Applications of Molecular Biology to Management of the Abalone Fishery

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Applications of Molecular Biology to Management of the Abalone Fishery

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List of Abbreviations

bp	base pair (referring to complementary bases in dsDNA)
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
kbp	kilobase pairs
microsatellite	(syn. VNTR; also a STR) a DNA repeat of 2-4bp
minisatellite	(*syn*. VNTR) a DNA repeat of ~15-35bp
mtDNA	mitochondrial DNA
nDNA	nuclear DNA
PCR	polymerase chain reaction
RAPD	random amplified polymorphic DNA
RFLP	restriction (enzyme) fragment length polymorphism
ssDNA	single-stranded DNA
STR	short tandem repeat of DNA (*syn*. microsatellite)
Taq	heat-stable enzyme used to polymerise DNA in PCR
UV	ultraviolet light
VNTR	variable number of tandem repeats (of a DNA sequence)

Non-technical Summary

A problem exists in the management of the economically important Australian abalone fishery, viz a clear definition of the constitution of a population. If small populations are separated through the non-transfer of the planktonic larval stage, then the populations could remain genetically distinct. As a consequence, extensive removal of adults might lead to abalone depleted reefs.

As well, there is an increasing demand from fisheries compliance managers to be able to identify the origin of poached abalone. Information of this nature would need to be acquired by methods that were accepted by industry, relevant, user-friendly, cost-effective, relatively rapid, reproducible, quality assured and recognised in a court of law.

Aquaculture developments are also putting a demand on wild stock identity. Firstly, large-scale culture of abalone has the potential to alter the genetic make-up, which is different to local stocks. Secondly, compliance measures may be compromised by illegally caught wild abalone being sold as farmed animals.

Our research set out to develop new molecular biology techniques to determine the extent of genetic differences between populations of Victorian blacklip abalone. This would provide data for managers controlling the fishery, including the reseeding of reefs and ranching, compliance managers, and aquaculturists. We utilised four DNA assays, all of which were based on amplifying segments of DNA into billions of copies by PCR (polymerase chain reaction), and are commonly termed RAPD (random amplified polymorphic DNA), VNTR (variable number of tandem repeat), STR (short tandem repeat) and mtDNA (mitochondrial DNA) sequence comparisons.

DNA segments generated by the first three assays were examined after separation by gel electrophoresis, and are fundamentally dominant DNA markers. The sizes of DNA segments generated by each method were analysed by a relatively new statistical software package known as AMOVA

(analysis of molecular variance). It basically examines the variation within and between population samples. RAPD and STR showed the best discriminations between the populations and delineated the abalone originating from each of the Victorian abalone management zones. They also showed abalone from Port Phillip Bay to be genetically separated from oceanic stocks. The RAPD method was found to be easy to perform but much care had to be taken to ensure that data was reproducible, and it was considerably more difficult than the other methods in data recording. By far the most useful, was the STR technique, which is commonly used in human forensic testing. This method showed a high degree of homozygosity (i.e. individuals with two segments the same), implying limited larval dispersion and inbreeding, but further studies are required to resolve these findings.

In regard to mtDNA, segments of two genes, oxidase I and oxidase II, have been cloned and sequenced. PCR primers, based on these sequences, have been produced and tested successfully in amplifying abalone mtDNA. A sequence map of the whole mtDNA is being completed.

DNA tests were also performed on canned abalone. However, it was found that the DNA was so degraded that only very short segments (about 150 bp) could be reliably amplified by PCR. Therefore, the choice of which STR or mtDNA test to use, needs to be considered carefully. Consequently, the origin of cooked blacklip abalone could be determined only with tests using short PCR products, and comparison of results against a database.

Our results are available in scientific journals, and the PCR primers available from GenBank on the internet. The results highlight three areas of future work. They include molecular studies of the high degree of homozygosity found in abalone populations, an extended study to include more population samples of Victorian abalone, and finally, development of a comprehensive national DNA database with all abalone producing states participating in data acquisition.

Background

Across the southern temperate coastline of Australia there are eight species of abalone, genus *Haliotis* (Wells and Bryce, 1988). Of these, *H. rubra* (blacklip abalone*), H. laevigata* (greenlip abalone), *H. conicopora* (brownlip abalone) and *H. roei* (Roe's abalone), are important as commercial or recreational resources in Tasmania, Victoria, New South Wales, South Australia and Western Australia (Prince and Shepherd, 1992). The blacklip and greenlip abalone (Figure 1) constitute most of the commercial harvest, which is valued at approximately $152 million in 1997/8 (ABARE, 1998). This value increases to nearly $192 million of export product with added-value by canning.

Figure 1. The two abalone species that make up the largest proportion of the annual abalone harvest.

DNA techniques utilised

Three molecular techniques were assessed using the criteria of result reproducability, experimental simplicity and cost effectiveness. All three were based on amplification of segments of DNA by a technique known as PCR. The PCR technique is described in detail in Appendix 1, but is essentially a

method by which short segments of double stranded DNA are copied into billions of copies. The short fragments generated by PCR can then be separated by gel electrophoresis and compared.

The three molecular techniques used included:

(1) random amplified polymorphic DNA (RAPD);
(2) variable number tandem repeat (VNTR); and
(3) short tandem repeat (STR).

As well, a genetic map of abalone mitochondrial DNA (mtDNA) was needed so that sections of the DNA could be amplified from different abalone species. Once analysed to reveal DNA sequence changes, comparisons can be made to determine genetic markers (i.e. changes) for species identification and population differences. Some of these changes are determined by in a technique known as restriction fragment length polymorphism (RFLP). This involves cutting the PCR amplified pieces of DNA by enzymes to produce DNA fragments of different size, and these new DNA fragments can be compared after separation by gel electrophoresis.

Description and Use of RAPD

The RAPD method is a PCR-based technique to identify DNA polymorphism (Williams *et al*., 1990; Welsh and McClelland, 1990), and is based on the fact that DNA sequences of individuals within a species contains subtle differences, commonly resulting from mutations. It is not dependent on having a prior knowledge of DNA sequences. RAPD markers are based on the amplification of unknown DNA sequences using a single, short random oligonucleotide (normally 10 bases; a 10-mer), which acts as both a forward and reverse primer in PCR. During PCR, the single primer binds to sites on opposite strands of genomic DNA and discrete DNA segments are produced, so long as the sites are within an amplifiable distance of each other (e.g. up to a few thousand nucleotides). The

disruption or displacement of homologous target sites between individuals results in the loss of a product. The presence or absence of this specific product, although amplified with an arbitrary primer, will be diagnostic for the oligonucleotide-binding sites on the genomic DNA. In practice, DNA samples are amplified with several different primers in separate reactions. Polymorphic bands are noted between samples and population groups. This method largely generates dominant markers (Milbourne *et al*., 1997).

The RAPD method was considered to have considerable promise for population genetic and evolutionary studies (Hedrick, 1992). As no previous sequence information is needed in the target organism practically all organisms are accessible to the technique. In addition, many markers can be readily identified, as is required for the reconstruction of phylogenetic history and the investigation of population genetics.

The RAPD data can be entered into Microsoft Excel spreadsheets and then analysed using a relatively new statistical program, AMOVA (Analysis of Molecular Variation), developed by Excoffier *et al*. (1992). AMOVA calculates a series of Φ-statistics that are analogs of hierarchical *F*-statistics, and there are a number of advantages in using AMOVA in the analysis of RAPD, minisatellite and microsatellite data (Excoffier *et al*., 1992; Huff *et al*., 1993; Morell *et al*., 1995). The data can then be presented in dendrogams showing genetic relatedness, using software programs such as the RAPDistance Package (Armstrong *et al.*, 1994), or NTSYS-PC (Exeter Software).

Description and Use of Satellite DNA (VNTR and STR)

Satellite DNA, a type of tandemly arranged highly repetitive sequence, was first found in calf thymus DNA by Meselson and co-workers in 1957. It was then found to exist widely in animals and plants (Beridze, 1986). Of these, the minisatellites are known as variable number of tandem repeats (VNTRs) (Budowle *et al*., 1991) whereas microsatellites are known as short tandem repeats (STRs) or simple sequence repeats (SSRs) (Kimpton *et al*.,

1993; Rafalski *et al*., 1996). Some researchers use the term VNTRs for minisatellites and microsatellites synonymously, as both of them are DNA sequences of variable number of tandem repeats but differ in length (Fregeau and Fourney, 1993). In this study, the term VNTR is used to describe a minisatellite (approximately 15-35 bp repeat) and STR is used to describe a microsatellite (2-4 bp repeat).

Minisatellite VNTRs are usually located in non-coding regions known as introns (Griffiths *et al*., 1993), or 3' end non-translated regions (Ludwig *et al*., 1989). There are a considerable numbers of VNTRs in animals and plants (Jeffreys *et al*., 1991; Rafalski *et al*., 1996). Initially, VNTRs were characterized by Southern analysis with a restriction enzyme that has its recognition sequence in the flanking regions of the repeat units (Decorte *et al*., 1993). This procedure was laborious, demanding relatively large amounts of DNA and did not provide the resolution for small size differences of one repeat unit between two relatively large fragments (Budowle *et al*., 1991; Decorte *et al*., 1993). With the innovation of PCR techniques, it has become possible to analyse VNTR loci rapidly and more accurately. VNTRs, and STRs, are now analysed by PCR amplification of genomic DNA. Two unique oligonucleotide primers are designed to flank the repeat unit region so that small differences in size of PCR products can be resolved in agarose gels (Horn *et al*., 1989), or polyacrylamide gels (Ludwig *et al*., 1989; Budowle *et al*., 1991). As each eukaryotic organism has two sets of chromosomes, one set from the mother (maternal) and the other set from the father (paternal), then the repeat units at a given site (i.e. a locus) in the DNA within a chromosome will also be inherited from each parent. Thus, PCR amplification products result in only one band (i.e. homozygous for the repeat size) or two bands (i.e. heterozygous for the repeats) when separated by gel electrophoresis. This method generates codominant markers.

VNTRs are widely employed in human identity, paternity and other forensic areas (Ludwig *et al*., 1989; Budowle *et al*., 1991; Balazs *et al*., 1992; Morling and Hansen, 1993; Hansen and Morling 1993). They have also been

used successfully in linkage analysis (Nakamura *et al*., 1987), antenatal diagnosis (Jeffreys *et al*., 1985), population genetic studies (Balazs *et al*., 1992), and fish stock identification (Taggart and Ferguson, 1990; Beacham *et al*., 1995). However, recent trends to automate DNA analyses has seen a move towards greater use of STRs.

Two VNTR's have already been identified, in untranscribed sequences of abalone DNA during recent research concerning the genetic cloning of growth factors, and used in VNTR analyses (Chai, 1994; Huang *et al.*, 1997). They showed a typical 18 base pair repeat in one VNTR and a 33 base pair repeat in the other. Pairs of oligonucleotides, complementary to DNA sequences flanking these two repeat regions, were constructed for use in PCR amplification of each VNTR. Their use in PCR of abalone DNA showed clear differences between individual abalone, but at that time, they had not been applied to analyses of the genetic structure of abalone populations.

STR dinucleotide repeat polymorphic DNA was found by Weber and May (1989), and later, trinucleotide and tetranucleotide repeats were discovered (Schumm, 1996). Microsatellites are often dispersed throughout the genome of eukaryotic organisms (Hamann *et al*., 1995). They have been reported in both genic and extragenic regions of the genome (Kimpton *et al*., 1993). Those in genic regions are present not only in intron and flanking sequences, but also within coding regions (Edwards *et al*., 1992). It is estimated that there are 50,000-100,000 interspersed (CA)n blocks in the human genome, with the range of n being roughly 15-30. Uniform spacing of the (CA)n blocks throughout the genome would place them every 30-60 kb (Weber and May, 1989). For humans, it is estimated that there is a total of 500,000 STRs including 6,000 to 10,000 trimeric and tetrameric repeats (Fregeau and Fourney, 1993). In the European flat oyster (*Ostrea edulis*), it is estimated that there are 12,700 (GA)n and 3,900 (AC)n microsatellite DNA markers (Naciri *et al*., 1995). In the snail (*Bulinus truncatus*), it is estimated that on average there is a dinucleotide microsatellite block every 40 kb and a tetranucleotide microsatellite block every 60 kb (Jarne *et al*., 1994).

Abundance, polymorphic nature, and amenability to amplification by PCR, make STRs ideal markers for genomic mapping and genetic linkage analysis (Sheffield *et al*., 1995; Bosch *et al*., 1993). Numerous STR loci have been used in predicative diagnosis of genetically inherited diseases, including myotonic dystrophy (Mully *et al*., 1991), cystic fibrosis (Morral *et al*., 1992), Duchenne/Becker muscular dystrophy (Oudet *et al*., 1991) and Huntington's disease (Weber *et al*., 1992). A panel of STRs consisting of ten tetranucleotide microsatellites is used in human identification and forensic medicine (Promega's GenePrint™, 1996). Microsatellites are also used in plant and animal breeding (Rafalski and Tingey, 1993; Lewin and Holm, 1994), and population genetic structure studies (Viard *et al*., 1996; Paetkau *et al*., 1995; Ciampolini *et al*., 1995; Slatkin, 1995; Deka *et al*., 1995; Michalakis and Excoffier, 1996; Takezaki and Nei, 1996; Primmer *et al*., 1996). STRs are also dominant markers.

Microsatellite sequences are normally obtained by screening genomic DNA libraries (Jarne *et al*., 1994; Naciri *et al*., 1995). However, it is a tedious process to construct and screen genomic DNA libraries, and to purify and sequence the clones containing STRs. More recently, it has been found that some of the bands amplified with a single arbitrary primer (i.e. RAPDs) contain microsatellite DNA sequences (Richardson *et al*., 1995; Ender *et al*., 1996). Therefore, RAPD products can be used as a start point to search for microsatellites, but newer methods are now available for the rapid isolation of STRs and other tandem repeated sequences in genomic DNA (Refset *et al.*, 1997).

Brief Description of Using mtDNA

Mitochondria are cell organelles (often many per cell) in which a cell generates most cellular energy through the oxidation of many small molecules. These molecules are encoded by mitochondrial DNA (mtDNA),

which is typically a circular molecule and with a conserved number of genes, including 2 rRNA genes, 22 tRNA genes and 13 protein genes. As well, there is a control region, making a total size in mammals and many invertebrates of a bit less than 20kb, although it is considerably bigger in some invertebrates. The mtDNA is mostly inherited through the maternal line and has a higher rate of mutational change than the nuclear DNA (nDNA), resulting in highly resolved differences of DNA sequences in different populations.

PCR and sequencing of mtDNA has been used for nearly a decade to solve problems of evolutionary and population biology (Innes *et al.*, 1990). In particular, bases within amplified sequences of animal and plant mtDNA are now being applied to differentiate species. N. Sweijd (*pers. comm.*) is now exploiting such changes in the control region of abalone species to produce species markers using PCR and subsequent RFLP (restriction fragment length polymorphism).

The total mtDNA genome of abalone has not been sequenced and a map would provide for PCR amplification and sequencing of its many genes. Three mtDNA genes, amongst others, that would be studied, are the oxidase I and oxidase II genes, and the cytochrome b gene. Evidence from other animals indicates that these genes, like many others, contain conserved DNA sequences that relate to functionality. DNA primers are commonly synthesised to these conversed regions for PCR amplification, and subsequent sequencing of intervening variable regions. Comparison of derived DNA sequences are made by alignment of sequences (via software programs), which can be then translated into population differences.

Need

There is a growing demand to distinguish differences between populations of Australian abalone. Although considerable research effort has gone into populations studies, including recruitment processes, and dispersal

of larvae and mortality (McShane *et al.*, 1988; Shepherd *et al.*, 1992), we are still unable to clearly define an abalone population. Previous studies, utilising allozymes, have indicated that blacklip abalone stock size is approximately 500 km, and the Victorian blacklip abalone management zones are regarded as metapopulations consisting of several blacklip abalone stocks (Brown, 1991; Shepherd and Brown, 1993).

Differentiation between populations is important in the implementation of sound fisheries management programs. For example, there is considerable debate as to the dispersal distance of larvae from one area to another. One view is that considerable dispersion of larvae occurs during the free-swimming stage, which lasts for several days, depending on species and environmental conditions. Therefore, the removal of reproductively mature adults from a particular area is not harmful to the overall fishery as new individuals are recruited from distant populations. The alternative opinion and stronger view is that larvae are predominantly recruited into local areas, and severe removal of adults lowers the numbers of new individuals from being produced and recruited within the population. This later view implies restricted, or little transfer, of genes between populations resulting in reduction of genetic diversity.

The proposed research project was set up to establish DNA-based molecular DNA assays for the DNA fingerprinting (i.e. profiling) of abalone. This would provide tools for examining populations differences at a molecular genetic level.

Objectives

1. To apply modern molecular biology techniques to identifying the genetic composition and structure of blacklip abalone populations;

2. To transfer the results of techniques that best identify abalone populations to abalone fishery managers; and
3. To utilise the techniques developed to identify abalone populations in enforcement situations.

Methods

As planned at the commencement of the project, details of the methods and results of the project would be published in copyrighted scientific journals, and a PhD thesis (see reference Huang, 1999), so as to distribute the information to a wide audience. The publications arising from the research are reproduced with the kind permission of the publishers in Appendices 5 - 8. Therefore, the following are general descriptions of the project.

Abalone samples

Samples of 10 abalone from 10 populations were obtained from Harry Gorfine, Marine and Freshwater Institute, Fisheries Victoria, Queenscliff. They were taken from sites being monitored as part of the Victorian abalone monitoring program. Nine populations were from sampling sites along the Victorian coastline, and another was from near Eden in New South Wales. Additional abalone were collected (under permit), as need be, to establish the DNA techniques for abalone.

DNA extraction

DNA was extracted from using a microwave/phenol technique (Goodwin and Lee, 1993), as it initially produced the best quality DNA (Fig. 2). However, in later PCR reactions, DNA extractions using DNAzol™ (Gibco

BRL) were necessary to get amplification of some STRs. Extracted DNA was stored at 4ºC until analysed.

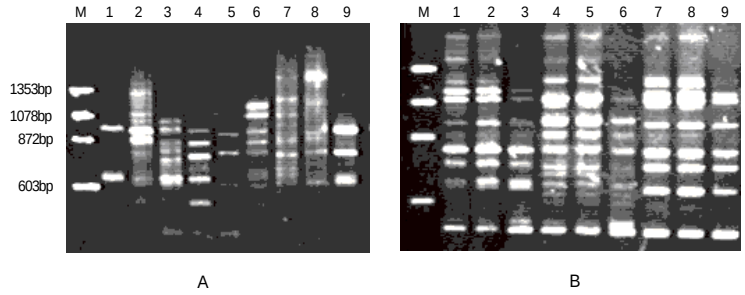

Figure 2. Effects of DNA extraction from blacklip abalone tissues on RAPD.
A: RAPD profiles of DNAs extracted using Chelex-100 method. Lanes 1, 2 and 3 represent DNA isolated from muscle, gonad and blood from one abalone; in the same tissue order, lanes 4, 5 and 6 represent abalone 2; lanes 7, 8 and 9 represent abalone 3.
B: The origin of DNAs is the same as Gel A, except the DNAs were extracted using the microwave/phenol method. The amount of DNA added into each reaction was not quantified. Due to the small amounts of DNA extracted from blood samples some of the high molecular weight bands in the lanes 3, 6 and 9 (i.e. blood) were not amplified as well as that of muscle and gonad samples. Lane M for both gels represent Phi X174 *Hae* III digested DNA marker (Promega).

PCR (polymerase chain reaction) was used to amplify blacklip abalone DNA samples in a thermal cycler, using primers which had already shown to react well with abalone DNA. The single primers used in RAPD analyses are described fully in the published papers and the results section. The pairs of primers used to amplify the microsatellite STRs and minisatellite VNTRs, are described fully in the published papers, but can also be accessed from GenBank (http://ncbi.nlm.nih.gov/) using the following Accession Numbers.

Locus	DNA repeat	GenBank Accession Number
Microsatellite RUBGT1	$(GT)_n$	AF027572
Microsatellite RUBCA1	$(CA)_n$	AF027573
Microsatellite RUBGACA1	$(GACA)_n$	AF027574
Minisatellite MIPR	18 bp repeat	AF027575
Minisatellite GHR	33 bp repeat	AF027576

mtDNA analysis

The mtDNA methodology had two main components. One was to develop abalone primers for the PCR amplification of mtDNA segments of the oxidase I and II genes, and cytochrome b gene. The other was to develop protocols for the isolation of good quality mtDNA for cutting with restriction enzymes and cloning to produce a complete genomic sequence map of the blacklip abalone mtDNA.

In the first of these, degenerate primers developed for the amplifying the land snail mtDNA were used, but no products were obtained for cytochrome b. PCR products of abalone oxidases I and II were cloned into a pCR2.1 vector using an AT cloning kit (Invitrogen), and subsequently sequenced so as to obtain non-degenerate primers for abalone mtDNA.

Results and Discussion

Three DNA techniques were used in the molecular analyses of the genetic composition and structure of Victorian blacklip abalone populations, viz RAPD, VNTR and STR.

Summary of the analyses of genetic variation in blacklip abalone (*H. rubra*) populations using random amplified polymorphic DNA (RAPD)

[See Appendices 7 & 8 for full details of the methods and results:
Hanna, P. J., Chai, Z., Cummins, S. and Huang, B. (1999). Applications of molecular biology to abalone fisheries and aquaculture. J. Med. Appl. Malacol., in press.]

Huang, B. X., Hanna, P. J. and Peakall, R. (1999). Molecular analyses of population genetic structure of blacklip abalone, *Haliotis rubra*, using RAPDs, minisatellite and microsatellite markers. Mar. Biol., in press.

In RAPD primer screening, 5 of 50 decamer oligonucleotides of a set purchased from the University of British Columbia gave highly polymorphic banding (Table 1). As well, a 15-mer M13 core repeat oligonucleotide (i.e. 5'-GAGGGTGGTGGCTCT-3') also gave highly polymorphic banding.

Table 1. Details of oligonucleotides used as primers in RAPD analysis of blacklip abalone populations. (UBC, University of British Columbia)

Primers	Number of amplified bands	Range of bands in individual	Monomorphic bands (≥95% shared)	Number of polymorphic markers	(%) Polymorphism
UBC-101	14	2-13	1	13	92.85
UBC-135	13	3-10	1	12	92.3
UBC-149	14	3-11	2	12	85.7
UBC-159	15	1-12	0	15	100.0
UBC-169	16	4-12	1	15	93.7
RM13	18	5-16	1	17	94.4
Total	90		6	84	Mean = 93.3

The polymorphic bands obtained from the six primers were collectively used to assess the population genetic structure of blacklip abalone. Initially, the genetic variation of the 10 blacklip abalone populations was analysed as a single group under the null hypothesis that there was no genetic

differentiation among the populations in an AMOVA analysis. This produced a value of Φ_{PT}=0.074, with significance level p<0.001, indicating that the null hypothesis was not true, and showing instead, that blacklip abalone populations along the coast were not homogeneous. That is, some population subdivision existed (Table 2). A further AMOVA analysis was applied to the populations by dividing them into three regions according to their location within the three blacklip abalone management zones of Victoria (Gorfine and Walker 1996). The AMOVA results of population divergence among zones, among populations within zones, and within populations, showed that there was significant population divergence among management zones (Φ_{RT}= 0.033; p=0.039), and highly significant population divergence among populations within zones (Φ_{PR}= 0.055; p<0.001), and within populations (Φ_{PT}= 0.086; p<0.001).

Table 2. AMOVA analysis of blacklip abalone populations of the Victorian coast

Source of variation	Degree of freedom (df)	Sums of squares (SS)	Mean squares (SS/df)	Expected mean squares	Estimated variance components	Φ_{PT}	Significance
Among Population	(P-R)=9	229.35	25.48	$\sigma^2_w+10\sigma^2$	1.130	0.074	p<0.001***
Within Population	(N-P)=90	1276.90	14.19	σ^2_w	S^2_w=14.19		
Total	(N-1)=99	1506.25					

Note: P is the number of populations (P=10); R is the number of regions (R=1); N is the number of samples (N=100).

A dendrogram (Fig. 3) clustered the 10 populations into 4 groups. The first group comprised 6 of the eastern populations, extending from Eden in New South Wales to Hicks Point in Victoria. The second group, was the western population from Portland. The third group included Apollo Bay and

Cape Schanck in the central fishery zone, and the last, but distinct group, was a population from Port Phillip Bay.

A correlation of distances between populations, based on shortest distance of population GSP plots, and genetic distances (Φ_{PT}), was highly significant ($r=0.71$, $p<0.001$) (Fig. 4). Results from this study, which excluded the population from Port Phillip Bay, indicated that genetic distance was influenced by geographic distance. The results of a pairwise population analysis suggest that significant genetic divergence can be expected among all blacklip abalone populations that are separated by more than 70 km.

