hm*D59, *Hm*NR106D, *Hm*NR120T, *Hm*NS19L, *Hm*NR20M, *Hmid*PS1.818C and *Hmid*PS1.870D were chosen for the ploidy verification multiplex. *Hm*LCS7T and *Hm*NR20M have identical fluorescent markers and allele size ranges. Only one of these can be added to the multiplex, but is interchangeable. *Hmid*PS1.305T was later discarded after difficulty in allele calling. The final multiplex therefore consisted of 7 markers.

5.5.3.2) Statistical Analysis

One-way ANOVA analysis showed no significant difference ($P > 0.05$) between the two groups of loci in terms of observed number of alleles ($P = 0.09$) and expected heterozygosity ($P = 0.16$). A significant difference ($P < 0.05$) was seen for observed heterozygosity ($P = 0.01$; average triploid loci = 0.76; average diploid loci = 0.52) showing higher levels for markers which identified triploid controls correctly.

5.5.3.3) Triploidy Verification Protocol

The KAPA Quick Extract kit (*KAPA Biosystems*) yielded high quality DNA to be used in a microsatellite multiplex reaction. An example of a genotyping electropherogramme of a diploid and triploid individual is shown in Figure 5.5.1. Thirty-one ploidy-unknown samples were classified as triploid and 11 as diploid. The control samples were also correctly assigned.

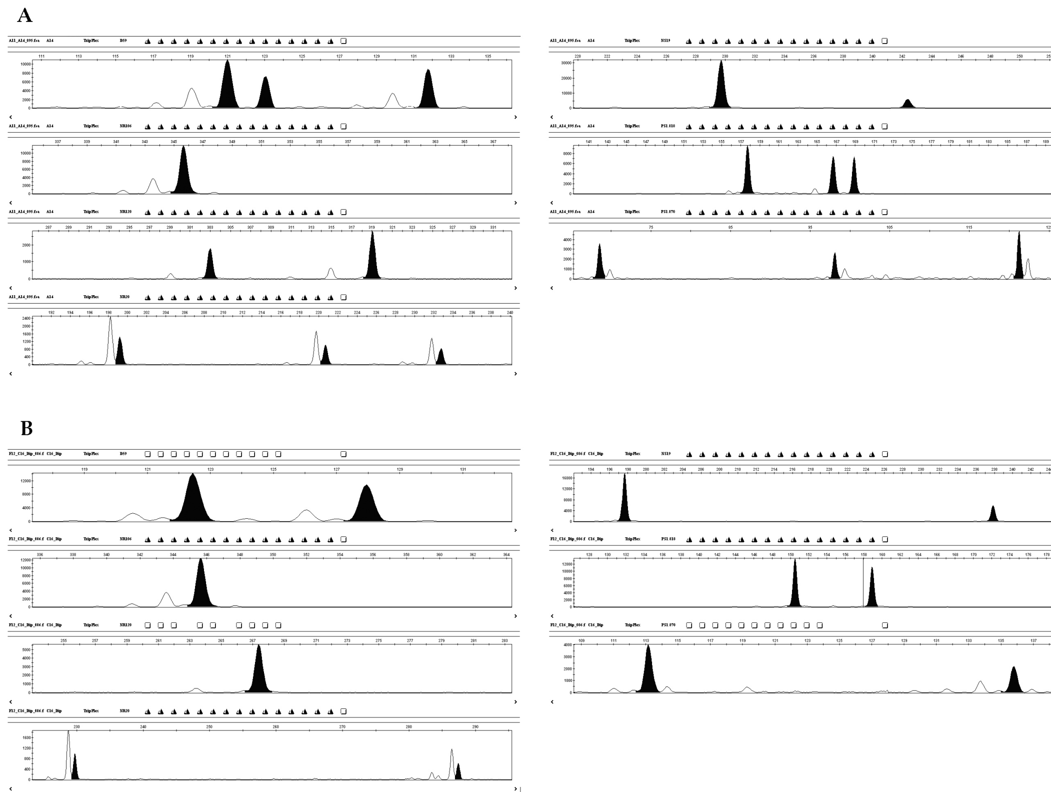

Figure 5.5.1: Electropherogrammes showing the alleles of each of the seven loci used for triploid verification for a triploid (A) and a diploid (B) individual. Three alleles can be observed for the triploid individual in some loci, while all loci of the diploid individual have no more than two alleles.

5.5.4) Discussion

Nine microsatellite loci (Table 5.5.2) were adequate for molecular verification of the level of ploidy of *Haliotis midae*. The original 30 loci were chosen because they had no duplication in the genome. Duplication is found in the abalone genome and was already reported for 17 *H. midae* loci (Slabbert *et al.,* 2010) and in *H. rubra* loci as well (Baranski, 2006). Our results suggest that markers can be chosen based on observed heterozygosities. Loci with high levels of observed heterozygosity (average = 0.76) were more likely to identify a triploid individual correctly. It is very important to have reliable locus characterisation data available when selecting markers for ploidy verification, since any errors in allele scoring can either over- or underestimate this statistic. Another technical point to consider is the position and coverage of the loci within the genome of an organism. Chance duplication events can be avoided by using multiple loci covering different linkage groups (chromosomes). The loci identified for triploid verification in the current study were mapped to six different linkage groups, while three loci remained ungrouped (Table 5.5.1).

Seven loci, namely *Hm*D59, *Hm*NR106D, *Hm*NR120T, *Hm*NS19L, *Hm*NR20M, *Hmid*PS1.818C and *Hmid*PS1.870D, were placed into a multiplex reaction and applied to identify the ploidy of individual abalones within a mixed experimental population of diploids and induced triploids. The PCR-multiplex and a closed tube extraction method were used to set up the triploid verification protocol. Closed tube methodologies are fast, do not require much tissue and minimise the times a sample is handled prior to PCR-analysis. Multiplex PCR reactions increases data per reaction, making it cheaper and faster compared to single locus PCR reactions. This protocol was applied to identify samples of unknown ploidy level. All 42 ploidy-unknown samples were assigned as either diploid (11 samples) or triploid (31 samples) and the six control samples were also assigned correctly.

Table 5.5.2: Genotypes of diploid and triploid controls for ploidy verification microsatellite panel.

Individual	Locus Genotypes								
	***Hm*D59**	***Hm*LCS7T**	***Hm*NR20M**	***Hm*NR106D**	***Hm*NR120T**	***Hm*NS19L**	***Hmid*PS1.305T**	***Hmid*PS1.818C**	***Hmid*PS1.870D**
Dilpoid1	124/128	233/233	221/233	386/386	291/307	178/224	096/096	157/157	098/098
Dilpoid2	111/124	261/263	227/239	381/381	248/339	231/238	103/127	151/157	109/115
Dilpoid3	153/155	261/263	221/233	-/-	248/303	185/237	096/125	144/161	115/122
Dilpoid4	111/130	-/-	193/209	373/386	287/307	237/250	103/127	157/161	130/130
Dilpoid5	124/146	229/229	193/227	345/350	263/291	224/244	099/127	157/167	098/122
Dilpoid6	124/136	229/243	227/245	388/388	315/315	237/245	096/099	159/167	111/117
Dilpoid7	124/130	231/231	227/233	381/381	267/331	215/267	099/127	157/157	098/111
Dilpoid8	122/124	210/233	230/233	379/386	248/303	209/242	096/099	151/157	107/122
Triploid1	117/119/124	233/240/243	245/245	375/386	248/260	231/253/272	102/103/127	157/161	122/124
Triploid2	117/124	208/229	187/202/230	375/383/386	283/307/323	238/250	096/099/103	157/159/169	098/128

Triploid3	124/124	208/229	202/233/245	373/386	248/263/323	190/198/235	096/099	155/157/167	098/100/115
Triploid4	113/121/128	208/221/243	230/233	375/381	307/339	244/253	096/099/127	159/167	098/113/126
Triploid5	113/115	243/243	233/233	375/381/392	255/299/307	231/253	103/103	157/157	126/128
Triploid6	119/121/126	208/240	193/202/230	345/345	260/295/323	191/196/259	099/099	152/157/165	098/115
Triploid7	113/124/128	221/233/243	233/233	375/390/392	275/307	231/238/253	099/099	165/167	098/122/126
Triploid8	111/113/128	243/243	227/233	345/345	267/299/307	231/238/253	096/099/103	146/146	098/100/126

This study shows the utility of microsatellite markers as tools for verifying the ploidy of individual abalones. The protocol can be applied in larger animals where the isolation of nuclei and metaphase chromosomes from tissues are difficult (pers. comm.: Dr. P. Franchini, Molecular Aquatic Research Group). Individual ploidy verification can be performed quickly and regularly and could facilitate the studying of, for example, any reverting from the triploid state to the diploid state over time (Dew *et al.,* 2003; Dunstan *et al.,* 2007).

CHAPTER 6

CONCLUDING REMARKS, RECOMMENDATIONS AND FUTURE DIRECTIONS

6.1) Background

The South African abalone industry went into partnership with government and academic institutions with the aim of increasing the productivity and profitability of farms. A genetics improvement programme was undertaken and consists of two major parts. The first was the quantitative section that looked at abalone growth rate variation between various farm locations and also investigated chromosome manipulation techniques such as triploid induction. This was later expanded to the creation of full-sib families for the calculation of trait heritabilities and broodstock breeding values. Ultimately the fastest growing animals would be further subscribed at age four, to a second round of controlled mating designs. The second aspect of the improvement programme focuses on molecular genetics. The main aim of the molecular section was the development of molecular markers, particularly microsatellite loci isolation. Other markers such as SNP's are currently (2009-2010) being targeted for development. These markers were primarily required to create the first framework linkage map for *Haliotis midae* and identify QTL associated to growth. These QTL would ultimately be used in marker assisted selection strategies for breeding high quality abalone. The focus of the group has recently shifted towards the characterisation of the transcriptome and gene expression profiles to add to the power of identifying superior abalone stocks. The sequence data generated will add to the resource for marker development. Reproductive technologies also became a part of the larger selective breeding programme. This aspect attempts to add information for selection of superior broodstock in terms of for example egg and sperm quality, larval quality, sperm competition and gonadal development.

6.2) The Contributions of the Current Study to the Programme

In this dissertation the focus fell on microsatellite loci isolation and the utility of these markers in identifying QTL associated with growth related traits; in this case shell length, shell width and wet weight. Framework linkage map construction formed part of two MSc and one postdoctoral project and was not essentially part of this current study.

Before the onset of this work, only 11 microsatellite loci were characterised within this species by Bester *et al.* (2004). A total of 125 loci was characterised and added to the growing pool of markers for *H. midae*. A total of 82 of these loci were isolated using a second generation sequencing platform, a first for any abalone species. Although microsatellite enriched libraries resulted in the generation of 125 microsatellite markers for *H. midae*, it would seem that newer high-throughput technologies and alternative marker types have reduced the usefulness of this resource for future application and marker development in this species. The development of second generation sequencing is allowing for greater data generation capabilities and EST-based markers are becoming more popular since coding regions can be studied. In terms of data generated and time and money spent it would be more sensible in future to sequence transcriptomes or EST-libraries (e.g.: Quilang *et al.*, 2007; Sha *et al.*, 2010) instead of microsatellite-specific libraries and use these for marker development.

