Development of tools for the sustainable management of genetics in polyploid Pacific oysters (*Crassostrea gigas*)

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Penny Miller

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Thesis Abstract

The commercial production of triploid Pacific oysters (*Crassostrea gigas*) has grown rapidly in recent years. There is now a push to move away from commonly used mass spawning techniques towards single pair cross selective breeding programs in an effort to improve growth and disease resistance within the triploid product. Before this can be achieved, there is a need to understand some of the fundamental genetics behind polyploid production and to develop molecular tools and techniques that can be used in establishing breeding programs. This dissertation developed and utilised suites of microsatellite markers to determine the baseline diversity of native, naturalised and cultured diploid oysters. It was found that the high diversity within naturalised oysters may provide a genetic reservoir for future breeding programs. The same microsatellite markers were used to determine diversity and pedigree assignment within a mass spawned tetraploid population across two successive generations. The first generation showed a high diversity, which significantly decreased in the second generation produced via mass spawning. This was most likely due to a low number of effective broodstock and skewed parental contributions and highlights the benefits of using single pair crosses over mass spawning to control inbreeding. A method for assigning pedigrees in triploids, produced by crossing diploids with tetraploids, was developed. This method will allow the pedigree of strongly performing triploids to be traced back so that the same or closely related broodstock can be used in single pair cross selection programs to produce future generations. To determine the long term stability of tetraploid oysters, the aneuploid frequency was analysed using flow cytometry across three generations. No difference was observed which suggests that either aneuploidy is occurring at a lower rate than previously predicted or that aneuploid oysters are being removed from the system

through hatchery grading or early mortality. Flow cytometry is not sensitive enough to detect small scale chromosome loss. Hence, fluorescent in situ hybridisation (FISH) using microsatellite markers was trialled. This was unsuccessful due to the inconsistency of the markers. The molecular tools, techniques and results described within this dissertation will aid in the development of single pair cross selective breeding programs for the improvement of triploid oysters.

Contents

Chapter 1

Introduction

The Pacific oyster (*Crassostrea gigas*) is an economically important species. Worldwide, the 2011 Pacific oyster aquaculture industry produced over 600 000 tonnes of oysters valued at over 1 billion dollars (FAO, 2010). The leading producers of oysters (*Crassostrea* spp.) are China, Korea, Japan, France and Taiwan. The Australian oyster industry is currently one of the largest aquaculture sectors in the country and has shown rapid growth over the past decade. The Australian industry is traditionally based on the culture of diploid individuals originating from Japan. In recent years, there has been a move towards the culture of polyploid oysters. Triploid oysters produced from tetraploid and diploid crosses show increased growth compared to their diploid counterparts (Piferrer et al., 2009). More importantly, the majority of triploids are genetically sterile and give higher quality meat (i.e. whole soft tissue) all year round, whereas diploids yield lower quality meat during the reproductive season due to a reduction in gonad size after spawning (Beaumont and Fairbrother, 1991). Sterility is also important for translocation between states and overseas. This is due to some regions declaring the Pacific oyster as feral species and restricting production to triploids only. Despite the potential of triploid oysters, there has been little research on their biology or that of their tetraploid parents.

1.1 What is polyploidisation?

An organism that has one or more additional sets of chromosomes in respect to the most frequent or 'natural' chromosome number for its species is termed a polyploid (Piferrer et al., 2009). Polyploidy is common within plants and, whilst less frequent in the animal kingdom, has been found to be viable in some invertebrate and lower

vertebrate taxa (Gong et al., 2004). Many commercial plants are human induced polyploids including sugar cane, sugar beet, banana, apple, some citrus fruits, cotton, potato, wheat, leek, peanut, tobacco, garlic, kiwi, plum and strawberry (Piferrer et al., 2009).

As the names imply, triploids have three sets of chromosomes in their somatic cells and tetraploids have four sets, rather than the normal diploid number of two sets (Benfey, 2001). In the Pacific oyster, the diploid number of chromosomes is 20 (Hubert and Hedgecock, 2004). Triploidy has been found to occur spontaneously at low frequencies, often from the egg failing to exclude the second polar body during meiosis (Collarespereira et al., 1995). This occurs in nearly all animal species and is often lethal in mammals and birds (Gong et al., 2004). Whilst there are some fish species that are naturally occurring triploids, for most animals triploidy is not a natural condition (Benfey, 2001).

Commercial culture of triploid oysters began in America in 1985. Initially, a chemical method was used to induce triploidy. 'Chemical' triploids were produced by a shock treatment (normally with cytochalasin B, heat or pressure) applied to a recently fertilised egg from a standard diploid cross to stop the first (Meiosis I triploids) or second polar body (Meiosis II triploids) from separating from the embryo (Fig. 1.1). Meiosis I triploids are likely to be more heterozygous; however, they are more difficult to produce. This is because treatment application must be timed highly precisely and the embryo has very little time to recover before meiosis II occurs. Additionally, meiosis I triploids are considered to have a higher aneuploid (detailed below) frequency than meiosis II triploids (Gerard et al., 1999). In 1993, 4Cs Breeding Technologies Inc patented a process which created the first tetraploid oyster

(Guo et al., 1994; Piferrer et al., 2009). The process involved finding a female triploid with usable sex cells (a very small percentage of triploids are fertile), crossing it with a sperm from a diploid and shocking to retain polar body I (Fig 1.2). Tetraploid oysters could now be used to create 'natural' triploids via crosses with diploids. These triploids showed better growth and survival rate compared to 'chemical' triploids and soon became the main method used in the culture of triploid Pacific oysters. More importantly, tetraploid x diploid crosses result in nearly 100% triploid offspring. This is opposed to the chemical induction methods that results in 50%-100% triploids with a mean of 83% (Beaumont and Fairbrother, 1991). Additionally, the production of 'natural' triploids has far better consumer acceptance (McCombie et al., 2005a).

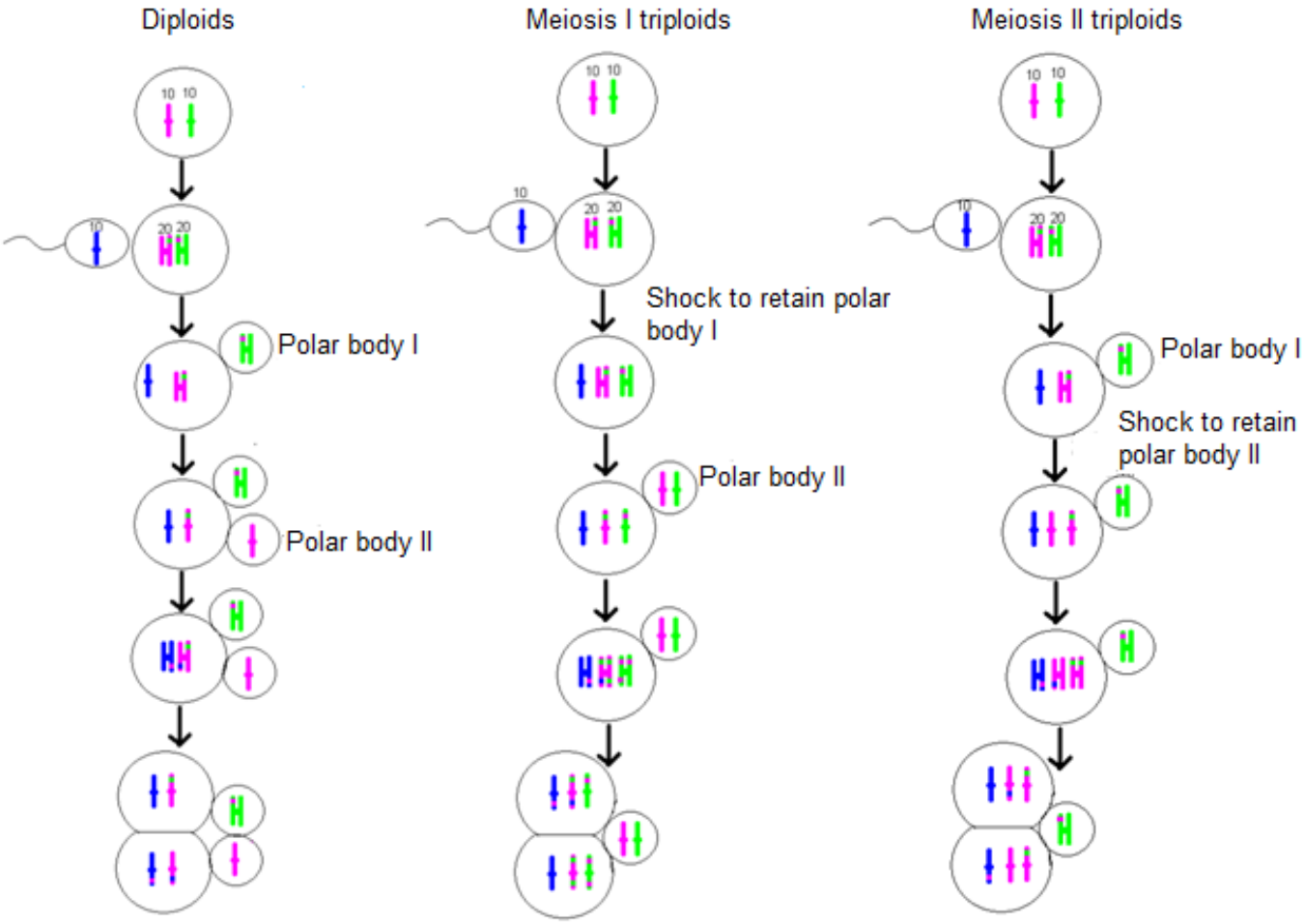

Figure 1.1: Standard diploid meiosis is shown on the left. Meiosis I 'chemical' triploid production process is shown in the centre. Here the recently fertilised egg is shocked at meiosis I to retain the first polar body. Meiosis II 'chemical' triploid production process is shown on the right. Here the recently fertilised egg is shocked at meiosis II to retain the second polar body.

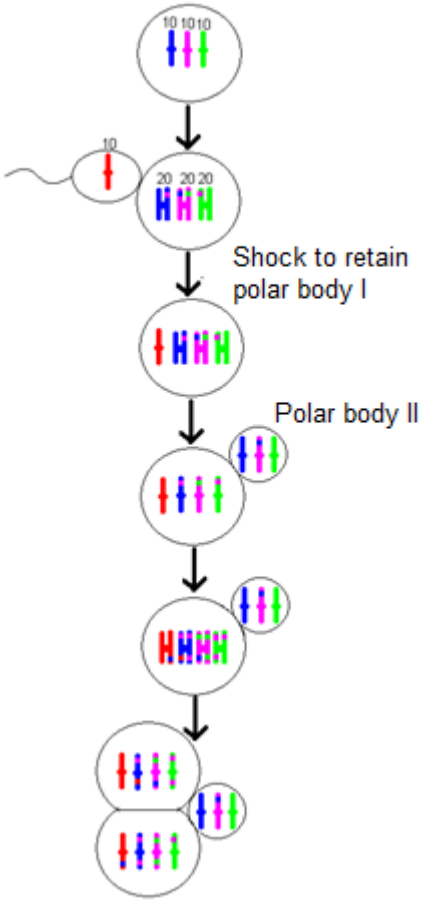

Fig 1.2: The 4Cs tetraploid Pacific oyster production method. Recently fertilised triploid eggs are shocked to retain polar body I

1.2 Polyploidisation in molluscs

Polyploidisation is considered simpler in shellfish compared to other animal species due to the fact that they are often broadcast spawners who rely on sperm activation of eggs. This means that eggs and sperm can easily be collected, that the correct division (e.g. meiosis I or II) can be targeted and egg maturation can be controlled (Beaumont and Fairbrother, 1991). However, polyploidy has been successfully achieved in other aquatic species such as salmonoids and prawns (Benfey and Sutterlin, 1984; Sellars et al., 2009).

Polyploidisation has been performed, with varying degrees of success, on a number of shellfish including oysters, mussels, scallops, clams and abalone (Table 1.1). Due to the Pacific oyster's global economic importance, this species is the most commonly farmed triploid shellfish. Commercial culture of triploid Pacific oysters is much further advanced than any other polyploid invertebrate species (Piferrer et al., 2009).

Table 1.1: A summary of some of the different shellfish species that ploidy manipulation has been completed on and the effects on growth, survival, fecundity and sex-ratio of triploidy. 2N = diploid and 3N = triploid.

Species	Growth	Survival	Fecundity	Sex ratio	Other	References
Pearl oyster, *Pinctada martensii*					First viable tetraploid for a bivalve mollusc	(He et al., 2000) (He et al., 1999)
American oyster, *Crassostrea virginica*	3N bigger than 2N after 8 months	3Ns had better survival rates against parasite *Haplosporidium nelsoni*	Similar to the Pacific oyster	No difference between 2Ns and 3Ns		(Guo et al., 2002) (Mattiessen and Davis, 1992) (Allen, 1987)
Sydney Rock oyster, *Saccostrea commercialis*	3Ns larger than 2Ns after the first year	No difference between 2Ns and 3Ns	High sterility rates in both sexes		Brown discolouration of 3N gonads over summer	(O'Connor and Dove, 2009) (Hand et al., 1999)
Common mussel, *Mytilus edulis*	3Ns bigger than 2Ns after 4 months		No mature gametes found in 3Ns	No difference between 2Ns and 3Ns		(Brake et al., 2004)
Lion-paw scallop, *Nodipecten subnodosus*	No difference between 2Ns and 3Ns	No difference between 2Ns and 3Ns	3Ns were 100% sterile during first maturation peak and 95%-99.95% sterile during second maturation peak			(Maldonado-Amparo et al., 2004) (Racotta et al., 2008)

Table 1.1 cont.

Species	Growth	Survival	Fecundity	Sex ratio	Other	References
Catarina scallop, *Argopecten ventricosus*	3Ns bigger than 2Ns after 3 months		(Hermaphrodite) Triploidy resulted in the female and male portions of the gonads being asynchronous	Triploidy resulted in altered sexuality		(Ruiz-Verdugo et al., 2000)
Dwarf Surfclam *Mulinia lateralis*	3Ns bigger than 2Ns		3N relative fecundity was found to be 59% for females and 80% for males	No difference between 2Ns and 3Ns		(Guo and Allen, 1994c)
Blacklip Abalone, *Haliotis rubra*	No difference between 2Ns and 3Ns (only measured for 37 months)	No difference between 2Ns and 3Ns	Gametogenesis severely retarded in 3Ns	No difference between 2Ns and 3Ns	Brown discolouration of male 3N gonads	(Dunstan et al., 2007)
Pacific Abalone, *Haliotis discus hannai*	No difference between 2Ns and 3Ns after 6 months		Female 3Ns had normally maturing oocytes, but spermatozoa were not detected in male 3Ns			(Okumura et al., 2007) (Yan et al., 2005)

1.3 The history of Pacific oysters in Australia

Throughout the late 1940s to the early 1950s the Pacific oyster was deliberately introduced from Japan into Australian temperate waters. The oysters were released at Oyster Harbour in southwest Western Australia and Pittwater in Tasmania (English et al., 2000). Only the oysters at the Tasmanian site survived. Spatfall was low and in 1953 this population was moved to the more optimal site of Port Sorrell where they flourished and spread, likely via human translocation as opposed to natural population expansion, to other Tasmanian locations (Stasko, 2000). Today, there are several naturalised populations of Pacific oyster in Tasmania and, due to illegal introductions, the southern coast of the mainland (English et al., 2000). Given it is an introduced species and has the ability to rapidly colonise and form dense aggregations in the intertidal zone, the Pacific oyster is considered a feral species in Australia. It was declared noxious in 1985 in New South Wales (NSW), with the Agriculture and Fisheries Department making the presence of the Pacific oyster on a shellfish lease illegal in NSW (NIMPIS, 2010). However, due to the prolific number of oysters present, in 1991 farmers from Port Stephens (NSW) were granted permission to culture both triploid and diploid Pacific oysters with cultivation later extending to the Georges and Hawkesbury Rivers (NSW) (O'Connor and Dove, 2009). In Victoria, Western Australia and parts of NSW, culture of Pacific oyster may only occur if individuals are sterile (Gavine and McKinnon, 2002).

Currently, the Australian oyster industry is the 4th largest aquaculture sector in the country and is worth AUD$ 89 million. In 2011-2012, the Tasmanian Pacific oyster industry was worth AUD$ 24 million (ABARES, 2012). Commercial aquaculture of this species in Tasmania began in the 1970s (Appleyard and Ward, 2006). In 1997, a successful national selective breeding program designed to improve growth rate was established (Ward et al.,

2005). Other traits such as shell shape, condition/quality and survival have been included in the breeding objective (Kube et al., 2011). More recently, disease resistance against the internationally important OsHV virus (Degremont, 2011), locally referred to as Pacific oyster mortality syndrome, has been incorporated into the breeding program. (Peter Kube, CSIRO, pers. comm.).

Since the introduction of Pacific oysters into Australia over 60 years ago, studies have suggested that this species has lost little genetic diversity compared to the native Japanese populations from where they originated (English et al., 2000; Appleyard and Ward, 2006). These studies, however, are limited by the type and/or number of markers used and the sample sites studied. A stronger understanding of the diversity of naturalised and hatchery produced oysters is needed to minimise the risk of inbreeding depression within breeding programs.

1.4 Triploid Pacific oyster production

The Pacific oyster is, in general, a diploid species that has no known history of polyploidisation in the wild, except, like most animal species, the rare spontaneous triploid. This is opposed to some species, such as sturgeon (*Acipenser naccarii)*, where tetraploidy is natural. Whilst, triploidy in mammals and birds is often lethal or results in harmful abnormalities, in the majority of invertebrates, amphibians and fish, a triploid individual is biologically viable and, in many cases, beneficial for scientific and commercial purposes (Gong et al., 2004).

Within many invertebrates and lower taxa, triploids are almost impossible to morphologically differentiate from diploids (Gong et al., 2004). However, some triploid molluscs, including the Pacific oyster, tend to grow faster than their diploid counterparts (Garnier-Gere et al., 2002; Gong et al., 2004; Nell and Perkins, 2005; Mallia et al., 2006; Liu et al., 2009;

Normand et al., 2009). This phenomenon is called polyploid gigantism. Several hypotheses have been proposed to explain this phenomenon including increased heterozygosity, larger cell size of polyploids and sterility (i.e. less energy expended on reproduction) (Gong et al., 2004; Piferrer et al., 2009). In regards to the larger cell size hypothesis, Benfey (2001) found that an increase in cell size led to a decrease in cell number in triploid Atlantic salmon (*Salmo salar)*. This decreased cell number in triploids was also found to be true for amphibians (Wang et al., 1999). However, Guo and Allen (1994c) found no decrease in cell number to compensate for larger cell size in polyploid dwarf surfclams (*Mulinia lateralis)*. To date, cell number has not been calculated in polyploid Pacific oysters.

Triploid Pacific oysters have been produced for aquaculture since 1986 (Guo and Allen, 1994b). A significant proportion (20-30%) of the current Pacific oyster culture in Australia involves the production of triploid oysters via diploid and tetraploid crosses (Michel Bermudes, pers. comm.). Between 2007 and 2008 in NSW, triploid Pacific oysters accounted for 40% of total production (O'Connor and Dove, 2009). In the USA, triploids account for approximately 50% of the total Pacific oyster production; in Europe, it is 20% (Piferrer et al., 2009). In France, triploids accounted for 80% of the 800 million spat sold by hatcheries in 2005 (Buestel et al., 2009).

The benefits of triploids are likely to be species-specific (Piferrer et al., 2009). In Australia, the main benefit of triploid Pacific oysters is their genetic sterility. The high genetic sterility allows for the translocation of this species between states and for Pacific oyster aquaculture industries to develop in Victoria and expand in NSW.

Despite having retarded gonadal development, triploids are not 100% sterile (Guo and Allen, 1994b; Gong et al., 2004; Normand et al., 2008). Guo and Allen (1994b) estimated that the fecundity of female triploid Pacific oysters to be about 2% of diploid fecundity. When

triploids were mated together, offspring survival rates were 0.0008% that of normal diploid crosses, with 90% of the surviving progeny also being triploid. When triploids were crossed with diploids, the reproductive potential of the triploids was estimated at 0.0046% of normal diploids (Guo and Allen, 1994b). Gong et al. (2004) more conservatively estimated the reproductive potential of triploid Pacific oysters to be 1 in 1000. These estimates are relatively low suggesting that, whilst triploidy cannot provide complete genetic containment of cultured oysters, it can help to reduce 'genetic pollution' caused by escapees from aquaculture (Wang et al., 1999; Gong et al., 2004). It has also been suggested that, whilst a number of triploid eggs may be viable, the resulting progeny could have a high aneuploidy percentage (described below) and, thus, a high mortality rate (Guo and Allen, 1994b; Normand et al., 2008). In the rainbow trout (*Oncorhynchus mykiss*), progeny produced from triploid males were not viable indicating that these males are 100% sterile despite having secondary sex characteristics (Benfey et al., 1986). Similar findings have been reported for shellfish (Table 1.1). The viability rate of progeny from triploid Pacific oysters in unknown.

1.5 Potential issues associated with polyploids

Whilst triploids are commercially beneficial, there are several issues associated with their production to be considered. These include the increased difficulty of breeding triploids compared to diploids as well as the biological constraints of sustainably maintaining polyploid populations.

1.5.1 Breeding

Producing adequate numbers of tetraploids and maintaining them in a way that maximises diversity and reduces inbreeding is an issue affecting polyploid production. To chemically induce a tetraploid, a triploid with viable eggs must first be found. As discussed previously, triploids only have about 2% of the fecundity of diploids (Guo and Allen, 1994b). Hence,

chemically producing a large number of genetically diverse tetraploids is time consuming. Once the tetraploids have been produced, they are continually mated together to maintain the tetraploid population, thus potentially increasing inbreeding (discussed below). Within diploid breeding programs, breeding lines are regularly infused with wild caught naturalised oysters to increase diversity (Scott Parkinson, pers. comm.), a practice that is more time consuming in tetraploids due to the need for chemical induction.