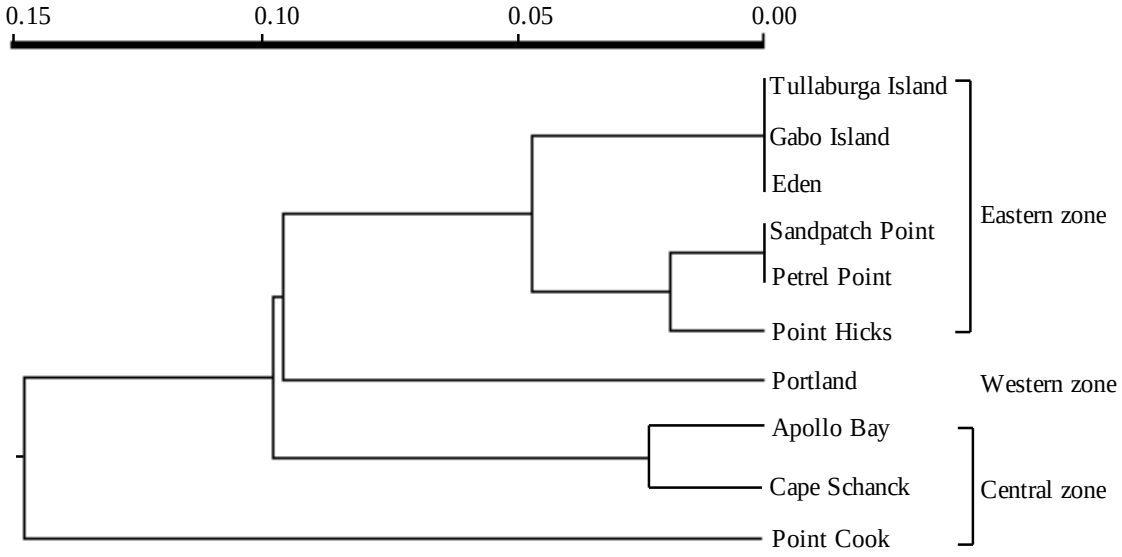

Figure 3. The relatedness of blacklip abalone populations along the Victorian coastline using RAPD. Genetic distances (Φ_{PT}) were calculated with AMOVA and then clustered with NTSYS-pc to produce the dendrogram.

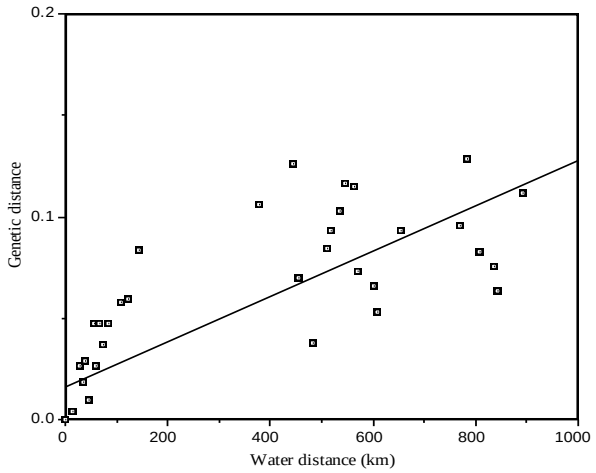

Figure 4. Correlation between shortest water distance between populations and genetic distance of populations (y=0.0172 + 0.000114x; r=0.71, p<0.001). Data of Point Cook is excluded.

Summary of the analyses of population genetic structure of the blacklip Abalone, *Haliotis rubra*, using variable number of tandem repeats (VNTRs)

[See Appendices 5 & 8 for full details of the methods and results:
Huang, B., Chai, Z., Hanna, P. J. and Gough, K. H. (1997). Molecular sequences of two minisatellites in blacklip abalone, *Haliotis rubra*. Electrophoresis, 18, 1653-1659.
Huang, B. X., Hanna, P. J. and Peakall, R. (1999). Molecular analyses of population genetic structure of blacklip abalone, *Haliotis rubra*, using RAPDs, minisatellite and microsatellite markers. Mar. Biol., in press.]

Two minisatellites were identified in the 3' untranscribed region of putative black-lip abalone growth hormone (designated GHR) and the insulin-like growth factor (designated MIPR) genes (Chai, 1994). They possessed 33 bp and 18 bp tandem repeat units, respectively. Primers were synthesised

according to flanking sequences of the two loci and the corresponding VNTRs of the black-lip abalone samples (as per RAPD analyses) amplified by PCR. The PCR products were separated using 6% polyacrylamide gel flat-bed electrophoresis and then visualised by silver staining. The gels were then dried for permanent records and subsequent analyses.

Characterisation of the VNTR within the putative growth hormone (GH) gene showed that repeat-number polymorphisms could be obtained using primers 2788 (forward) and 2923 (reverse). Initially one set of primers were synthesised and designated primers 2788 and 2789. Every measure was taken to optimise the PCR conditions, but they still generated multiple bands, or very faint bands if the annealing temperature was raised. Subsequently, another reverse primer 2923 was synthesised and found to work well in combination with primer 2778 to produce good band patterns. Thirteen different-sized VNTRs (designated alleles), were observed in 100 blacklip abalone samples collected from nine Victorian sites, and one southern NSW location at Eden. The largest allele was estimated to be 20 repeats (810 bp) in length and the smallest allele 7 repeats (381 bp) in length. Allele 8 has not yet been observed.

Determination of VNTRs within the insulin-like growth factor (MIP) gene repeat utilised two primers, viz 2786 and 2849. Amplified genomic DNA of the 100 different individuals (as per other analyses) showed the presence of four different-sized alleles, of which the largest allele was 7 repeats (177 bp) in length, and the smallest allele was 4 repeats (123 bp) in length.

The observed heterozygosities were 64.0% and 65.0% at the GHR and MIPR loci, respectively. The expected heterozygosities were 64.9% and 57.2% at GHR and MIPR loci, respectively. An analysis showed that there was no significant difference between the observed and expected heterozygosities at the GHR and MIPR loci. Consequently, there was no significant departure from the Hardy-Weinberg Equilibrium at the both loci.

A dendrogram of the population relatedness among the population pairs, measured using Nei's genetic distances (Nei 1973), is illustrated in Fig.

5. The eastern zone populations clustered closely. However, the population of Point Cook, collected from Port Phillip Bay, did not cluster with the other two central management zone populations of Apollo Bay and Cape Schanck, with their splitting point at Nei's genetic distance (D) being approximately 0.10. This indicated that some genetic divergence between the Point Cook population and the other two central management zone populations (genetic distance D>0.05).

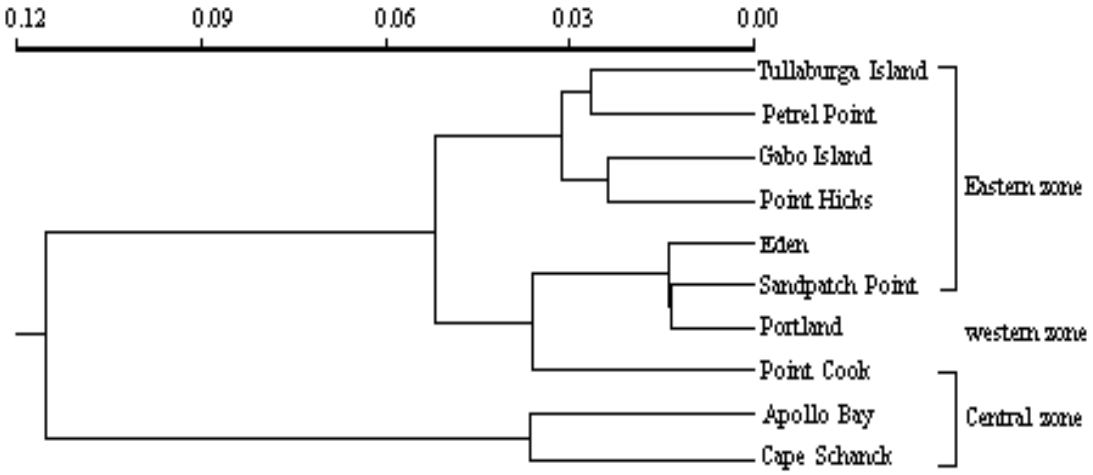

Figure 5. Population relatedness clustered using Nei's genetic distances in the VNTR loci, GHR and MIPR.

Summary of the analyses of population genetic structure of the blacklip abalone, *Haliotis rubra*, using short tandem repeats (STRs)

[See Appendices 6 & 8 for full details of the methods and results:

Huang, B. and Hanna, P. J. (1998). Identification of three microsatellites in blacklip abalone, *Haliotis rubra* (LEACH), and detection in other abalone species. J. Shellfish Res., 17, 795 - 799.

Huang, B. X., Hanna, P. J. and Peakall, R. (1999). Molecular analyses of population genetic structure of blacklip abalone, *Haliotis rubra*, using RAPDs, minisatellite and microsatellite markers. Mar. Biol., in press.]

Five STRs were isolated by probing RAPDs and genomic libraries. Of these, three have been fully sequenced, and flanking PCR primers synthesised for amplifying the STRs from genomic DNA. The STRs have been called RUBGT1, RUBCA1 and RUBGACA1, to designate the first blacklip abalone STR loci corresponding to repeats of (GT)n, (CA)n and (GACA)n, respectively. All three loci were polymorphic in the blacklip abalone samples collected from the Victorian coast and Eden, New South Wales. There were 41 alleles identified at the RUBGT1 locus, 30 at the RUBCA1 locus, and 8 at the RUBGACA1 locus.

It has been found that there were excessive homozygotes across the three loci, and the ten sampled blacklip abalone populations (Table 3). Consequently, the significant departure of Hardy-Weinberg Equilibrium was observed across the three loci and the populations. The causes may be limited larval dispersal and asynchronous spawning.

Table 3. Observed and expected heterozygosities and homozygosities of the blacklip abalone samples at the RUBGT1, RUBCA1 and RUBGACA1 loci

Locus	Observed heterozygosity (%)	Expected heterozygosity (%)	Observed homozygosity (%)	Expected homozygosity (%)
RUBGT1	37.0	95.52	63.0	4.48
RUBCA1	38.0	95.48	62.0	4.52
RUBGACA1	19.0	81.43	81.0	18.57

Molecular variation of the three microsatellite loci in the ten populations were analysed under the null hypothesis, using AMOVA. The value of Φ_{PT} was 0.067 and $p < 0.001$ (Table 4). Therefore, the three microsatellite loci revealed that there was highly significant variation among the ten blacklip abalone populations. This indicated that the blacklip abalone populations along the coast were not homogeneous and population subdivision existed.

Further analysis in the three management zones found there was some genetic differentiation among populations within zones (Φ_{PR}=0.05, p<0.001) and within populations (Φ_{PT} =0.078, p<0.001), but no significant molecular variation among zones (Φ_{RT} =0.029, p>0.05). Significant molecular variation based on microsatellite data started to show with the shortest water distance 84 km between Eden and Sandpatch Point (Φ_{PT}=0.04, p=0.009).

Table 4. AMOVA analysis of blacklip abalone populations using microsatellite markers

Source of variation	Degree of freedom (df)	Sums of squares (SS)	Mean squares (SS/df)	Expected mean squares	Estimated Variance components	Φ_{PT}	Significance
Among Population	(P-R)=9	65.64	7.29	$\sigma^2_w+10\sigma^2$	0.303	0.067	p<0.001***
Within Population	(N-P)=90	383.30	4.26	σ^2_w	S^2_w=4.26		
Total	(N-1)=99	448.94					

Note: P=10; R=1; and N=100; ***, significance level at p<0.001.

A dendrogram show the genetic relatedness of the ten population using pairwise Φ_{PT} of the three microsatellite markers, is illustrated in Figure 5. The Point Cook population was distinct from the other two populations (Apollo Bay and Cape Schanck) within the central management zone.

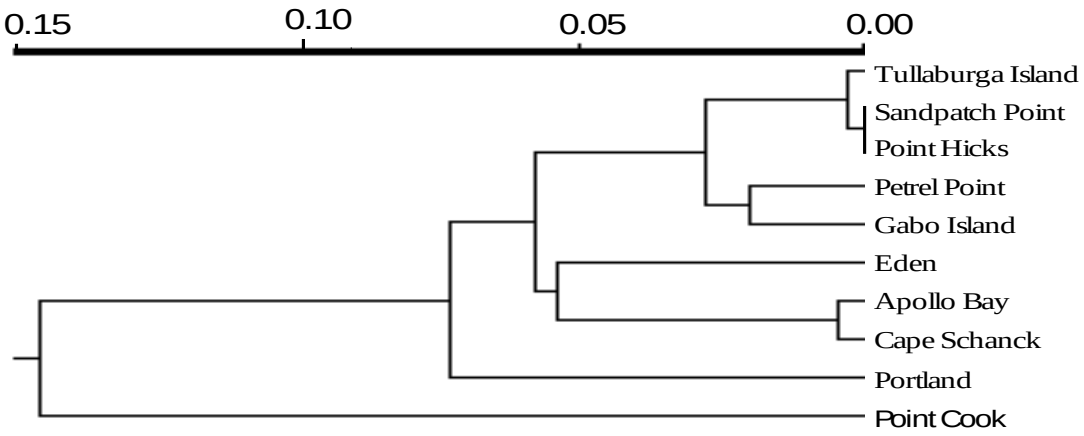

Figure 5. Relatedness of the ten blacklip abalone populations using STRs

Summary of species testing using PCR with the three STR and two VNTR primers sets

The five STR and VNTR markers developed for blacklip abalone were tested on 14 abalone species (Table 5), and no amplifications occurred for the overseas species, with the exception of the South Korean species *H. gigantea* and *H. sieboldi* at the RUBGT1 locus. The MIPR was found to be a species-specific marker for blacklip abalone, as there was no amplification detected in other species. Positive amplifications occurred mainly with the Australian sympatric species, *H. conicopora, H. roei* and *H. scalaris*. As well, *H. conicopora* amplified at four out of five loci, but *H. roei* and *H. scalaris* only amplified at two out of five loci. This data suggests that *H. conicopora* (brownlip abalone) is more closely related to blacklip abalone than the other three species. Similar results were observed in allozyme analyses by Brown (1991), in which the genetic distance of blacklip abalone compared with other species, from closest to least related, were *H. conicopora*, *H. roei*, *H. scalaris* and then *H. laevigata*. Surprisingly, no there were no amplifications of the loci for *H. laevigata* (greenlip abalone), as incidences of hybridization between

Table 5. PCR of commercial abalone species of the world using three STR and two VNTR primers sets

ORIGIN	SPECIES	STR			VNTR	
		(GAGA)n	(CA)n	(GT)n	*MIPR	**GHR
NORTH	*H. rufescens*	-	-	-	-	-
AMERICA		-	-	-	-	-
	H. cracharodii	-	-	-	-	-
		-	-	-	-	-
SOUTH	*H. pervum*	-	-	-	-	-
AFRICA	*H. midae*	-	-	-	-	-
		-	-	-	-	-
		-	-	-	-	-
		-	-	-	-	-
SOUTH	*H. discus* (A)	-	-	-	-	-
KOREA		-	-	-	-	-
	H. discus (B)	-	-	-	-	-
		-	-	-	-	-
	H. gigantea	-	-	28/28	-	-
	H. sieboldi	-	-	27/27	-	-
		-	-	27/27	-	-
	H. discus hannai	-	-	-	-	-
		-	-	-	-	-
	H. diversicolor	-	-	-	-	-
		-	-	-	-	-
SOUTH	*H. rubra*	5/8***	38/38	13/13	5/5	18/14
AUSTRALIA		2/5	32/32	10/19	5/6	20/14
	H. laevigata	-	-	-	-	-
		-	-	-	-	-
	H. roei	2/2	-	29/29	-	-
		2/2	-	19/19	-	-
	H. scalaris	-	7/7	11/28	-	-
		-	11/11	11/11	-	-
WESTERN	*H. conicopora*	5/5	37/41	28/32	-	+ve
AUSTRALIA		5/5	41/44	17/18	-	+ve

* ILR, VNTR insulin-like gene repeat of 18bp
** GHR, VNTR growth hormone gene repeat of 33bp
*** 5/8, refers to alleles of 5 and 8 repeats, respectively, in the diploid abalone

blacklip and greenlip abalone have been observed in nature, and produced in aquaculture. Additional STRs need to be characterised to assess more fully the evolutionary distances among abalone species.

Summary of the molecular characterisation of the blacklip abalone mtDNA

Research towards producing a sequence map of mtDNA was frustrating. The main problem was isolating good quality DNA from proteins and mucopolysaccharides. This meant that enzymatic cutting of the mtDNA, for cloning and subsequent sequencing, was difficult. Through patience and modifications to protocols, the problem was over come and we can reliably cut the mtDNA into fragments. As a consequence, we have now cloned portions of the abalone mtDNA and these are being sequenced. A sequence will provide for good PCR primers to be designed to PCR amplify segments of many genes.

Degenerate primers developed for amplifying the land snail mtDNA were used in an attempt to amplify segments of abalone mtDNA genes, oxidases I and II, and cytochrome b. No products were obtained for cytochrome b, but PCR products of 710 bp for oxidase I and 550 bp for oxidase II were produced from fresh and alcohol-fixed abalone. These segments were then AT cloned into the pCR 2.1 vector (Invitrogen). Sequences of the cloned segments have provided information to construct internal specific primers for amplifying abalone mtDNA. As a result, sub-fractionation of organelle DNA is no longer required in the PCR of mtDNA of the oxidase I and II genes because the specific primers (i.e. primers that do not recognise nuclear DNA coding regions) can be used. This in turn will allow amplification of variable regions that should give data on population differences and species markers.

Summary of the analysis of DNA extracted from canned blacklip abalone

Firstly, it should be noted that PCR amplification of mtDNA segments have been successfully used to identify 5 tuna species in 30 commercially

distributed fish cans (Unseld *et al.*, 1995). In the study, PCR amplified of cytochrome b segments using universal primers (i.e. normally amplify most species) was attempted, but not successful. This was attributed to the cooking process causing a degradation of DNA into fragments of approximately 100 bp. Therefore, new PCR primers were designed for amplification of small 59 bp segments of cytochrome b from the canned fish meat. This was a success, and the amplified segments were then cloned, and the DNA sequenced to produce species identity of the canned fish.

Total DNA (i.e. nDNA and mtDNA) extracted from canned blacklip abalone meat is very degraded and also showed a smear of low molecular weight sized DNA after separation by agarose gel electrophoresis (Fig. 6 left).

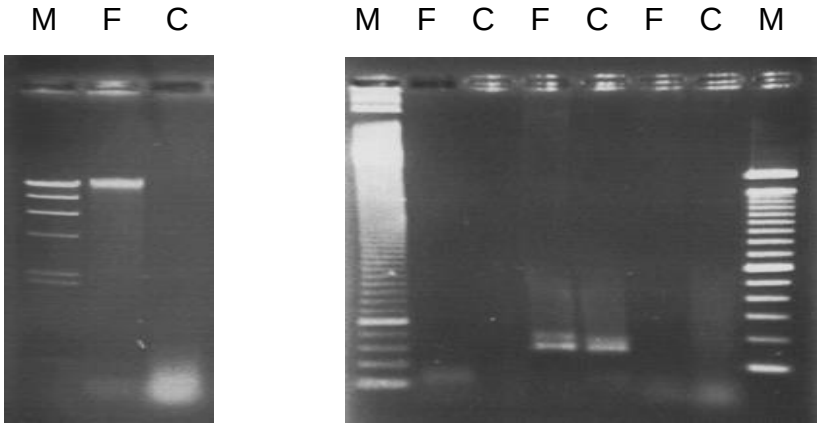

Figure 6. (Left): Comparison of total DNA extracted from fresh abalone (about 23 kbp; lane 2, F) and canned abalone (degraded into fragments of about 100 bp; lane 3, C), with DNA molecular weight markers (lane 1, M).

(Right): PCR amplification of STRs from degraded cooked abalone. Lanes: 1, 50 bp DNA ladder; 2&3, RUBGACA1; 4&5, RUBCA1; 6&7, RUBGT1; 8, 100 bp DNA ladder.

As a result, we have not been able to PCR amplify microsatellite DNA or mtDNA of >500 bp, due to the lack of large-sized template DNA remaining in the cooked meat. Amplification of smaller sized fragments was found to

require two rounds of PCR to produce good results. That is, a PCR was performed and the product then used in a second PCR. Target regions of 150 bp, or less, appeared to be the best for this type of work (Fig. 4 right). We have used primers to amplify two of the microsatellites (see RUBGACA1 and RUBCA1, Fig 4 right), and mtDNA, with success, but the ones amplifying alleles of >200 bp (e.g. minisatellite GHR with known PCR size ranges from 381-810 bp) just do not work.

We were able to show that samples of canned abalone supplied by local enforcement personnel were not black-lip abalone. For future compliance situations involving the identification of canned abalone, DNA tests need to be able amplify segments of low molecular weight DNA. This should be taken into consideration during the isolation and characterisation of microsatellites.

Benefits

As expected, the project has lead to better identification of genetic variation and population structure in blacklip abalone. The abalone populations were found to be more discrete and homozygous than once thought. We have attributed this to localised recruitment and inbreeding, and the implications are that that overfishing could wipe out a population.

At the start of the project we believed that the results would benefit the abalone fishery through the implementation of better management leading to a sustainable abalone fishery, rather than a major increase in productivity. Although abalone divers and processors benefit most from a sustained fishery, all Australians will benefit through a continuous and reliable level of export product. Identification of stocks also provides benefits in issues of compliance.

The flow of benefits to the state managed fisheries was expected to be mainly Victoria (50%), on which the study was centred, and flow-on benefits

to Tasmania (15%), South Australia (15%), Western Australia (15%), and New South Wales (5%). On the outcomes of the project, this was a reasonable breakdown of benefits.

Further Developments

- RAPDs gave good results for population studies and use in the identifying the origin of abalone samples. However, reliable repetition of results was difficult to control, due to many variables in the PCR conditions, and particularly temperature. As well, the analysis of bands was more tedious and difficult to interpret than microsatellites (STRs) or minisatellites (VNTRs). Minisatellites are rare and thus difficult to isolate. As they are are considerably larger in size compared with microsatellites, profiling tests in combination with microsatellites, are not easily automated.

Of the three DNA techniques used, microsatellites were the easiest to isolate and they also gave the very good discriminating data for the genetic structure of abalone populations. It is recommended they be used in future work of this type.

- Two additional microsatellites, a trinucleotide $(GGT)_n$ and a tetranucleotide $(AACT)_n$, were identified in the blacklip abalone genome during this study and further work is required to clone and sequence them for use in population studies. It should be noted that tri- and tetranucleotide microsatellites display greater separation differences between allelic bands in gel electrophoresis and lack of multi-banding (per allele), compared with dinucleotide microsatellites, making them easy to genotype.

Future studies should concentrate on these types of microsatellites.

- Some species-specific genetic markers were obtained using RAPDs, but this was not a main aim of the project. Recently, N. Sweijd (*pers. comm*.) has produced mtDNA markers for abalone species identification using PCR of mtDNA and subsequent restriction digestion of products.

Further work needs to be performed in this area to obtain tests and markers that can be used worldwide in compliance situations. However, species identification is only the first step, and what is much needed, is identification of where the abalone came from. This is population identification.

- Microsatellites were not isolated for the other Australian commercial species, *H. laevigata*, *H. roei* and *H. conicopora*.

Microsatellites now need to be obtained for these species and their degree of polymorphism tested for use in analysing abalone populations. This would need to cover abalone from all states of Australia to make the data of any real value.

- We have shown that PCR of microsatellites and mtDNA can be used in the DNA profiling of live, as well as processed samples (e.g. such as frozen, retort cooked, dried and canned abalone). The PCR products need to be relatively small, and preferably less than 150 bp for microsatellites for reliable results.

For compliance situations, small microsatellites need to be identified so that a battery of 5-7 tests standardised tests can be developed, and possibly automated.

- The DNA tests will need to be used in compliance situations.

Laboratory testing should be standardised and laboratories registered, such as occurs with DNA testing in humans.

- We have established a small be good DNA database for Victoria.

An extensive Australian survey of population samples, from all states, now needs to be set up in conjunction with state abalone fishery managers and compliance officers. A uniform sampling regime (e.g. at least 25 animals within a 100 m diameter of a given GPS recording) needs to be invoked so that data can be utilised in a national abalone DNA database. Tissue samples should also be stored from each site to act as standards.

- The high degree of homozygosity found in microsatellites used in the current study indicates an urgent need to analyse further the distribution of microsatellites in abalone populations.

Kinship and gene flow should be examined by studying abalone populations on reefs in close proximity. These studies could include local larval settlement traps to examine the DNA profiles of recruitments.

- It is likely that abalone populations will continue to show a high degree of homozygosity.

If this is the case, then important decisions for selection of broodstock in aquaculture need to be addressed.

Conclusions

The primary aim of this project was to apply modern molecular biology techniques to identifying the genetic composition and structure of blacklip abalone populations. In achieving this goal we developed, and tested, three molecular techniques, viz RAPDs, minisatellites and microsatellites. Of these, the microsatellite technique has been shown to be the best of the three

for simplicity and ease. It was shown that there were significant genetic differences among Victorian blacklip abalone populations of 60-70 km or more apart by RAPD markers, and 84 km or more by microsatellite markers. These data may suggest that neighbourhood sizes in this species rarely exceed 100 km. This estimate is smaller than the neighbourhood size of 500 km suggested by previous allozyme data (Brown 1991), but is consistent with the ecological evidence for limited dispersal and local blacklip abalone recruitment (McShane *et al*. 1988). The important issue arising from the results of this project is that, in order to maintain the resource, managers and divers need to consider carefully the effects of overfishing, especially on isolated populations.

