Genetic Diversity in Oysters

by

Louise Jasmins
Louise English, B.Sc., Hons.
(Flinders University of South Australia)

Submitted in fulfilment of the requirements

for the degree of

Doctor of Philosophy (Aquaculture)

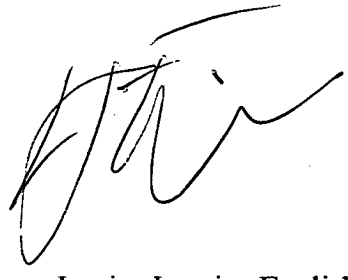
University of Tasmania (February, 2001)

DECLARATION

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the Thesis, and to the best of the candidate's knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the Thesis.

AUTHORITY OF ACCESS

This thesis may be made available for loan and limited copying in accordance with the *Copyright Act 1968.*



Louise Jasmins English

ABSTRACT

This project examined the effects on genetic diversity of oysters by hatchery techniques and selective breeding. The edible oyster industry in Australia comprises of two main species: *Crassostrea gigas* and *Saccostrea glomerata*, which are produced by hatcheries or natural spatfall, respectively. This study examined the levels of genetic diversity as a result of unintentional selection in *C. gigas*, and as a result of intentional selection (a selective breeding program to increase meat weight) in *S. glomerata*. This study has implications for aquaculture species worldwide as it examines the levels of genetic diversity in introduced (eg *C. gigas*) and a previously declining, native (*S. glomerata*) species, which comprise two of the main groups of species utilised for aquaculture.

The oyster aquaculture industry in Tasmania is based on Pacific oysters, *C. gigas*, and derived from imports of Japanese oysters made some 40 years ago. Fears were held that introduction and subsequent domestication had eroded the genetic diversity. As the industry wished to embark on selective breeding programs, a genetic audit of the hatchery stocks was required. Four Australian established (ie non-hatchery produced) populations, and two of Japanese populations that originally imported, were analysed to determine the amount of genetic diversity present. Three different genetic techniques were employed - allozyme electrophoresis, microsatellite DNA and mitochondrial DNA RFLP (restriction fragment length polymorphism) analyses.

Using 17 allozyme loci, three hatchery and four naturalised populations of *Crassostrea gigas* (Thunberg) in Australia were compared with one another and

with two endemic Japanese populations. All populations showed a high degree of genetic variability. The percent of polymorphic loci ranged from an average of 70.6% (hatcheries) through 73.5% (naturalised and Japan). Mean observed heterozygosities ranged from 0.267 (naturalised) through 0.285 (hatcheries) to 0.291 (Japan). Mean numbers of alleles per locus ranged from 3.0 (hatcheries) through 3.3 (naturalised) to 3.5 (Japan). Most loci and populations showed good fits to Hardy-Weinberg expectations; the few significant exceptions were heterozygote deficiencies. Tests of allele frequency differentiation among the nine populations revealed that 11 of the 16 variable loci showed significant (α reduced to 0.05/16 = 0.0031) inter-population heterogeneity after both χ^2 and G_{ST} analysis (Table 2.5). Four loci — *GDA*, *6PGDH*, *PEPS-1* and *PROT-2* — were non-significant for both analyses, and *EST-D* was significant for χ^2 analysis ($P <$ 0.001), but not after Bonferroni correction for G_{ST} analysis ($P = 0.004$). Five populations (BEA, BRI, DUN, SMI, SEN) conformed to Hardy-Weinberg equilibrium for all loci. A few populations and loci did not conform (after Bonferroni correction, using $\alpha = 0.0031$): HIR (*AK*, *DIA*, *PGI*), NSW (*PEPS-2*), PIT (*DIA*), and SWA (*DIA*) (data not shown). All the non-conforming samples showed heterozygote deficiencies, which were significant in two cases: HIR (*DIA*: $\chi^2 = 35.67$, $P < 0.001$; $D = -0.314$, $P < 0.001$); and SWA (*DIA*: $\chi^2 =$ 79.04, $P = 0.001$, $D = -0.397$, $P < 0.001$). Allele-frequency differences among populations were minor, although sometimes statistically significant: only about 1% of the allele frequency variation could be attributed to among-population differences. The levels of homozygous excess observed in this study were lower than that previously reported for this species, but may be due to the same reasons

such as gel scoring errors, null alleles, selection, inbreeding, population admixture or sampling error.

Four microsatellite loci (consisting of two dinucleotide, one tetranucleotide and one tetranucleotide/dinucleotide motifs) were used to analyse the populations. Primers were designed on clone sequence containing at least five repeats of a microsatellite motif. Ten sets of primers were trialled but only four sets had variability levels useful for population genetics analyses, as the others had between 23-34 alleles or were monomorphic. High levels of genetic variability were observed (mean polymorphism = 0.889, mean heterozygosity = 0.188). Tests of allele frequency differentiation among the nine populations revealed that three of the four variable loci showed significant (α reduced to 0.05/4 = 0.0125) inter-population heterogeneity after χ^2 analysis and G_{ST} analysis — *cmrCg17*, *BV59* and *cmrCg61*. The amount of differentiation among the populations was, however, small. Across all loci, only $\approx$ 4% of the genetic variation could be attributed to differences among populations. No population conformed to Hardy-Weinberg equilibrium for all loci. Where populations did not conform to Hardy-Weinberg equilibrium, a significant excess of homozygotes was observed. Although null alleles have been previously reported for the loci used in this study null alleles do not appear to explain the heterozygote deficiencies observed in this study, based on analysis by the NullTest program (W. Amos, pers. comm.) as the frequency of the proposed null alleles seems too high. Unbiased (Nei, 1978) genetic distances over the four loci were estimated between all pair-wise combinations of populations. All pair-wise population distances are very small (Nei D<0.03). However, the

standard errors of the distances are large (ranging to 0.0295±0.0867 between the SWA/BEA/BRI/HIR/PIT/NSW/SMI/SEN and DUN clusters), indicating that the groupings, based on only four loci, are not statistically meaningful. There were no significant changes of genetic diversity between the populations. Overall, the use of microsatellites confirmed the results of the allozyme study of these populations. - the introduction of oysters from Japan to Tasmania, and their subsequent domestication, have not substantially eroded the genetic basis of the Tasmanian stock.

Very little variation was found using mitochondrial DNA (mtDNA) RFLP analysis using 12 restriction enzymes in one hatchery and one endemic population. The mtDNA fragment used was found to contain two conserved genes explaining the lack of variability observed. The technical limitations and lack of knowledge of the oyster mtDNA genome made this approach inappropriate for population genetics analysis. However, the identification of the proximity of the 16srRNA, and COIII genes observed in this study gives further insight into the mtDNA gene order of *C. gigas*. Together with previous findings of the mtDNA RE site map (Oohara and Mori, 1989), the location of the cytochrome b gene (Li and Hedgecock, 1998), this study demonstrates a block of COIII, 16srRNA and cytochrome b mtDNA genes —a combination unique to bivalves.

Two generations of a selected breeding line for increased whole weight (using mass selection) and unselected group of the Sydney rock oyster *Saccostrea glomerata* were examined using allozyme electrophoresis (a total of 14 loci

analysed). Genetic variability levels were determined for each group - all were high and similar to one another (mean percentage polymorphic, 66.7, mean observed heterozygosity, 0.222; mean number of alleles, 2.5). Genotype frequencies at all loci in all groups conformed to Hardy-Weinberg equilibrium, except for the *ESTD-2* locus in the second generation ($P<0.001$), which had a large and significant excess of homozygotes (Selander index D = -0.451, $P<0.001$). Thus significant allele frequency differences were observed at seven loci between control and second generation, at eight loci between second and third generation, and at only one locus for control and third generation. This suggests that the second generation sample is responsible for most of the heterogeneity observed. Despite the difference between actual and estimated broodstock numbers, the expected numbers of alleles of the second and third generations of the selected breeding line were very close to the observed numbers in all cases, suggesting that random genetic drift (sampling variation) alone was the cause of allelic variation between the groups. The results of this allozyme survey indicate that the selective breeding for increased whole weight has not substantially eroded levels of genetic variation. There are high levels of genetic variation present in the control group and in the two generations of the selected breeding line. The limited, but statistically significant, heterogeneity between the second generation and other samples appears to be due to a sampling artifact; it is likely to be biologically unimportant. The type of selective breeding program used (mass selection) aided the retention of genetic diversity across the generations sampled. By using oysters from 4 different areas and using large numbers of oysters for spawning helped to achieve the levels of genetic diversity observed.

In summary, this study has not only substantially broadened the base of knowledge in the two oysters species investigated, but also shown that intentional (in the form of selective breeding) and unintentional (in the form of introduction and subsequent domestication) selection of aquaculture species need not result in major allele loss. Without major allele loss an aquaculture species then has the potential for successful selective breeding for desired traits, as the genetic diversity and hence likelihood of finding particular gene(s) of interest have not been diminished significantly, and has every chance of success. That the two commercially important edible oyster species (introduced and native) in this study have not shown a large magnitude of genetic diversity loss despite either introduction, naturalisation and domestication, or selective breeding serves as a encouraging example to the oyster industries and researchers involved as well as those in other countries and indeed other aquaculture species.

ACKNOWLEDGEMENTS

To the Cooperative Research Centre for Aquaculture Ltd and Tasmanian Oyster Research Council Ltd (TORC), for funding this project.

To my supervisors, Drs Bob Ward and Greg Maguire

To Dr John Benzie and Prof Ned Pankhurst for their feedback on drafts, encouragement and support.

To Drs Nick Elliot, Dan McGoldrick, Peter Rothlisberg and Vivienne Mawson and various anonymous referees for feedback on the paper versions of some of the chapters.

To the following for technical advice: Pat Graham (allozymes); Anne Reilly, Bronwyn Innes (microsatellites), Dr Peter Grewe (mtDNA).

To the following for the supplying or collecting oysters for this study: Colin Sumner (TORC), Dr Katsuyuki Numaguchi, Shellfish Hatcheries, Cameron of Tasmania Pty Ltd, Marine Shellfish Hatcheries, Coal River Oysters, Barilla Bay Oysters, Duck Bay Shellfish, Bolduans Bay Oysters Pty Ltd, Dr John Nell, Ros Hand and Ian Smith (NSW Fisheries, Port Stephens Research Centre), Greg Kent (University of Tasmania) and Brad Evans (CSIRO).

To my friends for their tireless encouragement, support and/or coffee, to mention just a few: Pat and the other Grahams, Assoc. Prof Sue Thomas, Drs Louise Goggin, Marnie Campbell, Jon Havenhand, Greg Kirby; Bronwyn Innes, Anne Reilly, Tracy Atherton, the Adelaide gang, the Port Norris set, Bob and Hazel Robins, Pat Preston, the Penguins, and anyone I've forgotten to mention – you know who you are!

Most of all, to my parents and family for their tireless emotional (and financial) support and encouragement.

TABLE OF CONTENTS

TABLE OF CONTENTS (Cont.)

LIST OF TABLES AND FIGURES

LIST OF TABLES

LIST OF TABLES (Cont.)

LIST OF FIGURES

Chapter 1: General Introduction

1.1 BACKGROUND

The increase in human consumption of fish products is in contrast with the state of the world's major marine fish stocks (FAO, 1998). At present, 44% of fish stocks are fully exploited, 16 % are overfished and 6 % are depleted (FAO, 1998). The contribution of the aquaculture sector to the total food fish and seafood supplies has quadrupled during the last 47 years (FAO, 1998). However, in order for an aquaculture species to be successful, it must have a high productivity in terms of growth but must not be too labour intensive. Several domestication issues must be resolved before any potential aquaculture species can undergo efficient hatchery production. Although most effort in fish culture has been on other conditions essential for domestication such as improved diet, health management and water quality management, genetics and breeding principles are also important in order to maximise the biological potential of the species (Tave, 1993).

Loss of genetic variation at an early stage of domestication of a species is a strong possibility leading to a likely restriction in future productivity, unless the process is properly managed. If fitness is positively correlated with important aquacultural traits, then domestication is beneficial to productivity – but domestication will decrease productivity if fitness is positively correlated with undesired traits in fish and shellfish (Doyle, 1983). Unintentional or unprogrammed selection – for example when broodstock are selected solely on particular traits such as gonad condition/weight/shape, without consideration of their genetic and/or environmental background – may eliminate potentially valuable alleles for aquacultural traits and be detrimental to future selective breeding programs (Tave, 1993).

Loss of genetic diversity in hatchery stocks has been documented in fin fish such as Atlantic salmon, brown trout, cutthroat trout, and channel catfish (Tave, 1993). Loss of genetic diversity has been observed as loss of mean number of alleles, mean observed heterozygosity or as a result of decreased effective mean population size (N_e, number of parents producing viable offspring). For example, a small overall loss of alleles and heterozygosity was observed in Tasmanian cultured Atlantic salmon when compared to their ancestral Canadian population (Reilly et al., 1998). Although there was no reduction in heterozygosity, loss of alleles was observed in one of two strains of reared rainbow trout (Butler and Cross, 1996).

In shellfish, similar losses of diversity have been observed in hatchery stocks. A hatchery population of *Panaeus vannemi* had fewer alleles and slightly lower levels of heterozygosity compared to wild populations, despite no reduction in effective population size or evidence of inbreeding (Sunden and Davis, 1991). Effective population size is important as it gives an indication of the genetic stability of a population as N_e is inversely related to both inbreeding and genetic drift (Crow and Denniston, 1988). Calculation of effective population size numbers were all found to be below 100, with 13 cases less than 50, in various shellfish hatchery stocks of hard clams, prawns, pearl oysters, the American oyster and Pacific oysters (Hedgecock et al., 1992). In most cases, the number of parents actually used as broodstock was substantially higher than the mean effective population size calculated (Hedgecock et al., 1992).

Inbreeding can lead to rare deleterious alleles having a greater likelihood of being paired and expressed, thus leading to a decrease in productivity (Tave, 1993). Studies of inbreeding in fish species (including rainbow trout (Calaprice, 1969; Aulstad and Kitelsen, 1971; Bridges, 1973, Kincaid, 1976a, 1976b, 1983; Davis 1976, Gjerde et al., 1983; Gjerde, 1988), Atlantic salmon (Ryman, 1970), brook trout (Cooper, 1961;

Davis, 1976), brown trout (Davis, 1976), common carp (Moav and Wohlfarth, 1968), *Tilapia mossambica* (Ch'ang, 1971), channel catfish (Bondari 1984; Bondari and Dunham, 1987), zebra danio (Pirion, 1978; Mrakovcic and Haley, 1979), eastern mosquitofish (Leberg, 1990) and convict cichlids (Winemiller and Taylor, 1982)), generally found changes detrimental to production such as growth and viability, and an increase in the number of abnormalities where the levels of inbreeding where 25-60% (see Tave, 1993). However, low levels of inbreeding may enhance production, as Kincaid (1977) found increased productivity without increasing developmental abnormalities or viability in rainbow trout with levels of inbreeding less than 18%.

A number of strategies have been developed to prevent high levels of inbreeding and loss of genetic diversity in fish (Tave, 1993). These strategies include maintaining a high number of parents (mean effective population size) per generation and using a 1:1 ratio of males to females in spawning. Despite theses attempts at reducing loss of diversity, a variety of other factors can lead to loss of variation; for example taking broodstock from wild populations where bottlenecks in population numbers have occurred, sampling error and selection, and differential fertilisation success of gametes from different individuals (Tave, 1993).

Since a range of factors can operate to reduce genetic diversity, and since management strategies that have been attempted do not always meet their goals, it is important to monitor genetic diversity, particularly in the early stages of domestication. However, despite the widespread evidence of uncontrolled inbreeding, there have been relatively few detailed descriptions of changes in genetic variation at early stages of domestication in shellfish. Most have been several years or decades after domestication e.g. in U.S. stocks of *Penaeus vannemi* (Sunden and Davis, 1991); in the European oyster, *Ostrea edulis*, in France (Saavedra, 1997; Bierne et al., 1998) and Spain (Saavedra, 1997); in the Pacific oyster *Crassostrea*

gigas stocks in the United States (Hedgecock and Sly, 1990; Hedgecock et al., 1992) and Britain (Gosling, 19981/82).

The issue of unintentional inbreeding, or loss of genetic diversity is likely to be particularly important for Pacific oyster aquaculture operations in many parts of the world. *C. gigas* has been introduced to Australia, Korea, US, France, China, Taiwan, Philippines and Canada, either to develop a new species for aquaculture, or to augment an existing oyster fishery in decline (Chew, 1990). Often this has involved the introduction of a relatively small number of individuals and there has been little research establishing the current genetic status of the introduced populations. The Australian populations, with good records of the source and number of introductions, provides an excellent case study with which to examine the genetic diversity in the ancestral, hatchery and naturalised stocks.

The oyster industry in Australia made sales worth approximately Australian $34.6 million in 1989/90 (Graham, 1991). The two main types of oyster farmed in Australia are the Sydney rock oyster (*Saccostrea glomerata*, note species name change from *S. commercialis*, see Anderson and Adlard, 1984) and the Pacific oyster (*C. gigas*) (Graham, 1991). The Sydney rock oyster differs from the Pacific oyster in various ways (Holliday and Nell, 1986); Pacific oysters have a faster growth rate, a thinner and lighter shell with long, spiky protrusions, lack of hinge teeth on the inside of the upper shell and darker-coloured mantle edges and adductor-muscle scar, than the Sydney rock oyster. The Sydney rock oyster is native to New South Wales, where it is farmed extensively. Smaller industries based on this species exist in Queensland and Victoria (Graham, 1991).

In contrast, the Pacific oyster was introduced to Australia in the late 1940s and early 1950s (Thomson, 1952, 1959). Given the relatively small number of introduced

animals, there could have been a bottleneck at the time, reducing genetic diversity. It is also possible that hatchery technique, using few individuals, could have reduced genetic variation. Specific attempts to selectively breed animals for example for shell colour or growth, and new approaches such as polyploid manipulation might also result in reduced genetic diversity. Loss of genetic variation at an early stage of domestication of a new species is a strong possibility leading to possible restriction in future productivity unless the process is managed. The Tasmanian oyster industry was concerned that anecdotal evidence from farmers of peculiarly-shaped oyster shells had indicated that inbreeding had occurred and that genetic diversity was severely limited and possibly inadequate to sustain planned genetic improvement programs.

The aims of this PhD project are to examine the effects of hatchery techniques on genetic diversity in *C. gigas*, and selective breeding in *S. glomerata*, using a variety of genetic techniques.

1.1.1 Genetic variation in the Pacific oyster *Crassostrea gigas*.

To improve the increasingly valuable oyster industry in Australia, oyster growers and researchers are interested in expanding selective breeding programs (Ward et al., 1999). The Australian Pacific oyster industry has the required hatchery base, but there were concerns that genetic variability might have been lost during the 50 or so years of naturalisation. Because of risk of disease, further importation of stock is not an option (Treadwell et al., 1992). Losses of genetic diversity in hatchery-produced Pacific oysters have been documented in Britain and the United States of America

(Gosling, 1982; Hedgecock and Sly, 1990, respectively), so an assessment of the genetic diversity of the Tasmanian hatchery stocks was requested, compared to that of naturalised Australian and endemic Japanese populations.

C. gigas samples from naturalised populations in Australia were obtained from two sites that were about 20 km apart along the Tamar River in Tasmania (Beauty Point and Swan Bay), from the Brid River, in Bridport in Tasmania, and from Port Stephens (≈ 200 km north of Sydney) in New South Wales. Three Tasmanian farmed populations, derived from hatchery-produced spat, were sampled: Smithton, Dunalley and Pittwater. Two endemic Japanese populations were sampled: Sendai and Hiroshima, representing two of the four ancestral populations of *C. gigas* in Australia. To prevent any geographic effect misrepresenting the genetic diversity levels in a group (naturalised, hatchery, Japanese), sites that were geographically different and distant were included in the sampling regime.

The null hypothesis is that no major loss in genetic diversity has resulted from a) introduction and subsequent naturalisation of the original founding population from Japan and/or b) hatchery production of *C. gigas*. This main study of genetic diversity levels in *C. gigas* naturalised, hatchery and endemic populations employs three techniques: allozyme electrophoresis, PCR-based mitochondrial DNA (mtDNA) restriction fragment length polymorphism analysis and microsatellite analysis. It has been suggested that the best way to examine levels of genetic variation is to use a variety of genetic techniques, as allozymes typically examine levels of broad-scale variation, whereas mtDNA and microsatellite techniques have greater potential to examine fine-scale population structure and/or recent bottlenecks in population

histories due to a smaller effective population size or more alleles observed, respectively (Burton, 1996; Hilbish, 1996; O'Connell and Wright, 1997; Hauser and Ward, 1998).

Each of the three techniques used in this study has advantages and disadvantages with respect to time, expense, amount of tissue needed, level of power and ease of use (Ward and Grewe, 1994; O'Connell and Wright, 1997). Allozyme analysis is the cheapest technique in terms of initial setup costs for reagents, labour and time followed by mtDNA and microsatellite analyses. However, the PCR based technology used in mtDNA and microsatellite analyses requires less tissue than allozyme analysis, which sometimes requires the death of the animal for tissue samples, and enables faster large scale screening (after initial setup stage). These techniques also vary in terms of frequency of application to oyster populations genetics, such that there are substantially more allozyme studies (Buroker et al., 1975; Buroker et al., 1979a; Gosling, 1982; Smith et al., 1986; Moraga et al., 1989; Hedgecock and Sly, 1990; Dupree, 1993;) than mtDNA ((whole mtDNA (Boome et al., 1994), SSCP (Li and Hedgecock, 1998), no PCR-based mtDNA RFLP population studies existed at the start of this study)) and microsatellite (Hedgecock et al, 1995; Magoulas et al., 1998) studies of *C. gigas*. Therefore, the first technique used for this study was allozyme analysis based on the fact it was the least expensive, and had highest frequency of use in the literature.

1.1.2 The effects of selective breeding on genetic diversity

At the commencement of the *C. gigas* study, no selective breeding programme had been developed for this species in Australia. However, a selective breeding programme was in progress for the native Sydney rock oyster, *S. glomerata* (Nell et al., 1996). Whereas the Pacific oyster study examined the effect on genetic diversity by naturalisation, domestication and possibly unintentional selection of an introduced oyster species, the examination of the *S. glomerata* selective breeding line gave an insight into the effects of intentional selection on genetic variation. As records of the number of parents spawned were available, the effective population sizes (N_K) were estimated from temporal variance in allele frequencies (Pollak, 1983; Waples, 1989; Hedgecock and Sly, 1990; Hedgecock et al., 1992). These calculations allowed the determination of whether any allele loss was due to random genetic drift alone. This calculation was not possible in the *C. gigas* study as the records of number of parents was not available and/or most importantly, assumptions essential for this calculation were violated.

In an attempt to increase profitability and to meet competition from the faster growing Pacific oyster in Tasmania, South Australia and New Zealand, a *S. glomerata* selective breeding program was established in 1990 (Nell et al., 1996). An increase in whole weight of 18% was gained by the third generation (Nell et al., 1999). Production of triploid Sydney rock oysters rates has also been examined for increasing whole weight (Nell et al., 1994).

Hence, the analysis of this *S. glomerata* selective breeding line offered a unique opportunity to examine of the effect of intentional selection on the genetic diversity of this endemic oyster species. Therefore, the genetic diversity of two generations and a control group of a selected breeding line for increased whole weight (Nell et al., 1996) was examined using allozyme analysis (see Chapter 5).

In summary, the aims of this PhD project are to examine the effects of hatchery techniques on genetic diversity in the introduced *C. gigas*, and selective breeding in endemic *S. glomerata* in Australia. Results of this study will not only represent the first comprehensive studies of genetic diversity in *C. gigas* hatchery stocks in Australia and effects of selective breeding in *S. glomerata*, but also give an insight into effects of hatchery practice and selective breeding on the levels of genetic diversity in an introduced and an endemic aquaculture species, respectively.

In terms of thesis structure, there is some planned overlap in the content of the thesis as a result of the following chapters being based on papers that have been accepted for publication:

- Chapter 2: "Genetic variation of wild and hatchery populations of the Pacific oyster, *Crassostrea gigas* (Thunberg), in Australia", by L.J. English, G.B. Maguire, R.D. Ward, Aquaculture, 2000, vol. 187, pages 283-298.
- Chapter 5: "No loss of allozyme variation in three generations of selection for whole weight in Sydney rock oysters (*Saccostrea glomerata*) by L.J. English, J.A. Nell, G.B. Maguire, R.D. Ward, Aquaculture, 2001, vol 193, pages 213-225.

Chapter 2: Genetic variation of wild and hatchery populations of the Pacific oyster, *Crassostrea gigas* (Thunberg), in Australia

2.1 INTRODUCTION

Pacific oysters *Crassostrea gigas,* were introduced into Australia from Japan by the Commonwealth Scientific Industrial Research Organization (CSIRO) to increase oyster cultivation in temperate waters in Australia (Thomson, 1952, 1959). In 1947-48, 50 cases of spat were shipped from Sendai (the Miyagi-strain), 50 from Hiroshima, and 5 from Kumamoto (about 15, 000 oysters per case, Thomson, 1952). The 30% of oysters that survived were set out at Oyster Harbour (south-western Western Australia) and Pittwater (Tasmania). Only the oysters at the latter site survived. Although they spawned, recruitment was not high enough to sustain the population. A further 80 cases were flown out from Japan to Pittwater during 1951-52 (Thomson, 1952; their origins not specified). In 1953, most of the oysters were moved to Port Sorrell in north Tasmania, where the higher summer temperatures were thought to be beneficial for spawning. Spatfall there appeared to be quite regular, and spat spread west to the Mersey River (16 km away) and east to the Tamar River (21 km away). There are now several naturalised populations in Tasmania and in New South Wales, the latter possibly resulting from deliberate (although illegal) transplants from Tasmania (Medcof and Wolf, 1975; Holliday and Nell, 1986).

The first commercial Tasmanian hatchery, in 1981, used oysters from the Tamar River (Colin Sumner, personal communication). Since then, other hatcheries have been established in Tasmania (Graham, 1991) and South Australia (Olsen, 1994). Tasmania is Australia's major producer of Pacific oysters, with production estimated at 3750 tonnes and A$19 million in 1995/96; South Australia is the next largest producer at

1000 tonnes and A$4 million in 1995/96 (Brown et al., 1997). All commercial production is hatchery based.

To improve the increasingly valuable Pacific oyster industry in Australia, growers are considering selective breeding programs. The Australian industry has the required hatchery base, but there were concerns that genetic variability might have been lost during the 50 or so years of naturalisation. Because of risk of disease, further importation of stock is not an option (Treadwell et al., 1992). Losses of genetic diversity in hatchery-produced Pacific oysters have been documented in Britain and the United States of America (Gosling, 1982; Hedgecock and Sly, 1990, respectively), so an assessment of the genetic diversity of the Tasmanian hatchery stocks was requested.

An allozyme study of hatchery-produced Tasmanian Pacific oysters (Deupree, 1993) had shown that the typically deep-cupped Tasmanian Pacific oysters were not derived from the similarly deep-cupped Kumamoto oysters (originally thought to be a strain of *Crassostrea gigas*, but now recognised as a separate species *Crassostrea sikamea* ; see Ahmed, 1975; Banks et al., 1994). Instead they are genetically more akin to the shallow-cupped Miyagi strain. However, side-by-side comparisons with Japanese stocks were not carried out, and the study was limited to a single Tasmanian stock.

The present study used allozyme electrophoresis to determine whether the genetic diversity of farmed oysters as a result of hatchery practices in Tasmania has decreased relative to the naturalised Australian populations and to native Japanese populations.

2.2 METHODS

2.2.1 Sample collection

Pacific oysters were collected from seven Australian and two Japanese sites (Table 2.1 and Figure 2.1). Samples from naturalised populations in Australia were obtained from two sites that were about 20 km apart along the Tamar River in Tasmania (Beauty Point and Swan Bay), from Bridport in Tasmania, and from Port Stephens (≈ 200 km north of Sydney) in New South Wales. Three Tasmanian farmed populations, derived from hatchery-produced spat, were sampled: Smithton, Dunalley and Pittwater. Two endemic Japanese populations were sampled: Sendai and Hiroshima. The Tasmanian samples were kept live on ice during transportation, while the Japanese and Port Stephens samples were air-freighted on dry ice. In the laboratory, all samples were stored at -80°C.

2.2.2 Allozyme analysis

Thirteen enzymes and general protein (17 loci) (Table 2.2) were examined in ≈ 100 oysters taken from each of the nine sites. Adductor muscle and digestive tissue (≈ 200 mg) were placed into separate 1.5 mL centrifuge tubes and homogenised in a few drops of distilled water before being microcentrifuged for 10 minutes at 13 500 *G*. The supernatant was subjected to cellulose acetate or starch gel electrophoresis. Helena Titan III cellulose acetate plates used either a tris glycine (0.02 M tris, 0.192 M glycine) or a 75 mM tris citrate (pH 7.0) buffer system (Hebert and Beaton, 1989; Richardson et al., 1986). The tris glycine gels were run at 200 volts and the tris citrate gels at 150 volts (Table 2.2). For starch electrophoresis, small pieces of Whatman No. 3 chromatography paper were soaked in the supernatant and placed in a gel made of 8% Connaught

Table 2.1 . Sample details.

Location	Type	Abbrev.	Approximate locality	Date of collection
Swan Bay (Tamar River, N. Tasmania)	nat	SWA	41°17'S,147°01'E	September 1994
Beauty Point (Tamar River, N. Tasmania)	nat	BEA	41°10'S,146°49'E	September 1994
Bridport (N. Tasmania)	nat	BRI	41°00'S,147°23'E	March 1995
Port Stephens (New South Wales)	nat	NSW	32°43'S,152°11'E	June 1995
Smithton (N. Tasmania)	hat	SMI	40°50'S,145°08'E	February 1995
Pittwater (S. Tasmania)	hat	PIT	43°36'S,151°18'E	March 1995
Dunalley (S. Tasmania)	hat	DUN	42°53'S,147°48'E	July 1995
Sendai Bay (N. Japan)	end	SEN	38°16'N,140°52'E	November 1995
Hiroshima Bay (S. Japan)	end	HIR	34°23'N,132°27'E	November 1995

nat = naturalised, hat = hatchery, end = endemic.