In Tasmania, the Pacific oyster industry mainly relies on hatchery produced seed; hence, it is well suited to selective breeding programs. In 1997, the Australian Government Fisheries Research and Development Corporation (FRDC) and the Aquaculture CRC established a selective breeding program of diploid Pacific oysters (Ward et al., 2000). Over 5 generations the breeding program showed an increase in growth rate, with the 4th generation showing a 1.6 x increase in growth compared to controls (Ward et al., 2005). Pacific oyster selective breeding programs in the USA have also reported gains. An increase in average survival, final individual weight and yield (16%, 9% and 33%, respectively) was reported after two generations of family based selection with the top 10% of families showing an increase of 56% for survival, 26% for individual weight and 83% for yield (Langdon et al., 2008). Langdon et al. (2008) also found shell colour to be a heritable trait ($h2 = 0.59$) that was surprisingly not substantially affected by the environment. Additionally, Leitao et al. (2001a) found a genetic basis for aneuploidy and suggested that selective breeding programs may one day be able to reduce its prevalence within oyster populations. Selective breeding programs, have yet to be researched for polyploid oysters; however, given the great success in diploid selective breeding, it is highly likely that improvements in the diploid and tetraploid lines could improve the triploid stock.

Currently, there is no selective breeding program in place for triploid Pacific oysters, despite the success of the diploid breeding program (Kube et al., 2011). This is partially due to the lack of genetic research that has been completed. Diversity, heterozygosity, effective population sizes, allele dosage and hybrid vigour are some of the some of the issues affecting triploid oysters that require further research before a successful breeding program can be implemented.

1.5.2 Inbreeding

As with any hatchery/domestication production system, inbreeding is a potential problem if not properly regulated. Tetraploid oyster populations may be particularly susceptible to inbreeding because of the difficulties in gaining unrelated broodstock caused by the low frequencies of triploids that produce viable eggs (Piferrer et al., 2009). However, the risk of inbreeding in tetraploids may be reduced by an increase in heterozygosity. Evans et al. (2004) found that, like many species, the Pacific oyster is adversely affected by low levels of inbreeding. This included a reduction in yield, body weight and fitness. Additionally, Pacific oysters are estimated to be heterozygous for 12 to 14 lethal recessive genes per individual, with most individuals carrying the identical by decent (IBD) lethal recessive genes (homozygous for a lethal gene) not surviving beyond 3 months of age (Launey and Hedgecock, 2001). Inbred families are more likely to carry the IBD lethal recessive genes, which could result in a further reduction of genetic diversity (Evans et al., 2004). Appleyard and Ward (2006) found that the number of effective broodstock used in breeding programs was significantly less than the census number. This is likely due to a variety of factors including skewed sex ratios, high fecundity, high juvenile mortality of some families and differences in gamete viability. Smaller than expected effective population sizes is a common occurrence in oyster mass-spawning breeding programs due to their enormous fecundity

which can result in unequal gametic contributions (Gaffney, 2006). The degree of tolerance to inbreeding is species dependent and it is not yet known if polyploids show greater or less tolerance to inbreeding compared to their diploid counterparts. Either way, a greater understanding of inbreeding is needed to maintain genetically healthy populations of tetraploid oysters.

The most common way to determine the diversity, and hence the predicted inbreeding within a population, is to use microsatellite markers. Given the economic importance of the Pacific oyster world-wide, a large number of genetic studies have been completed on this species including a comprehensive microsatellite marker linkage map (Hubert and Hedgecock, 2004; Hubert et al., 2009). To date, there has been numerous allozyme, microsatellite (150+), expressed sequence tags (ESTs) (4.6 million), single nucleotide polymorphisms (SNPs) and fluorescent *in situ* hybridisation (FISH) markers optimised for the Pacific oyster (Guo and Allen, 1997b; Huvet et al., 2000; McGoldrick et al., 2000; Wang et al., 2001; Li and Kijima, 2002; Li et al., 2003; Hedgecock et al., 2005; Yamtich et al., 2005; Appleyard and Ward, 2006; Sauvage et al., 2007; Yu and Li, 2007; Bouilly et al., 2008; Yu and Li, 2008; Fleury et al., 2009; Li et al., 2009a; Qiu et al., 2009; Sauvage et al., 2009; Wijga et al., 2009). In recent years, the entire Pacific oyster genome has been sequenced (Zhang et al., 2012). Despite this intense study on diploid Pacific oyster genetics, many features of polyploid oyster genetics remain unknown (Piferrer et al., 2009). The majority of polyploid research to date focuses on the phenotypic benefits displayed by triploids over diploids (Garnier-Gere et al., 2002; Mallia et al., 2006; Normand et al., 2009). Of the genetic markers published, the vast majority have only been trialled on diploid individuals. Only approximately 60 microsatellite markers have been successfully used on triploid Pacific oysters (Magoulas et al., 1998; Garnier-Gere et al., 2002; Hubert et al., 2009) and, in a singular study, only 40 have been used on tetraploids (Curole and Hedgecock, 2005). To date, none of these markers have been used to assess the

diversity, inbreeding or pedigree assignment of polyploid oysters. Furthermore software programs that can analyse polyploid data are infrequent.

Within Pacific oysters, a major difficulty associated with the development and use of microsatellite markers for assessing diversity, inbreeding, parental assignment etc. is null alleles. Null alleles are alleles that do not amplify during PCR for any number of reasons including mutations in the primer annealing site, differential amplification depending on allele size (partial nulls) and PCR failure (Dakin and Avise, 2004). Pacific oysters (*Crassostrea gigas*) have been shown to have high frequencies of null alleles, likely due to a high level of nucleotide polymorphism (Hedgecock et al., 2004). McGoldrick et al. (2000) reported a null allele frequency of 22% in Australian Pacific oyster stocks; hence it is important when assessing diversity or assigning parentage in Pacific oysters to take into account null alleles.

1.5.3 Aneuploidy

Aneuploidy is the loss or gain of one or more chromosomes so that the haploid set has unequal copies (Wang et al., 1999). Aneuploidy is often lethal in many higher order taxa, (e.g. humans can only tolerate a 6% gain and 3% loss) or results in severe growth retardation. Many plant species, however, have been found to have a tolerance of aneuploidy that is good enough to allow the production and maintenance of aneuploidy genetic stock for genetic analysis (Wang et al., 1999). The Pacific oyster, like many lower order taxa, has been found to tolerate a large range of different chromosome numbers (Guo and Allen, 1994b; Wang et al., 1999; Gong et al., 2004), however, aneuploid Pacific oysters have been found to have a smaller growth rate than their diploid counterparts (Leitao et al., 2001b; Gong et al., 2004; Landau and Guo, 2005).

A high aneuploidy rate could potentially be very problematic within polyploid oyster populations. McCombie et al. (2005a) found a somatic aneuploid rate of 53% in 2nd and 3rd generation tetraploids. This suggests that tetraploid oysters may not be a stable state and could result in increased sterility within the population through reversion from tetraploid to triploid. Chromosome loss is likely progressive, with an increase in aneuploid cells over time (Allen et al., 1999). More research is needed on aneuploidy in successive generations of tetraploid oysters to determine what long term effects it has on the populations and if reversion to a lower ploidy level is able and or likely to occur. High aneuploidy in successive tetraploid populations could also have carry over effects on the triploid stock. However, Allen et al. (1999) suggested that the frequency of cells in triploids that reverted to diploid cells was about 2-3 time higher in 'chemical' triploids induced from cytochalasin B than in 'natural' triploids. This is opposed to Wang et al. (1999) who found more aneuploids in 'natural' triploids compared to 'chemical' triploids. Wang et al. (1999) proposed that tetraploids were likely to suffer segregation errors during meiosis, thus producing aneuploid gametes. Allen et al. (1999) predicted that harsher environmental conditions could potentially exacerbate aneuploidy frequency. Additionally, Leitao et al. (2001c) found that chromosome loss was not random in aneuploid oysters, but rather a reflection of differential chromosome susceptibility. These, often conflicting, aneuploidy predictions are still relatively speculative and further research is needed on the stability of polyploid oyster populations.

1.6 Dissertation overview

Currently, genetic improvement in diploid oysters is fast outpacing that of polyploids and this gap could be a risk for the triploid sector of the industry in the future. Triploid oysters represent 20-30% of the Australian market and their demand is growing. While there has been considerable progress made in diploid oyster breeding, in particular the development of

selective breeding programs (Ward et al., 2005), very little genetic improvement exists in the triploid production. The primary aim of this dissertation is to help close the gap by researching some of the fundamental genetics of polyploid oysters. The following research covers many of the potential issues relating to the production of triploid oysters, including diversity, inbreeding, parental assignment and aneuploidy, whilst developing molecular tools for further study into polyploids. The information contained within this dissertation will be useful in the future development of a selective breeding program for triploid oysters.

The topics of this dissertation are as follows:

Chapter 1 - Genetic diversity of cultured, naturalized, and native Pacific oysters, *Crassostrea gigas*, determined from multiplexed microsatellite markers. Aims – to determine the current genetic diversity of diploid oysters, reveal if there has been any significant change in diversity since their introduction, discover if current hatchery stocks are at risk of inbreeding depression and develop suites of microsatellite markers that can be used to efficiently assess diversity in Pacific oysters.

Chapter 2 - Considerations for maintaining tetraploid Pacific oysters (*Crassostrea gigas*) as broodstock for triploids: a case study of diversity and pedigree assignment. Aims - to assess the genetic diversity and parental contributions in a population of tetraploid oysters using microsatellite markers.

Chapter 3 - Assignment of parentage in triploid species using microsatellite markers with null alleles, an example from Pacific oysters (*Crassostrea gigas*). Aims - to develop a method for accurately assigning parentage in triploid species and to test this method on triploid Pacific oysters to determine effective population size.

Chapter 4 - Stability in tetraploid Pacific oysters (*Crassostrea gigas*) across three generations using flow cytometry. Aims - to use flow cytometry to determine if large scale chromosome loss is occurring and if it progressive to the point where reversion of tetraploids to triploids may occur.

Chapter 5 - The possibilities and difficulties associated with counting and identifying polyploid Pacific oyster (*Crassostrea* gigas) chromosomes. Aims - to develop a method for fluorescent *in situ* hybridisation (FISH) in polyploid oysters that will allow individual chromosomes to be identified so that aneuploidy rates and chromosome susceptibility to loss can be determined.

1.7 Conclusion

The aquaculture demand for triploid Pacific oysters in Australia is growing. The improved growth, and more importantly, the high sterility rate of triploids make them a very desirable product that can potentially be cultured in areas where this species is considered a noxious pest. Despite this demand, however, there has currently been very little research compiled on polyploid oysters. Potential issues, namely aneuploidy and inbreeding, are not well understood in polyploids and could play an important role in maintaining viable tetraploid populations. In short, there is a need to close the gap between diploid and polyploid Pacific oyster research, this will help ensure genetically healthy polyploid populations and aid in the development of selective breeding programs for the improvement of the triploid product in the future.

Chapter 2

A genetic diversity study of cultured, naturalized and native Pacific oysters (*Crassostrea gigas*) determined from multiplexed microsatellite markers

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2.1 Introduction

Native to Northeast Asia, the Pacific oyster (*Crassostrea gigas*) has been deliberately introduced into North America (early 1900s), Australia (1940s and 1950s) and France (1970s) where it is now a very lucrative aquaculture species. Its ability to tolerate a variety of different salinities and temperatures, in conjunction with its high fecundity, has made it an economically important species worldwide. In 2008, world aquaculture produced over 600,000 tonnes of Pacific oysters valued at more than one billion US dollars (FAO, 2010).

In Australia, Pacific oysters were released in temperate waters at Oyster Harbour, in southwest Western Australia, and Pittwater, in Tasmania (English et al., 2000). Only the oysters at the Tasmanian site survived. Spatfall, however, was low and in 1953 this population was moved to the more optimal site of Port Sorrell, in Tasmania, where they flourished and spread to other Tasmanian locations (Stasko, 2000). Today, there are several populations of Pacific oyster in Tasmania and, due to illegal introductions, New South Wales (NSW) (English et al., 2000). These, now naturalized oysters, formed the basis of a number of commercial farms in Tasmania, South Australia and NSW. These farms use seed from two main commercial hatcheries that maintain their own mass selection breeding programs and also use some seed from the national family-based selective breeding program. All programs

have occasionally introduced additional wild caught naturalized oysters in an attempt to maintain or increase genetic diversity (Ward et al., 2000).

Over the past decade, there has been a move towards family-based selective breeding programs in aquaculture as opposed to mass selection. In many countries, including Australia, the Pacific oyster industry is entirely hatchery-based, meaning that it is in a position to benefit strongly from genetic improvement (Ward et al., 2000; Langdon et al., 2003; Degremont et al., 2010). Current selective breeding programs have been highly successful, particularly in improving growth rate (Ward et al., 2005). The future application of marker assisted selection (MAS) may provide further enhancement, particularly in relation to summer mortality resistance (Ward et al., 2005; Sauvage et al., 2010). Regardless of breeding technique, the maintenance of genetic diversity is fundamental to future improvement.

Determining the level of genetic variation in cultured, naturalized and native oysters will provide baseline information important to breeding programs, such as revealing whether there has been a significant change since introduction and indicate if poorly managed cultured stocks are at risk of inbreeding. English et al. (2000) found no significant genetic difference between Australian cultured (mass selected), Australian naturalized and Japanese native populations of Pacific oyster using allozyme markers, suggesting that there had been little genetic loss since the introduction of the Pacific oyster in the 1940s. The allozyme results of Appleyard and Ward (2006) were in agreement with this conclusion. However, Appleyard and Ward (2006) also used eight microsatellite markers to analyse the cultured (mass selected), naturalized and native populations. They discovered a drop in diversity within the cultured stock, which they attributed to the loss of rare alleles and bottleneck effects. Nevertheless, it was suggested that genetic diversity was still adequate and that inbreeding

was likely to be minimal. The authors recommended continual monitoring of diversity levels to determine if there is further decline. Kim et al. (2008) found a similar drop in diversity in Korean cultured Pacific oysters compared to wild ones using six microsatellite loci. A study by Li et al. (2006) investigated mass selected Pacific oysters within and among five Chinese farms. The results indicated high genetic diversity within cultured Chinese Pacific oysters and, surprisingly, large differences in allele frequencies between northern and southern farms. This suggests that population structuring can occur within this species.

Within closed breeding populations, some level of inbreeding is unavoidable, but this can be managed in family based programs. Oysters are a highly fecund animal, a trait that can increase the risk of inbreeding if breeders only use a small number of individuals as broodstock (Hedgecock et al., 2004). Additionally, Appleyard and Ward (2006) found that the effective number of broodstock was significantly less than the actual number of broodstock used, which could also result in greater inbreeding depression. This concurred with work by Boudry et al. (2002) who concluded that unbalanced parental contribution could be explained by both non-genetic and genetic effects. Tolerance to inbreeding is relatively species specific. Evans et al. (2004) found Pacific oysters to be sensitive to inbreeding depression, reporting an 8.8% decrease in average body weight and 4.3% decrease in survival with a 10% increase in inbreeding. Monitoring of inbreeding is highly important within shellfish culture to ensure that a healthy genepool is maintained.

In the Appleyard and Ward (2006) study, microsatellite markers proved more informative than allozymes at detecting genetic diversity in Pacific oysters. There have been numerous microsatellite markers developed for the Pacific oyster (Hubert and Hedgecock, 2004; Appleyard and Ward, 2006) many of which have been mapped using linkage analysis (Hubert and Hedgecock, 2004; Hubert et al., 2009; Plough and Hedgecock, 2011). Of these, only 15

microsatellite markers in five panels have been multiplexed within this species (Taris et al., 2005; Li et al., 2010). Multiplexing allows more rapid and cost-effective analysis. This study aimed to use at least 10 microsatellite markers, analysed within multiplexing suites, to determine genetic diversity and potential inbreeding of native, naturalized and cultured Pacific oysters. In addition to the native Japanese oysters used in the study by Appleyard and Ward (2006), oysters from Korea and France, as well as Australian naturalized and cultured populations, were analysed.

2.2 Materials and methods

2.2.1 Sample collection and DNA extraction

In total, 343 Pacific oyster samples were used in this study. Native samples were obtained from Hiroshima (n = 17) and Sendai (24) in Japan and the west coast of Korea (41). The Japanese samples were those used in the English et al. (2000) and Appleyard and Ward (2006) studies. Naturalized samples were collected from Australia and France. The French samples (50) were collected by L'Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER) from a number of different locations along the western coast. Within Australia, naturalized samples were collected from the Bridport Estuary (25) and Tamar Estuary (25) in Tasmania and at Port Stephens, in NSW (36). Cultured samples were provided from two Tasmanian hatcheries, Shellfish Culture (Clifton Beach, Tasmania; 50) and Cameron of Tasmania (Dunalley, Tasmania; 25). Shellfish Culture oysters were sampled across three mass selection breeding lines. Cameron samples were collected from a single mass selected commercial line. Additional cultured oysters were sampled from the Australian national selective breeding program, operated by Australian Seafood Industries (ASI; Hobart, Tasmania; 50). These oysters have been selectively bred for 15 years. The ASI oysters were

selected from 50 genetically diverse families (1 individual per family) across four year classes (2005-2008).

DNA was extracted from gill tissue (approx. 0.5cm^2), mostly using the extraction protocol with NaCl described by Lopera-Barrero et al. (2008), with the small modifications of 2µl of 20mg/ml proteinase K and resuspended in 100µl of TE buffer. The DNA was not treated with RNase. A 1 in 10 DNA dilution was performed prior to polymerase chain reaction (PCR). The Korean samples were extracted by Jung-Ha Kang (National Fisheries Research and Development Institute) using MagExtractor MFX-6100 (Toyobo). The Japanese samples were previously extracted by English et al. (2000). All DNA was stored at -20ºC.

2.2.2 Microsatellite analysis and PCR conditions

Originally, 29 previously published microsatellite markers, three from each linkage group (except L10 where there were only two), were selected from Hubert and Hedgecock's (2004) linkage map (for an updated linkage map see Plough and Hedgecock, 2011). Markers were selected based on high polymorphism, low frequency of null alleles and similar annealing temperatures as reported by the original designers of each marker (Magoulas et al., 1998; Huvet et al., 2000; Li et al., 2003). The 29 markers were reduced to the 13 that preformed best within a multiplex (e.g. non complimentary, distinct size ranges). The 13 microsatellite markers used to assess genetic diversity were: *um2Cg10, um2Cg48* (Huvet et al., 2000); *ucdCg120, ucdCg126, ucdCg129, ucdCg160, ucdCg166, ucdCg171, ucdCg175, ucdCg196, ucdCg198, ucdCg200* (Li et al., 2003); *imbCg49* (Magoulas et al., 1998). Primers for four of these markers (ucdCg129M F:GCATGCAGTGTATTGCTCTGTTAT, R:TGGCAAGAACTGGTGGTATG; *ucdCg160M* F:GAGATGGTTAGGCAGAACATTAAGA, R:TGTATCTCTTCCTTGTGCTCTCTC; *ucdCg196M* F:GCATCAGAAATTGAACTTGCAC, R:GTCGATCTTGCCATTTGCTTT; *um2Cg48M* R:TTCCAAATGCAACTGAGAGAGT) were redesigned using PRIMER3PLUS (Untergasser et al.,

2007) to generate larger or smaller fragment sizes to allow for multiplexing at 60°C . Markers were divided into four multiplex groups using MULTIPLEX MANAGER v. 1.1 (Holleley and Geerts, 2009) (Table 2.1). The markers were a mixture of di-, tri- and tetra-nucleotide repeats. An assortment of complex and simple microsatellites was utilised.

Table 2.1: Multiplexes of microsatellite markers for the Pacific Oyster (*Crassostrea gigas)*

Microsatellite	Primer conc. (μM)	Linkage Group	Repeat Array	5' Fluorescent label	Allele Size Range	Number of alleles observed
Panel 1; T_m 60°C						
ucdCg129M	2	10	GA	FAM	129-207	43
ucdCg166	3	9	TC	PET	184-264	34
ucdCg171	2	10	CAT	FAM	211-273	16
ucdCg200	1	1	GAT	HEX	227-284	18
um2Cg48M	3	3	GA	NED	107-202	71
Panel 2; T_m 60°C						
ucdCg160M	1	3	(GA)(GACA)	FAM	125-336	61
ucdCg196M	3	8	(GAC)(GAT)	HEX	311-430	34
ucdCg198	1	4	CAT	HEX	216-278	19
Panel 3; T_m 60°C						
um2Cg10	1	10	AG	HEX	95-240	49
ucdCg120	1	5	(CA)(GA)	PET	135-169	15
ucdCg126	1	2	(TCTA)	NED	92-233	34
Panel 4; T_m 55°C						
imbCg49	1	4	GT	PET	129-204	37
ucdCg175	3	8	CAT	PET	217-345	37

M indicates that primers have been changed from those originally published for each microsatellite. T_m is annealing temperature used in this study. Conc. = concentration. Linkage groups are in accordance with Plough and Hedgecock (2011).

PCR was performed in 5µl reactions using the Qiagen multiplex PCR kit as per the manufacturer's instructions (Qiagen, Australia). Primer concentrations were marker dependent (Table 2.1). Cycling conditions for PCR were as follows: 15 min at 95ºC; 30 cycles of 30 sec at 94ºC, 90 sec at annealing temperature, 90 sec at 72ºC; 30 min at 60ºC. A 1 in 200 dilution of PCR product was used as template for separation on an ABI 3770 (Applied Biosystems, Australia) sequencer using LIZ 500 (-250) size standard by the Australian Genome Research Facility (Adelaide). Alleles were scored using GENEMAPPER v.3.7 software (Applied Biosystems, Australia).