Therefore, an important outcome of the study is the need to do microsatellite analyses using greater samples within Victoria, and to also extend the study to all states with a blacklip abalone fishery. Even greater abalone population substructure may be revealed, than that shown by our study, and such findings would have important applications in compliance.

A notable outcome of the study was the amount of genetic difference shown by the Port Phillip Bay animals compared oceanic populations, and especially with nearby ones in the central abalone fishery zone. Additional differences are also shown in our recent physiological research (data unpublished). Overall, these data reflect a long-time isolation of possibly up to 8,000 years when the Bay was thought to have formed. As such, the Bay abalone could almost be considered a sub-species of the blacklip.

In reference to cross-species testing with the minisatellites and microsatellites, the Western Australia brownlip abalone was positive for 4 of the 5 tests, showing it to be closely related to the blacklip. This has already been postulated by Brown (1991), based on his allozyme study. It was interesting to find that none of the 5 minisatellites and microsatellites, that we used in the blacklip population study, were detectable in greenlip abalone tissues. This would appear to be a good test in compliance situations, but

certainly there is a need to test more populations of both species southern across Australia before a definite conclusion can be made.

There were two additional aims of the project, based on results of identifying the genetic composition and structure of blacklip abalone populations. These aims were to transfer the results of techniques that best identified abalone populations to abalone fishery managers, and to utilise the techniques developed to identify abalone populations in enforcement situations. At the onset of the project we indicated that the results would be published in scientific journals in order to reach a wide audience. This has been accomplished and the publications are listed in Appendix 1: Valuable Information. As well, the information has been presented at several conferences. The primers used to amplify the minisatellites and microsatellites have been lodged with GenBank and are accessible to everyone on the internet.

In reference to using test in enforcement situations, we performed a small separate study for the Western Australian Fisheries to determine the most likely origin of an impounded sample of greenlip abalone. As there were no microsatellites available for profiling greenlip DNA, and a quick result was wanted, the RAPD technique was used. It clearly showed the samples to be more similar to Esperance greenlip abalone than any others tested, albeit only relatively few.

On a final note, it should be emphasised that our tests for profiling of blacklip abalone DNA were specifically developed for studying genetic population structure. It is believed that the tests could now be used to distinguish from which Victorian fishery zone, blacklip abalone originated, but with caution. As stated earlier, an extensive database of many populations from all states with abalone fisheries, is needed for cross-reference. Without databases for all commercial species the results of any test would be open to considerable criticism.

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Appendix 1
Valuable Information

The valuable information generated by this project is two-fold. Firstly, there are 5 sets of PCR primers that amplify 2 minisatellites and 3 blacklip microsatellites of blacklip abalone. These are available for further DNA studies of blacklip abalone, including genetic structure of populations and profiling of processed tissues. Details of the primer sets can be obtained from GenBank (see Accession Numbers p. 18) and the publications cited below (Reproduced with permission of the publishers in Appendices 5 - 8).

Secondly, there is a set of DNA profiles for 10 populations of abalone obtained from the Victorian and New South Wales coastline. This information collectively starts an Australian database and can be used as a reference to compare results obtained from future analyses.

Publications Arising from the Research
(Copied with permission of the publishers in Appendices 5 – 8)

Huang, B., Chai, Z., Hanna, P. J. and Gough, K. H. (1997). Molecular sequences of two minisatellites in blacklip abalone, *Haliotis rubra*. Electrophoresis, 18, 1653-1659.

Huang, B. and Hanna, P. J. (1998). Identification of three microsatellites in blacklip abalone, *Haliotis rubra* (LEACH), and detection in other abalone species. J. Shellfish Res., 17, 795-799.

Hanna, P. J., Chai, Z., Cummins, S. and Huang, B. (1999). Applications of molecular biology to abalone fisheries and aquaculture. J. Med. Appl. Malacol., in press.

Huang, B. X., Hanna, P. J. and Peakall, R. (1999). Molecular analyses of population genetic structure of blacklip abalone, *Haliotis rubra*, using RAPDs, minisatellite and microsatellite markers. Mar. Biol., in press.

Appendix 2
Participants in the Project

Associate Professor Peter Hanna	Project Supervisor
Dr Bixing Huang	Full-time PhD student
Ms Lyndal Kerr	Full-time PhD student
Mr Harry Gorfine (MAFRI)	Collection of abalone samples

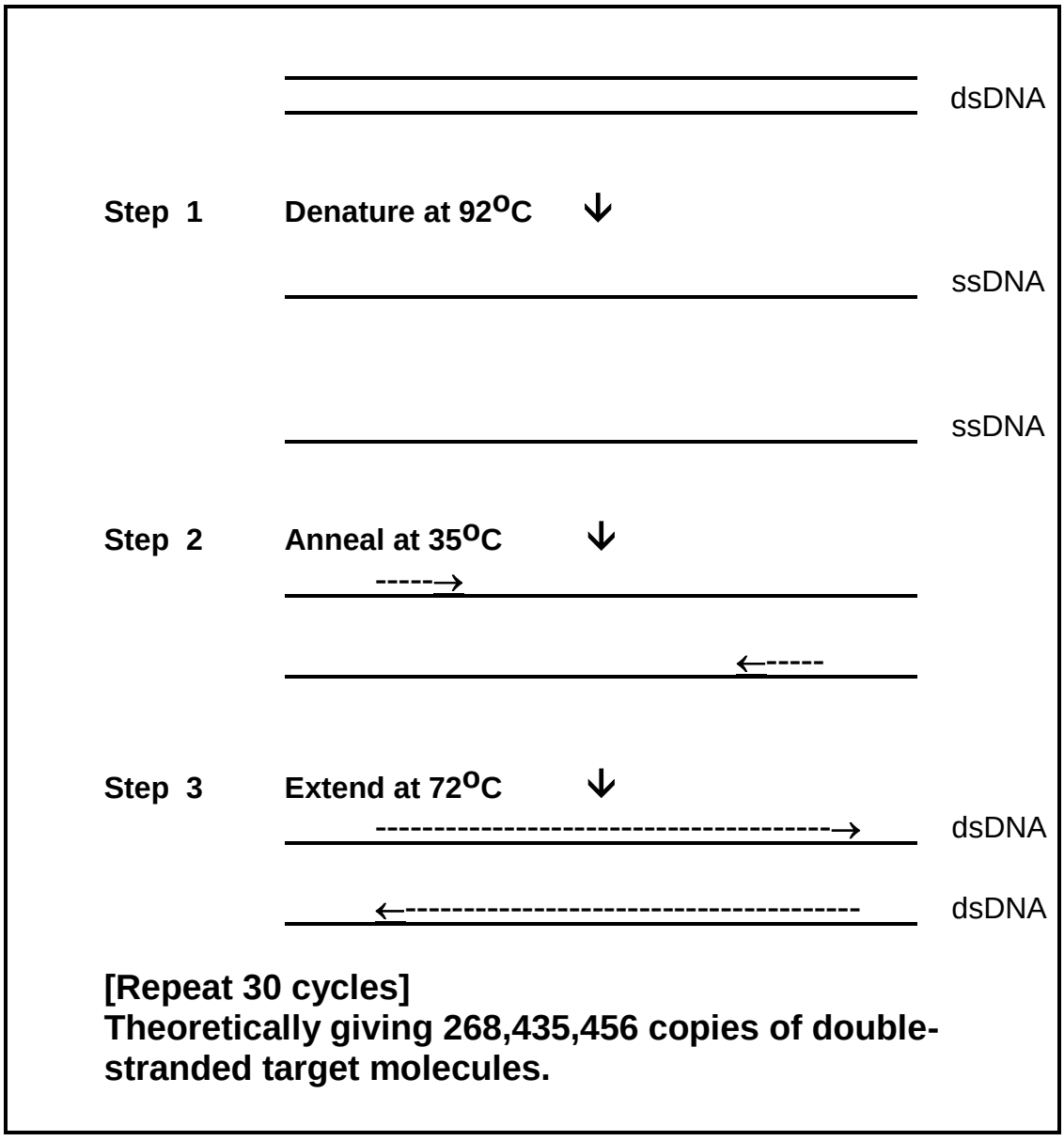
Appendix 3
Description of PCR (Polymerase Chain Reaction)

PCR (Polymerase Chain Reaction) is a technique for amplifying segments of double stranded DNA (dsDNA) into billions of copies. The fragments generated by this process can be analysed using gel electrophoresis. The principle of PCR is shown in the diagram (overleaf) and involves a 3-step reaction cycle, repeated many times.

In each cycle a small amount of extracted dsDNA (template DNA) is heated in a reaction mixture (buffer, nucleotides, heat-stable DNA polymerase such as *Taq*, oligonucleotide primers) until the dsDNA is denatured into single-stranded DNA (ssDNA). The reaction temperature is quickly lowered to allow annealing of the primers (lower temperatures for shorter primers), and the temperature is quickly raised to 72^{o}C, the optimum for the polymerase to synthesis new DNA making dsDNA. This is all done using an instrument called a thermal cycler. After about 30 cycles (each usually taking less than 10 min), there are billions of copies of the amplified section of DNA, between and including the primer sequences. Note that the primers anneal in opposite directions, in accordance with the alignment of the 2 strands in dsDNA.

Analysis of the PCR products commonly involves their separation in 0.8-25% agarose gels, followed by staining with ethidium bromide and visualisation of bands under UV light. Commercially available molecular weight DNA markers, or a mix of all allelic products in the case of VNTRs and STRs, run simultaneously on the gels with PCR products, allows size comparisons to be made.

Three-step cycle in the PCR amplification of DNA segments.



Appendix 4
Distribution List of the Final Report

This report will be distributed to each of the following organisations, as well as researchers and diver associations who have interests in abalone fisheries.

Australian Seafood Industry Council, PO Box 533, Curtin, ACT 2605

Australian Institute of Marine Science, PMB 3, Townsville MC, QLD 4810

CSIRO Division of Marine Research, GPO Box 1538, Hobart, TAS 7001

Commonwealth Research & Environment Committee, AFMA, PO Box 7051, Canberra Mail Centre, ACT 2601

NSW Fisheries, Port Stephens Research Station, Taylors Beach Road, Taylors Beach, NSW 2316

NSW Advisory Council on Fisheries Research, NSW FRI, PO Box 21, Cronulla NSW 2230

NT Fisheries Research & Development Advisory Committee, NT DPIF, GPO Box 990, Darwin, NT 0801

QLD Fishing Industry Research Advisory Committee, QDPI, Fisheries Group, GPO Box 3129, Brisbane, QLD 4001

SA Fisheries R&D Board, University of Adelaide, Zoology Dept., GPO Box 498, Adelaide, SA 5001

South Australian Fishing Industry Council, 22 The Parade, Norwood, SA 5067

South Australian Aquatic Sciences Centre, PO Box 120, Henley Beach, SA 5022

TAS Fisheries Research Advisory Board, TAS DPIF, GPO Box 192B, Hobart, TAS 7001

Tasmanian Fishing Industry Council, 117 Sandy Bay Road, Sandy Bay, TAS 7005

Fisheries Victoria, Department of Natural Resources and Environment, PO Box 500, East Melbourne, VIC 3002

VIC Fisheries Research Advisory Committee, MAFRI, PO Box 114, Queenscliffe, VIC 3225

Victorian Fishing Industry Federation, 7/20 Commercial Road, Melbourne, VIC 3004

WA Aquatic Resources R&D Advisory Committee, WARD, PO Box 55, Mt Hawthorn, WA 6016

Western Australian Fisheries Research Laboratories, West Coast Drive, Waterman, WA 6020

Appendix 5

Paper: Electrophoresis, 18, 1653-1659 (1997)

Molecular Sequences of Two Minisatellites in Blacklip Abalone, *Haliotis rubra*

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Abbreviations:

cDNA	complementary DNA
GH	growth hormone
GHR	growth hormone gene repeat
MIP	molluscan insulin-related peptide
MIPR	molluscan insulin-related peptide gene repeat
mRNA	messenger RNA
PB	phosphate buffer
RT	room temperature
SSC	sodium chloride and sodium citrate buffer

Key words: Minisatellite DNA, sequence, blacklip abalone

Summary

In the cloning and sequencing of growth promoting genes of the blacklip abalone, *Haliotis rubra* (Leach, 1814), two DNA variable number tandem repeats (VNTRs) were identified in abalone cDNA libraries. One contained a 33bp repeat unit (5'-CCCAAGGTCCCCAAGGTCAGGGAGGCGAAGGCT-3') located in the 3' untranslated region of a putative growth hormone (GH) gene, and the repeat was designated as GHR. The other contained an 18bp repeat unit (5'-ACCCGGCGCTTATTAGAG-3') located in the 3' untranslated region of a putative molluscan insulin-related peptides (MIP) gene, and was designated as MIPR. Primers flanking the two VNTR repeat regions were derived from sequence information. One hundred blacklip abalones were collected along the Victorian coastline and used in a preliminary population study. The range of GHR alleles containing the 33bp basic unit repeat motif included 7 to 20 repeats, with allele GHR 8 not being identified. The most frequent alleles contained GHR 16 and 17 repeats (56.0% and 16.5%, respectively). Four types of alleles were identified in MIPR, viz 4, 5, 6 and 7 repeats. The alleles containing 6 and 5 repeats were the most frequent ones (50.0% and 41.5%, respectively). Overall, the results indicate that these two DNA minisatellites have use in abalone studies, including paternity testing, triploid testing, population genetic structure and gene flow.

Introduction

Abalones are marine snails, belonging to the family *Haliotidae*. There are approximately 100 species of abalone found world wide in temperate waters, of depths ranging from the low tide line to in excess of 30 meters [5]. For centuries they have been a commercially important food source in China, Japan and USA. The blacklip abalone (*Haliotis rubra*) and greenlip abalone (*H. laevigata*) are the commercially important abalone species in Australia, and most of the Australian abalone products are exported to Asian countries. In one Australian state, Victoria, abalone exports were worth A$53 million (U$42.4 million) in 1994 [14]. The high

demand for abalone has increased pressure on natural stocks, which needs to be maintained if the fishery is to remain viable. To achieve this goal, there is a need for fisheries management to understand the genetic structure of abalone populations and to identify stocks of commercially important blacklip abalone.

It was reported that allozyme analyses had been used to study the genetic structure of blacklip abalone populations [6]. The results contradicted the previous ecological research [20, 23, 24, 29], which suggested that blacklip abalone larvae dispersal was generally on the scale of metres, and abalone juveniles and adults were entirely localized thereby minimizing gene flow.

Newer methods, involving molecular DNA analyses to assess abalone population structure, are now available. Analyses of the population structure has been revolutionized by the development of polymerase chain reaction (PCR) to amplify DNA [25], and the finding of a class of highly polymorphic DNA segment located in untranslated regions of genes. These DNA are commonly known as minisatellites or variable number tandem repeats (VNTRs) [2, 7, 16]. Minisatellite DNA is widely present in plant genomes [4, 10], humans [2], mammals [30], fish [3] and crustaceans [12, 31]. However, until this study, there had been no known minisatellite DNA sequences reported within the abalone family *Haliotidae*. During the cloning of blacklip abalone growth control genes, 2 minisatellite DNAs were found in abalone cDNA libraries. We now report their molecular characterisation and the usefulness of these minisatellite DNAs in a preliminary studies of the genetic structure of abalone populations.

Materials and Methods

Abalone samples: Blacklip abalone used in the construction of juvenile cDNA libraries were fast growing animals of 2 cm in length and artificially raised at Deakin University. Wild stock females were used to obtain tissues for construction of a cerebral ganglia cDNA library.

Ten adult blacklip abalone were collected from nine sites along the Victoria coast, and one site from the New South Wales coast (Fig. 1), making a total of 100.

Samples included six sites (60 animals) from the eastern zone and Eden, NSW, 3 sites (30 animals) including Port Phillip Bay from the central zone and 1 site (10 animals) from the western zone (see Fig. 1).

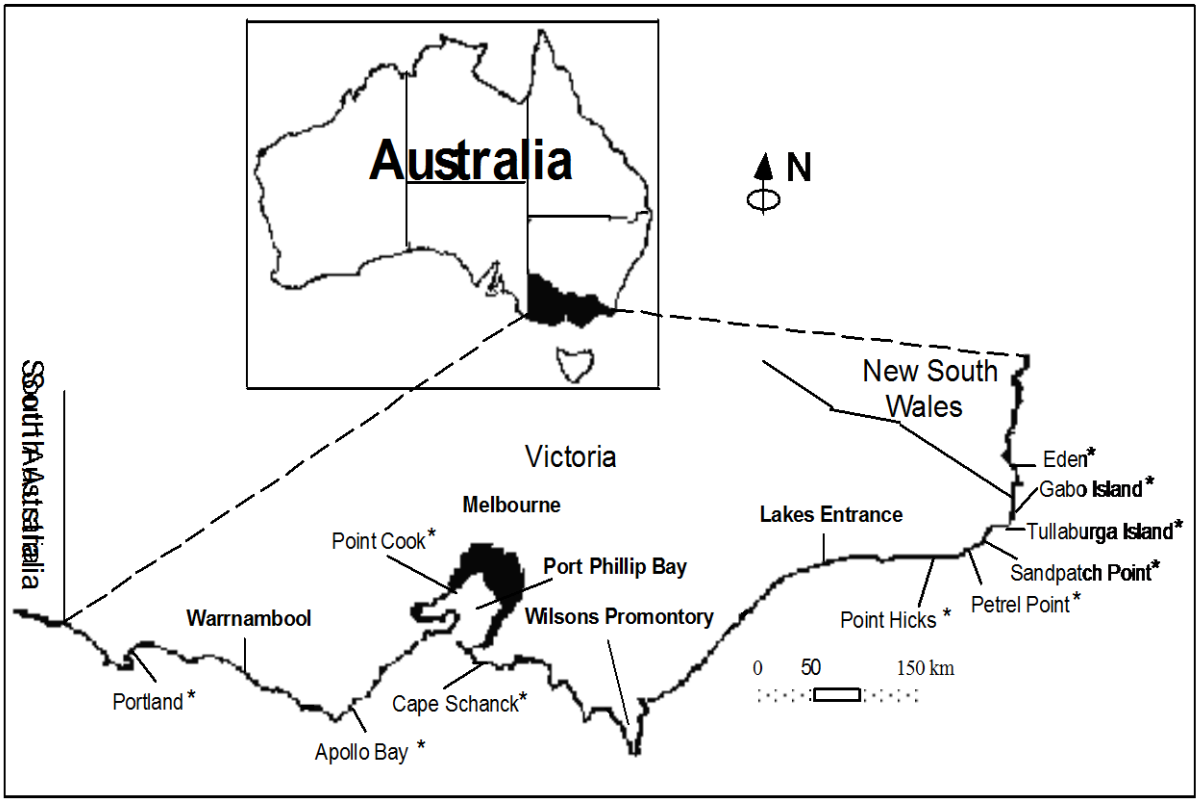

Fig. 1 Geographic sites where blacklip abalone (*H. rubra*) were sampled along the coast in Victoria and New South Wales, Australia. Asterisks show the study sampling sites.

After removing the animal from the shell, muscle tissue was taken from the region where the animal was attached to the shell. To prevent cross contamination of genetic material, a new pair of gloves and a new scalpel blade were used for the dissection of each abalone. Samples were stored at -70^{O}C prior to use.

Preparation of abalone lambda *gt*10 cDNA libraries: A procedure describing total RNA extraction from whole bodies of several juvenile abalone or the

cerebral ganglia adult abalone was followed [35]. The mRNA was enriched from total RNA using a mRNA purification kit (Pharmacia), after which cDNA was synthesised with a cDNA Synthesis System Plus (Pharmacia). An *Eco*RI/*Not*I linker (Invitrogen) with a phosphorylated blunt end and a non-phosphorylated *Eco*RI cohesive end was ligated to the cDNA using a DNA ligation system (Amersham). The cDNA was ligated into 1 μg lambda *gt*10 *Eco*RI arms using T_4 DNA ligase.

Probes and primers: 1. Probe N1041: 5'-GAACGCAGACAGCTG-3', common to some fish growth hormone (GH) genes in domain 5 [9, 15, 18]; 2. Probe N2072: 5'-CT(G/C)CACC(A/T)(G/A)CTGGCT-3', degenerate sequence of domain 1 of growth hormones from 20 vertebrate species [22, 28, 36]; 3. Probe N1577: 5'-GCACGGTTTCAGCAGCTTCACACACTAT-3', sequence of *Lymnaea stagnalis* molluscan insulin-related peptide-I (MIP-I) domain A [8, 32]; 4. Primers flanking the cloning site of lambda *gt*10 [8]: forward primer 5'-GAGGTGGCTTATGAGTATTTCTTCCAG-3' and reverse primer 5'-CTTTTGAGCAAGTTCAGCCTGGTTAAG-3'; 5. Primers for sequencing subcloned inserts in a M13 vector [8]: forward primer 5'-GTTGTAAAACGCGGCCAGTG-3' and reverse primer 5'-GAAACAGCTATGACCATGATT-3'; 6. Primers flanking GHRs [8]: forward primer N2788: 5'-ACCTCCAGAGGGCAATGAAC-3' and reverse primer N2923: 5'-CCTTCAGATCCTTGTTCCTTTTGC-3'; and 7. Primers flanking MIPRs [8]: forward primer, N2786, 5'-GCGAAAAAATGCAAAGGTCTAT-3' and reverse primer, N2849, 5'-AGGAGCAAACAACTACCGTATTTC-3'.

5' end labelling for oligonucleotide probes: In a 1.5 ml Eppendorf tube, 1 μ l oligonucleotides (100ng), 1 μl 10x PNK buffer, 3-4 μl (γ-^{32}P) ATP (10 pmol/6μl), 1 μl T4 PNK and water were added to make a final volume of 10 μl. The contents were mixed and incubated at 37^{o}C for 30 min. The enzyme was inactivated by heating at 65^{o}C for 20 min.

Library screening using nucleic acid probes: A lambda *gt*10 phage cDNA library ($5x10^4$-$1x10^6$pfu) was plated with *E. coli* C600Hfl onto plates which were

incubated until plaques were just visible. The plates were chilled at 4°C for 2 h and duplicate plaque lifts were performed using Hybond N nylon membrane discs (Amersham). The discs were placed DNA side up on Whatman 3MM paper saturated with denaturing buffer (1.5M NaCl, 0.5M NaOH) for 5 min, followed by neutralisation with neutralising buffer (1.5M NaCl, 1M Tris, pH 7.4) for 5 min and finally with 3x SSC for 5 min. The DNA was crosslinked to the membrane by UV light (Stratalinker). The plaque lift duplicates were subjected to hybridization with labelled probes. After autoradiography, the duplicates were compared and the matching spots were identified as hybridization positive.

The cDNA library constructed from adult abalone cerebral ganglia was screened using a probe N1041, derived from domain 5 in fish GH gene. The abalone cDNA library constructed from juvenile whole body was screened using probe N1577, derived from MIP-I domain A.

Southern transfer: Southern transfer of DNA was performed using the method described by Sambrook *et al*. [26].

Dot blot: Samples of 0.5-2.0 µl DNA were spotted onto a piece of Hybond N^+ nylon membrane (Amersham), denatured and crosslinked under alkaline conditions.

Hybridization: Dot blots, Southern blots or plaque lift discs were prehybridised in 5-30 ml prehybridization buffer (5x SSC, 20 mM PB, pH 7.0, 1% SDS and 10x Denhardt's solution). Labelled probes were added and hybridizations performed, using the same buffer as prehybridization, by rolling at 42°C-65°C for 2-24 h depending on the nature of the probe. The membranes were washed in 50-200 ml of 2x SSC, 0.5% SDS at 45°C for 1 h, with two changes of buffer. Washing at a higher temperature and more stringent buffer, (0.5x SSC, 1% SDS) was used, depending on the T_m of the oligonucleotide probe.

Analysis of DNA sequences: Nucleic acid sequences were determined by the dideoxynucleotide chain termination method [27]. The templates using manual sequencing in this study were ssDNA. Sequencing reactions were performed using

Sequenase version 2.0 (USB). The sequencing products (^{35}S-labelled) were separated on 6% polyacrylamide sequencing gel [26] and then dried before exposure to Kodak X-ray film in a cassette, overnight, or for a few days. After the film had been developed, the sequences were read and analysed by an IBI gel reader with a IBI Pustell program.

PCR products subcloned into pCR-Script^{TM} were sequenced by ABI automatic sequencer (Perkin Elmer).

Conventional PCR: If PCR products were to be sequenced, the proof reading enzyme Vent DNA polymerase (NEB) was used, otherwise *Taq* DNA polymerase (Perkin Elmer) was used. PCR reactions were carried out in 0.6 ml polypropylene thin wall tube (Scientific Plastics) in a volume of 15-100 μl reaction. For Vent DNA polymerase PCR, the reaction mixture contained 1x Vent DNA polymerase buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, pH 8.8, 2 mM $MgSO_4$ and 0.1% Triton X-100), 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, 0.5 μM (50 pmol/100 μl) of each primer, 1-2.5 units of thermostable Vent DNA polymerase (NEB) and 100 ng DNA template. For Taq DNA polymerase PCR, the reactions were the same as Vent, except the 1x Taq buffer contained 10 mM Tris-HCl, pH8.3, 50 mM KCl and 1.5 mM $MgCl_2$ and 1-2.5 units of Taq (Perkin Elmer). Amplifications were then performed with 30 cycles 3-step cycling reactions comprising DNA denaturing at 94°C, annealing of primers to templates at 45-65°C, and primer extension of the templates catalysed by the DNA polymerase at 72°C.