The newly isolated loci and those characterised by other members of the group were applied in a bulked segregant analysis in combination with selective genotyping to do a preliminary screen for QTL associated with the three abovementioned traits. This type of pooled analysis was found to be an effective method for preliminary screening purposes as also found by Baranski (2006) in *H. rubra*. This method was however sensitive to the presence of null alleles, genotyping errors and sampling errors. Therefore, markers showing significance for linkage was analysed further using single marker regression and interval mapping. Additional markers were also used for these two analyses and were chosen by using a low density framework map created in this study as well as a preliminary higher density map created by two other researchers. The same chromosomal segment was identified as carrying three potential QTL, one each for length, width and wet weight, in both the families under investigation. This suggests that these QTL are likely to be true QTL, but verification is needed to confirm this result.

The work described in this dissertation also contributed directly and indirectly to other projects within the programme. The high-throughput DNA isolation protocol can be applied in any future projects that need to analyse large numbers of samples. An improvement to the current protocol would be to add automated PCR steps into 384-well plate formats. The real-time PCR assay for hybrid verification can be applied in a future project where hybridisation between *H. midae* and *H. spadicea* is to be verified. The triploid verification protocol has already been used to identify triploid individuals within the current experimental population undergoing growth trials.

The multiplex setup has been used to create multiplex panels for parentage assignment and population diversity studies being done in other projects. These include the study of the genetic impact of escapees from farms on the genetic diversity of wild populations and sperm competition where males with superior reproductive qualities are identified in terms of number of offspring they contribute towards the next commercial generation. Some of the microsatellite loci described in this thesis is part of these multiplexes. The family specific multiplex design has also already been used to generate the data for linkage map construction, greatly saving on genotyping costs.

6.3) Challenges and Recommendations (QTL-Mapping)

An area identified for possible improvement is the collection of phenotypic data. The high number of individuals within a family makes phenotyping of traits very labour intensive. This problem was solved, but only for metric traits, when automated measuring and weighing equipment was acquired early 2010. Other traits may be more difficult to automate, but the volume of work in phenotyping is a part of research and the amounts of data collected should not be sacrificed just to save time. In this dissertation the continuous collection of phenotypic data was not possible due to high numbers of tags lost between planned measurement timeframes (originally every six months). Continuous phenotypic data collections are needed to verify the presence or absence of a trait for or within individuals or help in identifying time-dependant traits such as growth rate over a certain period of time. A reliable and cost-effective tagging method should therefore be pursued and implemented in future. In tests conducted thus far a resin-based adhesion method seems to be the most durable, but an identity tag must still be chosen. Currently radio

frequency identification tags are used, but these are incredibly expensive compared to other available tags such as bee-tags.

A challenge encountered during QTL-mapping was the unavailability of a framework linkage map; a preliminary linkage map only became available near the completion of this study. To compensate for this and to perform a more comprehensive analysis in terms of interval mapping, linkage groups were constructed using data generated during single marker regression analyses. Once the framework linkage map becomes available, it can be utilised for future QTL-mapping studies. For further fine-mapping of QTL, the marker density of linkage maps should however be increased. A recommendation in this regard would be to increase the number of families used, since a low number of markers was found to be informative in the full-sib abalone families used thus far (see Chapter 3 as an example; 43% and 64%). A further strategy to increase linkage map densities is the generation and incorporation of SNPs (Baranski *et al.*, 2008). The SNP frequencies thus far reported for *H. midae* range from one every 113bp - one every 185bp (Bester *et al.*, 2008; Rhode *et al.*, 2008; Rhode, 2010), suggesting that the incorporation of this marker type should greatly increase map density and coverage. SNP development is already underway within the research group.

Another problem to be aware of in future is family admixture. A number of families were either mixed together during grading on some of the farms or in some cases individuals from the commercial populations on the grow-out platforms were accidentally added to the baskets containing the genetic experimental animals. In pooling analysis the presence of DNA from unrelated animals will affect the results, especially if these individuals carry same sized alleles as the full-sibs. Such admixture will increase the costs of any studies relying on family structure, because individuals would need to be screened using molecular markers to infer their origin.

6.4) Future Directions in QTL Studies in *Haliotis midae*

The QTL identified in this study, which are associated with loci *Hmid*PS1.818 and *Hmid*PS1.1063 (in both families), need to be verified. Firstly, the marker density of the linkage group involved with the chromosomal region showing QTL association needs to be increased. If possible the target families should be phenotyped again and genotyped with

additional markers to see if the QTL are true or not. The same procedure holds true for the loci showing significance after single marker regression (*Hm*RS27 for Family 42A and *Hmid*PS1.551 for Family 7B). Interval mapping could then confirm (or not) the position of the potential QTL.

For future QTL-mapping studies multiple traits can be targeted, increasing the amount of information obtained from experimental families. When multiple traits are to be targeted the use of DNA pooling analysis should be reconsidered. The phenotypic extremes may not always consist of the same individuals, therefore necessitating the construction of a number of different DNA pools. Pooling analysis has a few sources of error including the technical difficulties and variation caused by DNA quantification, pool construction and differential amplification during PCR (Mariasegaram *et al.*, 2007). Even though pooling is a good initial screening tool and saves on costs, it could be labour intensive and complex when analysed. A suggested strategy would be to combine a high-density linkage map, a number of well phenotyped full-sib or half-sib families and individual genotypes of animals in these families. The loci used for the individual genotyping can be chosen by selecting markers covering each linkage group at regularly spaced intervals. This strategy would harness the power of interval mapping and even composite interval mapping when studying multiple traits. Information such as pleiotropic effects, genotype-environment interactions and the nature of the genetic correlations between traits can then be studied properly (Jiang and Zeng, 1995).

A final remark is relevant to the creation of additional genetic material and MAS. The full-sib families available for research are reaching maturity and new family structures should be designed such as inbred lines and back-cross designs. Such families will be very useful in establishing the QTL-marker phase by tracking the segregation of the marker with the QTL through generations (Soller and Medjugorac, 1999). This information will be useful for short-term MAS, until such a time that adequate genomic resources such as whole genome sequences and BAC libraries become available (Baranski, 2006), which will help in establishing population wide QTL-marker phases via haplotypes or candidate genes (Soller and Medjugorac, 1999).

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APPENDIX A

Microsatellite Loci Characterised by Myself (Ruhan Slabbert)

Table A.1: The following markers (total = 31) were developed by myself as part of the current dissertation (Section 2.2).

Locus	Accession Number
*Hm*AD102T	DQ785747
*Hm*G16D	DQ785744
*Hm*G46D	DQ785745
*Hm*G53T	DQ785746
*Hm*IF33M	DQ785769
*Hm*LCS1T	DQ825701
*Hm*LCS5M	DQ825705
*Hm*LCS7M	DQ825707
*Hm*LCS9M	DQ993214
*Hm*LCS18M	DQ993217
*Hm*LCS37M	DQ993229
*Hm*LCS47M	DQ993228
*Hm*LCS48M	DQ993227
*Hm*LCS55T	DQ993226
*Hm*LCS63T	DQ993223
*Hm*LCS67M	DQ993222
*Hm*LCS72M	DQ993220

*Hm*LCS73T	DQ993219
*Hm*RS27T	DQ785751
*Hm*RS36T	DQ785753
*Hm*RS37D	DQ785754
*Hm*RS38M	DQ785755
*Hm*RS54D	DQ785774
*Hm*RS61H	DQ785776
*Hm*RS62D	DQ785777
*Hm*RS80M	DQ785756
*Hm*RS83M	DQ785757
*Hm*RS88M	DQ785758
*Hm*RS90M	DQ785759
*Hm*RS117M	DQ785765
*Hm*RS129D	DQ785766

Table A.2: The following markers (total = 12) were developed by myself as part of the current dissertation (Section 2.3).

Locus	Accession Number
*Hm*LCS152M	GQ927139
*Hm*LCS34M	DQ993230
*Hm*R16T	GQ927138
*Hm*LCS71T	DQ993221
*Hm*LCS388M	GQ927140
*Hm*LCS58M	DQ993225
*Hm*LCS383T	GQ927141
*Hm*S104T	GQ927137
*Hm*2H6FT	GQ927136
*Hm*3A11FM	GQ927135
*Hm*LCS147T	GQ927134
*Hm*LCS175M	GQ927133

APPENDIX B

Multiplexes and Family Specific Multiplexes

Table B.1: Theoretical multiplexes set up during this study.

Multiplex #	Loci
Multiplex#1	*Hm*LCS9; *Hm*LCS47; *Hm*LCS48; *Hm*LCS63; *Hm*LCS67; *Hm*LCS72
Multiplex#2	*Hm*LCS7; *Hm*RS38; *Hm*RS80; *Hm*RS83; *Hm*RS88
Multiplex#3	*Hm*LCS5; *Hm*LCS18; *Hm*IF33; *Hm*LCS37; *Hm*RS90; *Hm*RS117
Multiplex#4	*Hm*LCS1; *Hm*G16; *Hm*RS27; *Hm*RS36; *Hm*RS37; *Hm*RS61; *Hm*AD102; *Hm*DL110
Multiplex#5	*Hm*DL34b; *Hm*G46; *Hm*G53; *Hm*RS62; *Hm*RS129; *Hm*DL151
Multiplex#6	*Hm*LCS55; *Hm*NR106; *Hm*NR185; *Hm*NR191; *Hm*NR258; *Hm*NR289
Multiplex#7	*Hm*DL34a; *Hm*RS54; *Hm*NS100; *Hm*DL123; *Hm*DL131
Multiplex#8	*Hm*DL50; *Hm*NR54; *Hm*NR120; *Hm*DL207; *Hm*DL214; *Hm*DL224
Multiplex#9	*Hm*NS6; *Hm*NS17b; *Hm*NR20; *Hm*NS56; *Hm*NR180
Multiplex#10	*Hm*NSS1; *Hm*NS14; *Hm*NS19; *Hm*NS31
Multiplex#11	*Hm*NS18; *Hm*NS58; *Hm*NR136; *Hm*NR281
Multiplex#12	*Hm*D14; *Hm*D33; *Hm*D36; *Hm*D44; *Hm*D55; *Hm*D59; *Hm*D60; *Hm*D61
Multiplex#13	*Hm*NS7; *Hm*LCS34; *Hm*NS59; *Hm*LCS152
Multiplex#14	*Hm*2H6FT; *Hm*NS9; *Hm*R16; *Hm*NSp31; *Hm*NSp42; *Hm*LCS58; *Hm*LCS71; *Hm*S104; *Hm*LCS383; *Hm*LCS388
Multiplex#15	*Hm*NSp6; *Hm*NS21; *Hm*NS32; *Hm*LCS147; *Hm*LCS175
Multiplex#16	*Hmid*PS1.38; *Hmid*PS1.42; *Hmid*PS1.95; *Hmid*PS1.138; *Hmid*PS1.147
Multiplex#17	*Hmid*PS1.195; *Hmid*PS1.332; *Hmid*PS1.469; *Hmid*PS1.1012; *Hmid*PS1.1036; *Hmid*PS1.1038; *Hmid*PS1.1066

