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JAMES COOK UNIVERSITY
AUSTRALIA

Population genetics, phylogeography and the effects of aquaculture on genetic diversity of the silver-lipped pearl oyster, *Pinctada maxima* (Jameson)

Thesis submitted by

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May 2009

For the degree of Doctor of Philosophy,

from the School of Marine and Tropical Biology, James Cook University, Townsville, Queensland, Australia

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Statement on the Contribution of Others

At the time of thesis submission, three manuscripts had been published in peer-reviewed academic journals. The contributions of co-authors to the respective manuscripts are outlined below:

Manuscript from Chapter 2:

Lind, C.E., Evans, B.S., Taylor, J.J.U., Jerry, D.R., 2007. Population genetics of a marine bivalve, *Pinctada maxima*, throughout the Indo-Australian Archipelago shows differentiation and decreased diversity at range limits. Molecular Ecology 16, 5193-5203.

Contributions: CEL - Project design; sample collection; data generation & analysis; manuscript preparation

BSE, JJUT, DRJ - Project design; sample collection; review of final manuscript

Manuscript from Chapter 4:

Lind, C.E., Evans, B.S., Knauer, J., Taylor, J.J.U., Jerry, D.R., 2009. Decreased genetic diversity and a reduced effective population size in cultured silver-lipped pearl oysters (*Pinctada maxima*). Aquaculture 286, 12-19.

Contributions: CEL - Project design; sample collection; data generation & analysis; manuscript preparation

BSE, JJUT - Project design; sample collection; review of final manuscript

JK - Sample collection

DRJ - Project design; review of final manuscript

Manuscript from Chapter 5:

Lind, C.E., Evans, B.S., Taylor, J.J.U., Jerry, D.R., The consequences of differential family survival rates and equalizing maternal contributions on the effective population size (Ne) of cultured silver-lipped pearl oysters, *Pinctada maxima*. Aquaculture Research. doi:10.1111/j.1365-2109.2009.0210.x

Contributions: CEL - Project design; sample collection; data generation & analysis; manuscript preparation

BSE, JJUT, DRJ - Project design; sample collection; review of final manuscript

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Abstract

The silver/gold-lipped pearl oyster, *Pinctada maxima* (Jameson 1901), is one of the most important pearl producing species throughout Southeast Asia and northern Australia. The commercial production of high value "South Sea" pearls is based on the culture of *P. maxima*, and stands to benefit substantially from the implementation of long-term selective breeding programs. Industry-wide interest towards genetic improvement of *P. maxima* is rapidly growing, however, several fundamental issues must be addressed before selection commences. To achieve sustained response to selective breeding, it is essential that adequate genetic diversity is present within the population; yet currently there is little known about how the culture process affects diversity in *P. maxima*. This thesis addresses key issues concerning the capture and maintenance of genetic diversity in cultured *P. maxima* populations, as well as identifying and understanding patterns of genetic structure and diversity distribution throughout its natural range.

As the source of broodstock to create base populations for selective breeding, it is important to understand the genetic properties of wild *P. maxima* populations. Analysis of microsatellite and mitochondrial DNA (mtDNA) variation in wild *P. maxima* populations throughout its natural distribution revealed a gradient in genetic diversity across its range, with decreasing levels of variation seen in peripheral populations when compared to those situated more centrally (i.e. central Indonesia). Significant genetic structuring and differentiation was also observed amongst populations, and is attributed to historic and contemporary biogeographic influences.

Comparisons between wild and cultured *P. maxima* populations indicate a high level of genetic erosion has occurred in hatchery-propagated populations, with effective populations sizes (N_e) as low as 3.5 and reductions in microsatellite variation as high as 44% occurring as early as two generations beyond wild progenitors. The practice of mass spawning was identified as a major factor in the reduction of diversity, although diversity was not necessarily maintained when a more controlled spawning approach was utilised. Subsequent investigation using DNA parentage analyses revealed highly variable broodstock contributions have played a significant

role towards an increase in genetic relatedness and low N_e in cultured *P. maxima* and is likely to be exaggerated by variable survival rates amongst different pearl oyster families. Upon further investigation and experimentation, it was determined that highly variable family survival will affect N_e in communally reared *P. maxima* and the practice of equalising family sizes in order to reduce family size variance (and maximise N_e) may only become consistently beneficial once further progress is made towards understanding and then reducing variation in family survival rates. Culture practices related to variation in growth, such as size grading, culling and mass-selection were assessed for the ability to contribute to diversity losses. It was cautioned that broodstock selection for subsequent generations was potentially far more influential on diversity maintenance than culling or grading.