Figure 2.1. Location of the established and farmed Tasmanian samples, and historically important sites (modified from Thomson, 1952).

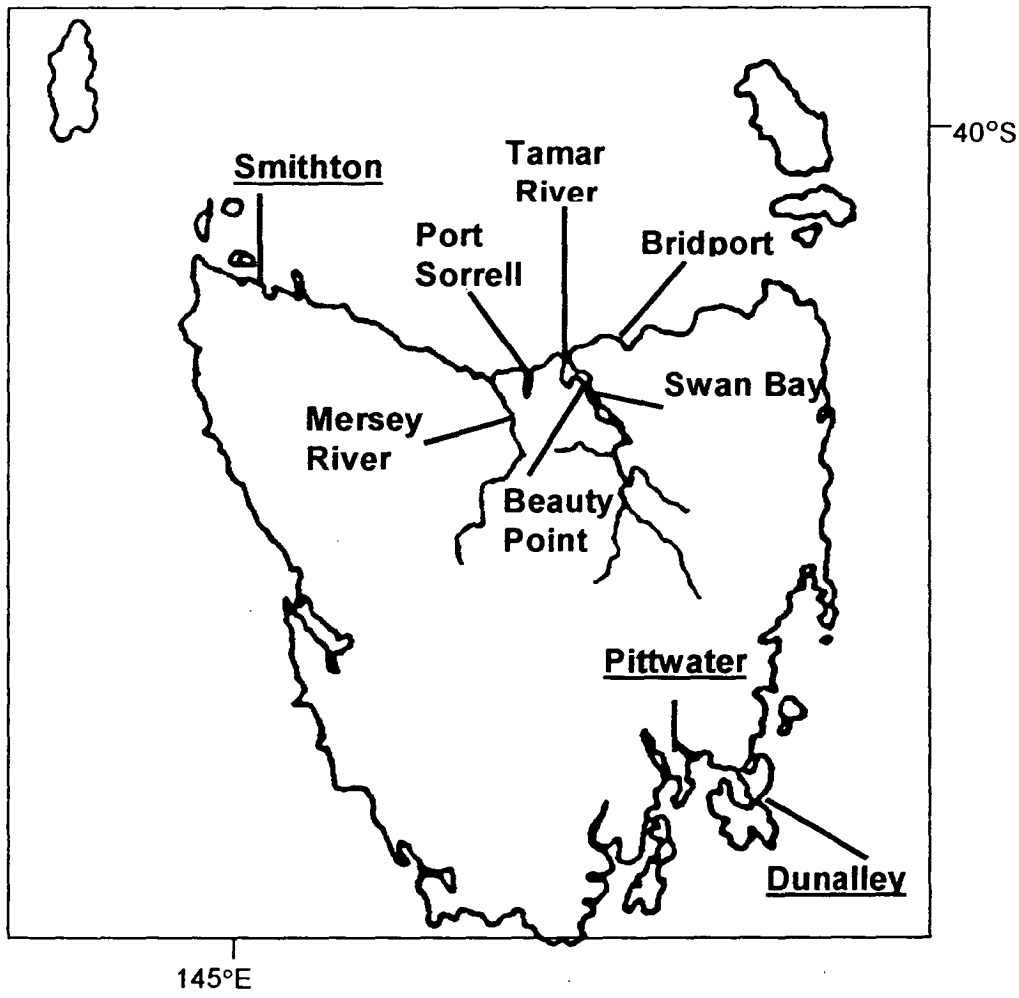


Table 2.2. List of allozymes used in this study.

Enzyme or protein name	Locus	EC number	Buffer	Time (mins)	Tissue	Structure
Aspartate aminotransferase	*AAT*	2.6.1.1	TG	35	a	dimer
Adenylate kinase	*AK*	2.7.4.3	LHTNC	300	a	dimer
Aldolase	*ALD*	4.1.2.13	TC	45	a	(invariant)
Diaphorase	*DIA*	1.6.*.*	LHTNC	300	d	monomer
Esterase-D	*ESTD*	3.1.1.1	LHTNC	300	a	dimer
Glyceraldehyde-3-phosphate dehydrogenase	*G3PDH*	1.2.1.12	TC	80	a	tetramer?
Guanine deaminase	*GDA*	3.5.43	TG	35	a	dimer
Isocitrate dehydrogenase	*IDH-1*	1.1.1.42	TC	70	a	dimer
	IDH-2	1.1.1.42	TC	70	a	dimer
Malate dehydrogenase	*MDH*	1.1.1.37	TC	90	a	dimer
6-Phosphogluconate dehydrogenase	*6PGDH*	1.1.1.44	TC	90	a	dimer
Phosphoglucose isomerase	*PGI*	5.3.1.9	LHTNC	300	a	dimer
Peptidase	*PEPS-1*	3.4.11/13?	TG	35	a	momer
	PEPS-2	3.4.11/13?	TG	35	a	dimer
General protein	*PROT-1*	-	TG	35	a	monomer
	PROT-2	-	TG	35	a	monomer
Superoxide dismutase	*SOD*	1.15.1.1	LHTNC	300	d	dimer

TC = Tris citrate, TG = Tris glycine, LHTNC = L-Histidine/Trisodium citrate (starch)
a = adductor muscle, d = digestive tissue
Structure determined from heterozygote band patterns. Note that *G3PDH* heterozygotes were diffuse and the five bands expected of a tetramer could not be resolved.

hydrolysed starch in a 5 mM l-Histidine HCl buffer (pH adjusted to 7.0 with 0.1 M sodium hydroxide). The starch gel was run in 0.41 M trisodium citrate electrode buffer (pH adjusted to 7.0 with 0.5 M citric acid) at 100 volts. The peptidase stain used phenylalanine-leucine as a substrate. General proteins were stained with Coomassie Blue. Where an enzyme had multiple loci, the locus encoding the fastest migrating allozyme was designated '1'. Alleles within loci were numbered according to the anodal mobility of their product relative to that of the most common allele, designated '100'.

2.2.3 Data Analysis

Mean sample sizes, numbers of alleles, heterozygosities and proportions of polymorphic loci were calculated by BIOSYS-1 (Swofford and Selander 1989). Genetic similarities between populations were assessed by Nei's (1978) unbiased genetic distance and Rogers' (1972) distance, clustering the distance matrices by unweighted pair group analysis or Wagner analysis respectively, again using BIOSYS-1.

Allele frequency heterogeneity across populations and comparison between groups of number of alleles per locus, percent loci polymorphic, mean expected Hardy-Weinberg heterozygosity were assessed using the randomised Monte Carlo chi square procedure of Roff and Bentzen (1989), thus avoiding the need to pool rare alleles. The data were randomised 1000 times per test. The number of times the randomised chi-squares were greater than or equal to the observed value divided by 1000 gave the probability value for homogeneity. Loci that showed significant differentiation were then compared by population, in pairs, to locate the source of the heterogeneity.

Testing of conformation of Hardy-Weinberg equilibrium per population and locus used 1000 replicates per test of the CHIHW program (Zaykin and Pudovkin, 1993). Again, this obviated the need to pool rare alleles. The Selander index [$(H_{obs}-H_{exp})/H_{exp}$, where H_{obs} is the observed and H_{exp} is the Hardy-Weinberg-expected heterozygosities] was estimated and tested for loci showing a significant deviation, again using the CHIHW program.

To allow for multiple tests of the same hypothesis, Bonferroni corrections of the preset significance level, α, were made by dividing by the number of tests. For example, where 16 variable loci were being tested, α levels were reduced from 0.05 to 0.05/16 = 0.0031.

Nei's gene diversity statistic G_{ST} (Nei, 1973) was used to determine the proportion of the total genetic variation observed that is attributable to variation between populations. The extent of variation that is attributable to sampling error alone, $G_{ST.null}$, was estimated by the BOOT-IT program, using 900 replicates (Ward and Grewe, unpublished).

2.3 RESULTS

Allele frequencies for the 17 loci examined in the nine populations were determined (Table 2.3). Heterozygote banding patterns for each of the enzymes showed the appropriate number of bands based on known subunit numbers (Ward et al., 1992). Only one locus (*ALD*) was invariant. Four loci (*PROT-1*, *MDH*, *IDH-2*, *SOD*) showed only rare variants. The remaining twelve loci showed medium to high levels of variability. Genetic variability levels were determined for each population (Table 2.4); all were high. The mean number of alleles per locus per population was 2.9, the mean percent loci polymorphic was 72.5, the mean observed heterozygosity per locus was 0.279, and

Table 2.3: Allele frequencies of 17 loci in nine populations of *C. gigas*.

Locus	Allele	SWA	BEA	BRI	SMI	PIT	DUN	NSW	SEN	HIR
AAT	1100	-	0.005	0.005	-	-	-	-	-	-
	800	0.291	0.313	0.335	0.270	0.392	0.455	0.170	0.237	0.212
	100	0.655	0.620	0.580	0.530	0.423	0.414	0.770	0.660	0.687
	-700	0.055	0.063	0.080	0.200	0.186	0.131	0.060	0.103	0.101
	n	110	104	100	100	97	99	100	97	99
AK	120	0.005	0.014	-	-	-	-	-	0.030	-
	110	0.171	0.168	0.147	0.091	0.195	0.186	0.050	0.130	0.105
	100	0.748	0.700	0.766	0.811	0.705	0.675	0.875	0.730	0.820
	85	0.032	0.064	0.065	0.085	0.084	0.062	0.045	0.070	0.060
	75	0.045	0.036	0.022	0.006	0.016	0.057	0.025	0.040	0.015
	65	-	0.018	-	0.006	-	0.021	0.005	-	-
	n	111	110	92	82	95	97	100	100	100
ALD	100	1	1	1	1	1	1	1	1	1
	n	112	109	100	96	93	99	100	99	99
DIA	120	0.018	0.009	0.070	-	0.010	-	-	-	0.005
	110	0.060	0.086	0.170	0.065	0.131	0.056	0.035	0.093	0.085
	100	0.532	0.536	0.470	0.380	0.434	0.475	0.510	0.469	0.480
	90	0.367	0.306	0.245	0.390	0.338	0.354	0.419	0.356	0.385
	70	0.023	0.045	0.040	0.160	0.086	0.111	0.025	0.082	0.045
	60	-	0.018	0.005	0.005	-	0.005	0.010	-	-
	n	109	111	100	100	99	99	99	97	100
ESTD	135	0.011	0.010	0.015	0.070	0.056	0.048	0.010	0.020	0.047
	120	0.115	0.167	0.135	0.225	0.202	0.149	0.165	0.179	0.146
	100	0.845	0.771	0.815	0.680	0.727	0.798	0.809	0.781	0.771
	55	0.029	0.052	0.035	0.025	0.010	0.005	0.015	0.020	0.036
	50	-	-	-	-	0.005	-	-	-	-
	n	87	105	100	100	99	94	97	98	96

Locus	Allele	SWA	BEA	BRI	SMI	PIT	DUN	NSW	SEN	HIR
G3PDH	120	0.005	0.004	0.010	-	-	-	-	-	-
	100	0.757	0.786	0.778	0.696	0.595	0.706	0.591	0.660	0.610
	80	0.239	0.210	0.207	0.304	0.405	0.294	0.409	0.340	0.385
	60	-	-	0.005	-	-	-	-	-	0.005
	n	111	112	99	97	100	97	99	100	100
GDA	135	-	-	-	-	-	-	-	0.005	0.017
	120	0.038	0.065	0.055	0.056	0.070	0.043	0.065	0.057	0.052
	100	0.920	0.925	0.915	0.918	0.890	0.957	0.930	0.923	0.924
	90	0.038	0.009	0.030	0.026	0.040	-	0.005	0.015	0.006
	75	0.005	-	-	-	-	-	-	-	-
	n	106	107	100	98	100	94	100	97	86
IDH-1	115	-	0.005	-	-	-	-	-	-	-
	110	0.023	0.027	0.015	0.035	0.030	0.041	0.052	0.075	0.087
	100	0.845	0.788	0.695	0.695	0.725	0.699	0.655	0.580	0.617
	85	0.132	0.180	0.285	0.265	0.245	0.260	0.294	0.345	0.296
	80	-	-	0.005	0.005	-	-	-	-	-
	n	110	111	100	100	100	98	97	100	98
IDH-2	110	0.040	0.067	0.015	0.080	0.015	0.048	0.026	0.055	0.010
	100	0.960	0.933	0.985	0.920	0.970	0.952	0.974	0.945	0.990
	95	-	-	-	-	0.015	-	-	-	-
	n	112	112	100	100	100	94	97	100	98
MDH	110	0.032	0.013	0.010	-	0.010	0.045	0.086	0.025	-
	100	0.968	0.987	0.985	1.000	0.990	0.955	0.914	0.960	0.995
	80	-	-	0.005	-	-	-	-	0.015	-
	70	-	-	-	-	-	-	-	-	0.005
	n	110	112	100	100	99	99	99	100	100

Locus	Allele	SWA	BEA	BRI	SMI	PIT	DUN	NSW	SEN	HIR
6PGDH	185	0.005	-	-	-	-	-	-	-	-
	165	0.074	0.035	0.021	0.005	0.005	-	0.042	0.040	-
	130	0.137	0.124	0.175	0.098	0.130	0.076	0.125	0.150	0.146
	100	0.774	0.797	0.804	0.875	0.859	0.924	0.833	0.800	0.837
	80	0.011	0.045	-	0.022	0.005	-	-	0.010	0.017
	n	95	101	97	92	96	86	12	100	89
PGI	175	0.005	0.009	0.015	-	-	-	0.015	0.005	-
	135	0.050	0.031	0.055	0.035	0.082	0.112	0.026	0.035	0.050
	110	-	0.004	-	-	-	-	-	0.015	0.025
	100	0.901	0.853	0.835	0.830	0.878	0.811	0.934	0.874	0.875
	90	-	0.054	0.010	0.010	0.015	-	0.010	0.005	0.010
	85	0.045	0.049	0.080	0.125	0.026	0.066	0.015	0.056	0.040
	50	-	-	0.005	-	-	0.010	-	0.010	-
	n	111	112	100	100	98	98	98	99	100
PEPS-1	125	0.005	0.015	0.010	0.026	-	0.053	0.015	0.020	0.025
	115	0.087	0.050	0.085	0.122	0.126	0.059	0.093	0.086	0.060
	100	0.784	0.775	0.780	0.740	0.793	0.809	0.763	0.803	0.795
	90	0.092	0.100	0.095	0.102	0.071	0.080	0.103	0.076	0.100
	85	0.032	0.060	0.030	0.010	0.005	-	0.026	0.015	0.020
	75	-	-	-	-	0.005	-	-	-	-
	n	109	100	100	98	99	94	97	99	100
PEPS-2	115	-	0.033	0.020	-	0.005	0.011	0.015	0.011	0.005
	105	0.376	0.400	0.362	0.330	0.162	0.462	0.263	0.381	0.389
	100	0.486	0.406	0.398	0.510	0.682	0.473	0.581	0.472	0.485
	95	0.133	0.161	0.189	0.140	0.131	0.054	0.136	0.125	0.101
	90	0.005	-	0.031	0.020	0.020	-	0.005	0.011	0.020
	n	109	90	98	100	99	93	99	88	99

Locus	Allele	SWA	BEA	BRI	SMI	PIT	DUN	NSW	SEN	HIR
PROT-1	135	-	-	-	-	-	-	-	-	0.006
	125	0.436	0.387	0.410	0.316	0.359	0.348	0.549	0.448	0.634
	100	0.564	0.613	0.590	0.684	0.641	0.652	0.451	0.552	0.360
	n	110	106	100	98	99	79	81	77	86
PROT-2	150	-	0.005	-	-	-	-	-	-	-
	100	1.000	0.995	0.990	1.000	1.000	1.000	1.000	0.990	0.989
	50	-	-	0.010	-	-	-	-	0.010	0.011
	n	112	100	100	98	99	87	98	98	93
SOD	165	0.004	-	-	-	-	-	-	-	-
	140	-	0.027	-	-	-	-	-	-	0.005
	100	0.996	0.973	1.000	0.995	1.000	1.000	1.000	1.000	0.990
	50	-	-	-	0.005	-	-	-	-	0.005
	n	112	112	100	100	99	98	100	100	100

Table 2.4: Genetic variability at 17 loci in all populations

Population	Type	Mean sample size per locus	Mean no. of alleles per locus	% loci poly-morphic*	Mean heterozygosity	
					Observed	Expected**
SWA	nat	108±1.6	3.2±0.3	70.6	0.254±0.078	0.278±0.050
BEA	nat	106.7±1.5	3.5±0.4	76.5	0.271±0.080	0.303±0.051
BRI	nat	99.2±0.5	3.5±0.4	70.6	0.282±0.085	0.302±0.056
NSW	nat	92.5±5.1	3.1±0.4	76.5	0.262±0.078	0.277±0.050
SMI	hat	98.0±1.2	3.1±0.3	76.5	0.303±0.093	0.310±0.056
PIT	hat	98.3±0.5	3.1±0.4	70.6	0.290±0.088	0.304±0.055
DUN	hat	94.4±1.4	2.8±0.3	64.7	0.296±0.090	0.296±0.055
SEN	end	97.6±1.6	3.5±0.4	76.5	0.287±0.086	0.315±0.054
HIR	end	97.0±1.3	3.4±0.3	70.6	0.267±0.082	0.295±0.054
Means for Population types						
Hatchery (n = 3)		290.7±1.1	3.0±0.3	70.6	0.285±0.055	0.303±0.055
Naturalised (n =4)		406.4±0.8	3.3±0.4	73.5	0.267±0.134	0.290±0.052
Endemic (n = 2)		194.6±1.4	3.5±0.4	73.6	0.291±0.051	0.305±0.054
Mean (n = 9)		99.1±1.7	3.2±0.2	72.7	0.279±0.093	0.298±0.004

nat = naturalised, hat = hatchery, end = endemic

* A locus is considered polymorphic if the frequency of the most common allele was <0.95

** Unbiased Hardy-Weinberg estimate (see Nei, 1978)

the mean Hardy-Weinberg expected heterozygosity per locus was 0.296. All alleles with frequencies of 0.1 or greater were present in all populations. Although some rare alleles were present only in Australian populations, the frequencies were very low (ranging from 0.004 – 0.021), suggesting that these alleles may not have been observed in other populations due to sampling error.

Differences in levels of variation among groups of populations — that is, hatchery versus naturalised versus endemic — were minimal (Table 2.4). The results of the Monte Carlo contingency χ^2 tests between groups also showed these minimal differences for the number of alleles per locus, percent loci polymorphic and mean expected Hardy-Weinberg heterozygosity. The numbers of alleles per locus (3.0±0.3, 3.3±0.4 and 3.5±0.4 respectively) were not significantly different between groups (P = 0.064). However, it is noteworthy that the endemic Japanese population had a higher average number of alleles per locus than the Tasmanian hatchery populations. In fact, the endemic samples showed a total of 63 alleles from an average sample size per locus of 194.6, while the hatchery samples showed a lower total of 59 alleles from a larger average sample size of 290.3. This difference is significant (P = 0.021), indicating that the hatchery samples are a little less variable than the endemic samples. There was no significant difference in percent loci polymorphic (P = 0.670) or mean expected Hardy-Weinberg heterozygosity (P = 0.305) among the hatchery, naturalised or endemic populations. There was a suggestion of heterogeneity for mean observed heterozygosity (P = 0.042), although Bonferroni correction of α level for these four tests of variability (α = 0.05/4 = 0.0125) renders this result non-significant.

Tests of allele frequency differentiation among the nine populations revealed that 11 of the 16 variable loci showed significant (α reduced to 0.05/16 = 0.0031) inter-population

Table 2.5: Summary of Chi square and G_{ST} analyses of 16 variable loci for nine populations.

Locus	No. of alleles	*n*	χ^2 analysis		G_{ST} analysis		
			χ^2	*P*	G_{ST}	$G_{ST.null}$±SD	*P*
AAT	4	906	130.37	<0.001	0.041	0.004±0.002	<0.001
AK	6	887	107.75	<0.001	0.016	0.005±0.002	<0.001
DIA	6	914	176.64	<0.001	0.013	0.004±0.002	<0.001
ESTD	5	876	63.07	<0.001	0.011	0.005±0.002	0.004
G3PDH	4	915	65.14	<0.001	0.026	0.004±0.002	<0.001
GDA	5	888	51.18	0.007	0.004	0.005±0.002	0.537
IDH-1	5	914	90.04	<0.001	0.023	0.004±0.002	<0.001
IDH-2	3	913	47.29	0.001	0.013	0.004±0.002	0.001
MDH	4	919	77.17	<0.001	0.025	0.004±0.002	<0.001
6PGDH	5	768	80.00	0.014	0.012	0.008±0.005	0.178
PGI	7	916	141.53	<0.001	0.013	0.004±0.001	<0.001
PEPS-1	6	896	67.99	0.004	0.005	0.005±0.002	0.466
PEPS-2	5	875	105.41	<0.001	0.025	0.005±0.002	<0.001
PROT-1	3	885	20.15	0.133	0.006	0.004±0.002	0.139
PROT-2	3	836	69.21	<0.001	0.038	0.005±0.002	<0.001
SOD	4	921	51.76	<0.001	0.014	0.004±0.002	<0.001
Across 17 loci					0.017	0.004±0.001	<0.001

heterogeneity after both χ^2 and G_{ST} analysis (Table 2.5). Four loci — *GDA*, *6PGDH*, *PEPS-1* and *PROT-2* — were non-significant for both analyses, and *EST-D* was significant for χ^2 analysis ($P < 0.001$), but not after Bonferroni correction for G_{ST} analysis ($P = 0.004$).

The amount of differentiation among the populations was, however, small. This can be seen in the G_{ST} analyses. For the four loci that were non-significant for both this and the χ^2 analysis, the observed degree of inter-population differentiation, G_{ST}, was very similar to that due to sampling error alone, $G_{ST.null}$. The two loci showing the most differentiation were *AAT* and *PROT-1*; for both of these loci, about 3.5% of the total amount of differentiation could be attributable to inter-population differences once sample effects had been allowed for (i.e. G_{ST} - $G_{ST.null}$). Across all loci, only $\approx$ 1% of the genetic variation could be attributed to differences among populations.

Populations were compared pair-wise to locate the source of this limited genetic differentiation. No significant differences in allele frequencies were observed between the two Tamar River populations (SWA and BEA), between two of the naturalised populations (BEA and BRI) or between one of the Japanese populations (SEN) and one of the Tasmanian hatchery populations (SMI). All other pair-wise population analyses showed significant differences for at least one locus (data not presented).

Five populations (BEA, BRI, DUN, SMI, SEN) conformed to Hardy-Weinberg equilibrium for all loci. A few populations and loci did not conform (after Bonferroni correction, using $\alpha = 0.0031$): HIR (*AK*, *DIA*, *PGI*), NSW (*PEPS-2*), PIT (*DIA*), and SWA (*DIA*) (data not shown). All the non-conforming samples showed heterozygote deficiencies, which were significant in two cases: HIR (*DIA*: $\chi^2 = 35.67$, $P < 0.001$; $D = - 0.314$, $P < 0.001$); and SWA (*DIA*: $\chi^2 = 79.04$, $P = 0.001$, $D = - 0.397$, $P < 0.001$).

Unbiased (Nei, 1978) genetic distances over 17 loci were estimated between all pair-wise combinations of populations, and from the resulting matrix a UPGMA dendrogram of population relationships was generated (Figure 2.2a). The three resident Tasmanian populations form a sub-cluster, as do the three farmed populations; a third sub-cluster is composed of the two Japanese populations and the New South Wales population. All pair-wise population distances are very small (Nei D<0.01). These three subgroups also form after Wagner tree analysis using Rogers (1972) distance (Figure 2.2b). Clustering does not appear to be dependent on the evolutionary model, since the UPGMA method assumes a constant rate of evolution, a constraint not present in the Wagner approach. However, the standard errors of the distances are large (ranging from Nei D = 0.0003±0.004 between SWA and BEA to 0.0101±0.0245 between the SWA/BEA/BRI/PIT/SMI/DUN and SEN/HIR/NSW clusters), such that the nodes of the clusters with their standard errors overlap, suggesting that the groupings are not biologically meaningful. This conclusion is supported by a consideration of allele distributions. For example, there were no alleles common to members of the SEN/HIR/NSW cluster that were not also found in one or more Tasmanian populations.

2.4 DISCUSSION

All populations, both Australian and Japanese, of Pacific oysters showed high genetic variability. Overall, variability levels (about 70% of loci polymorphic and with average heterozygosities per locus of around 0.29) were as high or higher than other surveys of this species (Table 2.6) — differences among the various studies may reflect real differences in variability among samples or the use of different samples of loci and different electrophoretic techniques.

Figure 2.2a: *C.gigas* Cluster Analysis: UPGMA using Nei (1978) distance.

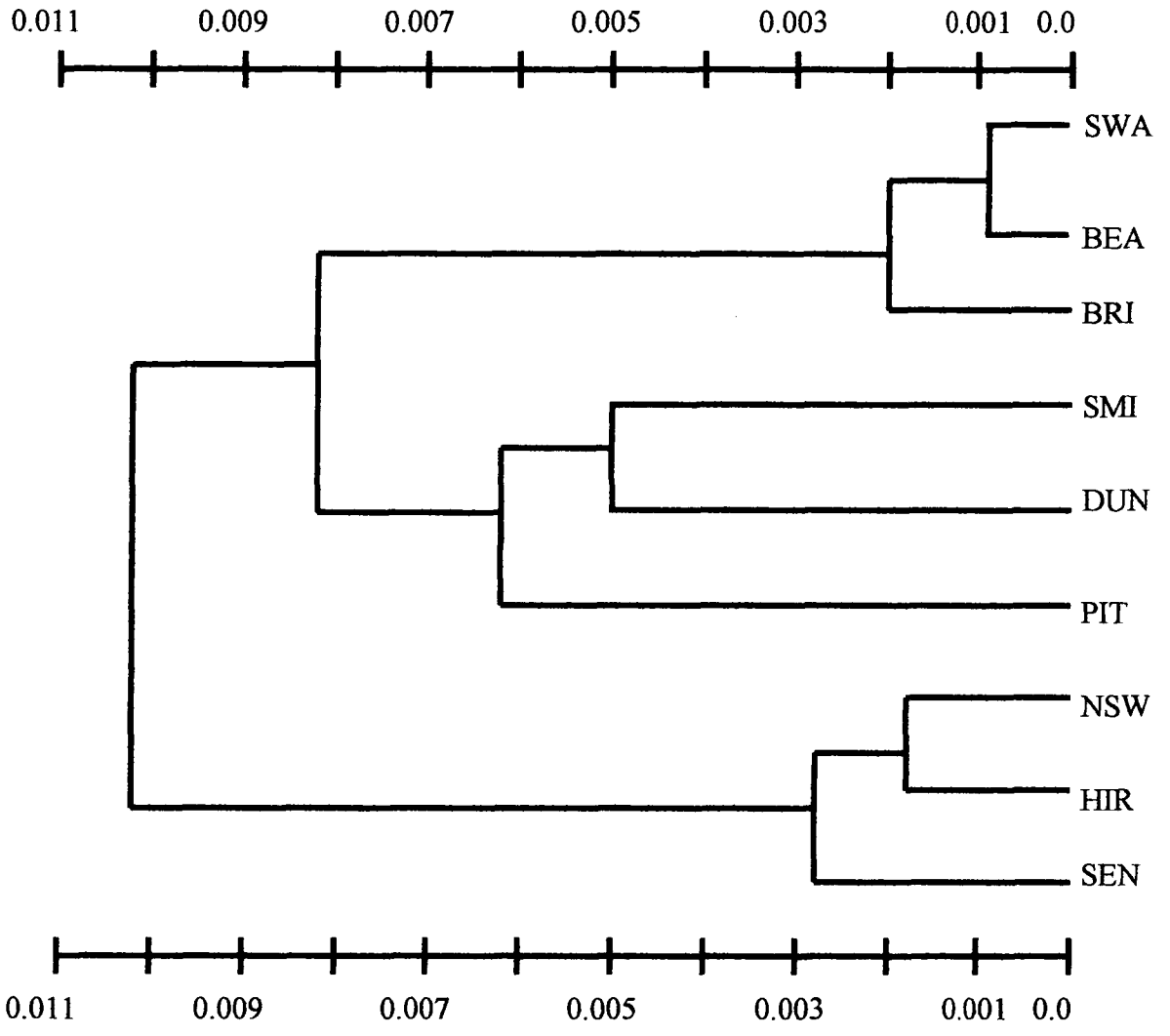


Figure 2b. *C.gigas* Rogers/Wagner Distance Tree

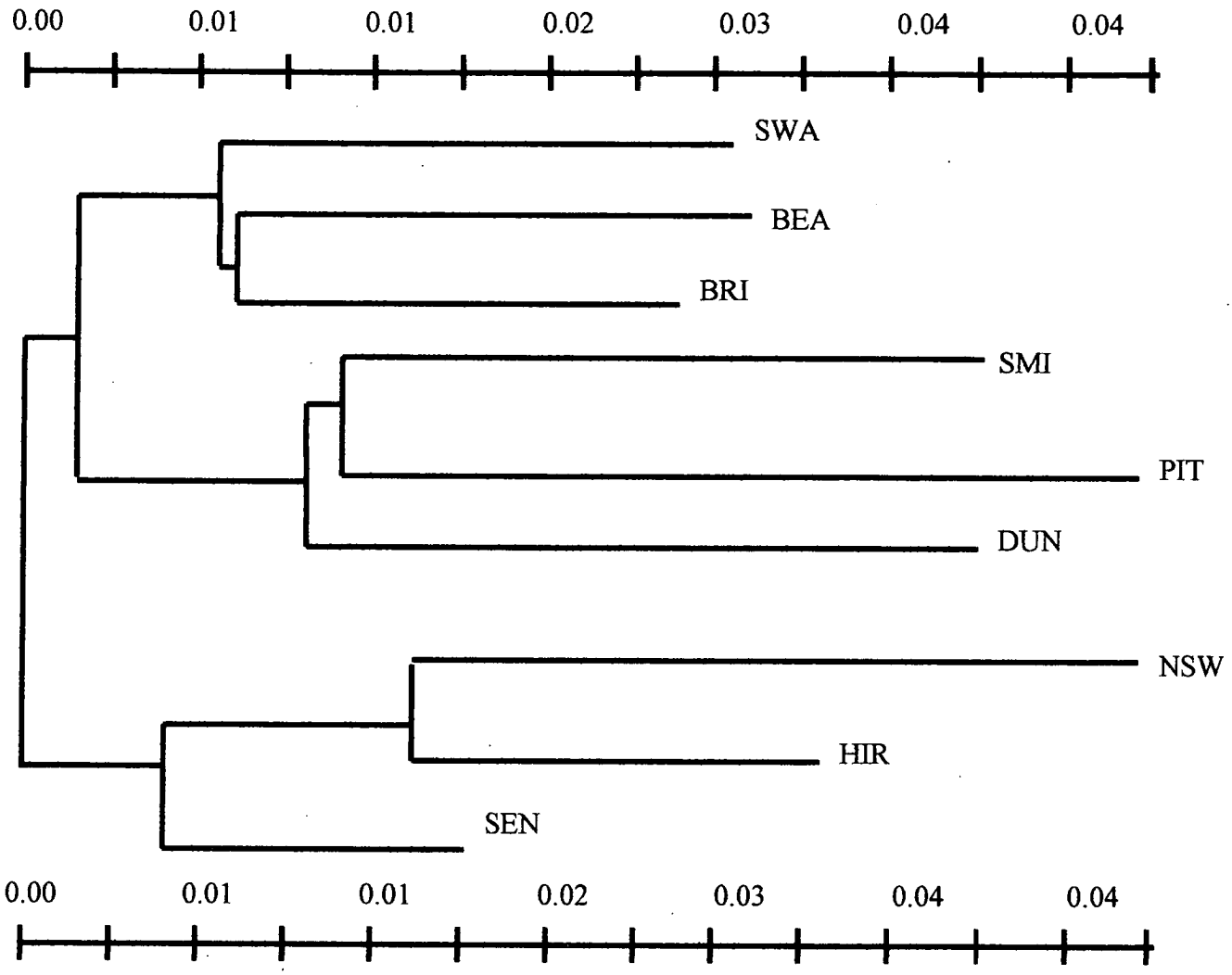


Table 2.6: Summary of previous allozyme studies of *C. gigas*, giving percentage polymorphism (% P) and observed and Hardy-Weinberg expected heterozygosities (*H*). "Kumamoto" refers to *C. sikamea*.