2.2.3 Data analysis

2.2.3.1 Marker performance and measures of genetic diversity

In order to assess the repeatability of each marker, approximately 10% of the samples were repeated (both within plate and across plates). The repeatability is the percentage of consistent allele scores across blindly scored, independent, repeated samples. The mean error rate per locus, as calculated in this study, is the most common repeatability measure used (Pompanon et al., 2005). This measure is appropriate to our study given the number of markers used and the high quality of the DNA samples analysed. Null allele frequency was calculated in INEst v. 1.0 using the individual inbreeding model (10,000 iterations; Chybicki and Burczyk, 2009). This program was also used to calculate a fixation index (F_{IS}) corrected for null alleles. Deviations from Hardy-Weinberg Equilibrium (HWE) for each microsatellite locus were tested using the Markov chain method (10 000 dememorization steps, 100 batches, 5000 iterations) in GENEPOP v. 4.0 (Rousset, 2008). Linkage disequilibrium was also calculated in this program. FSTAT v. 2.9.3.2 (Goudet, 1995) was used to calculate allele numbers and allelic richness across populations. Genetic subdivision (F_{ST}) across pre-defined sample populations was also analysed using this program. According to Jost (2008), G_{ST} and

its relatives (such as F_{ST}) may not be a good representation of genetic differentiation when there is high within population diversity, as often found with microsatellites. Therefore, the computer program SMOGD v. 1.2.5 (Crawford, 2010) was used to calculate an estimate of *D* (D_{EST}) (recommended by Jost, 2008) in order to validate the F_{ST} results (bootstrap replicates = 1000). GENALEX v. 6.4 (Peakall and Smouse, 2006) was used to determine the number of private alleles per population, observed (Ho) and expected (He) heterozygosity and to generate a Principal Coordinate Analysis (PCA) using co-dominant genotypic distance (Smouse and Peakall, 1999).

2.2.3.2 Bayesian analysis

Bayesian analysis of population structure was undertaken using the methods implemented in BAPS v. 5.1 (Corander et al., 2003) and STRUCTURE v. 2.2.3 (Pritchard et al., 2000). The Cameron family cross was excluded from Bayesian analysis due to the known relatedness between individuals. Using BAPS, clustering of groups of individuals was calculated using an admixture model (1,000 iterations, 200 reference individuals and 10 admixture coefficients). This method takes into account the *a priori* populations of the samples. For STRUCTURE analysis, the admixture model with allele frequencies correlated was used (100,000 iterations after a burn in period of 50, 000). This program uses an *a posteriori* method to group the samples into genetic clusters (populations) that reduce Hardy-Weinberg and gametic disequilibrium, thus reducing the biases associated with designations due to sampling locality. For each value of K (potential number of clusters) tested, 20 independent runs were performed. STRUCTURE HARVESTER v. 0.56.4 (Earl, 2009) was used to determine the delta K value using the Evanno et al. (2005) method, which indicates the number of clusters that best fits the data. CLUMPP v. 1.1.2 (Jakobsson and Rosenberg, 2007) was used to standardise the results across runs.

2.3 Results

2.3.1 Marker performance

In general, the markers performed well within each multiplex. The microsatellites with tetra- and tri-nucleotide repeats were easier to score than those with di-nucleotide repeats, primarily due to more stutter observed within the di-nucleotides. All 13 microsatellite markers were highly polymorphic, and had similar levels of repeatability (93.3% to 98.9% and missing data (0.03% - 5%; except *ucdCg196* at 16%; Table 2.2). Locus *um2Cg48M* had the most alleles (71), but showed a large degree of stutter and was sometimes difficult to score. Consequently, it had the lowest repeatability percentage (93.3%). Null alleles were estimated to exist in all 13 loci at frequencies from 3% to 32% across all samples. The three loci *ucdCg129, ucdCg200 and ucdCg196* were excluded from the rest of the analysis because they had predicted null allele frequencies higher than 25% (Table 2.2). Once these markers were excluded, the null allele frequencies were similar between native, naturalized and cultured populations (Table 2.3). The 10 remaining markers were dispersed into eight different linkage groups (Table 2.1). Despite the fact that the pairs *ucdCg171* - *um2Cg10* and *imbCg49* - *ucdCg198* were linked with 19.6 and 30.1 centiMorgan respectively between them, linkage disequilibrium was not significant for either pair. All other markers were located more that 50 centiMorgan apart and thus considered unlinked.

Table 2.2: Marker performance data. The performance statistics for 13 microsatellite markers (details in Table 2.1) of the Pacific Oyster (*Crassostrea gigas*) across 343 samples. Null allele percentage calculated in INEst v.1.0 (10,000 iterations; Chybicki and Burczyk 2009). Av = average

	Microsatellite marker													
	129	166	171	200	48	160	196	198	10	120	126	49	175	Av
# of alleles	43	34	16	18	71	61	34	19	49	15	34	37	37	36
Repeatability (%)	95.5	97	97	97	93.3	97.8	96.3	94.8	97.8	98.6	97.8	96.7	96.7	96.6
Missing data (%)	5	3	4.6	1.6	2.4	0.05	16	1.1	0.03	0.05	3.8	1.4	1.4	3.1
Null allele (%)	32	20	21	25	18	11	32	12	5	6	21	11	3	17
Frequency of null alleles within cultured samples	31.3	22.1	16.6	26.9	22.7	14.3	36.9	18.2	0.9	2.1	28.6	7.3	2.5	17.7
Frequency of null alleles within naturalized samples	34.2	20.3	20.1	24.6	18.1	9.3	29.8	10.9	5.2	2.2	23.8	11.6	2.5	16.4
Frequency of null alleles within native samples	2.7	20.3	24.8	1.6	12.9	9.0	3.2	11.7	4.7	5.2	19.4	12.1	1.1	9.9

2.3.2 Measures of genetic diversity

Private alleles were observed in all populations, with highest numbers in the native Korean (18) and Japanese (12) samples and the Tasmanian naturalised (17) samples. However, private allele frequencies were low, with the highest observed being 7%, and most being between 1 to 3%, therefore the private alleles were not biologically significant and were not considered further. The observed heterozygosity (*Ho)* in each population was significantly less than the expected (*He)* values (Table 2.3). Null alleles were estimated at similar frequencies across all populations and sub-groups (10.3% - 14.8%; Table 2.3). F_{IS} and null allele frequencies were similar across populations. Once null alleles were taken into consideration, the corrected F_{IS} was much lower than that originally stated (Table 2.3). The average number of alleles (*A*), allelic richness (*Ar*) and expected heterozygosity (*He*) were, as expected, lower in the cultured samples compared to the native and naturalized populations (Table 2.3). Specifically, the cultured populations showed a 34.5% decrease in the average *Ar* and a 5.6% decrease in average *He*. The family based breeding population (ASI) showed slightly higher values of *Ar* and *He* than the mass selected populations. There were no discernible differences in allele statistics between the native and naturalized oysters.

Table 2.3: Sample size (*N*), average number of alleles (*A*), allelic richness (*Ar*; based on 24 individuals), observed heterozygosity (*Ho*), expected heterozygosity (*He*), inbreeding coefficient (F_{IS}), corrected F_{IS}, number of private alleles, and frequency of null alleles (Null %) calculated using 10 microsatellite markers in the eight Pacific Oyster (*Crassostrea gigas*) populations.

Population	*N*	*A*	*Ar*	*Ho*	*He*	F_{IS}	Corrected F_{IS}	Null (%)
Cultured								
ASI	50	15.2	12.8	0.64	0.84	0.25	0.01	12.6
Cameron	25	12.2	12.1	0.61	0.84	0.30	0.01	14.8
S. Culture	50	14.6	11.9	0.59	0.83	0.30	0.02	13.9
Naturalized								
France	50	25.0	19.1	0.66	0.91	0.28	0.02	13.2
Tasmania	50	24.3	19.2	0.67	0.90	0.27	0.02	12.5
NSW	36	20.1	17.6	0.64	0.88	0.29	0.03	13.5
Native								
Japan	41	23.4	19.3	0.71	0.88	0.21	0.01	10.3
Korea	41	22.7	18.5	0.62	0.89	0.32	0.04	14.4

ASI = Australian seafood industries; Cameron = Cameron Oysters Tasmania mass selected line; NSW = New South Wales, Australia; S. Culture = Shellfish Culture Tasmania

The overall F_{ST} value among all populations was low (0.05), as were the F_{ST} values among the naturalized, native and cultured groups (0.01-0.06). The F_{ST} between native and naturalized populations was negligible (0.00). The F_{ST} between native and cultured or naturalized and cultured populations was slightly higher (0.02-0.03). The D_{EST} values (0.04 -0.30), whilst higher than the F_{ST} values, showed the same patterns as the F_{ST} results (data not shown). The Principal Coordinate Analysis (PCA) showed a close clustering of native and naturalized populations, compared to the hatchery derived samples (Fig. 2.1). The percent of variation explained by PC1 was 46% and by PC2 22%.

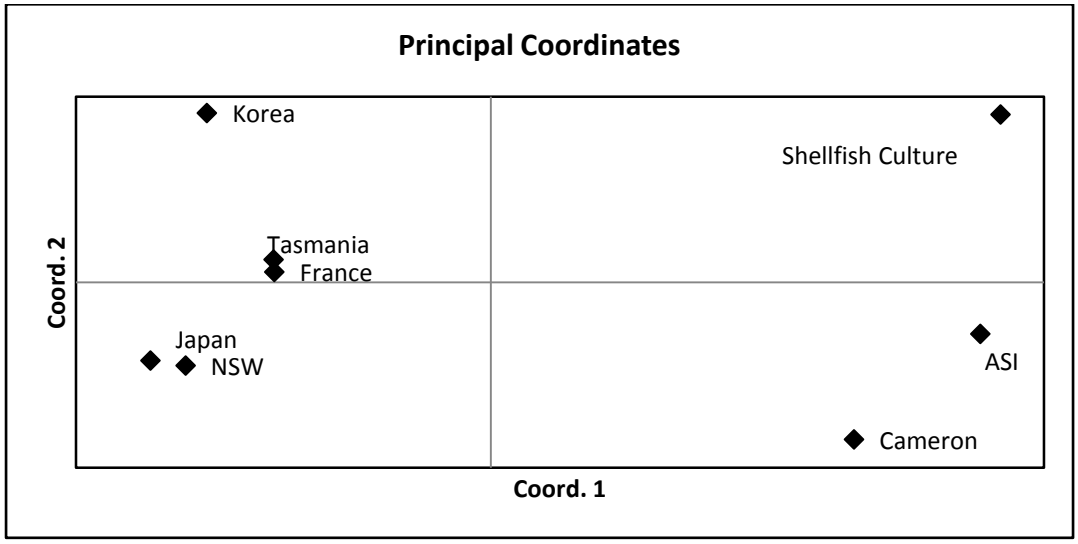

Figure 2.1: Principal Coordinates Analysis (PCA) matrix of genetic distance between 8 populations of the Pacific Oyster (*Crassostrea gigas*) calculated from 10 microsatellite markers. The percent variation explained by PC1 is 46.6% and by PC2 22.0%. Native populations are Japan and Korea. Naturalized populations are France, New South Wales, Australia (NSW) and Tasmania, Australia. Cultured populations are Australian Seafood Industries, (ASI), Cameron and Shellfish Culture, (all Tasmania, Australia).

2.3.3 Bayesian analysis

The output from BAPS indicated two genetic clusters (probability of two clusters = 0.99). One cluster grouped the native and naturalized populations and the other cluster grouped the family breeding population and mass selected populations (data not shown). The STRUCTURE output showed a large degree of log likelihood variance, particularly within the K = 3 runs, and this was relatively unaltered by an increase in the number of burn-in iterations. To reduce this variance, and thus improve the accuracy of the Evanno et al. (2005) method at finding the "true" K, the five runs with the highest average log likelihood value were selected and analysed for each value of K tested. This greatly reduced the variance and produced a Delta K plot that concurred with the BAPS outcome (i.e. two genetic clusters present). The Q matrix plot (bar plot showing individual population assignment) also concurred with the BAPS output by assigning the native and naturalized oysters to one cluster and the cultured oysters to a second (data not shown). Within the native and naturalized populations, the samples from Korea, NSW and, to a lesser extent, Tasmania showed a small number of individuals possessing genotypes that were consistent with the cultured oysters. Within the cultured oyster populations, the Cameron samples had the most individuals with genotypes similar to the native/naturalized cluster.

2.4 Discussion

The ten microsatellite markers used in this study performed well within the multiplexes, suggesting that they could be useful tools for cost-effective genotyping of Pacific oysters in the future. Neutral markers, such as microsatellites, have been shown to be effective for analysing diversity among populations (Pfrender et al., 2000). All of the populations analysed in this study showed disagreement with HWE, as shown by the large difference between expected and observed heterozygosity. This could be explained by the high number of non-

amplifying null alleles that have been shown to occur in this study and others in oysters (McGoldrick et al., 2000; Hedgecock et al., 2004). Fortunately, the presence of null alleles is unlikely to have significantly impacted on the population assignment analysis of this study because all the populations showed similar frequencies of null alleles.

In general, this study concurred with the microsatellite findings of Appleyard and Ward (2006). The results indicated a distinct difference in genetic diversity between cultured and native/naturalized populations, supporting the hypothesis of an initial hatchery induced bottleneck. A loss of genetic variation is a common phenomenon within mass selection breeding programs, particularly in molluscs (Appleyard and Ward, 2006), where often only a small number of broodstock are used due to their high fecundity. A number of studies in oysters have shown that the effective size of a population (*Ne*) is generally smaller than the number of spawned individuals (Hedgecock and Sly, 1990; Li and Hedgecock, 1998; Boudry et al., 2002; Appleyard and Ward, 2006), hence a large number of broodstock are generally needed to maintain high diversity levels. It is important for oyster breeding to manage genetic diversity, as high diversity is an indicator of future selective breeding potential and reduced risk of inbreeding depression. A decrease in diversity is expected within breeding programs (both mass and family) as hatcheries need to balance selection intensity with inbreeding (Bentsen and Olesen, 2002). As yet, there is no definitive threshold to inform oyster farmers what the minimal diversity index should be. Pacific oyster growth and survival have been shown to be significantly adversely affected by relatively low levels of inbreeding (F_{IS} = 0.2), therefore diversity should be maximised (Evans et al., 2004).

The Australian ASI family-based breeding program was established in 1997 to primarily improve growth rate within cultured Pacific oysters. One of the main goals of the breeding program was to balance genetic gain and inbreeding. As a result, the inbreeding coefficient has been calculated over each generation and the program aims for an average rate of

inbreeding less than 1% per generation. Such a rate of inbreeding is considered acceptable for long term sustainable breeding programs (Ward et al., 2005; Kube et al., 2011). It is important to note that the commercial lines from Shellfish Culture and Cameron showed no significant difference in allelic richness or inbreeding coefficients compared to the ASI population. This suggests that the current mass-selected breeding programs within these two Australian hatcheries have inbreeding levels similar to that of the family-based ASI program, which has been monitored to ensure that the inbreeding rate per generation is less than 1%. This suggests that the mass-selected populations are not currently experiencing inbreeding depression.

Our observed 34.5% decrease in *Ar* within the cultured populations is lower than the 60.4% decrease reported by Xiao et al. (2011) within cultured populations of *C. ariakensis*. Our observed reduction in *He* is also less than that observed (10.7%) by Appleyard and Ward (2006) for previous Australian hatchery year-classes. Bottleneck effects are common within breeding programs due to small effective population sizes. It has been shown that, within populations that have undergone a bottleneck, there is a time delay before significant changes to *He* are observed (Hedgecock and Sly, 1990; Leberg, 1992), and therefore the difference observed between the two studies may be an artefact of the samples and loci in each.

Bayesian analysis could not differentiate between native and naturalized populations. This, coupled with the similar results observed in allelic richness and heterozygosity, indicate that naturalized populations in France and Australia have lost very little diversity since introduction. This concurs with Appleyard and Ward (2006). The Principal co-ordinates figure showed Tasmanian and French naturalized samples clustered together suggesting they may have originated from a similar native population. In Australia, many oyster hatcheries, including Shellfish Culture and Cameron, regularly use naturalized individuals as broodstock in an attempt to infuse greater genetic diversity into their lines. The results of this study

suggest that naturalized populations of Pacific oysters in France and Australia are good reservoirs of diversity for breeding programs or hatcheries.

Chapter 3

Genetic diversity and pedigree assignment in tetraploid Pacific oysters (*Crassostrea gigas*)

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3.1 Introduction

Tetraploid Pacific oysters (*Crassostrea gigas*) were first produced in the 1990s using chemical induction methods that inhibit polar body I in the eggs of triploid oysters crossed with diploid males (Guo and Allen, 1994a). Whilst triploid oysters are mostly sterile, there are a small number of individuals that can produce eggs (Guo and Allen, 1994b; Gong et al., 2004; Normand et al., 2008). A complimentary method that inhibits polar body II in diploid females crossed with tetraploid males has also been developed (McCombie et al., 2005b). The ability to generate tetraploid oysters allowed 100% triploids to be produced by crossing tetraploids with diploids, a method that is considerably more effective than previous chemical induction techniques (Guo et al., 1996). The high sterility rate in triploid oysters is thought to lead to increased growth, as less energy is expended on reproduction (Guo and Allen, 1994b; Nell and Perkins, 2005). Sterility also has the important benefit of allowing high quality triploid meat to be harvested all year round, whereas diploid meat quality is lower during reproductive seasons (Allen and Downing, 1986). Triploid Pacific oysters account for 20-30% of total production in Australia (O'Connor and Dove, 2009). Despite the increase in production of triploid oysters, many features of their biology and genetics still remain uncertain (Piferrer et al., 2009).

3.1.1 Selection in polyploid oysters

A successful selective breeding program, run by Australian Seafood Industries (ASI), for diploid Pacific oysters in Australia has been in operation since 1997. This program has resulted in oysters that show significantly better growth than mass selected individuals (Ward et al., 2005; Kube et al., 2011). However, only diploid oysters are currently bred. In a number of Australian estuaries, production of Pacific oysters is restricted by law to triploids due to this species "noxious" status. With triploid Pacific oysters accounting for 40% of total production in New South Wales alone between 2007 and 2008 (O'Connor and Dove, 2009), there is a desire to improve growth rate and disease resistance of triploids. To date, very little research has occurred in relation to selection in tetraploid oysters to improve the triploid product.

3.1.2 The importance of diversity within tetraploid oysters

Tetraploids are generally produced *de novo* from a few fertile triploid females, automatically limiting diversity (Eudeline et al., 2000). This is because fertile triploids, produced from either chemical induction or tetraploid diploid crosses, used to make tetraploids are infrequent (Piferrer et al., 2009) and the induction treatment, difficult. Thus, there tends to be a limited number of inductions to produce *de novo* tetraploids, after which these are used to perpetuate the tetraploid population. Further reductions in genetic variability are possible in tetraploid spawns if small numbers of parents participate in the production of progeny. Li et al. (2009) showed that the effective population size, that is the number of broodstock contributing to the next generation, for diploid Pacific oysters, was significantly lower than the census (i.e. sampled) number of broodstock, owing to large variance in reproductive success. It is unknown whether tetraploid oysters show similar patterns. Within some tetraploid plants, greater sterility rates have been observed compared to diploids (Bishop,

1947; Buyukkartal and Colgecen, 2007). If a similar phenomenon were to occur within oysters, it could reduce the effective population size.

A high level of diversity can indicate a robust population and may reduce the chance of inbreeding depression becoming an issue. Diploid Pacific oysters have been shown to be sensitive to inbreeding depression which causes significant decreases in weight and survival (Evans et al., 2004). It is unknown if tetraploid oysters show the same sensitivity. In plant species, it has been proposed by Husband and Schemske (1997) and Soltis and Soltis (2000) that autotetraploids are less prone to inbreeding depression then their diploid counterparts. Other studies, however, show autotetraploid inbreeding depression to be similar or greater than diploids (Lamkey and Dudley, 1984; Bingham et al., 1994; Galloway and Etterson, 2007). Very little research has been undertaken on inbreeding depression in polyploid animals. Additionally, it can be argued that inbreeding depression within the tetraploid population will have negligible effect on the triploid product due to hybrid vigour and triploid oysters generally being a terminal line. Often referred to as heterosis, hybrid vigour is the phenomenon where progeny produced from two different lines (i.e. two unrelated pedigrees) show greater fitness, such as, better growth, development, or fertility (Birchler et al., 2003). Autopolyploids tend to show greater hybrid vigour than diploids (Comai, 2005). This suggests that, even if a tetraploid oyster population was to become significantly inbred, crossing with an unrelated line of diploids could produce genetically healthy triploids for commercial use.