Multiplex#18	*Hmid*PS1.208; *Hmid*PS1.227; *Hmid*PS1.484; *Hmid*PS1.487; *Hmid*PS1.551; *Hmid*PS1.711; *Hmid*PS1.807; *Hmid*PS1.811
Multiplex#19	*Hmid*PS1.124; *Hmid*PS1.150; *Hmid*PS1.155; *Hmid*PS1.197; *Hmid*PS1.860
Multiplex#20	*Hmid*PS1.171; *Hmid*PS1.179; *Hmid*PS1.188; *Hmid*PS1.193
Multiplex#21	*Hmid*PS1.206; *Hmid*PS1.207; *Hmid*PS1.222; *Hmid*PS1.228
Multiplex#22	*Hmid*PS1.324; *Hmid*PS1.331; *Hmid*PS1.353; *Hmid*PS1.355
Multiplex#23	*Hmid*PS1.370; *Hmid*PS1.375; *Hmid*PS1.379; *Hmid*PS1.382
Multiplex#24	*Hmid*PS1.398; *Hmid*PS1.405; *Hmid*PS1.433; *Hmid*PS1.457
Multiplex#25	*Hmid*PS1.549; *Hmid*PS1.559; *Hmid*PS1.1009; *Hmid*PS1.1018
Multiplex#26	*Hmid*PS1.160; *Hmid*PS1.1026; *Hmid*PS1.1058; *Hmid*PS1.1063
Multiplex#27	*Hmid*PS1.305; *Hmid*PS1.374; *Hmid*PS1.521; *Hmid*PS1.561
Multiplex#28	*Hmid*PS1.629; *Hmid*PS1.635; *Hmid*PS1.638; *Hmid*PS1.678
Multiplex#29	*Hmid*PS1.805; *Hmid*PS1.831; *Hmid*PS1.840; *Hmid*PS1.844
Multiplex#30	*Hmid*PS1.692; *Hmid*PS1.728; *Hmid*PS1.768; *Hmid*PS1.792
Multiplex#31	*Hmid*PS1.859; *Hmid*PS1.870; *Hmid*PS1.873; *Hmid*PS1.890
Multiplex#32	*Hmid*PS1.895; *Hmid*PS1.961; *Hmid*PS1.967; *Hmid*PS1.972
Multiplex#33	*Hmid*PS1.9; *Hmid*PS1.156; *Hmid*PS1.247; *Hmid*PS1.981; *Hmid*PS1.982
Multiplex#34	*Hmid*PS1.254; *Hmid*PS1.490; *Hmid*PS1.588; *Hmid*PS1.789
Multiplex#35	*Hmid*PS1.818; *Hmid*PS1.868; *Hmid*PS1.874; *Hmid*PS1.906
Multiplex#36	*Hmid*PS1.952; *Hmid*PS1.1007

Table B.2: Family specific multiplexes used for linkage mapping in Section 2.3.

Multiplex #	Loci
LinkageMulti#1	*Hm*LCS1; *Hm*G16; *Hm*NS18; *Hm*NR20; *Hm*RS27; *Hm*D55; *Hm*NS56; *Hm*D59; *Hm*RS62; *Hm*LCS67; *Hm*AD102; *Hm*DL110; *Hm*NR136; *Hm*NR224; *Hm*NR281; *Hm*LCS383; *Hm*LCS388
LinkageMulti#2	*Hm*NS6; *Hm*D14; *Hm*LCS18; *Hm*NS19; *Hm*NS32; *Hm*D33; *Hm*DL34a; *Hm*RS36; *Hm*LCS37; *Hm*DL50; *Hm*NS100; *Hm*NR120; *Hm*DL123; *Hm*RS129; *Hm*DL131; *Hm*LCS152; *Hm*NR185
LinkageMulti#3	*Hmid*PS1.150; *Hmid*PS1.247; *Hmid*PS1.805; *Hmid*PS1.807; *Hmid*PS1.860; *Hmid*PS1.874; *Hmid*PS1.961; *Hmid*PS1.1009; *Hmid*PS1.1012; *Hmid*PS1.1058
LinkageMulti#4	*Hmid*PS1.147; *Hmid*PS1.188; *Hmid*PS1.208; *Hmid*PS1.332; *Hmid*PS1.551; *Hmid*PS1.638; *Hmid*PS1.870; *Hmid*PS1.967; *Hmid*PS1.1063
LinkageMulti#5	*Hmid*PS1.193; *Hmid*PS1.206; *Hmid*PS1.305; *Hmid*PS1.379; *Hmid*PS1.405; *Hmid*PS1.1038
LinkageMulti#6	*Hmid*PS1.38; *Hmid*PS1.382; *Hmid*PS1.487; *Hmid*PS1.549; *Hmid*PS1.559; *Hmid*PS1.588; *Hmid*PS1.711; *Hmid*PS1.859; *Hmid*PS1.1018
LinkageMulti#7	*Hmid*PS1.370; *Hmid*PS1.374; *Hmid*PS1.457; *Hmid*PS1.484; *Hmid*PS1.768; *Hmid*PS1.818; *Hmid*PS1.873; *Hmid*PS1.981; *Hmid*PS1.1026

Table B.3: Family specific multiplexes used for single marker regression analysis in Chapter 3.

Multiplex #	Loci
MultiFam42A	*Hm*NR20; *Hm*RS27; *Hm*D55; *Hmid*PS1.247; *Hmid*PS1.433; *Hmid*PS1.818; *Hmid*PS1.860; *Hmid*PS1.972; *Hmid*PS1.1063; *Hmid*PS1.1066
MultiFam7B	*Hm*D55; *Hm*D59; *Hm*RS62; *Hm*NR120; *Hmid*PS1.179; *Hmid*PS1.551; *Hmid*PS1.860; *Hmid*PS1.981; *Hmid*PS1.1009; *Hmid*PS1.1012; *Hmid*PS1.1058; *Hmid*PS1.1063

APPENDIX C

Articles Published (Chronological)

Slabbert, R., Roodt-Wilding, R., 2006. Non-destructive sampling of juvenile abalone using epipodial tentacles and mucus: method and application. African Journal of Marine Science 28:719-721.

Slabbert, R., Ruivo, N.R., Van den Berg, N.C., Lizamore, D.L., Roodt-Wilding, R., 2008. Isolation and characterisation of 63 microsatellite loci for the abalone, *Haliotis midae*. Journal of the World Aquaculture Society 39:429-435.

Slabbert, R., Hepple, J., Venter, A., Nel, S., Swart, L., Van den Berg, N.C., Roodt-Wilding, R., 2010. Isolation and segregation of 44 microsatellite loci in the South African abalone *Haliotis midae* L. Animal Genetics 41:332-333.

Slabbert, R., Prins, N., Brink, D., 2010. A microsatellite panel for triploid verification in the abalone, *Haliotis midae*. African Journal of Marine Science *in press*.

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Short Communication

Non-destructive sampling of juvenile abalone using epipodial tentacles and mucus: method and application

R Slabbert* and R Roodt-Wilding

Department of Genetics, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa
** Corresponding author, e-mail: rslabbrt@sun.ac.za*

Good-quality biological material is needed to obtain intact deoxyribonucleic acid (DNA) for use in molecular techniques such as the polymerase chain reaction (PCR). Non-destructive sampling protocols of juvenile abalone *Haliotis midae* (7–15 months old) were tested in order to collect material for DNA extraction. DNA was successfully extracted from epipodial tentacles and mucus samples. PCR results confirmed the good quality of the DNA and the reliability of the method.

Keywords: abalone, DNA extraction, DNA quality, epipodial tentacles, mucus, non-destructive sampling, PCR

Introduction

Deoxyribonucleic acid (DNA) quality is important for applications such as polymerase chain reactions (PCR), endonuclease restriction enzyme digests, Southern blot analysis, genomic library construction and DNA sequencing. The first step in these molecular genetic studies is DNA extraction. For abalone, the quality of DNA obtained is dependent on the biological material used for the extraction. The quality of DNA can be tested by agarose gel electrophoresis. Degraded DNA is characterised as a smear whereas intact DNA is usually viewed as a single band (Sarsfield *et al*. 2000).

Non-destructive sampling is desirable for, among others, endangered species (e.g. Wasko *et al*. 2003) or for organisms that are needed for future studies, such as those in breeding programmes (Chaline *et al*. 2004). Withler *et al*. (2001) reported non-destructive sampling of epipodial tentacles in adult abalone *Haliotis kamtschatkana* and Maynard *et al*. (2004) reported the sampling of such tentacles from *H. laevigata* exceeding 100mm in length. Li *et al*. (2004) sampled the hemolymph of wild *H. discus hannai* and Elliott *et al*. (2002) used mucus as a sampling medium for *Haliotis* sp. Sweijd *et al*. (1998) extracted DNA from meat from canned *Haliotis* sp. for forensic analysis. However, such studies on juvenile abalone have been mostly done on dead specimens (e.g. Evans *et al*. 2004a, Li *et al*. 2004, Gutierrez-Gonzales and Perez-Enriquez 2005) by dissecting the animal for muscle or gill tissue.

This study reports on the extraction of DNA from epipodial tentacles and mucus from live juvenile abalone *H. midae*. The DNA quality and application of these samples for PCR and genotype analysis were also determined.

Material and Methods

Epipodial tentacles and mucus from 12 juvenile abalone (7–15 months old; 19–35.8mm long and weighing 1.08–7.11g) were collected from the Irvin and Johnson abalone hatchery at Danger Point near Gansbaai, South Africa. The animals were placed upside down in a Petri dish containing water in such a way that their tentacles extruded. The tentacles were cut using dissection scissors, and were stored in 99.9% ethanol until the DNA extractions. Mucus samples were taken by putting the animal on sterile Whatman No. 1 filter paper and allowing the animal to deposit mucus. The mucus was allowed to dry and the filter paper was sealed and stored at 4°C. Each animal was placed in a separate tank to assess survival after sampling.

DNA extractions

DNA extractions were performed according to the CTAB-method described by Saghai Maroof *et al*. (1984), using 300µl of extraction buffer per epipodial tentacle sample (one tentacle) and 500µl per mucus sample (1cm^2 piece of mucus-containing filter paper).

DNA quality was tested using agarose (0.7%) gel electrophoresis. Microsatellite DNA markers were used to assess the applicability of the DNA with regards to PCR and genotyping. Undiluted DNA was used for PCR reactions. Two highly polymorphic DNA markers, HmD55 and HmD59 (Bester *et al*. 2004), were used for genotyping. A 10µl multiplex PCR reaction (1x Flexi Buffer [*Promega*], 0.8mM $MgCl_2$, 0.5mM of each dNTP, 1pmol of each primer, 0.25U *GoTaq*® [*Promega*]) was performed using a touch-down protocol. An initial denaturising step of 94°C for 5min was

followed by 2 cycles of 94°C for 30s, 65°C for 30s and 72°C for 30s. Thereafter, the annealing temperature was lowered by 1°C in every two consecutive cycles, until an annealing temperature of 55°C was reached and maintained for 30 cycles of 94°C for 1min, 55°C for 1min, 72°C for 1min, with a final elongation step at 72°C for 7min.