Outcomes of this thesis have provided a substantial advancement in the understanding of factors influencing genetic diversity in wild and cultured *P. maxima* populations. Population structuring and differentiation found in wild *P. maxima* provides grounds for further investigation into possible hybrid vigour or outbreeding depression when crossbreeding different stocks, and whether population genetic differences translate into phenotypic variation in commercially significant traits that could be exploited by selective breeding. This thesis also highlights important culture practices that must be improved (or avoided) in order to capture and retain genetic diversity and reduce inbreeding within closed populations, which will increase the likelihood of sustained response to selection programs. It is recommended that to ensure the maintenance of genetic variation and long-term sustainability of future *P. maxima* selection programs, culturists should employ the use of molecular tools for parentage assignment of candidate broodstock to avoid mating related individuals, or implement structured breeding designs intended to conserve genetic variability whilst maximising genetic response to selection.

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Chapter 1 General Introduction

1.1 Selective breeding and the significance of genetic diversity in aquaculture

Once the complete closure of an aquaculture species' life-cycle in captivity has been achieved the opportunity for selective breeding to boost productivity becomes possible. By comparison to terrestrial livestock production, however, the implementation of long-term captive breeding programs to genetically improve desirable production traits in aquaculture species has been limited (Gjedrem, 2005). Early attempts at genetic improvement in aquaculture saw poor responses to selection, and were believed to be due to inefficient selection methods, a rapid accumulation of inbreeding and inadequate control of environmental influences affecting traits of interest (Moav & Wohlfarth, 1976 ; Hulata *et al*., 1986 ; Huang & Liao, 1990). More recently, successful selective breeding efforts in aquaculture have been achieved but are restricted to only a handful of species (reviewed in Hulata, 2001). Despite this, in the vast majority of aquaculture operations domestication has not surpassed 3-4 generations beyond wild progenitors, with regular and readily sourced broodstock obtained from local wild populations.

Although domestication in aquaculture is still young, the potential to capture and exploit high levels of naturally occurring genetic diversity by utilising wild-sourced broodstock is a significant advantage when developing new selective breeding programs. Within a population, high levels of genetic diversity is associated with greater general fitness and resilience/adaptability to fluctuating environmental pressures (Reed & Frankham, 2003) and thus is favoured for any aquaculture operation. Further to this, a sustained response to selective breeding efforts is dependent on the presence of sufficient genetic diversity within a population, and will also allow continued selection if breeding objectives shift over time (Davis & Hetzel, 2000). Therefore the capture and subsequent retention of genetic diversity within successive generations of captive populations should be of high priority to aquaculturists.

A significant stumbling block to selection programs in aquaculture, however, is that genetic diversity can be easily and rapidly lost - often unbeknownst to the culturist - by poor husbandry

methods and culture techniques, therefore limiting potential response to selection. More concerning is that substantial loss of genetic diversity within closed aquaculture populations have been consistently reported over the last 25 years, strongly indicating that advances in aquaculture technologies and culture practices during this period have not translated into better maintenance of genetic resources (Wada, 1986 ; Vuorinen, 1984 ; Verspoor, 1988 ; Sekino *et al*., 2003 ; Sbordoni *et al*., 1986 ; Saavedra, 1997 ; Porta *et al*., 2007 ; Norris *et al*., 1999 ; Nielsen & Gan, 1994 ; Mgaya *et al*., 1995 ; Lundrigan *et al*., 2005 ; Lind *et al*., 2009 ; Li *et al*., 2004 ; Launey *et al*., 2001 ; Frost *et al*., 2006 ; Evans *et al*., 2004a ; Eknath & Doyle, 1990 ; Durand *et al*., 1993 ; Cross & King, 1983 ; Campton, 2004 ; Brown *et al*., 2005 ; Benzie & Williams, 1996 ; Appleyard & Ward, 2006 ; Alarcon *et al*., 2004 ; Aho *et al*., 2006).