Having a high diversity within a tetraploid population increases the probability that genotypes linked to desired breeding objectives (such as, disease resistance, increased growth, etc.) will be present, but it does not ensure it. Having a high genetic diversity within a tetraploid population increases the probability that genotypes linked to desired breeding objectives (such as, increased disease resistance, increased growth, etc.) will be present, but it does not

ensure it. Having the linked genotype present within the tetraploid population will allow selection of tetraploid broodstock to improve the triploid product. On the other hand, if the genotype is common, reduced genetic diversity within the tetraploid population may result in a more consistent triploid product. Regardless of whether it is optimal to maximise diversity or not, an understanding of the genetic variation within a tetraploid population is needed

To the best of our knowledge, genetic diversity in tetraploid oysters has not been examined. One of the major factors influencing the degree of genetic diversity in any population is parental contribution. Within oyster culture, mass spawning is the most common breeding technique. Generally, a number of potential broodstock are strip spawned and the gametes combined in a single tank for fertilisation and rearing. Factors, such as, gamete quality, sperm quantity and differential growth rates can bias parental contributions and may decrease the effective population size (N_e), which is the number of broodstock contributing to the next generation. Previous studies have shown that the effective population size in mass spawned diploid Pacific oysters is less than the census population (Boudry et al., 2002; Appleyard and Ward, 2006; Li et al., 2009b), however no such research has been completed on tetraploid oysters. It could be that tetraploid populations show an increased bias in parental contributions due to greater differential survival rates, possibly due to low quality sperm (Suquet et al., 2010). During the growth stages, oysters are continually graded to remove the smaller, slower growing individuals, which may further bias parental contributions (Taris et al., 2006; Lind et al., 2009). The objective of this study is to assess the genetic diversity and parental contributions in successive generations of a hatchery population of tetraploid oysters.

3.2 Materials and methods

3.2.1 Sample collection

All oysters used in this study were supplied by Shellfish Culture Ltd (Pipeclay, Tasmania, Australia). Two generations of tetraploid oysters were sampled. The base generation for this study (TN08) were spawned in January 2009 from at least 70 putative broodstock in four mass spawned crosses. The next generation (TN11) were spawned in November 2011. They were spawned from six mass spawned crosses using 42 TN08 broodstock (Table 3.1). Some TN08 broodstock were used in more than one cross. To produce the commercial tetraploid cohort, at 24 h post fertilisation, the surviving larvae from all six crosses were combined into a single tank. This study is divided into two sections, diversity and pedigree. For diversity analysis, 50 random TN08s (not previously used in the pedigree analysis) were sampled along with a random 50 three month old TN11s. The 42 TN08s used as broodstock (for 2011 spawns) were also included in the diversity analysis (total n = 92 for TN08). For pedigree assignment, a random 370 three month old TN11 progeny (50 of which were used in the diversity analysis) were sampled. Over the following three months, the tetraploid oysters were commercially graded several times to remove smaller individuals. To study the effects of grading, a random 94 TN11 progeny were sampled at six months of age. This consisted of 47 from a large size class (>12mm screen size) and 47 from a small size class (<5mm screen size).

Table 3.1: Tetraploid Pacific oyster broodstock spawned in 2008 (TN08) used in six crosses to generate the TN11 tetraploid progeny.

Cross	Male	Female
1	M1, M2, M3, M4	F1, F2, F3, F4
2	M1, M2, M3, M4	F5, F6, F7, F8, F9, F10, F11, F12, F13, F14, F15, F16
3	M5, M6, M7	F5, F6, F7, F8, F9, F10, F11, F12, F13, F14, F15, F16
4	M8, M9, M10	F17, F18, F19, F20, F21
5	M11, M12, M13, M14	F22, F23, F24, F25, F26
6	M1, M2, M3, M4	F27, F28
Total	14	28

3.2.2 DNA extraction and PCR

DNA was extracted from gill tissue (approx. 0.5cm^2) using the modified extraction protocol with salt (NaCl) described by Lopera-Barrero (2008), with the small modification of 2µl of 20mg/ml proteinase K. The DNA was not treated with RNAse. A 1 in 10 DNA dilution was performed prior to polymerase chain reaction (PCR). All DNA was stored at -20ºC.

The multiplex suites described by Miller et al. (2012) were used to assess diversity [8 markers (*ucdCg120, ucdCg126, ucdCg160, ucdCg166, ucdCg175, ucdCg198, um2Cg10* and *imbCgi49*) chosen based on scoring performance and minimal missing data]. For pedigree assignment, additional markers (*ucdCg138*: suite 4, *ucdCg174*: suite 4, *ucdCg184*: suite 3, *ucdCg186*: suite 1, *imbCgi44*: suite 1; Li et al., (2003)) were added to the multiplex suites. Approximately 5% of the samples were independently repeated within and across plates to calculate an error rate. PCR was performed in 5µl reactions using the Qiagen multiplex PCR

kit as per the manufacturer's instructions (Qiagen, Australia). Cycling conditions for PCR were as follows: 15 min at 95ºC; 30 cycles of 30 s at 94ºC, 90 s at 60°C, 90 s at 72ºC; 30 min at 60ºC. A 1 in 160 dilution of PCR product was used as template for separation on an ABI 3770 (Applied Biosystems, Australia) sequencer using LIZ 500 (-250) size standard by the Australian Genome Research Facility (Adelaide, Australia). Alleles were scored using GENEMAPPER v.3.7 software (Applied Biosystems, Australia). More than 10% of the broodstock samples were independently repeated to ensure consistent scoring.

3.2.3 Statistical methods

3.2.3.1 Software

There have been numerous software programs developed to analyse diploid diversity and pedigree assignment (Jones and Ardren, 2003; Hoban et al., 2012), however, very few programs have been developed that can handle autotetraploid data. The primary difficulty associated with autotetraploid microsatellite data is the uncertainly of the dosages to determine allele frequencies (Van Puyvelde et al., 2010). For example, a heterozygote with the genotype AABB is recorded as AB and it is not known if the actual genotype is AABB, AAAB or ABBB. There is also uncertainty associated with heterozygotes that have three unique alleles (i.e. AABC, ABBC, ABCC). To the best of our knowledge, there is no one program available that can calculate the statistics required for both diploid and autotetraploid data.

The Windows based program wHDP v.1.1 (Galli et al., 2011) was used to determine allele numbers, frequencies, and pedigree assignments in all tetraploid samples. This program does not calculate expected heterozygosity (*He*), hence the software ATETRA v.1.0 (Van Puyvelde et al., 2010) was employed for this purpose (using the Monte Carlo method, 10000 simulations). Due to the uncertainty of the genotypes, observed heterozygosity (*Ho*) was

calculated by only classing the individuals with four identical alleles (i.e. AAAA) as homozygotes, with all other genotypes (e.g. AAAB, AABB) classed as heterozygotes. For pedigree assignment, wHDP employs both the exclusion method and likelihood approach. An issue encountered with the wHDP software was its inability to deal with many microsatellite markers that likely contained errors (e.g. scoring error and null alleles). Due to the potentially high number of null alleles and the possibility of errors within the data, it was assumed that a progeny could be assigned to a broodstock pair if it matched in five or more loci, a conservative threshold considering the variability of the markers (Lind et al., 2009). The issue with wHDP was that it does not allow the researcher to set this threshold. Instead it gave the option of "no match locus" where, if selected, any individual that did not match at more than two loci was discarded from the analysis. This implied that, if all 13 markers were used at once to analyse the tetraploid dataset, then only individuals that matched at ten or more loci could be determined. To overcome this issue, and thus set the threshold to match at five or more loci, various combinations of the 13 microsatellite markers (a minimum of seven markers with 'no match locus' selected) had to be run through the program numerous times. The likelihood method employed by this program to assign progeny in the case of multiple allocations was relatively uninformative with likelihood values generally being identical. It was more informative to increase the number of markers analysed until only a single allocation remained.

A t-test was carried out in Microsoft Excel 2007 to determine if the three month old oyster diversity differed significantly from the six month old diversity, which would indicate a grading effect.

3.2.3.2 Parental assignment

The effective number of males based on reproductive success was calculated using N_{em} = $(N_m\bar{k}_m - 1) / [\bar{k}_m + (\sigma^2_{km} / \bar{k}_m) - 1]$, where N_m is the actual number of males, $\bar{k}_m$ is the average number of offspring sired by a single male and σ^2_{km} is the variance of $\bar{k}_m$ (Lande and Barrowclough, 1987). This same method was used to calculate the effective number of females. Using these results, the effective population size based on reproductive success was calculated using $N_e = 4N_{em}N_{ef}/(N_{em}+N_{ef})$. In the past, these measures have generally only been used on diploid individuals and the assumptions may be different for tetraploids. However, given that these statistics only require the contribution frequencies of each parent it is expected that they are appropriate for tetraploid analysis. T-tests were carried out in Microsoft Excel 2007 to determine the significances.

3.3 Results

3.3.1 Tetraploid diversity

The average number of alleles observed in 50 individuals from the tetraploids spawned in 2008 (TN08) was 16.7 and for the tetraploids spawned in 2011 (TN11) it was 10.0 (Table 3.2). The loss of allelic diversity between the TN08 and TN11 tetraploids was significant (-40.1%, $P = 0.01$). The tetraploid broodstock used to generate the TN11 tetraploids contained 66.5% of the TN08 population allelic diversity. The resulting TN11 progeny showed a 9.9% decrease in the number of alleles compared to their parents, which was not significant ($P > 0.05$). Within both tetraploid populations, observed heterozygosity (*Ho)* was greater than expected heterozygosity (*He)* (Table 3.2).

Table 3.2: Number of individuals (*N*), average number of alleles (*A*), average observed heterozygosity (*Ho*) and average expected heterozygosity (*He*) across eight markers in diploid (2N) and tetraploid (TN08 = tetraploids spawned in 2008, TN08x25 = 30 subsamples of 25 randomly selected TN08s, TN08 broodstock = tetraploids spawned in 2008 and used as broodstock for TN11 and TN11 = tetraploids spawned in 2011 from TN08 broodstock) Pacific oysters.

Population	*N*	*A*	*Ho*	*He*
TN08	50	16.7	0.94	0.85
TN08 broodstock	42	11.1	0.97	0.83
TN11	50	10	0.94	0.83

3.3.2 Pedigree analysis

The broodstock genotypes confirmed that all TN08 broodstock used were tetraploid because of the presence of four different alleles in at least one marker. This result was previously determined via flow cytometry prior to crossing (Andy Day, Shellfish Culture, pers. comm.). Over the 13 microsatellite loci and 137 alleles observed, only three alleles, were observed in the progeny that were not present in the broodstock. Each of these alleles was present at frequencies of less than 2%. Private alleles were present at a frequency of 13.8% within the broodstock. Eight broodstock individuals had one private allele within their genotypes and two individuals had two private alleles.

Using the software wHDP, 84 % of the three month old progeny and 70% of the six month old progeny were able to be assigned to two unique parents. The unassigned 16% three month old progeny consisted of 10% non-assignments (i.e. not able to be assigned to any parent) and 6% ambiguous assignments. An ambiguous assignment (progeny assigned to parents from different crosses) implies that either the sire or the dam was correct, but not both.

Determining which parent was correct could not be calculated with wHDP, hence these samples were excluded from further analysis. The unassigned 30% of the six month old progeny consisted of 21% non-assignments and 9% ambiguous assignments. Out of the 94 six month old progeny sampled, an equal number (32) from each size class were able to be assigned. One third of unassigned offspring were repeated via PCR and analysed to ensure correct microsatellite peak scoring. Scoring error among the repeats was 0%.

At three months of age, all six crosses contributed to the population. At six months, no contribution was recorded for cross one. Given that only 2% of the progeny were from cross one at three months of age, the fact that cross one was not represented at six months of age is likely indicative of fewer individuals being sampled at this time. Two crosses contributed the most to the population in both the three month (cross 3 = 55%, cross 5 = 27%) and six month (cross 3 = 45%, cross 5 = 39%) old progeny. These two crosses showed similar frequencies in both the large (cross 3 = 44%, cross 5 = 47%) and small (cross 3 = 48%, cross 5 = 31%) size grades (Fig. 3.1).

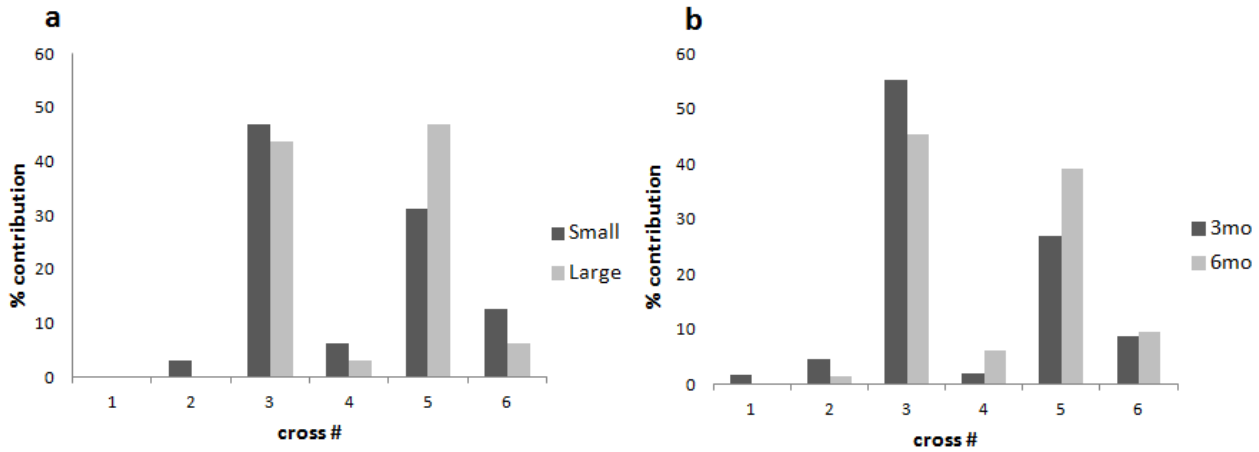

Figure 3.1: Percent contribution of six Pacific oyster crosses to the next generation between small and large size classes at six months of age (a) and at three (3mo) and six (6mo) months of age (b).

Over all the crosses, one male (M5, present only in cross three) contributed the most to the three month (46% contribution) and six month (42% contribution) old progeny. The same four broodstock (two males: M5 from cross three and M14 from cross five and two females: F6 from crosses two and three and F25 from cross five) contributed the most (> 15%) to the next generation in both the three and six month old progeny (Fig. 3.2). Within cross five, one male (M14) contributed 73% and one female (F25) contributed 70% to the number of progeny (n) produced by cross five (n=82). This equated to M14 contributing 21% and F25 contributing 19% to the TN11 population. A similar pattern was observed in cross three with one male (M5) contributing to 79% of the progeny produced by cross three (n=124), which equates to 46% of the TN11 population. However, within cross three, contribution frequencies were more even amongst females, with F6 contributing the most (35%) to the progeny (equates to 21% of the TN11 population). Seven individual broodstock (one male and six females) did not contribute to the population. No difference ($P > 0.05$) was observed in the broodstock contribution between the small and large size classes of the six month old progeny over all six crosses.

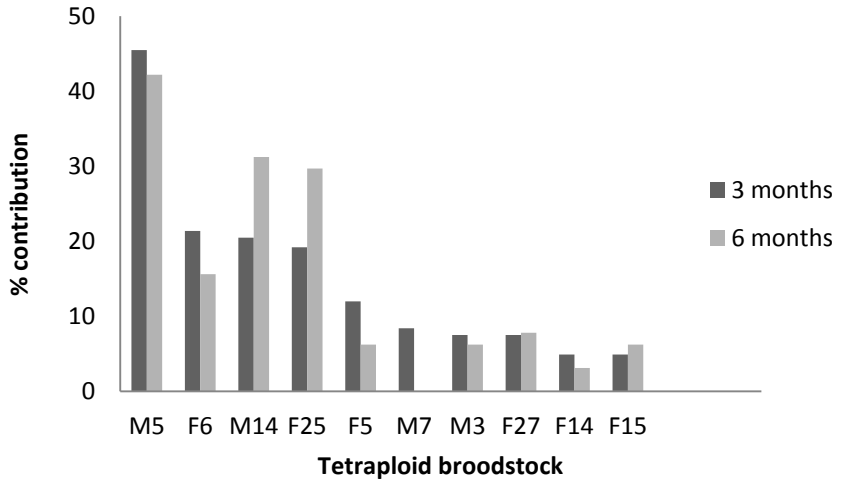

Figure 3.2: The percentage contribution from the ten TN08 tetraploid Pacific oysters that contributed the most to the TN11 progeny population over six crosses at three months of age and their corresponding frequencies at six months of age. F = female and M= male.

The effective population size based on reproductive success was lower than the actual population size (60 - 78% less). The number of effective males was 25 - 83% less than the actual number of males and 0 - 63% less than the actual number of contributing males. A difference (43 - 74%) was also observed in the effective number of females compared to the actual number. Cross 1 showed a higher number of effective females compared to the actual number of contributing females, however, the remaining crosses showed the same patterns as the males (22 - 65% decrease; Table 3.3).

Table 3.3: The census population size, number of broodstock that contributed to the TN11 population (contributors), effective population size based on reproductive success (N_e), actual number of males (N_m), number of males that actually contributed to the TN11 population (male contributors), effective number of males (N_{em}), actual number of females (N_f), number of females that actually contributed to the TN11 population (female contributors) and effective number of females (N_{ef}) across six crosses of Pacific oysters. N_e, N_{em} and N_{ef} were calculated using pedigree results from 308 three month old TN11 progeny. Entire stands for the entire TN08 broodstock population.

	Cross						
	1	2	3	4	5	6	Entire
Pop. Size	8	16	15	8	9	6	42
Contributors	3	8	15	4	8	5	37
N_e	2.2	3.6	4.4	3.0	2.9	2.4	11.7
N_m	4	4	3	3	4	4	14
Male contributors	1	3	3	2	4	3	13
N_{em}	0.7	1.2	1.3	1.7	1.5	3.0	4.3
N_f	4	12	12	5	5	2	28
Female contributors	2	5	12	2	4	2	22
N_{ef}	2.3	3.9	6.0	1.3	1.4	0.8	9.1

3.4 Discussion

3.4.1 Tetraploid diversity

The diversity of the tetraploid populations, calculated via number of alleles, was less than that previously observed for diploids. The average number of alleles observed in a random sample of 50 diploid commercial Pacific oysters from the same hatchery, using the same suite of microsatellites as in this study, was 18.3 (Miller et al., 2012). This is not significantly different to the observed 16.7 value for the equivalent sized random sample from the TN08 population that was derived from mass spawnings of more than 70 putative tetraploid broodstock. However it is debatable whether it is useful or valid to compare microsatellite genetic diversity between tetraploid and diploid populations due to the uncertainty of allele dosage in tetraploids. If we crudely account for ploidy (analyse an equal number of alleles as opposed to an equal number of individuals), the decrease in diversity observed in the TN08 sample would be significant, and suggestive that uncommon or rare alleles may be occurring at lower frequencies or have been lost due to a low effective population size. We can only speculate without knowing allele dosage.

Reasons why the overall number of alleles within the TN08 population was similar to a diploid sample could be due to increased heterozygosity within tetraploids and/or good husbandry practices. The tetraploid oysters showed a much greater *Ho* (> 30%) compared to the diploids. Autotetraploids undergo tetrasomic inheritance, which can lead to an increase in heterozygosity (Soltis and Soltis, 2000). Similar increases in *Ho* have been observed in other species that exhibit tetrasomic inheritance. Mahy et al. (2000) found more than a 30% increase in the *Ho* of tetraploid cranberry (*Vaccinium oxycoccos) and Wolf et al. (1990) showed a similar (36%) increase in Ho for the naturally occurring herbaceous tetraploid Heuchera grossulariifolia* compared to their diploid counterparts. Within aquaculture, triploids show similar increases. Leary et al. (1985) found a 30% increase in heterozygosity

within triploid Rainbow trout (*Oncorhynchus mykiss*) and Taniguchi et al. (1987) found a 60% increase in triploid Ayu (*Plecoglossus altivelis*) compared to diploids.

Due to the difficulties involved in generating a chemically induced tetraploid, many tetraploid populations could start off with closely related triploid individuals (i.e., possible siblings). Tetraploids are then bred with one another to maintain the population, thus increasing the risk of inbreeding depression. The level of diversity observed within this study suggests that the induced tetraploids used to breed the TN08 population were not closely related as hypothesised; rather they were from individuals that captured a wide variety of the available diploid diversity. Hence, the risk of inbreeding depression within this line of tetraploid oysters is likely lower than previously predicted. Furthermore, studies in autotetraploid plants have shown a 50% decrease in immediate inbreeding depression compared to related diploid species (Husband and Schemske, 1997; Galloway et al., 2003; Galloway and Etterson, 2007).

The loss of diversity in the TN11 generation compared to its progenitor population (TN08) was significant. This indicates that, if similar numbers of broodstock and mass selection protocols are used in future spawnings, the diversity may continue to decline at a significant rate, thus increasing the risk of inbreeding depression within the tetraploid population in future generations. Our results clearly show that under the commercial hatchery conditions employed to produce the TN11 population there was a significant difference between the effective population size and the broodstock numbers conditioned and spawned. It is recommended from these results that controlled single paired mating rather than mass spawning is undertaken to ensure genetic diversity is maximised.

3.4.2 Tetraploid assignment

The effective population size, calculated by taking into account reproductive performance, was much lower than the census population. This is not uncommon in marine organisms (Hedgecock, 1994; Frankham, 1995) and has been found previously in farmed diploid oysters (Gaffney et al., 1992; Appleyard and Ward, 2006; Li et al., 2009b). This study found that the difference between the effective population size based on reproductive success compared to the census population size (62%) was much higher than that previously reported (16%) for diploids by Li et al. (2009), but similar to that reported by Boudry et al. (2002) (38% and 68%). Li et al. (2009) sampled the progeny in larval form, before they had been strongly influenced by differential survival or grading, which may have contributed to the difference observed. Boudry et al. (2002), however, recorded effective population size over a period of 90 days, finding a continual decline. Tetraploid oysters may show different fertilisation success rates or juvenile mortality rates compared to diploids. They may also show a greater variation in family survival and growth rates, all of which could influence effective population size.