	Samples	Type	Loci (*n*)	Average per locus (*n*)	% P*	Observed *H*	Expected *H*	Mean alleles (*n*)
Buroker et al., 1975**	Mud Bay, USA	nat	15	≤262	53.3	0.210	0.245	2.7
Buroker et al., 1979	Kumamoto	end	27	≤156	59.3	0.195±0.006	0.214±0.006	2.5
	Hiroshima	end	24	≤164	58.3	0.198±0.007	0.201±0.007	2.5
	Miyagi	end	27	≤222	63	0.222±0.006	0.238±0.006	2.6
Dupree, 1993	Miyagi	nat	21	102.6	85.7	0.177	0.205	3.0
	Tasmania	hat	21	107.3	71.4	0.178	0.177	2.5
	Kumamoto	hat	21	105.3	71.4	0.202	0.191	2.5
Gosling, 1982	UK	hat	19	≤70	52.6	0.176	0.203±0.003	1.41
Smith et al., 1986**	East NZ	nat	17	≤52	47.1	0.195	0.238	2.82
	West NZ	nat	17	≤42	47.1	0.168	0.222	2.76
	Mangokuura, Japan	hat	17	≤67	47.1	0.189	0.229	2.82
Moraga et al., 1989	Miyagi, Japan	end	13	50	76.9	0.146	0.189	2.77
	Naturalised pops	nat	13	≤250	57	0.157	0.168	3.17
	Arcachon	hat	13	≤250	59.2	0.157	0.162	2.9
	La Trinité	hat	13	≤250	63.6	0.150	0.152	3.21

Table 2.6 (Cont.): Summary of previous allozyme studies of *C. gigas*, giving percentage polymorphism (% P) and observed and Hardy-Weinberg expected heterozygosities (*H*). "Kumamoto" refers to *C. sikamea*.

	Samples	Type	Loci (*n*)	Average per locus (*n*)	% P*	Observed *H*	Expected *H*	Mean alleles (*n*)
Moraga et al., 1989 (cont.)	Hatchery	hat	13	≤50	62.5	0.155	0.158	2.58
Hedgecock and Sly, 1990**	Dabob Bay, USA	nat	14	57.4±1.0	64.3**	0.262±0.053	0.283±0.058	3.50±0.39
	Willapa Bay, USA	hat	14	57.9±0.1	78.6**	0.268±0.065	0.254±0.056	3.07±0.27
	Humboldt Bay, USA	hat	14	57.1±1.2	57.1**	0.256±0.071	0.252±0.065	2.43±0.31
This study	Hatchery (Tas.)	hat	17	290.7±1.1	70.6	0.292±0.055	0.303±0.055	3.0±0.3
	Naturalised (Tas. and NSW)	nat	17	406.4±0.8	73.5	0.267±0.047	0.290±0.052	3.3±0.4
	Endemic (Japan)	end	17	194.6±1.4	73.6	0.279±0.051	0.305±0.054	3.5±0.4

nat = naturalised, hat = hatchery, end = endemic

*loci for which frequency of the most common allele is 0.99

**loci for which frequency of the most common allele is ≤0.95

NSW = New South Wales, NZ = New Zealand, UK = United Kingdom, USA = United States of America

Differences in numbers of alleles per locus, percentage of loci polymorphic, or heterozygosity levels between the Tasmanian hatchery populations, the Tasmanian and New South Wales naturalised populations, and the Japanese endemic populations were very small or non-existant (Table 2.4). Thus the Australian hatchery stocks showed very little reduction in genetic variation compared with the naturalised or Japanese stocks. Smith et al. (1986) made the same finding when comparing introduced New Zealand wild stocks with cultured oysters from Mangokuura, Japan. However, American hatchery stocks of Pacific oysters are reported to have lost variation for *AAT* (Hedgecock and Sly, 1990) and British stocks to have lost variation for *PGI* (Gosling, 1982).

One of the first casualties of inbreeding is the loss of rare alleles, and changes in allelic diversity after bottlenecks may be more striking than changes in heterozygosity (Allendorf and Ryman, 1987). The reduction in mean numbers of alleles per locus from 3.5 in Japan to 3.0 in Australian hatchery stocks ($P = 0.021$), suggests that some allele loss might have occurred. This would be worth examining further with hyper-variable loci such as microsatellites; such loci typically have far higher numbers of alleles per locus and would be more powerful monitors of genetic variation than allozymes.

Most samples showed good agreement with Hardy-Weinberg expectations, but some significant deviations — all resulting from significant heterozygote deficiencies — were observed. These deviant samples included those from endemic (HIR, one case) and naturalised populations (SWA, one case), but not from hatchery-reared populations. Eight of the nine populations had observed mean heterozygosities per locus less than that expected from Hardy-Weinberg equilibrium — in one population, the two heterozygosities were equal. Similar deficits have been widely observed in *C. gigas* (Buroker et al., 1979 a & b; Fujio, 1979; Gosling, 1982; Smith et al., 1986; Moraga et

al., 1989; Deupree, 1993) and in *C. virginica* (Zouros et al., 1980). Sixteen other studies have observed mean heterozygosities less than that expected from Hardy-Weinberg equilibrium (Table 2.6). The causes of these deviations are not understood, and could be different from case to case, but could include gel scoring errors, null alleles, selection, inbreeding, or population admixture (see Foltz, 1986; Lavie and Nevo, 1986; Gaffney et al., 1990; Borsa et al., 1991; Gardner, 1992; Fairbrother and Beaumont, 1993; Ríos et al., 1996).

While all populations showed high variability, there were small but significant differences in allele frequencies at one or more loci in 33 of the 36 pair-wise population comparisons, including most of those within Tasmania. This indicates that there is some fragmentation of the genetic structure within Tasmania, and that careful husbandry will be necessary to avoid losses of genetic variation in the longer term. At present, Nei's (1978) genetic distances between populations are all very small (≤ 0.011).

Both *Crassostrea gigas* and *C. sikamea* (formerly referred to as the 'Kumamoto' strain of *C. gigas)* were originally imported into Tasmania; the former in much larger quantities than the latter (Thomson, 1952, 1959). Five largely diagnostic allozyme loci (*IDH-1, IDH-2, AAT, MDH,* and *MPI)* distinguish the two species (Buroker et al., 1979b; Deupree, 1993; Banks et al., 1994), and the genetic distance between the two species is high at 0.440 (Banks et al., 1994). There is a one-way gametic incompatibility between the two species, such that *C. gigas* sperm will fertilize *C. sikamea* eggs but not vice versa) (Banks et al., 1994). The four diagnostic loci we used (*IDH-1, IDH-2, AAT* and *MDH*) failed to detect any *C. sikamea* genotypes in any of the Tasmania oysters, corroborating the earlier findings of Deupree (1993) from a single Tasmanian hatchery population. The lack of evidence of *C. sikamea* genotypes suggests that: (a) *C. sikamea* oysters now exist in such low numbers in Tasmania that they were undetected by the

sampling regime used; or (b) *C. sikamea* genotypes were lost due to higher mortality rates than *C. gigas* [as observed during shipment (Thomson, 1952)]; or (c) the one-way gametic compatibility with the more prevalent *C. gigas* eroded the *C. sikamea* genetic structure.

It is possible to estimate effective population numbers (N_K) from temporal variance in allele frequencies (Pollak, 1983; Waples, 1989; Hedgecock and Sly, 1990; Hedgecock et al., 1992). The basic rationale is that the smaller the N_K, the larger the variance in allele frequencies between generations. Various assumptions underlie the calculations (Hedgecock et al., 1992), and at least two of these assumptions were violated in this study, rendering any such calculations invalid. The two assumptions in question are a) that samples are drawn from different generations of a single population and b) that it is a closed population. In order to do these calculations we would have to assume that the genotype frequencies in the present-day Tamar River populations, which we consider the ancestral population of the hatchery stocks, remained representative of the frequencies in the original hatchery stocks that came from this region (i.e. that there have been no genetic changes in the Tamar River from the first commercial spawning in 1981 to our sampling in 1995). Secondly, non hatchery-derived animals have, occasionally and sporadically, been used in industry spawnings since 1981, hence violating the assumption of a closed population. Therefore is was decided that the results of these calculations on the populations in this study would not accurately depict the level of temporal variation present and were not included in the present study.

The results of this allozyme survey indicate that the introduction of oysters from Japan to Tasmania, and their subsequent domestication, have not substantially eroded the genetic basis of the Tasmanian stock. The standard breeding practices of the Tasmanian hatcheries, which in a spawning typically uses several dozen males and females, often

from different farms and hatcheries, appear to have minimised loss of genetic variability levels. However, it is possible that hatchery practices have depressed genetic variation underlying specific traits which are not reflected by the allozymes used in this study. But the high levels of genetic variation suggest that there may be variation present for genes coding for traits that may be of commercial interest; so the Tasmanian industry can now begin a selective breeding program with every expectation of success.

Chapter 3: Mitochondrial DNA analysis of *Crassostrea gigas* populations

3.1 INTRODUCTION

Mitochondrial DNA (mtDNA) is a useful tool for examining population genetics and phylogeny, depending on the variability of the region of mtDNA being examined, especially with the introduction of PCR technology which has simplified lab analyses because the mtDNA no longer has to be purified (Kocher et al., 1989; Palumbi et al., 1991; Folmer et al., 1994; Simon et al., 1994; Hilbish , 1996; see reviews by Meyer, 1993; Ward and Grewe, 1994; Hauser and Ward, 1998). The unique properties of mtDNA that make it a valuable tool are as follows: it is inherited maternally, hence it has an effective population size that is one quarter of that available for nuclear DNA; it undergoes only limited DNA repair therefore having a higher mutation rate than nuclear DNA. It is more valuable as a genetic marker therefore, as theoretically it is likely to accumulate differences more quickly and be a more sensitive measure of genetic drift (Meyer, 1993).

MtDNA studies have been used widely in wild fisheries and aquaculture species, for example: lake trout (Grewe et al., 1993; Grewe et al., 1994; Vitic and Strobeck, 1996); brown trout (Hansen and Mensberg, 1996; Estoup et al., 1998); brook trout (Jones et al., 1996); far east salmonids (Shed'ko et al., 1996); *Thunnus* species (Chow and Inoue, 1993; Chow and Ushiama, 1995; Ward et al., 1994; Ward et al., 1997; Grewe and Hammond, 1998); west Atlantic snappers (Chow et al., 1993); Chinook salmon (Cronin et al., 1993); chum salmon (Cronin et al., 1993; Park et al., 1993); white sturgeon (Buroker et al., 1990); rainbow trout (Danzmann and

Ferguson, 1995); minnow (Broughton and Dowling, 1994); Atlantic salmon (Tessier et al., 1995; Reilly et al., 1998) and orange roughy (Smith et al., 1997).

The use of restriction fragment length polymorphism (RFLP) analysis of mtDNA in fishes had been made easier by the knowledge of the conserved gene order of mtDNA in these organisms together with the development of PCR technology (Meyer, 1993). PCR technology allowed the PCR amplification of the mtDNA fragment, once the region of interest had been sequenced and PCR primers developed, substantially decreasing the amount of time required for the RFLP procedure. Knowledge of the gene order allowed amplification of known genes across gene boundaries and/or amplification of many fragments, increasing the likelihood of finding a region containing variation suitable for RFLP analysis (Kocher et al., 1989). RFLP analysis involves the restriction enzyme (RE) digestion of a fragment of mtDNA, which then undergoes gel electrophoresis, thus separating the mtDNA fragments. Different sizes of fragments are produced by RE digestion when the mutation of the mtDNA sequence results in the absence or presence of the RE target sequence.

Even among the few mtDNA RFLP studies in molluscs and bivalves, most population studies do not use PCR technology (Reeb and Avise, 1990; Brown and Paynter, 1991; Boom et al., 1994; Blake et al., 1997). PCR technology has been used in phylogenetic and/or interspecific studies in *Mytilus* (Hoffmann et al., 1992; Geller et al., 1994), *Crassostrea* (Banks et al., 1993; Ó Foighil et al., 1995), and in starfish (Asakawa et al., 1995; Evans et al., 1998), and molluscans and other metazoan invertebrates (Folmer et al., 1994; Beagley et al., 1998; Kojima et al., in press).

Most mtDNA studies of molluscs, and oysters in particular, have focussed on interspecies analysis. Ó Foighil et al. (1995) found that 16srRNA mtDNA gene sequence in *Crassostrea virginica*, was 85.5% genetically similar to *C. gigas* and 84% to *C. ariakensis*. Banks et al. (1994) found a diagnostic difference between *C. gigas* and *C. sikamea* by restriction enzyme (RE) digest of part of the 16srRNA gene. Native and inbred *C. virginica* were examined and found that the common native haplotype was not present in the inbred oysters, whereas six out of the seven inbred haplotypes were not present in the native oysters (Brown and Paynter, 1991).

In the few population genetic studies examining spatial variation in the frequencies of mtDNA genotypes, two have successfully demonstrated significant differences between populations where allozyme studies did not. Differentiation of mtDNA was observed between populations of *C. virginica* in the Gulf of Mexico compared to Atlantic stocks (Reeb and Avise, 1990), contrasting with results from previous allozyme surveys which showed no differentiation (Buroker, 1983). Another study using mtDNA examined wild *C. gigas* populations in British Columbia (Boom et al., 1994) and found that there was very low levels of divergence between, but substantial genetic variance within populations.

Unlike fishes, the gene order of bivalves and molluscs is not well known, so mtDNA RFLP analysis in not a widespread technique (Boore and Brown, 1994). PCR amplification of mtDNA benefits from a knowledge of the gene order and/or sequence data flanking the region of interest. Of the few molluscs whose gene order is known - *Mytilus edulis*, *Katharina tunicata* – there appears to be no real

conservation of gene order across the order (Boore and Brown, 1994). Currently there is no gene order available for oysters, although there is a restriction site map available for *C. gigas* (Oohara and Mori, 1989).

Previously, allozyme analysis of nine populations of *C. gigas* revealed high levels of variation in endemic Japanese and Australian established and hatchery stocks, with little evidence of loss of variation in the Australian samples (see Chapter 2). As mtDNA techniques have been found to detect more discrete population structure than that observed by allozyme analysis in some cases (Reeb and Avise, 1990), mtDNA RFLP analysis was attempted, to compare and/or contrast with the allozyme results of the same populations. This study was aimed at finding a variable region of mtDNA suitable for PCR -based RFLP analysis of genetic diversity in *C. gigas* populations in Australia.

3.2 MATERIALS AND METHODS

3.2.1 Sample collection

C. gigas samples were taken from Beauty Point (BEA, Tasmanian naturalised); Sendai Bay (SEN, Japanese endemic); and Group A oysters were courtesy of Cameron's of Dunalley. *Saccostrea glomerata* samples were supplied by Dr J. Nell (NSW Fisheries). The Tasmanian samples were kept live on ice during transportation, while the Japanese and *S. glomerata* samples were air-freighted on

dry ice. In the laboratory, all samples were stored at -80°C.

3.2.2 DNA extraction

DNAs from the oysters were extracted as per Grewe et al. (1993), except 50 µg of tissue was used and DNAs were resuspended in 300 µl of sterilised double-distilled water.

3.2.3 PCR reagents and profiles

PCR solutions and PCR programs were performed as per Tables 3.1 and 3.2. Table 3.3 shows the list of primer pairs trialled in search for mtDNA fragment. Table 3.4 shows the sequences of the primers trialled. PCR reagents were stored on ice while setting up the PCR reactions and all reagents were pulse microfuged before use. PCR mixes were mixed by pipetting up and down after addition of reverse primer and pulse microfuged after addition of Amplitaq (Perkin Elmer). DNA samples were added after addition of PCR mix to each tube. Reactions were performed in a Geneamp PCR system 9600 thermocycler (Perkin Elmer).

3.2.4 16srRNA

Part of the 16SrRNA mitochondrial gene was amplified in *S. glomerata* , *C. gigas* samples (Group A, BEA), using 16sar-L and 16sbr-H primers. Amplifying part of the 16srRNA gene allowed comparison with previously published DNA sequence of this gene (Banks et al., 1993a); enabling the identification of the fragment amplified and hence acting as a quality control of the PCR and sequencing methods. Amplification

of the 16srRNA region also allowed identification of levels of similarity between *C. gigas* and *S. glomerata* 16srRNA genes. PCR reagents and profile followed those of Banks et al. (1994) except as noted in Tables 3.1 and 3.2.

3.2.5 *C. gigas* putative RFLP fragments

Initially, the combinations of PCR primer pairs trialled were based on combinations that should amplify successfully in *Mytilus edulis*, based on the published mtDNA gene order (Hoffmann et al, 1992), followed by combinations successfully amplified in fish, specifically tuna (Kessing et al., 1991; Chow and Inoue, 1993; Park et al., 1993; 1994), then random combinations of primers were trialled (Table 3.3). Table 3.4 lists all the primers trialled in this study. 16srRNA PCR amplifications and profiles were as per Banks et al. (1994). PCR profiles in Table 3.2 were the optimum conditions for amplification of the respective mtDNA fragments. *cmrCg*-H.1/16sar-L PCR conditions were as per *cmrCg*-H.1/*cmrCg*-L.1 PCR reaction mix, except 16sar-L primer were used instead of *cmrCg*-L.1 (Table 3.1) and using PCR profile as per 16srRNA were used (Table 3.2).

3.2.5.1 Purification of PCR products

PCR products were purified to remove excess primers prior to sequencing, with Wizard PCR preparations (Promega) used as per manufacturer's instructions, steps "B. Sample purification for direct purification from PCR reactions" and "VI. PCR product purification without a vacuum manifold", (p3). Where PCR products were extracted from agarose gels, Wizard PCR preparations were again used, as per manufacturer's instructions, p2, step "A. Purification of DNA from agarose".

Table 3.1: List of volumes for PCR reactions for different mtDNA fragments.

Reagent	16srRNA PCRs (x10 mix, µl)	Trp/ATCO (H9432) PCRs (x10 mix, µl)	Asn-H/ 16sar-L PCRs (x10 mix, µl)	Ile-L/ 16sar-L PCRs (x10 mix, µl)	*cmrCg*-H.1/ *cmrCg*-L.1 PCRs (x10 mix, µl)
ddH_20	310	309	349	369	379
10X PCR Buffer II	50	50	50	50	50
$MgCl_2$ (25 mM)	30 (1.5 mM total)	60 (3 mM total)	60 (3 mM total)	40 (2 mM total)	30 (1.5 mM total)
dNTP	40 (using 2.5 mM stock)	10 (using 10 mM stock)	10 (using 10 mM stock)	10 (using 10 mM stock)	10 (using 10 mM stock)
Forward primer (10µM stock)	16SaR-L: 10	Trp: 10	16sar-L: 10	16sar-L: 10	*cmrCg*-L.1: 10
Reverse primer (10µM stock)	16SbR-H: 10	ATCO (H9432): 10	Asn-H: 10	Ile-L: 10	*cmrCg*-H.1: 10
DNA*	5 µL per sample	5 µL per sample	1 µL per sample	1 µL per sample	1 µL per sample
Amplitaq (5 U/µL)	1	1	1	1	1
TOTAL	50 µL per tube	50 µL per tube	50 µL per tube	50 µL per tube	50 µL per tube

ddH_20 = sterilised double-distilled water,
10X PCR Buffer II (100 mM Tris-HCl, pH 8.3 (at 25°C); 500 mM KCl), Amplitaq and $MgCl_2$ are all products by Perkin Elmer; dNTP by Promega

Table 3.2: PCR reaction profiles for amplification of various *C. gigas* mtDNA fragments.

Program No.	PCR Step	mtDNA fragment			
		16rRNA	Trp/ ATCO (H9432)	Asn-H/ 16sar-L	Ile-L/ 16sar-L
1	Denaturing	95°C, 4 mins	95°C, 4 mins	95°C, 4 mins	95°C, 4 mins
	Annealing	50°C, 45 secs	57.5°C, 45 secs	60°C, 45 secs	60°C, 45 secs
	Extension	72°C, 2 mins	72°C, 2 mins	72°C, 2 mins	72°C, 2 mins
	No. of cycles	1	1	1	1
2	Denaturing	94°C, 30 secs	94°C, 30 secs	94°C, 30 secs	94°C, 30 secs
	Annealing	50°C, 45 secs	57.5°C, 45 secs	60°C, 45 secs	60°C, 45 secs
	Extension	72°C, 2 mins	72°C, 2 mins	72°C, 2 mins	72°C, 2 mins
	No. of cycles	35	35	40	40
3	Extension/ Hold	72°C, 10 mins	72°C, 10 mins	72°C, 10 mins	72°C, 10 mins
4	Hold	4°C, forever	4°C, forever	4°C, forever	4°C, forever

°C = degrees Celsius, mins = minutes, secs = seconds

Table 3.3: Primer pairs trialled in BEA 4,5 and Group A 2268 and results.

Results	Primer pairs
No product	12SA-L/12SAR-H*, 12SA-L/16sbr-H*, CO1e-H/12SAR-H*, ATCO (H9432)/LAT6 (4, 5 only), His-H/LAT6, Asn-H/ LAT6, Pro-L/ATCO (H9432), Phe-H/12SAR-H (4 only)
Weak, multiple products	CO1e-H/CO1f-L**, Glu-H/Leu-L, CO1e-H/LAT6, ATCO (H9432)/LAT6 (2268 only), His-H/CO1f-L, Pro-L/Asn-H, Pro-L/His-H, Phe-H/12SAR-H (5, 2268 only)
One very strong product, weak multiple products	Trp/ATCO (H9432), Asn-H/16sar-L (5 only), Ile-L/16sar-L (5 only)
One very strong product	16sar-L/16sbr-H*, *cmrCg*-H.1/*cmrCg*-L.1, 16sar-L/*cmrCg*-H.1

*Also trialled in *S. glomerata* , with similar results.

** Also trialled in *S. glomerata* , but with different size products.

Table 3.4: List of sequences of primers used in this study.

Primer name	**DNA Sequence (5'-3')**	**Reference**
12SA-L	AAACTGGGATTAGATACCCCACTAT	Kessing et al., 1991
12SAR-H	ATAGTGGGGTATCTAATCCCAGTT	Kessing et al., 1991
16sar-L	CGCCTGTTTATCAAAAACAT	Kessing et al., 1991
16sb-L	ACGTGATCTGAGTTCAGACCGG	Kessing et al., 1991
16sbr-H	CCGGTCTGAACTCAGATCACGT	Kessing et al., 1991
Asn-H	CGCGTTTAGCTGTTAACTAA	P.Grewe (pers. comm.)
CO1f-L	CCTGCAGGAGGAGGAGAYCC	Kessing et al., 1991
CO1e-H	CCAGAGATTAGAGGGAATCAGTG	Kessing et al., 1991
cmr*Cg*-L.1	TCGATCATAGGAGAAGTTACGCC	This study
cmr*Cg*-H.1	CTGCCATATACACCATCAGAAATAG	This study
Glu-H	CAACGGTGGTTCTTCAAGTC	Park et al., 1993
ATCO (H9432)	GCCATATCGTAGCCCTTTTTG	Chow & Inoue, 1993
His-H	AGAATCACAATCTAATGTTT	Park et al., 1993
Ile-L (nd2)	CCGGATCACTTTGATAGAGT	P.Grewe (pers. comm.)
ATCO (L8562)	CTTCGACCAATTTATGAGCCC	Chow & Inoue, 1993
Phe-H	TCTTCTAGGCATTTTCAGTG	Kessing et al., 1991
Pro-L	CTACCTCCAACTCCCAAAGC	Kessing et al., 1991
Trp	CTGAGGGCTTTGAAGGCCC	Park et al., 1993

All primers manufactured by Bresatec Pty Ltd.

3.2.5.2 Sequencing of mtDNA fragments

Sequencing of the mtDNA fragments was done to allow confirmation of the mitochondrial DNA identity of the fragments with existing mitochondrial DNA sequences in the Genbank database and to allow restriction enzyme site identification for use in the RFLP study. Mitochondrial DNA fragments and pGEM-T vectors containing the mtDNA fragments were sequenced using the "ABI Prism dye terminator cycle sequencing ready reaction kit" as per manufacturer's instructions (August 1995, Perkin Elmer), in an ABI 377 DNA Sequencer (Perkin Elmer). The primers used in sequencing were the same as those used to amplify the fragment during PCR. One exception was the use of the 16sb-L (Kessing et al, 1991) and M13

primers (Promega) to sequence part of the "Ile-L/16sar-L"-pGEM-T vector (see Figure 3.1 for primer site). The 16sar-L primer could not be used for sequencing, due to the apparent amplification of the original "Ile-L/16sar-L" fragment with only the 16sar-L primer, resulting in 16sar-L primer sites at both ends of the fragment (see Results). A total of 4 fragments were sequenced (*C. gigas* unless otherwise stated): Trp/ATCO (H9432), Asn-H/16sar-L, Ile-L/16sar-L, 16sar-L/16sbr-H (also for *S. glomerata*).

3.2.5.3 DNA sequence comparison

DNA sequences were viewed using "Sequence Navigator" version 1.0 software (Perkin Elmer). DNA sequence comparisons with other mitochondrial DNA sequences in the Genbank database were performed at the NCBI using the BLAST network service, namely BLASTN 1.4.9MP (Altschul et al., 1990).

3.2.5.4 Cloning of mtDNA fragments

Extraction and purification of three PCR-amplified mtDNA fragments (Trp/ATCO (H9432), Asn-H/16sar-L, Ile-L/16sar-L) from an agarose gel were not sufficient for successful DNA sequencing of these fragments. As a result, these three fragments were then cloned into the pGEM-T vector (Promega), then the inserts (the fragments) of the vector were sequenced using M13 primers, which bind to the vector, either side of the insert, which should enable the inserts to be successfully sequenced. Cloning and sequencing of these fragments into pGEM-T were performed as per manufacturer's instructions (Promega), except the vector was transformed into XL1-blue cells (Stratagene, as per manufacturer's instructions for transformation).

3.2.5.5 Primer design

Primers for the "Ile-L/16sar-L" fragment were designed using "DNAstar" software (DNAstar, Inc.). As the 16sar-L primer had annealed to both ends of the fragment in the original PCR amplification, a primer was developed to anneal to the 3' end of the amplified "Ile-L/16sar-L" fragment (cmr*Cg*-H.1). Another primer, cmr*Cg*-L.1, was developed so that the region of the fragment comprising the unknown region and COIII genes (possibly containing a variable region) could be amplified without using the 16sar-L primer, thus eliminating any potential for non-specific binding by 16sar-L (previously observed in original amplification of fragment). Table 3.4 shows the sequence of the primers (see Figure 3.1 for binding sites on the mtDNA fragment). These primers were synthesised by Bresatec Pty Ltd.

3.2.5.6 RFLP analysis of "Ile-L/16sar-L" fragment

The "Ile-L/16sar-L" fragment was the only fragment cloned into the pGEM-T vector that could identified as being of mitochondrial DNA origin (see Results). Together with the identification of an unknown region within the fragment, it was possible that this fragment may contain variation useful in RFLP analysis, so trial restriction enzyme digests were performed in an Australian and Japanese populations (BEA and SEN). These populations were used, as previous allozyme analysis (see Chapter 2) had determined that these were two of the most variable populations observed, so there was an expectation that variation should be observed using RFLP analysis.

Restriction enzyme (RE) digests were performed on ice as per manufacturer's instructions (NEB) at 37°C, using the following X10 reaction mix per RE used : 15 μL of appropriate X10 RE buffer (NEB: for RE's AluI,

HpaII, RsaI - NEB1; AflII, HinfI, HinPII - NEB2, DdeI - NEB3; ScrfI - NEB4; BamHI - BamHI buffer); 30 µL of sterilised double-distilled water; 5 µL of appropriate RE (NEB). This mix was pulse microfuged before and after addition of RE (which had also been pulse microfuged before use), 5 µL of X10 RE mix was added to each tube containing 10 µL of DNA.

3.3 RESULTS

3.3.1 16srRNA intra- and interspecific comparison

Figure 3.2 shows the 16srRNA DNA sequence (516 bp) for three *C. gigas* (one from BEA and one Group A, the third from Banks et al, 1993a, fourth from Ó Foighil et al., 1995)) and a Sydney rock oyster (*S. glomerata,* this study). There was no difference between the two *C. gigas* sequences from this study or between these, Banks et al., (1993a) and the Ó Foighil et al. (1995) sequences.