Autoradiography: Dot blots, Southern blots, discs or fixed sequencing gels were placed between two sheets of polyethylene film. A piece of X-ray film (Kodak) was placed in direct contact in a Kodak X-ray cassette. Exposures were carried out for a few hours to a few days, before developing.

Genomic DNA extraction: Genomic DNA used in population studies was extracted using a microwave DNA extraction method described by Goodwin *et al*. [13].

Electrophoresis and Silver staining: Routine electrophoretic analyses of DNA, including plasmids and PCR products, were performed in 1% agarose gels. Gels were stained with ethidium bromide and visualised under UV light. PCR amplified minisatellite products were separated by 6% (for GHR) or 7.5% (for MIPR) flat-bed PAGE, and visualised by silver staining [1].

Results

The finding of a GHR (putative growth hormone gene repeat) from a blacklip abalone cerebral ganglia cDNA library: Of the clones that were hybridization positive with probe N1041, fifteen were selected and their insert sizes checked by PCR using lambda *gt*10 primers flanking the cloning site. Various sizes of inserts were found, and the longest was approximately 1.2kb (see Fig. 2A). After the amplified DNA inserts had been transferred onto Hybond N by Southern transfer onto membranes, and hybridised using a second probe N2072 derived from domain 1 of GH, only one lambda insert, clone 8 (L8), was positive (Fig. 2B).

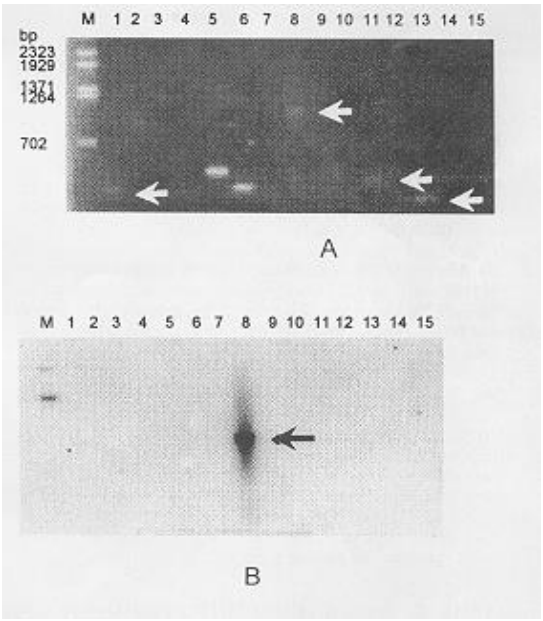

Fig. 2 Hybridization of abalone cDNA inserts with a GH oligonucleotide probe N2072. A: agarose gel electrophoresis of the abalone cDNA inserts amplified by PCR using primers flanking the lambda *gt*10 cloning site. The clones in lanes 1-15 were isolated from an adult abalone cerebral ganglia cDNA library. Lane M, DNA markers; B: Southern blots transferred from gel A and hybridised with the oligonucleotide probe N2072, washed with 2x SSC at 42°C and exposed for 4h at RT. Arrows indicate clone 8 strongly hybridised with the probe N2072 (B, lane 8).

The insert from L8 was subcloned into M13 sequencing vector to analyse its nucleic acid sequence. Fig. 3 shows part of the sequence of L8 (only the sequence of 3' untranslated region with repeat motif is given). The sequence of L8 was found to be a typical mRNA with a poly(A) tail. At positions 80-94 the sequence was found to be 100% homologous to the probe N2072, being 5'-CTCCACCAACTGGCT-3' (Fig. 3). In addition, there were 13 repeat sequences containing a 33bp basic repeat unit found in the 3' untranslated region. Although some minor variations of the basic repeat sequence were found, the 33bp basic repeat consensus sequence was:
5'-CCCAAGGTCCCCAAGGTCAGGGAGGCCAAGGCT-3'.

To determine DNA polymorphism in GHR, two primers N2788 and N2923 were synthesised, according to sequence information, and used to amplify the repeat region of several blacklip abalone genomic DNAs. Only one band or two bands were amplified from every sample of DNA, and band-size variations were observed. This met the theoretical profiles, either one band (if the sample was homozygous) or two bands (if the sample was heterozygous).

One amplified fragment from an abalone designated as B4, was calculated to have 7 repeats in its sequence by the estimated PCR product size 360bp. It was subcloned into pCR-Script^{TM} then sequenced. The sequence was compared with the L8 sequence (Fig. 3) and confirmed that the calculated number of repeats were present in the sequence.

```
L8     1 AGACTTTGTTGGTACTACTTGCGCTCTCCTTTG TTFCCACATGTTGGTCGCAACCCCCTCAGGGAA

L8    67 GGCGAGGGCCCAC*CTCCACCAACTGGCT*GTCCT CCCCCTAATCAGGGTATGGGAAAATCCCGCCAA
                     Probe N2072
                                                                Primer N2788 -->
L8   133 CGCAGACAACAGCGGCCAGAGGGTGACGTCCAC CTCCACCAACAGGATGCCCTCC*ACCTCCAGAGG*
B4     1                                                        -----------

L8   199 *GCAATGAAC*AATCCCGACCACGCAGACAAGCACAAC
B4    12 ------------------A-----------------

L8   235 AACAAGGTCCCCAAGGTCAGGGAGGCGAAGGCT CCCAAGGTCCCCAAGGTCAGGGAGGCGAAGGCT
B4    48 -----------G--------------C------ --G----------------------------

L8   301 CCCAAGGTCCCCAAGGTCAGGGAGGCCAAGGCT CCGAAGGTCCCCAAGGTCAGGGAGGCGAAGGCT
B4   114 ---------G---------------------- --------------------------------

L8   367 CCCAAGGTCGCCAAGGTCAGGGAGGCCAAGGCT CCCAAGGTCCCCAAGGTCAGGGAGGCCAAGGCT
B4   180 ---------C---------------------- --------T-----------------------

L8   433 CCGAAGGTCCCCAAGGTCAGGGAGGCGAAGGCT CCCAAGGTCGCCAAGGTCAGGGAGGCGAAGGCT
B4       ............................... ...............................

L8   499 CCGAAGGTCCCCAAGGTCAGGGAGGCGAAGGCT CCCAAGGTCCCCAAGGTCAGGGAGGCGAAGGCT
B4       ............................... ...............................

L8   565 CCCAAGGTCCCCAAGGTCAGGGAGGCCAAGGCT CCCAAGGTCGCCAAGGTCAGGGAGGCCAAGGCT
B4       ............................... ...............................

L8   631 *CGAAAGGTCAGAGGGGTGATGGCT* CCCAAGGTCAGAAAGGCGAAGGTTCCGAAGGTC
B4   246 ------------------------- -----------------A---------------

L8   688 AGAAAGGTGAAAGATCCTAAGGTCAGAGAGGCG AAGGCGGCAAAGGCCCCGAAGG*GCAAAAGGAAC*
B4   303 -----------------G---------A-A--- -----------------A---------------

L8   754 *AAGGATCTGAAGG*TTCCGAAGAAAGGCCCAGAA GACCCCCTCCACAAAGACGATGATTCCTGTGAA
B4   369 -------------
         <-- Primer N2923
L8   820 AGAAATAAAACAAATGAATACCAAAAAAAAAAA
```

Fig. 3 Nucleotide sequences of the abalone cDNA clone L8 and a PCR product B4 amplified from genomic DNA using primers N2788 and N2923 (sequences underlined and italicised). The repeat regions are given in bold type. B4 aligned well with L8, except in the variations specified. The dashes represents identical nucleotides of B4 to L8. The dots are gaps. The sequence clearly indicated that the variation of size of DNA amplified from primers N2788 and N2923 was due to the number of 33bp basic repeats in the sequence. There was a 24bp non-repeat (italicised) located before the last repeat. Probe N2072 hybridised to L8 at 80-94 (underlined and italicised).

The finding of a MIPR (putative molluscan insulin related peptide gene repeat) from a blacklip abalone juvenile whole body cDNA library: Screening of the abalone cDNA library, constructed from juvenile whole body, with probe N1577, derived from MIP-I domain A, resulted in 4 positive hybridization clones at a low stringency of 2x SSC and 42^{o}C. They were designated as MIPa1, MIPa2, MIPa3 and MIPa4. Insert sizes estimated by PCR using lambda *gt*10 primers showed inserts of MIPa1 and MIPa2 to be approximately 1200bp long. Inserts of MIPa3 and MIPa4 were approximately 1400bp and 1800bp, respectively (Fig. 4A). The four PCR products were subsequently used in dot hybridization with probe N1577 and all were found to be positive (Fig. 4B). After the four inserts were subcloned into the M13 sequencing vector and sequenced, a motif of an 18bp core repeat was found in MIPa1 (see Fig. 5 in which only the 3' untranslated region with the repeat motif is depicted). The consensus repeat sequence was 5'-GCCCGGCGCTTATTAGAG-3' and was designated MIPR.

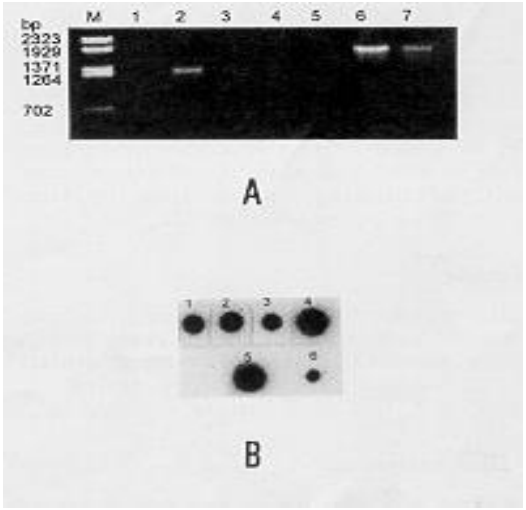

Fig. 4 A: Agarose gel electrophoresis of the abalone cDNA inserts amplified by PCR from the lambda clones screened from abalone juvenile whole body cDNA library using the MIP domain A oligonucleotide probe N1577. Lane M, DNA markers; each lane is the PCR product of abalone cDNA clones. 1 and 2, MIPa1; 3 and 4, MIPa2; 5, MIPa3; 6 and 7, MIPa4. B: Hybridization of PCR products of abalone cDNA inserts

with a MIP domain A oligonucleotide probe N1577, at washing stringency of 2x SSC, 42°C. Spot 1, MIPa1; 2, MIPa2; 3 and 6, MIPa3; 4 and 5, MIPa4.

Amplification of a genomic MIPR region and sequence comparison was subsequntly made using DNA of another animal and a pair of primers, N1860 and N1983, flanking the MIPa1 repeat sequence (Fig. 5). When the PCR product was subcloned into M13 sequencing vector and sequenced, it aligned with the cDNA MIPa1 sequence with the exception of an 18bp gap (Fig. 5). This can be explained by the use of different animals to produce the cDNA library and test in the PCR of genomic DNA.

```
                Primer N1860 -->
cDNA     451 TGAAGTTATG ATTTAAGCGA CCATTCCTTA ATATATTTAT TTTTAGACCA
genomic    1 ..----------------------------------------------------T

                          Primer N2786 -->
cDNA     501 AGGGAGAATA CATTTGCGAA AAAATGCAAA GGTCTAT
genomic   49 -T-------- -G-------- ---------- -------

cDNA     538 ACCCGGCGCTTATTAGAG ACCCGGTGCTTATTAGAG GCCCGGCGCTTATTTCTT
genomic   86 .................. ----A------------ ------------------

cDNA     592 AAAGCACTACTTCAT CACCGACGGTTATTAGAG GCCCGGCGCTTAATAGAG
genomic  122 ---------------- -C-G-G------------ ------------------

cDNA     643 GAAATACGGT AGTTGTTTGC TCCTTGTGTT TGTGGAGAAT CGTCACAACA
genomic  173 ---------- ---------- ---------- ---------- ----------
                   <-- Primer N2849

cDNA     693 CGTATTGTTA CTGAGAGGAT TGTGTGTGGA TGCTGCTTGT ACCTGTAAGT
genomic  223 ---------- ---------- ---------- ---------- ----------
                                  N1577 probe region

cDNA     743 GTGTTGAGGA TGGCATAAAA TCATTGAAAT CTGAAAAAAA AAAAAAAAA
genomic  273 ---------- ---------- ---------- ----
                              <-- Primer N1983
```

Fig. 5 Comparison of the sequences of abalone cDNA MIPa1 and a product amplified from genomic DNA, using primers N1983 and N1860 (based on the cDNA MIPa1 sequence). Genomic sequence with nucleotides identical to the cDNA sequence MIPa1 (cDNA) are represented by dashes. Only the substitute nucleotides are shown. The 18 dots in the middle of the genomic sequence is the gap of one repeat difference of the sequence in the genomic DNA. The repeat regions are given in bold type.

Distribution of GHR alleles in the population: In this study, each amplified fragment was completely resolved based on increments of the repeat unit of the VNTR locus using 6.0% flat-bed PAGE with subsequent silver staining. Fig. 6 shows some representatives of the GHR polymorphisms from 8 individual animals. The GHR polymorphism was examined using the primers N2788 and N2923, producing patterns with either one band or two bands. Overall, thirteen different-size fragments were observed in 100 blacklip abalones from 10 sites (see Fig. 1). The fragment sizes were determined by comparison to the DNA markers and the number of repeats was calculated (see the legend in Fig. 6). The alleles were designated as GHR7 to GHR20 according to the number of their basic repeats, except allele GHR8, which has not yet been observed.

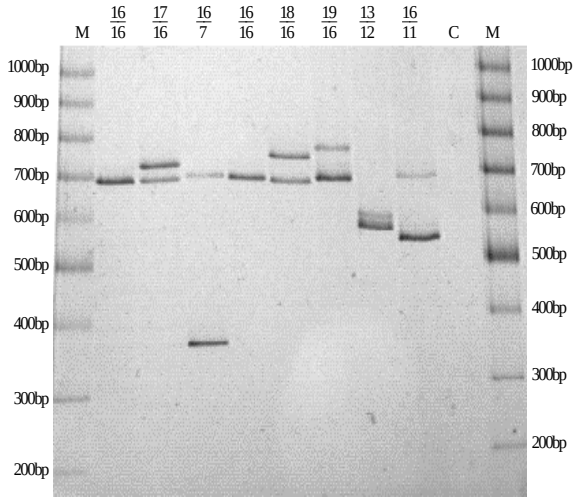

Fig. 6 Silver-stained PAGE gel displaying GHR profiles. The number of hypervariable repeats is equal to the size of the band in bases minus the sum of the 5' and 3' nonrepetitive flanking sequences divided by 33, the repeat length; eg., GHR 16 is 680 - 150, divided by 33 = 16.0. Lane C is a negative control and lane M is a standard 100 bp DNA ladder (Gibco BRL).

The distribution of observed genotypes of GHR in 100 samples is listed in Table 1. GHR16 and GHR17 were the most prevalent alleles, with frequencies of 56.0% and 16.5%, respectively. The observed heterozygosity was 64.0%.

Table 1. The distribution of observed GHR genotypes in 100 blacklip abalone samples from 10 sites.

Genotype	Observed (n=100)
7- 9	1
7-16	1
9-16	1
10-14	1
10-16	1
11-16	1
12-13	1
12-16	1
12-17	2
12-18	1
13-13	2
13-14	2
13-16	1
13-17	1
14-16	3
14-17	2
14-20	1
15-16	6
15-17	3
16-16	33
16-17	20
16-18	6
16-19	4
16-20	1
17-17	1
17-18	3
Total	100
Homozygosity	36.0%
Heterozygosity	64.0%

Distribution of MIPR alleles in the population: To examine the MIPR length polymorphism, two primers N2786 and N2849 (see Fig. 5) were used to amplify the MIPR from genomic DNAs. The products, which were electrophoresed on a 7.5% flat-bed PAGE and visulised by silver staining, showed alleles differing by

only 18bp in length (Fig. 7). Four different-size fragments were apparent, and their sizes ranged from 133bp to 187bp. Occasionally, there were non-specific 400bp to 700bp bands in a few samples. The alleles were designated MIPR4 to MIPR7, where allele MIPR4 is the 4-repeat VNTR and alleles MIPR7 is the 7-repeat VNTR.

Distribution of observed MIPR genotypes from 100 samples, as used in the GHR study, is shown in Table 2. MIPR6 and MIPR5 were the commonest alleles, with frequencies of 50.0% and 41.5%, respectively. The observed heterozygosity was 65.0%.

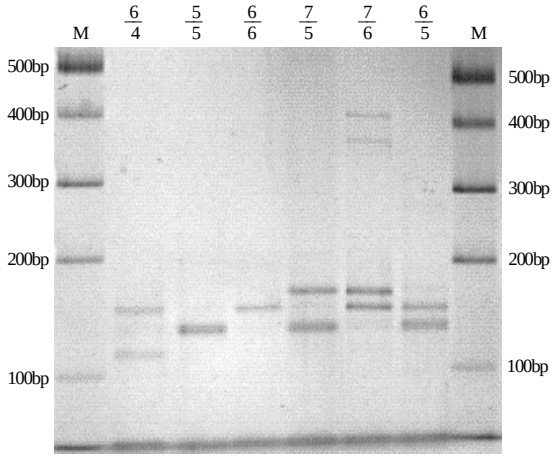

Fig. 7 Silver-stained PAGE gel displaying MIPR profiles. The number of hypervariable repeats is equal to the size of the band in bases minus the sum of the 5' and 3' nonrepetitive flanking sequences and spacer divided by 18, the repeat length; eg., MIPR 5 is 149 - 61, divided by 18 = 5.0. Lane M is a standard 100 bp DNA ladder (Gibco BRL).

2

Genotype	Observed (n=100)
4-6	1
5-5	14
6-6	21
5-6	48
5-7	7
6-7	9
Total	100
Homozygosity	35.0%
Heterozygosity	65.0%

Distribution of GHR and MIPR alleles in abalone regions: To evaluate regional variation of the GHR and MIPR, the allele frequencies of the eastern, central, western regions and Port Phillip Bay, were tabulated (Table 3). Some of the rare alleles were only observed in abalones from certain regions. Alleles GHR 7, 9 and 19 were detected in eastern region abalones, allele GHR 20 in central region abalones, allele GHR 11 only in western region abalones, and allele GHR 10 only in central and Port Phillip Bay abalones. Allele MIPR 4 was only detected in eastern region abalones. The MIPR 5 allele frequency in central region abalones was greater than that of other region abalones, but MIPR 6 allele frequency was lower than that of other region abalones. These results suggested that blacklip abalones along the Victorian coast could be divided into 4 main sub-populations: eastern, central, western and Port Phillip Bay populations.

Table 3. The distribution of GHR and MIPR repeat phenotypes in different regions.

Allele Frequency (%)	Sample regions			
	Eastern Zone (n=60)	Central Zone (n=20)	Port Phillip Bay (n=10)	Western Zone (n=10)
GHR 7	1.7%	-	-	-
GHR 9	1.7%	-	-	-
GHR 10	-	2.5%	5.0%	-
GHR 11	-	-	-	5.0%
GHR 12	1.7%	5.0%	-	5.0%
GHR 13	5.0%	5.0%	-	5.0%
GHR 14	0.8%	12.5%	15.0%	-
GHR 15	4.2%	5.0%	-	10.0%
GHR 16	56.7%	57.5%	50.0%	55.0%
GHR 17	18.3%	5.0%	25.0%	20.0%
GHR 18	7.5%	2.5%	5.0%	-
GHR 19	3.3%	-	-	-
GHR 20	-	5.0%	-	-
MIPR 4	0.8%	-	-	-
MIPR 5	34.2%	62.5%	40.0%	50.0%
MIPR 6	55.0%	32.5%	55.0%	45.0%
MIPR 7	10.0%	5.0%	5.0%	5.0%

Discussion

Of the 100 blacklip abalones from 9 coastal sites in Victoria and 1 in New South Wales (Fig. 1), the heterozygosities for the GHR locus and the MIPR locus were 64.0% and 65.0%, respectively. These values are substantially higher than the average 13.6% heterozygosity revealed in a 12 polymorphic allozyme study by Brown [6]. For a natural and assumed random mating population, 13.6% is a very low figure, and would indicate a conclusion of inbreeding within a population (Brown, 1991). The observed range of GHR and MIPR heterozygosity is within the 50-86% heterozygosity obtained in populations analyses of human ApoB, D2S44 and D17S79 VNTR loci [2, 19].

Genetic variation rate of DNA is not constant along the molecule. The variation rate in non-coding regions is much higher than that in coding regions [21, 34]. GHR and MIPR are located within 3' untranslated regions and exhibit obvious variations. For example, the observed variations included 3 homozygote genotypes and 23 different heterozygote genotypes within the GHR locus, and 2 homozygote genotypes and 4 different heterozygote genotypes detected in the MIPR locus (see Tables 1 and 2). Since allozymes are translated from relatively stable DNA coding regions, the population difference and total genetic diversities can not be effectively revealed by an allozyme study. In the teleost *Hoplostethus atlanticus* (orange roughy) from southern Australia and New Zealand waters, allozyme analysis results little genetic difference among populations, but DNA RFLP analysis indicated that stock separation was evident [11, 33]. The previous blacklip abalone allozyme results showed little genetic difference along the coast and no group of samples were genetically discrete [6]. The data obtained from GHR and MIPR loci along the coast show that the blacklip abalones could be initially divided into 4 sub-populations, eg. eastern, central, western and Port Philip Bay, and possibly into local populations (Huang, unpublished). Overall, observed DNA results here support the previous blacklip abalone larval recruitment research [20, 23, 24, 29] and suggest that blacklip

abalone local populations are relatively isolated. However, further studies and analyses are required to obtain greater details of population structure.

Acknowledgements

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Appendix 6

Paper: Journal of Shellfish Research, 17, 795-799 (1998)

IDENTIFICATION OF THREE POLYMORPHIC MICROSATELLITE LOCI IN BLACKLIP ABALONE, *HALIOTIS RUBRA* (LEACH), AND DETECTION IN OTHER ABALONE SPECIES

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ABSTRACT Three microsatellites were obtained from the screening of RAPD products and a genomic DNA library of blacklip abalone (*Haliotis rubra* Leach). They were assigned RUBGT1 (for *H. rubra* GT1) containing (GT)n repeats, RUBCA1 containing (CA)n repeats, and RUBGACA1 containing (GACA)n repeats. All were polymorphic in 100 blacklip abalone samples collected from the Victorian coast and Eden, New South Wales. There were 41 alleles identified at the RUBGT1 locus, 30 at the RUBCA1 locus, and 8 at the RUBGACA1 locus. PCR primers of the three microsatellites, together with primers of two minisatellites, a growth hormone gene repeat (GHR) and a molluscan insulin-related peptide gene repeat (MIPR), were used in cross-species amplification of 14 abalone species from the USA, South Africa, South Korea and Australia. Most amplifications occurred in the Australian species, *H. conicopora*.

KEY WORDS: Microsatellite sequences, polymorphism, abalone, *Haliotis*.

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INTRODUCTION

Satellite DNA, a type of tandemly arranged highly repetitive sequence, has been found to exist widely in animals and plants (Beridze, 1986). By definition, the repeat unit size within the range 7-70 bp is known as a minisatellite or variable number of tandem repeat (VNTR), whilst the repeat unit size between 1-6 bp is known as a microsatellite or short tandem repeat (STR) (Budowle *et al*. 1991, Kimpton *et al*. 1993). Minisatellites are usually located in introns (Griffiths *et al*. 1993), or 3' end non-translated regions (Budowle *et al*. 1991, Huang *et al*. 1997), and a considerable number have been identified in animals and plants (Jeffreys *et al*. 1991, Rafalski *et al*. 1996). There are abundant microsatellites located in both genic and extragenic regions of the eukaryotic cell genome (Kimpton *et al*. 1993). For example, in the snail, *Bulinus truncatus*, it is estimated that on average there is a dinucleotide microsatellite locus every 40,000 bp and a tetranucleotide microsatellite locus every 60,000 bp (Jarne *et al*. 1994).

The abundance of VNTRs and STRs, their polymorphic nature, and amenability to amplification by PCR, have made them ideal markers for genetic studies, including genetic linkage analysis, antenatal diagnosis, forensic medicine, population genetic structure and evolutionary studies (Bosch *et al*. 1993, Jeffreys *et al*. 1991, Budowle *et al*. 1991, Deka *et al*. 1995, Primmer *et al*. 1996). There is only one report concerning two minisatellites in blacklip abalone (*Haliotis rubra* Leach), viz a growth hormone gene repeat (GHR), and a molluscan insulin-related peptide gene repeat (MIPR) (Huang *et al*. 1997). There are no reports concerning microsatellites in abalone, and there is a need for them, particularly for use in population studies and aquaculture.

We describe in this report three microsatellites obtained from screening a blacklip abalone genomic DNA library and random amplified polymorphic DNA (RAPD) products of blacklip abalone. As well, we report on three microsatellite alleles identified in blacklip abalone samples from Victorian and New South Wales waters, together with cross-species amplification of DNA from additional abalone species using three microsatellite primer sets and two minisatellite primer sets.