Pedigree analysis suggested a strong bias in contributions from both individuals and crosses. This concurs with previous studies on diploid Pacific Oysters (Boudry et al., 2002; Li et al., 2009b). Interestingly, a similar number of males and females were the main contributors to the next generation in each cross, suggesting that, unlike many other species (Hedgecock, 1994), sex is not the reason for this observed bias. Additionally, no significant difference was observed in the contribution frequencies of parental oysters at three and six month old or between oysters from the small and large size classes. This suggests that the bias occurs at the time of fertilization or within the first three months and is related to gamete quality, sperm competition, differential genotype survival, etc., and is less influenced by the hatchery

grading out smaller individuals. However, it would be interesting to sample oysters prior to three months of age to determine the effect of initial gradings.

3.4.3 Unassigned tetraploids

Within the pedigree assignment analysis, there were a number of individuals unable to be assigned to potential broodstock. Reasons why these progeny could not be assigned include scoring error, null alleles, or contamination. In this case, scoring error is unlikely to be the reason for non-assignment. Scoring error can occur when there is poor amplification, misprinting, incorrect identification of alleles, contamination or labelling errors within the genotype dataset (Selkoe and Toonen, 2006). In our study, negative controls were used to ensure there was no contamination among samples, samples that showed poor amplification were discarded, and one third of the unassigned individuals were repeated via PCR and independently rescored.

Pacific oyster stocks have been shown to have high frequencies of null alleles (McGoldrick et al., 2000). It is understood that null alleles with a frequency of more than 5% can reduce the efficiency of microsatellite markers to determine parentage in diploid individuals (Marshall et al., 1998). To overcome this difficulty, a large number of microsatellite markers were used for the analysis. Traditionally, four to six markers are considered adequate for pedigree assignment in mixed diploid populations (Li et al., 2009b). It is also likely, that null alleles have less effect on pedigree analysis in tetraploids due to the uncertainty of the genotypes and lower frequency of homozygotes. Hence, null alleles are unlikely to be the major mechanism explaining the presence of unassigned individuals.

A further mechanism to explain the presence of unassigned individuals is double reduction. Tetraploid oysters are classed as autopolyploids, meaning that they originate from within a single species. Autotetraploids generally undergo tetrasomic inheritance, the paring of four

homologous chromosomes during meiosis, and can often experience double reduction. This phenomenon occurs when two sister chromatids enter the same gamete (Ronfort et al., 1998), meaning that a heterozygous individual (e.g. ABCD) can produce homozygous gametes (e.g. AA). Double reduction has been commonly observed in many autotetraploid plant species including maize and potatoes (Levings and Alexande, 1966; Haynes and Douches, 1993). Curole and Hedgecock (2005) calculated the double reduction coefficient in diploid Pacific oysters and found eight cases out of a possible twelve likely exhibiting this phenomenon. The pedigree assignment software used in this study is unable to account for double reduction. Given that Curole and Hedgecock (2005) determined that tetraploid Pacific oysters do experience double reduction, it is possible that some of the unassigned individuals could be the result of this phenomenon. However, given the large number of markers used in this study (i.e. double reduction would have to occur in a number of loci at once), it is unlikely that double reduction had a significant impact on the results.

Contamination at spawning appears to be the best hypothesis to explain the non-assigned individuals since this would also explain the presence of three alleles in the progeny that were not present in the broodstock. Approximately one third of the unassigned broodstock carried one or more of these extra alleles. The frequency of these alleles (1-2%) indicated that they were not likely to be the result of spontaneous mutations. It is important to note that, in samples that were repeated, scoring was consistent across all the markers (i.e., observed scoring error = 0%). Additionally, 13 markers were used for assignment, with an individual only having to match in five to be assigned. Given that oyster spawning is a wet system, the opportunity for accidental transfer of eggs and/or sperm is high. In this case broodstock oysters were not strip spawned, rather they were housed in a single tank with other tetraploids (not used as broodstock) right up to the point of spawning. Once the oysters began to spawn, they were washed and moved to individual tanks, that eggs and sperm were later collected

from. During the washing process, however, eggs/sperm from other oysters could have remained trapped within the shell. Additionally, contamination could have arisen from inadequate washing of equipment. It is important to note, that only three unique alleles were observed in the progeny. This, coupled with the low frequency of private alleles present in the broodstock, suggests that if contamination did occur, it was likely from only a small number of individuals.

All the progeny from the six crosses were pooled 24 h after fertilisation. Hence, when sampling, there was no way to identify which individual belonged to which cross. If the pedigree assignment was incorrect, you would expect to see individuals allocated to broodstock in separate crosses (e.g. a sire of cross 1 and a dam of cross 6). However, the probability that the assigned progeny were paired with the correct sire and dam is high. This is because the wHPD program allocated every assigned individual to the correct cross (i.e. assigned parents from the same cross).

3.4.4 Conclusion

The results of this study suggest that, with respect to the diploid population, the overall number of alleles within the TN08 tetraploid population was high, but dropped significantly in the TN11s, likely due to less broodstock used to create the TN11 population, compared to the TN08 population, which would be the primary reason for the observed diversity decrease. Additionally, low effective population sizes due to differential parental contributions would have decreased diversity further. Individual pair crossing is proposed to ensure maximum diversity in producing tetraploid generations in the future.

Chapter 4

Assignment of parentage in triploid species using microsatellite markers with null alleles, an example from Pacific oysters (*Crassostrea gigas*)

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4.1 Introduction

Mass spawning, a common technique used within aquaculture programs, typically involves the simultaneous spawning of many individuals in a single tank. Whilst this is more time, space and cost effective than single pair cross-spawning (Perez and Alfonsi, 1999), the exact pedigree of the resulting progeny is unknown and cannot be determined without molecular analysis. For this reason, numerous parental assignment software programs have been developed for traditional diploid breeding programs (Cercueil et al., 2002; Piry et al., 2004; Coombs et al., 2010). However, when it comes to analysing polyploid data, very few programs are available.

There are at least two software programs developed to analyse tetraploid pedigree data (Van Puyvelde et al., 2010; Galli et al., 2011), but to our knowledge, there have been no programs designed to assign triploid progeny, produced by crossing autotetraploids and diploids, to their corresponding parents. The primary difficulty in assigning pedigrees in autopolyploid populations is genotype uncertainty (De Silva et al., 2005). For example, a triploid may have the genotype AAB, however DNA profiling using microsatellite markers is unable to determine if the correct genotype is AAB or ABB, because the duplicate allele is masked. Whilst it might be possible to determine dosage using either microsatellite DNA allele counting-peak ratios (MAC-PR) (Esselink et al., 2004) or PCR product intensities (Buteler et

al., 1999; McQuown et al., 2002), these techniques are often laborious and caution must be taken to rule out potential artefacts (Landergott et al., 2006). Determining dosage is further complicated by the presence of null alleles: alleles that do not amplify during PCR for reasons including mutations in the primer annealing site, differential amplification depending on allele size (partial nulls) and PCR failure (Dakin and Avise, 2004). They generally occur at low frequencies meaning they are unlikely to significantly bias parental assignment results in diploid populations (Dakin and Avise, 2004). In some species however, null alleles have been reported at high frequencies. Pacific oysters (*Crassostrea gigas*) have been shown to have very high frequencies of null alleles, likely due to a high level of nucleotide polymorphism (Hedgecock et al., 2004). McGoldrick et al. (2000) reported a null allele frequency of 22% in Australian Pacific oyster stocks. Dakin and Avise (2004) reported that high frequencies of null alleles can lead to an increase in false parentage exclusions and result in substantial errors. Hence, it is valuable for pedigree assignment programs to allow for null allele errors.

One software program that performs parental assignment in tetraploids is w-HDP (Galli et al., 2011), which uses both an exclusion method and a likelihood approach to assign progeny to potential broodstock. W-HDP was designed for use in autotetraploid sturgeon (*Acipenser naccarii*) populations. A study by Miller et al. (in press) used w-HDP to successfully assign pedigree in a tetraploid population of Pacific oysters. W-HDP cannot be used with other levels of ploidy and is unable to correct for null allele errors. Thus, no parental assignment program has yet been available for the analysis of triploid oysters.

Triploid production in aquaculture is of major international commercial interest, due to their sterility and production characteristics (Allen and Downing, 1986; Benfey, 2001; Garnier-Gere et al., 2002; Maldonado-Amparo et al., 2004; Piferrer et al., 2009). Commercial

triploids are mostly produced directly from diploid broodstock crosses and a stress shock (e.g. pressure, temperature) at either meiosis I or meiosis II (Piferrer et al., 2009). Within oyster aquaculture however, the common practice is to cross diploid female broodstock with tetraploid male broodstock to obtain triploids (Guo et al., 1996). The tetraploid oysters arise from chemical inductions but, unlike triploids, are fully fertile and can be perpetuated to maintain a tetraploid broodstock population. Between 2007 and 2008 in New South Wales (Australia), triploid Pacific oysters accounted for 40% of production (O'Connor and Dove, 2009). In Europe and the USA, it represents 20-50% of production (Piferrer et al., 2009). In France, triploids accounted for 80% of the 800 million spat sold by hatcheries in 2005 (Buestel et al., 2009). Demand for triploid oysters is growing rapidly (Nell, 2002), yet very little research has been completed on genetically improving these oysters.

Given that triploids are mostly sterile (i.e. an end product, not contributing to a subsequent generation), coupled with the fact that having a high degree of diversity and low degree of inbreeding is not necessarily important within triploid populations (because they will not be used as broodstock), it can be asked, what is the importance of determining triploid pedigrees? The answer lies within selective breeding to enhance triploid performance.

Currently, family selection programs only exist within diploid Pacific oysters (Ward et al., 2000; Kube et al., 2011). Crossing a selected diploid oyster with a mass-spawned tetraploid does not guarantee that the resulting triploids will have the desired traits. If, however, triploid and tetraploid pedigree can be determined, the breeder will be able to pick out strongly performing triploid families (e.g. better growth, disease resistance, and morphology) and, by applying quantitative genetic analysis, manage the diploid and tetraploid populations and select the best combinations to spawn for more desirable commercial triploids. The same

principle could apply to salmonids and other commercial breeding programs producing triploid cohorts.

This study uses a likelihood approach to assign parentage to triploid oysters (i.e. triploids arising from tetraploid cross diploid broodstock), however, with minor modification, this method could have applications in other triploid species including watermelons (Omran et al., 2008), salmonids, bream, prawns and mud loach (Piferrer et al., 2009). The results of the parentage analysis are used to determine the effective population sizes (i.e. the number of individuals contributing to the next generation) and potential biases (i.e. different contribution rates between males and females) associated with triploid Pacific oyster populations.

4.2 Materials and methods

4.2.1 Sample collection, DNA extraction and PCR

All oysters used in this study were supplied by Shellfish Culture Limited, Tasmania. Two separate mass-spawned crosses were used for analysis (line 1 = five tetraploid sires and ten diploid dams; line 2 = five tetraploid sires and eight diploid dams). At approximately three months of age, 105 triploid progeny from each cross were randomly sampled.

DNA was extracted from all 28 broodstock and 105 randomly selected progeny from each cross. DNA was extracted from gill tissue using the modified extraction protocol with NaCl described by Lopera-Barrero (2008), with the small modification of 2µl of 20mg/ml proteinase K added instead of that stated. The DNA was not treated with RNAse. A 1-in-10 DNA dilution was performed prior to polymerase chain reaction (PCR). All DNA was stored at -20ºC.

The multiplex suites described by Miller et al. (2012) [eight microsatellie markers (*ucdCg120, ucdCg126, ucdCg160, ucdCg166, ucdCg175, ucdCg198, um2Cg10* and *imbCgi49*) chosen based on scoring performance and minimal missing data], with the inclusion of four additional markers, *ucdCg138* (suite 4) *ucdCg174* (suite 4) *ucdCg184* (suite 3) *ucdCg186* (suite 1) (Li et al., 2003), were used for this study. PCR was performed in 5µl reactions using the Qiagen multiplex PCR kit as per the manufacturer's instructions (Qiagen, Australia). Cycling conditions for PCR were as follows: 15 min at 95ºC; 30 cycles of 30 s at 94ºC, 90 s at 60°C, 90 s at 72ºC; 30 min at 60ºC. A 1 in 160 dilution of PCR product was used as template for separation on an ABI 3770 (Applied Biosystems, Australia) sequencer using LIZ 500 (-250) size standard by the Australian Genome Research Facility (Adelaide, Australia). Alleles were scored using GENEMAPPER v.3.7 software (Applied Biosystems, Australia). Approximately 5% of the samples were independently repeated within and across plates. More than 10% of the broodstock samples were independently repeated to ensure consistent scoring.

4.2.2 Statistical method

The method consists of two major components: estimation of the likelihood for each sire/dam/offspring trio, and the determination of the appropriate threshold for declaring parentage. Similar to Marshall et al. (1998), the likelihood is essentially a function of the number of inconsistencies between a set of feasible genotypes for progeny for the parent pair and the genotype of progeny under the assumption of random segregation. In the method of Marshall et al. (1998) population allele frequencies are used in calculating the likelihood, but these are difficult to estimate in polyploid populations (Galli et al., 2011), so in our method allele frequency estimates are not used. Unlike the autotetraploid pedigree analysis software w-HDP (Galli et al., 2011), probabilities of different types of errors are explicitly modelled.

A worked example of the likelihood calculations appears in the appendix. In order to estimate the likelihood for each sire/dam/offspring trio, the first step is, for each individual, for each marker, to form a vector (**U**) of length *n* representing our best estimate of the underlying genotype, where *n* is the number of alleles. Elements of **U** are estimates of allele frequencies within the individual (and hence sum to 1.0), based on the observed genotype, the range of possible genotypes, and assumptions regarding the distribution of genotyping errors. **U** is proportional to the sum $\mathbf{X} + \mathbf{G} + \mathbf{B}$ where **X**, **G** and **B** (described in more detail below) are vectors relating to the observed genotype, the probabilities of null alleles, and the probabilities of allele base pair shift errors respectively (to assist with readability we omit subscripts for the individual and marker).

In the derivation of $\mathbf{X} = (X_1, \dots, X_i, \dots X_n)$ we assume that dosage cannot be measured, alleles are observed or not observed. So, if a total of *q* alleles were observed to be present for an individual,

$$X_i = \begin{cases} 0, & \text{allele } i \text{ was not observed} \\ \dfrac{1}{q}, & \text{allele } i \text{ was observed} \end{cases}.$$

The first form of error we include in the model is the probability that an individual carries an allele although that allele was not observed to be present, i.e. probabilities of null alleles, represented by the vector $\mathbf{G} = (G_1, \dots, G_i, \dots G_n)$. We model the probability of a null allele in a genotype vector as constant whenever fewer alleles are observed than the degree of ploidy, regardless of the number of alleles at the marker and the number of alleles observed. For an individual with degree of ploidy *m*, the vector of null allele probabilities **G** is formed where

$$G_i = \begin{cases} 0, & m = q \\ 0, & \text{allele } i \text{ was observed} \\ \dfrac{\theta}{n-q}, & \text{otherwise} \end{cases}$$

where θ is a parameter representing the rate of null alleles. The appropriate value for this parameter varies between species, and most likely between markers.

The second form of error we include in the model is that of incorrectly assigning an observed genotype, which we refer to as an allele base pair shift error. For microsatellite alleles scored as integer counts of base pairs, we assume that the probability of allele *i* being incorrectly called as allele *j* declines as the distance between alleles *i* and *j* increases. We model this decline using a geometric distribution with parameter *p*. As with the null allele rate, the appropriate value for the parameter *p* may not be known with any degree of certainty. The vector of base pair shift allele probabilities $\mathbf{B} = (B_1, \ldots, B_i, \ldots B_n)$ is formed where

$$B_i = \begin{cases} 0, & \text{allele } i \text{ was observed} \\ \sum h_{ij}, & \text{allele } i \text{ was not observed} \end{cases}$$

where, if *d* is the distance in base pairs between alleles *i* and *j*, $h_{ij} = (1-p)^d p$ if allele *j* was observed, and 0.0 otherwise.

Alternative functions for the distribution of null alleles and for the distribution of allele base pair shift errors are easily implemented and would be appropriate if the true distributions are known or suspected to be different to those described here. Having to assign values to the two parameters θ and *p*, possibly based on little empirical data, is not ideal, but in practice it is no more arbitrary than assigning an error rate parameter in the method of Marshall et al. (1998). Additional error terms, such as probabilities of sample swaps, could also be included in calculating **U**.

The second step in estimating the likelihood for each sire/dam/offspring trio is, for each marker, given vectors $\mathbf{U^s}, \mathbf{U^d}$ and $\mathbf{U^o}$ relating to the sire, dam and offspring respectively, to estimate the contribution of the marker to the likelihood. If both parents were diploid then the matrix $\mathbf{T^{sd}} = \mathbf{U^s U^{d'}}$ would contain the ordered genotype probabilities expected from that pairing, so $\mathbf{T^{sd}}$ is the transmission matrix for the marker from parents s and d. In our case, where the sire is tetraploid and the dam diploid, instead of an (*n* x *n*) transmission matrix, $\mathbf{T^{sd}}$ is an (*n* x *n* x *n*) 3-dimensional transmission array; $\mathbf{T^{sd}} = \mathbf{T^s} \otimes \mathbf{T^d}$ where $\otimes$ indicates the outer product, $\mathbf{T^s}$ is a 2-dimensional transmission matrix for the sire, and $\mathbf{T^d}$ is a 1-dimensional transmission matrix for the dam. The transmission matrix for the diploid dam is simply $\mathbf{T^d} = \mathbf{U^d}$ and the (*n* x *n*) sire transmission matrix $\mathbf{T^s}$ is proportional to $\mathbf{U^s U^{s'}}\text{diag}(w_{1,} \ldots, w_n)$, where diagonal elements $\text{diag}(w_{1,} \ldots, w_n)$ relate to the probability of there being two copies of the allele available to transmit to the offspring. The derivation of $\text{diag}(w_{1,} \ldots, w_n)$ is illustrated through example. Ignoring for the moment errors due to null alleles and allele base pair shifts, possibilities are:

1. If four alleles are observed in the sire (i.e. $X_i = \frac{1}{4}$ for these alleles), then he does not have two copies of any of them to pass on to progeny, w_i should be zero for all alleles.
2. If three alleles are observed in the sire (i.e. $X_i = \frac{1}{3}$ for these alleles), then the sire has two copies of one allele, and one copy of each of the others. Assuming equal allele frequencies, there is a $\frac{1}{3}$ chance of there being two copies available for any particular allele, so w_i should equal $\frac{1}{3}$ for each of the three alleles observed, and zero elsewhere.
3. If two alleles are observed in the sire (i.e. $X_i = \frac{1}{2}$ for these alleles), then there are two possible genotype configurations: one copy of one allele and three of the other, or two

copies of each allele. Assuming equal frequencies for the alleles, enumerating the combinations of the two configurations gives 14 combinations, of which 10 contain two or more copies of one allele, and 10 contain two or more copies of the other allele. So, w_i should equal $\frac{10}{14}$ for each of the two alleles observed, and zero elsewhere.

4. If only one allele is observed in the sire (i.e. $X_i = 1$ for this allele), then he has four copies of that allele, and w_i should equal one for that allele, and zero elsewhere.

Because elements of vector $\mathbf{U^s}$ have been perturbed to account for the probabilities of null alleles and base pair shifts, it is necessary to estimate values for w_i for values of U_i that lie between the four possible values of X_i listed above, and for this we use a linear interpolation. Finally, $\mathbf{T^s}$ is produced by normalising $\mathbf{U^s U^{s'}}\text{diag}(w_1, \ldots, w_n)$ to sum to one.

To estimate the likelihood of the offspring allele probability vector $\mathbf{U^o}$ given the transmission array for the parents $\mathbf{T^{sd}}$, we first build the 3 dimensional ordered genotype array for the offspring; $\mathbf{R^o} = \mathbf{U^o} \otimes \mathbf{U^o} \otimes \mathbf{U^o}$. Introducing a superscript (*m*) for the marker, the likelihood at marker *m* for sire s, dam d and offspring o then is the sum of the entrywise product of $\mathbf{R^{(m)o}}$ and $\mathbf{T^{(m)sd}}$; $L^{(m)sdo} = \sum_i \sum_j \sum_k R_{ijk}^{(m)o} T_{ijk}^{(m)sd}$. This likelihood is estimated for all markers, and the product calculated to provide the likelihood (L^{sdo}) for the hypothesis that sire (s) and dam (d) are the parents of offspring (o); $L^{sdo} = \prod_m L^{(m)sdo}$.

4.2.3 Simulated dataset

To help validate the software developed, a data set consisting of four sires, four dams, 1000 offspring and ten microsatellite markers was generated. Real dam and sire data was used to create the dataset, with tetraploid sire allele dosages invented. Whilst allele dosages were

known for the construction of the dataset, they were removed for testing the method. The progeny genotypes were created using a random number generator. Once run, the dataset was perturbed to simulate the presence of null alleles, distributed evenly across all markers. This new dataset was analysed using three different null allele frequency settings (0.01, 0.1 and 0.2) to determine the effect of changing this setting on the accuracy of the program. A second dummy dataset containing an average null allele frequency of 10% across all markers was created and tested with null allele frequency settings of 0.01 and 0.1.

4.2.4 Declaring parentage thresholds

As with Galli et al (2011), progeny were simulated from real parent genotypes using Mendelian inheritance rules appropriate to the levels of ploidy of parents and offspring, and these pedigrees were used to determine appropriate thresholds for declaring parentage. Unlike Galli et al (2011), the simulations and thresholds are experiment specific, to account for the degree of relationship between prospective parents. This is similar to the approach of Marshall et al. (1998) except that they use allele frequencies rather than simulated meioses to generate the simulated progeny. In addition, by comparing the distribution of likelihoods from the simulated progeny to the distribution of likelihoods from real progeny, the error parameters can be fine-tuned.