A 16.7 % difference (86 bases) between the *C. gigas* and *S. glomerata* 16srRNA DNA sequence was observed (Figure 3.2). Nucleotide substitutions and deletions were observed between the two oyster species: a deletion of 5 bp (starting at the 44bp position) and an insertion of 4 bp (starting at the 308 bp position) in the *S. glomerata* 16srRNA with respect to the *C. gigas* 16srRNA region.

Figure 3.1: Diagram of *C. gigas* mtDNA fragment used in RFLP analysis. Nucleotide positions of genes and primer sites are indicated in the middle figure and the bottom figure denotes RE sites of variable enzymes: * - partial digestion sites; ** - speculated digestion sites based on fragment length. Arrows denote primer binding sites and direction. BamHI site is shown to allow comparison with *C. gigas* RE map by Oohara and Mori (1989).

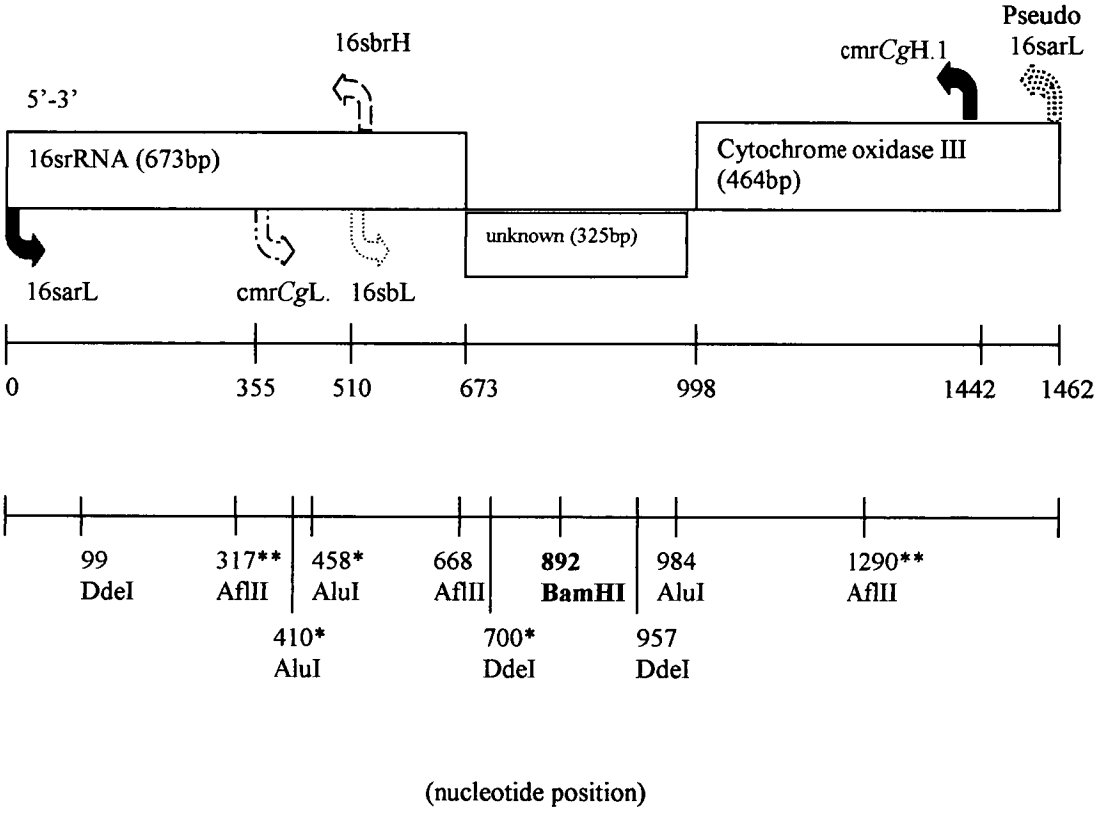


Figure 3.2: Alignment of DNA sequence of 16srRNA from *C. gigas* (this study, Banks et al., 1993a, Ó Foighil et al., 1994), and *S.glomerata* (this study). Dashes refer to gaps inserted by the CLUSTAL sequence alignment procedure, Ns refer to undetermined nucleotides, letters refer to differences in *S. glomerata* sequence with respect to *C .gigas*.

```
SWs5                1    CATAGAAGTC AAAG-ATTTT AGCAATACTG CCCAGTGCGA AATATTACTG TAAACGGCCG CCCTAGCGTG AGGGTGCTAA
G2268                    ********** ****-***** ********** ********** ********** ********** ********** **********
Banks et al.,1993a                                                     ********* ********** ********** **********
Ó Foighil et al.,1994                                 ******* ********** ********** ********** ********** **********
S. glomerata             *****G-*** **TTT**C** ****G***** ********** ***-----** ********** ********** *******C**

SWs5               81    GGTAGCGAAA TTCCTTGCCT TTTGATTGTG GGCCTGCATG AATGGTTTAA CGAGGGTTTG ACTGTCTCTA AATTTTTT-A
G2268                    ********** ********** ********** ********** ********** ********** ********** ********-*
Banks et al.,1993a       ********** ********** ********** ********** ********** ********** ********** ********-*
Ó Foighil et al.,1994    ********** ********** ********** ********** ********** ********** ********** ********-*
S. glomerata             ********** ********** ***A*****A ********** ********** *******CAA G********T GC***GAGC*

SWs5               161   TTGAAATTGT ACTGAAGGTG AAGATACCTT CATTTAAAAG TTAGACAAAA AGACCCCGTG CAACTTTGAA AATTAACTTT
G2268                    ********** ********** ********** ********** ********** ********** ********** **********
Banks et al.,1993a       ********** ********** ********** ********** ********** ********** ********** **********
Ó Foighil et al.,1994    ********** ********** ********** ********** ********** ********** ********** **********
S. glomerata             GC*******G ********** ********** ***A****** ********G* ********** **G****T** **A*TTA***

SWs5               241   ATTCAGGAGT AAAAGATTTT TAGGTGGGGC GCCTAGAAAG CAAGTCTAAC CTTTCTGAAT AACTAACT-- --CTTTCCGG
G2268                    ********** ********** ********** ********** ********** ********** ********-- --********
Banks et al.,1993a       ********** ********** ********** ********** ********** ********** ********-- --********
Ó Foighil et al.,1994    ********** ********** ********** ********** ********** ********** ********-- --********
S. glomerata             ***A****** GT******** ********** ******G*** T**AA**T*A *C****T*T* TGT***AAGC AA**G**T**

SWs5               321   ATTTGACCCG ATTATATTCG ATCATAGGAG AAGTTACGCC -GGGGATAAC AGGCTAATCC TTTAGTAGAG TTCGTATTGG
G2268                    ********** ********** ********** ********** -********* ********** ********** **********
Banks et al.,1993a       ********** ********** ********** ********** -********* ********** ********** **********
Ó Foighil et al.,1994    ********** ********** ********** ********** -********* ********** ********** **********
S. glomerata             TG*A****** GCA*G-*C** ********** ********** N********* *****A***T A********* C***N*****
```

Figure 3.2 (Cont.): Alignment of DNA sequence of 16srRNA from *C. gigas* (this study, Banks et al., 1993a, Ó Foighil et al., 1994), and *S. glomerata* (this study).

```
SWs5                401   CTAAAGGGAT TGGCACCTCG ATGTTGAATC AGGGATAATA GCTTCAAGGC GTAGAGGCTT TGAAAGTAGG
G2268                     ********** ********** ********** ********** ********** ********** **********
Banks et al.,1993a        ********** ********** ********** ********** **N***NN** ****
Ó Foighil et al.,1994     ********** ********** ********** ********** ********** ********** ***
S. glomerata              N***TA**** ********** ****N****T *A****G*** A***T*N*** *******N** *A*****G*N
```

3.3.2 Cloning, sequence analysis and comparison of mtDNA fragments

Sequence analysis showed that the 16sar-L primer site was located on both ends of the "Ile-L/16sar-L" mtDNA fragment , with no Ile-L primer site observed (Figure 3.3). The sequence of the "Ile-L/16sar-L" fragment, minus the pseudo 16sar-L site (total length of fragment of 1442 bp) was compared to other sequences in Genbank. Figure 3.1 depicts the gene organisation of the "Ile-L/16sar-L" fragment. Similarities with 16srRNA (see previous section) and cytochrome oxidase three genes in other organisms were revealed (Table 3.5).

Table 3.5: Similarities between the "Ile/16sar-L" fragment and cytochrome oxidase sub unit three (COIII) genes reported for other organisms.

Organism	**Identity of genes in sequence**	**% similarity**	**Range (nt position) of similarity in "Ile/16sar-L" fragment**
Saccharomyces cerevisiae (yeast)	oxi2, COIII, Val - tRNA	54	980-1439
Anopheles gambiae (mosquito)	complete mitochondrial DNA	54	990-1439
Perognathus longimembris	COIII	55	1010-1277
L. squarrosum	COIII mRNA	59	1033-1232
Chaetodipus hispidus	COIII	54	1012-1310
Salmo salar (Atlantic salmon)	COIII	53	993-1296
Mytilus trossulus	COIII	55	1063-1211

nt = nucleotide

The region consisting of the 16srRNA gene (first 673 bp) was removed from the "Ile-L/16sar-L" fragment and the resulting sequence (nucleotides 674-1442, Figure 3.2), consisting of an unknown region and putative cytochrome oxidase three (COIII) gene, was subjected to further comparison with the Genbank sequence database. Approximately the last 400 bp of this region showed between 53-59% similarity with other COIII genes from *S. cerevisiae*, *A. gambaie*, *S. salar*, *M. trossulus* (Table 3.5).

Figure 3.3: DNA sequence of *C. gigas* "Ile-L/16sar-L" mtDNA fragment used in RFLP analysis

```
1     CGCCTGTTTATCAAAAACATCACTAGAAGATAAAGACTTTTAGCAATACC
51    TGCCCAGTGCGAAATATTACTGTAAACGGCCGCCCTAGCGTGAGGGTGCT
101   AAGGTAGCGAAATTCCTTGCCTTTTGATTGTGGGCCTGCATGAATGGTTT
151   AACGAGGGTTTGACTGTCTCTAAATTTTTTATTGAAATTGTACTGAAGGT
201   GAAGATACCTTCATTTAAAAGTTAGACAAAAAGACCCCGTGCAACTTTGA
251   AAATTAACTTTATTCAGGAGTAAAAGATTTTTAGGTGGGGCGCCTAGAAA
301   GCAAGTCTAACCTTTCTGAATAACTAACTCTTTCCGGATTTGACCCGATT
351   ATATTCGATCATAGGAGAAGTTACGCCGGGGATAACAGGCTAATCCTTTA
401   GTAGAGTTCGTATTGGCTAAAGGGATTGGCACCTCGATGTTGAATCAGGG
451   ATAATAGCTTCAAGGCGTAGAGGCTTTGAAAGTAGGTCTGTTCGACCTTT
501   AATACCCTACGTGATTTGAGTTCAGACCGGCGTAAGCCAGGTTGGTTTCT
551   ATCTGCTTTACATAAACTTATCTCGGCTGTACGAAAAGACGTTGATACTG
601   GAAAGCAGTAAATCCAGAAAAAAATTGCAGAATGCATCAATTTAATGCGT
651   TAATAAATTTTATAAACTTAAGCGCCGAAATAGTGTAAAAGAAGTTGGTG
701   ATGTGCAGCACATTTAGGGGCGCTTACGTTCCAAGTTTAGAATATTAATA
751   TAATTGTTGAATTTAAAAATGAAGACGGTTAGTAATAAATTTTTAGTGCA
801   GTGCAAAACGTTTTGCTGCTTATTTTTTTACGGGAAAACAATCTGTGGTC
851   TATTACAGAACATCTGTTGTTCGTACCCCATATCATGTNGTGGATCCTAN
901   ACCATGACCAATAATTATGGGTGCAAACTTATGGGGAGTTGCCGAATGTT
951   CATTTGCTGAGTTAATGAGATTAGGCTTGATAGCTTATATTGAGGTATTC
1001  CTCTTNTNGTACTAACTTTTTATAGTTGAGTACGTGACATNATTAATGAN
1051  GCAACATNTCAGGGGTTTCATACTGAAAAAGTTCAGTCAGGGCTTACTTT
1101  GGGTTTTATTTTGTTCCTAATNTCTGAGTTNATATTATTTTTNTCATTNT
1151  TTTGAGCATTTTTCCATAGGGCTTTGTCATCTTCTGTTGAGATTGGGTGC
1201  TGCTGACCACCAGTCGGGCTAGAGTGTTTAGACTGAAGAAAAGTGCCATT
1251  ACATAATACAGCATTATTAGTAGCATCTTCTGCAAGTATTACTTTAAGAC
1301  ATAACTACCTGCAATGGGGGGACATTTGGGTAGCAACAGCAACGTATATT
1351  GCTACTTTAGGCTTGTCGGTAATATTTATTAAGAATCAATACGAAGAGTA
1401  TGCCTGATCTAGGTTTTCTATTTCTGATGGTGTATATGGCAG
```

3.3.3 C. gigas RFLP analysis

The "Ile-L/16sar-L" fragment was analysed to determine the best regions where variability might be expected to be observed. Although

cytochrome oxidase genes are known to be conserved, no sequence currently exists for these genes in *C. gigas* in the available databases. Hence it was decided, that together with the approximately 325 bp region that did not correspond with any known sequence in the databases, this section of the fragment represented the best option for possibility of polymorphism between individuals. A 1442 base pair fragment was amplified using the *cmrCg*-H.1/16sar-L primers, compared to a 1039 bp fragment amplified using the *cmrCg*-H.1/*cmrCg*-L.1 primers. Despite previous concerns, the 16sar-L primer did not appear to bind non-specifically when used in conjunction with the *cmrCg*-H.1 primer. The larger fragment was preferred for use in RFLP analysis, so that the longer fragments produced by restriction enzyme (RE) digestion of the 1442 bp fragment would be easier to visualise with agarose gel electrophoresis.

RE site analyses were performed in "Map Draw" (part of the DNAstar program, DNAstar, Inc). Table 3.6 lists the REs found to give the numbers of fragments and fragments lengths best suited to agarose electrophoresis and Table 3.7 shows RFLP haplotypes. Some restriction enzymes did not always cut to completion: DraI, HaeII, RsaI and ScrFI, but these enzymes partially cut consistently.

Only three (AflII, AluI, DdeI) of the twelve REs showed variation and only in the Tasmanian population, BEA (Table 3.7). In each case, variability was low, with only one other haplotype observed other than the common haplotype (AAA). No oyster showed variation for more than one RE. Further analysis of more oysters from these and other populations were abandoned due to the lack of variation

observed in BEA and SEN, together with the identification of the conserved genes in this region.

3.4 DISCUSSION

Both populations, established and endemic, showed very little mtDNA variation (Table 3.7). Three individual Tasmanian oysters each displayed a rare haplotype at one restriction site, demonstrating more variation than the Japanese population. A general statement of the genetic diversity of mtDNA in SEN and BEA cannot be made on the basis of this study. More haplotypes may have been observed, had the sample size been larger. However, the main aim of analysing mtDNA was to detect genetic differences not observed using allozymes. Given the mtDNA diversity was so low, it was decided to discontinue the study. The fact that the segment of mtDNA contains two highly conserved genes is reflected in the little variation observed in this study. However, a region containing variable genes was not amplified in this study. Although this study was by no means definitive in terms of the primers trialled whilst looking for a variable region, the difference in primer binding site between *C. gigas* and other animals previously studied appears to be quite substantial. The primers used in this study have been used with success in a variety of animals (Kessing et al., 1991), various species of tuna (Chow and Inoue, 1993; Park et al., 1993) and would have been expected to be successful should the mtDNA gene order of *C. gigas* be similar to that of *M. edulis* and/or similar to that of other molluscs (Boore and Brown, 1994). However, in piscines,

Table 3.6: List of restriction fragment lengths for "Ile-L/16sar-L" *C. gigas* mtDNA fragment. Rare haplotype (B) in brackets. Fragment lengths are approximate and based on agarose gel electrophoresis and restriction site map of fragment generated by "Map Draw" software.

Restriction enzyme (units)	**No. of cut sites**	**Fragment lengths (bp)**
AflII (10 U/μL)	1	668, 774 (626, 318, 250,150, 100)
AluI (8 U/μL)	2	1032*, 526, 458, 458 (1032*, 984, 458, 410)
BamHI (20 U/μL)	1	524, 927
DdeI (10 U/μL)	3	600, 317, 258,168, 99 (600, 485, 258, 99)
DraI (20 U/μL)	2	678, 548, 217
HaeII (20 U/μL)	3	718, 381, 294, 49**
HhaI (20 U/μL)	3	720, 382, 292, 47**
HinfI (5 U/μL)	2	941, 443, 58
HinPI (10 U/μL)	3	722, 382, 290, 48**
HpaII (10 U/μL)	3	914, 335, 150, 42
RsaI (10 U/μL)	5	600*, 410, 389, 294, 192, 136, 21**
ScrfI (5 U/μL)	2	904, 548*, 379, 161

All enzymes produced by New England Biolabs (NEB)

*results of partial digestion

Table 3.7: List of RFLP haplotypes for "Ile-L/16sar-L" *C. gigas* mtDNA fragment.

Composite haplotypes	AflII	AluI	DdeI	BEA (n = 30)	SEN (n = 24)*
1	A	A	A	27	24
2	A	A	B	1	0
3	B	A	A	1	0
4	A	B	A	1	0

*n = 23 for AluI

All other enzymes: AAAAAAAAA = 30 (BEA), or AAAAAAAAA = 24 (SEN).

the gene order of mtDNA is conserved (Meyer, 1993) and that does not appear to be the case with molluscs, certainly not with respect to the few molluscs whose gene order has been determined thus far. But variation is present in mtDNA of *C. gigas*, as observed by Boom et al. (1994) using RE digestion of purified mtDNA. It appears, therefore, the technical limitations with PCR-based analysis are the cause for the lack of variation seen in this study, in that the only genes that can be amplified are the relatively conserved ones. Until the mtDNA of *C. gigas* is sequenced and a gene order determined, the tedious and time-consuming method of extraction of whole mtDNA and subsequent digestion with restriction enzymes is the only successful way to determine mtDNA RFLP analysis or using single

strand conformational polymorphisms (SSCP) analysis (Li and Hedgecock, 1998).

RFLP analysis of the entire mitochondrial DNA molecule by Boom et al. (1994) showed that there was a low level of divergence between populations but substantial genetic variance within populations. Native and inbred *C. virginica* were examined and the common native haplotype was found not to be present in the inbred oysters, whereas six out of the seven inbred haplotypes were not present in the native oysters (Brown and Paynter, 1991). These previous studies indicate that there is variation present in the mtDNA molecule, but that the region used in this study may too conserved to observe this.

DNA sequence database comparisons found that the mtDNA region used for RFLP analysis in this study contained sections of two conserved genes: 16srRNA and cytochrome oxidase three (COIII), and one region of approximately 300 bp of unidentified sequence. The low level of variation suggests that this unidentified region does not vary considerably either. This is not the only case where part of the mtDNA sequence could not be identified - such regions have been reported in the blue mussel, *M. edulis* (Hoffmann et al., 1992).

Boore and Brown (1994) suggested possible gene orders for molluscs: this contrasts with the gene order indicated by this study. The gene order of 16srRNA within 400 bp of COIII as seen in this study has not been observed previously. These genes are usually separated by several transfer RNA and protein genes in other organisms whose mtDNA gene orders are known: another mollusc, *Mytilus edulis* (Hoffmann et al., 1992); sea anemone, *Metridium*

senile (Beagley et al., 1998); starfish *Asterina pectinifera* and sea urchin (see Asakawa et al., 1995); fish (Meyer, 1993), Drosophila and vertebrates (see Simon et al., 1994). *Katharina tunicata*, also a mollusc (Class Polyplacophora), has the only other mtDNA with close proximity of these two genes, as only 12srRNA gene and eight transfer RNAs are separating the 16srRNA and COIII genes (Boore and Brown, 1994). The similarity of the proximity between 16srRNA and COIII in *C. gigas* (this study) and *Katharina tunicata* (Boore and Brown, 1994) is puzzling, and contrasts with the arrangement of six transfer RNA genes, 4 proteins genes and an unknown region between these genes seen in another bivalve, *M. edulis* (Hoffmann et al., 1992) - further suggesting evidence of lack of conservation of mtDNA gene order in molluscs (Boore and Brown, 1994).

A BamHI site at the 892 bp position in the 1442 bp mtDNA fragment generated in this study accords with the same restriction enzyme site (only one BamHI site in *C. gigas* mtDNA) in a restriction enzyme (RE) site map of *C. gigas* mtDNA that had been previously developed (Oohara and Mori, 1989). The orientation of the 1442 bp fragment with respect to the RE site map cannot be determined as the fragment is too short to match up with other RE sites on the map, which are approximately 1 kb away. However, Li and Hedgecock (1998) found a mtDNA fragment (segment 8) of which 524bp shows high homology with the cytochrome b gene and it was located near the XbaI RE site according to the Oohara and Mori (1989) RE site map. The XbaI RE site is 1.58 kb away from the BamHI site according to the Oohara and Mori (1989) RE site map. Depending on the orientation of the 1442 bp fragment found in the present study, comparison with the Li and Hedgecock (1998) and Oohara and Mori

(1989) results may give examples of possible gene orders. The 1442 bp fragment containing the 16srRNA and COIII genes separated by 300bp of unidentified sequence, (present study) is within approximately 200-1000bp (depending on orientation) from the cytochrome b fragment determined by Li and Hedgecock (1998). Two possibilities exist for the gene order: a) 16srRNA, 300bp unidentified sequence, COIII, approximately 1 kb of unknown sequence, then cytochrome b; or b) COIII, 16srRNA, approximately 200bp of unknown region, then cytochrome b. Cytochrome b has been observed next to either 16srRNA or COIII in other species: 16srRNA is separated from cytochrome b, by 1.158kb of unidentified sequence in *M. edulis* (Hoffmann et al., 1992); COIII and cytochrome b are separated only by a tRNA gene, leucine (CUN) in the nematode *Ascaris suum* (Okimoto et al., 1992). However, the total order of these three genes, either 16srRNA/COIII/cytochrome b or COIII/16srRNA/cytochrome b, appears to be unique. In lieu of the sequencing of the entire mtDNA genome in oysters, future work to determine the orientation of the fragment (COIII/cytochrome b or 16srRNA/ cytochrome b) using PCR, could involve the PCR amplification using the respective conserved primers for these mtDNA genes from other organisms (Palumbi et al., 1991; Simon et al., 1994).

A 516 nucleotide fragment of the 16srRNA gene was amplified in *C. gigas* and *S. glomerata* (Figure 3.2). There was a 16.7 % difference (86 bases) between the *C. gigas* and *S. glomerata* 16srRNA gene sequence. Ó Foighil et al. (1995) examined a 443 nucleotide region of 16srRNA in *C. gigas* (Figure 3.2), *C. ariakensis*, and *C. virginica*, which matches the 43 - 586 bp region in the 1442 bp mtDNA fragment (Figure 3.3) in this study. As well as nucleotide

substitutions, there was a deletion of 5 bp (starting at the 44bp position) and an insertion of 4 bp (starting at the 308 bp position) observed in the *S. glomerata* 16srRNA with respect to the *C. gigas* 16srRNA region. When *C. gigas* and *S. glomerata* 16srRNA were compared over the same region as Ó Foighil et al. (1995) (corresponding to the region 22 - 465 bp in the 16srRNA fragments determined in this study, Figure 3.2), an 82.4% similarity between the mtDNA sequence was observed (this study), which was similar to the level of mtDNA sequence similarity (85.5%) observed between *C. gigas* and *C. virginica*, but not as high as the 95.1% similarity observed between *C. gigas* and *C. ariakensis*. The greatest sequence similarity seen in Crassostrea thus far was 98.3% between *C. gigas* and *C. sikamea* in a 400 bp region of 16srRNA (Banks et al., 1993a), corresponding to the 62 - 462 bp region in the 1442 bp *C. gigas* mtDNA fragment in this study. Again, the level of sequence similarity seen between *S. glomerata* and *C. gigas* 16srRNA (82.4 %, this study) was similar to that Banks et al. (1993a) observed between *C. gigas* and *Ostrea lurida* (85 %) and between *C. sikamea* and *O. lurida* (84.5 %), over the same region of 16srRNA.

The lack of variability observed in the PCR-based RFLP analysis of the 1442 bp mtDNA fragment developed in this study, despite previously high levels of genetic variability observed by allozyme and microsatellite techniques (see chapters Two and Four, respectively), meant that this technique was unlikely to yield further information on inbreeding and genetic diversity of Tasmanian stocks. With the technical limitations and lack of knowledge of the oyster mtDNA genome, the whole mtDNA restriction digest method (Boom et al., 1994) and SSCP analysis (Li and Hedgecock, 1998) appear more

informative than PCR-based mtDNA RFLP analysis techniques in *C. gigas* as this time. Time constraints prevented their application to the present study. The identification of the proximity of the 16srRNA, and COIII genes observed in this study gives further insight into the mtDNA gene order of *C. gigas*. Together with previous findings of the mtDNA RE site map (Oohara and Mori, 1989), the location of the cytochrome b gene (Li and Hedgecock, 1998), this study demonstrates a block of COIII, 16srRNA and cytochrome b mtDNA genes —a combination unique to bivalves.

Chapter 4: Microsatellite variation of Pacific oysters, *Crassostrea gigas* (Thunberg): naturalised and hatchery populations in Australia and endemic populations in Japan

4.1 INTRODUCTION

Microsatellite analysis has gained popularity within the last decade in fisheries and aquaculture population genetics for the determination of stock structure, paternity and mapping (see Wright and Bentzen, 1994, O'Connell and Wright, 1997, Hauser and Ward, 1998). Microsatellites are composed of di-, tri-, or tetranucleotide repeats, arranged in tandem in short stretches (10-100's of bp) of DNA, and assumed to be inherited in a Mendelian fashion (see Wright and Bentzen, 1994). Some of the species studied for stock structure using microsatellites include Northern pike (Miller and Kapuscinski, 1996), Atlantic cod (Bentzen et al., 1996), Atlantic salmon (McConnell et al., 1995; Tessier et al., 1995; Fontaine et al., 1997; Beachem and Dempson, 1998; Reilly et al., 1999), brown trout (Estoup et al., 1998) and bigeye tuna (Grewe and Hampton, 1998). Microsatellites have the added advantage of being useful for mapping, such as in Atlantic salmon (Slettan et al., 1997) and sea bass (Garcia de Leon et al., 1998). The popularity of microsatellites lies not in its technical development — which is more expensive and more time consuming than most other techniques, including allozyme and mitochondrial DNA analyses — but in its increased sensitivity to detect vagaries in population structure, caused by factors such as inbreeding. This is due to the greater variability of such markers compared with allozymes; typically there are large numbers of alleles per microsatellite locus and heterozygosities are often extremely high (O'Connell and Wright, 1997). Also, the potential access to a much larger number of microsatellite markers available using

PCR technology has made microsatellite analysis a popular technique (O'Connell and Wright, 1997.

Microsatellite analysis has also been able to resolve differences between hatchery and wild or naturalised stocks to a greater extent than that of other techniques, e.g. in Tasmanian hatchery and ancestral Canadian Atlantic salmon stocks (Reilly et al., 1999), and between natural and hatchery stocks of brown trout (Estoup et al., 1998). But this is not always the case, as microsatellite analysis has also been shown to be no more definitive than other markers such as allozymes at discriminating stock structure in populations of Pacific herring from the Gulf of Alaska and Bering Sea (O'Connell et al., 1996b). Although significant differentiation was revealed using microsatellites and mitochondrial DNA analyses, the latter technique was more diagnostic than any single microsatellite locus of a first generation hatchery and a wild stock of Atlantic salmon from Quebec (Tessier et al., 1995), and in discriminating stocks of bigeye tuna (Grewe and Hampton, 1998).

The use of microsatellite markers in aquaculture species other than fish is less widespread. However, microsatellites have proved useful in population genetic studies of prawns (Brooker et al., 2000; Tassanakajon, 1999) and freshwater crayfish (Imgurnd et al., 1997). Bagshaw and Buckholt (1997) found a novel satellite/microsatellite combination in *Penaeus vannamei.*

Clabby et al. (1996) identified and located a satellite in the Pacific oyster, *C.gigas* and microsatellites have been used to examine hybrid vigor of *C.gigas* (Hedgecock et al., 1995). Moreover, few populations studies of oysters using microsatellites exist (Magoulas et al., 1998). Naciri et al. (1997) developed one microsatellite for population genetic analysis and 2 highly polymorphic microsatellite loci for paternity testing in *Ostrea edulis.* Another study in *O. edulis*

(Bierne et al., 1999) also found a significant departure in Mendelian expectation in microsatellite loci when examining the effect of inbreeding on growth and survival in the early developmental phase of this oyster. Magoulas et al. (1998) developed three microsatellites in *C.gigas* which demonstrated codominant autosomal inheritance in family studies and were used successfully to confirm ploidy status in putative triploids.

Previously, allozyme electrophoresis had been used to determine whether the genetic diversity of farmed oysters in Tasmania had decreased relative to naturalised Australian populations and to native Japanese populations (Chapter 2/English et al., 2000). There was little difference in genetic variability between these groups. As different results had been seen depending on which markers had been used in *C. virginica* and in other fisheries and aquaculture species (Reeb and Avise, 1990, McDonald et al., 1996, Hare and Avise, 1998; Tessier et al., 1995, Estoup et al., 1998, Reilly et al., 1999, see review O'Connell and Wright, 1997) it was decided to also use microsatellite analysis on the populations previously analysed by allozymes in Chapter 2, to enable a more definitive examination into the genetic diversity levels of the populations.

Therefore, the present study used microsatellite analysis to determine whether the genetic diversity of farmed oysters in Tasmania has decreased relative to the naturalised Australian populations and to endemic Japanese populations. Results are compared to those of a previous allozyme study.