In many instances, past and present, the relative ease of producing large populations from very few broodstock has meant that the long-term genetic implications of utilising a small number of breeders is regularly overlooked in favour of short-term production gains. Consequently, by using only limited numbers of breeders, the availability of favourable genes for future generations is directly affected; and subsequent broodstock selection from a pool containing many closely related individuals will significantly increase the risk of future inbreeding depression. Simply using more broodstock, however, will not necessarily assure increased genetic diversity if inefficient spawning techniques such as mass-spawning are employed, where broodstock contributions are often highly skewed in favour of a single parent-pair (Frost *et al.*, 2006 ; Herlin *et al*., 2008 ; Sekino *et al.*, 2003). Other culture practices that have been traditionally thought to improve productivity, such as culling of smaller ‘inferior’ individuals, have recently been shown to reduce genetic variation within cultured populations through the disproportionate removal of smaller families (e.g. Taris *et al*., 2006).

Rearing many different families communally (i.e. in the same pond/tank) is often unavoidable in aquaculture, particularly if broodstock require social stimuli for successful spawning (e.g. barramundi, *Lates calcarifer* (Frost *et al.*, 2006) or Atlantic cod, *Gadus morhua* (Herlin *et al.*, 2008)), or if limited infrastructure restricts separate rearing of different full-sib families. This has led to difficulties retaining pedigree information of communally reared individuals, and is a

significant factor contributing to inadvertent diversity losses in aquaculture. When different families are communally reared, several questions essential to the effective management and maintenance of genetic diversity arise. How is the culturist to know which broodstock have contributed and how much are their progeny represented after mass-spawning? Have all families created at spawning survived the entire culture process? Are candidate broodstock related? Without knowledge of pedigree, such questions cannot be reliably answered, and severely restrict the adequate monitoring of genetic diversity within aquaculture populations.

The advent of molecular genetic tools that permit DNA parentage assignment has provided a promising technique for pedigree determination of communally reared aquaculture organisms *a posteriori* (Liu & Cordes, 2004). Indeed, the use of polymorphic microsatellite markers in particular have been highly effective in parentage assignment and genetic management of aquaculture populations (e.g. Borrell *et al*., 2004 ; Boudry *et al*., 2002 ; Jackson *et al*., 2003 ; Jerry *et al*., 2004 ; Selvamani *et al*., 2001), and are emerging as an invaluable tool for identifying critical stages of the culture process that contribute to genetic diversity loss. If selective breeding is to make a significant impact to the improvement of commercially important traits in aquaculture, it is now realised that particular attention must be given to understanding and improving how effective current culture practices are at 1) capturing available genetic diversity from wild progenitors; and 2) maintaining genetic variation within closed populations once it has been captured. Through the utilisation of molecular tools and microsatellite DNA parentage analyses, the major theme of this thesis will focus on these two key issues, with particular attention given to factors affecting the capture and maintenance of genetic diversity within cultured populations of the silver-lipped pearl oyster, *Pinctada maxima*.

1.2 Pearl culture overview

Since the development of artificial pearl culture techniques in the early 1900's, the production of cultured marine pearls has become a significant industry throughout Southeast Asia, northern Australia and Pacific island nations (Southgate, 2007). The marine pearl industry is primarily based on culture of pearl oysters from the genus *Pinctada spp* or *Pteria spp* (Family: Pteriidae) (Gervis & Sims, 1992), with an estimated annual global value of over US$500m (2004 data,

Torrey & Sheung, 2008). The silver/gold-lipped pearl oyster, *Pinctada maxima* (Jameson 1901), is the largest pearl oyster species and, due to its size, is valued for its ability to produce large (>15 mm), round, high-quality silver and gold pearls. As a marketing strategy implemented to differentiate between the smaller Japanese Akoya pearls, silver and gold pearls produced using *P. maxima* have become known as "South Sea pearls". South Sea pearls can be cultured throughout the tropical Southeast Asia and northern Australia, although the majority of production is carried out in northwest Australia, the Philippines, Indonesia and Myanmar (Southgate, 2007 ; Shor, 2007).