MATERIALS AND METHODS

Abalone samples

Fourteen species of abalone were collected from Australia, Korea, USA and South Africa (see Table 2). In addition, 100 blacklip abalone collected for a population genetic structure study were from nine sites along the Victorian coast, and one site from New South Wales. The site details have been described by Huang *et al*. (1997), and the abalone were provided frozen at -20^{o}C, or preserved in 75% ethanol. Each abalone was dissected using a new set of latex gloves and surgical blades in order to prevent cross contamination. After removing the shell, 1.5 g of soft inner muscle or gonad was taken, and stored in sterile 1.5 mL Eppendorf tubes at -70^{o}C.

DNA extraction and purification

Abalone DNA was extracted using a method of microwaving tissue in lysis buffer followed by phenol:chloroform extraction (Goodwin and Lee, 1993). DNA to be cloned and sequenced was purified using a QIAquick^{TM} Gel Extraction kit (QIAGEN), according to the manufacturer's instructions.

Detection of microsatellites in blacklip abalone RAPD products and a genomic library

RAPD products were generated from abalone genomic DNA using primers UBC 101 ($^{5'}$GCGGCTGGAG$^{3'}$), UBC135 ($^{5'}$AAGCTGCGAG$^{3'}$) and M13 ($^{5'}$GAGGGTGGCGGTTCT$^{3'}$), in 50µL volumes according to Williams *et al.* (1990). RAPD products were transferred to membranes by Southern transfer described in Sambrook *et al*. (1989), and subsequently hybridized with labelled probes to detect bands containing microsatellite sequences. The procedures for hybridization and detection of microsatellites utilized 3' end fluorescein labelled oligonucleotide probes produced with a RENAISSANCE^{TM} kit (Du Pont). Labelled probes used in this study included: $(CA)_{10}$, $(GA)_{10}$, $(CT)_{10}$, $(GT)_{10}$, $(GGT)_{7}$, $(GATA)_{5}$, $(GACA)_{5}$,

$(GGGT)_5$, and $(AACT)_5$. Positive bands were excised from additional agarose gels, DNA fragments extracted and cloned into a pCR-Script Vector (Stratagene) for sequencing, following the manufacturer's instructions. The inserts were sequenced with T3 and T7 primers.

A blacklip abalone genomic library was obtained from Dr. Z. Chai (Medical School, Monash University). It was constructed using *Hae*III digestion of whole juvenile blacklip abalone genomic DNA and subsequent ligation of 1-3 kb fragments into lambda *gt*10 phage. Double plaque lifts were prepared and membranes hybridized with labelled probes. Positive plaques were subcultured, and inserts amplified by PCR using lambda primers. PCR products were purified from gels and sequenced in both directions using lambda sequencing primers.

Cloned RAPD products, or purified PCR products, were sequenced utilizing a Perkin Elmer ABI PRISM^{TM} Dye terminator cycle sequencing kit and autosequencer (Model 377). Alleles of microsatellites were designated by the number of repeats units contained within sequences; for example, allele RUBGACA1-5 contained 5 units of a GACA repeat.

Electrophoresis and staining of gels

Minisatellites (GHR and MIPR) were analyzed using 0.4mm ultrathin 6% polyacrylamide gel, following the method of Budowle *et al*. (1991). Microsatellites (RUBGT1, RUBCA1 and RUBGACA1) were analyzed using standard 6% denaturing polyacrylamide sequencing gels. Gel preparation and electrophoresis conditions followed the manufacturer's instructions (Bio-Rad). A non-mutagenic silver stain method, described by Budowle *et al*. (1991), was used to visualize separated PCR fragments in both types of gels.

PCR conditions

Protocols for PCR amplification of MIPR and GHR minisatellites followed Huang *et al*. (1997). PCR conditions for analyzing variation amongst individuals was performed using 30x 3-step cycling reactions, consisting of a hot start at 94^{o}C for 5

min, followed by 30 cycles of denaturing at 94^{o}C for 30 sec, annealing step for 1 min, and extension at 72^{o}C for 15 sec. The primer sequences and annealing temperatures are listed in Table 1.

Table 1. Primer sequences and annealing temperatures (Ta) used in the analysis of microsatellite loci.

Locus	Ta	Primer designation	Primer sequence
RUBGACA1	50^{o}C	RUBGACA1F (forward)	$^{5'}$CGCCGTTTTATTCGTCACCAATC$^{3'}$
		RUBGACA1R (reverse)	$^{5'}$CCACATATACAAATAAATATATC$^{3'}$
RUBCA1	61^{o}C	RUBCA1F (forward)	$^{5'}$CCAATTTTACTTGAAGACTTGTGATGC$^{3'}$
		RUBCA1R (reverse)	$^{5'}$ATGTGTACGCGTTGGTGGATGG$^{3'}$
RUBGT1	61^{o}C	RUBGT1F (forward)	$^{5'}$AGGGTGGCGGTTCTGGTCCTAAATC$^{3'}$
		RUBGT1R (reverse)	$^{5'}$GGCAGTGATGATATAGCGTTGTTCG$^{3'}$

RESULTS

Identification of a (GT)n microsatellite in blacklip abalone

Abalone RAPD fragments, amplified with the M13 primer, are shown in Fig 1A. Subsequent Southern transfer of the RAPD products and hybridization with a 3' end fluorescein labelled $(GT)_{10}$ probe resulted in hybridization positive bands of 900-1000bp (Fig. 1B). A hybridization positive band from A7 (corresponding to lane 7 in Fig. 1A) was found to contain 38 GT repeats. The sequence was submitted to GenBank (Accession Number AF027572). This microsatellite locus was designated RUBGT1 (for *H. rubra* GT1).

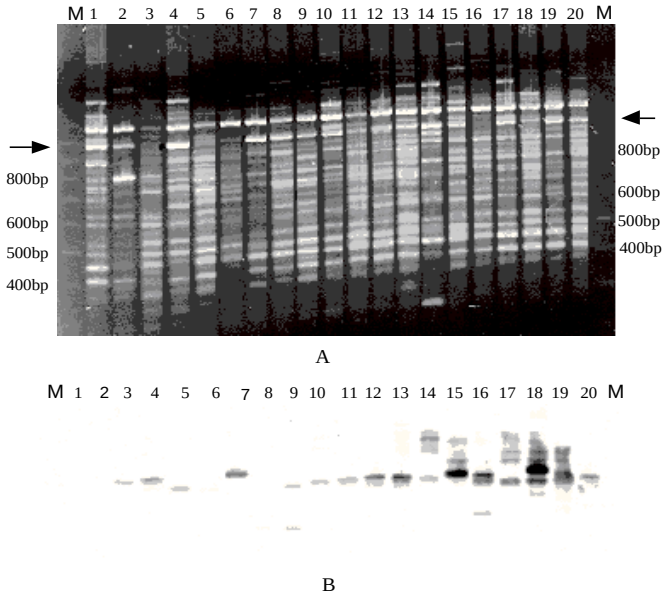

Figure 1. Identification of a (GT)n repeat in blacklip abalone RAPD products. (A) RAPD products of blacklip abalone DNA amplified with a M13 random primer (5'-GAGGGTGGCGGTTCT-3'). Arrows indicate location of hybridizing bands in B. Lanes: 1-10, animals from Tullaburga Island; 11-20, animals from Eden; M, 100 bp DNA ladder (Promega). (B) Hybridization of a $(GT)_{10}$ probe to RAPD products shown in A.

Identification of a (CA)n microsatellite in blacklip abalone

Abalone RAPD products, amplified with primers UBC 101 and UBC 135, are displayed in Fig. 2A. Hybridization of Southern transferred RAPD products with a 3' end fluorescein labelled $(CA)_{10}$ probe resulted in positive bands of 800-900bp (Fig. 2B; Lanes 1 to 16). Positive bands were present in the RAPD products amplified with primer UBC 101, but not in RAPD products amplified with primer UBC 135. Sequencing of a hybridization positive band from Petrel Point (corresponding to lane 1 in Fig. 2A) showed the presence of a 30 repeat microsatellite, composed of $(CA/G)_{18}(CA)_{12}$. The sequence was submitted to GenBank (Accession Number AF027573). This microsatellite locus was designated RUBCA1.

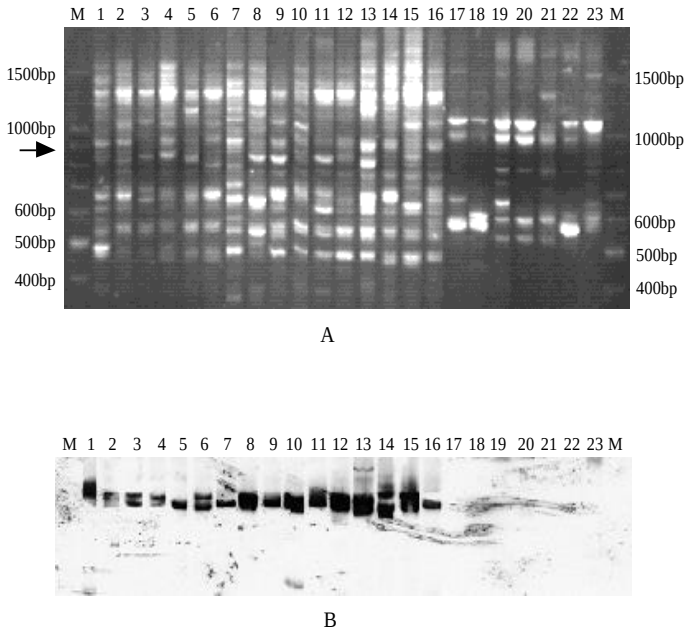

Figure 2. Identification of a (CA)n repeat in blacklip abalone RAPD products. (A) RAPD products, with arrows indicating the location of hybridizing bands in B. Lanes: 1-16, RAPD products amplified with UBC-101 (5'-GCGGCTGGAG-3'), in which 1-7 are RAPD products of animals from Sandpatch Point, and 8-16 from Petrel Point; 17-23, RAPD products from Sandpatch point amplified with UBC-135 (5'-AAGCTGCGAG-3'); M, 100 bp DNA ladder (Promega). (B) Hybridization of a $(CA)_{10}$ probe to RAPD products of A.

Identification of a (GACA)n microsatellite in blacklip abalone

Screening of a lambda *gt*10 blacklip abalone genomic DNA library produced one hybridization positive plaque in 2,500 plaques screened. Subsequent sequence analysis of the insert showed that there were 6 repeats of GACA tetranucleotides. The sequence was submitted to GenBank (Accession Number AF027574). This microsatellite locus was designated RUBGACA1. Fig. 3 shows amplified blacklip abalone microsatellite alleles containing known GACA repeats, with the exception of the RUBGACA1-13 allele.

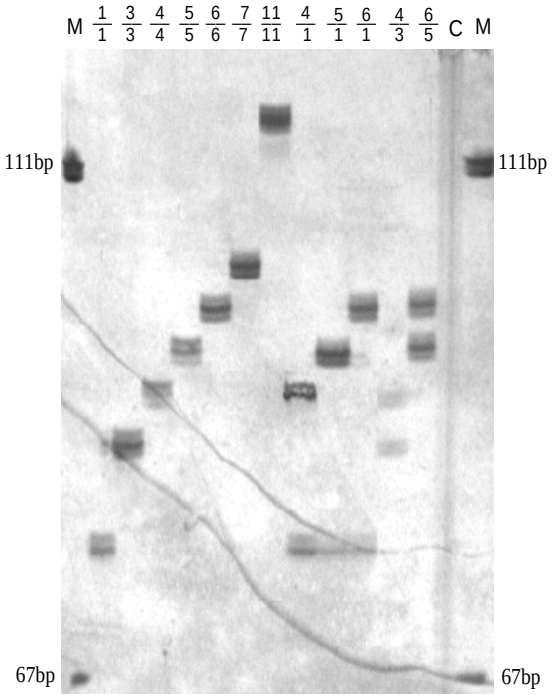

Figure 3. Alleles of the RUBGACA1 locus identified in blacklip abalone. Alleles are designated by number of repeat units. Genotypes of animals are given at the top of each lane, and indicate the alleles present.

Allelic frequencies of three microsatellites in blacklip abalone

Amplification of DNA extracted from 100 blacklip abalone collected along the Victorian coastline and Eden, New South Wales, using the three microsatellite primer sets, showed that all three microsatellite loci were polymorphic. There were 41 alleles found at the RUBGT1 locus, which were defined as RUBGT1-1 to 51. Alleles RUBGT1-2, 4, 6, 7, 8, 9, 10, 16, 28 and 50, were not observed. There were 30 alleles identified at the RUBCA1 locus. These alleles ranged from RUBCA1-13 to 47, with the exception of alleles RUBCA1-14, 16, 17, 19 and 46, which were not observed. There were eight alleles identified at the RUBGACA locus, ranging from RUBGACA1-1 to 13, except alleles RUBGACA1-2, 8, 9, 10 and 12, which were not observed. A summary of the allele frequencies of the 3 microsatellite loci for the 100 abalone are displayed in Fig. 4. The allele frequencies of all three loci were found to depart significantly from Hardy Weinberg equilibrium ($p<0.001$).

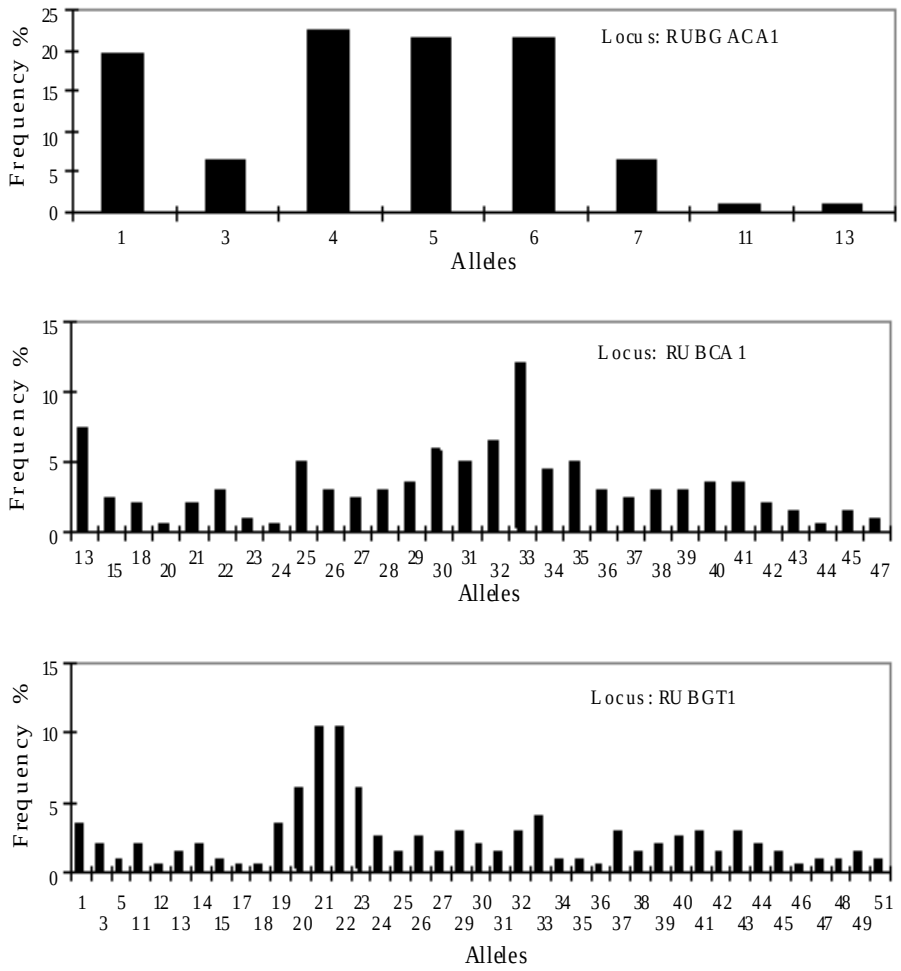

Figure 4. Allelic frequencies of three microsatellites observed in 100 blacklip abalone of the Victorian coast and Eden, New South Wales.

The application of the five sets of mini-/microsatellite primers to other abalone species

Fourteen abalone species collected from four countries (USA, South Africa, South Korea and Australia), were used in a study utilizing all five primer sets developed for minisatellite and microsatellite studies of blacklip abalone populations. The results listed in Table 2 show that there were no amplifications of DNA from four species, *H. rufescens*, *H. cracharodii*, *H. pervum*, and *H. midae*, from USA and South Africa. However, primers for amplifying the RUBGT1 locus amplified the DNA of the two South Korean species, *H. gigantea* and *H. sieboldi*. Of the other four

Australian abalone species, DNAs from two species, *H. roei* and *H. scalaris*, were amplified with 2 sets of primers. DNA from *H. conicopora* was amplified with four sets of primers, but was not amplified with the MIPR locus primers. None of the five primer sets could amplify DNA from *H. laevigata*.

Table 2 The use of mini- and microsatellite primer sets on DNA of different abalone species.

Country	Species	Sample No.	Microsatellites			Minisatellites	
			A	B	C	D	E
USA	*H. rufescens*	1	-	-	-	-	-
		2	-	-	-	-	-
	H. cracharodii	1	-	-	-	-	-
		2	-	-	-	-	-
SOUTH AFRICA	*H. pervum*	1	-	-	-	-	-
	H. midae	1	-	-	-	-	-
		2	-	-	-	-	-
		3	-	-	-	-	-
		4	-	-	-	-	-
SOUTH KOREA	*H. discus**	1	-	-	-	-	-
		2	-	-	-	-	-
	*H. discus***	1	-	-	-	-	-
		2	-	-	-	-	-
	H. gigantea	1	-	-	28/28	-	-
	H. sieboldi	1	-	-	27/27	-	-
		2	-	-	27/27	-	-
	H. discus hannai	1	-	-	-	-	-
		2	-	-	-	-	-
	H. diversicolor	1	-	-	-	-	-
		2	-	-	-	-	-
AUSTRALIA	*H. rubra*	1	4/5***	37/37	13/13	5/5	14/18
		2	1/4	31/31	9/19	5/6	14/20
	H. laevigata	1	-	-	-	-	-
		2	-	-	-	-	-
	H. roei	1	3/3	-	29/29	-	-
		2	3/3	-	19/19	-	-
	H. scalaris	1	-	7/7	11/28	-	-
		2	-	11/11	11/11	-	-
	H. conicopora	1	1/1	36/39	20/24	-	16/16
		2	1/1	40/43	9/11	-	16/17

Note: A = RUBGACA1; B = RUBCA1; C = RUBGT1; D = MIPR and E = GHR
*, *H. discus* was sampled from a hatchery; **, *H. discus* was sampled from the sea; ***, alleles present in a diploid individual are expressed in the number of repeats (e.g. 4 and 5); -, no amplification. Samples of *H. rubra*, *H. laevigata*, *H. roei* and *H. scalaris* were from South Australia, and samples of *H. conicopora* were from Western Australia.

DISCUSSION

Microsatellite sequences are normally obtained by screening genomic DNA libraries (Jarne *et al*. 1994). However, it is a tedious process to construct and screen genomic DNA libraries, and to purify and sequence the clones containing STRs. Recently, it has been found that the bands amplified with a single arbitrary primer (i.e. RAPDs) often contain microsatellite DNA sequences (Richardson *et al*. 1995). As the RAPD method does not require any DNA sequence knowledge of the organisms, and is relatively easy to perform, the products provide a good source and start point to search for microsatellites. This approach circumvents the construction of genomic DNA libraries, but has limitations. Firstly, a number of RAPD primers need to be tested in order to determine those showing reliable band variations among population samples. Secondly, there is also some difficulty extrapolating from hybridization positive bands back to the complementary band in agarose gels, so that cloning and sequencing of DNA can be achieved.

In this study, three microsatellites were successfully cloned and sequenced. Of these, two dinucleotide microsatellites, RUBCA1 and RUBGT1, were identified and cloned from two RAPD products of blacklip abalone. The microsatellite motif (CA)n/(GT)n may be the most frequent microsatellites in the blacklip abalone genome, as hybridizations of other microsatellite probes (i.e., $(GA)_{10}$, $(CT)_{10}$, $(GGT)_7$, $(GATA)_5$, $(GGGT)_5$, and $(AACT)_5$), with RAPD products and a lambda *gt*10 library, were all negative. Interestingly, the (CA)n/(GT)n microsatellites are reported to be the most frequently occurring dinucleotide microsatellites in mammalian genomes (Rafalski *et al*. 1996). The polymorphism and codominant features of the three microsatellites have made them useful in population differentiation and genetic structure studies of blacklip abalone (Huang 1997). In particular, they have revealed significant molecular variation in a study of 10 blacklip abalone populations (Φ_{PT}=0.067, $p<0.001$).

Minisatellite and microsatellite primers developed for blacklip abalone were applied to some of the abalone species in the world to determine whether

microsatellite marker systems established here could be transferable to other abalone species. It has been reported that the detection of polymorphic STR loci in birds, using the primers developed for swallow, is related to evolutionary distance (Primmer *et al*. 1996). Therefore, the evolutionary relatedness of abalone species could possibly be assessed using the five mini- and microsatellite markers developed for blacklip abalone. Of the 14 species tested, no amplifications occurred for the overseas abalone species, with the exception of the South Korean species *H. gigantea* and *H. sieboldi* at the RUBGT1 locus. The minisatellite MIPR was a species-specific locus for blacklip abalone, as there was no amplification detected in other species. Amplifications occurred mainly in the Australian sympatric species, *H. roei*, *H. scalaris* and *H. conicopora*. It is expected that abalone species in Australia would be closer in evolutionary terms than overseas species, and of the species in Australia, *H. conicopora* displayed amplification at four out of five loci, and *H. roei* and *H. scalaris* at two out of five loci. Therefore, *H. conicopora*, also named brownlip abalone, appears to be more closely related to blacklip abalone than the other three species. Similar results were observed in allozyme analysis by Brown (1991), in which the genetic distance of blacklip abalone to other species, from closest to least related, were *H. conicopora*, *H. roei*, *H. scalaris* and then *H. laevigata*. It was unexpected that there was no amplifications at the five mini- and microsatellite loci for greenlip abalone, as the observed incidences of hybridization between blacklip and greenlip abalone in nature indicates that a close evolutionary correlation exists between these two species (Brown, 1991). Additional microsatellites need to be characterised to assess more fully the evolutionary distances among abalone species.

ACKNOWLEDGMENTS

We sincerely thank the following people for providing abalone samples in this study. They are Dr. S. Shepherd of the South Australian Department of Fisheries, Dr. D. Blackman and Mr. B. Hancock of the Fisheries Department of Western Australia, Dr. J. Je of the Korea Ocean Research and Development Institute, Prof. D. Morse of the University of California in Santa Barbara, Dr. P. Britz of Rhodes University and

Mr. H. Gorfine of the Victorian Marine and Freshwater Research Institute, Queenscliff. This project was funded by the Fisheries Research and Development Corporation.

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Appendix 7
Paper: Journal of Medical and Applied Malacology
(in press)

APPLICATIONS OF MOLECULAR BIOLOGY TO ABALONE FISHERIES AND AQUACULTURE

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ABSTRACT

In many countries of the world abalone are in high commercial demand as a seafood. Some of the abalone fisheries have maintained a relatively constant supply, whereas supplies of others have decreased to a level where closure has occurred. This has put pressure on maintaining supply by better management of the fisheries or supplementation of supply through aquaculture. As a result, there has been an increasing number of molecular biology projects related to abalone fisheries and aquaculture, several of which overlap both areas. These include genetic markers obtained from RAPDs, minisatellites, microsatellites and mtDNA, all of which are importance in determining genetic variation and structure within wild populations, and identifying poached abalone. Others include the isolation, cloning and expression of abalone genes to produce products for use in spawning, settlement and enhancing growth rates, and production of transgenic animals. A current review of these projects is presented.

KEY WORDS - *Haliotis* spp., molecular biology, DNA profiling, larval settlement, egg-laying hormone, developmental genes

INTRODUCTION

Abalone are herbivorous gastropods belonging to the family Haliotidae, phylum Mollusca. Of the 60 to 70 known species, about 10 to 20 are of commercial importance, and of these only a few make up the major part of the world production. Although commercial fishing of the mollusc began in Japan and China over 1500 years ago, the Australian abalone industry is now the largest producer of abalone. The main abalone product is the delicately flavoured meat of the large muscular foot, and worldwide demand for this valuable resource has risen steadily (Hooker and Morse, 1990).

Abalone fishing around the world has lead to major decline of some stocks, and in some cases closure (Davis *et al*., 1992; Guzman del Proo, 1992; McShane, 1992). Increasing incidences of poaching have also imposed pressures on the natural stocks. These factors, plus a number of others, have resulted in a growing need for research to conserve the valuable resources, and to maintain the sustainable production of the commercially important abalone. As well, there is an increasing pressure to supplement diminishing or depleted stocks by aquaculture. Therefore, there are an increasing number of molecular biology projects related to abalone fisheries and aquaculture. Several of theses are described in this paper.