4.2 METHODS

4.2.1 Sample collection

Pacific oysters were collected from seven Australian and two Japanese sites (Chapter 2: Table 2.1 and Figure 2.1). Samples from naturalised populations in Australia were obtained from two sites that were about 20 km apart along the Tamar River in Tasmania (Beauty Point and Swan Bay), from Bridport in Tasmania, and from Port Stephens (≈ 200 km north of Sydney) in New South Wales. Three Tasmanian farmed populations, derived from hatchery-produced spat, were sampled: Smithton, Dunalley and Pittwater. Two endemic Japanese populations were sampled: Sendai and Hiroshima. The Tasmanian samples were kept live on ice during transportation, while the Japanese and Port Stephens samples were air-freighted on dry ice. In the laboratory, all samples were stored at -80°C.

4.2.2 Genomic Library Construction

DNA was extracted from five Beauty Point oysters as per Grewe et al. (1994), except 50 µg of tissue per oyster was used. Each of the five DNAs (300µl, DNA concentrations ranged from 15-78 ng/µl) was RNAsed treated with 20 units of RNAse One ™ (Promega) as per manufacturer's instructions. Treatment of RNAse is needed because the presence of RNA a) interferes with the restriction enzyme digestion of DNA and b) does not allow accurate estimations of DNA concentration using a fluorimeter. Fragments of DNA that were 500 bp long were required to ensure sequencing of entire fragment in both forward and reverse

directions. In order to get DNA fragments of 500 base pairs, a double restriction enzyme digest of 22µl (approximately 8.5 µg) of one of the five oyster DNAs was done with 5.5 µl of AluI (80U/µl, New England Biolabs (NEB)) and 5.5 µl HaeIII (10U/µl, NEB) in a reaction mix of 10 µl of Buffer 2 (NEB), 43 µl of sterile double-distilled water, with a total reaction volume of 100µl and incubated at 37°C for three hours. The remaining four DNAs were pooled to ensure that a large amount of DNA of all possible size fragments was generated by the restriction enzyme digestion, such that theses fragments could be easily viewed on an agarose gel after electrophoresis. These 4 pooled DNAs were digested in the following volumes: 20 µl (approximately 33 µg) of DNA, 22 µl of AluI (80U/µl, New England Biolabs (NEB)) and 22 µl HaeIII (10U/µl, NEB) in a reaction mix of 44 µl of Buffer 2 (NEB), 332 µl of sterile double-distilled water, in a total reaction volume of 440 µl and incubated at 37°C for three hours. The digested fragments from the two digests were then loaded on a 0.7% TAE agarose gel (using sterile 1X TAE buffer) and electrophoresed for 3 hrs at 50 V and 1hr at 80V. A gel fragment containing digested DNA fragments from the 300-700 base pair range (according to the λ\HindIII DNA standard (New England Biolabs) and PCR standard (Promega)) was extracted from the gel using the Qiaex kit (Qiagen) as per manufacturers instructions, assuming 8.5µg of DNA was present. These DNA fragments were then ethanol-precipitated to a final concentration of 14 ng/µl and size-checked on a 1% TAE gel with a pBR322/BstN1 DNA standard; sizes were found to be the correct range.

The cloning vector pGEM 3zf+ (19µg, Promega) was digested with 80 units of SmaI (NEB, 10U/µl) in a reaction mix (100 µl total) containing 10µl 10X Buffer

4 (NEB) and 66µl sterilised double distilled water, as per manufacturer's instructions for two hours, before a further 20 units of SmaI were added and the reaction proceeded for another two hours. SmaI was used to generate a blunt end cut of the plasmid, to be compatible with the blunt ends of the inserts. The digested vector was pulse-microfuged before being stored at 4°C overnight, then at -20°C. The SmaI-digested pGEM 3zf(+) was then treated with calf intestine phosphatase (CIP, Pharmacia) as per manufacturer's instructions. CIP treatment ensures that the plasmid cannot rejoin with itself and can only ligate with an insert (oyster DNA) fragment. The DNA concentration quantitated using Hoefer fluorometer as 117ng/µl. The ligation protocol varied from standard methods by the following: 14 Weis U of ligase (approx. 2 µl , as per Pharmacia Technical notes) were used and 0.5 mM ATP (Sambrook et al, 1989; also within 0.1-1 mM range suggested by Pharmacia) as recommended for blunt end ligations. For the ligations, the following ratios of insert (oyster DNA) to vector were as follows (to determine optimal ratio): 200ng vector + 25ng insert, 400ng Vector + 50ng insert (1:1); 400ng Vector + 150ng insert (1:3); 400ng Vector + 17 ng insert (3:1). UnCIPed vector, CIPed vector with reaction mix without ligase added, and pUC18 were used as controls for CIP activity, ligase activity and plasmid, respectively. The reaction mix contained the vector, insert, 2µl 10X ligation buffer (Pharmacia), 4µl 0.5 mM ATP (0.22µM filter-sterilised, Sigma), 2µl ligase (Pharmacia, added last) and sterile double-distilled water to total volume of 20µl. Before ligase was added, 4µl was removed from the mix to act as the non-ligated control. The reactions were incubated in a Perkin Elmer 9600 thermocycler at 16°C hold for approximately 16 hours before the reaction was stopped by heating tubes to 65°C for 10 minutes in a thermocycler before storage at -20°C.

XL-1 Blue cells (Stratagene) were made competent by the rubidium chloride method (Hanahan, 1985). These treated cells were tested for competency using pBR322 (NEB), then transformed with the pGEM 3zf(+)-oyster DNA (Hanahan, 1985). Selective media (containing Xgal, IPTG (both Promega), ampicillin (Sigma), as per Stratagene instructions) was warmed to 37°C, then 100 µl of transformation mix was plated onto the pre-warmed agar plates, with control plates containing 100 µl of cells only or 10 µl of DNA only. Selective media should ensure that only bacteria containing the plasmid with ampicillin resistance, grow. Detection of potential clones is possible by the colour of the bacterial colony, as the insertion of the DNA in the plasmid will disrupt the production of the ß-glactosidase gene (white = insert, blue = no insert).

4.2.3 Colony screening and hybridisation

Colonies were screened for microsatellites using the Colony Hybridisation DIG kit (Boehringer Mannheim). A $(CA)_8$ oligonucleotide (UBC primers) was labelled as per the DIG Labelling kit (Boehringer Mannheim). Plasmids from positive clones were extracted as per Sambrook et al. (1989) and sequenced as per "ABI Prism –Dye Terminator Cycle Sequencing Ready Reaction kit Revision A August 1995" manual.

4.2.4 Primer design and microsatellite gel electrophoresis

Primers were designed using Oligo (NBL) software on clone sequence containing at least five repeats of a microsatellite motif. Ten sets of primers were trialled but only four sets had variability levels useful for population genetics analyses, as the others had between 23-34 alleles (Table 4.1).

Table 4.1: List of microsatellite loci used in this study.

CLONE/ PRIMER NAME	MOTIF	PRODUCT LENGTH (base pairs)	NO. OF ALLELES	PRIMERS (5'-3')
cmrCg61	$(CA)_6TACATGTA(TACA)_4TGTA(TACA)_3TTCA(TACA)_4$	195-228	10	GATTGGTTGAAAAAATCACACG, TAACAGCAGCGCTACCATGC(F)
cmrCg141	$(CA)_9(CT)_6$ $TT(CT)_{20}$	143-238	32	ACCATTTGCACCTTTCCAAC, TGACACTTGAAGCCTTGCAC(F)
cmrCg143	$(TG)_8$ $(G)_{16}$	141-167	23	CTTGCCATATTGCCATGTGT, CTTTTACATGGAATTGTCACAGG(F)
cmrCg151	$(AC)_{10}AT(TC)_{24}$	248-306	23	TGCTTCATTGTTTGTTGTATGG, CATACACAAATTGCACTTATAGCA(F)
cmrCg17	$(TAAA)_2$	242-324	6	GACAGTTTCCCAAAGCAGTCCTAA, CCCACGGATGAGCACAGGT(F)
*BV59**	$(GA)_{12}$	155-195	11	AAGCAACTATCAGTTTTTGGTAGC, AATGAGCTGCACGTTCATAGGC(F)
cmrCg64	$(TC)_2(TG)_2(CA)_2$	142-182	4	TTGAGTCCGATAAAACTTAGCGTA, AAGGACGTTGATGTTAAAGTAGGC(F)

*supplied by Dr Dan McGoldrick, Genbank acccession no. #AF051172

The PCR amplification reaction mix per individual 25 µl PCR reaction per locus was as follows: 2.5 µl 10X PCR Buffer II (100mM Tris-Hcl, pH 8.3 (at 25 °C); 500 mM KCl, Perkin Elmer), 1.5 µl 25 mM $MgCl_2$ (Perkin Elmer), 0.5 µl 10 mM dNTP, 0.5 µl 10 µM fluorescently labelled primer, 0.5 µl 10 µM unlabelled primer, 10 ng DNA template , 0.2 µl Amplitaq (5U/µl, Perkin Elmer). The PCR reaction profiles were as follows: the first cycle involved an initial denaturation at 95 °C for 4 min., followed by annealing at 60 °C for 45 secs, followed extension at 72 °C for 1 min.; then samples were subjected to 35 cycles of denaturation at 95 °C for 30 secs., followed by annealing at 60 °C for 45 secs, followed extension at 72 °C for 1 min.; with a final extension step at 72 °C for 10 min.. For the *cmrCg141*, *143*, *151* primers, the previous conditions were used, except for a final extension step at 72 °C for 2 hours, to reduce formation of split peaks.

Microsatellite analyses used an ABI 377 DNA sequencer (Perkin Elmer Applied Biosystems) with Genescan 2.1 software as per manufacturer's instructions. The gel mix was 4.8% Page-Plus/6M urea containing 6 mL 40% Page-Plus gel stock solution (Astral), 18 g urea (Astral), 5 mL 10 X TBE mix (Astral), 30 µl Temed (Astral), 300 µl 10% ammonium persulphate (Sigma), and double distilled water to 49.5 mL. Genescan 500 TAMRA standard (0.5 µL, Perkin Elmer) was added to each diluted sample before loading, together with formamide (2.22 µL, Astral) and gel–loading blue (0.48 µL, Perkin Elmer). The amount of sample/formamide/blue/standard mix loaded onto the gel varied depending on the different number of wells used per gel (which altered the volume of the wells): 1.1 µL on a 64 well gel and 2 µL on a 34 well gel. The gels were electrophoresed for 2.5 hours as per manufacturer's instructions.

4.2.5 Data analysis

4.2.5.1 Microsatellite genotyping

Genotyper 1.1.1r6 software (Perkin Elmer Applied Biosystems) was used to assign alleles. Allele categories or bins were created for the loci (see Table 4.2).

4.2.5.2 Statistical analyses

Mean sample sizes, numbers of alleles, heterozygosities and proportions of loci polymorphic were calculated by BIOSYS-1 (Swofford and Selander 1989). Genetic similarities between populations were assessed by Nei's (1978) unbiased genetic distance, and Rogers' (1972) distance, clustering the distance matrices by unweighted pair group analysis or Wagner analysis respectively, again using BIOSYS-1 (Figures 4.1a and 4.1b).

Testing of conformation of Hardy-Weinberg equilibrium for each polymorphic locus in each population used 1000 replicates per test of the CHIHW program (Zaykin and Pudovkin, 1993). The Selander index [$D = (H_{obs}-H_{exp})/H_{exp}$, where H_{obs} and H_{exp} are observed and Hardy-Weinberg expected heterozygosities respectively] was estimated and tested for loci showing a significant deviation, again using the CHIHW program. Allele frequency heterogeneity across populations was assessed using the CHIRXC program (Zaykin and Pudovkin, 1993). Both of these programs are based on Monte Carlo randomisations of the data and this obviates the need to pool rare alleles.

Table 4.2: Allele frequencies and numbers of individuals (*n*)

Locus	Allele	SWA	BEA	BRI	SMI	DUN	PIT	NSW	SEN	HIR
LECg17	242	-	0.029	-	0.075	-	0.047	-	0.014	0.041
	272	0.208	-	-	-	-	-	-	-	0.031
	286	0.052	0.029	0.021	0.088	0.078	0.116	-	0.114	0.082
	306	0.010	-	0.011	0.013	-	0.023	0.021	0.029	-
	316	0.729	0.943	0.968	0.825	0.856	0.814	0.979	0.843	0.847
	324	-	-	-	-	0.067	-	-	-	-
	n	48	35	47	40	45	43	47	35	49
BV59	145	-	-	-	-	-	0.024	-	-	-
	155	-	-	0.037	0.015	0.014	0.012	-	0.033	0.021
	157	0.207	0.098	0.159	0.212	0.386	0.220	0.115	0.150	0.085
	161	0.146	0.174	0.159	0.182	0.229	0.110	0.250	0.117	0.245
	169	0.537	0.565	0.451	0.424	0.286	0.390	0.404	0.600	0.500
	177	0.073	0.109	0.085	0.015	0.029	0.085	0.173	0.067	0.064
	179	0.012	0.020	0.049	0.136	0.057	0.061	-	0.017	0.011
	183	0.024	-	-	-	-	0.049	0.019	-	0.053
	187	-	0.011	-	-	-	0.012	0.019	0.017	0.011
	189	-	0.022	0.012	-	-	-	-	-	0.011
	193	-	-	0.037	-	-	0.037	-	-	-
	195	-	-	0.012	0.015	-	-	0.019	-	-
	n	41	46	41	33	35	41	26	30	47
LECg61	195	-	-	-	-	-	0.028	0.010	0.025	-
	199	0.023	0.026	0.020	0.170	0.054	0.042	0.010	0.050	0.031
	203	-	0.051	0.031	-	-	0.083	-	-	0.020
	206	0.011	0.013	0.010	0.011	-	0.014	0.020	0.063	0.010
	212	0.023	0.013	0.020	-	-	-	-	0.050	-
	218	0.818	0.795	0.796	0.574	0.924	0.750	0.950	0.588	0.673
	223	0.114	0.064	0.122	0.245	0.022	0.083	-	0.213	0.143
	228	0.011	0.026	-	-	-	-	0.010	0.013	0.010
	232	-	-	-	-	-	-	-	-	0.082
	241	-	0.013	-	-	-	-	-	-	0.031
	n	44	39	49	47	46	36	50	40	49
LECg64	142	-	-	-	-	-	-	-	0.015	-
	168	-	0.013	-	-	-	0.034	0.020	-	0.020
	174	1.000	0.987	1.000	1.000	1.000	0.966	0.980	0.985	0.939
	182	-	-	-	-	-	-	-	-	0.041
	n	47	38	15	39	45	29	25	34	49

In order to allow for multiple tests of the same hypothesis, Bonferroni corrections of the preset significance level, a, were made by dividing a by the number of tests. For example, where four variable loci were being tested at the 0.05 significance level, a levels were reduced from 0.05 to 0.05/4 = 0.0125.

Homozygote excesses were commonly observed. These can be caused by non-amplifying or null alleles, so that some apparent homozygotes are really null allele/expressed allele heterozygotes. Null allele frequencies were estimated using the "NullTest" program (W. Amos, pers. comm.). This estimates best fit null allele frequencies from microsatellite data sets. 95% confidence intervals are estimated for any null allele as 1.96 standard deviations based on 100 randomisations of the data set. This program estimates the frequency of null alleles based on the scored genotypes as any unscored animals were exempt from analysis. A population that is exactly in Hardy-Weinberg equilibrium will have an estimated null allele frequency of zero.

Nei's gene diversity statistic G_{ST} (Nei, 1973) was used to determine the proportion of the total genetic variation observed that is attributable to variation between populations. The extent of variation that is attributable to sampling error alone, $G_{ST.null}$, was estimated by the Boot-it program, using 1000 replicates (Ward and Grewe, unpublished).

4.3 RESULTS

4.3.1 Genomic library construction

A Pacific oyster genomic library has been constructed. Approximately 5500 clones were screened with DIG-labelled $(CA)_8RT$ microsatellite probe (later found to be faulty), of which 39 clones underwent a secondary screen. Approximately another 2500 clones were screened with the $(CA)_8RT$ microsatellite probe. Although 194 clones were selected for purification, only 86 clones grew. Plasmids were extracted from 44 of these 86 clones, which were then sequenced. Twenty-eight clones were sequenced in both directions and 16 sequenced in one direction. A total of 28 clones contained microsatellites, two clones contained inserts but not microsatellites, three clones didn't contain inserts and eight clones were not sequenced successfully. Five different perfect dinucleotide repeat motifs, fifteen different trinucleotide motifs, eight different tetranucleotide motifs and one motif containing five bases have been identified by sequencing analysis. The following imperfect repeats were found: five dinucleotide repeats, one trinucleotide and one tetranucleotide repeat.

4.3.2 Primer design

Primers were designed for clones containing microsatellite sequence of greater than 5 dinucleotide repeats, or if containing at least two tri- or tetranucleotide repeats. All loci that would PCR amplify successfully were used to analyse an Australian hatchery (SMI) and a Japanese endemic population (SEN), which were deemed to represent the groups which should be the most genetically different, thus giving an overview of the levels of diversity of the loci. Results of the initial analysis of the loci *cmrCg:61, 141, 143, 151* are shown in Table 4.3b and 4.4b. The high numbers of alleles observed in all loci but *cmrCg61* made reliable scoring difficult due to the close proximity of the alleles (< 2 bp) and/or difficulty

Table 4.3a: Genetic variability at 4 loci (*cmrCg 61, 17, 64, BV59*) in all populations (standard errors in parentheses).

Population	Type	Mean sample size per locus	Mean no. of alleles per locus	% loci polymorphic*	Mean heterozygosity	
					Observed	Expected**
SWA	nat	45.0±1.6	4.3±1.2	75	0.172±0.123	0.349±0.135
BEA	nat	39.5±2.3	5.0±1.5	100	0.155±0.095	0.284±0.137
BRI	nat	38.0±7.9	4.8±1.8	75	0.179±0.108	0.290±0.169
SMI	hat	39.8±2.9	4.0±1.2	75	0.204±0.147	0.408±0.162
PIT	hat	37.3±3.1	5.5±1.7	100	0.231±0.127	0.400±0.148
DUN	hat	42.8±2.6	3.3±1.0	75	0.230±0.146	0.282±0.156
NSW	nat	37.0±6.7	4.0±1.2	100	0.145±0.119	0.231±0.172
SEN	end	34.8±2.1	5.0±1.2	100	0.152±0.056	0.381±0.140
HIR	end	48.5±0.5	6.0±1.5	100	0.204±0.098	0.400±0.126
Means for Population types						
Hatchery (n = 3)		40.0±1.6	4.3±0.6	83.3	0.222±0.009	0.363±0.041
Naturalised (n = 4)		39.9±1.8	4.5±0.2	87.5	0.163±0.008	0.289±0.024
Endemic (n = 3)		41.7±6.9	5.5±0.5	100	0.178±0.026	0.391±0.010
Mean (n = 9)		40.3±1.5	4.7±0.3	88.9	0.186±0.011	0.336±0.022

nat = naturalised, hat = hatchery, end = endemic

* A locus is considered polymorphic if more than one allele was detected.

** Unbiased Hardy-Weinberg estimate (see Nei, 1978).

Table 4.3b: Genetic variability at 4 microsatellite loci (*cmrCg 61, 141, 143, 151*) in two populations (n=50) of Pacific oysters from Sendai Bay (Japan, established) and Smithton (Tasmanian, farmed).

Population	Mean sample size per locus	Mean no. of alleles per locus	Mean Heterozygosity/locus	
			observed	expected
Sendai	35.3±11.6	16.3±8.6	0.458±0.089	0.849±0.069
Smithton	49.5±1.9	17.3±7.3	0.384±0.181	0.847±0.136

Table 4.4a: Summary of Chi square and G_{ST} analyses of four variable microsatellite loci (*cmrCg61, 17, 64; BV59*) in nine populations of *C.gigas*.

	number		χ^2 analysis		*GST* analysis		
Locus	**of alleles**	***n***	χ^2	***P***	***GST***	***GST.null*±SD**	***P***
cmrCg17	6	390	221.101	<0.001	0.053	0.010 ± 0.003	<0.001
BV59	11	344	261.480	<0.001	0.032	0.012 ± 0.003	<0.001
cmrCg61	10	400	253.547	<0.001	0.064	0.010 ± 0.003	<0.001
cmrCg64	4	330	42.568	0.016	0.022	0.014± 0.005	0.0630
Across 4 loci					0.043	0.0116 ± 0.002	<0.001

Table 4.4b : Summary of interpopulation heterogeneity observed at four microsatellite loci (*cmrCg61, 141, 143, 151*) between Sendai and Smithton populations of Pacific oysters.

Locus	**No. of alleles**	χ^2	
		χ^2	P
cmrCg61	10	25.845	<0.001
cmrCg141	32	50.289	0.004
cmrCg143	23	35.459	0.003
cmrCg151	23	57.268	<0.001

in determining allele bins. Therefore, analyses of nine populations with these loci was deemed to be too technically demanding with regard to the time limitations of the study. Hence, only loci *cmrCg17, 64* (developed in this study) and *BV59* (courtesy of Dr D. McGoldrick) were used in the analyses of the nine populations (n = 50 per population) (Table 4.1).

It is assumed that the primers used detect the microsatellite motifs seen in Table 4.1. This should mean that the allele sizes should vary by the factor of the number of repeat seen in the motif, i.e., by a two or a number divisible by two for the *BV59* and *cmrCg64*, or by four or a number divisible by four for *cmrCg17*. *cmrCg61* contains both di- and tetranucleotide repeats, so may display the effects mention previously, or a combination of the two. However, in practise, as the allele bins are often one or more alleles than have been pooled due to their close proximity to each other (< 2bp) and hence difficulty in scoring reliably, so the length difference between two alleles may not be a factor of the length of the nucleotide repeat (ie two alleles containing a dinucleotide repeat may be more than a factor of two different in size). If there is more than one type of microsatellite repeat unit in a motif, or a di – and trinucleotide repeat in the same motif, or an insertion or deletion in the fragment being amplified, the detection of which repeat is causing the length variation is less defined. Time limitations prevented the sequencing of fragments to determine the repeat responsible for the length variation, and sequencing of same size fragments from different location to determine if they were the same allele.

4.3.3 Data analysis

4.3.3.1 Statistical analyses

Due to the high number of alleles observed for loci *cmrCg141,143* and *151*, these loci were dropped for analysis of remaining populations in study. Trial data for these loci and *cmrCg61* for SEN and SMI is listed in Table 4.3b. Allele

frequencies for the four loci (*cmrCg17*, *61*, *64*; *BV59*) examined in the nine populations were determined (Table 4.2). One locus, *cmrCg64*, was nearly monomorphic. The remaining three loci showed medium levels of variability. Genetic variability levels were determined for each population (Table 4.3a); all were moderate. The mean number of alleles per locus per population was 4.7±0.3, the mean percent loci polymorphic was 88.9%, the mean observed heterozygosity per locus was 0.186±0.011, and the mean Hardy-Weinberg expected heterozygosity per locus was 0.336±0.022. Differences in levels of variation among groups of populations — that is, hatchery versus naturalised versus endemic — were minimal (Table 4.3a).

Tests of allele frequency differentiation among the nine populations revealed that three of the four variable loci showed significant (α reduced to 0.05/4 = 0.0125) inter-population heterogeneity after χ^2 analysis and G_{ST} analysis — *cmrCg17*, *BV59* and *cmrCg61* (Table 4.4). The amount of differentiation among the populations was, however, small. This can be seen in the G_{ST} analyses. The three loci showing the most differentiation were *cmrCg17*, *BV59* and *cmrCg61*; for all of these loci, about 4.7% of the total amount of differentiation could be attributable to inter-population differences once sample effects had been allowed for (i.e. G_{ST} - $G_{ST.null}$). Across all loci, only ≈ 4% of the genetic variation could be attributed to differences among populations.

Populations were compared pair-wise to locate the source of this limited genetic differentiation (α reduced to 0.05/4 = 0.0125, data not shown). There were no significant differences between any of the populations for the *cmrCg64* locus. There were no significant differences between BEA/BRI, and BEA/HIR for any

of the four loci – all other populations were significantly different with any given population for at least one of three loci (*cmrCg17*, *BV59* and *cmrCg61*). Some populations were significantly different from all other populations at certain loci: SWA (*cmrCg17*); PIT (*BV59*) and SMI (*cmrCg61*). In terms of differences within the hatchery (DUN, PIT, SMI), naturalised (BEA, BRI, SWA, NSW) and endemic (Japanese – HIR, SEN) groups, at least one member of any group was significantly different to another member of the same group for at least one locus. The hatchery group were the most heterogeneous group, with significant differences between DUN and PIT at 3 loci (*cmrCg*17, BV59, *cmrCg*61), and at two loci between DUN and SMI (*cmrCg17*, *cmrCg61*) and between PIT and SMI (*BV59*, *cmrCg61*). However, there were no significant differences observed between populations within the naturalised Tasmanian groups (SWA, BEA, BRI) for three out of four loci – *BV59*, *cmrCg61*, *cmrCg64*. There were no significant differences between members of the naturalised (SWA, BEA, BRI, NSW) and endemic Japanese (HIR, SEN) groups at two loci – *BV59* and *cmrCg64*; but both groups were significantly different from members of the hatchery group at *BV59*.

No population conformed to Hardy-Weinberg equilibrium for all loci. All the non-conforming samples showed significant heterozygote deficiencies (data not shown). Only one population (BRI) conformed to Hardy-Weinberg equilibrium for *cmrCg17* ($\chi^2 = 0.051$, $P = 1.000$). SEN was the only population that showed a significant deviation from Hardy-Weinberg equilibrium and a significant heterozygote deficiency for *BV59* ($\chi^2 = 65.477$, $P = 0.005$; $D = -0.554$, $P = <0.001$). Only three populations conformed to Hardy - Weinberg equilibrium for *cmrCg61* — BEA ($\chi^2 = 96.367$, $P = 0.015$), DUN ($\chi^2 = 46.160$, $P = 0.015$) and HIR ($\chi^2 = 63.936$, $P = 0.05$). Four populations were invariant for *cmrCg64* — SWA, BRI, DUN and SMI; HIR was the only population that did not conform to Hardy-Weinberg equilibrium at this locus due to a significant excess of homozygotes ($\chi^2 = 98.0$, $P = <0.001$; $D = -1.0$, $P = <0.001$).

The estimations of null allele frequencies using NullTest (W. Amos, pers. comm.) are shown in Table 4.5. The highest mean frequencies of null alleles across all populations were observed at *cmrCg17* (0.483±0.081) and *cmrCg61* (0.410±0.054). Calculations determined the absence of null alleles at some populations for each locus: 7 cases at *cmrCg64* (SWA, BEA, BRI, SMI, DUN, SEN, NSW), 2 cases at *BV59* (SWA, DUN) and 1 case for both *cmrCg17* (BRI) and *cmrCg61* (DUN). Assuming the frequency of the null allele is p, then the number of animals expected to be null homozygotes and hence would be unscorable in a sample size of 50 animals is p^2 x50. Calculations of the expected number of null homozygotes were much higher than the number of unscorable samples for all but one population (DUN) for *cmrCg17*, at four populations for *cmrCg61* (SWA, BRI, SMI, HIR) and at one population for *cmrCg64* (HIR). Conversely, the number of null homozygotes were much lower than the number of unscorable samples at 3 loci: *cmrCg17*(DUN), *BV59* (BRI, PIT, NSW, SEN) and *cmrCg61* (BEA, PIT).