Results and Discussion

Non-destructive methods of sampling juvenile abalone were successful. All 12 juvenile abalone survived for the two-month study. DNA extraction from epipodial tissue and mucus was also successful. The agarose gel electrophoresis results for DNA quality are shown in Figure 1. All DNA samples are shown as single bands, indicating good-quality DNA. Some protein impurities can be seen in the mucus samples of animals 11 and 12. Mucus sample extractions tended to have more impurities than epipodial samples. The DNA was also successfully used in PCR analysis by means of polymorphic microsatellite markers. The PCR products for both sample types within an individual abalone sample amplified identical alleles (Figure 2). The DNA obtained can therefore be successfully used in PCR and genotyping analysis. Unlike the method used in the Elliott *et al*. (2002) study, the mucus samples in this study were dried before DNA extraction and the DNA was not diluted before PCR reactions.

Non-destructive sampling methods are cost effective for commercial abalone farms, especially for older animals because they are not harmed or killed. This method is also useful for research purposes, because a large number of commercial animals can be sampled for genetic population or parentage studies. Further, non-destructive sampling methods could be used effectively for identifying genetically superior abalone at an early age, after the development of technologies to enable marker-assisted selection, for use in future selective breeding programmes. This sampling method will also have application in broodstock and offspring monitoring, with the ultimate goal of re-introduction of abalone into the wild. This will become an important alternative in view of the rapid decline in natural abalone stocks in South Africa (Tarr *et al*. 1996). Monitoring will enable selection of a highly heterozygous reseeding stock, resulting in offspring with increased heterosis, thus increasing the fitness of the offspring or masking the effects of deleterious alleles (Ingvarsson and Whitlock 2000, Whitlock *et al*. 2000). However, the introduction of foreign alleles may also lead to outbreeding depression. In view of the significant genetic differentiation between West and East Coast populations of wild *H. midae* (Evans *et al*. 2004b), it is important to select reseeding stock that is not too divergent from the local population, so limiting the number of foreign alleles that are introduced. Non-lethal sampling can also be used in transgenic studies on abalone. The

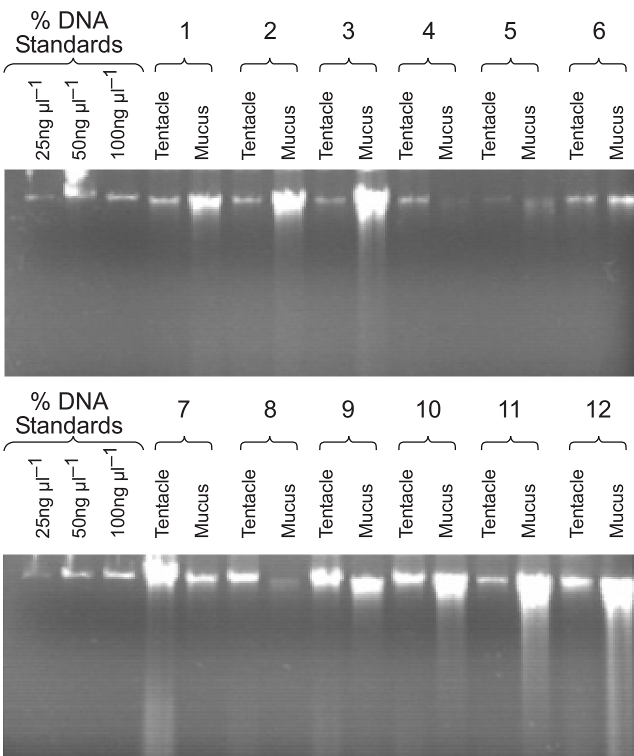

Figure 1: Results of agarose gel electrophoresis (0.7%), showing the DNA concentrations and quality of tentacles and mucus samples from the 12 juvenile abalone under study

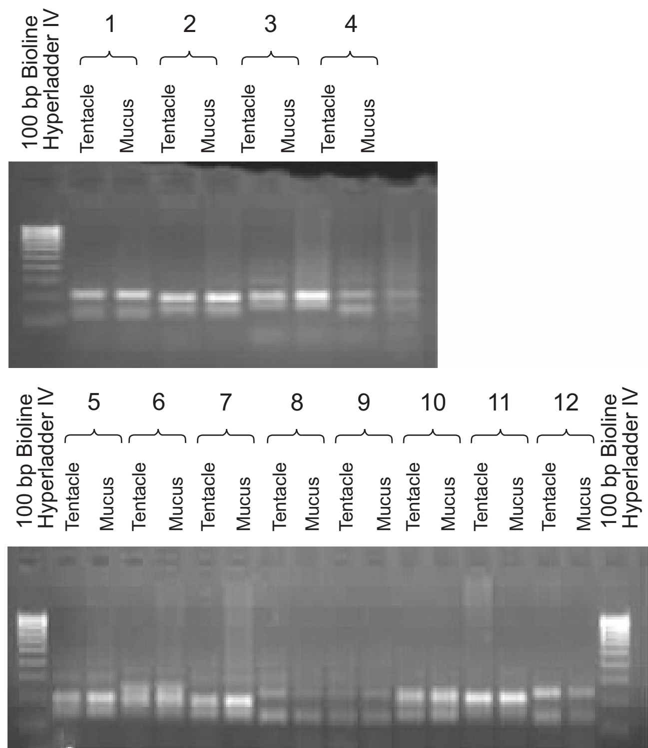

Figure 2: Results of PCR reactions for tentacles and mucus samples from the 12 juvenile abalone under study, using HmD55 and HmD59

current method of collecting and processing mucus samples can be applied in forensic science, where mucus is found on clothes or equipment of alleged poachers. This would ensure reliable molecular identification of *H. midae* trace elements (Sweijd *et al*. 1998).

In summary, non-destructive sampling protocols allow for the collection of ample tissue to isolate DNA and perform molecular studies of abalone, which are beneficial to both abalone farmers and researchers alike.

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Isolation and Characterization of 63 Microsatellite Loci for the Abalone, *Haliotis midae*

RUHAN SLABBERT[1], NICOLA R. RUIVO, NICOL C. VAN DEN BERG,
DARRELL L. LIZAMORE, AND ROUVAY ROODT-WILDING

Department of Genetics, Stellenbosch University, JC Smuts Building, Private Bag X1, Matieland 7602 South Africa

Haliotis midae is an important species within the fisheries industry of South Africa, with an output of almost 750 tonnes in 2005 (Loubser 2005). *H. midae* has been cultured in captive conditions since 1981 (Genade et al. 1985, 1988), but the first real effort to establish commercial abalone farms was made in 1990 by industrial and academic institutions (Sales and Britz 2001). The species is as yet undomesticated, and few breeding programs have been undertaken. Genetic characterization could play a large role in further development of the resource. Currently, molecular work done on *H. midae* has been limited to population structure studies and some parentage analyses. To assist the industry in genetic improvement programs to enhance their stocks, more molecular markers are required. These will be used to construct the first linkage map for this species, identify quantitative trait loci (QTLs), and perform accurate parentage assignments. To date, 11 polymorphic microsatellite loci have been reported for *H. midae* (Bester et al. 2004). Here, we report the characterization of a further 63 microsatellite loci that will facilitate future molecular studies and breeding programs.

Materials and Methods

DNA Extractions

Genomic DNA was isolated from mantle tissue following a standard N-cetyl-N,N,N-trimethyl ammonium bromide (CTAB) extraction method (Saghai Maroof et al. 1984). Tissue was homogenized in 500 μL of CTAB lyses buffer containing 0.5 mg/mL proteinase K and incubated at 60 C. Following chloroform : isoamyl alcohol (24:1) extractions, the supernatants were precipitated with two-third volume of 100% cold isopropanol. DNA was washed with 200 μL of 70% ethanol, redissolved in 100 μL of distilled water, and stored at −20 C.

Microsatellite Enrichments

In this study, microsatellite repeat sequences were isolated using an enrichment technique (FIASCO) (Zane et al. 2002). Enriched partial genomic libraries were constructed using DNA from four individuals. For this, 250 ng DNA was simultaneously digested with *Mse*I and ligated to *Mse*I amplified fragment length polymorphism (AFLP) adaptors. DNA was selectively amplified using a mixture of four adaptor-specific primers (*Mse*I-N) and hybridized independently with a biotinylated $(AC)_{12}$, a $(GATC)_6$, a $(CAA)_8$, and a $(GTGC)_6$ probe. Repeat-containing fragments were recovered by streptavidin magnetic particles and cloned into a Qiagen p-Drive vector (Qiagen, Cape Town, South Africa) in order to produce a highly enriched microsatellite library.

Clones were sequenced using an ABI 3100 Automated Sequencer to verify the presence of repeat motifs. Primer sets were designed using Oligo version 4.1 (Rychlik and Rhoads 1989). All primer information was submitted to GenBank (Table 1).

Genotyping

A total of 32 individuals from Black Rock, on the east coast of South Africa, were genotyped to test the level of polymorphism of the markers. For each primer pair, one of the primers was labeled with FAM, NED, VIC, or PET dyes.

[1] Corresponding author.

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TABLE 1. *Primer sequences and characteristics of 63* Haliotis midae *microsatellite loci.*