DNA Profiling of Abalone by Randomly Amplified Polymorphic DNA (RAPD)

RAPDs provide a means of investigating the genetic structure of populations, an understanding of which could assist in abalone management program. In a pilot study using RAPDs, greenlip abalone (*H. laevigata* Donovan) samples were sourced from three states, Western Australia (WA; via Mr. D. Blackman of Fisheries Western Australia), South Australia (SA; via Dr. S. Shepherd of the South Australian Research and Development Institute) and Victoria (VIC; via Mr. H. Gorfine of the Victorian Marine and Freshwater Research Institute). The sample sizes collected from populations (see Fig. 1) were as follows: Augusta, WA, 4; Hamelin Bay, WA, 6;

Esperance, WA, 6; West Island, SA, 4; Point Cook, VIC, 4. Six poached abalone samples, seized by WA Fisheries WA, were also supplied (location unknown).

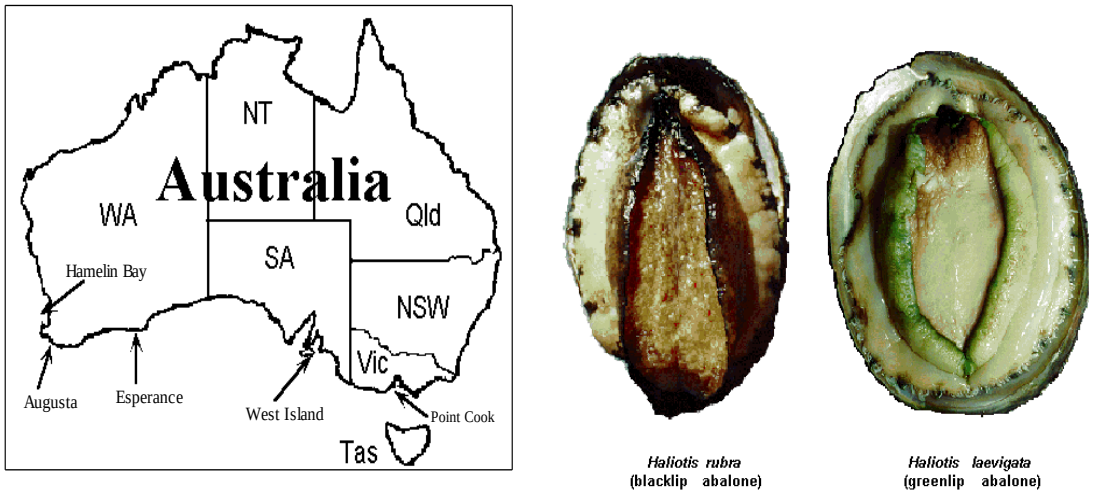

Figure 1. Locations of greenlip abalone sample collections along the southern coast of Australia, together with pictures of the blacklip and greenlip abalone.

Extraction of DNA from samples and the RAPD-PCR conditions are published elsewhere (Huang *et al.*, 1999). The RAPD profiles of greenlip abalone were generated by three primers, UBC-159 (GAGCCCGTAG), UBC-169 (ACGACGTAGG) and RM13 (GAGGGTGGCGGTTCT), although 14 primers obtained from the University of British Columbia (Set No. 2) showed potential for RAPD analyses of greenlip abalone. The results are given in Table 1. If a RAPD band was shared by 95% or more individuals, it was regarded as a monomorphic band, and was eliminated from analysis (Hartl and Clark, 1989). The three primers produced 51 polymorphic bands out of a total of 57 bands, giving an average polymorphism of 89.47%. These polymorphic bands were collectively used to analyse the genetic structure of the greenlip populations.

Table 1. Details of primers used in RAPD analysis of greenlip abalone populations

Primer	Number of amplification bands	Range of bands in individuals	Monomorphic bands (≥95% shared)	Number of polymorphic markers	Polymorphism (%)
UBC-159	18	1-12	2	16	88.89
UBC-169	16	4-12	0	16	100.00
RM13	23	5-16	4	19	82.61
Total	57		6	51	Average 89.47

The five greenlip abalone populations were analysed under the null hypothesis, viz that there was no genetic differentiation among the populations, and treated as a single group in an initial analysis of molecular variation determined with AMOVA software (Table 2), resulting in a Φ_{PT} value of 0.184 ($p<0.001$). Therefore, there was significant molecular variation along the southern Australian coastline.

Table 2. AMOVA analysis of greenlip abalone populations along the coast

Source of variation	Degree of freedom (df)	Sums of squares (ss)	Mean squares (ss/df)	Expected mean squares	Estimated variance components	Φ_{PT}	Significance
Among Population	(P-R)=4	59.0	14.75	$\sigma^2_w+4.8\sigma^2$	$S^2_R=1.61$	0.184	p<0.001***
Within Population	(N-P)=19	135.33	7.12	σ^2_w	$S^2_w=7.12$		
Total	(N-1)=23	194.33					

Note: P is the number of populations (P=5); R is the number of regions (R=1); N is the number of samples (N=24); ***, highly significant.

A further AMOVA analysis results of three greenlip abalone state groups are listed in Table 3, indicating that there was significant molecular variation within greenlip abalone populations along the southern Australian coast ($\Phi_{PT}=0.223$, $p<0.001$). The greenlip abalone from the three states showed significant molecular variation ($\Phi_{RT}=0.131$, $p=0.031$). Although only three populations were collected

from WA, the AMOVA results indicate that there was significant molecular variation among the three populations within a region (Φ_{PR} =0.107, p=0.011).

Table 3. AMOVA analysis of greenlip abalone from three states (VIC, SA and WA)

Source of variation	Degree of freedom	Sums of squares	Mean squares ss/df	Expected mean squares	Expected variance components	Φ-statistics	Significance
Among groups	(R-1)=2	35.83	17.92	$\sigma^2_w+\tilde{n}_2\sigma^2_p+\tilde{n}_3\sigma^2_R$	S^2_R = 1.197	Φ_{RT} =0.131	p=0.031*
Among populations/ group	(P-R)=2	23.17	11.58	$\sigma^2_w+\tilde{n}_1\sigma^2$	S^2_p = 0.849	Φ_{PR} =0.107	p=0.011*
Within Population	(N-P)=19	135.33	7.12	σ^2_w	S^2_w=7.12	Φ_{PT} =0.223	p<0.001***
Total	(N-1)=23	194.33					

Note: R=3; P=5; N=14; $\tilde{n}_1$ = 5.25, $\tilde{n}_2$ = 4.25 and $\tilde{n}_3$ = 6.0; *, significant (p<0.05), ***, highly significant.

A dendrogram of the five populations sampled from VIC, SA, and WA (Fig. 2), were clustered by NTSYS-pc software using a pairwise Φ_{PT} matrix among them calculated by AMOVA. The dendrogram shows that the greenlip abalone populations from Port Phillip Bay, VIC and West Island, SA clustered independently from WA populations, while WA populations clustered very closely. Overall, the greenlip abalone populations clustered into three major groups, VIC, SA, and WA.

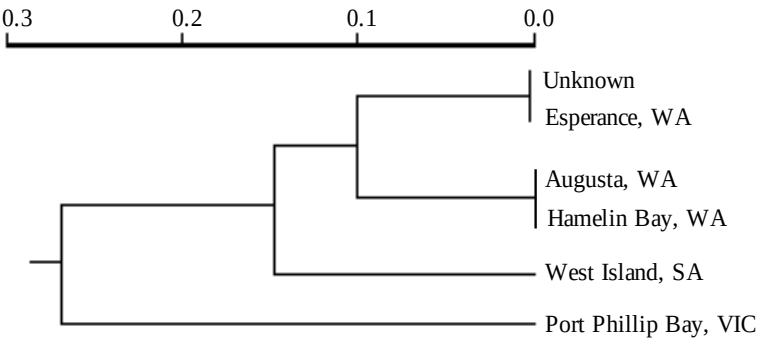

Figure 2. Relatedness of poached greenlip abalone with the greenlip abalone populations from WA, SA and VIC.

A genetic analysis was performed on a group of poached greenlip abalone in order to estimate its genetic relatedness to greenlip populations already analysed. The RAPD profiles of the poached greenlip abalone were analysed together with the RAPD profiles of greenlip abalone samples from WA, SA and VIC. The poached greenlip abalone clustered with the greenlip abalone samples from Esperance in WA (Fig. 2). A principal component analysis (PCA) of the individual greenlip samples has supported that the likely origin of the poached greenlip abalone as Esperance, WA. Five out of the six samples in the poached group clustered closely with five out of six samples taken from Esperance, WA. They did not cluster with the other samples taken from West Island, SA, and Port Phillip Bay, VIC (Fig. 3).

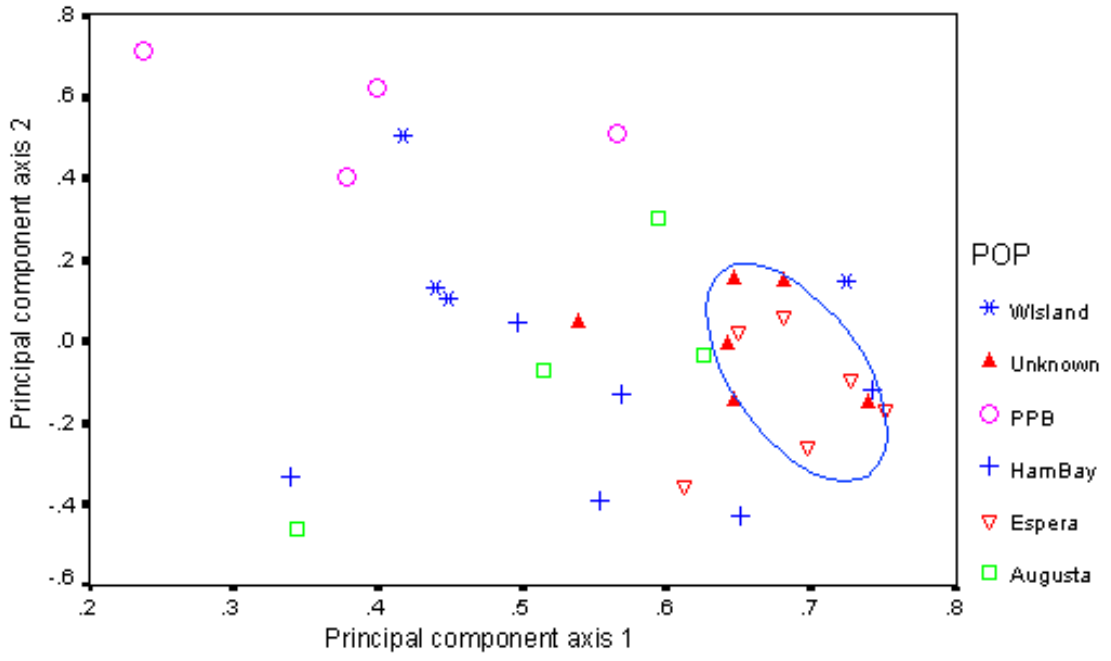

Figure 3. A principal component analysis of greenlip abalone samples. (∴) WIsland, West Island, SA; (σ) Unknown, poached samples; (O) PPB, Port Phillip Bay, VIC; (+) HamBay, Hamelin Bay, WA; (∇) Espera, Esperance, WA; (□) Augusta, Augusta, WA.

Applications of Additional DNA Markers

Minisatellite and microsatellite markers, have demonstrated considerable promise for population genetic and evolutionary studies. This is particularly important in studies of populations in which there is high selfing and a lack of allozyme polymorphisms (Hedrick 1992; Huff *et al*. 1993; Hughes and Queller 1993; Peakall *et al*. 1995; Viard *et al*. 1996). Minisatellite and microsatellite markers have now been identified in *Haliotis rubra* Leach, the Australian blacklip abalone (Huang *et al*., 1997; Huang and Hanna, 1998). A study combining RAPD, minisatellite and microsatellite markers to analyse population genetics has now been carried out on blacklip abalone populations in the state of Victoria (Huang *et al*., 1999), showing that there was significant subdivision in the populations along the coastline. This was attributed to the relatively short pelagic duration and limited dispersion of abalone larvae. The study also found that a population sampled from Port Phillip Bay, a semi-enclosed bay, was genetically discrete. There was an excess homozygosity observed in genotypes of the microsatellite loci, and was thought to be due to localised larval recruitment pattern (Prince *et al*., 1987; McShane *et al*., 1988), and asynchronous spawning of populations (McShane *et al*., 1986). Overall, the results show a need for further studies to confirm whether local populations are differentiated by larval recruitment, asynchronous spawning and effects of overfishing of adults. Management programs may need to consider these issues.

The minisatellite and microsatellite markers can also be used in tracing relationships of broodstock with progeny in aquaculture situations. Several single-locus tests can reveal these relationships, and it is possible to store samples of gametes from parents for DNA testing at later dates. The same tests can be used to study kinship in wild populations.

Mitochondrial DNA (mtDNA) is also commonly used in DNA typing. This is fundamently due to there being many copies per cell, thus enabling better PCR amplification of mtDNA sectors compared with nuclear loci. However, it often requires sequencing after a PCR to allow comparisons to be made. In recent studies of abalone mtDNA, it has been shown that PCR amplification of the mtDNA control

region, and subsequent restriction enzyme digestion of PCR products, can give good species specific markers (N. Sweijd, *pers. comm.*). This has already provided useful information in the identification of abalone meat.

Abalone Egg-Laying Hormone

Egg-laying in the two gastropods, *Aplysia californica* Cooper and *Lymnaea stagnalis* L. has been shown to be a hormone response to expression of genes located in neural ganglia. These hormone-producing genes have been previously cloned and characterised (Scheller *et al*., 1983; Vreugdenhil *et al*., 1988). Conserved DNA probes and PCR primers were made for the identification and amplification of an abalone egg-laying hormone (aELH) gene in the Australian blacklip abalone (*H. rubra*). PCR amplification of genomic blacklip abalone DNA with *Pfu* polymerase, and subsequent DNA sequencing identified an abalone ELH (Wang and Hanna, 1998). Further cloning and sequencing studies have shown a very high nucleotide homology (99.1%) between the two Australian abalone species *H. rubra* and *H. laevigata*, and with *Haliotis discus hannai* Ino and *Haliotis gigantea* Gmelin (provided by Dr Je, KORDI, from Korea) (Fig. 4). This homology was shared with *L. stagnalis* more than *A. californica*, although *L. stagnalis* is more closely related taxonomically. In regard to the amino acid sequences, all share the same number (i.e. 36), and there is a very high homology in comparison to CDCH in *L. stagnalis*. Although *A. californica* has 20 changes (44.4% homology), the abalone species examined have no more than 2 amino acid differences (94.4% homology), indicating the conserved nature of the hormone amongst gastropods.

A

```
ELH     A.C..C....A.C.G...T..AA.......A.A....TGCTG..TACA..
CDCH    CTTTCGATCACCAATGACCTGCGGGCTATCGCTGACAGTTACCTGTACGA
HRUB    ..................................................
HLAE    ............C...................C.................
HDISH   ..............................C.................
HGIG    ..............G.................C.................
                .         .         .         .         .
```

```
ELH     G..----..T.C....A....AAAG.T.TCTC.CTG.....C.CCA....C..T.G..AAAG
CDCH    CCAGCACAAGCTGAGAGAGCG----GCAAGAAGAGAACCTAAGAAGACGTTTCCTAGAGCTC
HRUB    ......................----..............................GAGA...
HLAE    ......................----..............................GAGA...
HDISH   ......................----..............................GAGA...
HGIG    ......................----..............................GAGA...
                 *         *             *         *         *
```

B

```
ELH     I..NQ..K..T.ML.TE.IRE.Q.YLAD..Q.L..K
CDCH    LSITNDLRAIADSYLYDQHKLRERQEENLRRRFLEL
HRUB    ................................R.
HLAE    ....M...........................R.
HDISH   ................................R.
HGIG    ....M...........................R.
                 *         *         *
```

Figure 4. Nucleotide (A) and amino acid (B) sequences of the egg-laying hormone of various gastropods. *A. californica* (from Scheller *et al*., 1983); CDCH, *L. stagnalis* (from Vreugdenhil *et al*., 1988); *H. rubra* (HRUB), *H. laevigata* (HLAE); *H. discus hannai* (HDISH) and *H. gigantea* (HGIG). Dots represent similarity with CDCH and dashes represent gaps for best alignment. * represents groups of 10 nucleotides or amino acids.

With further data concerning the egg-laying prohormone sequences of haliotids, it may be possible to examine phylogeny of the genus *Haliotis* using molecular data. A study of this type was carried out by Lee and Vacquier (1995) using DNA sequence data of abalone sperm lysin. They found that 22 of the 27 species were clearly distinguishable by greater than 20 nucleotide differences, and that there were three groupings which were aligned with the previously recognised sub-genera *Nordotis*, *Paua* and *Padollus*.

The egg-laying genes of all the commercially important abalone species from around the world are now being cloned and sequenced. Expression of the genes in appropriate expression vectors is also being carried out for use in bioassays. It would be expected to find that, due to the high homology of the peptides, a peptide from one species might be effective in another. Two pilot bioassays found the expressed egg-laying peptide of *H. discus hannai* induced spawning of two lots of 3 female red abalone (*Haliotis rufecscens* Swainson) in approximately 190 min.

Abalone Larval Settlement

There is a general lack of knowledge concerning the nature of abalone larval settlement inducers in the wild. Therefore, with the development of aquaculture there has been a growing number of molecular investigations in this important area. Many studies indicate that GABA (gamma-butyric acid), and GABA-like compounds released from crustose coralline algae, cause competent free-swimming abalone larvae to cease swimming and metamorphose (Morse *et al.*, 1979; Morse and Morse, 1984). As a result, GABA is now used in some aquaculture facilities for inducing the settlement of larvae. However, Seki and Kan-No (1981) have shown that abalone larvae settle on the mucous trails of juvenile and adult abalone of conspecifics, but will not settle on the mucus of other species. This implies that there is a settlement inducer present in the mucous. However the question of separate cues attachment and metamorphosis has been raised (Roberts and Nicholson, 1997). Studies have begun to isolate and characterise effector molecules.

Abalone Growth Genes

Efficient aquaculture requires fast growing animals, so there is a strong interest in identifying genes that control animal growth. However, in order to clone growth regulating genes of abalone, it is an important first step to know what to identify. In a study, efforts were made to determine the presence of the abalone genes related to GH (growth hormone), IGFs (insulin-like growth factors) and insulin, by Southern and Northern hybridizations, and PCR-based techniques (Chai, 1996). No positive results were obtained from both abalone Southern and Northern hybridizations using reference cDNA probes. This was not surprising if the abalone genes for growth regulation had very little nucleic acid sequence homology with the reference genes. However, RT-PCR, 5' and 3' RACE techniques were used to successfully amplify abalone cDNA fragments, using primers designed to anneal (i.e. hybridise) to the conserved regions of growth hormone genes. These fragments were subsequently

analysed for their properties and identities with known reference genes, that have been stored in the GenBank data base.

Abalone cDNA libraries were prepared using the mRNA from the cerebral ganglia and whole bodies of juvenile abalones, as well as the cerebral ganglia of adult female abalones. The juvenile abalones were sampled when they were at a fast growing stage, and presumed to contain greater numbers of mRNA transcripts of the growth promoting genes to be cloned. Several clones were examined, of which two are described.

One abalone cDNA clone, designated L8, isolated from the lambda *gt10* abalone cDNA library, contained an open reading frame of 54 amino acid residues (Fig. 5). The nucleic acid sequence showed a general structure of mRNA with a poly(A) tail and a polyadenylation signal sequence AATAAA, 7 bases away from the poly(A) tail. A minisatellite (i.e., containing repeated DNA sequences) was identified in the 3' untranscribed region, and subsequently characterised (Huang *et al.*, 1997). The amino acid sequence LHQLA, encoded by the probe region was identified, and was conserved between all known GHs. The region at 80-94 shows the probe annealing site. However, the remaining sequence is relatively short in length (54 amino acids) has a lack of homology with other GH's. Further investigations, such as expression of this peptide and bioassays of growth rates, are required.

```
1                AGACTTTGTTGGTACTACTTGCGCTCTCCTTTGTTGCCAC

 41  ATG TTG GTC GCA ACC CCC TCA GGG AAG GCG AGG GCC CAC
  1   M   L   V   A   T   P   S   G   K   A   R   A   H

 80  CTC CAC CAA CTG GCT GTC CTC CCC CTA ATG AGG GTA TGG
 14   L   H   Q   L   A   V   L   P   L   M   R   V   W

119  GAA AAT CCC GCC AAC GCA GAC AAC AGC GGC CAG AGG GTG
 27   E   N   P   A   N   A   D   N   S   G   Q   R   V

158  ACG TCC ACC TCC ACC AAC AGG ATG CCC TCC ACC TCC AGA
 40   T   S   T   S   T   N   R   M   P   S   T   S   R

197  GGG CAA TGAACAATCCCGACCACGCAGACAAGCACAACAACAAGGTCCC
 53   G   Q   *
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```
246  CAAGGTCAGGGAGGCGAAGGCTCCCAAGGTCCCCAAGGTCAGGGAGGCGAA
297  GGCTCCCAAGGTCCCCAAGGTCAGGGAGGCCAAGGCTCCGAAGGTCCCCAA
348  GGTCAGGGAGGCGAAGGCTCCCAAGGTCGCCAAGGTCAGGGAGGCCAAGGC
399  TCCCAAGGTCCCCAAGGTCAGGGAGGCCAAGGCTCCGAAGTCCCCAAGGTC
450  AGGGAGGCGAAGGCTCCCAAGGTCGCCAAGGTCAGGGAGGCAAGGCTCCGA
501  AGGTCCCCAAGGTCAGGGAGGCGAAGGCTCCCAAGGTCCCCAAGGTCAGGG
552  AGGCGAAGGCTCCCAAGGTCCCCAAGGTCAGGGAGGCCAAGGCTCCCAAGG
603  TCGCCAAGGTCAGGGAGGCCAAGGCTCGAAAGGTCAGAGGGGTGATGGCTC
654  CCAAGGTCAGAAAGGCGAAGGTTCCGAAGGTCAGAAAGGTGAAAGATCCTA
705  AGGTCAGAGAGGCGAAGGCGGCAAAGGCCCCGAAGGGCAAAAGGAACAAGG
756  ATCTGAAGGTTCCGAAGAAAGGCCCAGAAGACCCCCTCCACAAAGACGATG
807  ATTCCTGTGAAAGAAATAAAACAAATGAATACCAAAAAAAAAAAAAA
```

Figure 5. Abalone cDNA clone L8 nucleic acid sequence and deduced amino acid sequence. The primer annealing site is in bold and underlined, the stop codon is indicated by an asterisk, and the polyadenylation signal sequence at the 3' end is underlined. Repeat sequences in the 3' UTR are either underlined or double underlined to show the two types of repeats.

Another open reading frame encoding 150 amino acid residues was identified in an abalone cDNA clone, designated L10 (Fig. 6). It was not clear if the first methionine at the deduced residue 65 was an internal methionine or the leading residue. The deduced amino acid sequence was found to have homology with several sequences in the GeneBank data base. Two proteins with the highest amino acid homology with the abalone clone L10 were the *Drosophila* dorsal-ventral patterning gene *TOLLOID* (Shimell *et al.*, 1991), with a 51.351% similarity and 28.378% identity, and the human bone morphogenetic protein (BMP-1) (Wozney *et al.*, 1988), with 52.3% similarity and 27.2% identity. Other homologous proteins with lesser homology included BMP-2A, BMP-2B and BMP-3. Cysteine residues (C) at 43, 67, 94 and 108 were present in the homologous sequences, but *TOLLOID* and human

BMP have more than 1000 amino acid residues, and are much longer than the amino acid sequence deduced from the abalone cDNA clone L10. Whether L10 is a full length cDNA, and whether the observed open reading frame is a complete open reading frame, are still to be determined. Overall, the amino acid sequence homology with the human BMPs implied that the protein encoded by the abalone cDNA clone L10, could be related to the shell growth regulation, since the growth of both the abalone shell and human bone, involves similar metabolism, calcium deposition.

```
   1                CGAAAACAACCAACACTGTGACATCACTATCCGCTCTCCCCC

  43  CACCCCTCACATCACCACTGTCAACTTTACCCGTTTTGACATCGAAGACGA

  94  TGCGGGCTGCGACTATGACAGTGTGTCAGTGACAAGTGGCGGCACCACTGA
                                                         *

 145  GAA ACT GGA GCT AAA GAG TCC TTC ATC CGA CAA TAC AAT
   1   E   T   G   A   K   E   S   F   I   R   Q   Y   N

 184  GGG CCA ACA GTT GAC ATT ACC TTT ACC AGT GAC GCA AGT
  14   G   P   T   V   D   I   T   F   T   S   D   A   S

 223  GTT GTG GGT AGC GGC TTC TCC TTT AAC TAT GTC ACT GAA
  27   V   V   G   S   G   F   S   F   N   Y   V   T   E

 262  CTA GTC TAC TGC AAT GCA ACC ATA AAC GGC ACC TCC GGT
  40   L   V   Y   C   N   A   T   I   N   G   T   S   G

 301  ACC TTT GAG TCA CAG CCT GGT GGC TAC GAC AGG AAT ATG
  53   T   F   E   S   Q   P   G   G   Y   D   R   N   M

 340  TAC TGC ACC TAC ACC ATT AAC ACC CCT GGT CCT GGA GAA
  66   Y   C   T   Y   T   I   N   T   P   G   P   G   E

 379  CTT GAC TTC ATC TTC CCA CTG TTT GAC GTT GAG AGA GGC
  79   L   D   F   I   F   P   L   F   D   V   E   R   G

 418  ACC AGC TGC CCA TAC GAC GCC GTG GAG ATG GGA GGA GAC
  92   T   S   C   P   Y   D   A   V   E   M   G   G   D

 457  AGG CTG TGT GGA ACA AAC GTG GAA GAC AAG ACC TAC AGT
 105   R   L   C   G   T   N   V   E   D   K   T   Y   S

 496  ACT GAT GGT CAT TTC CAG TTC ACC TTC ACA AGC GAT GGA
 118   T   D   G   H   F   Q   F   T   F   T   S   D   G
```

```
535  TCC GTA AAT GGA GAC GGT TTC AAG ATA GAC TTC GTC TTC
131   S   V   N   G   D   G   F   K   I   D   F   V   F

574  CTC CCT GAA GAA TTT CAG AAC TAGACACGTGGACCATTTACCAC
144   L   P   E   E   F   Q   N   *

618  CAGATGGAAAACATGCTCAATAAACAACTGCAAAAAAAAAAAAAAAAA
```

Figure 6. Abalone cDNA clone L10 nucleic acid sequence and deduced amino acid sequence, which has homology with the *Drosophila* dorsal-ventral patterning gene *TOLLOID* and the human bone morphogenetic protein (BMP-1). The first codon ATG for methionine in the open reading frame is double underlined. The stop codons are indicated by asterisks and the polyadenylation signal sequence is underlined.