The method was implemented in the R statistical computer language (http://www.r-project.org/), but can be easily implemented in any computer language that supports matrix algebra.

4.2.5 Statistical analysis of triploid oysters

The genotypes from the two triploid lines were analysed separately to avoid confusion (despite test runs showing that the program was able to successfully separate the crosses). Each line was analysed using three different null allele frequencies (0.05, 0.1 and 0.2). Null allele frequencies were set based on results from Miller et al. (in press) who found null allele frequencies between 0.03 and 0.21 (average 0.17) in eight of the twelve markers used in this study. The likelihoods obtained from the different null allele frequency runs for each cross were compared. Any sample that showed inconsistencies across the three runs was removed.

The effective population size (N_e) was determined based on reproductive success. Effective number of sires was calculated using $N_{em} = (N_m\bar{k}_m - 1) / [\bar{k}_m + (\sigma^2_{km}/\bar{k}_m) - 1]$, where N_m is the actual number of sires, $\bar{k}_m$ is the average number of progeny sired by a single sire and σ^2_{km} is the variance of $\bar{k}_m$ (Lande and Barrowclough, 1987). This same method was used to calculate the effective number of dams. Using these results, the effective population size was calculated using $N_e = 4N_{em}N_{ef}/(N_{em}+N_{ef})$.

4.3 Results

4.3.1 Software performance

Within the independently repeated (via PCR) samples, scoring error was 0%. For the simulated dataset, assignments were consistent across all null allele frequencies (0.01, 0.1 and 0.2). All simulated progeny were assigned to the correct sire and dam. When the 10% null allele simulated dataset was analysed at the first null setting (0.01), the error rate was 0.45%. When this same dataset was run at the true null setting (0.1), the error rate reduced to 0.05%. Within the real data, comparison of the three software runs for different null allele frequencies (0.01, 0.1 and 0.2) resulted in a 4% error rate (i.e. 16 inconsistent assignments

out of 420). Inconsistent samples were removed from further analysis. This brought the final sample sizes for each line down to 92 progeny for line 1 (the dam assignment of 10 progeny, the sire assignment of two progeny and both assignments in one progeny were inconsistent) and 101 progeny for line 2 (the dam assignment of two progeny and the sire assignment of two progeny were inconsistent). This means that the program was able to unambiguously assign 92% of the triploid progeny.

4.3.2 Triploid parental assignment

Figure 4.1 graphically displays output for an example of the line 1 dataset with the null allele frequency set at 0.1. Each graph shows the maximum log likelihood (x) vs. Δ (the difference in likelihood between the most likely parent and second most likely parent). The colours on this figure represent simulated data and the black points are the actual data. The line 1 and line 2 datasets showed very similar outputs. The data spread for both lines lay somewhere between figures 4.1c and 4.1d across all null allele frequencies. This indicates that many of the assignments are likely correct, however there could possibly be some incorrect dams. The vast majority of samples were between the 5^{th} and 95^{th} percentiles, which were determined from simulated data.

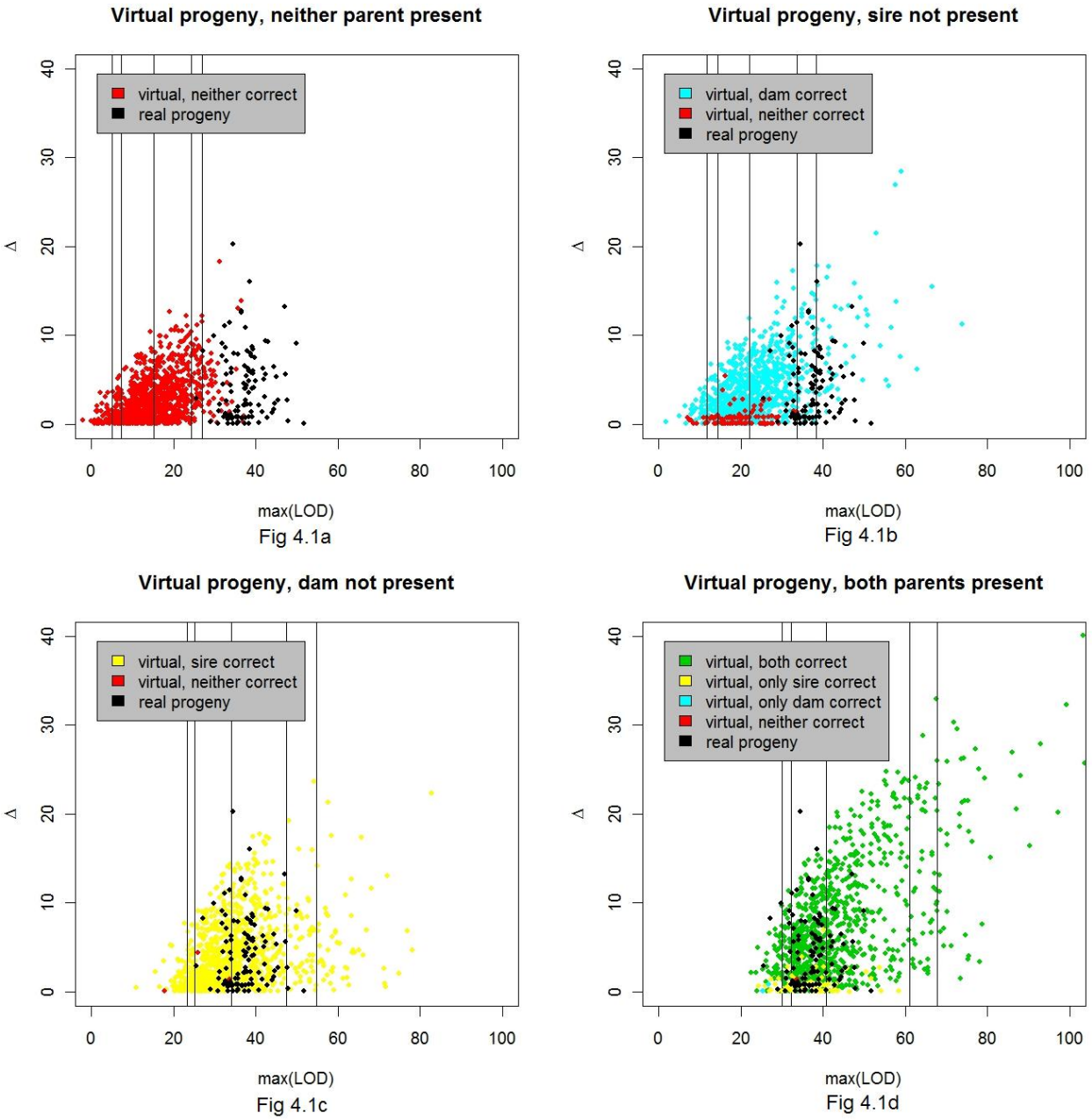

Figure 4.1: Graphical display results output from R for triploid parental assignment. Maximum log likelihood vs. Δ (the difference in likelihood between the most likely parent and second most likely parent), for 105 Pacific oyster progeny (actual data in black). Null allele frequency was set at 0.1 and number of simulations used to create median and percentiles was 1000. The coloured points indicate simulated data used to set the median (third vertical line from axis) and percentiles (the first vertical line is the 1st percentile, the second is the 5th, the fourth is the 95th and the last is the 99th). In Fig. 4.1a the red points indicate the distribution of the expected data if neither parent assigned to the progeny were correct. Fig. 4.1b is the expected spread if most of the

individuals were assigned to the correct dam, but incorrect sire. Fig. 4.1c is the expected spread if the sire was correct and the dam incorrect and fig. 4.1d is the expected spread if most of the individuals were assigned to the correct parents.

The parental contribution frequencies for both lines showed biases. Within the line 1 cross, two sires (male 3 = 33 progeny (36% of progeny have this sire) and male 4 = 40 progeny (43%)) and three dams (female 1 = 20 progeny (22%), female 2 = 20 progeny (22%)) and female 3 = 18 progeny (20%)) contributed the most to the next generation. Within the line 2 cross three sires (male 2 = 20 progeny (20%), male 4 = 27 (27%) and male 5 = 39 (39%) and one dam (female 1 = 34 progeny (34%)) contributed the most to the next generation (Fig. 4.2). Within both crosses, only one individual broodstock (female 10 from line 1) did not contribute to the progeny. Chi-square tests showed that the observed parental contributions were significantly different from expected (the hypothesis was equal contribution of each parent) for both sires and dams in each line ($P > 0.001$).

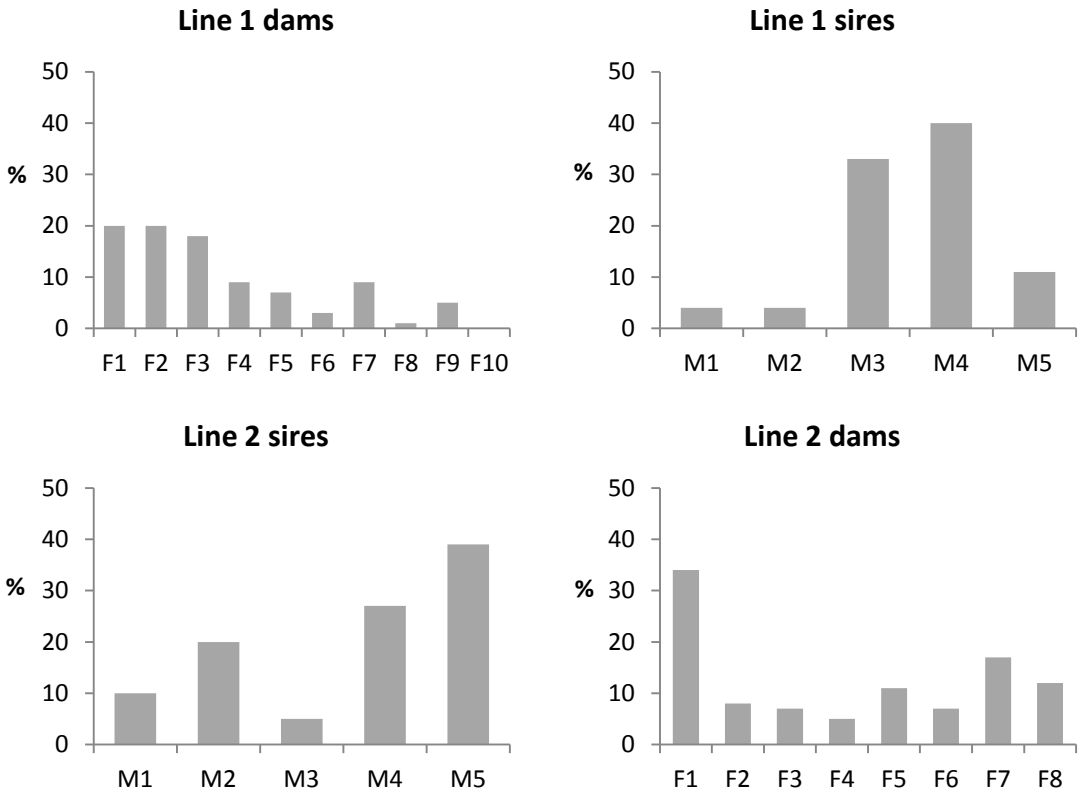

Figure 4.2: The contribution of each tetraploid sire (line 1 M1-5 and line 2 M1-5) and diploid dam (line 1 F1-10 and line 2 F1-8) to the triploid population within two mass spawned crosses (line 1 and 2) of Pacific oyster

The effective population size of each sire and dam was 49% lower than the actual population size for the line 1 cross and 34% lower for the line 2 cross. Effective numbers of males and females were also lower than the actual number, but similar between crosses. Despite having more broodstock, the line 1 cross had a smaller effective population size based on reproductive success than the line 2 cross (Table 4.1).

Table 4.1: Population size, effective population size based on reproductive success (N_e), actual number of tetraploid sires (N_m), effective number of tetraploid sires (N_{em}), actual number of diploid dams (N_f) and effective number of diploid dams (N_{ef}) in two mass spawned crosses (line 1 and line 2) of triploid Pacific oysters

	Line 1	Line 2
Pop. size	15	13
N_e	7.7	8.5
N_m	5	5
N_{em}	2.8	3.5
N_f	10	8
N_{ef}	6.3	5.4

4.4 Discussion

The parental assignment method was able to unambiguously assign 92% of the Pacific oyster triploid progeny to their most likely tetraploid and diploid broodstock using three different null allele frequencies. The remaining 8% of the progeny assignments were ambiguous, which could be a result of null alleles, genotyping errors or mutations. The effects of the latter two, however, were likely minimal because no scoring error was detected, suggesting that null alleles were probably the major cause of ambiguous assignments. If the actual null allele rate was known, assignment accuracy would have been increased.

When no null alleles were present, all simulated progeny could be assigned to the correct broodstock. When null alleles were included this accuracy dropped to 0.05%, suggesting that the method is highly accurate at parental assignment in triploid oysters when the null allele is known. However, statistical methods for estimating null allele frequencies from microsatellite data are not perfect (Girard and Angers, 2008), which is why for the real data, three putative null allele frequencies were used, with inconsistent assignments removed. Our results are comparable to the tetraploid pedigree assignment program, w-HDP (Galli et al. (2011), which

had an accuracy of 93.7% unambiguously correct assignments in virtual progeny. The accuracy in the simulated data was likely higher than in the real data due to the actual null allele frequency being unknown and an increase in the number of broodstock being analysed.

This study uses a likelihood approach and threshold determination method as opposed to an exclusion or combination of exclusion and likelihood approaches. A limitation of using an exclusion approach for parental assignment is erroneous exclusions due to genotyping errors, null alleles and mutations (Jones and Ardren, 2003). This is particularly problematic in Pacific oyster studies due to their known high null allele frequency and rapid mutation rate (McGoldrick et al., 2000; Hedgecock et al., 2004). A likelihood approach is more informative to a breeding manager as it allows situation specific exclusions to be made. For example, from the graphical output, the spread of both the triploid cohorts data (line 1 and line 2) suggested that the majority of the progeny were assigned to the correct parent, but some of the dam assignments may be incorrect. For a more precise result, a percentage of the progeny with the lowest likelihood values could be removed from the analysis, however this could result in correct assignments being excluded. In the case of the line 1 and line 2 cohorts, there were no outliers and all data lay close to the median (well within the 5^{th} and 95^{th} percentile range) for correct assignment. This suggests that most assignments made are correct. If an individual has been assigned to the wrong dam, as the spread suggested, it is likely that the incorrectly assigned dam is closely related to the real progenitor. Relatedness of broodstock is common within commercial mass spawnings (Scott Parkinson, per. comm.), as parental pedigrees are unknown and broodstock are generally selected based on family breeding values and physical appearance as opposed to diversity. Strip spawning is the most commonly used method in oyster breeding. Given that it is destructive, the original broodstock can no longer be used as progenitors. This means that closely related individuals will be required as future broodstock for selectively bred triploids.

One of the primary benefits of our method is the ability to incorporate a null allele frequency. Null alleles are a common cause of erroneous parental exclusion, or a reduction in likelihood, in many pedigree analysis studies (Dakin and Avise, 2004). Within oysters, high null allele frequencies have previously been reported (McGoldrick et al., 2000; Miller et al., 2012) which indicates a likely decrease in the probability that progeny will be assigned to the correct parent. By including a null allele error rate, assignment is more flexible, which decreases the rate of erroneous exclusion. Galli et al. (2001) gave the option of "no match locus" as a way to account for null alleles. If selected, any individual that did not match at more than two loci were discarded from the analysis. This implied that, if all 12 markers were used at once to analyse the dataset, then only individuals that matched at nine or more loci could have their parentage determined. This approach works well in datasets where there is a low null allele frequency. For datasets, particularly oysters, which are known to have a high null allele frequency, this method may result in an increase in erroneous exclusions (Dakin and Avise, 2004). The benefit of including a null allele error can be seen with the results of the simulated dataset which contained a 10% null allele frequency. When run without correcting for null alleles the error rate was 0.45%. This error rate was reduced to 0.05% when null alleles were accounted for.

Whilst oysters are the best known and advanced aquaculture triploid, they are not alone. Triploid Atlantic salmon (*Salmo salar*) have been developed since the 1980s (Benfey and Sutterlin, 1984; O'Flynn et al., 1997). Unlike oysters, triploidy is typically induced in salmon through hydrostatic pressure and may use masculinised females (i.e. males with a XX-genotype) to create an all female triploid population (Benfey, 2001). As with oysters, triploid Atlantic salmon are generally produced via mass spawning, hence parentage is often unknown. Other triploid salmonoids (induced in a similar way to the Atlantic salmon), such as the rainbow and brown trout, have also been trialled for aquaculture. Similar to salmon,

triploidy has been successfully induced in many prawn species (Bao et al., 1994; Sellars et al., 2009; Burge et al., 2011). However, triploidy has not been implemented at a commercial scale due to the fragility of the embryos. Studies are taking place to overcome this difficulty. With some small changes, such as altering the data input to reflect that both parents are diploid and the female contributing twice the genetic material to the triploid offspring, our method should be able to successfully analyse data from these species. Given that the majority of parental assignment software programs do not allow for polyploid data, our method will be a useful tool in improving triploid breeding in aquaculture.

The ability to measure and identify triploids with desirable traits and trace their pedigree back to their broodstock should appeal to the industry. In Australia selection programs have been in place for diploid oysters for the past 16 years (Kube et al., 2011). Over this time, the diploid lines have shown performance improvements (Ward et al., 2005). No such selection program currently exists for tetraploid or triploid oysters. Allele dosage and ploidy effects (such as segregation and dominance in polyploids), are currently poorly understood in oysters, and likely play a strong role in determining trait expression in triploids. Hence, simply crossing a diploid from the selection program with a standard tetraploid, without progeny testing, will not guarantee that triploid will have the desired trait. However, through triploid progeny testing and pedigree assignment, the opportunity exists for selecting diploid and tetraploid broodstock to ensure optimal commercial triploid performance.

Similar to mass spawned cohorts of diploid and tetraploid Pacific oysters (Li et al., 2009b; Miller et al., in press), triploid cohorts also show a strong bias in parental contribution leading to the effective population size being much smaller than the census population. This highlights the haphazardness of mass spawning, especially if highly valuable and selected broodstock are being used. To enhance the production of triploid oysters in the future, there is

a need to develop an effective of breeding program. This will likely involve both family selection and a shift from mass spawning to controlled single pair crossing. Accurate parental assignment in triploid offspring will be a valuable tool in the selection of diploid and tetraploid broodstock for triploid production. Whilst the initial investment in changing the breeding design will be high (e.g. increased infrastructure costs, more time consuming), the benefits, already observed in a number of diploid breeding programs, should be significant (Gjedrem et al., 2012b).

Chapter 5

Chromosome stability in successive generations of tetraploid oysters using flow cytometry

5.1 Introduction

Tetraploid oysters have been an important part of aquaculture for many years due to their role as progenitors of commercial triploids (Guo et al., 1996). The technique of crossing tetraploid with diploid oysters to produce triploids is more reliable and effective than previous chemical induction methods (Guo and Allen, 1994b; Guo et al., 1996). Tetraploid oysters can also be crossed with each other to maintain a tetraploid breeding population. The long term stability and sustainability of a tetraploid population, however, has been the focus of much speculation (McCombie et al., 2005a; Zhang et al., 2010b).

Polyploidy is not a natural state for the Pacific oyster and, hence, may potentially be unstable (McCombie et al., 2005a). Within this species, chromosomal mosaic individuals (those with variable chromosomal numbers in different cells) and aneuploidy (abnormal number of chromosomes within a cell) have been described (Allen et al., 1999; Wang et al., 1999). Additionally, previous research has shown that the Pacific oyster is able to tolerate a variety of chromosome numbers (Gong et al., 2004), indicating that aneuploid and/or mosaic oysters can be viable. Low levels of somatic aneuploidy are a natural occurrence within many species of plants and animals (Wenger et al., 1984), however, aneuploidy rates within diploid Pacific oysters have been shown to exceed this natural level (Thiriot-Quievreux et al., 1992; Leitao et al., 2001b). Likewise, high aneuploid frequencies have also been observed in triploid Pacific and Sydney Rock oysters (*Saccostrea commercialis*) (Allen et al., 1999; Hand et al., 1999), but has not been well documented. These studies suggest an increase in chromosome loss

with age and/or environmental stress such as pollutants. The long term impact of this loss on growth, survival and reproduction is currently unknown.

Having a robust, highly fecund population of tetraploid oysters is vital for a commercial breeding company producing triploids. The tetraploid broodstock should be maintained and selected to produce fast growing and high survival triploids for commercial consumption, and to perpetuate future generations of equally robust and enhanced tetraploid broodstock. . Research by Wang et al. (1999) reported that 18 of the 90 (20%) triploid oyster progeny examined from three tetraploid by diploid crosses were aneuploid, likely resulting from segregation errors during meiosis. Studies on plants have shown that aneuploid individuals produce high frequencies of aneuploid progeny (Doyle, 1986). If the same occurs in oysters, then a high aneuploid frequency within the tetraploid population could have carry-over effects to the next generation of tetraploid broodstock and the subsequent commercial triploid product.

A number of studies have documented a slower growth rate of aneuploid individuals within diploid oysters (Thiriot-Quievreux et al., 1988; Zouros et al., 1996; Leitao et al., 2001b), but very little research has be conducted on growth and survival of aneuploids from triploid and tetraploid production. Additionally, the effect that aneuploidy has on the sterility rate of triploid oysters is unknown. Genetic sterility is believed to be linked to increased growth as less energy is expended on reproduction and has the important benefit of allowing high quality triploid meat to be harvested all year round, whereas diploid meat quality is low during reproductive seasons (Wang et al., 1999). Additionally, in Australia, the Pacific oyster is regarded as a feral species, with restrictions in place limiting areas to the production of triploid oysters only. If high aneuploid frequency within the tetraploid breeding population has carry over effects to the triploid product, this could potentially affect the sterility rate, and ultimately reduce growth and meat quality.