Locus	Repeat sequence	Primer sequence (5′–3′)	n	Size range (bp)	T_a (P^a)	H_O	H_E	n_a	P	Null	Accession number
HmAD102T	$(ACTC)_{15}$	**F** ACATTGGGGTTCTCAATCA **R** TAACGGGACAATGAATAAACTA	28	199–263	TD (5)	0.32	0.93	16	***	Y	DQ785747
HmDL34aR	$(ATC)_{13}$	**F** TGCAAGTCCAGAGTATGTGG **R** TGTGCTTGAGAGAGATGGTG	25	230–320	60 C (4)	0.56	0.94	20	***	Y	EF054860
HmDL34bT	$(CAGA)_{16}$	**F** CATCACCATCTCTCTCAA **R** TAAATCACATAATCATGAACCTG	32	104–166	TD (5)	0.84	0.94	16	***	N	EF054860
HmDL50M	$(TGTC)_{11}(GGTC)_6$	**F** TTATTGCGCCATACAGTTCG **R** TCAGGCAGACAGACATACCG	25	202–280	60 C (4)	0.80	0.93	18	0.022	Y	EF054861
HmDL110T	$(TCAC)_{23}$	**F** TCCTAAAAGCTGCATAACACCA **R** TGAAGGGGATAAAACCAGGA	32	138–278	55 C (4)	0.75	0.92	17	0.027	Y	EF054864
HmDL123D	$(CT)_{20}$	**F** ATCCCCTAAATCTGCGTCAA **R** CGCCTGTAAAATGCACAAGA	27	266–374	60 C (4)	0.22	0.95	20	***	Y	EF054865
HmDL131M	$(AC)_{14}(TC)_8$	**F** GTGACAGAAGGTGGAAGTGGA **R** TTACGACAAGCATGGGTTACTG	22	195–277	55 C (4)	0.50	0.73	10	0.027	Y	EF054867
HmDL151T	$(CTGA)_{14}$	**F** AATAAGCCAAAACACGGAGCA **R** GGGTTCGATTCCCCACAA	30	156–234	55 C (4)	0.53	0.54	8	0.675	N	EF054868
HmDL207M	$(CT)_7(TG)(CT)_5(TT)(CT)_6(CA)_6$	**F** CATACACACGCATTCACATACA **R** CATCAACAGGTCCAAGGAAG	25	211–283	55 C (4)	0.80	0.90	15	0.279	N	EF054869
HmDL214T	$(TGAG)_{15}$	**F** CCTGCATCCATTTAGCTCTGAT **R** GTTGTGCTGGATTGGGATGT	32	199–249	60 C (4)	0.28	0.31	5	0.126	N	EF054871
HmG16D	$(GT)_{13}$	**F** ATATTGCTGAATGAGGGGTA **R** CACCACCACTACCACCATAC	32	285–321	TD (5)	0.94	0.88	9	0.066	N	DQ785744
HmG46D	$(TG)_{17}$	**F** GATGAGTCCTGAGTAAGTAAATAAT **R** ATCCGTGTACACACTCACTG	30	221–237	TD (5)	0.60	0.60	6	0.755	N	DQ785745
HmG53T	$(CACT)_{31}$	**F** TGCTGTTGAAGTCTTTGTCC **R** TATCAGTCCCGCATCTATTG	25	104–200	TD (5)	0.32	0.96	20	***	Y	DQ785746
HmIF33M	$(GATA)_3(GACA)_{30}$	**F** ATGGATAGCTAGCGAGATATAGA **R** TAGTGATTTTACGGAAACGG	30	104–310	TD (5)	0.23	0.85	17	***	Y	DQ785769
HmLCS1T	$(CGTG)_6$	**F** TTGAAAAACACAGGAAATGC **R** AGTAAAGGTTGTTTCGTGAAAG	27	154–174	TD (5)	0.67	0.63	9	0.895	N	DQ825701
HmLCS5M	$(GCTC)_4(ACTC)_3$	**F** AACTCAATCCCATCTATGGC **R** CTTTGACCACTAGGCTACCC	24	472–482	TD (5)	0.00	0.08	2	0.006	N	DQ825705
HmLCS7M	$(GT)_7(GCGT)_6(GT)_7$	**F** ATGATGCTATTCAGCTCTCG **R** ATGATGAAAGTGGCGTAAAA	29	196–270	TD (5)	0.69	0.89	16	***	Y	DQ825707

TABLE 1. *Continued*

Locus	Repeat sequence	Primer sequence (5′–3′)	n	Size range (bp)	T_a (P^a)	H_O	H_E	n_a	P	Null	Accession number
HmLCS9M	$(GC)_2(GT)_2(GCGTGT)_2(GCGT)_2(GC)$	**F** TTGGCATAGGATGGACTTGT **R** GATGCGGCCACAGGC	30	281–299	TD (5)	0.27	0.51	4	0.001	Y	DQ993214
HmLCS18M	$(CAC)_2(GAC)(TAC)_3(CAC)_4(TAC)_3(CACGAC)(TAC)_{12}(CAC)_3(TAC)_4(AAC)(TAC)(CAC)_2(TAC)_4(CAC)_2(TAC)_4(CAC)$	**F** CGGTGATAACGATAGTTGGT **R** GGTAGTTGCAGTAATGGTATTC	26	144–266	55 C (1)	0.81	0.91	19	0.083	N	DQ993217
HmLCS37M	$(GA)_{13}(CA)(GA)_8(CA)(GA)_4(CAGA)_9(CA)_5$	**F** ACCTTGAGGTCCTGTCAGTC **R** AAGTATTCCAGAAACGCTTCT	21	194–376	55 C (1)	0.76	0.96	23	***	Y	DQ993229
HmLCS47M	$(AC)_6(AG)(AC)_{15}$	**F** CAAAACAAAAAAACAACAAC **R** ATCACGTATTGATTGATTCTAT	32	172–208	TD (5)	0.78	0.85	13	0.044	N	DQ993228
HmLCS48M	$(CT)_{14}(CA)_9$	**F** ATGTGTGAGCACGTGTTTCT **R** AGTCACAAGCTACATCGAATCT	21	334–352	TD (5)	0.52	0.55	9	0.466	N	DQ993227
HmLCS55T	$(GTGA)_5$	**F** ATTGTTGATAATGGCATTGG **R** TTCATTACACGTCTAAATCCAA	32	268–286	TD (5)	0.25	0.43	7	0.006	Y	DQ993226
HmLCS63T	$(CACT)_4$	**F** AGTCTTCCTCCAGTTCTCCA **R** AGCAAACATACGTGACTTGG	26	208–230	TD (5)	0.04	0.54	4	***	Y	DQ993223
HmLCS67M	$(GAGT)_3(GT)_5(GC)_4$	**F** ATGGCGGAGGATATAATGAT **R** GAAGCCTATTTCTGGTGTCC	31	258–296	TD (5)	0.45	0.73	8	***	Y	DQ993222
HmLCS72M	$(TG)_5(CGTG)_2$	**F** TGTGACAGGAAAGCCTAAAG **R** GTGATAGAGGGAGAAAGTATGG	28	261–281	TD (5)	0.25	0.76	7	***	Y	DQ993220
HmLCS73T	$(GAGT)_{10}$	**F** CCATGGCTCAGAATATTGAA **R** CATGTTGGAGATCTGGTTTG	26	151–245	TD (5)	0.31	0.76	15	***	Y	DQ993219
HmNR20M	$(TCC)_5(TAC)_7$	**F** CTACAACAAACGCCGATG **R** TGCAGTAATAGGGGTACCAG	27	187–289	60 C (2)	0.85	0.85	11	0.716	N	EF063097
HmNR54H	$(TTAGGG)_4$	**F** TAACACTAAGTCCCTCACCC **R** CATTCTACATTCGACATTCG	32	329–407	60 C (2)	0.78	0.76	10	0.217	N	EF063103
HmNR106D	$(TG)_{15}$	**F** TCCTTGGCCAGAATAACC **R** TATATGGTCTGCATCGCTG	31	329–389	60 C (2)	0.81	0.88	16	0.076	N	DQ825709
HmNR120T	$(TGAG)_{23}$	**F** TTGAGCATGAGTCGTTGAGC **R** ACCTGCTCTTTAGCTCAGATGG	29	235–347	62 C (2)	0.90	0.94	24	0.197	N	EF121745
HmNR136D	$(CA)_{11}$	**F** GAGTAATATGGGCACCTCG **R** GTTTGGAATGTCTGATTGGA	32	211–309	60 C (2)	0.75	0.80	20	0.156	N	DQ825710
HmNR185D	$(GT)_{13}$	**F** TAGAGTTCATGTGTGTACGTGTGC **R** TACCTGTAACGCGCTTGCT	31	132–160	65 C (2)	0.61	0.88	11	0.010	Y	EF121750

Table 1. *Continued*

Locus	Repeat sequence	Primer sequence (5′–3′)	n	Size range (bp)	T_a (P^a)	H_O	H_E	n_a	P	Null	Accession number
HmNR191T	$(GAGT)_6$	**F** CCACATGGGTACAAAGTCC **R** TTAGTTTTACGCCGCACTC	31	241–497	62 C (2)	0.81	0.86	16	0.136	N	EF121752
HmNR180D	$(GT)_{24}$	**F** ACAAGGAGGCGTGAAATCTGC **R** GCATTGTTACCCCCTACAAAGACC	31	269–297	65 C (2)	0.52	0.91	12	***	Y	EF121748
HmNR224T	$(CATA)_{18}$	**F** TGTCCATAGCAGCCCCTTAC **R** ACATCTTGTTGCCGTTGTTG	22	444–540	65 C (2)	0.95	0.95	20	0.529	N	EF512269
HmNR258R	$(CAA)_{11}$	**F** GCATCGCCTGATTTGATTC **R** CAGAAGGGTGGGTTGTAGTATG	32	239–257	62 C (2)	0.72	0.76	6	0.627	N	EF512272
HmNR281P	$(CTCAA)_{24}$	**F** AACCTTCAGTAACCCATGC **R** TGAATAGGCACCATAAAGGG	28	225–375	60 C (2)	0.71	0.92	21	0.026	Y	EF512274
HmNR289P	$(GTTGT)_5$	**F** GCAAGACAGACATCCAAGAC **R** TACAAATCCCGACACAAGAG	32	301–316	65 C (2)	0.25	0.26	4	0.487	N	EF512275
HmNS6T	$(ACGC)_6$	**F** TGAGAGACATTTGAAGCATTTA **R** AACACTCACGTACGCATACAC	32	186–230	55 C (3)	0.59	0.78	9	0.031	Y	EF367117
HmNST7T	$(CACT)_{26}$	**F** CACATGGGTACAATGTGTGAAG **R** GGTAGCACTGTTTCTCACGA	32	228–328	60 C (3)	0.94	0.95	21	0.565	N	EF455618
HmNS14R	$(TTG)_5$	**F** GCTCTGGTGTATGTTGTGTCA **R** TTGATCAAGTTGCACATGAAT	31	252–261	60 C (3)	0.03	0.21	4	***	Y	EF367115
HmNS19L	$(AACACCC)_9$	**F** ACAACAACAAAGGTGGTCAA **R** CAATGAATAGCTATGGGTCG	32	178–252	55 C (3)	0.56	0.95	25	***	Y	EF033330
HmNS28D	$(CA)_{16}$	**F** CAGTCAATTTTCATCGCATT **R** AGGTCGTTTTTCTCCTTCAG	32	123–185	55 C (3)	0.91	0.94	19	0.450	N	EF033332
HmNS31D	$(GT)_4(CT)(GT)_8$	**F** CTCGGGTTCAGTTACCTACA **R** CTTGCTGACTTCGATCACAC	32	238–288	55 C (3)	0.34	0.80	14	***	Y	EF033333
HmNSa34D	$(AC)_7$	**F** CATTCCACGCTGAAGAAATC **R** TGAGATGAGCGTGAAAATGT	31	185–189	50 C (3)	0.10	0.36	3	***	Y	EF367118
HmNS38T	$(TCAC)_{10}$	**F** CTGAGACCCAAAGTTTTCTTTA **R** ATCTATGTTCAGGGTGTCAGTG	31	402–474	55 C (3)	0.74	0.83	11	0.596	N	EF367113
HmNS56D	$(CA)_{20}$	**F** TTCGGCAAGTGAATGTCTAG **R** CCGAGTTTGGAATGTCTGAT	31	211–253	55 C (3)	0.84	0.85	16	0.528	N	EF455619
HmNS58D	$(GTT)_8$	**F** TGCCACTCAAATGTTCCTTA **R** CTATTTCAGGTGTCCCCAGT	32	233–272	60 C (3)	0.78	0.86	10	0.034	N	EF367119
HmNS100T	$(GAGT)_{16}$	**F** CAGTTTTTGTTAGGGATTTCAT **R** GAAAAAGACTGTTGATGGGG	32	232–454	60 C (3)	0.53	0.92	14	***	Y	EF367114