Additional Developmental Genes

Homeobox genes which control cell fate and formation of body pattern in the development of arthropods and mammals have now been identified in the red abalone, *H. rufescens*, by Degnan and Morse (1993). They cloned and sequenced RT-PCR products following amplification with RNA degenerate homeobox primers. Eight unique transcripts were identified prior to and during metamorphosis of abalone larvae, but not in eggs or embryos, and were designated *Hrox1-8*, corresponding to *Haliotis rufescens* homeobox. Five sequences appeared to be homologs of the genes in the *Drosophila* HOM-C complex/*Hox* clusters.

More recently, a homeobox-containing cDNA from *Haliotis rufescens*, called *HruMox*, has been identified (Degnan *et al.*, 1997), and was shown to be expressed in larvae, but not in early embryos. It was found to be most similar to members of the Mox homeobox gene class, particularly vertebrates (i.e the sequence was 85% identical to mouse and frog Mox-2 homeodomains).

The fast-growing tropical abalone, *H. asinina*, is currently being used a model for investigations of factors regulating growth in abalone (Counihan *et al.*, 1998). They have produced cDNA clones of the tropomysin gene, whose sequence when

compared with that of *H. rufescens*, revealed a high degree of homology. Nucleotide and amino acid sequences were 95.0% and 96.6% homologous, respectively.

During development of metazoan animals the molecular mechanisms involved in differentiated patterns of gene expression are highly conserved (Davidson, 1991). In particular, many proto-oncogene families have been shown to have a conserved structurally and functionally role. Of these, the *ets* multigene family, which was identified originally through sequence homology to the *v-ets* gene of the avian erythroblastosis virus E26, has been shown to be conserved throughout the metazoa (Degnan *et al.*, 1993). Their research was based on sequences derived from cloned PCR products of 12 marine invertebrate species, including the red abalone (*H. rufescens*), and several lower metazoans. The results, together with other molecular studies of invertebrates and vertebrates, imply that the *ets* genes may play an essential role in development of a wide range of taxa.

Future Directions and Transgenic Abalone

Further studies will occur in the molecular areas already discussed. However, two relatively new areas drawing attention are the identification of genes controlling heat shock proteins (Hsp's) and one for production of an abalone pheromone. Hsp's are found in both prokaryotes and eukaryotes. They are a family of genes that are typically expressed when organisms become stressed, and in doing so, produce proteins which help to stabilise the internal cellular environment. It has been found that abalone from more variable environments (e.g. Port Phillip Bay versus open ocean habitats) respond differently to heat and salinity changes (Hanna, Toop and Miller, unpublished). The notion that this difference is due to expression of Hsp's is now being investigated.

A water-borne pheromone released during egg-laying of the gastropod, *Aplysia californi*a, has recently been identified and the encoding gene cloned (Fan *et al*., 1997; Painter *et al*., 1998). This pheromone attracts breeding gastropods into aggregations, and coordinates male and female reproductive behaviour within the aggregation. Similar aggregations of abalone during spawning periods have been

noticed by abalone divers (unpublished), and the adaptive significance of aggregation for spawning has been reported (Breen, and Adkins, 1980; Shepherd, 1986). A molecular search is in progress for a gene encoding an abalone pheromone.

Foreign DNA has already been introduced successfully into fertilized eggs of the red abalone using electroporation (Powers *et al.*, 1995). The DNA was a plasmid containing the *Drosophila* β-actin promoter coupled to a β-galactosidase cassette, and was retained in 70-100% of all abalone sampled, with an average retention rate of 72% in 3-7 month old juveniles. They reported that they electroporated fertilized abalone eggs in 1992 with the coho salmon growth hormone gene coupled with the same promoter. The transgenic abalone should have now had time to mature sexually and produce progeny that can be tested for accelerated growth. Additional reports indicate that successful electroporation of eggs of *Haliotis asinina* Linnaeus has been carried out using a pEGFP plasmid (R. Counihan, *pers. comm.*), and that sperm has been used as a carrier to introduce an exogenous DNA fragment into oocytes of the Japanese abalone *Haliotis diversicolor supertexta* Lischke (Tsai *et al.*, 1997). The way in now open for some exciting new abalone research.

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Appendix 8
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Analysis of population genetic structure of blacklip abalone (*Haliotis rubra*) using RAPD, minisatellite and microsatellite markers

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Abstract

We investigated the utility of three PCR-based DNA molecular markers in the analysis of population genetic structure of blacklip abalone, *Haliotis rubra* of

Victoria, Australia. The DNA markers included 84 polymorphic RAPD bands amplified using 6 random primers, two minisatellites, GHR (putative growth hormone gene repeat) and MIPR (putative molluscan insulin-like peptide gene repeat), and three microsatellites, RUBGT1 (containing $(GT)_n$ repeats), RUBCA1 (containing $(CA)_n$ repeats) and RUBGACA1 (containing $(GACA)_n$ repeats). All three types of DNA markers revealed significant subdivision in the blacklip abalone populations along the coastline. This was postulated to be related to the abalone's relatively short pelagic duration and limited dispersion. Further analysis found that a Point Cook population sampled from within the semi-enclosed Port Phillip Bay was distinct from two other central zone populations (Apollo Bay and Cape Schanck). The genotypes of microsatellites indicated excessive homozygotes across all the populations at all three microsatellite loci, and possible causes, such as larval recruitment pattern, asynchronous spawning, have been discussed. The excessive homozygotes for the three microsatellite loci contrast with those observed in the minisatellite loci, GHR and MIPR, of which heterozygosities were within Hardy-Weinberg equilibrium.

Key words: Blacklip abalone, population genetic structure, RAPD, minisatellite, microsatellite

Introduction

Ecological studies have found that the movement of juvenile and adult blacklip abalone is limited to less than 100 metres over their life time (Shepherd 1973). Therefore, the population genetic structure of abalone, like other marine sedentary benthic molluscan animals, largely depends on the larval dispersal ability and recruitment pattern (Hunt 1993). Free-swimming abalone larvae are lecithotrophic and have a relatively brief pelagic phase of 3-11 days (McShane 1992), before they settle, and metamorphose. Their settlement is activated by factors both in the mucus of conspecific parents and juveniles (Seki and Kan-no 1981), as well as inducing factor(s) released from the crustose red algae broken by adult and juvenile abalone

during feeding (Morse et al. 1979). This may result in higher concentrations of the inducer in the vicinity where abalone cluster and therefore larvae tend to settle near their parents (Prince et al. 1988; McShane et al. 1988). Under limited dispersal, it is expected that abalone populations will exhibit some genetic differentiation among populations.

The population genetic structure of blacklip abalone has previously been studied using allozyme analysis (Brown 1991). Of 15 loci examined, 12 were polymorphic with an average heterozygosity of 13.6%. However, of these 12 loci, 9 were dominated by a single high frequency allele (frequency ≥ 0.90) despite often extensive sample sizes (30-126 animals with an average sample size 84). Thus, for most analyses the results largely reflect the outcome at the 3 most informative loci. Significant genetic differentiation among populations was detected at the 3 loci (F_{ST}= 0.008 ~ 0.032, $p<0.001$). At the population level, some loci showed significant heterozygote deficiencies, relative to Hardy-Weinberg expectations. Rogers' genetic distance was positive and significantly correlated with geographic (shortest across the water) distance ($r=0.828$, $p<0.01$). Therefore, the patterns were consistent with those predicted by an "isolation by distance" model, with an estimated neighbourhood size of approximately 500 km.

The newer DNA techniques, such as RAPD, minisatellite and microsatellite markers, have the potential to provide additional information for abalone population studies, and have demonstrated considerable promise for population genetic and evolutionary studies in other species (Hughes and Queller 1993; Peakall et al. 1995). However, despite the increasing use of these PCR based genetic markers, their application to the study of marine organisms generally lags behind terrestrial plants and animals (Liu and Furnier 1993; Powell et al. 1996). This paper describes the application of three types of DNA markers to the same set of individuals in order to verify the relative merits of the different methods in marine animals. Our findings have important implications for future abalone studies.

The specific objectives of this paper were (1) to conduct a preliminary population genetic analysis with each molecular assay using an existing blacklip

abalone sample set collected from the coastline reefs of the state of Victoria; (2) to compare and contrast the population genetic findings among the DNA-based assays, and also with previous more extensive allozyme studies in order to provide further insights into the biology of the species; and (3) to assess the utility and limitations of allozyme and DNA-based molecular assays for further studies of abalone.

Materials and methods

Blacklip abalone samples

One hundred blacklip abalone had been collected at nine sites along the Victorian coast and one from Eden, New South Wales as part of the Victorian Abalone Management Monitoring Program of the Victorian Marine and Freshwater Research Institute, Queenscliff. Ten adult abalone were available from each site, and had been taken from a 60 metre diameter sampling area. The sampling sites and methods are detailed in a previous paper (Huang et al. 1997).

DNA extraction and PCR conditions

Abalone DNA was extracted using the method described by Goodwin and Lee (1993). Fifty 10-mer RAPD primers in a random primer Kit No. 2 (The University of British Columbia, Canada), ranging from the odd numbered primers UBC 101 to 199, were screened for informative RAPD profiles. A 15-mer M13 core repeat was also used as a random primer in this study (Stenlid et al. 1994). The sequence was 5'-GAGGGTGGCGGTTCT-3' and the primer was assigned the code RM13.

The PCR conditions for RAPD followed the protocols of Williams et al. (1990). RAPD reactions were performed in a 50 μL reaction volume, containing 50 mM KCl, 10mM Tris-HCl (pH 8.3, 25^{o}C), 0.2 μM random primer, 200 μM of each dNTP (dATP, dCTP, dGTP and dTTP), 0.1% gelatin, 2.0 mM $MgCl_2$, 1 unit of Ampli*Taq* DNA polymerase LD (Perkin Elmer), and 100 ng DNA. The initial cycle was set at 94^{o}C for 5 min, 38^{o}C for 2 min and 72^{o}C for 2 min, then followed by 44 cycles at 94^{o}C for 5 sec, 38^{o}C for 30 sec and 72^{o}C for 60 sec. Finally, samples were

incubated for 10 min at 72°C then held at 4°C until analysis. PCR conditions were the same for RM13, except that 45°C was used as the annealing temperature (Stenlid et al. 1994). The RAPD profiles were reproducible from run to run, nevertheless, all RAPD reactions were performed in a single run for each primer using a specially designed 100 tube holder.

Primers and PCR conditions for two minisatellites, GHR and MIPR, and three microsatellites, RUBGT1, RUBCA1 and RUBGACA1, are detailed in Huang et al. (1997), and Huang and Hanna (1998), respectively.

Gel electrophoresis and visualisation

The RAPD products were separated by electrophoresis in 2.0% agarose gels (0.5x TBE buffer at 6-8Vcm^{-1}) for 1-1.5 h, followed by ethidium bromide staining and visualisation under UV light.

The minisatellite fragments were separated in ultrathin layer acrylamide gels attached to Gelbond (FMC, USA) using a discontinuous buffer system (0.07 M Tris-sulfate, pH 9.0 and 0.14 M Tris-borate, pH 9.0 as the trailing ion) following the methods of Ludwig et al. (1989). PCR amplified minisatellite products were separated by 6% (for GHR) or 7.5% (for MIPR) PAGE for 2-4 h at 120V. The microsatellite fragments were separated in 6% polyacrylamide sequencing gels. Both minisatellite and microsatellite fragments were visualized by non-mutagenic silver staining of ultra thin layer polyacrylamide following the procedure of Ludwig et al. (1989).

Sizes of amplified minisatellite DNAs were determined by comparison of their distance migrated with standard 100 bp DNA ladders (Promega) on each side of the gel. The microsatellite DNA sizes were determined using an Eagle Eye™ II Still Video System with Eagle Sight™ 3.0 Image Capture and Analysis Software (Stratagene) and comparison with pUC19/*Hpa* II digested DNA marker (GeneWorks, Australia).

Statistical analysis

Total genetic variation was partitioned by Analysis of Molecular Variance (AMOVA) (Excoffier 1995) into three levels: among regions, among populations within regions,

and among individuals, and then summarised as Φ-statistics (Excoffier et al. 1992; Peakall et al. 1995). The correlation of individuals from the same region was expressed as Φ_{RT}, the correlation between individuals within a population, relative to that of individuals from the same region, as Φ_{PR}, and the correlation between individuals within a population, relative to that of individuals from the species, as Φ_{PT}. The three level Φ-statistics, using the following equations, is shown in Table 1, where:

$$\Phi_{RT} = \frac{s^2_R}{s^2_w + s^2_P + s^2_R}$$

$$\Phi_{PR} = \frac{s^2_P}{s^2_w + s^2_P}$$

$$(1-\Phi_{PT}) = (1- \Phi_{PR})\ (1- \Phi_{RT})$$

Table 1 The three-level AMOVA where R is the number of regions; P is the number of populations; N is the total sample number.

Source of variation	Degrees of freedom (df)	Sums of squares (ss)	Mean squares (ss/df)	Estimated variance components	Φ-ratios
Among regions	R-1	SSR	MSR	s^2_R	Φ_{RT}
Among populations/region	P-R	SSP	MSP	s^2_P	Φ_{PR}
Individuals/Within population	N-P	SSW	MSW	s^2_w	Φ_{PT}

An important feature of the AMOVA procedure is that it can be used to analyse both multilocus dominant data such as provided by RAPD and single locus codominant data such as revealed by allozyme, minisatellite or microsatellite data

(Peakall et al. 1995). This is possible because the AMOVA analysis is performed on a pairwise genetic distance matrix that can be generated with appropriate procedures for both dominant and codominant data. AMOVA analysis followed the procedures of Excoffier et al. (1992), and Peakall et al. (1995) using the software WINAMOVA (http://acasunl.unige.ch/LGB/Software/Windoze/amova) with 999 permutations for significance testing. Pairwise population distances (Φ_{PT}) were also calculated among all pairs of populations and used in subsequent analyses performed within NTSYS-pc (Numerical Taxonomic System) version 1.80 (Exeter Software 1993) to cluster the populations.

For the RAPD data, the presence or absence of a particular band was designated as 1 or 0, respectively. Therefore, each individual was represented by a vector of 1s and 0s. If a RAPD band was shared by 95% or more individuals, it was regarded as a monomorphic band and was excluded from the genetic distance analysis since only polymorphic bands contribute to the estimation of genetic distance (Hartl and Clark 1989; Peakall et al. 1995). The RAPDistance Package version 1.04 (Armstrong et al. 1994) was used to calculate the pairwise genetic distance matrices required for subsequent AMOVA.

The alleles of minisatellite and microsatellite loci were scored by inferring the number of repeat units, based on the size of the allele. The calculation of a genetic distance matrix between pairs of individuals for these codominant markers followed the method of Peakall et al. *(*1995). For a single-locus analysis of minisatellite or microsatellite marker, with *i*-th, *j*-th, *k*-th and *l*-th alleles being different, a set of squared distances is defined as $d^2(ii, ii)=0$, $d^2(ij, ij)=0$, $d^2(ii, ij)=1$, $d^2(ij, ik)=1$, $d^2(ij, kl)=2$, $d^2(ii, jk)=3$, and $d^2(ii, jj)=4$. Genetic distances were summed across loci under the assumption of independence. The pairwise genetic distances were calculated using a beta release of the GenAlEx software (Peakall and Smouse 1996). AMOVA was then performed as for the RAPD analysis.

The expected heterozygosity of *H. rubra* was estimated, using observed allele frequencies of minisatellite and microsatellite loci, according to the formulae of Nei (1972):

$$h = 1 - \sum_{i=1}^{k} p_i^2$$

where h is the expected heterozygosity, k is the number of alleles in the population and p_i is the frequency of the *i*th allele.

Chi-square tests for estimating Hardy-Weinberg equilibrium (HWE) of the minisatellite and microsatellite population data, followed the method described by Budowle et al. (1991, in which rare alleles with less than four events are pooled.

The correlation between genetic distance and the corresponding geographic distances, were assessed using the Mantel test (Mantel 1967). Relationships among population and individuals was assessed using principal component analysis (PCA). Both Mantel tests and PCA analysis were performed using NTSYS-pc software.

Results

RAPD, minisatellite and microsatellite markers found in blacklip abalone

The screening of 51 RAPD primers identified 14 informative and reliable primers. Five of the 14 primers were randomly selected for further study: UBC-101 (GCGGCTGGAG), UBC-135 (AAGCTGCGAG), UBC-149 (AGCAGCGTGG), UBC-159 (GAGCCCGTAG), UBC-169 (ACGACGTAGG) plus RM13 (see Table 2). These 5 primers, combined with RM13, produced 84 polymorphic bands out of a total of 90 bands.

Alleles of minisatellites and microsatellites identified in the same samples were described in two previous reports (Huang et al. 1997; Huang and Hanna, 1998). The two minisatellites, GHR and MIPR, had 13 and 4 alleles, respectively, and the three microsatellites RUBGT1, RUBCA1 and RUBGACA1, had 41, 30 and 8 alleles, respectively.

Table 2 Results of primers used in RAPD analysis of blacklip abalone populations

Primers	Number of amplified bands	Range of bands in individual	Monomorphic bands (≥95% shared)	Number of polymorphic markers	Polymorphism (%)
UBC-101	14	2-13	1	13	92.85
UBC-135	13	3-10	1	12	92.30
UBC-149	14	3-11	2	12	85.71
UBC-159	15	1-12	0	15	100.00
UBC-169	16	4-12	1	15	93.75
RM13	18	5-16	1	17	94.44
Total	90		6	84	Mean 93.33

Analysis of Molecular Variance

Analysis of molecular variance revealed significant molecular differentiation among populations for the RAPD (Φ_{PT}=0.074, p<0.001), and microsatellite markers (Φ_{PT}=0.067, p<0.001), but not the minisatellite markers (Φ_{PT}=0.001, p=0.445). These results indicated that the blacklip abalone populations were not homogeneous and that some population subdivision existed.

To determine if there was any regional subdivision, a regional AMOVA analysis was performed by dividing populations into their three Victorian abalone management zones (Gorfine and Walker 1996). For RAPD significant population divergence among management zones (p<0.05), and among populations within zones, (p<0.001) was revealed (Table 3). For microsatellites genetic differentiation among populations within zones (p<0.001), and within populations (p<0.001), was also detected whereas for the minisatellites there was significant molecular differentiation among the three management zones (p<0.05), but not among populations within zones (Table 3). Similar results were found when the region containing a single population (Portland) was excluded (data not shown), indicating a lack of population replication in this zone had little effect on the overall patterns.

Table 3 AMOVA analyses of blacklip abalone populations of three management zones of the Victoria coast

Source of variation	Degrees of freedom (df)	RAPD		Minisatellites		Microsatellites	
		Φ-statistics	Significance	Φ-statistics	Significance	Φ-statistics	Significance
Among zones	(R-1)=2	Φ_{RT}=0.033	p=0.039*	Φ_{RT}=0.039	p=0.034*	Φ_{RT}=0.029	p=0.074
Among populations /zone	(P-R)=7	Φ_{PR}=0.055	p<0.001***	Φ_{PR}=-0.023	p=0.807	Φ_{PR}=0.050	p<0.001***
Within populations	(N-P)=90	Φ_{PT}=0.086	p<0.001***	Φ_{PT}=0.016	p=0.403	Φ_{PT}=0.078	p<0.001***

R (Regions) =3, P (Populations) =10, N (Number of samples) =100.

Genetic relatedness of the ten blacklip abalone populations

Cluster analysis of the pairwise Φ_{PT} population matrix generated from the RAPD AMOVA shows that geographically close populations tended to cluster together, with the exception of the three populations sampled from the central zone which were split into two groups (Fig. 1A). It also showed Point Cook in one branch, and Apollo Bay and Cape Schanck in another branch. Similar separation of the Point Cook population from the other central management zone populations was apparent with minisatellite and microsatellite data (Figs. 1B and 1C).

Additional PCA analysis based on the RAPD data showed that Point Cook population was clearly separated from the two other central populations (Fig. 2). Similarly, microsatellite allelic frequencies at Point Cook population were considerably different from other two central zone populations at the RUBGACA1 locus, with only 2 kinds of alleles found in the population of Point Cook. There were 4 kinds of alleles found in the other 2 central populations (Table 4).

Table 4 Frequencies of RUBGACA1 microsatellite alleles in the 3 central zone populations

Allele	Apollo Bay	Cape Schanck	Point Cook
1	-	0.30	0.80
3	0.05	-	-
4	0.15	-	-
5	0.60	0.40	-
6	0.2	0.2	0.2
7	-	0.1	-

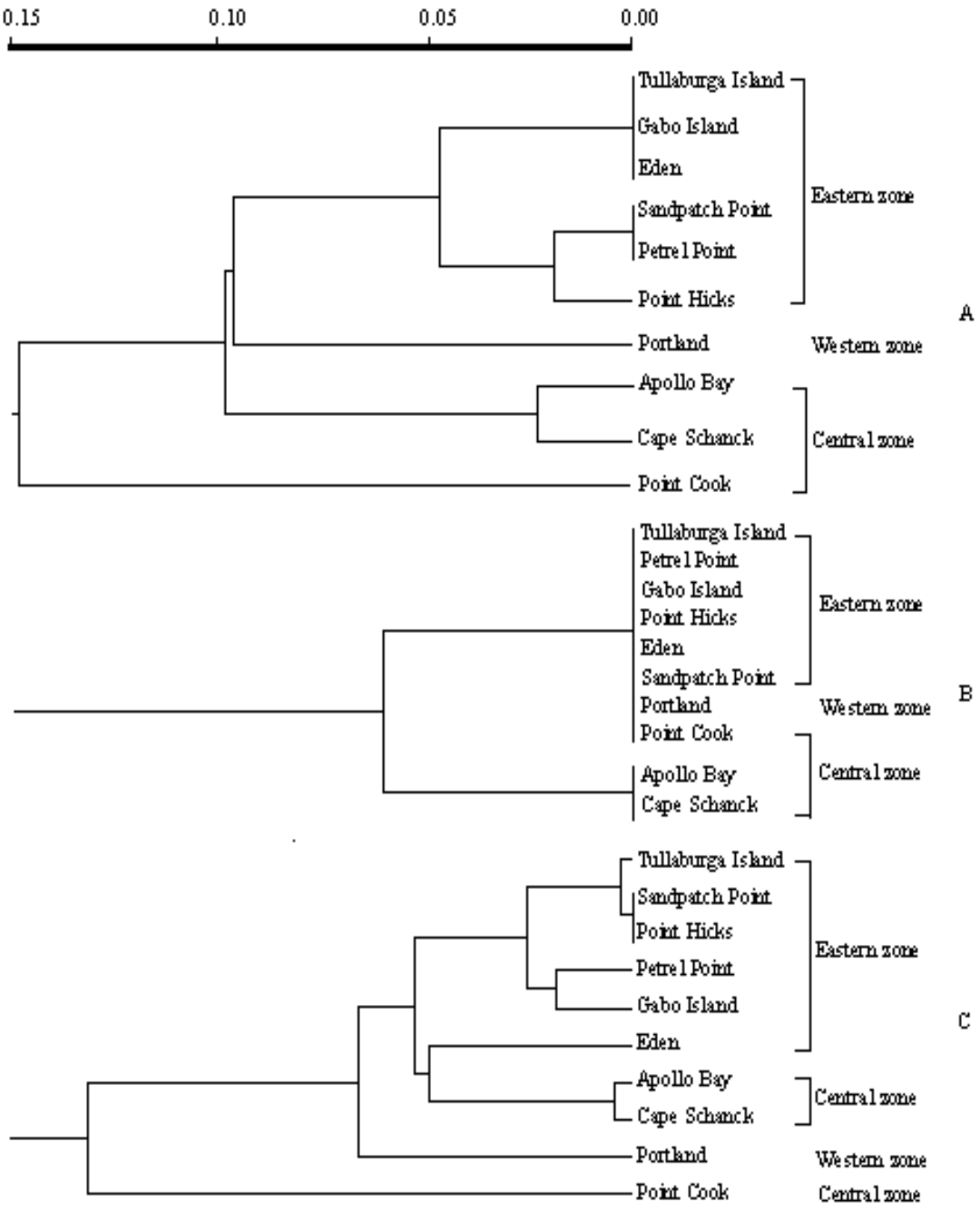

Fig. 1 The relatedness of blacklip abalone populations along the coast using RAPD, minisatellites and microsatellites. The genetic distances (Φ_{PT}) were calculated with AMOVA, and the dendrograms produced with NTSYS-pc. Analyses: A, RAPD; B, minisatellites; and C, microsatellites.