A study by McCombie et al. (2005a) used chromosome counts to determine the aneuploid frequency of tetraploid Pacific oysters from six bi-parental families over one year. A high aneuploid frequency was observed within the tetraploids (53%), with differences in chromosome loss between families suggesting a genetic basis for aneuploidy. No difference however, was observed in samples taken at four months of age compared to one year of age. McCombie et al. (2005) was unable to analyse oysters older than one year. If aneuploid frequency increases with time, it could be that a longer time frame is needed to observe significant differences. This current study aims to use flow cytometry to determine if aneuploid frequency increases over subsequent generations in tetraploid oysters.

Flow cytometry is a laser based method to estimate DNA quantity in cell nuclei to determine ploidy. Whilst it is a less sensitive method of detecting aneuploidy frequency compared to chromosome counting, it is more time and cost effective allowing a greater number of samples to be analysed. Flow cytometry has previously been shown to be sensitive enough to detect aneuploidy in plants (Pfosser et al., 1995; Dolezel and Bartos, 2005). Within the Sydney Rock oyster, Hand et al. (1999) reported a 5% variation between flow cytometry and chromosome counting. Our study aims to determine if large scale genetic loss is occurring in tetraploid oysters and if it is progressive. Rather than determining the exact number of chromosomes present, the use of flow cytometry is being examined as an appropriate tool for this analysis.

5.2 Materials and methods

5.2.1 Sample Collection

Ninety tetraploid Pacific oysters comprised of three different year-classes (30 per class) were provided by Shellfish Culture Ltd (Pipeclay, Tasmania, Australia). The year-classes consisted of five year old oysters spawned in 2008, two year old oysters spawned in 2011 and

one year old oysters, spawned in 2012. The five year old oysters were spawned by a mixture of tetraploid crosses and direct inductions methods. The tetraploid broodstock used to spawn the one and two year old oysters were from the five year old population. Oysters over five years of age are generally not used within aquaculture.

To avoid sample degradation over time which can effect flow cytometry results (Dolezel and Bartos, 2005), oysters were collected and processed in batches, ensuring that no oyster was more than three days out of the water before being processed. Oysters were refrigerated prior to processing. Two pieces of gill tissue (independently collected) were sampled from each oyster. Three replicates of each tissue sample was analysed by flow cytometry. A maximum of 15 oysters per run (five from each year-class) were analysed, 12 runs were completed in total. For each run, the order that the oyster, tissue and repetition were analysed was randomised.

5.2.2 Standard

Sperm from two diploid oysters was used as a control. The first eight runs used sperm from one individual and the remaining four runs used sperm from another individual. The reason why two individuals were used instead of one was tissue degradation which can affect flow cytometry results. Sperm was collected fresh from the refrigerated diploid individual prior to each run and diluted in Cystain DNA 1 step (Partec, Munster, Germany) before being added to each sample.

5.2.3 Flow cytometry

Gill tissue (approx 0.5cm^2) was added to 1.8ml Cystain DNA 1 step (Partec) and mixed for 5 min. The resulting cell suspension was aspirated and passed through a 20µm Celltrics® (Partec) filter. Samples were analysed on a Partec CyFlow® Ploidy Analyzer "DAPI" one

parameter (FL) | UV LED excitation. The standard was used to set the gain (average 760) and the speed varied (0.4-1.0µl/s) depending on cell concentration. Due to the repetitions required, 50µl was used per sample.

5.2.4 Statistical methods

The software ModFit LT (Verity Software House Inc., USA) was used to determine the control peak and G_0G_1 peak (the major peak of fluorescence) for each sample and calculate the coefficient of variance (CV) of the peak area. From the CV value, a standard deviation for each sample was determined.

The DNA index (DI), which is the relative DNA content of a sample was calculated as follows:

$$DI = \frac{mean\ of\ the\ relevant\ DNA\ content\ of\ the\ G_0G_1\ peak\ of\ the\ sample}{mean\ of\ the\ relevant\ DNA\ content\ of\ the\ G_0G_1\ peak\ of\ the\ control}$$

A linear mixed effects model was run with CV and DI as a function of age accounting for run, individual, tissue, control and replication variation.

5.3 Results

Measurable peaks were obtained for all samples, tissues and replicates, with the goodness of fit model used producing low to medium reduced chi-square (RCS) values (Fig. 5.1). An artefact of using haploid sperm from a diploid as a control was that a small number of diploid cells were present in each sample producing a slight peak. This peak was consistently small and in the same location (channel number 50), hence did not impact the results. The peaks (CV and DI values) from the two different sperm controls were compared using a standard t-test, with no significant difference observed ($P<0.001$).

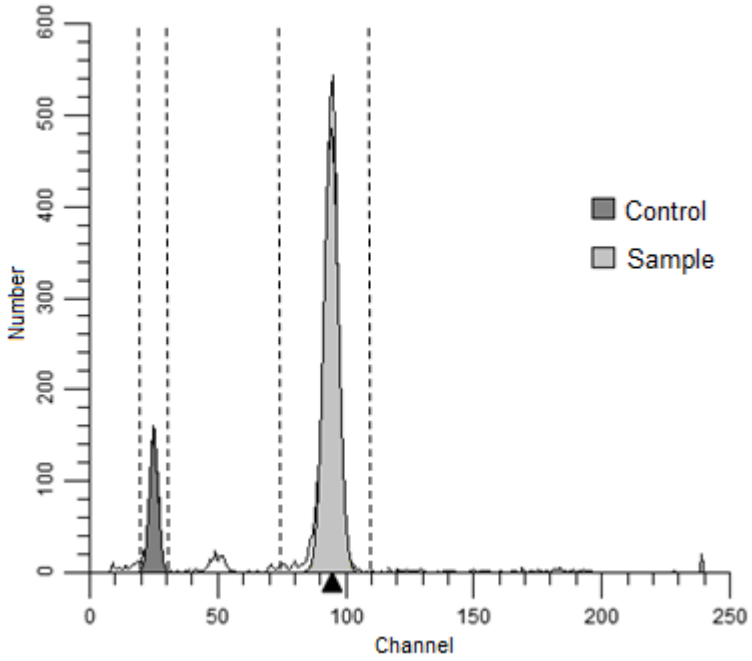

Figure 5.1: An example of a tetraploid Pacific oyster flow cytometry output. The peak around channel 25 is a diploid sperm control and the larger peak around channel 95 is the tetraploid individual. The shading is a goodness of fit model used to calculate the coefficient of variance (CV). The small diploid peak observed at 50 is from the control individual and was consistently present when the standard was run independently of the sample. The broken vertical lines indicate the variation observed in peak location across all samples.

Of the total variation observed across all the samples, the effect of runs (7%), tissue samples (0%) and replicates (0%) were negligible and less than the natural variance observed between individuals (31%) and residual effects (61%).

The CV and DI values were consistent across age classes (Table 5.1). The linear mixed effects model showed that the CV and DI values did not significantly differ across the year-classes (CV: $\chi^2 = 0.5$, $p > 0.05$, DI: $\chi^2 = 2.2$, $p > 0.05$) indicating that that the null hypothesis, no difference between year-classes, should be accepted

Table 5.1: Flow cytometry summary for 90 tetraploid Pacific oysters. The coefficient of variance for peak area including variance between individuals (CV) and DNA index (DI) was recorded for three year-classes (one year old, two year old and five year old), thirty oysters per year-class were sampled.

Year-class	CV	**DNA index**
1 year old	3.2	3.8 ± 0.02
2 year old	3.2	3.8 ± 0.01
5 year old	3.1	3.8 ± 0.01

5.4 Discussion

Flow cytometry, whilst fast and cost efficient, is not as sensitive as chromosome counting. In the study by McCombie et al. (2005) 87% of the tetraploid oysters had chromosome numbers ranging from 35-42. It could be that flow cytometry was not sensitive enough to detect this small scale loss of chromosomes, however, a loss of 4 chromosomes is equal to 10% and should have been observable if present. McCrombie et al. (2005), who sampled oysters at one year of age, used both "good" and "poor" ploidy broodstock to determine the effect of parental ploidy status on the subsequent generation. The samples used in our study would have all arisen from broodstock of "good" ploidy, as it is industry protocol to run a flow cytometry analysis on all parental tissue prior to spawning to ensure that they are tetraploid. However, if aneuploidy frequency were to follow the predicted progressive loss hypothesis, a difference should be able to be observed in the two or five year old year-classes. Instead, no difference was observed.

Within this study, no difference within DNA content was observed. This suggests that no large scale loss or gain of chromosomes is occurring within the tetraploid oysters. Furthermore, no significant difference was found between year-classes, indicating that the aneuploid frequency within tetraploid oysters may not be as high or progressive as originally hypothesised (McCombie et al., 2005a).

A lack of difference observed between year-classes could be due to differential survival. For instance, most aneuploid oysters may not have survived past one year of age. The survival rates of aneuploid oysters are currently unknown. Whilst the Pacific oyster has been shown to tolerate a variety of different chromosome numbers (Guo and Allen, 1994b; Wang et al., 1999; Gong et al., 2004), it could be that aneuploid oysters have lower survival rates. Aneuploid individuals often show impaired fitness (Siegel and Amon, 2012). A study by Zhang and Arai (1999) crossed induced triploid loach with tetraploids. The resulting aneuploid progeny showed poor survival and severe malformation compared to the triploid and tetraploid controls. It could also be that aneuploid oysters are removed from the system through sorting (removing deformed or undesirable broodstock) and grading (removing smaller individuals) methods. Within diploid oysters, aneuploidy has been linked to slower growth rates (Thiriot-Quievreux et al., 1992; Leitao et al., 2001b), however, this has not been shown to occur in polyploids (Guo and Allen, 1994b; Wang et al., 1999).

Studies on autotetraploid maize have shown that 30-40% of their progeny have been aneuploid (Doyle, 1986; Comai, 2005). Similarly, Li et al. (2012) found high frequencies of aneuploids in the embryonic progeny of tetraploid loach. Guo and Allen (1997a) found that Pacific oysters follow a similar pattern, with tetraploids producing more aneuploid progeny than diploids. However, Guo and Allen (1997) only looked at the progeny during embryonic stage. The one and two year old oysters used in our study were 2nd and 3rd generation tetraploid. The high aneuploid frequency observed in tetraploid progeny was not found,

which could imply that either advanced hatchery produced tetraploid Pacific oysters are more stable than originally predicted or aneuploid individuals are being removed from the system early via poor survival or grading.

Due to sampling constraints, this study only looked at somatic gill tissue. It could be that other tissues, such as gametic, could show a different aneuploid frequency. However, this study did look at generations of oysters, that is oysters that were spawned directly from older year-classes analysed. Hence, if the gametic tissue did have a high aneuploid frequency, one would expect to see an effect in the somatic cells of the progeny. Instead, no difference was observed between the broodstock population (five year old oysters) and the resulting progeny populations (one and two year old oysters) in the Shellfish Culture breeding program.

Chapter 6

Counting and identifying polyploid Pacific oyster (*Crassostrea* gigas) chromosomes

6.1 Introduction

A practice in the Pacific oyster aquaculture industry is to crossbreed tetraploid and diploid individuals to produce fast growing, mostly sterile triploids for commercial production. Given that tetraploidy and triploidy are not normal genomic states in oysters, they are possibly unstable with a high potential for aneuploidy; the state of having a chromosome number that is not an exact multiple of the haploid number, through either loss or gain (Wang et al., 1999).

Aneuploidy is often lethal in many higher order taxa, (e.g. humans can only tolerate a 6% gain and 3% loss) or results in severe growth retardation (Wang et al., 1999). Many plant species, however, have a good tolerance to aneuploidy and have been highly useful in genetic analysis and breeding research (Riley and Law, 1985). The Pacific oyster, like many lower order taxa, has been found to tolerate a large range of chromosome numbers (Guo and Allen, 1994b; Wang et al., 1999; Gong et al., 2004), however, aneuploid triploid Pacific oysters have been found to have a smaller growth rate than their diploid and triploid counterparts with full karyotype (Leitao et al., 2001b; Gong et al., 2004; Landau and Guo, 2005).

A high aneuploidy rate could therefore potentially be problematic within commercial polyploid oyster populations resulting in lower survival and/or production. McCombie et al. (2005a) found a somatic aneuploid rate of 53% in 2nd and 3rd generation tetraploids. This suggests that tetraploidy in oysters may not be a stable state and reversion likely progressive, with an increase in aneuploid cells over time (Allen et al., 1999). Therefore, further research

is needed on aneuploidy in successive generations of tetraploid oysters to determine what long term effects it has on the populations.

High aneuploidy in successive tetraploid populations could also have carry over effects on the success of the resulting commercial triploid stock. However, it has been reported that frequency of cells in triploids that reverted to diploid cells was about 2-3 times higher in 'chemical' induced triploids by cytochalasin B than in 'natural' triploids, those produced by crosses of tetraploids and diploids (Allen et al., 1999). This is in contrast to another report on the same species where more aneuploids were found in 'natural' triploids compared to 'chemical' induced triploids (Wang et al. 1999). Based on this, Wang et al. (1999) proposed that tetraploids were likely to suffer segregation errors during meiosis, thus producing aneuploid gametes. Additionally, using chromosome banding techniques, (G-banding), Leitao et al. (2001c) found that chromosome loss was not random in aneuploid oysters, but rather a reflection of differential chromosome susceptibility; that is particular pairs of chromosomes were lost more frequently than others. These, often conflicting, aneuploidy predictions are still relatively speculative and further research is needed on the stability of polyploid oyster populations.

The Pacific oyster has a small genome with an estimated haploid genome containing approximately 824 million nucleotide base pairs (Hedgecock et al., 2005). A diploid oyster contains 10 chromosome pairs (2N=20), all of which are relatively small and of similar size. This means that it is not possible to distinguish each chromosome's identity without the use of a banding or labelling technique. Labelling methods use probes, such as microsatellite markers, to bind to the chromosomes and produce a fluorescent signal that can be viewed through a microscope. Depending on the probe, the signal location, pattern or colour can be used to individually identify chromosomes; this is important in aneuploid studies to determine individual chromosome susceptibility to loss. The study of aneuploidy in tetraploid

oysters by McCombie et al. (2005a) used chromosome counting and did not use any labelling methods, so it is unknown if particular chromosomes were being lost at a faster rate than others as predicted by Leitao et al. (2001c).

The Pacific oyster is of great economic importance worldwide. This means that a number of chromosome counting, banding and fluorescence *in situ* hybridisation (FISH) studies, using telomere, centromere, RNA and microsatellite probes, have previously been performed, with mixed success (Guo and Allen, 1997b; Wang et al., 2001; Leitao et al., 2004; Bouilly et al., 2008; Zhang et al., 2010a; Zhang et al., 2010b). FISH using fluorescent probes generally produces clearer signals, giving better resolution for karyotyping than other banding methods, such as enzyme digestion or G-banding.

In a study by Bouilly et al. (2008), three oligonucleotide markers, $(GGAT)_4$, $(GT)_7$, and $(TA)_{10}$ were used to fluorescently label the diploid oyster karyotype. These markers produced distinct patterns on the chromosomes that allowed for identification. This was the first, and currently only, study to use microsatellite FISH analysis for chromosome identification and karyotyping in oysters. In this present study, probes and methods detailed by Bouilly et al. (2008) were used to label tetraploid Pacific oyster chromosomes with the goal of determining aneuploid frequency and individual chromosome susceptibility. This will help to inform oyster producers of the current stability within tetraploid oysters and what degree of risk aneuploidy may pose to future production. In order to undertake the FISH analysis, usable metaphase spreads (i.e. complete, minimal chromosome overlap etc.) from tetraploid oysters will need to be obtained.

In addition to using microsatellite markers, this study trials the peptide nucleic acid (PNA) telomere probe $(TTAGGG)_n$ in tetraploid oysters. This probe regularly produces signals at the ends of chromosome arms; however, in many species it also shows signals at internal sites

(interstitial telomeric repeats [ITRs]) indicating areas of evolutionary meiotic recombination (Meyne et al., 1990; Mondello et al., 2000; Demin et al., 2011) as well as chromosome fusion. The PNA probe is much larger than microsatellite probes; hence it should provide a stronger signal. Additionally, PNA probes are more stable and versatile than DNA or microsatellite probes (Zhang and Appella, 2010). To the best of my knowledge, the PNA probe has not previously been mapped in the Pacific oyster. If enough ITRs are present, the PNA probe could potentially be used to individually identify chromosomes as well as providing valuable insights into oyster evolution.

6.2 Materials, methods and results

6.2.1 Sampling

Three month old tetraploid oysters (2^{nd} and 3^{rd} generation), supplied by Shellfish Culture Ltd, were used for analysis. Preliminary work showed that older oysters had fewer metaphase spreads, likely due to the fact that three month old oysters were still growing rapidly, whereas the growth rate in older oysters (>one year) had decreased. With oysters younger than three months, samples of gill tissue (without contamination by other tissues) were difficult to obtain due to their small size. Oysters were sampled in late spring and early summer when warmer environmental conditions increase growth rate. Oysters sampled in winter had less metaphase spreads than those sampled in the warmer months. Diploid oysters from the same company were used as controls.

6.2.2 Metaphase spreads

Live oysters were incubated in seawater containing 0.005% colchicine for 7 h; shorter time frames resulted in less metaphase spreads. Placing the oysters in colchicine for more than 7 h resulted in highly condensed chromosomes, which could potentially mask any FISH signals.

After colchicine treatment, a small fragment of gill tissue was extracted and immediately placed in 0.075M potassium chloride for 15 min. Longer or shorter time periods in the hypotonic solution resulted in less metaphase spreads, as did using sodium citrate instead of potassium chloride. Tissue samples were fixed in 3:1 methanol acetic acid and refrigerated overnight.

Fixed tissue, with three drops of 70% acetic acid, was crushed for 30s before the resulting cell suspension was aspirated and washed three times in fixative (3:1 methanol acetic acid). Dissolving methods tested resulted in less metaphase spreads than crushing.

The washed cell suspension was dropped on to a clean slide at room temperature and allowed to dry before being aged overnight at -80°C. Height of the drop made little difference to the metaphase spreads. Heating, cooling or increasing the humidity to the slide prior to dropping resulted in similar or less metaphase spreads and had no discernible effect on spread quality. Slides were viewed with a Zeiss Axio Scope A1 epifluorescence microscope (Carl Zeiss Ltd.). Analysis of images was performed using ISIS software (Metasystems).

The metaphase spreads from the tetraploid oysters using this method were not ideal. The vast majority had chromosomes overlapping (Fig. 6.1). In many cases, individual chromosomes could not be easily identified, making repeatable counting problematic.

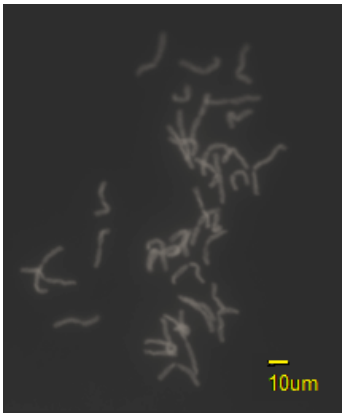

Figure 6.1: An example of a metaphase spread from a tetraploid Pacific oyster

6.2.3 FISH using microsatellite motifs

The FISH protocol using three probes, $(GGAT)_4$, $(GT)_7$ and $(TA)_{10}$, described in Bouilly et al. (2008) was tested, with no fluorescent signals on the chromosomes observed. Changing probe concentrations, hybridisation times, and denaturing times and temperatures had no observable effect on the results. The FISH protocol by O'Meally et al. (2009) was also trialled using the same three markers, with varying concentrations of probes, denaturing times and hybridisation times. Again no observable signals were present.

In order to increase signal intensity, the probes were redesigned to make them longer: $(GGAT)_8$, $(GT)_{15}$ and $(TA)_{15}$. Using the protocol described by Cioffi et al. (2011), signals on some metaphase spreads in some samples was observed (Fig. 6.2).

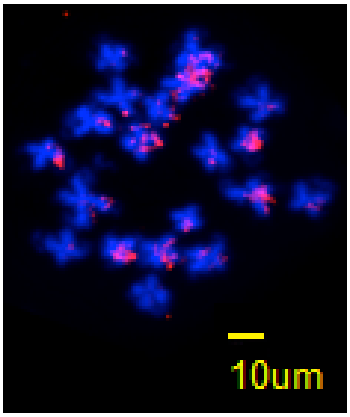

Figure 6.2: Fluorescent *in situ* hybridisation of the microsatellite $(GT)_{15}$ on diploid Pacific oyster chromosomes.

The inconsistency of signals within and between samples was high. Slides rarely had more than three metaphase spreads that showed signals; many had no signals present. When signals were present, they were often faint. Additionally, there was background fluorescence from cell debris and un-annealed probe (present even with extra washing cycles and when lower probe concentrations were trialled), which often disguised signals. The frequent overlapping of chromosomes, lack of signals in many metaphases and weakness of the signal strength made mapping of these probes impossible for a detailed aneuploid study.

6.2.4 FISH using PNA probe

Aged metaphase spreads were labelled with the PNA probe at a concentration of 3µg/ml. The probe was added to a hybridisation buffer containing 70% deionised formamide, 1x Denhardt's solution, and 10mM Tris (pH 7.2). To each slide, 10µl of this solution was added with a coverslip, sealed with rubber cement, and denatured then at 80°C for 3min. The slides were then placed in a hybridisation chamber for 2h at 37°C. Slides were washed twice for 15min each in a solution of 70% formamide, 10mM Tris (pH 7.2) and 0.1% BSA. The slides were further washed three times for 5min each in a solution of 0.1M Tris, 0.15M NaCl and

0.08% IGEPAL. The slides were stained with 4',6-diamidino-2-phenylindole (DAPI), dehydrated and stored at 4°C overnight.