TABLE 1. *Continued*

Locus	Repeat sequence	Primer sequence (5′–3′)	n	Size range (bp)	T_a (P[a])	H_O	H_E	n_a	P	Null	Accession number
HmRS27T	$(TCAC)_{30}$	**F** TACCGGTATAAACCGAACAC **R** GTTCAGCAAGAAATCAGTCG	29	224–428	TD (5)	0.41	0.93	23	***	Y	DQ785751
HmRS36T	$(CTCA)_7$	**F** TCAACTCACTCAACCAACCA **R** TAGTCTATGTTGCGGTCTGC	25	348–366	TD (5)	0.52	0.67	6	0.398	N	DQ785753
HmRS37D	$(AC)_{15}$	**F** AACTTTCAGGACGAAAGGG **R** ATATGTTAGATGTGCGGCAA	29	335–353	TD (5)	0.55	0.69	7	0.001	N	DQ785754
HmRS38M	$(GT)_{14}(GA)_9$	**F** ATCAAGATATCTCCCAAGGG **R** CACACATACACACAAACACACA	31	229–269	TD (5)	0.58	0.89	13	***	Y	DQ785755
HmRS54D	$(AC)_{13}$	**F** TTTGTGAAATAGCATGGAGC **R** TGTAAATAATCGAGCCTGGA	31	224–236	TD (5)	0.84	0.59	4	0.022	N	DQ785774
HmRS61H	$(GAGATA)_3$	**F** GGTTTACTCAGGGTTTAGGG **R** AAATTTTGGGGAGTTTACAAC	32	507–549	TD (5)	0.91	0.86	12	0.017	N	DQ785776
HmRS62D	$(GT)_{12}$	**F** ATCCACTTTGACTTGTTTATTTG **R** GTGTGTACTGATGTTCTGCCA	26	262–300	TD (5)	0.42	0.86	14	***	Y	DQ785777
HmRS80M	$(GAGT)_{17}(GA)_3(GAGT)$	**F** AATGGTTCTTTTGATCCCTT **R** TCATTATAACATCTGGCCTTG	31	178–240	TD (5)	0.71	0.92	18	0.005	Y	DQ785756
HmRS83M	$(GTTT)_2(GT)_{34}(TTTG)_6$	**F** TGACTCTCAGTTTCACATCCA **R** ATATGTCACATATCACAAATGCA	31	192–362	TD (5)	0.65	0.96	27	***	Y	DQ785757
HmRS88M	$(GT)_{10}(GCGT)_2(GT)(GCGT)(GT)_2$	**F** TCAGAATATTGCACCCAAAC **R** CATGAACCATCAATACTGCC	30	311–349	TD (5)	0.80	0.87	12	0.282	N	DQ785758
HmRS90M	$(GT)(GTGC)_2(GT)_4(GTGC)$ $(GT)(GTGC)$	**F** ATTTGATACCTTGTCTCGCTT **R** TGAGATCGAAAATCCCACTAT	31	434–458	TD (5)	0.23	0.54	4	***	Y	DQ785759
HmRS117M	$(GAGT)_{33}(GCGT)_3$	**F** GAGCACACGAATACCAAGAG **R** AATTCAACCCCTCCTCACT	30	171–307	TD (5)	0.43	0.91	22	***	Y	DQ785765
HmRS129D	$(GT)_{15}$	**F** TTGAATCTGACTGAACTGGG **R** TATAAGCCACATTCTGAGGAA	29	251–295	TD (5)	0.52	0.91	16	***	Y	DQ785766

n = sample size; T_a = optimal annealing temperature; TD = touchdown polymerase chain reaction; H_O = observed heterozygosity; H_E = expected heterozygosity; n_a = number of alleles; Y = null alleles present; N = null alleles not present.

[a] Polymerase chain reaction program.

*** $P < 0.0001$ (for Hardy-Weinberg Equilibrium).

All polymerase chain reactions (PCRs) were conducted in a Geneamp 2700 thermal cycler (Applied Biosystems, Cape Town, South Africa) in 10 μL reactions containing 20 ng DNA, 0.2 μM of each primer, 200 μM deoxyribonucleotide triphosphates (dNTPs), 0.1 unit of GoTaq polymerase (Promega, Cape Town, South Africa), 1 × GoTaq Flexi Buffer (Promega), and 2 mM $MgCl_2$. Various PCR programs were used. Program 1: initial denaturing step at 94 C for 5 min followed by 25 cycles of 30 sec at 94 C, 30 sec at 55 C, and 30 sec at 72 C, and a final extension for 7 min at 72 C. Program 2: initial denaturing step at 94 C for 5 min followed by 35 cycles of 45 sec at 94 C, 45 sec at 55–65 C, and 45 sec at 72 C, and a final extension for 7 min at 72 C. Program 3: initial denaturing step at 94 C for 5 min followed by 30 cycles of 30 sec at 94 C, 1 min at 55 –65 C, and 1 min at 72 C, and a final extension for 10 min at 72 C. Program 4: initial denaturing step at 94 C for 5 min followed by 25 cycles of 45 sec at 94 C, 45 sec at 55 C, and 45 sec at 72 C, and a final extension for 10 min at 72 C. Program 5 (touchdown): an initial denaturing step of 94 C for 5 min followed by two cycles of 30 sec at 94 C, 30 sec at 65 C, and 30 sec at 72 C. Thereafter, the annealing temperature was lowered by 1 C in consecutive cycles, until an annealing temperature of 55 C was reached and maintained for 30 cycles for 1 min at 94 C, 1 min at 55 C, and 1 min at 72 C, with a final extension for 7 min at 72 C. PCR products were separated on an ABI 3100 Automated Sequencer and analyzed using the GeneMapper software program (Applied Biosystems).

Statistical Analyses

Observed and expected heterozygosities, null allele frequencies, and the probability of Hardy–Weinberg equilibrium were calculated using CERVUS version 3.0.3 (Kalinowski et al. 2007), GENEPOP version 4 (Raymond and Rousset 1995), and MICRO-CHECKER version 2.2.3 (Van Oosterhout et al. 2004). Corrections of the significance levels for multiple tests were performed following the sequential Bonferroni procedure (Rice 1989).

Results and Discussion

A total of 983 recombinant clones were sequenced of which 47% contained repeat motifs. A total of 192 primer pairs were designed and 63 were found to amplify polymorphic loci. The PCR primer sequences, optimal annealing temperature, repeat motif, and allele size ranges are shown in Table 1.

Loci showed an average of 13.1 (range 2–27) alleles per locus and mean observed and expected heterozygosities of 0.573 (range 0–0.955) and 0.775 (range 0.082–0.958), respectively. A total of 25 loci did not conform to Hardy–Weinberg expectations (Table 1). This could be caused by null alleles (24 of these loci exhibited null alleles) but can also be attributed to other factors such as nonrandom sampling. Null alleles, caused by homozygote excess, were detected in a total of 33 loci (Table 1).

The high levels of polymorphism and heterozygosity exhibited at these loci provide an invaluable tool for population and kinship studies. The reported markers are currently being applied in parentage as well as QTL studies. The new markers should also be very useful in future mapping studies and molecular breeding programs for *H. midae*.

Acknowledgments

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Isolation and segregation of 44 microsatellite loci in the South African abalone *Haliotis midae* L.

R. Slabbert, J. Hepple, A. Venter, S. Nel, L. Swart, N. C. van den Berg and R. Roodt-Wilding

Molecular Aquatic Research Group, Department of Genetics, Stellenbosch University, Stellenbosch, 7600, South Africa

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Source/description: Microsatellite loci were isolated from *Haliotis midae* genomic DNA using either the FIASCO method[1] or the SNX Unilinker method.[2] The FIASCO method was modified to accommodate additional restriction enzymes, namely MseI, EcoRI and MspI (New England Biolabs). New adaptor and primer sequences were designed for EcoRI and MspI (Table S1). AluI and RsaI were used for the SNX method. The DNA libraries were enriched using an AC-rich probe and cloned using the QIAGEN PCR Cloning kit. Sequencing was performed on an ABI 3730 × l DNA analyser. Sequences were edited using SEQUENCE SCANNER version 1 (Applied Biosystems). Primers were designed for repeat containing sequences using BATCHPRIMER version 1[3] and labelled using fluorescent dyes.

PCR conditions: PCR reactions were carried out in a final volume of 10 μl containing 20 ng DNA, 0.2 μM of each primer, 200 μM deoxyribonucleotide triphosphates (dNTPs), 0.1 unit of GoTaq polymerase (Promega), 1× GoTaq Clear Flexi Buffer (Promega) and 2 mM $MgCl_2$. PCR programs are shown with Table S2. Genotyping was performed using an ABI 3730 × l DNA analyser and GENEMAPPER version 4 (Applied Biosystems).

Isolation results: A total of 978 recombinant clones were sequenced, and 49% of these contained repeat motifs. A total of 222 primer pairs were designed of which 44 were found to be polymorphic (Table S2).

Polymorphism and segregation analysis: DNA from 32 individuals from each of two full-sib families (Family7B and Family42A) was extracted. The Mendelian segregation patterns (1:1:1:1, 1:2:1, 1:1) of the 44 markers were examined in these families using the chi-square test. Among a total of 88 marker-family combinations (44 × 2), 38 were informative, 18 combinations were monomorphic, eight combinations had three or more alleles and 24 combinations could not be reliably scored or amplified (Table S2). Of the 38 informative marker-family combinations, 10 did not conform to expected Mendelian segregation patterns ($P < 0.05$). Of these 10 combinations, three (*Hmid0006M* for Family42A and *Hmid2044T* and *HmLCS147* for Family7B) could be explained by the presence of null alleles. *Hmid0006M* and *HmLCS147T* conformed to Mendelian segregation after we corrected for the null alleles (Table S2). The distortion of the other combinations (*Hmid0053D*, *Hmid0310D*, *Hmid4018D*, *HmLCS71T* and *HmNSp31M* for Family42A and *Hmid0310D* and *Hmid0065M* for Family7B) could possibly be explained by PCR errors or scoring difficulties.[4] These markers should be used with caution.

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Correspondence: R. Slabbert (rslabbrt@sun.ac.za)

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Primer and adaptor sequences of EcoRI and MspI restriction enzymes which were used for the FIASCO method.

Table S2 Marker information, PCR conditions, segregation analyses and accession numbers of 44 novel microsatellite loci for *Haliotis midae*.

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Short Communication

A microsatellite panel for triploid verification in the abalone *Haliotis midae*

R Slabbert*, N Prins and D Brink

Aquaculture Division, Department of Genetics, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa
** Corresponding author, e-mail: rslabbrt@sun.ac.za*

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A method for ploidy verification of triploid and diploid *Haliotis midae* was developed using molecular microsatellite markers. In all, 30 microsatellite loci were tested in control populations. A final microsatellite multiplex consisting of seven markers were optimised and a complete protocol is reported. This protocol was successfully applied in identifying the ploidy levels of 42 ploidy-unknown abalone, showing the utility of microsatellite markers as tools for verifying the ploidy of individual abalone. The protocol can be applied in larger animals for which the isolation of nuclei and metaphase chromosomes from tissues is difficult.