Historically, underwater divers collected all seed stocks used for *P. maxima* pearl production from naturally occurring oyster beds. In addition to diver-collected stocks, spat collectors that encourage the settlement of newly metamorphosed pearl oyster larvae (spat) onto artificial substrates can also be deployed across ocean sites. In these instances settled spat are then on-grown until they attain a size suitable for the pearl production process (>110mm). Although the collection of wild pearl oyster has been largely successful (and still a significant source of operational stock in Australian pearling (Wells & Jernakoff, 2006)), fluctuations and unpredictability in annual recruits plus concerns about the sustainability of wild fisheries led a drive to develop hatchery-based propagation techniques as an additional source of production stock. Early experimentation on artificial culture of *P. maxima* was first reported in the late 1960s, but with limited success (Minaur, 1969). In subsequent years, advancements in artificial propagation techniques for *P. maxima* were not widely published due to intense secrecy amongst pearling companies and any research outcomes were kept in-house. A few works were published in this period, however, and remain seminal publications on the hatchery culture of *P. maxima* (Gervis & Sims, 1992 ; Rose *et al*., 1990 ; Rose & Baker, 1994 ; Tanaka & Kumeta, 1981; Taylor *et al*., 1997a ; Taylor *et al*., 1997b ; Taylor *et al*., 1997c ; Taylor *et al*., 1998a ; Taylor *et al*., 1998b). Since the late 1980s the importance of hatchery-produced pearl oysters has rapidly increased from being a negligible component of a company's working stock, to becoming the entire source of seeded shell in most Southeast Asian pearling operations and comprising up to 50% of many Australian operations (Fletcher *et al*., 2006 ; Shor, 2007).

Although husbandry and management will invariably differ from farm to farm, a typical pearl oyster hatchery will use broodstock that has been either collected from the wild or from previously produced hatchery stocks. At the time this thesis commenced, the mass-spawning approach was predominantly used for broodstock fertilizations, whereby a pool of sexually ripe broodstock (up to 100-150 individuals) are placed in a single, large spawning tank/tray and induced to spawn after a series of water temperature manipulations and tank drainages. Fertilization between male and female gametes will occur in the water-column and then zygotes are collected on a fine mesh sieve beneath the tank outlet valve (J. J. U Taylor *pers. comm.*) and reared in fibreglass or concrete tanks until settlement/metamorphosis onto artificial substrates like nylon ropes or mesh. Throughout the pearl production process, the period where animals are reared in the hatchery is the only time when supplemental food is provided, by maintaining high concentrations of microalgae within hatchery tanks enabling larvae and spat to filter feed. Pearl oysters are not transferred to open ocean growout sites until approximately one month post-fertilization (~10-14 days post-settlement). Typically, the grow-out phase of pearl oysters is the same for hatchery produced and wild-collected animals, where oysters are grown in pocketed mesh panels that are suspended from longlines moored in open ocean sites. This allows the pearl oysters to filter-feed on naturally occurring plankton. To allow efficient feeding rates, pearl oysters are regularly cleaned of biofouling marine organisms using high-pressure water guns; and to prevent overcrowding they are periodically moved to lower density individually pocketed panels, as the animals grow larger. Size grading of pearl oysters can also occur, which categorises animals into size classes in order to assist management decisions such as timing of pearl nucleus implantation.

To initiate pearl formation, each oyster is surgically implanted with a spherical shell bead nucleus alongside a small graft of mantle “saibo” tissue (known as ‘seeding’) cut from a donor oyster, which will deposit lustrous nacre layers around the nucleus and form a pearl. Seeding can usually commence once individuals have attained approximately 110mm dorso-ventral shell height (J.J.U Taylor *pers. comm.*). Seeded oysters are usually ongrown in the same manner as pre-seeded oysters (known as virgin oysters) for a further 12-18 months before the pearl is harvested, allowing the nacre layer to reach 1-2mm thick.

Once a pearl has been harvested, it is then classified based on several factors that, in combination, will determine its quality and therefore its market value. Variations in five factors - shape, colour, lustre, skin perfection and size - can have enormous influence on a pearl's value and consequently, the income of a pearl culture operation. Such is the variation in pearl quality generally observed in most farms that only a small proportion of pearls produced are categorized as 'gem' quality, yet it is these pearls that can contribute the majority of an operation's revenue. What is promising, however, is that if variation in pearl quality traits has a genetic basis, selective breeding for favourable traits may permit a substantial shift in the proportion of high-quality pearls produced by hatchery-cultured pearl oysters, significantly improving profitability. This of course is also true for many other traits of commercial significance, such as growth and survival, which can also influence the success of a pearling operation. Currently, there has been no significant genetic improvement in *P. maxima* from selective breeding despite these potential benefits; therefore, this forms a driving influence behind this thesis. Before long-term genetic improvement programs can commence, however, it is important to understand several key factors related to the culture process itself. These issues form the basis of this thesis, and are discussed in further detail in the following section.