The success rate of the PNA probe was much higher than that observed for the microsatellites, but still inconsistent, both within and between slides. The majority of the chromosomes, if not all, appeared to have no ITRs, with signals only present on the chromosome ends (Fig 6.3).

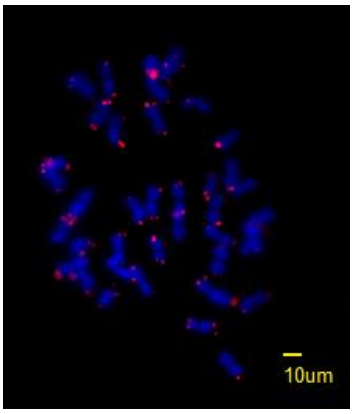

Figure 6.3: Fluorescent *in situ* hybridisation of the peptide nucleic acid (PNA) telomere probe $(TTAGGG)_n$ on tetraploid Pacific oyster chromosomes

The planned study of aneuploidy was not completed due to the extra time that would be needed to perfect the tetraploid metaphase spreads and the strength of the PNA probe signal.

6.3 Discussion

Obtaining optimum metaphase spreads, with minimal overlapping, can be difficult and is often a major limitation in cytogenetic studies (Deng et al., 2003). Humidity, slide temperature, drying time and the height from which the cells are dropped have all been reported to potentially effect chromosome spreading (Spurbeck, 1996; Hlics, 1997; Deng et al., 2003). All these factors were taken into account in this study when trying to optimise the

condition for metaphase spreads preparation, and various combinations were tested. However, I was unable to obtain sufficient numbers of optimal spreads for analysis. It is speculated that some minor modification to the techniques trialled plays an important role in achieving successful tetraploid spreads, but this was not identified.

The methods used in this study for obtaining metaphase spreads within Pacific oysters was previously developed for diploid oysters (Guo and Allen, 1997b; Wang et al., 2001; Bouilly et al., 2008). As a result, signals were more often observed in the diploid controls as opposed to tetraploids. This may be due to the increased overlapping of chromosomes hiding signals within the tetraploid samples or could suggest that alternative methods for polyploid oysters may be required. The Pacific oyster is an estuarine species and, as such, its cells have a strong tolerance to salinity. When a common dissolving method was trialled to obtain spreads, it appeared that the cell membrane was not breaking despite the cells being swollen from the hypotonic solution. For this reason, the crushing method was utilised. This may have also influenced the spread of the chromosomes.

The age of the oyster and the season it was sampled in appears to play an important role in determining the number of metaphases present per slide. It was observed that young oysters, 3-4 months old, had at least double the number of metaphases spreads present compared to oysters older than a year. This could most likely be contributed to by the fact that young oysters have a much faster growth rate than older oysters. Similarly, oysters sampled in spring-summer months appeared to produce more metaphase spreads than those sampled in winter. An increase in feeding, and hence growth, during the warmer months and a reduction during the cooler months when oysters are more dormant is the most probable reason for this observation.

The metaphase spreads obtained were not optimal for accurate chromosome counting without fluorescent labelling. Despite replicating the Bouilly et al. (2008) method and trying numerous optimisations, microsatellite signals were not visible on the chromosomes until the probes were redesigned. Even then, the number of metaphase spreads that had visible signals was much less than those that had no signals. The reason for the inconsistency is unknown. It was not subject to the potential issues stated in the methods as individual slides contained metaphases spreads both with and without signals. It appeared that some other factor was inhibiting the probe binding to the chromosome as excess probe was visible despite reducing concentration and/or adding extra washing steps.

The PNA telomere probe is unlikely to be useful in identifying aneuploidy in Pacific oysters in the future. To the best of my knowledge, no previous studies have looked at ITRs in Pacific oysters. Despite my inability to map this probe due to the poor metaphase spreads (i.e. limited number of spreads and overlapping of chromosomes) in the tetraploid samples, it appears that this species does not contain many, if any, ITRs. This means that signals are present only on the telomere ends of the chromosomes, thus individual identification of chromosomes cannot be determined.

A bacterial artificial chromosome (BAC) library containing 10x coverage has been established for the Pacific oyster. FISH using BACs allows for larger sections of DNA to be labelled, thus resulting in stronger signals. Additionally, multiple probes with different colours can be used, which allows for easier identification of chromosomes. BAC analysis is more expensive than microsatellite analysis and requires specialised equipment; hence it was not trialled during my study. It has however proved very successful in other species (Young et al., 2013). Given the lack of success with the microsatellites, BACs may be the best way to determine aneuploid frequency in Pacific oysters in the future.

Chapter 7

Conclusion

The production of triploid Pacific oysters for commercial consumption has been steadily growing nationally and internationally and a continual increase is predicted in the future. The high sterility and increased growth rate make triploid oysters a desirable commercial product. Currently, research on the genetics of polyploid oysters is lagging behind the growth of the industry. This knowledge gap could be detrimental to future production as poorly understood factors, such as inbreeding and aneuploidy, may negatively impact on the sustainability of the industry. The research undertaken in this dissertation will help bridge the gap between industry growth and research required.

In 2008, the Food and Agriculture Organisation (FAO) of the United Nations reported that the maximum potential from wild caught fisheries has most likely been reached (FAO, 2008), which means that the increase in future seafood demand will need to be met by aquaculture. Most aquatic species generally have a high fecundity and short generation interval (i.e. quick to mature to breeding age), traits that are highly beneficial for selective breeding programs. However, in comparison to terrestrial agriculture, aquaculture has been slow on the uptake of implementing breeding programs for improvement of commercial stock, with many hatcheries still using wild caught broodstock. In comparison to terrestrial plant and animal industries, selective breeding programs in aquaculture are relatively rare, particularly for shellfish. Despite the fact that high genetic gains from such breeding programs have been reported for aquatic species (Gjedrem et al., 2012a; Stear et al., 2012; Alajmi et al., 2014; Zak et al., 2014), only around 10% of aquaculture production is based on genetically improved stocks (Gjedrem et al., 2012a).

Family selective breeding programs utilise single pair crosses of broodstock and retain the progeny within their separate family lines until they can be individually tagged when they are then grown collectively; this is opposed to mass selection where multiple broodstock are used and progeny are grown collectively throughout their production cycle. Family selective breeding allows for better management of inbreeding and for multiple commercially important traits (growth, survival) to be targeted for improvement in future generations (as opposed to mass selection that generally targets a single trait). In Australia, a selective breeding program, run by Australian Seafood Industries (ASI), for diploid Pacific oysters has been in place since 1997 (Ward et al., 2000). Initially, this program focused primarily on increasing growth rate, which was successful, with the fourth generation of selected oysters showing a 1.6x faster growth rate than the control population (Ward et al., 2005). In later years, this program was hampered by a negative correlation between increased growth rate and shell width index (the ratio of shell width to shell length), which resulted in the production of long, skinny oysters, unsuitable for market. In turn this led to a loss of industry confidence in the program and resulted in the need for a more comprehensive breeding plan design to be implemented (Kube et al., 2011). A lack of understanding of the basic genetic architecture behind commercially important traits led to an inappropriate selection emphasis being placed on shell width index within the diploid oyster breeding scheme, and in the following years, other traits of interest, such as shape and condition, and disease resistance were included in the program. This highlights the need for thorough research to be conducted prior to the implementation of selective breeding programs.

Selected diploid individuals from the ASI program are used to create commercial triploids, when crossed with a non-selected tetraploid. Currently, little is understood about the underlying genetics in polyploid oyster breeding (Dufresne et al., 2014). This lack of

understanding of the basic genetic framework behind polyploids may result in serious consequences arising in future selective breeding programs and commercial production.

Breeding programs will be essential to the improvement and expansion of triploid oyster production. It is predicted that breeding programs incorporating tetraploids to improve the triploid product will soon be implemented (Peter Kube, pers. comm.). When implementing a selective breeding program, it is important to have a high level of genetic variation available to optimise selection potential and minimise inbreeding (Billington, 1996). The diploid population analysis undertaken in chapter two of this dissertation implies that naturalised populations of the Pacific oyster in Australia may provide a genetic reservoir of diversity for Australian breeding programs since they have lost little diversity since their introduction. This concurred with genetic diversity studies undertaken previously on diploid Pacific oyster populations (English et al., 2000; McGoldrick et al., 2000; Appleyard and Ward, 2006; Li et al., 2006). The tetraploid population, spawned in 2008, showed comparable diversity levels to the cultured diploids. The presence of this relatively high diversity will be beneficial in the implementation of a selective breeding program. However, a significant diversity drop in the following tetraploid generation was observed. This is most likely due to a small number of effective broodstock being used and skewed parental contributions. It implies that the tetraploid population, produced via mass spawning, is at risk of suffering inbreeding depression in the future if it is not managed. Family selection and paired crossing would help to reduce this risk, whilst potentially increasing growth rates and/or disease resistance, traits that will have carry over effects to the commercial triploid product.

Pedigree assignment will play an important role in the implementation of polyploid oyster breeding programs. Using the novel methods described in chapter four, strongly performing triploids can now be traced back to their respective broodstock. This means that the same or closely related broodstock can be used within the commercial breeding program to generate

more desirable triploids. Additionally, full siblings of the triploid may be used to induce new tetraploids. The process of using estimated triploid breeding values for tetraploid and diploid broodstock based on triploid progeny testing will be less haphazard than using ASI selected diploids to cross with un-pedigreed tetraploids, as it is unknown what the effects dosage will have on the triploid product. Additionally, this method will assist the development of a family selective breeding program for tetraploids, as described in chapter three, to be developed in parallel with the triploid improvement program.

Improvements to polyploid oyster breeding programs will require further research. The effect of dosage and its role in improving the triploid product is currently poorly understood. Dosage affects have been shown to influence growth rate in triploid Coho salmon (Devlin et al., 2014) and transgenic tilapia (Martinez et al., 1999).The microsatellite markers, designed in chapter two, offer good coverage of the chromosomes and hence will be useful in dosage research. Additionally, the pedigree methods set out in chapters three and four will aid in identifying or confirming relationships between the oysters. Further research is also needed on aneuploidy and the long term sustainability of tetraploid populations, along with the carry over effects to the triploid product.

Flow cytometry (chapter five) found no difference in chromosome numbers across three generations of tetraploid oysters. This suggests that if, as hypothesised, large scale aneuploidy is occurring, then the aneuploid oysters are being removed from the system, either via hatchery grading or early mortality. It could also be that only small scale chromosome loss is occurring, in which case flow cytometry is likely not sensitive enough to detect this frequency. Chromosome counting is a more accurate method of calculating aneuploidy. However, as demonstrated in chapter six, identifying chromosomes in polyploid Pacific oysters is highly time consuming, technique specific and inconsistent. Improved methods for fluorescent *in situ* hybridisation (FISH) analysis are needed to successfully and accurately

identify the aneuploid frequency and individual chromosome loss rate of polyploid oysters. Microsatellite markers with fluorescent probes proved inefficient at correctly identifying chromosomes, likely due to their small base pair repeat size. More success would be expected by using BAC markers. Now that the Pacific oyster genome has been mapped, the number and availability of such markers will likely increase.

The next logical step for research into the genetics underlying polyploid oyster breeding for the establishment of a selective breeding program is the construction and screening of a comprehensive bacterial artificial chromosome (BAC) library. A preliminary BAC library has already been established (Cunningham et al., 2006) for the Pacific oyster, however, this needs to be expanded and screened so that an informative suite of FISH markers can be developed. The benefit of using BACs over microsatellite markers for FISH analysis is their larger size, which results in stronger signal intensity on the chromosomes, and the ability to use multiple fluorochromes. Given the small size of the oyster chromosomes and the difficulties associated with obtaining usable metaphase spreads (as discussed in chapter six), an informative suite of BAC markers will be vital for accurately accessing aneuploidy in polyploid oysters.

The benefits of having an informative suite of BAC markers do not stop at detecting aneuploidy. BACs are commonly used to locate and map genes linked to traits of interest such as growth and disease resistance (Wang et al., 1996; Nam et al., 1999; Peterson et al., 2000; Tomkins et al., 2004). Additionally, having an informative suite of BAC markers will allow research into the relatively new area of copy number variation (CNV). CNV is defined as sections of DNA that display different copy numbers in populations (Feuk et al., 2006). Recent studies on CNV have demonstrated that it plays an important role in genetic variation in humans, particularly in disease susceptibility (Henrichsen et al., 2009; Stankiewicz and Lupski, 2010). These studies have prompted research on CNV in other species such as maize

(Springer et al., 2009), rice (Yu et al., 2011) and the tetraploid potato (Iovene et al., 2013). It is likely that the effects of CNV are dosage dependant (Iovene et al., 2013), hence its role in genetic and phenotypic variation within polyploid species may be highly significant and play an important role in future oyster breeding programs.

Research into allele dosage and its role in the phenotypic expression of important traits is another vital area of study for the successful implementation of polyploid oyster breeding programs. Polyploidisation has been shown to affect gene expression in a number of plant species(Adams and Wendel, 2005). For dosage analysis in oysters, the best approach will likely involve the use of single nucleotide polymorphism (SNP) markers. Numerous SNP markers have already been described for the Pacific Oyster (Bai et al., 2009; Zhong et al., 2013; Jin et al., 2014). Whilst SNPs are often considered less informative than microsatellites, they have been shown to be less prone to errors (Ball et al., 2010; Hauser et al., 2011). More importantly, previous studies have demonstrated the ability to predict dosage in polyploid species using the signal intensity of the SNP markers (Voorrips et al., 2011; Cuenca et al., 2013). Whilst these dosage studies have currently only been conducted on plant species, the methods should theoretically be easily transferable to polyploid oysters.

Developing a panel of high density SNP markers will also allow research into whole genome association (WGA) and whole genome association selection (WGS) using molecular estimated breeding values (MEBV) to be undertaken. In previous studies WGA has been used to map complex traits and help infer the underlying genetic architecture associated with plant and animal breeding programs (Goddard and Hayes, 2009; Riedelsheimer et al., 2012; Zhang et al., 2014). For future polyploid breeding programs, WGA/WGS will potentially be a powerful tool for improving the selection process associated complex traits. This is particularly the case with triploid oysters that are a terminal line, where the high performing individuals cannot be used for future breeding. The ability to use WGA/WGS for within-

family selection of the best diploid and tetraploid parents based on their within-ploidy performance and their performance as triploid parents will significantly enhance genetic gains for commercial triploid production.

Family selection breeding programs for the production of commercial triploids is the way of the future for the Pacific oyster aquaculture industry. This dissertation has provided some of the fundamental information required for the establishment of such a program and developed some of the genetic tools and techniques that will aid in its implementation.

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Appendix

Example of likelihood calculations

In this example we assume a single marker with $n = 6$ alleles, with base pair counts (207,209,215,223,227,259), a null allele frequency parameter of 0.1 and an allele base pair shift parameter of 0.7. The tetraploid sire genotype is $(1,0,0,1,0,0)$ where 0 and 1 indicate that the allele was not or was observed respectively. Table A.1 shows the derivation of the vector of allele probabilities for the sire. The first and fourth alleles are observed in the sire, so the vector X^S contains 0.5 for each of these alleles. With a null allele frequency parameter $\Theta = 0.1$, for the second allele, with base pair count 207, the null allele probability is $G_2^S = \frac{\Theta}{(\mathrm{n-q})} = \frac{0.1}{(6-2)} = 0.025$. With a base pair shift error parameter $p = 0.7$, for the second allele the base pair shift error is $B_2^S = h_{1,2} + h_{4,2} = (1-0.7)^{(209-207)}(0.07) + (1-0.7)^{(223-209)}(0.07) = 0.063 + 3.3\text{E-}8 \cong 0.063$. The vector of genotype probabilities $U^S = \frac{X^S+G^S+B^S}{\mathrm{sum}(X^S+G^S+B^S)}$. For the first allele, the probability that the sire has two copies to pass on to progeny is $w_1 = \frac{1}{3} + \left(\frac{10}{14} - \frac{1}{3}\right) * \frac{(0.428-\frac{1}{3})}{(\frac{1}{2}-\frac{1}{3})} = 0.549$.

Table A.1. Derivation of the vector of genotype probabilities U^s and vector W^s of probabilities of having two copies of an allele to transmit to progeny, for a sire with alleles 209 and 223 observed, given a null allele frequency parameter $\Theta = 0.1$ and a base pair shift error parameter $p = 0.7$.

Allele	$\mathbf{X^s}$	$\mathbf{G^s}$	$\mathbf{B^s}$	$\mathbf{U^s}$	$\mathbf{W^s}$
207	0.500	0.000	0.000	0.428	0.549
209	0.000	0.025	0.063	0.075	0.000
215	0.000	0.025	<0.001	0.021	0.000
223	0.500	0.000	0.000	0.428	0.549
227	0.000	0.025	<0.001	0.026	0.000
259	0.000	0.025	<0.001	0.021	0.000

The 6 x 6 transmission matrix for the sire $\mathbf{T^s} = \frac{\mathbf{U^s U^{s'}}\,\mathrm{diag}(\mathbf{W^s})}{\mathbf{sum}(\mathbf{U^s U^{s'}}\,\mathrm{diag}(\mathbf{W^s}))}$.

For a diploid dam with only the second allele seen, the 6 x 1 transmission vector $\mathbf{T^d} = \mathbf{U^d} = (0.071, 0.859, 0.018, 0.017, 0.017, 0.017)'$ and the 6 x 6 x 6 joint sire, dam transmission array is $\mathbf{T^{sd}} = \mathbf{T^s} \otimes \mathbf{T^d}$.

For a triploid progeny with alleles 2 and 4 seen, the 6 x 1 genotype probability vector is $\mathbf{U^p} = (0.075, 0.428, 0.022, 0.428, 0.026, 0.021)'$ and the 6 x 6 x 6 genotype probability array $\mathbf{R^o} = \mathbf{U^o} \otimes \mathbf{U^o} \otimes \mathbf{U^o}$. The likelihood at this marker is

$L^{sdo} = \sum_i \sum_j \sum_k R^o_{ijk} T^{sd}_{ijk} = 0.021.$

R code to perform the calculations for this example

```
# Example of triploid parentage likelihood calculation



n <- 6 # 6 alleles


alleles <- c(207,209,215,223,227,259)       # vector of alleles known to exist at
                                            # this marker


theta <- 0.1   # null allele frequency
p  <- 0.7      # parameter for allele base pair shift error


ploidy.sire <- 4      # Tetraploid sire
ploidy.dam <- 2       # Diploid dam
ploidy.offspring <- (ploidy.sire + ploidy.dam)/2
                      # Triploid offspring


# S, observed genotype vectors, logical (n x 1)
S.sire <- c(T,F,F,T,F,F)
S.dam  <- c(F,T,F,F,F,F)
S.offspring <- c(F,T,F,T,F,F)




#####################################################################
# Function to estimate U, the vector of estimated allele frequencies
#####################################################################


S2U <- function(S,m,theta,p) {
        # Function to estimate U
        # Input parameters:
                # S, dimension(n), logical, observed genotype vector, where
```

```
                # n is the number of alleles
        # m, integer, degree of ploidy
        # theta, real, null allele rate parameter
        # p, real, allele base pair shift parameter
# Returns
        # U, dimension(n), real, estimated allele frequency within
                # the individual



n <- length(S)


# Vector X
X <- as.integer(S)/sum(S)


# Null alleles
if (sum(S) < m) {     # if fewer alleles observed than ploidy
  G <- array(1,n) - as.integer(S)
  G <- G / sum(G)
  G <- G * theta
} else {
  G <- array(0,n)
}


# Base pair shifts
B <- array(0,n)
for (i in 1:n) {
  if (!S[i]) {  # allele not seen
    for (j in 1:n) {
      if (S[j]) {  # allele seen
       B[i] <- B[i] + (1 - p)^(abs(alleles[i] - alleles[j])) * p
      }
    }
  }
```

```
        }



        # U is the normalized sum, as allele frequency estimates sum to one
        U <- (X + G + B) / sum(X + G + B)

        # Return U
        U

} # end function S2U

######################################################################

# Estimate U
U.sire <- S2U(S.sire,ploidy.sire,theta,p)
U.dam  <- S2U(S.dam,ploidy.dam,theta,p)
U.offspring <- S2U(S.offspring,ploidy.offspring,theta,p)

# Td   Transmission vector from dam, (n x 1)
Td <- U.dam

# Ts   Transmission matrix from sire (n x n)

Ts <- U.sire %*% t(U.sire)

# Diagonals have to be scaled by probability of there being two copies
# in the sire (Ws).  Linear interpolation between points on X,W plane.

Ws <- array(NA,n)

for (i in 1:n) {
  ui <- U.sire[i]
  if (ui <= 1/4) {
```

```
    Ws[i] <- 0
  } else if (ui <= 1/3) {
    Ws[i] <- 1/3 * (ui - 1/4)/(1/3-1/4)
  } else if (ui <= 1/2) {
    Ws[i] <- 1/3 + (10/14 - 1/3) * (ui - 1/3)/(1/2-1/3)
  } else {
    Ws[i] <- 10/14 + (1 - 10/14) * (ui - 1/2)/(1 - 1/2)
  }
}

diag(Ts) <- diag(Ts) * Ws
Ts <- Ts/sum(Ts)



# Tp, 3D Transmission array from sire and dam (n x n x n)


Tp <- Ts %o% Td  # outer product



# Progeny  genotype probability array
GPA.offspring <- U.offspring %o% U.offspring %o% U.offspring


# Likelihood
L <- sum(Tp * GPA.offspring)
```