Keywords: abalone, *Haliotis midae*, microsatellites, triploidy

Introduction

Five abalone species are found in South African coastal waters, but only one, *Haliotis midae*, is cultivated and exported. The farm production for 2008 for this species amounted to 870 t, fetching around US$38 per kg (W Barnes, Abalone Farmers Association of South Africa, pers. comm.). One strategy for increasing production output is triploid induction (Elliott 2002). Triploid abalone can be produced using various methods including pressure shock (e.g. Arai et al. 1986), thermal shock (e.g. Yang et al. 1998), 6-dimethylaminopurine treatment (e.g. Norris and Preston 2003), caffeine treatment (e.g. Okumura et al. 2007) and cytochalasin B treatment (e.g. Li et al. 2007). Triploidy was successfully induced in *H. midae* using either cytochalasin B (Stepto and Cook 1998) or hydrostatic pressure (De Beer 2004).

It is important to verify the ploidy level of the experimental population after induction. There are various methods for such verification, including flow cytometry, karyotyping, nucleolar organiser region analysis, and particle size analysis (Harrell et al. 1995). The use of flow cytometry has thus far been the preferred direct method for triploid verification in abalone (Stepto and Cook 1998, Norris and Preston 2003, De Beer 2004, Liu et al. 2004, Li et al. 2007, Okumura et al. 2007), because hundreds of samples can be analysed at any one time and the relative percentage of triploid induction can also be analysed for an experimental population (Harrell et al. 1995). Another direct method of triploidy verification is the use of polymorphic DNA markers such as microsatellites (Magoulas 1998). Triploidy in molluscs are induced by suppressing the formation of either the first or second polar body, resulting in an extra set of chromosomes from the female (Gérard et al. 1999). The usefulness of the molecular markers therefore relies on the heterozygosity of a locus within the female and on the allele inherited from a male having a different size than those of two female alleles. A large number of loci need to be tested to identify the combination of markers that can reliably identify triploid individuals. This became practical for *H. midae* after the development of 200 microsatellite markers for this species (Bester et al. 2004, Slabbert et al. 2008, 2010).

In this study, we tested 30 polymorphic microsatellite loci for their application in ploidy verification. The development of an accurate protocol for triploidy verification for *H. midae* using appropriate polymorphic microsatellite markers is described followed by its application in identifying diploid and induced triploid individuals within an experimental population.

Material and methods

Triploidy induction and sample preparation

Abalone were spawned under farming conditions (I&J Abalone) and triploidy was induced according to De Beer (2004). Tentacles were taken from eight triploid and eight diploid abalone to be used as control samples for the initial genotyping. DNA extractions were performed using the CTAB extraction protocol of Saghai-Maroof et al. (1984). A further 42 ploidy-unknown, three triploid and three diploid juvenile abalone were sampled by taking two tentacles. All tissue samples were stored in 100% ethanol for transportation to the laboratory.

Initial genotyping

The 16 control samples were genotyped with 30 microsatellite loci (Table 1). Polymerase chain reaction (PCR) amplification was performed according to the conditions described in Bester et al. (2004) and Slabbert et al. (2008, 2010). Fluorescent analysis was performed with a 3730xl DNA Analyzer (Applied Biosystems) and genotyping with GeneMapper version 4 (Applied Biosystems). An individual was classified as triploid if three alleles were detected for a locus. Markers for the triploidy verification multiplex were chosen based on amplification success, unambiguous identification between ploidy levels and the number of correct triploid individuals assigned; the cut-off being three out of eight control samples. Any loci showing four or more alleles were discarded from further analyses.

Statistical analysis

Population estimates such as number of alleles and observed and expected heterozygosities (Table 1) were taken from Bester et al. (2004) and Slabbert et al. (2008, 2010). A one-way analysis of variance (Statistica 8) was performed to ascertain if markers for ploidy verification should be chosen based on number of observed alleles, observed heterozygosity or expected heterozygosity. Loci that correctly identified (three alleles) three out of eight triploid controls were compared to loci that did not identify (two or less alleles) any triploid controls. Each parameter was tested separately.

Multiplex set-up and experimental triploidy verification protocol

The triploid verification multiplex reaction was optimised using the QIAGEN Multiplex Kit (QIAGEN). PCR reactions were performed using a 2720 Thermal Cycler (Applied Biosystems) in a final volume of 10 µl and contained 20 ng of genomic DNA, 1× QIAGEN Multiplex PCR Master Mix and 2 µl of the primer mix (4 µM each of HmD59 and HmNR106D and 2 µM each of HmNR20M, HmNR120T, HmNS19L, HmidPS1.305T, HmidPS1.818C and HmidPS1.870D). The PCR programme was as follows: an initial activation step was performed at 95 °C for 15 min followed by 35 cycles of 30 s at 94 °C, 90 s at 57 °C and 1 min at 72 °C, with a final extension step at 60 °C for 30 min. Fluorescent genotyping was done using a 3730xl DNA Analyzer (Applied Biosystems).

This optimised protocol was then applied to identify 42 ploidy-unknown samples, and three triploid and three diploid control samples, to verify their ploidy status. DNA was

Table 1: Microsatellite loci tested for suitability for triploidy verification. Bold loci indicate those used for triploid verification in this study

Locus	n_a	H_o	H_e	Account number	Group[d]
HmAD102T[b]	16	0.32	0.93	DQ785747	15
HmD55[a]	9	0.68	0.80	AY303337	Ungrp
HmD59[a]	15	0.78	0.84	AY303338	5
HmLCS7M[b]	16	0.69	0.89	DQ825707	Ungrp
HmLCS47M[b]	13	0.78	0.85	DQ993228	Ungrp
HmLCS48M[b]	9	0.52	0.55	DQ993227	Ungrp
HmLCS63T[b]	4	0.04	0.54	DQ993223	Ungrp
HmLCS67M[b]	8	0.45	0.73	DQ993222	Ungrp
HmNR20M[b]	11	0.85	0.85	EF063097	6
HmNR106D[b]	16	0.81	0.88	DQ825709	Ungrp
HmNR120T[b]	24	0.90	0.94	EF121745	4
HmNR224T[b]	20	0.95	0.95	EF512269	Ungrp
HmNR258R[b]	6	0.72	0.76	EF512272	Ungrp
HmNR281P[b]	21	0.71	0.92	EF512274	1
HmNS19L[b]	25	0.56	0.95	EF033330	2
HmNS58D[b]	10	0.78	0.86	EF367119	Ungrp
HmidPS1.305T[c]	5	0.71	0.76	GU256679	Ungrp
HmidPS1.332D[c]	18	0.56	0.95	GU256680	14
HmidPS1.549D[c]	10	0.81	0.89	GU256696	8
HmidPS1.811C[c]	11	0.46	0.84	GU256710	Ungrp
HmidPS1.818C[c]	11	0.63	0.74	GU256711	8
HmidPS1.870D[c]	15	0.88	0.94	GU256718	1
HmidPS1.874C[c]	14	0.63	0.93	GU256720	7
HmPS1.1058C[c]	11	0.60	0.90	GU256735	13
HmPS1.1063C[c]	11	0.53	0.90	GU256736	3
HmRS27T[b]	23	0.41	0.93	DQ785751	13
HmRS36T[b]	6	0.52	0.67	DQ785753	1
HmRS37D[b]	7	0.55	0.69	DQ785754	Ungrp
HmRS80M[b]	18	0.71	0.92	DQ785756	Ungrp
HmRS83M[b]	27	0.65	0.96	DQ785757	Ungrp

[a] Bester et al. (2004)
[b] Slabbert et al. (2008)
[c] Slabbert et al. (2010)
[d] RS, unpublished data
Ungrp = Not assigned to a linkage group

extracted from tentacle tissue using the KAPA Quick Extract kit (KAPA Biosystems). Extractions were performed using a 2720 Thermal Cycler (Applied Biosystems) in a final volume of 100 µl. The reaction contained 1x KAPA Quick Extract Buffer, 2U KAPA Quick Extract Enzyme and a 1 mm² piece of epipodial tentacle. The PCR programme was as follows: an incubation step was performed at 60 °C for 15 min followed by a heat-inactivation step at 95 °C for 5 min. The multiplex PCR reaction was then performed followed by fluorescent genotyping. An individual was classified as triploid if three alleles were detected for at least two loci. Samples that had any loci showing four or more alleles were excluded from further analyses because of possible DNA contamination from other sources.

Results

Initial genotyping

In this study, nine loci met the minimum criteria set for triploid verification: HmD59, HmLCS7T, HmNR20M, HmNR106D, HmNR120T, HmNS19L, HmidPS1.305T, HmidPS1.818C and HmidPS1.870D identified all diploid individuals correctly and identified at least three out of eight triploid individuals correctly (Table 2). The remaining 21 loci identified all diploid individuals correctly, but only two or less triploid individuals. Loci HmD59, HmNR106D, HmNR120T, HmNS19L, HmNR20M, HmidPS1.818C and HmidPS1.870D were chosen for the ploidy verification multiplex. HmLCS7T and HmNR20M have identical fluorescent markers and allele size ranges. Only one of these can be added to the multiplex, but is interchangeable. HmidPS1.305T was later discarded after difficulty in allele calling. The final multiplex therefore consisted of seven markers.

Statistical analysis

There was no significant difference ($p > 0.05$) between the two groups of loci in terms of observed number of alleles ($p = 0.09$) and expected heterozygosity ($p = 0.16$). However, a significant difference ($p < 0.05$) was found for observed heterozygosity ($p = 0.01$; average triploid loci = 0.76; average diploid loci = 0.52) with higher levels for markers that identified triploid controls correctly.

Triploidy verification protocol

The KAPA Quick Extract kit (KAPA Biosystems) yielded high-quality DNA for use in a microsatellite multiplex reaction. An example of a genotyping electropherogram of a diploid and triploid individual is shown in Figure 1. In all, 31 ploidy-unknown samples were classified as triploid and 11 as diploid. The control samples were also correctly assigned.