Unbiased (Nei, 1978) genetic distances over the four loci were estimated between all pair-wise combinations of populations, and from the resulting matrix a UPGMA dendrogram of population relationships was generated (Figure 4.1a). There is no clear cut structure to the clusters: the different groups (naturalised, hatchery, endemic) and locales (Tasmania, NSW and Japan) are not reflected in the clusters, except for BEA and BRI (both Tasmanian naturalised sites) forming a sub-cluster. All pair-wise population distances are very small (Nei D<0.03). The only similarity between this dendrogram and that generated by Wagner tree analysis using Rogers (1972) distance (Figure 4.1b) is the sub-cluster of SMI (hatchery, Tasmania) and SEN (endemic, Japan). The UPGMA method assumes a constant rate of evolution, a constraint not present in the Wagner approach. However, the standard errors of the distances are large (ranging to 0.0295±0.0867

Table 4.5: Frequency of null alleles (calculated by NullTest (W. Amos, pers. comm.)) in nine populations of *C. gigas* at four microsatellite loci. Null hom. exp = number of null homozygotes expected.

	cmrCg17		**BV59**		**cmrCg61**		**cmrCg64**	
	Allele		**Allele**		**Allele**		**Allele**	
SWA	272	0.065	157	0.187	199	0.007	174	1.000
	286	0.017	161	0.129	206	0.007		
	306	0.006	169	0.501	212	0.007		
	316	0.242	177	0.063	218	0.280		
			179	0.012	223	0.040		
			183	0.025	228	0.007		
	Nulls	0.670		0.083		0.656		0.000
	95% CI	0.122		0.100		0.121		0.000
	Null hom. exp.	22.0		0.3		21.5		0.0
BEA	242	0.006	157	0.083	199	0.023	168	0.014
	286	0.006	161	0.127	203	0.023	174	0.986
	316	0.237	169	0.446	206	0.011		
			177	0.072	212	0.011		
			179	0.020	218	0.520		
			187	0.010	223	0.046		
			189	0.020	228	0.011		
					241	0.011		
	Nulls	0.774		0.223		0.342		0.000
	95% CI	0.148		0.084		0.123		0.111
	Null hom. exp.	30.0		2.5		5.8		0.0
BRI	286	0.021	155	0.036	199	0.009	174	1.000
	306	0.011	157	0.125	203	0.018		
	316	0.967	161	0.112	206	0.009		
			169	0.375	212	0.009		
			177	0.060	216	0.009		
			179	0.036	218	0.479		
			189	0.012	223	0.081		
			193	0.036				
			195	0.012				
	Nulls	0.000		0.196		0.387		0.000
	95% CI	0.094		0.085		0.112		0.000
	Null hom. exp.	0.0		1.9		7.5		0.0

	cmrCg17 Allele		**BV59 Allele**		**cmrCg61 Allele**		**cmrCg64 Allele**	
DUN	286	0.054	155	0.014	199	0.055	174	1.000
	316	0.654	157	0.367	218	0.805		
	324	0.054	161	0.207	223	0.011		
			169	0.303				
			177	0.029				
			179	0.044				
	Nulls	0.238		0.037		0.130		0.000
	95% CI	0.135		0.099		0.139		0.000
	Null hom. exp.	2.8		6.8		0.8		0.0
PIT	242	0.018	145	0.024	195	0.025	168	0.011
	286	0.065	155	0.012	199	0.025	174	0.381
	306	0.018	157	0.185	203	0.051		
	316	0.418	161	0.101	206	0.012		
			169	0.348	218	0.493		
			177	0.062	223	0.064		
			179	0.049				
			183	0.037				
			187	0.012				
			193	0.037				
	Nulls	0.481		0.134		0.330		0.630
	95% CI	0.137		0.073		0.108		0.151
	Null hom. exp.	11.6		0.9		5.4		19.8
SMI	242	0.019	155	0.015	199	0.052	174	1.000
	286	0.025	157	0.201	206	0.006		
	306	0.006	161	0.182	218	0.213		
	316	0.240	169	0.370	223	0.093		
			177	0.015				
			179	0.129				
			195	0.015				
	Nulls	0.712		0.072		0.635		0.000
	95% CI	0.122		0.080		0.115		0.000
	Null hom. exp.	25.3		0.3		20.0		0.0

	cmrCg17 Allele		**BV59 Allele**		**cmrCg61 Allele**		**cmrCg64 Allele**	
NSW	306	0.008	157	0.077	195	0.008	168	0.020
	316	0.506	161	0.207	199	0.008	174	0.980
			169	0.335	206	0.008		
			177	0.140	218	0.533		
			183	0.019	223	0.008		
			187	0.019				
			195	0.019				
	Nulls	0.502		0.185		0.440		0.000
	95% CI	0.096		0.078		0.101		0.085
	Null hom. exp.	12.6		1.7		9.7		0.0
SEN	242	0.011	155	0.014	195	0.010	142	0.015
	286	0.054	157	0.089	199	0.020	174	0.985
	306	0.011	161	0.073	206	0.031		
	316	0.407	169	0.363	212	0.020		
			177	0.043	218	0.342		
			179	0.014	223	0.130		
			187	0.014	228	0.010		
	Nulls	0.520		0.388		0.436		0.000
	95% CI	0.137		0.103		0.105		0.102
	Null hom. exp.	13.5		7.5		9.5		0.0

	cmrCg17 Allele		**BV59 Allele**		**cmrCg61 Allele**		**cmrCg64 Allele**	
HIR	242	0.025	155	0.021	199	0.018	168	0.004
	272	0.016	157	0.064	203	0.018	174	0.219
	286	0.042	161	0.180	206	0.009	190	0.009
	316	0.469	169	0.432	218	0.442		
			177	0.053	223	0.085		
			179	0.010	228	0.009		
			183	0.031	232	0.056		
			187	0.010	241	0.028		
			189	0.010				
	Nulls	0.449		0.189		0.334		0.793
	95% CI	0.105		0.081		0.089		0.055
	Null hom. exp.	10.1		1.8		5.6		31.5
Mean null freq. (n=9 pops)	0.483±0.081		0.167±0.034		0.410±0.054		0.158±0.105	

Figure 4.1a: *C. gigas* cluster analysis: UPGMA using Nei (1978) distance.

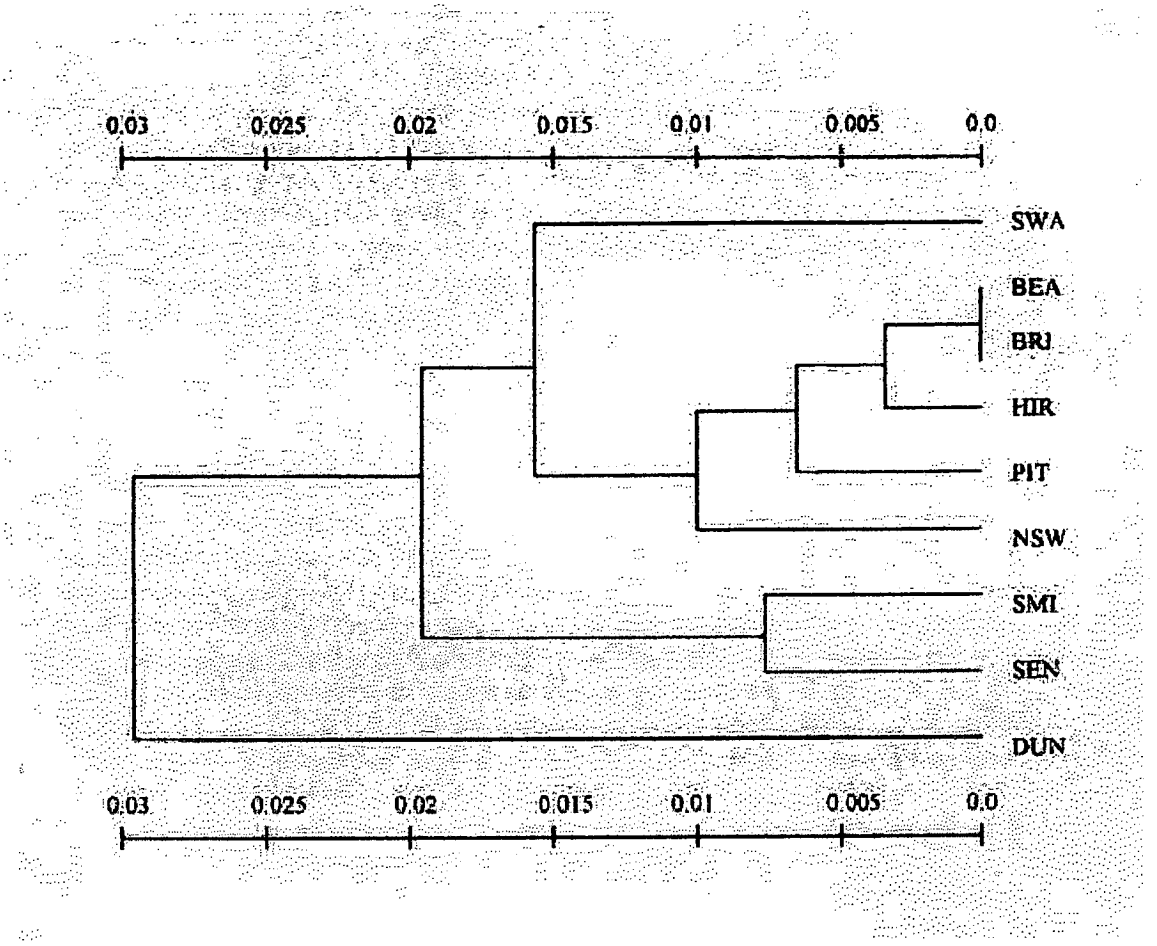
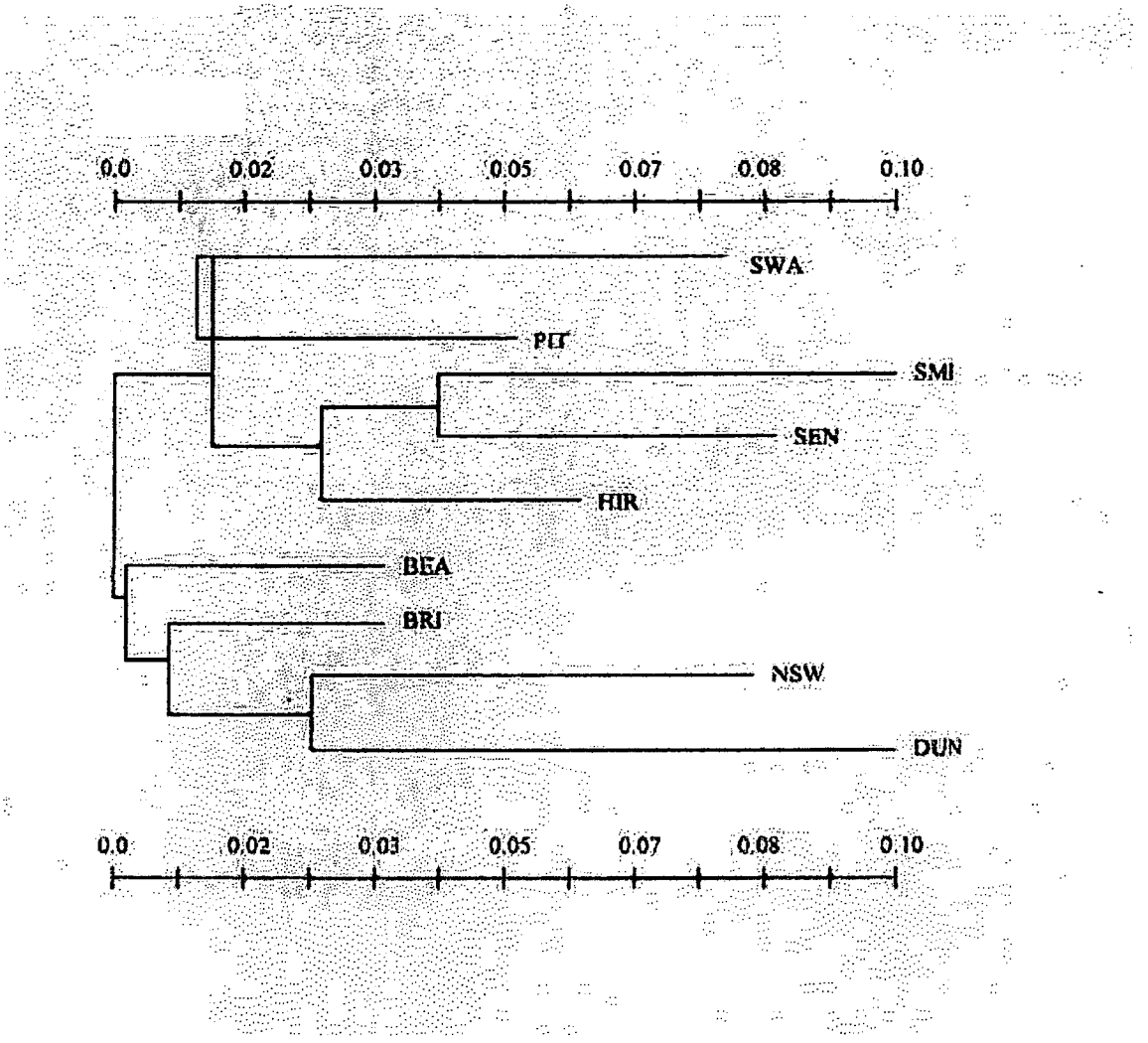


Figure 4.1b: *C. gigas* Rogers/Wagner tree



between the SWA/BEA/BRI/HIR/PIT/NSW/SMI/SEN and DUN clusters), indicating that the groupings, based on only four loci, are not statistically meaningful.

4.4 DISCUSSION

All populations, both Australian and Japanese, of Pacific oysters showed moderate genetic variability. Across the nine populations sampled, variability levels (about 89% of loci polymorphic, average observed heterozygosities per locus of around 0.188 and average number of alleles around 4.7) were higher than that determined using allozyme analysis on the same populations (about 72.5 % of loci polymorphic, average observed heterozygosities per locus of around 0.279 and average number of alleles around 2.9; Table 2.5, Chapter 2), except for average observed heterozygosity. The values of microsatellite variation in this study were not as high as that previously recorded for oysters. In the only other *C.gigas* microsatellite population genetics study available, Magoulas et al. (1998) reported that the observed heterozygosities in the unrelated wild parents from the Thau lagoon, France, ranged from 0.6-1.0 and the number of alleles ranged from 12- 15 for three microsatellite loci. A microsatellite developed for population genetic analysis in *O. edulis* (Naciri et al., 1997) had a higher expected heterozygosity (H = 0.664; this study: 0.336) but a lower effective number of alleles = 2.98 (this study: 4.7). However, the levels of genetic variation were on average higher for the two microsatellite loci developed for paternity testing in *Ostrea edulis* (Oedu.B11 - expected H = 0.969, effective number of alleles = 31.72, Oedu.C6 - expected H = 0.910, effective number of alleles = 11.14) than that observed for the highly variable loci trialled in the present study (Table 4.3b). It is likely that the lower levels of genetic variation observed in this study are indicative of the lower polymorphism of the microsatellites used, rather than the

levels of variation present in the populations, as both the allozymes and highly polymorphic microsatellite loci (SEN and SMI only) demonstrated higher values in the same populations. The use of more polymorphic loci with an effective number of alleles of about 10-15 alleles would of course demonstrate higher variability levels – note that the three most variable microsatellites were dropped from this study due to scoring difficulties.

One of the first casualties of inbreeding is the loss of rare alleles, and changes in allelic diversity after bottlenecks may be more striking than changes in heterozygosity (Allendorf and Ryman, 1987). The reduction in mean numbers of alleles per locus from 5.5 in Japan to 4.1 in Australian hatchery stocks, suggests that some allele loss might have occurred. An allozyme study on these populations did not demonstrate a significant loss of mean number of alleles (3.1 and 3.0 respectively, Chapter 2/English et al, 2000). In this case, the loss of alleles was higher using microsatellites than that observed with allozymes. However, the difference between the naturalised samples, with a mean number of alleles of 4.5 is within the variance range for the mean number of alleles present in the hatchery samples. Given that the animals from naturalised populations have been used in hatchery spawnings, the time frame of the loss of approximately 0.4 of an allele cannot be determined, as it is not known whether the actual allele loss is greater but being masked by the occasional use of naturalised oysters. However, as this represents a small and insignificant loss of alleles, and given that there was no total loss of variation in the loci observed, the drop of mean number of alleles in the hatchery samples is not a cause for concern. This study can provide baseline data for any future comparison of Tasmanian hatchery stocks, enabling monitoring to determine if the loss of alleles is greater and/or over what timeframe the loss is occurring. In other studies comparing hatchery and natural populations of fish, for example, Atlantic salmon, Tessier et al. (1995) also found losses or decreases in the frequency of less common alleles and lower diversity in hatchery

fish compared to natural populations in Lake St Jean, Quebec, and loss of alleles was also observed in the Tasmanian hatchery stock when compared with the ancestral Canadian stock (Reilly et al., 1999). Loss of alleles contrasts with the findings of Estoup et al. (1998), that the samples of brown trout from the Vosges hatchery had a greater number of alleles, including many private alleles than the natural populations surveyed from the Moselle and Ill drainages.

Most samples showed good agreement with Hardy-Weinberg expectations at two loci (*BV59*, *cmrCg64*; 8 cases at each, with either of the Japanese samples showing deviations). At the remaining two loci, there were some significant deviations — all resulting from significant heterozygote deficiencies, with few populations meeting Hardy – Weinberg expectations (*cmrCg17*, one case; *cmrCg61*, three cases). Eight of the nine populations had observed mean heterozygosities less than that expected from Hardy-Weinberg equilibrium — only in one population were the two heterozygosities similar (DUN). Similar deficits have been observed in Atlantic salmon (Reilly et al., 1999) and have been attributed to null alleles. Although null alleles have been previously reported for the loci used in this study (*cmrCg61*: Boaprasertkul , 1998), null alleles do not appear to explain the heterozygote deficiencies observed in this study, based on analysis by the NullTest program (W. Amos, pers. comm.). The frequency of the proposed null alleles seems too high. At that frequency, more null homozygotes would be expected and more samples should not amplified than were actually observed. This phenomenon has also been observed in the French populations of *C. gigas* (A. Huvet, pers. comm.). Allozyme study of the same populations (see Chapter 2) also demonstrated heterozygote deficiencies, and probably occurred as a result of a combination of gel scoring errors, selection, inbreeding and the Wahlund effect (see Foltz, 1986; Lavie and Nevo, 1986; Gaffney et al., 1990; Borsa et al., 1991; Gardner, 1992; Fairbrother and Beaumont, 1993; Ríos et al., 1996). However, not all microsatellite studies show heterozygote deficiencies -

heterozygote excesses have been reported in *O. edulis* (Naciri et al., 1995; Bierne et al., 1998) as a result of zygotic selection and co-segregation of microsatellite markers with fitness-associated genes, respectively. Also, a brown trout hatchery stock conformed to Hardy - Weinberg equilibrium, as did the natural stocks after Bonferroni correction for multiple tests (Estoup et al., 1998).

The G_{ST} results were higher using microsatellite analysis ($\approx$ 4%), compared to allozyme analysis of the same populations (see Chapter 2, $\approx$ 1%), but were still relatively low compared to other studies in fish, where 14-34 % of variation seen was due to variation between populations of Atlantic salmon (Tessier et al., 1995). This means that the populations examined in this study show more within-population than between-populations variation, also indicating the lack of difference between the hatchery, endemic and naturalised populations.

Overall, the use of microsatellites confirmed the results of the allozyme study of these populations. - the introduction of oysters from Japan to Tasmania, and their subsequent domestication, have not substantially eroded the genetic basis of the Tasmanian stock, thus agreeing with an earlier allozyme study (Chapter 2/English et al, 2000). The standard breeding practices of the Tasmanian hatcheries, which in a spawning typically use several dozen males and females, often from different farms and hatcheries, appear to have maintained genetic variability levels. However, the levels of genetic variability should be monitored, to detect any possible subsequent loss of alleles. The high levels of genetic variation suggest that variation for quantitative trait loci might also be maintained in Tasmanian stocks; industry and scientists have subsequently initiated a selective breeding program for traits such as growth rate (R. D. Ward, pers. comm.).

Chapter 5: Allozyme variation in three generations of selection for whole weight in Sydney rock oysters (*Saccostrea glomerata*).

5.1 INTRODUCTION

Production of the native Sydney rock oyster, *Saccostrea glomerata* (note species name change from *S. commercialis*, see Anderson and Adlard, 1984), like many other native oysters, has declined in recent years. The Pacific oyster, *Crassostrea gigas*, now dominates world oyster production (Shatkin et al., 1997). Sydney rock oyster production has declined from about 14.5 million dozen oysters in the 1970s to 8.5 million dozen oysters in 1995/1996 (NSW Fisheries, 1998). Farming of the Sydney rock oyster, which relies on natural spatfall, began on the Australian east coast (New South Wales and southern Queensland) in the 1870s and on the west coast in the early 1980s (Nell, 1993). Declining production rates have resulted from oyster mortalities by infection of protoctistan parasites (winter mortality, *Mikrocytos roughleyi* and QX, *Marteilia sydneyi* (Nell, 1993)), adverse effects of viral outbreaks among consumers, and labour-intensive methods that have reduced economic viability.

In an attempt to increase profitability and to meet competition from the faster growing Pacific oyster in Tasmania, South Australia and New Zealand, a selective breeding program (using mass selection) was established in 1990 (Nell et al., 1996). Equal numbers of oysters were taken in equal numbers from each of four estuaries in New South Wales: Georges (32°0'S, 151°0'E) and Hawkesbury (33°30'S, 151°15'E) Rivers, Wallis Lake (32°10'S, 152°29'E), Port Stephens (32°45'S, 152°10'E). Efforts were made to ensure that only locally caught oysters were sampled, not those that had been transferred from another estuary. Oysters from each of the four estuaries were divided equally between eight groups for spawning (100 oysters/group). Of the 100 oysters in each of the eight spawning groups, not all of the 100 oysters spawned - only those that spawned profusely were used. Once oysters began to spawn, they were rinsed and continued to spawn in separate containers. Prior to fertilisation, all eggs were pooled separately for each mass spawning, and this process was repeated for all the sperm

samples. The complete spawning procedure outlined previously was repeated for the spawning of subsequent generations. Control, or non selected oysters, were taken from the same four estuaries as the original base population. An increase in whole weight of 18% was gained by the third generation and the selection intensity was 7% for the loose oysters and 10% for the slat oysters (Nell et al., 1999). Production of triploid Sydney rock oysters rates has also been examined for its potential for increasing whole weight and it was found that triploid oysters were on average 41% heavier than their diploid counterparts after 2.5 years of growth (Nell et al., 1994).

Selective breeding programs may result in the loss of genetic variation through inadequate numbers of parents, leading to inbreeding (Tave, 1993). Farming has led to the reduction of genetic diversity in Pacific oysters (*Crassostrea gigas*) in the United States of America (Hedgecock and Sly, 1990) and in United Kingdom (Gosling, 1982). Hedgecock and Sly (1990) also found that the effective population sizes of the U.S. stocks were several orders of magnitude smaller than the number of broodstock actually used by industry. In Tasmania, farming has not led to the loss of appreciable amounts of genetic variation (Chapter 2/English et al., 2000).

A previous study of Sydney rock oysters from three sites in New South Wales found heterozygosities ranging from 0.17-0.19 (Buroker et al., 1979a). Ours is the first study to examine the genetic variability in a selected line. Allozyme electrophoresis was used to determine the levels of genetic diversity of the second and third generation oysters and of a control, unselected, line.

5.2 METHODS

5.2.1 Sample collection

A mass selection program was set up in Port Stephens, New South Wales (Nell et al., 1996). Spawning numbers for each of the groups are shown in Table 5.1. Samples of adductor muscle from approximately 100 individuals from each of a control line and the

Table 5.1: Details of broodstock used to found the various generations of *S. glomerata* Georges River Slat 2 selection line and controls (I.Smith, personal communication).

Sample	Spawning date	No. of spawning groups used	Max. total number of broodstock used*
1st generation/ Control group	February 1990	8	800
2nd generation	January 1992	3	22
3rd generation	January 1994	4	89

*over total no. of spawning groups.

Table 5.2. List of allozymes used in this study.

Enzyme or protein name	Locus	EC number	Buffer	Time (mins)	Structure
Aspartate aminotransferase	*AAT*	2.6.1.1	TG	35	dimer
Aldolase	*ALD*	4.1.2.13	TC	45	(invariant)
Esterase-D	*ESTD-1*	3.1.1.1	LHTNC	300	dimer
	ESTD-2	3.1.1.1	LHTNC	300	dimer
Glyceraldehyde-3-phosphate dehydrogenase	*G3PDH*	1.2.1.12	TC	80	tetramer?
Isocitrate dehydrogenase	*IDH*	1.1.1.42	TC	70	(invariant)
Malate dehydrogenase	*MDH*	1.1.1.37	TC	90	dimer
6-Phosphogluconate dehydrogenase	*6PGDH*	1.1.1.44	TC	90	dimer
Phosphoglucose isomerase	*PGI*	5.3.1.9	LHTNC	300	dimer
Peptidase	*PEPS-1*	3.4.11/13?	TG	35	dimer
	PEPS-2	3.4.11/13?	TG	35	dimer
General protein	*PROT*	-	TG	35	monomer
Superoxide dismutase	*SOD-1*	1.15.1.1	LHTNC	300	(invariant)
	SOD-2	1.15.1.1	LHTNC	300	monomer

TC = tris citrate, TG = tris glycine, LHTNC = L-Histidine/trisodium citrate (starch)
a = adductor muscle
Structure determined from heterozygote band patterns - note that *G3PDH* heterozygotes were diffuse and the five bands expected of a tetramer could not be resolved.

second and third generations of the slat 2 Georges River Sydney rock oyster line (Nell et al., 1996) were air-freighted on dry ice to the laboratory, where all samples were stored at -80°C. Equal numbers of oysters from the same four estuaries (Georges River, Hawkesbury River, Port Stephens, Wallis Lake) that founded the original base population were used as the control group (Nell et al., 1996), and were sampled for this study at the same time as the second generation of the selected line. No samples of the original controls or the first generation were analysed in this study as no oysters from these groups had been saved.

5.2.2 Allozyme analysis

Eleven enzymes (13 loci) and one general protein locus (Table 5.2) were examined in about 100 oysters from each of the three groups. Adductor muscle (about 200 mg) was placed into separate 1.5 ml centrifuge tubes and homogenised in a few drops of distilled water before being microcentrifuged for 10 minutes at 13 500 G. The supernatant was subjected to cellulose acetate or starch gel electrophoresis. For cellulose acetate electrophoresis, Helena Titan III cellulose acetate plates were used with either a tris glycine (0.02 M tris, 0.192 M glycine) or a 75 mM tris citrate (pH 7.0) buffer system (Hebert and Beaton, 1989; Richardson et al., 1986). The tris glycine gels were electrophoresed at 200 volts, the tris citrate gels at 150 volts (Table 5.2). For starch gel electrophoresis, small pieces of Whatman No. 3 chromatography paper were soaked in the supernatant and placed in a gel made of 8% Connaught hydrolysed starch in a 5 mM l-Histidine HCl buffer (pH adjusted to 7.0 with 0.1 M sodium hydroxide). The starch gels were electrophoresed in 0.41 M trisodium citrate electrode buffer (pH adjusted to 7.0 with 0.5 M citric acid) at 100 volts. Standard enzyme staining protocols (Hebert and Beaton, 1989; Richardson et al., 1986) were used; the peptidase stains used glycyl-leucine (PEPS-1*) and phenylalanyl-proline (PEPS-2*) as substrates. General proteins were stained with Coomassie Blue.

Where an enzyme had multiple loci, the locus encoding the fastest migrating allozyme was designated '1'. Alleles within loci were numbered according to the anodal mobility of their product relative to that of the most common allele (designated '100').

5.2.3 Data Analysis

Mean sample sizes, numbers of alleles, heterozygosities and proportions of loci polymorphic were calculated by BIOSYS-1 (Swofford and Selander, 1989). Genetic similarities between populations were assessed by Nei's (1978) unbiased genetic distance, again using BIOSYS-1.

Tests of conformation to Hardy-Weinberg equilibrium for each polymorphic locus in each population used 1000 replicates per test of the CHIHW program (Zaykin and Pudovkin, 1993). The Selander index [$(H_{obs}-H_{exp})/H_{exp}$, where H_{obs} and H_{exp} are observed and Hardy-Weinberg expected heterozygosities, respectively] was estimated and tested for loci showing a significant deviation from Hardy-Weinberg equilibrium, again using the CHIHW program. Allele frequency heterogeneity across populations was assessed using the CHIRXC program (Zaykin and Pudovkin, 1993). Both of these programs are based on Monte Carlo randomisations of the data, obviating the need to pool rare alleles.

To allow for multiple tests of the same hypothesis, Bonferroni corrections of the preset significance level, α, were made by dividing α by the number of tests. For example, where 11 variable loci were being tested at the 0.05 significance level, α levels were reduced from 0.05 to $0.05/11 = 0.0045$.

The effective population sizes (N_K) of the groups were estimated from allele frequency variances, using the methods of Pollak (1983) and Hedgecock and Sly (1990). The number of alleles at a given locus is k, F_k is the temporal allele frequency variance at a given locus, and F_K is the multi-locus estimate of allele frequency variance, weighted by the number of independent alleles. As oysters from the control group were used as broodstock for the first generation, the control group oysters were considered the

progenitors of second and third generation populations. When the control group was the progenitor, the harmonic mean effective population sizes, N_e, values were calculated by multiplying N_K by two, as there were two lines of descent (control line and selected line, see Hedgecock et al. (1992)). An analysis comparing the second and third generations was also done, as oysters from the second generation were used directly as broodstock for the third generation. Data from loci that were monomorphic in all three groups (*ALD*, *IDH* and *SOD-2*) were excluded from the calculations.

5.3 RESULTS

Allele frequencies for the 14 isozyme loci were determined for control, second and third generation of selectively-bred Sydney rock oysters (Table 5.3). Heterozygote banding patterns for each of the enzymes showed the appropriate number of bands based on known subunit numbers (Ward et al., 1992). Three loci (*ALD*, *IDH*, *SOD-2*) were invariant, and five loci (*6PGDH*, *ESTD-2*, *G3PDH*, *PROT*, *SOD-1*) showed only rare variants. The remaining six loci showed medium to high levels of variability. Genetic variability levels were determined for each group (Table 5.4); all were high and similar to one another. All alleles with sample frequencies of 0.2 or greater were present in all groups.

Tests of allele frequency differentiation among the three groups revealed that seven of the eleven variable loci - *AAT*, *ESTD-2*, *6PGDH*, *PEPS-1*, *PEPS-2*, *PGI* and *SOD-1* - showed significant (α reduced to 0.05/11 = 0.0045) interpopulation heterogeneity after chi-square analyses (Table 5.5).

Allele frequencies of groups were compared pairwise to locate the sources of the genetic differentiation (data not presented). Chi-square tests showed significant differences for seven loci (*AAT*, *ESTD-2*, *MDH* , *6PGDH* , *PEPS-2*, *PGI* , *SOD-1*) between: a) the second generation and both the control and third generation, and b) across all groups. *PEPS-1* showed significant differences across all groups and between the second and third generation. Genotype frequencies at all loci in all groups conformed to Hardy-

Table 5.3: Allele frequencies and numbers of individuals (*n*).

Locus	Allele	Control	2nd gen.	3rd gen.
AAT	1100	0.035	0.000	0.010
	800	0.356	0.281	0.479
	100	0.584	0.701	0.510
	-700	0.025	0.018	0.000
	n	101	112	97
ALD	100	1.000	1.000	1.000
	n	99	88	97
ESTD-1	120	0.006	0.000	0.005
	100	0.575	0.653	0.582
	55	0.420	0.347	0.413
	n	87	111	92
ESTD-2	120	0.000	0.000	0.016
	100	0.969	0.820	0.964
	80	0.031	0.180	0.021
	n	98	111	96
G3PDH	100	0.994	1.000	1.000
	80	0.006	0.000	0.000
	n	80	99	98
IDH	100	1.000	1.000	1.000
	n	102	112	95
MDH	110	0.080	0.188	0.106
	100	0.915	0.812	0.889
	80	0.005	0.000	0.006
	n	100	109	90
6PGDH	165	0.005	0.000	0.000
	130	0.005	0.091	0.005
	100	0.985	0.909	0.990
	80	0.005	0.000	0.005
	n	102	111	99
PGI	125	0.006	0.000	0.035
	100	0.289	0.256	0.260
	80	0.250	0.356	0.260
	70	0.278	0.106	0.330
	65	0.139	0.281	0.115
	50	0.039	0.000	0.000
	n	90	80	100

Locus	Allele	Control	2nd gen.	3rd gen.
PEPS-1	155	0.150	0.150	0.069
	145	0.100	0.233	0.161
	100	0.600	0.568	0.592
	75	0.144	0.049	0.144
	50	0.006	0.000	0.034
	n	90	103	87
PEPS-2	110	0.029	0.014	0.028
	100	0.676	0.804	0.608
	90	0.276	0.173	0.318
	80	0.018	0.009	0.045
	n	85	107	88
PROT	150	0.000	0.000	0.006
	100	1.000	1.000	0.994
	n	100	112	79
SOD-1	140	0.005	0.000	0.000
	100	0.995	0.875	1.000
	50	0.000	0.125	0.000
	n	102	112	99
SOD-2	100	1.000	1.000	1.000
	n	103	112	112

Table 5.4: Summary of genetic diversity levels of two generations of a *S. glomerata* selective breeding line and control and genetic diversity measures published previously for this species.