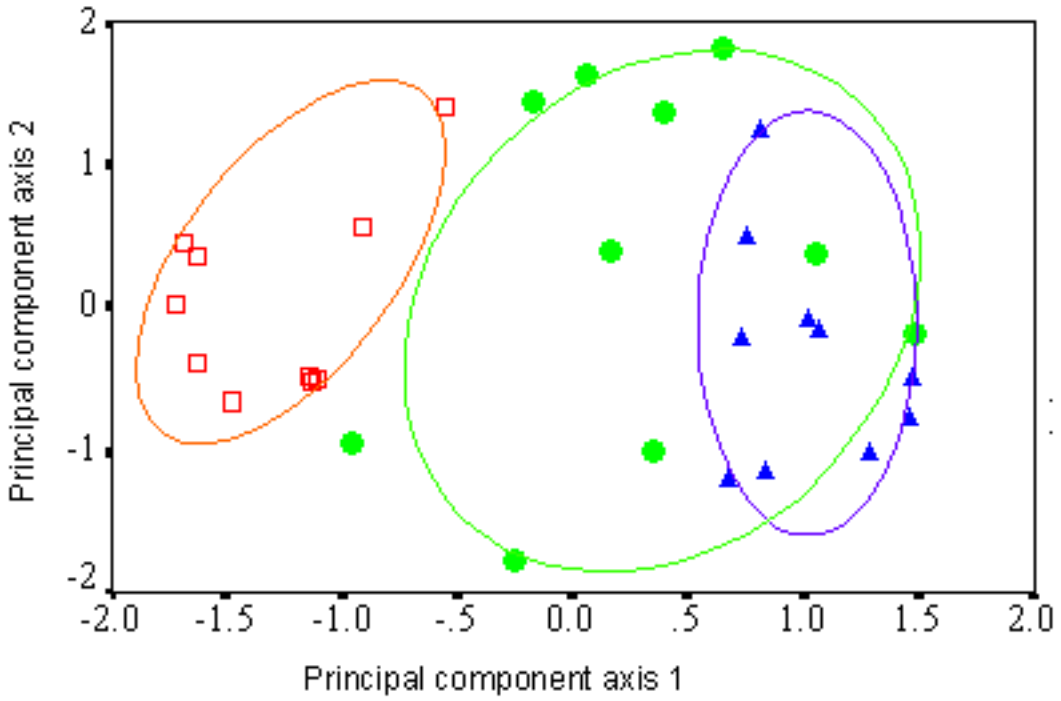

Fig. 2 Principal component analysis of the RAPD data in the central zone populations. σ, Apollo Bay; λ, Cape Schanck; θ, Point Cook.

The relationship between molecular variation and geographic distance in blacklip abalone populations

A Mantel test, excluding the Point Cook population within the semi-enclosed bay, indicated that population genetic distances based on RAPD increased with increasing water distances ($r=0.71$ and $p<0.001$). A similar result was obtained using microsatellite markers ($r=0.42$, $p<0.05$), but not minisatellite markers ($r=0.28$, $p>0.05$). RAPD and microsatellite data suggest that oceanic populations along the Victorian coast best fit an "isolation by distance" model.

The shortest water distance between sampling sites giving significant molecular differences based on RAPD was between Tullaburga Island and Petrel Point, with a water distance of 55 km (Table 5). With the exception the population pairs of Gabo Island and Sandpatch Point (61 km), and Portland and Apollo Bay (236 km), all the population pairs over 60 km showed significant molecular variation.

Therefore, in general populations tended to exhibit significant molecular difference when approximately 60-70 km apart. Molecular variation based on microsatellite data started to show significant differentiation with the shortest water distance 84 km between Eden and Sandpatch Point (Φ_{PT}=0.04, p=0.009) (data not shown). However, a similar relationship was not observed in minisatellite markers.

Table 5 Correlation between shortest water distance (km) of blacklip abalone sampling sites (excluding Point Cook) and molecular variation significance level.

	Shortest water distance (km)								
Sites	Eden	Gabo Island	Tullaburga Island	Sandpatch Point	Petrel Point	Point Hicks	Cape Schanck	Apollo Bay	Portland
Eden	-								
Gabo	48†								
Island	p=0.243	-							
Tullaburga	54	6							
Island	p=0.999	p=0.999	-						
Sandpatch	84*	36	29						
Point	p=0.025	p=0.151	p=0.129	-					
Petrel	109***	61	55***	25					
Point	p<0.001	p=0.096	p<0.001	p=0.999	-				
Point	121*	74***	67***	38	13				
Hicks	p=0.013	p<0.001	p<0.001	p=0.123	p=0.381	-			
Cape	864***	816***	810***	774*	749***	736***			
Schanck	p<0.001	p<0.001	p<0.001	p=0.02	p<0.001	p<0.001	-		
Apollo	973***	925***	919*	883***	858***	845***	109***		
Bay	p<0.001	p<0.001	p=0.013	p<0.001	p<0.001	p<0.001	p<0.001	-	
	1209***	1161***	1155***	1119***	1094***	1081***	345***	236	
Portland	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p=0.5425	-

†, distances were calculated using readings of global positioning system (GPS) of the populations of sampling sites and expressed in round numbers; *, p<0.01-0.05; **, p<0.01-0.001; ***, p<0.001.

Tests for Hardy-Weinberg equilibrium at the minisatellite and microsatellite loci

The observed heterozygosities at the GHR and MIPR were close to the expected heterozygosities (Table 6). Consequently, Chi-square tests showed that the distributions of the genotypes of GHR and MIPR were consistent with Hardy-Weinberg equilibrium (HWE) in the pooled population (p>0.05) (see Table 7). All 10 subpopulations were within HWE at both GHR and MIPR loci as well (all p>0.05 with n=10 samples in each population, data not shown).

Table 6 The observed and expected heterozygosities and homozygosities of the blacklip abalone samples at the two minisatellite and three microsatellite loci.

	Locus	Observed heterozygosity	Expected heterozygosity	Observed homozygosity	Expected homozygosity
Mini-satellites	GHR	64.0%	64.9%	36.0%	35.1%
	MIPR	65.0%	57.1%	35.0%	42.9%
Micro-satellites	RUBGT1	37.0%	95.5%	63.0%	4.5%
	RUBCA1	38.0%	95.5%	62.0%	4.5%
	RUBGACA1	19.0%	81.4%	81.0%	18.6%

At the population level a consistent and unexpected excess of homozygotes was apparent across the three microsatellite loci (see Table 6). Excess homozygosity remained when samples were pooled according to their management zone, increasing sample size to 60 for the eastern zone and 30 for the central zone (Table 8). Similarly, no change was found when the most frequent alleles in each locus were used in the analysis without the effect of rare alleles (Table 9). If the three microsatellites are coincidentally located on the sex chromosome of blacklip abalone (assumed to be a XX/XY sex type), genotypes in the half of the animals of XY type will be typed to be homozygous. If this were the case, the observed heterozygosities of the three microsatellite loci in Table 6 would be underestimated. However, there was still significant difference between the observed and expected heterozygosities, even when the figures of observed heterozygosities in Table 6 was doubled (data not shown). Thus, all analyses consistently revealed a significant excess of homozygotes which cannot be attributed to the effects of sex linkage, small sample size, or rare alleles.

Excess homozygosity can sometimes be explained by null alleles and mistyping of the results. Neither appears likely explanations in this study. At the GT locus, a new set of flanking primers were developed, and samples initially homozygous with the first set of primers, remained homozygous with the new primers. Mistyping of the results is also unlikely since high resolution electrophoresis and subsequent digital analysis of the gel images was used to score the results.

Table 7 Hardy-Weinberg equilibrium tests at the GHR and MIPR loci. "The rest" are pooled from all classes with less than four observations; Xo are the observed genotypes; Xe are the expected genotypes. For GHR locus, df = 1, χ^2 values of $P_{0.25}$ =1.32, $P_{0.10}$ =2.71; therefore $P_{0.25} < \chi^2 < P_{0.10}$. For MIPR locus df = 1; χ^2 values of $P_{0.10}$ = 2.71, $P_{0.05}$ = 3.84; $P_{0.10} < \chi^2 < P_{0.05}$.

Locus	Phenotype	Observed (Xo)	Expected (Xe)	$(Xo - Xe)^2/Xe$
GHR	15-16	6	5.04	0.183
	16-16	33	31.36	0.085
	16-17	20	18.48	0.125
	16-18	6	5.60	0.028
	16-19	4	2.24	1.382
	The rest*	31	26.88	0.631
				$\chi^2 = 2.434$
MIPR	4-6	1	0.5	0.50
	5-5	14	17.2	0.595
	6-6	21	25	0.640
	5-6	48	41.5	1.01
	5-7	7	6.6	0.024
	6-7	9	8	0.125
				$\chi^2 = 2.894$

Table 8 Tests of Hardy-Weinberg equilibrium at the three microsatellite loci in the three abalone fishery management zones.

Locus		Eastern zone (n=60)	Central zone (n=30)	Western zone (n=10)
RUBGT1	P-value	0.000	0.000	0.001
	F_{IS}	0.545	0.826	0.372
RUBCA1	P-value	0.000	0.000	0.000
	F_{IS}	0.597	0.587	0.673
RUBGACA1	P-value	0.000	0.000	0.003
	F_{IS}	0.768	0.818	0.489

Table 9 Tests of Hardy-Weinberg equilibrium in the pooled population using the most frequent alleles at the three microsatellite loci. All χ^2 at df = 1, $p<0.01$.

Locus	Phenotypes	Observed	Expected	$(Xo - Xe)^2/Xe$	χ^2
RUBGT1	Homozygotes	7	1.10	31.65	
(RUBGT1 21)	Heterozygotes	7	18.80	7.39	39.04
RUBCA1	Homozygotes	10	1.44	50.88	
(RUBCA1 33)	Heterozygotes	4	21.10	13.85	64.73
RUBGACA1	Homozygotes	20	5.06	44.11	
(RUBGACA1 4)	Heterozygotes	5	34.87	25.60	69.71

Correlation of Φ_{PT} distance matrix generated by RAPD, minisatellite, and microsatellite markers

The Mantel test for the correlation of distance matrixes of the 10 blacklip abalone populations based on RAPD, minisatellites, and microsatellite markers is displayed in Table 10. The results show that there was significant correlation in the Φ_{PT} distance matrix generated by RAPD and microsatellite markers ($r=0.514$, $p<0.05$). Therefore, the Φ_{PT} distance matrix of RAPD and microsatellite markers were significantly related. However, the Φ_{PT} distance matrix of RAPD and minisatellites, and minisatellites and microsatellites, were not correlated (both $p\geq0.1$).

Table 10 Correlation coefficients of the distance matrix created by RAPD, minisatellite, and microsatellite markers. Note: *, $p<0.05$.

Distance matrix	Distance matrix	r	p-value
RAPD	Microsatellites	0.514	p=0.012*
RAPD	Minisatellites	0.266	p=0.100
Minisatellites	Microsatellites	0.060	p=0.367

Discussion

Population divergence of blacklip abalone

In this study, RAPD, minisatellites and microsatellites, has identified significant molecular differentiation in blacklip abalone populations sampled from Victoria and Eden, New South Wales. Significant genetic variation among the management zones was detected with the RAPD and minisatellite markers, and the significant genetic variation among population within zones and within populations was detected with both RAPD and microsatellite markers (see Table 3).

An allozyme analysis conducted previously by Brown (1991), has also demonstrated some population differentiation among blacklip abalone populations, although the extent of sampling differed from our study. Despite sampling over a smaller scale, our study revealed more genetic differentiation with both RAPD ($\Phi_{PT}=0.086$, $p<0.001$), and microsatellites ($\Phi_{PT}=0.077$, $p<0.001$) markers, than

allozymes (Φ_{PT} analogue F_{ST}=0.022, p=0.002). By contrast, the minisatellites showed no significant population differentiation, although there was some regional differentiation (Φ_{RT} =0.039, p=0.034). Overall, these findings are consistent with the observations in other organisms that RAPD and microsatellite markers reveal greater molecular variation than allozyme analysis (Peakall et al. 1995; Hughes and Queller 1993). Despite having larger sample sizes and more populations, the capacity to detect significant genetic differences may have been limited in the allozyme study since only 3 allozyme loci provided useful allele frequencies out of the 12 polymorphic loci, the remaining loci being dominated by a single high frequency allele (Brown 1991). Similarly, the lack of genetic differentiation detected by the minisatellites may also reflect the high frequency of just 2 alleles (GHR 16 and 17) at GHR, and 3 alleles (MIPR 5, 6 and 7) at MIPR loci, even though an inspection of the allelic data shows unique alleles in some populations.

RAPD and microsatellite analyses have revealed that shortest water distances and genetic distances among population pairs is correlated (see Table 5). This is also consistent with the previous allozyme data (Brown 1991). A question that arises is how large is the stock unit detectable by RAPD and microsatellite analysis? Shepherd and Brown (1993) have defined the abalone stock unit as "a group of individuals that share a common gene pool and more or less isolated during reproduction from all other conspecifics". In geographic terms, the boundary of a stock unit is the point at which the molecular genetic variation starts to exhibit significant differences from each other and the divergence increases with distance (Slatkin 1993; Pfenninger et al. 1996). The results of the pairwise population analyses along the coast, excluding the Point Cook population (see Table 5), showed significant genetic differences among all pairs of populations 60-70 km or more apart by RAPD markers, and 84 km or more by microsatellite markers. These data may suggest that neighbourhood sizes in this species rarely exceed 100 km. This estimate is smaller than the neighbourhood size of 500 km suggested by previous allozyme data (Brown 1991), but is consistent with the ecological evidence for local blacklip abalone recruitment and limited dispersal (McShane et al. 1988; Prince et al. 1988).

Significant genetic differentiation of the Point Cook blacklip abalone population

All three DNA markers, RAPD, minisatellites and microsatellites revealed significant molecular divergence between the Point Cook population and two other central zone populations from oceanic locations at Apollo Bay and Cape Schanck (Figs. 1 and 2; Table 4). Physiological differences have also been observed in the blacklip abalone from the Port Phillip Bay region; (1) the shell shape and topology of the blacklip abalone in Port Phillip Bay has subtle differences compared with those of oceanic blacklip abalone; (2) they bleed longer than the oceanic blacklip abalone when wounded; and (3) they are able to tolerate higher seawater temperatures (unpublished data).

The semi-enclosed feature of Port Phillip Bay, estimated to be formed approximately 8,000 years ago (Smith 1990), most likely plays a major role in the molecular divergence of the Point Cook population from the other central zone populations in this study. As Port Phillip Bay is linked to the ocean by only a narrow rocky entrance, the water exchange is limited, making it predominantly a very large tidal embayment. The flushing time estimated by various methods is approximately 12-15 months (Black and Mourtikas 1992). It is likely that blacklip abalone larval exchange between Port Phillip Bay and central zone oceanic populations is reduced, resulting in a genetic divergence and associated with physical differences. This suggests that from a management point of view, the abalone in Port Phillip Bay should be treated as a distinct metapopulation from the other central oceanic populations.

Hardy-Weinberg equilibrium at the minisatellite and microsatellite loci

The significant heterozygote deficiencies detected at the 3 microsatellite loci cannot be attributed to technical or statistical artefacts, suggesting instead that inbreeding is contributing to this pattern. Ecological evidence indicates potential for inbreeding in black-lid abalone. As noted earlier, black lip abalone larval recruitment is likely to be very restricted. Furthermore, the spawning of blacklip abalone is affected by local environmental factors that can lead to asynchnonous spawning over a short distance. For example, independent spawning seasons have been observed between two

Victorian blacklip abalone populations only four km apart, and similar patterns have been observed in South Australian waters (McShane et al. 1986; Shepherd and Laws 1974).

Allozyme data for some populations of blacklip abalone also show heterozygote deficiencies (Brown 1991). Extensive heterozygote deficiency has also been observed at allozyme loci in natural populations of Japanese Pacific abalone *H. discus hannai* natural populations (Fujino 1978). In this abalone species, an allozyme study showed that there was an excess of homozygotes in all natural populations collected from sampling sites 2-10 km apart, suggesting extensive inbreeding in this species (Hara and Kikuchi 1992).

Microsatellite loci may be particularly sensitive to inbreeding effects in small and isolated population. In a study of eight $(CA)_n$ microsatellite loci in isolated human populations, average heterozygosity was significantly lower than expected heterozygosity as some of the loci (Deka et al. 1995). Collectively, the evidence points to inbreeding as the cause of heterozygote deficiency at microsatellite loci in blacklip abalone. However, evidence for inbreeding is not supported by the minisatellite analyses, in which the 10 populations were all within HWE for both loci. However, both loci were located within the exons of putative blacklip abalone growth hormone and molluscan insulin-related peptide genes, respectively (Huang et al. 1997). Consequently, these loci may be directly or indirectly influenced by selection. Size constraints are known in other tandem repeats that are located within the coding regions of exons (La Spada et al. 1991), and constraints on allele size may also influence the genetic patterns at these loci. It would be of interest to determine if the patterns hold in other minisatellites not closely linked to coding regions. Alternatively, due to different mutation rates, selection may have different effects on minisatellite and microsatellite markers. It has been observed that selection has variable effects on different allozyme genotypes of sea bass (*Dicentrarchus labrax*) (Allegrucci et al. 1994).

Other studies have reported contrasting patterns between loci. For example, a study of five microsatellite loci in four human ethnic populations showed that HUMARA[AGC]n in the American black population, and HUMTH01[AATG]n in

Asians, departed significantly from HWE, respectively, while the other microsatellite loci in the American black and Asian population studied were within HWE, respectively (Edwards et al. 1992). The reasons for these discrepancies remain unknown, and clearly further studies in both human and blacklip abalone populations are required to elucidate the cause for these unexpected patterns.

Utility and limitation of allozyme and PCR-based DNA molecular assays

It is well established that allozyme analysis is cost effective, but often gives less information compared to DNA methods (Liu and Furnier et al. 1993). We have found that among the three DNA methods, the RAPD is the simplest assay, requiring no DNA sequence knowledge, to produce polymorphic DNA profiles. However, this procedure may be sensitive to experimental conditions, and the multilocus profile can be difficult and time consuming to score. Both minisatellites and microsatellites are multiallelic, single-locus codominant markers that are easy to score, potentially transferable to closely related species and amenable to automation. However, minisatellites are more difficult to obtain due to sequence complexity in the repeat unit and the relatively small number in the eukaryotic genome. On the other hand, microsatellites are much easier to obtain due to their sequence simplicity and abundance in the eukaryotic genome. Novel cloning methods using enriched libraries (eg. Refseth et al. 1997) make the isolation of microsatellites simpler and easier, and therefore, as in other organisms, microsatellites are likely to be the marker of choice for further population genetic study of abalone.

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Appendix 1 Allelic data of minisatellites and microsatellites of blacklip abalones studied

The allelic data of each blacklip abalone is arranged in the order of GHR, MIPR, RUBCA1, RUBGACA1, and RUBGT1, respectively. POP stands for population

POP	POP
Eden, 1717 0506 3737 0505 2034	Point Hicks, 1516 0606 3640 0404 3343
Eden, 1616 0507 1315 0606 1213	Point Hicks, 1617 0506 3434 0707 2121
Eden, 1617 0506 2727 0303 2742	Point Hicks, 1517 0506 3030 0404 2224
Eden, 0716 0506 2541 0103 3737	Point Hicks, 1616 0606 3333 0606 2222
Eden, 1616 0506 2932 0303 2222	Point Hicks, 1618 0506 3333 0506 2020
Eden, 1617 0506 3333 0606 2121	Point Hicks, 1616 0606 3232 0506 1113
Eden, 1616 0506 3939 0606 1922	Point Hicks, 1619 0506 3333 0607 3939
Eden, 1317 0506 2935 0405 2222	Point Hicks, 1617 0606 3131 0707 4747
Eden, 1616 0606 3939 0606 2237	Point Hicks, 1316 0506 2743 0404 2121
Eden, 1616 0507 3838 0606 3240	Point Hicks, 1617 0607 2632 0101 3131

POP	POP
Gabo Island, 1616 0507 3535 0404 4848	Apollo Bay, 1616 0506 3434 0505 4040
Gabo Island, 1617 0406 2929 0404 2929	Apollo Bay, 1216 0506 3844 0505 2121
Gabo Island, 0709 0506 3535 0505 1315	Apollo Bay, 1616 0505 1313 0606 0505
Gabo Island, 1517 0607 1829 0404 3333	Apollo Bay, 1416 0506 4242 0506 2323
Gabo Island, 1616 0606 3240 0606 1424	Apollo Bay, 1620 0506 3238 0404 2323
Gabo Island, 1516 0506 2640 0506 1414	Apollo Bay, 1617 0506 1836 0305 2121
Gabo Island, 1618 0506 2323 0404 2222	Apollo Bay, 1218 0507 3939 0505 4141
Gabo Island, 1616 0506 3335 0404 2323	Apollo Bay, 1016 0506 3342 0506 2126
Gabo Island, 1718 0606 3333 0606 1421	Apollo Bay, 1516 0505 3636 0505 2626
Gabo Island, 1313 0506 3335 0101 3337	Apollo Bay, 1616 0505 2440 0405 1720
POP	POP
Tullaburga Island, 1617 0606 4343 0404 2929	Cape Schanck, 1314 0606 2528 0505 3746
Tullaburga Island, 1718 0606 3232 0506 1129	Cape Schanck, 1616 0506 2838 0707 5151
Tullaburga Island, 1617 0506 3235 0404 1111	Cape Schanck, 1517 0505 2727 0606 4444
Tullaburga Island, 1617 0507 3742 0506 2020	Cape Schanck, 1416 0505 3535 0606 3535
Tullaburga Island, 1618 0507 1315 0404 2124	Cape Schanck, 1616 0506 2525 0505 4444
Tullaburga Island, 1618 0607 1315 0506 3437	Cape Schanck, 1420 0505 3434 0505 2121
Tullaburga Island, 1616 0506 4141 0505 2020	Cape Schanck, 1616 0506 2137 0101 3232
Tullaburga Island, 1616 0506 2929 0505 2133	Cape Schanck, 1616 0506 1313 0101 2525
Tullaburga Island, 1616 0506 3030 0707 1922	Cape Schanck, 1616 0607 3845 0101 4141
Tullaburga Island, 1616 0606 3140 0606 2727	Cape Schanck, 1314 0505 3636 0505 4040
POP	POP
Sandpatch Point, 1616 0606 4545 1313 1943	Point Cook, 1417 0606 2828 0606 3232
Sandpatch Point, 1516 0505 4747 0101 4545	Point Cook, 1014 0507 1818 0101 4343
Sandpatch Point, 1616 0607 3133 0606 2424	Point Cook, 1617 0606 3232 0101 3838
Sandpatch Point, 1718 0505 2025 0606 4242	Point Cook, 1616 0506 3037 0101 1523
Sandpatch Point, 1616 0506 2125 0506 0101	Point Cook, 1417 0606 3131 0101 2323
Sandpatch Point, 1617 0606 1313 0707 0119	Point Cook, 1617 0506 2222 0101 1919
Sandpatch Point, 1619 0506 3333 0101 0101	Point Cook, 1616 0506 4141 0606 3030
Sandpatch Point, 1617 0506 2222 0404 0101	Point Cook, 1617 0506 2630 0101 2323
Sandpatch Point, 1416 0506 3030 0505 4343	Point Cook, 1618 0506 1330 0101 2138
Sandpatch Point, 1217 0506 3434 0404 2022	Point Cook, 1616 0505 2828 0101 2222
POP	POP
Petrel Point, 1616 0607 3030 0505 2121	Portland, 1617 0506 3333 0105 3945
Petrel Point, 1616 0507 3131 0404 3030	Portland, 1617 0606 3333 0101 2022
Petrel Point, 0916 0606 3333 0304 2349	Portland, 1213 0506 2525 0303 4141
Petrel Point, 1619 0506 2121 0505 2222	Portland, 1516 0505 1313 0104 0303
Petrel Point, 1617 0506 4040 0404 3236	Portland, 1617 0506 1526 0303 0333
Petrel Point, 1618 0607 3334 0404 3149	Portland, 1516 0506 3333 0101 3333
Petrel Point, 1619 0506 2230 1111 2020	Portland, 1116 0607 2526 0303 0321
Petrel Point, 1313 0606 2626 0404 2222	Portland, 1616 0505 4141 0106 1819
Petrel Point, 1217 0505 1315 0404 3949	Portland, 1616 0606 3232 0104 2626
Petrel Point, 1616 0606 1313 0404 2229	Portland, 1617 0505 2122 0707 2125