Discussion

Nine microsatellite loci (Table 2) were adequate for molecular verification of the level of ploidy of *H. midae*. The original 30 loci were chosen because they had no duplication of alleles in the genome. Duplication is found in the abalone genome and was already reported for 17 *H. midae* loci by Slabbert et al. (2010). Our results suggest that markers can be chosen based on observed heterozygosities. Loci with high levels of observed heterozygosity

Table 2: Genotypes of diploid and triploid controls for ploidy verification microsatellite panel

Individual	Locus genotypes								
	HmD59	HmLCS7T	HmNR20M	HmNR106D	HmNR120T	HmNS19L	HmidPS1.305T	HmidPS1.818C	HmidPS1.870D
Dilpoid1	124/128	233/233	221/233	386/386	291/307	178/224	096/096	157/157	098/098
Dilpoid2	111/124	261/263	227/239	381/381	248/339	231/238	103/127	151/157	109/115
Dilpoid3	153/155	261/263	221/233	–/–	248/303	185/237	096/125	144/161	115/122
Dilpoid4	111/130	–/–	193/209	373/386	287/307	237/250	103/127	157/161	130/130
Dilpoid5	124/146	229/229	193/227	345/350	263/291	224/244	099/127	157/167	098/122
Dilpoid6	124/136	229/243	227/245	388/388	315/315	237/245	096/099	159/167	111/117
Dilpoid7	124/130	231/231	227/233	381/381	267/331	215/267	099/127	157/157	098/111
Dilpoid8	122/124	210/233	230/233	379/386	248/303	209/242	096/099	151/157	107/122
Triploid1	117/119/124	233/240/243	245/245	375/386	248/260	231/253/272	102/103/127	157/161	122/124
Triploid2	117/124	208/229	187/202/230	375/383/386	283/307/323	238/250	096/099/103	157/159/169	098/128
Triploid3	124/124	208/229	202/233/245	373/386	248/263/323	190/198/235	096/099	155/157/167	098/100/115
Triploid4	113/121/128	208/221/243	230/233	375/381	307/339	244/253	096/099/127	159/167	098/113/126
Triploid5	113/115	243/243	233/233	375/381/392	255/299/307	231/253	103/103	157/157	126/128
Triploid6	119/121/126	208/240	193/202/230	345/345	260/295/323	191/196/259	099/099	152/157/165	098/115
Triploid7	113/124/128	221/233/243	233/233	375/390/392	275/307	231/238/253	099/099	165/167	098/122/126
Triploid8	111/113/128	243/243	227/233	345/345	267/299/307	231/238/253	096/099/103	146/146	098/100/126

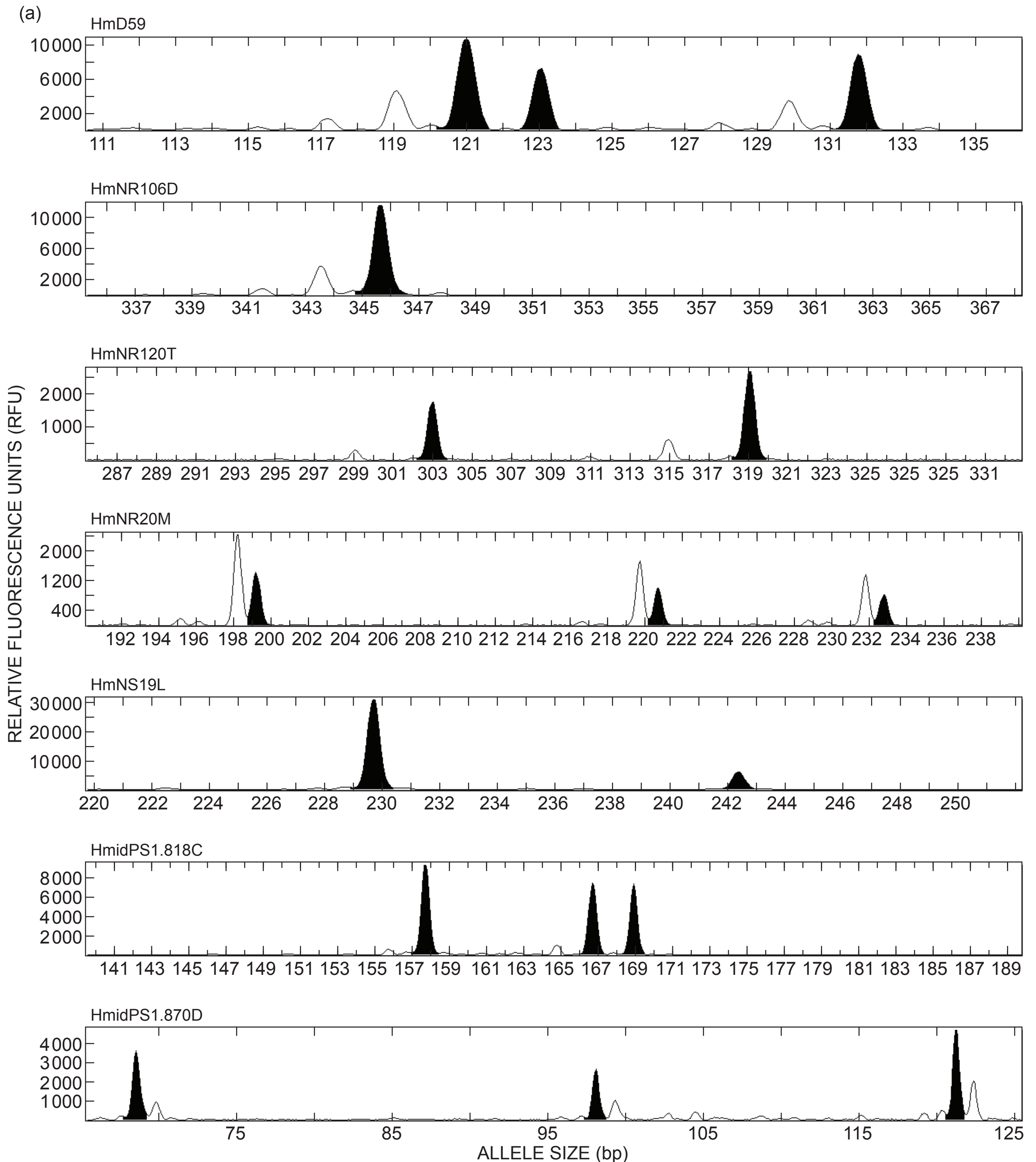
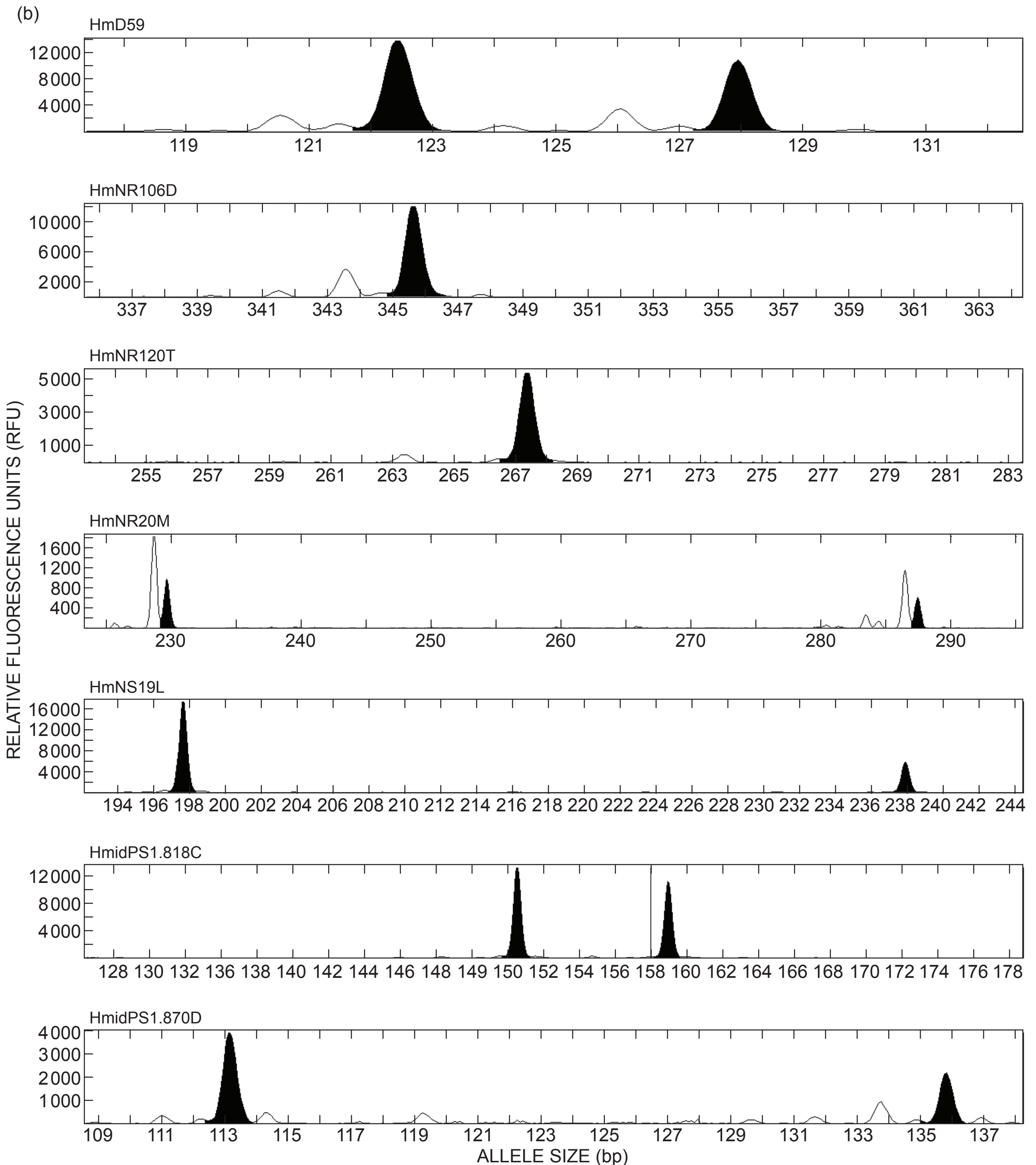


Figure 1: Electropherograms showing the alleles of each of the seven loci used for triploid verification for (a) a triploid and (b) a diploid individual. Three alleles can be observed for the triploid individual in some loci, whereas all loci of the diploid individual have no more than two alleles

(average = 0.76) were more likely to identify a triploid individual correctly. It is important to have reliable locus characterisation data available when selecting markers for something like ploidy verification, because any errors in allele scoring can either over- or underestimate this statistic. Another (technical) point to consider is the position and coverage of the loci within the genome of an organism. Chance duplication events can be avoided



by using multiple loci covering different linkage groups (chromosomes). The loci identified for triploid verification in the current study were mapped to six different linkage groups, whereas three loci remained ungrouped (Table 1; RS unpublished data).

Seven of these — HmD59, HmNR106D, HmNR120T, HmNS19L, HmNR20M, HmidPS1.818C and HmidPS1.870D — were placed into a multiplex reaction and applied to identify the ploidy of individual abalone within a mixed experimental population of diploids and induced triploids. The PCR-multiplex and a closed-tube extraction method were used to set up the triploid verification protocol. Closed-tube methods are fast, do not require much tissue and minimise the times a sample is handled prior to PCR analysis. Multiplex PCR reactions

increase data per reaction, making it cheaper and more rapid compared to single locus PCR reactions. This protocol was applied to identify samples of unknown ploidy level. All 42 ploidy-unknown samples were assigned as either diploid (11 samples) or triploid (31 samples), and the six control samples were also assigned correctly.

This study shows the utility of microsatellite markers as tools for verifying the ploidy of individual abalone. The protocol can be applied to larger animals for which the isolation of nuclei and metaphase chromosomes from tissues are difficult. Individual ploidy verification can be performed quickly and regularly and could facilitate in studying, for example, any reverting from the triploid state to the diploid state over time (Dew et al. 2003, Dunstan et al. 2007).

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