	Populations	Loci (n)	Average per locus (n)	% P*	Mean Heterozygosity		Mean no. alleles (n)
					Observed H	Expected H	
This study	Control	14	95.4±2.1	71.4	0.197±0.065	0.222±0.074	2.8±0.4
	2nd generation	14	105.6±2.7	64.3	0.240±0.073	0.241±0.066	2.1±0.3
	3rd generation	14	92.8±1.5	64.3	0.230±0.079	0.227±0.075	2.6±0.6
	Mean	14	97.9±1.5	66.7	0.222±0.087	0.223±0.087	2.5±0.1
Buroker et al., 1979a	Tweed River	28	141.4	46.4	0.195±0.006	0.196±0.006	2.5
	George's River	28	158.6	46.4	0.172±0.006	0.184±0.006	2.5
	Merimbula Lake	28	159.1	46.4	0.173±0.006	0.191±0.006	2.4

*loci for which frequency of the most common allele is 0.99

Table 5.5: Summary of Chi square analyses of 11 variable loci for three populations

Locus	number of alleles	*n*	χ^2 analysis	
			χ^2	P
AAT	4	310	31.220	<0.001
ESTD-1	3	305	4.219	0.441
ESTD-2	3	290	51.047	<0.001
G3PDH	2	277	2.467	0.307
MDH	3	299	13.067	0.007
6PGDH	4	312	33.453	<0.001
PGI	6	261	62.556	<0.001
PEPS-1	5	280	37.628	<0.001
PEPS-2	4	280	21.370	0.001
PROT	2	291	2.324	0.292
SOD-1	3	313	54.585	<0.001

Weinberg equilibrium, except for the *ESTD-2* locus in the second generation ($P<0.001$), which had a large and significant excess of homozygotes (Selander index D = -0.451, $P<0.001$). Thus significant allele frequency differences were observed at seven loci between control and second generation, at eight loci between second and third generation, and at only one locus for control and third generation. This suggests that the second generation sample is responsible for most of the heterogeneity observed.

Unbiased genetic distances (Nei, 1978) over 14 loci were estimated between pairwise combinations of groups. All pairwise population distances are very small (Nei $D<0.013$). The genetic distance between the control and third generation is 0.0007, whereas the distance between these and the second generation is 0.0122.

Temporal variation (F_K) in allele frequencies was determined, and used to estimate effective population sizes (N_K) and the expected number of alleles (n_t) remaining in the populations as a result of random genetic drift (Table 5.6). The N_k of the third generation was calculated twice, once using the control group and once using the second generation as the progenitor population, to determine if the sampling variation in the second generation sample (see 5.4 Discussion) would underestimate the N_K value for the third generation.

One allele from the second generation and two alleles from the third generation were not seen in the control group (Table 5.3). Six alleles in the third generation were not present in the second generation sample. Alleles that were not in the progenitor group were rare (frequencies of less than 0.125). These rare alleles were assumed to be present in the progenitor group but not detected in the sampling regime; they were omitted from the calculations. Allele frequency variances, F_k, for variable loci, ranged from 0.007 to 0.138 for the Control - 2nd generation analysis, from 0.006 to 0.250 for 2nd generation - 3rd generation, and from 0.002 to 0.041 for Control - 3rd generation (Table 5.6). The weighted multi-locus estimates of allele frequency variance, F_K, were lower for the Control - 3rd generation comparison, 0.019, than for those including the second generation: 0.059 (Control - 2nd); 0.077 (2nd generation - 3rd generation). The harmonic

Table 5.6: Estimates of allele frequency variance (F_k), multilocus estimates of temporal variance (F_K), the harmonic mean effective population sizes (N_e) between the progenitor population - derived population for control group and selective breeding line, and the effective population sizes (N_K) of the second and third generation of the selected breeding line of *S. glomerata*. n_t and n_o: numbers of alleles in the derived stocks expected (given N_K) and observed, respectively (ignoring alleles present in the derived but not in the progenitor stocks).

	F_k		
Locus	**Control - 2nd**	**2nd - 3rd**	**Control - 3rd**
AAT	0.037	0.219	0.124
ESTD-1	0.018	0.030	0.001
ESTD-2	0.018	0.307	0.036
G3PDH	0.012	-	0.012
MDH	0.109	0.065	0.008
6PGDH	0.007	0.012	0.010
PGI	0.065	0.506	0.127
PEPS-1	0.053	0.249	0.128
PEPS-2	0.029	0.197	0.036
PROT	-	0.012	0.036
SOD-1	0.138	0.250	0.010
F_K	0.059	0.077	0.019
N_K (± s.e.)		7.5±2.5	
N_e (± s.e.)	41.4±14.0		383.8 ± 249
$n_t.(n_o)$	25.8 (25)	22.0 (24)	31.0 (30)

mean effective population sizes (N_e ± s.d.) over both lines of descent (control and selected generation) varied considerably: 41.4±14.0 for the second generation effective population size (Control as progenitor) to 383.8 ± 249 for the third generation (Control as progenitor). The indirect estimate of effective population size (N_K± s.d.) for the third generation was 7.5 ± 2.5 (second generation as progenitor). Numbers of alleles remaining in the selective breeding lines were generally very similar to those expected (Table 5.6), given the estimated N_k (effective population sizes).

5.4 DISCUSSION

All groups - controls and two selected generations - of Sydney rock oysters showed a high degree of genetic variability; overall variability levels (about 68% of loci polymorphic and average heterozygosities per locus around 0.22) were somewhat higher than in an earlier survey of this species that examined 28 loci (Buroker et al., 1979a) (Table 5.4). The differences may reflect real differences in variability among samples and/or the use of different samples of loci and different electrophoretic techniques. The two generations of the selected line showed no reduction in genetic variation compared with the control group. Smith et al. (1986) made the same finding when comparing introduced New Zealand wild stocks of Pacific oysters with cultured oysters from Mangokuura, Japan. English et al. (2000/Chapter 2) also found little reduction in genetic diversity when comparing hatchery and established Australian stocks with ancestral, native Japanese stocks, respectively. However, American and British hatchery stocks of Pacific oysters are reported to have lost variation for *AAT* (Hedgecock and Sly, 1990) and *PGI* (Gosling, 1982) respectively.

The reduction in mean numbers of alleles per locus in Sydney rock oyster populations from 2.8 in the controls to 2.1 in the second generation and 2.6 in the third generation, while not statistically significant, suggests that some allele loss might have occurred. The lower value for the second generation sample is probably due to sampling variation alone. However, any loss of alleles could be examined further using hypervariable loci such as

microsatellites; such loci typically have far higher numbers of alleles per locus and would be more powerful markers of genetic variation than allozymes (Wright and Bentzen, 1994; Reilly et al., 1999).

Almost all genotype frequencies showed good agreement with Hardy-Weinberg expectations – the only exception was a significant heterozygote deficiency at *ESTD-2* in the second generation. Buroker et al. (1979 a) observed mean heterozygosities less than Hardy-Weinberg expectations, but these were not significant for two of their three sites (including Georges River, a site contributing to the control group in this study). The conformation to Hardy-Weinberg expectations of almost all genotype frequencies observed in the present study (Table 5.4) differ from studies of other oyster species such as *Crassostrea gigas* (Buroker et al., 1975; Buroker et al., 1979 a & b; Fujio, 1979; Gosling, 1982; Smith et al., 1986; Moraga et al., 1989; Deupree, 1993, English et al., 2000) and *C. virginica* (Singh and Zouros, 1978; Zouros et al., 1980), where heterozygosity deficits have been widely observed.

The second generation sample is responsible for most of the (limited) heterogeneity among groups. It was the only group to show a deviation from Hardy-Weinberg equilibrium for genotype frequencies, albeit for just one locus. It appears to have lost six alleles that were present in both the third generation and control samples. As second generation oysters were used as broodstock for the third generation, these six alleles must have been present in the second generation, although absent from the sample. The oysters collected for the second generation sample could not have been representative of the true genetic status of this line, and this sampling error has had a significant influence on estimates of gene diversity. While important, this effect is not very substantial: all pairwise Nei's (1978) genetic distances between groups are <0.013.

Effective population numbers (N_K) can be estimated from temporal variance in allele frequencies (Pollak, 1983; Waples, 1989; Hedgecock and Sly, 1990; Hedgecock et al., 1992). The basic rationale is that the smaller the N_K, the larger the allele frequency

variance between generations. The second generation had six fewer alleles than the third generation, and may therefore underestimate the N_K value for the third generation. Also, given that the third generation appeared more similar genetically to the control group in other calculations of genetic variability, the N_K of third generation was also calculated using the control group as the progenitor population. Comparing these results with the actual numbers of parents used (I. Smith, personal communication), the mean effective population number (N_e) for the second generation, 41.4±14.0, is higher than the maximum possible number of first-generation oysters contributing to the second generation, 22. For the third generation, the estimated N_K of 7.5 ± 2.5 is substantially lower than the maximum possible number of 89 second-generation oysters contributing to the third generation. However, the estimated N_K of 383.8±249 for the third generation, using the controls as the progenitors, is substantially higher than the previous estimate, and higher than maximum possible number of immediate parents of 89. The control sample was collected contemporaneously with the second generation sample, so has undergone one less generation than the third generation sample, and this effect may be inflating the N_e.

This high N_e, together with the high F_K values observed between the second generation oysters and the other populations, suggests that the sampling error in second generation oysters is underestimating the N_K of the third generation when using the second generation sample as progenitors. Estimated effective population sizes larger than the number of actual parents used have been observed in a hatchery stock of hard clams (*Mercenaria mercenaria*) and four selected lines of pearl oysters (*Pinctada martensii*) (Hedgecock et al., 1992). On the other hand, Hedgecock and Sly (1990), found effective population sizes of two United States farmed stocks of Pacific oysters to be 40.6±13.9 (Willapa Bay) and 8.9±2.2 (Humboldt Bay), the latter stock having become fixed for an otherwise rare allele at one locus, even though much larger numbers of broodstock are routinely used by industry.

Despite the difference between actual and estimated broodstock numbers, the expected numbers of alleles of the second and third generations of the selected breeding line were

very close to the observed numbers in all cases, suggesting that random genetic drift (sampling variation) alone was the cause of allelic variation between the groups. This was also true for two United States farmed stocks (Hedgecock and Sly, 1990) of Pacific oysters, a hard clam hatchery stock and four selected lines of pearl oysters (Hedgecock et al., 1992).

The results of this allozyme survey indicate that the selective breeding for increased whole weight has not substantially eroded levels of genetic variation. There are high levels of genetic variation present in the control group and in the two generations of the selected breeding line. The limited, but statistically significant, heterogeneity between the second generation and other samples appears to be due to a sampling artifact; it is likely to be biologically unimportant. The type of selective breeding program used (mass selection) aided the retention of genetic diversity across the generations sampled. By using oysters from four different areas and ensuring large numbers of oysters for spawning helped to achieve the levels of genetic diversity observed. . Not all of the oysters collected for spawning actually spawned. But separating spawning oysters and by mixing equal amounts of each of the sperm and eggs, ensured that any given oysters sperm or eggs could not dominate the total amounts present, thereby could not homogenize the levels of genetic variability of the subsequent generation.

Chapter 6: General Discussion

The effects of domestication and selection on levels of genetic diversity have been documented in fish, and to a lesser extent, shellfish (see Chapter 1). One of the major risks that domestication and/or selection entails is the erosion of genetic diversity for commercially important traits (Tave, 1993). Most of the commercially important oyster industries and perhaps even aquaculture industries worldwide, are either introduced species or native species suffering a decline due to overfishing and/or disease (Chew, 1990). By investigating the two major commercially important edible oyster species in Australia, the opportunities existed to examine genetic diversity levels in a species which had undergone domestication (in the case of the *C. gigas* industry) and/or intentional selection (*S. glomerata*). Each species offers potential opportunities to examine different aspects of genetic diversity than the other species. Reports in the literature cite loss of alleles as a more definitive marker of loss of genetic diversity than average heterozygosity (Allendorf and Ryman, 1987; Waples, 1989; Hedgecock and Sly, 1990; Hedgecock et al., 1992). By knowing the number of parents used for the various generations in this study enables the examination of potential loss of alleles in selectively-bred *S. glomerata*. Whereas the analysis of the *C. gigas* samples allows the examination of the effects of introduction, naturalisation and domestication (unintentional selection) on the levels of genetic diversity. This study used with markers such as allozymes, mtDNA and microsatellites to determine the levels of genetic diversity.

6.1 Genetic variation in nine populations of *Crassostrea gigas*

6.1.1. Allozyme and microsatellite analysis

As mitochondrial DNA analysis proved to be unsuccessful in this study, revealing minimal levels of genetic variation, the results from the allozyme and microsatellite analysis will be compared and discussed.

Using 17 allozyme loci and four microsatellite loci, three hatchery and four naturalised populations of *Crassostrea gigas* (Thunberg) in Australia were compared with one another and with two endemic Japanese populations (see Chapters 2 and 4 respectively). Sample sizes varied for the two techniques, with 100 oysters from each population sampled for allozyme analysis and only 50 sampled from each population for the microsatellite analysis. Both techniques showed moderate to high genetic variability in all populations and some minor loss in the number of alleles in the hatchery populations compared to the endemic and naturalised populations.

The levels of genetic variability differed slightly depending on the technique – with microsatellite analysis showing higher levels than allozyme analysis, except for mean observed heterozygosities (microsatellite vs allozyme: 0.163: 0.267 (naturalised) through 0.178: 0.291 (Japan) to 0.222:0.285 (hatcheries)). The percentage of polymorphic loci was higher for microsatellites (an average of 83.3%:70.6% (hatcheries) through 87.5%:73.5% (naturalised) to 100%:73.5% (Japan)), as was the mean numbers of alleles per locus (ranging from 4.3:3.0

(hatcheries) through 4.5:3.3 (naturalised) to 5.5:3.5 (Japan)). But the overall trends in the levels of genetic variability were the same, regardless of technique, for the percentage of polymorphic loci and mean number of alleles (lowest to highest: hatcheries, naturalised, Japan). The trend in the mean observed heterozygosities was slightly different depending on the technique used (allozymes, lowest to highest: naturalised, hatcheries, Japan; microsatellites: naturalised, Japan, hatcheries). Other studies using allozyme and microsatellite analysis of hatchery and wild/ancestral fish populations have observed greater heterogeneity levels using microsatellites than allozymes (Tessier et al, 1995; Estoup et al., 1998; Reilly et al., 1999). However, Naciri et al. (1995) observed similar expected heterozygosity levels between the microsatellite locus *Oed.B0* and allozyme locus *PGM* in *O. edulis* from Quiberon Bay. Microsatellites and allozymes were equally informative in terms of resolution of stock structure in the Pacific herring (O'Connell et al., 1996b) and prawns (Brooker et al., 1999).

Allele-frequency differences among populations in this study were minor, although sometimes statistically significant: Allozyme analysis showed that only about 1% of the allele frequency variation could be attributed to among-population differences, whereas this value was 4% when determined by microsatellite analysis. Presumably, this difference is due to the higher resolution of microsatellites than allozymes due to the higher number of alleles (O'Connell and Wright, 1997; Hauser and Ward). However, the value of allele frequency variation is still low, compared to the 14-34% allele frequency variation observed between populations of Atlantic salmon (Tessier et al., 1995).

Both allozyme and microsatellite analyses indicated minor loss of alleles in the hatchery groups, compared to the naturalised and endemic groups. Microsatellite analysis has indicated greater loss of alleles than allozymes in Tasmanian Atlantic salmon, compared to ancestral Canadian stocks, due to the greater number of alleles available from microsatellite analysis (Reilly et al., 1999). Tessier et al. (1995) also found losses or decreases in the frequency of less common alleles and lower diversity in hatchery fish compared to natural populations in Lake St Jean, Quebec. Loss of alleles contrasts with the findings of Estoup et al. (1998), that the samples of brown trout from the Vosges hatchery had a greater number of alleles, including many private alleles than the natural populations surveyed from the Moselle and Ill drainages. Loss of variation observed by allozyme analysis has been seen previously in *C. gigas* : American hatchery stocks of Pacific oysters are reported to have lost variation for *AAT* (Hedgecock and Sly, 1990) and British stocks to have lost variation for *PGI* (Gosling, 1982).

Allozyme analysis in the present study demonstrated that most loci and populations showed good fits to Hardy-Weinberg expectations; the few significant exceptions were heterozygote deficiencies; this contrasts with previous allozyme studies in this species, where much higher levels of heterozygote deficiencies have been observed (see Chapter 2,Table 2.6; Buroker et al., 1975; Buroker et al., 1979; Gosling, 1981/82; Smith et al., 1986; Moraga et al., 1989; Hedgecock and Sly, 1990; Deupree, 1993) — differences among the various studies may reflect real differences in variability among samples or the use of different samples of

loci and different electrophoretic techniques. Similar heterozygosity deficits using microsatellite analysis have been observed in Atlantic salmon (Reilly et al., 1999) and have been attributed to null alleles. Although null alleles have been previously reported for the loci used in this study (*cmrCg61*: Boaprasertkul, 1998), null alleles do not appear to completely explain the heterozygote deficiencies observed in this study, based on analysis by the NullTest program (W. Amos, pers. comm.). The frequency of the proposed null alleles seems too high. At that frequency, more null homozygotes would be expected and more samples should not amplified than were actually observed. This phenomenon has also been observed in the French populations of *C. gigas* (A. Huvet, pers. comm.). Allozyme study of the same populations (see Chapter 2) also demonstrated heterozygote deficiencies, but at a lower level than the microsatellite study (Chapter 4) and also lower than previously reported for *C. gigas* allozyme studies. Previously reported higher levels of heterozygote deficiencies have been attributed to a combination of gel scoring errors, selection, inbreeding and/or the Wahlund effect (see Foltz, 1986; Lavie and Nevo, 1986; Gaffney et al., 1990; Borsa et al., 1991; Gardner, 1992; Fairbrother and Beaumont, 1993; Ríos et al., 1996). Although gel scoring errors cannot be completely ruled out, it does not seem likely that selection, inbreeding or the Wahlund effect is responsible for the heterozygote deficiencies in this study. Heterozygote excesses, not deficiencies, have been shown to occur as a result of zygotic selection and co-segregation of microsatellite markers with fitness-associated genes in *O. edulis* (Naciri et al., 1995; Bierne et al., 1998, respectively), so it seems that these factors are not the likely explanation of heterozygote deficiencies in this study. A conclusive explanation for the low

levels of heterozygote deficiencies observed cannot be made on the basis of this study, but as combination of sampling error and null alleles seems the most likely explanation. The low levels of heterozygote deficiencies seen in the *C. gigas* allozyme study (Chapter 2/ English et al., 2000) are similar to those observed in the *S. glomerata* selective breeding line study (Chapter 5/ English et al., 2001) which were attributable to sampling error.

Hence, differences in numbers of alleles per locus, percentage of loci polymorphic, or heterozygosity levels between the Tasmanian hatchery populations, the Tasmanian and New South Wales naturalised populations, and the Japanese endemic populations were very small or non-existent. Thus the Australian hatchery stocks showed very little reduction in genetic variation compared with the naturalised or Japanese stocks. Smith et al. (1986) made the same finding when comparing introduced New Zealand wild stocks with cultured oysters from Mangokuura, Japan using allozyme analysis. This contrasts with previous microsatellite studies of brown trout (Estoup et al., 1998) and Atlantic salmon (Tessier et al., 1995; Reilly et al., 1999) where hatchery stocks showed higher or lower levels of mean genetic diversity and mean number of alleles compared to wild/ancestral stocks, respectively.

Based on the minor loss of genetic diversity in the Australian *C.gigas* hatchery stocks and established populations, it appears that introduction and subsequent domestication of *C. gigas* have retained most of the genetic variation present in the Japanese populations – although there is some minor loss of alleles in the

hatchery stock, evident by allozyme analysis and to a greater extent with microsatellite analysis.

6.1.2. PCR-based mitochondrial DNA RFLP analysis

Given the disparity between results of allozyme (Buroker et al., 1975) and mtDNA (Brown and Paynter, 1991; Reeb and Avise, 1990) studies of population structure observed in *C. virginica*, the use of a mtDNA RFLP analysis technique was desired to give further insight into the population genetics of *C. gigas*. Very little variation was found using mitochondrial DNA (mtDNA) RFLP analysis in one Tasmanian hatchery and one Japanese endemic population (see Chapter 3). This contrasts with another mtDNA study in *C. gigas* in British Columbia using RFLP analysis of whole mtDNA, which showed substantial genetic variation (Boom et al, 1994). RFLP analysis of the entire mitochondrial DNA molecule by Boom et al. (1994) showed that there was a low level of divergence between populations but substantial genetic variance within populations. Native and inbred *C. virginica* were examined and the common native haplotype was found not to be present in the inbred oysters, whereas six out of the seven inbred haplotypes were not present in the native oysters (Brown and Paynter, 1991). These previous studies indicate that there is variation present in the mtDNA molecule, but that the region used in this study may too conserved to observe this.

The lack of variability observed in the PCR-based RFLP analysis of the 1442 bp mtDNA fragment developed in this study, despite previously high levels of genetic variability observed by allozyme and microsatellite techniques (see chapters Two and Four, respectively), meant that this technique was unlikely to yield further information on inbreeding and genetic diversity of Tasmanian stocks. With the technical limitations and lack of knowledge of the oyster mtDNA genome, the whole mtDNA restriction digest method (Boom et al., 1994) and SSCP analysis (Li and Hedgecock, 1998) appear more informative than PCR-based mtDNA RFLP analysis techniques in *C. gigas* as this time. Time constraints prevented their application to the present study. The identification of the proximity of the 16srRNA, and COIII genes observed in this study gives further insight into the mtDNA gene order of *C. gigas*. Together with previous findings of the mtDNA RE site map (Oohara and Mori, 1989), the location of the cytochrome b gene (Li and Hedgecock, 1998), this study demonstrates a block of COIII, 16srRNA and cytochrome b mtDNA genes — a combination unique to bivalves.

6.1.3. Comparison of the 3 techniques

In terms of choice of marker, when considering the time and financial aspects of marker analysis, allozyme would have to be the first choice. Microsatellite markers, though costly in financial and temporal terms to develop, do have the advantages of deployment in selective breeding programs, quantitative trait loci analyses and not requiring the sacrifice of the animal being investigated. At the time of the

microsatellite study, no literature existed on microsatellite studies in *C. gigas*. In fact, the only oyster microsatellite study that existed was in *Ostrea edulis* (Naciri et al., 1997). Since this study was completed, *C. gigas* microsatellites have become available (Margoulis et al., 1998; McGoldrick et al., 2000). Due to the time and financial constraints, theses microsatellite markers could not be applied to this study., With the development and publication of more microsatellite markers in oysters and especially *C. gigas*, microsatellite markers may become as widely used in these species as they are in other fisheries and aquaculture species (see Wright and Bentzen, 1994, O'Connell and Wright, 1997, Hauser and Ward, 1998).

Obviously, PCR based RFLP analysis of mtDNA will not be as popular a technique as it is in fish populations genetics until the gene order of the *C. gigas* and/or bivalve mtDNA is discovered. Although this study was unable to develop PCR primers of a variable mtDNA region suitable for population genetics analysis, the discovery of the 16S/COIII/cyt b block of genes has added to the (albeit, scant) knowledge of the mtDNA gene order in *C. gigas* and hence bivalves.

Both mtDNA and microsatellite techniques have the potential to supercede allozyme analysis in C.gigas and oysters to the same extent as that in other aquaculture species (see Wright and Bentzen, 1994, O'Connell and Wright, 1997, Hauser and Ward, 1998). However, more research is required in the case of microsatellites and substantially more so for PCR based RFLP mtDNA analysis in terms of basic knowledge of the *C. gigas* (and bivalve) mtDNA gene order. This is one of the first studies to increase the amount of knowledge available for these techniques.

6.2. The effect of selective breeding on genetic variation in Saccostrea glomerata

Whereas the Pacific oyster study examined the effect on genetic diversity by naturalisation, domestication and possibly unintentional selection of an introduced oyster species, the examination of the *S. glomerata* selective breeding line gave an insight into the effects of intentional selection on genetic variation in a native species. As records of the number of parents spawned were available, the effective population sizes (N_K) were estimated from temporal variance in allele frequencies (Pollak, 1983; Waples, 1989; Hedgecock and Sly, 1990; Hedgecock et al., 1992). These calculations allowed the determination of whether any allele loss was due to random genetic drift alone. This calculation was not possible in the *C. gigas* study as the records of number of parents was not available and/or most importantly, assumptions essential for this calculation were violated.

Genetic variability in second and third generations of a selective breeding line of Sydney rock oysters (*Saccostrea glomerata*) was compared with one another and a control group, using 14 allozyme loci. All groups showed a similar and high degree of genetic variability. Overall, the percentage of polymorphic loci ($P< 0.99$ criterion) was 66.7%, the mean observed (and expected) heterozygosity (±s.d.) was 0.222±0.087 (0.223±0.087), and the mean number of alleles per locus (±s.d.) was 2.5±0.6. While selection for increased whole weight does not appear to have

significantly diminished genetic variation of the two generations of the selected breeding line relative to the controls, there were some significant allele frequency differences among groups (n = 100 per group). Unexpectedly, the allele frequencies from the third generation sample was more similar to those of the control sample than the second generation sample, due to sampling error in the latter samples. This was reflected in estimated effective population numbers of 41.4±14.0 for the second generation line and 383.8±249 for the third generation lines, when compared to the control group, and 7.5±2 for the third generation when compared to the second generation. Numbers of alleles remaining in all cases were similar to numbers expected if allele loss occurred through genetic drift or sampling variation only.

The results of this allozyme survey indicate that the selective breeding for faster growth rate has not substantially eroded levels of genetic variation. There are high levels of genetic variation present in the control group and in the two generations of the selected breeding line. The limited, but statistically significant, heterogeneity between the second generation and other samples appears to be due to a sampling artefact; it is likely to be biologically unimportant.

The levels of genetic diversity in selective breeding program of *S. glomerata* observed in this study not only has implications for the success of the program but as an example of retention of the majority of genetic diversity in this native species. Only one other example of the assessment of the genetic diversity using allozymes in this species exists (Buroker et al., 1979a). The effects of selective breeding on the level of genetic diversity in this species has not been examined prior to this study.

The implications of this study are that enough genetic diversity is available to find a particular trait of interest. This study also serves as an example of a commercially-important native species of oyster that has not lost diversity as a result of intentional selection.

6.3 Conclusions

- *Acceptance of the null hypothesis – no major loss in genetic diversity resulting from introduction, subsequent domestication and possibly unintentional selection of C. gigas.*

Allozyme (17 loci, n = 100 per population) and microsatellite (4 loci, n = 50 per population) analyses of 2 Japanese endemic, 3 Tasmanian hatchery and 4 Australian naturalised populations of *C. gigas* found no major loss of genetic variation, although minor loss of alleles was observed. Six microsatellite loci were developed as part of this study. This is the first study to examine the genetic diversity in the Australian populations compared to Japanese populations of C. gigas and one of the first studies to use allozyme and microsatellite analyses on the same populations of *C. gigas*.

- *Acceptance of the null hypothesis –no major loss in genetic diversity in a S. glomerata selective breeding line.*

Allozyme (14 loci, n = 100 per population) analysis of control, second and third generations of a *S. glomerata* selective breeding line, found no major loss of genetic variation, where the minor loss of alleles was shown to be attributable to

random genetic drift or sampling error. This is the first study to examine the genetic diversity in a selective breeding line in this species, and only the second ever allozyme survey of this species.

- *Demonstration of a block of the 16srRNA, COIII and cytochrome b genes in the C. gigas mtDNA gene order*

The PCR-based mtDNA RFLP analysis in this study revealed very little mtDNA variation. Sequence analysis of the mtDNA fragment revealed the presence of the 16srRNA and cytochrome oxidase III (COIII) genes, both highly conserved genes, hence the little mtDNA variation seen. Together with the *C. gigas* restriction map (Oohara and Mori, 1989) and the location of the cytochrome b (cyt b) gene (Li and Hedgecock, 1998), the proximity of the 16srRNA/COIII unit to cyt b was shown, although the exact orientation of the 16srRNA/COIII (i.e. 16srRNA/COIII/cyt b or COIII/16srRNA/cyt b) cannot be determined on the basis of this study. The block of the 16srRNA, COIII and cyt b genes in the *C. gigas* mtDNA gene order appears to be unique to bivalves.

In summary, this study has not only substantially broadened the base of knowledge in the two oysters species investigated, but also shown that intentional (in the form of selective breeding) and unintentional (in the form of introduction and subsequent domestication) selection of aquaculture species need not result in major allele loss. Without major allele loss an aquaculture species then has the potential for successful selective breeding for desired traits, as the genetic diversity and hence likelihood of finding particular gene(s) of interest have not been diminished significantly, and has

every chance of success. That the two commercially important edible oyster species (introduced and native) in this study have not shown a large magnitude of genetic diversity loss despite either introduction, naturalisation and domestication, or selective breeding serves as a encouraging example to the oyster industries and researchers involved as well as those in other countries and indeed other aquaculture species.

Chapter 7: References

Ahmed, M., 1975. Speciation in living oysters. Adv. Mar. Biol., 13: 375-397.

Allendorf, F.W., Ryman, N., 1987. Genetic management of hatchery stocks. In: N. Ryman and F. Utter (Eds), Population Genetics & Fishery Management. University of Washington Press, pp 141-159.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215: 403-410

Anderson, T.J., Adlard, R.D., 1994. Nucleotide sequence of a rDNA internal transcribed spacer supports synonymy of *Saccostrea commercialis* and *S. glomerata*. J. Molluscan Stud., 60: 196-197.

Asakawa, S., Himeno, H., Miura, K., Watanabe, K., 1995. Nucleotide sequence and gene organization of the starfish *Asterina pectinifera* mitochondrial genome. Genetics, 140: 1047-1060.

Aulstad, D, Kitelsen, A., 1971. Abnormal body curvatures of rainbow trout (*Salmo gairdneri*) inbred fry. J. Fish. Res. Board Can., 28: 1918-1920.

Bagshaw, J.C., Buckholdt, M.A., 1997. A novel satellite/microsatellite combination in the genome of the marine shrimp, *Penaeus vannemi.* Gene, 184: 211-214.

Banks, M.A., Hedgecock, D., Waters, C., 1993. Discrimination between closely related Pacific oyster species (*Crassostrea*) via mitochondrial DNA sequences coding for large subunit rRNA. Molec. Mar. Biol. Biotech., 2: 129-136

Banks, M.A., McGoldrick, D.J., Borgeson, W., Hedgecock, D., 1994. Gametic incompatibility and genetic divergence of Pacific and Kumamoto oysters, *Crassostrea gigas* and *Crassostrea sikamea* . Molecul. Biol., 121: 127-135.

Beachem, T.D., Dempson, J.B., 1998. Population structure of Atlantic salmon from the Conne River, Newfoundland as determined from microsatellite DNA. J. Fish Biol., 52: 665-676.

Beagley, C.T., Okimoto, R., Wolstenholme, D.R., 1998. The mitochondrial genome of the sea anemone *Metridium senile* (Cnidaria): introns, a paucity of tRNA genes, and a near-standard genetic code. Genetics, 148 : 1091-1108.

Bentzen, P., Taggart, C.T., Ruzzante, D.E., Cook, D., 1996. Microsatellite polymorphism and the population structure of Atlantic cod (*Gadus morhua*) in the northwest Atlantic. Can. J. Fish. Aquat. Sci., 53: 2706-2721.

Bierne, N., Launey, S., Naciri-Graven, Y., Bonhomme, F, 1998. Early effect of inbreeding as revealed by microsatellite analyses on *Ostrea edulis* larvae. Genetics, 148: 1893-1906.

Blake, S.G., Blake, N.J., Oesterling, M.J., Graves, J.E., 1997. Genetic divergence and loss of diversity in two cultured populations of the bay scallop, *Argopecten irradians* (Lamarck, 1819). J. Shellfish Res., 16: 55-58.

Boaprasertkul, P., 1998. DNA Microsatellite analysis of Mendelian inheritence in Pacific oyster families. Unpublished Masters thesis. University of Tasmania, Launceston.

Bondari, K., 1984. Efficiency of male reproduction in channel catfish. Aquaculture, 35: 79-82.

Bondari, K., Dunham, R.A., 1987. Effects of inbreeding on economic traits of channel catfish. Theor. Appl. Genet., 74: 1-9

Boom, J.D.G., Boulding, E.G., Beckenbach, A.T., 1994. Mitochondrial DNA variation in introduced populations of Pacific oyster, *Crassostrea gigas*, in British Columbia. Can. J. Fish. Aquat. Sci., 51: 1608-1614.

Boore, J.L., Brown, W.M., 1994. Mitochondrial genomes and the phylogeny of mollusks. Nautilus, Suppl. 2: 61-78.

Borsa, P., Zainuri, M., Delay, B., 1991. Heterozygote deficiency and population structure in the bivalve *Ruditapes decussatus*. Heredity, 66: 1-8.

Bridges, W.R., 1973. Rainbow trout breeding projects. Progress in Sport Fishery Research, 1971. Div. Fish. Res. Bur. Sport Fish. Wild Res. Pub., 121: 60-63.

Brooker, A.L., Benzie, J.A.H., Blair, D., Versini, J.J., 2000. Population structure of the giant tiger prawn *Penaeus monodon* in Australian waters, determined using microsatellite markers. Mar. Biol., in press.

Broughton, R.E., Dowling, T.E., 1994. Length variation in mitochondrial DNA of the minnow *Cyprinella spiloptera*. Genetics, 138: 179-190.

Brown, B.L., Paynter, K.T., 1991. Mitochondrial DNA analysis of native and selectively inbred Chesapeake Bay oysters *Crassostrea virginica*. Mar. Biol., 110: 343-352.

Brown, D., van Landeghem, K., Schuele, M., 1997. Australian Aquaculture: Industry Profiles for Selected Species. ABARE Research Report 97.3, ABARE, Canberra.

Buroker, N.E., Hershberger, W.K., Chew, K.K., 1975. Genetic variation in the Pacific oyster. J. Fish. Res. Board Can., 32: 2471-2477.

Buroker, N.E., Hershberger, W.K., Chew, K.K., 1979a. Population genetics of the family Ostreidae. I Intraspecific studies of *Crassostrea gigas* and *Saccostrea commercialis*. Mar. Biol., 54: 157-169.

Buroker, N.E., Hershberger, W.K., Chew, K.K., 1979b. Population genetics of the Family Ostreidae. II Interspecific studies of *Crassostrea gigas* and *Saccostrea commercialis*. Mar. Biol., 54: 171-184.

Buroker, N.E., Chanley, P., Cranfield, H.J., Dinamani, P., 1983. Systematic status of two oyster populations of the genus *Tiostrea* from New Zealand and Chile. Mar. Bio., 77: 191-200.

Buroker, N.E., Brown, J.R., Gilbert, T.A. O'Hara, P.J., Beckenbach, A.T., Thomas, WlK., Smith, M.J., 1990. Length heteroplasmy of sturgeon mitochondrial DNA: an illegitimate elongation model. Genetics, 124: 157-163.

Burton, R.S., 1996. Molecular tools in marine ecology. J. Expt. Mar. Biol. Ecol., 85-101.

Butler, A., Cross, T.F., 1996. Genetic differences between successive year classes of two strains of reared rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquaculture Res., 27: 643-649.

Calaprice, J.R., 1969. Production and genetic factors in managed salmonid populations. In: T.G. Northcote (Ed.) Symposium on Salmon and Trout in Streams, Institute of Fisheries, The University of British Columbia, Vancouver.

Ch'ang, M.T., 1971. Influence of inbreeding on tilapia (*Tilapia mossambica* Peters). Sov. Genet., 7: 1277-1282.

Chew, K.K., 1990. Global bivalve shellfish introductions. World Aquaculture 21: 9-22.

Chow, S., Inoue, S., 1993. Intra- and interspecific restriction fragment length polymorphisms in mitochondrial genes of *Thunnus* tuna species. Bull. Nat. Res. Inst. Far. Seas Fish., 30: 207-225.

Chow, S., Clarke, M.E., Walsh, P.J., 1993. PCR-RFLP analysis on thirteen western Atlantic snappers (subfamily Lutjaninae): a simple method for species and stock identification. Fish. Bull. 91: 619-627.

Chow, S., Ushiama, H., 1995. Global population structure of albacore (*Thunnus alalunga*) inferred by RFLP analysis of the mitochondrial ATPase gene. Mar. Biol. 123: 39-45.

Clabby, C., Goswami, U., Flavin, F., Wilkins, N.P., Houghton, J.A., Powell, R., 1996. Cloning, characterization and chromosomal location of a satellite DNA from the Pacific oyster, *Crassostrea gigas*. Gene, 168: 205-209.

Cooper, E.L., 1961. Growth of wild and hatchery strains of brook trout. Trans. Am. Fish. Soc., 90: 424-438.

Cronin, M.A., Spearman, W.J., Wilmot, R.L., Patton, J.C., Bickham, J.W., 1993. Mitochondrial DNA variation in chinook (*Oncorhyncus tshawytscha*) and chum salmon (*O. keta*) detected by restriction enzyme analysis of polymerase chain reaction (PCR) products. Can J. Fish. Aquat. Sci., 50: 708-715.

Crow, J.F., Denniston, C., 1988. Inbreeding and variance effective population numbers. Evolution, 42: 482-495

Danzmann, R.G., Ferguson, M.M., 1995. Heterogeneity in the body size of Ontario cultured rainbow trout with different mitochondrial DNA haplotypes. Aquaculture, 137: 231-244.

Davis, R.H., 1976. Evaluation of growth of inbred lines and their F_1 hybrids in brook trout, *Salmo gairdneri*. Unpublished Doctoral thesis. Pennsylvania State University, University Park.

Deupree, Jr., R.H., 1993. Genetic characterization of deep-cupped Pacific oysters (*Crassostrea gigas*) from Tasmania, Australia. Unpublished Masters thesis. University of Washington, Washington.

Doyle, R.W., 1993. An approach to the quantitative analysis of domestication selection in aquaculture. In: Tave, D., 1993. Genetics for fish hatchery managers. 2nd. Ed. Van Nostrand Reinhold, New York, p 154.

English, L.J., Maguire, G.B., Ward, R.D., 2000. Genetic variation of wild and hatchery populations of the Pacific oyster, *Crassostrea gigas* (Thunberg), in Australia. Aquaculture, 187: 283-298.

English, L.J., Maguire, G.B., Ward, R.D., 2000. Genetic variation of wild and hatchery populations of the Pacific oyster, *Crassostrea gigas* (Thunberg), in Australia. Aquaculture, 187: 283-298.

English, L.J., Nell, J.A., Maguire, G.B., Ward, R.D., 2001. No loss of allozyme variation in three generations of selection for whole weight in Sydney rock oysters (*Saccostrea glomerata*). Aquaculture, 193: 213-225.

Estoup, A., Rousset, F., Michalakis, Y., Cornuet, J.M., Adriamanga, M., guyomard, R., 1998. Comparative analysis of microsatellite and allozyme markers: a case study investigating microgeographic differentiation in brown trout (*Salmo trutta*).

Evans, B.S., White, R.W.G., Ward, R.D., 1998. Genetic identification of asteroid larvae from Tasmania, Australia, by PCR-RFLP. Molec. Ecol., 7: 1077-1082.

Fairbrother, J.E., Beaumont, A.R., 1993. Heterozygote deficiencies in a cohort of newly settled *Mytilus edulis* spat. J. Mar. Biol. Ass. U.K., 73: 647-653.

FAO, 1998. The state of world fisheries and aquaculture 1998. Food and agriculture organization of the United Nations, Rome.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molec. Mar. Biol. Biotech., 3: 294-299.

Foltz, D.W., 1986. Null alleles as possible causes of heterozygote deficiencies in the oyster *Crassostrea virginica* and other bivalves. Evolution, 40: 869-870.

Fontaine, P., Dodson, J.J., Bernatchez, L., Slettan, A., 1997. A genetic test of metapopulation structure in Atlantic salmon (*Salmo salar*) using microsatellites. Can. J. Fish. Aquat. Sci., 54: 2434-2442.

Fujio, Y., 1979. Enzyme polymorphism and population structure of the Pacific oyster, *Crassostrea gigas*. Tohoku J. Agric. Res., 30: 32-42.

Gaffney, P.M., Scott, T.M., Koehn, R.K., Diehl, W.J., 1990. Relationships of heterozygosity, growth rate and heterozygote deficiencies in the Coot Clam, *Mulinia lateralis*. Genetics, 124: 687-699.

Gardner, J.P.A., 1992. Null alleles and heterozygote deficiencies among mussels (*Mytilus edulis* and *M. galloprovincialis*) of two sympatric populations. Malacologia, 34: 99-106.

Garcia de Leon, F.J., Canonne, M., Quillet, E., Bonhomme, F., Chatain, B., 1998. The application of microsatellite markers to breeding programmes in the sea bass, *Dicentrarchus labrax*. Aquaculture, 159: 303-316.

Geller, J.B., Carlton, J.T., Powers, D.A., 1994. PCR-based detection of mtDNA haplotypes of native and invading mussels on the northeastern Pacific coast: latitudinal patter of invasion. Mar. Biol., 119: 243-249.

Gjerde, B., 1988. Complete diallele cross between six inbred gropus of rainbow trout, *Salmo gairdneri.* Aquaculture, 75: 71-87.

Gjerde, B., Gunnes, K., Gjedrem, T., 1983. Effect of inbreeding on survival and growth in rainbow trout. Aquaculture, 34: 327-332.

Gosling, E.M., 1982. Genetic variability in hatchery-produced Pacific oysters (*Crassostrea gigas* Thunberg). Aquaculture, 26: 273-287.

Graham, M.A, (1991). Situation Analysis of the Australian Oyster Industry with Particular Reference to the Tasmanian Pacific Oyster. A Report for the Tasmanian Aquaculture Co-operative Society and the Business School, University of Tasmania. 88 pp.

Grewe, P.M., Kreuger, C.C., Aquadro, C.F., Bermingham, E., Kincaid, H.L., May, B.,1993. Mitochondrial DNA variation among lake trout (Slavelinus namaycush) strains stocked in to Lake Ontario. Can. J. Fish. Aquat. Sci., 50: 2397-2403.

Grewe, P.M., Kreuger, C.C., Aquadro, C.F., May, B., 1994. Stability of allozyme and mitochondrial DNA markers among three year-classes of lake trout propogated from Seneca Lake, New York. N. Amer. J. Fish. Management., 14: 467-474.

Grewe, P.M., Hampton, J., 1998. An assessment of bigeye (Thunnus obesus) population structure in the Pacific Ocean, based on mitochondrial DNA and DNA microsatellite analysis. CSIRO Marine Research, Hobart, Australia.

Hanahan, D., 1985. In: Glover, D. (ed.) DNA cloning, volume 1. IRL Press, London.

Hansen, M.M., Mensberg, K.D., 1996. Founder effects and genetic population structure of brown trout (*Salmo trutta*) in a Danish river system. Can. J. Fish. Aquat. Sci., 53: 2229-2237.

Hare, M.P., Avise, J.C., 1998. Population structure in the American oyster as inferred by nuclear gene genealogies. Mol. Biol. Evol., 15: 119-128.

Hauser, L., Ward, R.D., 1998. Population identification in pelagic fish: the limits of molecular markers. In: :Carvalho, G.R., (ed.), 1998. Advances in molecular ecology. IOS Press, p 191-224.

Hebert, P.D.N., Beaton, M.J., 1989. Methodologies for Allozyme Analysis Using Cellulose Acetate Electrophoresis. Helena Laboratories. Beaumont, Texas.

Hedgecock, D., Sly, F., 1990. Genetic drift and effective population sizes of hatchery-propagated stocks of the Pacific oyster, *Crassostrea gigas*. Aquaculture, 88: 21-38

Hedgecock, D., Chow, V., Waples, R.S., 1992. Effective population numbers of shellfish broodstock estimated from temporal variance in allelic frequencies. Aquaculture, 108: 215-232

Hedgecock, D., McGoldrick, D.J., Bayne, B.L., 1995. Hybrid vigor in Pacific oysters: an experimental approach using crosses among inbred lines. Aquaculture, 137: 285-298.

Hilbish, T.J., (1996). Population genetics of marine species: the interaction of natural selection and historically differentiated populations. J. Exp. Mar. Biol. Ecol., 200: 67-83.

Hoffmann, R.J., Boore, J.L., Brown, W.M., 1992. A novel mitochondrial genome organization for the blue mussel, *Mytilus edulis*. Genetics, 131: 397-412.

Holliday, J.E., Nell, J.A., 1986. Concern over Pacific oyster in Port Stephens. Aust. Fish., 159: 29-31.

Jones, M.W., Clay, D, Danzmann, R.G., 1996. Conservation genetics of brook trout (Salvelinus fontinalis): population structuring in Fundy National Park, New Brunswick, and eastern Canada. Can. J. Fish. Aquat. Sci., 2776-2791.

Kessing, B.H., Croom, H., Martin, A., McIntosh, C., McMillan, W.O., Palumbi, S., 1989. The simple fool's guide to PCR, version 1.0. Department of Zoology, Unversity of Hawaii, Honolulu.

Kincaid, H.L., 1977. Rotational line crossing: an approach to the reduction of inbreeding accumulation in trout brood stocks. In: Tave, D., 1993. Genetics for fish hatchery managers. 2nd. Ed. Van Nostrand Reinhold, New York, p 218.

Kincaid, H.L., 1976a. Effects of inbreeding on rainbow trout populations. Trans. Am. Fish. Soc., 105: 273-280.

Kincaid, H.L., 1976b. Inbreeding in rainbow trout, *Salmo gairdneri.* J. Fish. Res. Board Can., 33: 2420-2426.

Kincaid, H.L., 1983. Inbreeding in fish populations used for aquaculture. Aquaculture, 33: 215-227.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Pääbo, S., Villabanca, F.X., Wilson, A.C., 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA, 86: 6196-6200.

Kojima, S., Segawa, R., Kobayashi, T., Hashimoto, T., Fujikura, K., Hashimoto, J., Ohta, S. Phylogenetic relationships among species of *Clayptogena* (Bivalvia: Vesicomyidae) collected around Japan revealed by nucleotide sequences of mitochondrial genes. In press.

Lavie, B., Nevo, E., 1986. Genetic selection of homozygote allozyme genotypes in marine gastropods exposed to cadmium pollution. Sci. Total Environ. 57: 91-98.

Leberg, P.L., 1990. Influence of genetic variability on population growth: implications for conservation. J. Fish Biol., 37(Suppl A): 193-195.

Li, G., Hedgecock, D., 1998. Genetic heterogeneity, detected by PCR-SSCP, among samples of larval Pacific oysters (*Crassostrea gigas*) supports the hypothesis of large variance in reproductive success. Can. J. Fish. Aquat. Sci., 55: 1025-1033.

Magoulas, A., Gjetvaj, B., Terzoglou, V., Zouros, E., 1998. Three polymorphic microsatellites in the Japanese oyster, *Crassostrea gigas* (Thunberg). Animal Genetics, 29: 69-70.

McConnell, S., Hamilton, L., Morris, D., Cook, D., Paquet, D., Bentzen, P., Wright, J., 1995. Isolation of salmonid microsatellite loci and their application to the population genetics of Canadian east coast stocks of Atlantic salmon. Aquaculture, 137: 19-30.

McDonald, J.H., Verrelli, B.C., Geyer, L.B., 1996. Lack of geographic variation in anonymous nuclear polymorphisms in the American oyster, *Crassostrea virginica*. Mol. Biol. Evol., 13: 1114-1118.

McGoldrick, D.J., Hedgecock, D., English, L.J., Baoprasertkul, P., Ward, R.D., 2000. The transmission of microsatellite alleles in Australian and North American stocks of the Pacific oyster (*Crassostrea gigas*): selection and null alleles. J. Shellfish Res., 19: 779-788.

Medcof, J.C., Wolf, P.H., 1975. Spread of Pacific oyster worries NSW culturists. Aust. Fish., 34: 1-7.

Meyer, A., 1993. Evolution of mitochondrial DNA in fishes. In: Hochachka, P.W, Mommsen, T.P., (eds) The biochemistry and molecular biology of fishes. Vol.2., Elsevier Press, New York, p1-53.

Miller, L.M., Kapuscinski, A.R., 1996. Microsatellite DNA markers reveal new levels of genetic variation in northern pike. Trans. Amer. Fish., 125: 971-977.

Moav, R., Wohlfarth, G.W., 1968. Genetic improvement of yield in carp. Proceedings of the World Symposium on Warm-water Pond Fish Culture. FAO Fish. Rep. No. 44: 12-29.

Moraga, D., Osada, M., Lucas, A., Nomura, T., 1989. Génétique biochemique de populations de *Crassostrea gigas* en France (côte atlantique) et au Japon (Miyagi) (Biochemical genetics of *Crassostrea gigas* populations from France (Atlantic coastline) and from Japan (Miyagi)). Aquat. Living Resour., 2: 135-143.

Mrakovcic, M., Haley, L.E., 1979. Inbreeding depression in the zebra fish *Brachydanio rerio* (Hamilton Buchanan). J. Fish Biol., 15: 323-327.

Naciri, Y., Vigouroux, Y., Dallas, J., Desmarais, E., Delsert, C., Bonhomme, F., 1997. Identification and inheritance of $(GA/TC)_n$ and $(AC/GT)_n$ repeats in the European flat oyster, *Ostrea edulis* (L.). Molec. Mar. Biol. Biotechnol., 4: 83-89.

Nei, M., 1973. Analysis of gene diversity in subdivided populations. Proc. Nat. Acad. Sci. U.S.A., 70: 3321-3323.

Nei, M., 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics, 89: 583-590.

Nell, J.A., 1993. Farming the Sydney rock oyster (*Saccostrea commercialis*) in Australia. Rev. Fish. Sci., 1: 97-120.

Nell, J.A., Cox, E., Smith, I.R., Maguire, G.B., 1994. Studies on triploid oysters in Australia. I. The farming potential of triploid Sydney rock oysters *Saccostrea commercialis* (Iredale and Roughley). Aquaculture, 126: 243-255.

Nell, J.A., Sheridan, A.K., Smith, I.R., 1996. Progress in a Sydney rock oyster *Saccostrea commercialis* (Iredale and Roughley) breeding program. Aquaculture, 144: 295-302.

Nell, J.A., Smith, I.R., Sheridan, A.K., 1999. Third generation evaluation of Sydney rock oyster *Saccostrea commercialis* (Iredale and Roughley) breeding lines. Aquaculture, 170: 195-203.

NSW Fisheries, 1998. 1997 NSW oyster industry report In: Status of fisheries resources 1996/1997. p91. NSW Fisheries, Sydney.

O'Connell, M., Skibinski, D.O.F., Beardmore, J.A., 1996a. Allozyme and mtDNA divergence between Atlantic salmon populations in North Wales. J. Fish. Biol. 48: 1023-1026.

O'Connell, M., Dillon, M.C., Wright, J.M., 1996b. Development of microsatellite markers for genetic discrimination of Prince William Sound herring populations. In: O'Connell, M., Wright, J.M., 1997. Microsatellite DNA in fishes. Rev. Fish. Biol. Fish., 7: 331-363.

O'Connell, M., Wright, J.M., 1997. Microsatellite DNA in fishes. Rev. Fish. Biol. Fish., 7: 331-363.

Ó Foighil D., Gaffney, P.M., Hilbish, T.J., 1995. Differences in mitochondrial 16S ribosomal gene sequences allow discrimination among American [*Crassostrea virginica* (Gmelin)] and Asian [*C. gigas* (Thunberg), *C. ariakensis* Wakiya] oyster species. J. Exp. Mar. Biol. Ecol., 192: 211-220.

Okimoto, R, Macfarlane, .J.L., Clary, D.O., Wolstenholme, D.R., 1992. The mitochondrial genomes of two nematodes, *Caenorhabditis elegans* and *Ascaris suum*. Genetics, 130:471-498.

Olsen, A.M., 1994. The history of the development of the Pacific oyster, *Crassostrea gigas* (Thunberg) industry in South Australia. Trans. R. Soc. S. Aust., 118: 253-259.

Oohara, I., Mori, K., 1989. Cloning and characterization of mitochondrial DNA from the Japanese oyster, *Crassostrea gigas*. Bull. Natl. Res. Inst. Aquaculture, 16: 89-99.

Palumbi, S., Martin, S., Romano, S., McMillan, W.O., Stice, L., Grabowski, G., 1991. The simple fools guide to PCR, version 2.0. Department of Zoology, University of Hawaii, Honolulu.

Park, L.K., Brainard, M.A., Dightman, D.A., Winans, G.A., 1993. Low levels of intraspecific variation in mitochondrial DNA of chum salmon (*Oncorhychus keta*). Molec. Mar. Biol. Biotech. 2: 362-370.

Pirion, R.D., 1978. Spontaneous skeletal deformities in the Zebra Danio (*Brachydanio rerio)* bred for fish toxicity tests. J. Fish Biol., 13: 79-83.

Pollak, E., 1983. A new method for estimating the effective population size from allele frequency changes. Genetics, 104: 531-548.

Reeb, C.A., Avise, J.C., 1990. A genetic discontinuity in a continously distributed species: mitochondrial DNA in the American oyster, *Crassostrea virginica*. Genetics, 124: 397-406.

Reilly, A., Elliott, N.G., Grewe, P.M., Clabby, C., Powell, P. & Ward, R.D, 1999. Genetic differentiation between Tasmanian cultured Atlantic salmon (*Salmo salar* L.) and their ancestral Canadian population: comparison of microsatellite DNA and allozyme and mitochondrial DNA variation. Aquaculture, 173: 457-467.

Richardson, B.J., Braverstock, P.R., Adams, M., 1986. Allozyme Electrophoresis: A Handbook for Animal Systematics and Population Studies. Academic Press, Sydney, 410 pp.

Ríos, C., Canales, J., Peña, J.B., 1996. Genotype dependent spawning: evidence from a wild population of *Pecten jacobaeus* (L.) (Bivalvia: Pectinidae). J. Shellfish Res. 15: 645-651.

Roff, D.A., Bentzen, P., 1989. The statistical analysis of mitochondrial DNA polymorphisms: χ^2 and the problem of small samples. Molec. Biol. Evol., 6: 539-545.

Rogers, J.S., 1972. Measures of genetic similarity and genetic distance. In: Studies in Genetics VII. University of Texas, Austin, pp. 145-153.

Ryman, N., 1970. A genetic analysis of recapture frequencies of released young of salmon (*Salmo salar* L.). Hereditas, 65: 159-160.

Saavedra, C., 1997. Low effective population sizes in hatchery populations of the European oyster (*Ostrea edulis*): implications for the management of genetic resources. J. Shellfish Res., 16: 441-446.

Sambrook, J., Fritsch, E.F., Maniatis, T. (eds.), 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor, New York.

Shatkin, G., Shumway, S.E., Hawes, R., 1997. Considerations regarding the possible introduction of the Pacific oyster (*Crassostrea gigas*) to the Gulf of Maine: a review of global experience. J. Shellfish Res., 16: 463-477.

Shed'ko, S.V., Ginatulina, L.K., Parpura, I.Z., Ermolenko, A.V., 1996. Evolutionary and taxonomic relationships among Far-Eastern salmonid fishes inferred from mitochondrial DNA divergence. J. Fish Biol., 49: 815-829.

Sheridan, A.K., Smith, I.R., Nell, J.A., 1996. Reducing the impact of environmental variation in a growth rate improvement program for the Sydney rock oyster *Saccostrea commercialis*. Aquaculture, 143: 145-154.

Simon, C., Fratl, F., Beckenbach, A., Crespi, B., Liu, H., Flook, P., 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am., 87: 651-701.

Singh, S.M., Zouros, E.,1978. Genetic variation associated with growth rate in the American oyster (*Crassostrea virginica*). Evol., 32: 342-353.

Slettan, A., Olsaker, I., Lie, O., 1997. Segregation studies and linkage analysis of Atlantic salmon microsatellites using haploid genetics. Heredity, 78: 620-627.

Smith, P.J., Ozaki, H., Fujio, Y., 1986. No evidence for reduced genetic variation in the accidentally introduced oyster *Crassostrea gigas* in New Zealand. N.Z. J. Mar. Res., 20: 569-574

Sunden, S.L.F., Davis, S.K., 1991. Evaluation of genetic variation in a domestic population of *Panaeus vannemi* (Boone): a comparison with three natural populations. Aquaculture, 97: 131-142.

Swofford, D.L., Selander, R.B., 1989. BIOSYS-1: A Computer Program for the Analysis of Allelic Variation in Population Genetics and Biochemical Systematics (Release 1.7). Illinois Natural History Survey, Champaign, 39 pp.

Tassanakajon, A., Tiptawonnukul Al., Supungul, P., Rimphanitchayakit V., Cook, D., Jarayabhand, P., Klinbunga, S., Boonsaeng, V., 1998. Isolation and characterization of microsatellite markers in the black tiger prawn *Penaeus monodon*. Molec. Mar. Biol. Biotechnol., 5: 230-238.

Tave, D., 1993. Genetics for fish hatchery managers. 2nd. Ed. Van Nostrand Reinhold, New York.

Tessier, N., Bernatchez, L., Presa, P., Angers, B., 1995. Gene diversity analysis of mitochondrial DNA, microsatellites and allozymes in landlocked Atlantic salmon. J. Fish. Biol., 47: 156-163.

Thomson, J.M., 1952. The acclimation and growth of the Pacific Oyster (*Gryphaea gigas*) in Australia. Aust. J. Mar. Freshwater Res., 3: 64-73.

Thomson, J.M., 1959. The naturalization of the Pacific Oyster in Australia. Aust. J. Mar. Freshwater Res., 10: 144-149.

Treadwell, R., McKelvie, L., Maguire, G.B., 1992. Potential for Australian Aquaculture. (Australian Bureau of Agricultural and Resources Economics (ABARE) research report). ABARE, Canberra.

Vitic, R., Strobeck, C., 1996. Mitochondrial DNA analysis of populations of lake trout (Slavelinus namaycush) in west-central Canada: implications for stock identification. Can. J. Fish. Aquat. Sci., 53: 1038-1047

Waples, R.S., 1989. A generalized approach for estimating effective population size from temporal changes in allele frequency. Genetics, 121: 379-391.

Ward, R.D., Skibinski, D.O.F., Woodwark, M., 1992. Protein heterozygosity, protein structure and taxonomic differentiation. Evol. Biol., 26: 73-159.

Ward, R.D., Grewe, P.M., 1994. Appraisal of molecular genetic techniques in fisheries. Rev. Fish Biol. Fish., 4: 300-325.

Ward, R.D., Elliott, N.G., Innes, B.H., Smolenski, A.J., Grewe, P.M., 1997. Global population structure of yellowfin tuna, *Thunnus albacares*, inferred from allozyme and mitochondrial DNA variation. Fish. Bull., 95: 566-575.

Ward, R.D., English, L.J., McGoldrick, D.J., Maguire, G.B., Nell, J.A. and Thompson, P.A. 1999. Genetic improvement of the Pacific oyster, *Crassostrea gigas*, in Australia. *Aquaculture Research*, in press.

Winemiller, K.O., Taylor, D.H., 1982. Inbreeding depression in the convict cichlid, *Cichlasoma nigrofasciatum* (Baird and Girard). J. Fish Biol., 21: 399-402.

Wright, J.M., Bentzen, P., 1994. Microsatellites: genetic markers for the future. Rev. Fish. Biol. Fish., 4: 384-388.

Zaykin, D.V., Pudovkin, A., 1993. Two programs to estimate significance of chi-square values using pseudo-probability tests. J. Hered., 84: 152.

Zouros, E., Singh, S.M, Miles, H.E., 1980. Growth rate in oysters: an overdominant phenotype and its possible explanations. Evolution, 34: 856-867.