1.3 Thesis aims and structure

This thesis encompasses a core component of the Australian Research Council Linkage Project LP0560298 "*Towards selective breeding of the silver-lipped pearl oyster, Pinctada maxima*", developed and undertaken in collaboration with industry partner, Atlas South Sea Pearl Ltd. With a major focus on understanding factors affecting genetic diversity and structure of natural *P. maxima* populations, along with identifying critical stages of the aquaculture process affecting the maintenance of genetic diversity in closed populations, the thesis addresses key issues that are necessary to understand before selection can commence, and provides a solid foundation for the future implementation of long-term genetic improvement programs in *P. maxima*.

Chapters 2 and 3 describe population genetic aspects of wild *P. maxima* populations spanning the majority of its natural distribution. As a source of broodstock to establish a selection

program, it is important to understand the genetic characteristics of wild *P. maxima* populations. Many pearling operations (such as the industry partner) have several hatchery and grow-out sites spread over broad geographic regions, therefore it is important to ascertain whether wild broodstock collected from various regions/sites are of the same or different genetic stocks. However, to date studies of this nature are limited to only a few regions in northwest Australia and small regions of Indonesia (Benzie *et al*., 2003 ; Benzie & Smith-Keune, 2006 ; Johnson & Joll, 1993). Using mitochondrial DNA and microsatellite DNA markers, Chapters 2 and 3 investigate the genetic structure and patterns of genetic diversity distribution in wild *P. maxima* populations throughout the Indo-West Pacific and northern Australia. This represents the largest and most comprehensive genetic survey of this species to date.

Once baseline levels of genetic diversity have been established in wild *P. maxima* populations (Chapters 2 and 3), an opportunity arises to assess the extent of genetic diversity losses in cultured populations. To determine the degree of genetic erosion occurring in cultured populations, Chapter 4 examines genetic diversity levels in hatchery-produced populations and the wild Indonesian populations from which their broodstock were sourced. In artificially spawned aquaculture species, broodstock contributions are often highly skewed towards only a few breeders, which can significantly restrict genetic diversity levels (e.g. Frost *et al.*, 2006 ; Herlin *et al.*, 2008 ; Sekino *et al.*, 2003). Through DNA parentage assignment methods, Chapter 4 also investigates how different spawning approaches affect the degree of variability in broodstock contributions, and its subsequent influence on genetic diversity reductions in cultured populations.

Chapter 5 investigates whether different *P. maxima* families exhibit variable survival rates when reared communally, and the potential influence this may have on differential broodstock contributions in cultured populations. Relative contributions of communally reared families were determined between 72 days and 18 months of age using DNA parentage assignment, which allowed individual survival rates to be calculated. A manipulative stocking experiment was also conducted, to determine whether equalising maternal broodstock contributions prior to

communal rearing would reduce uneven broodstock contributions and therefore maximise genetic diversity, as indicated by the effective population size (N_e).

Chapter 6 examines whether variable growth rates occur amongst communally reared pearl oyster families and how genetic diversity may be affected by growth-related culture practices such as culling of smaller individuals or broodstock selection based on superior growth. By combining morphometric data of 18-month-old pearl oysters with its pedigree (determined using DNA parentage analyses), it was determined whether some families were disproportionately affected by the culture practices of indiscriminate culling and broodstock selection based solely on phenotypic superiority.

A synopsis of the five data chapters is given in Chapter 7 alongside a general discussion relating to how effective genetic diversity is captured and maintained in cultured *P. maxima* populations. Particular attention is given to implications of these outcomes in the context of future selective breeding programs in *P. maxima*.

Each of the five data chapters in this thesis contains a stand-alone Introduction, Materials & Methods, Results and Discussion section. At the time of thesis submission, several chapters have been accepted for publication in peer-reviewed journals and are presented as published herein, with minimal modification and minor re-formatting. It should be noted at this point that in answering key questions related to the major theme of this thesis, several broader ecological and evolutionary issues were also addressed (particularly Chapter 2 and 3 investigating wild *P. maxima* populations). In order to improve publication success and provide a greater appeal to the broader scientific community, these chapters were written with a focus on their ecological application; however, the implications of these outcomes in relation to aquaculture and selective breeding are discussed in more detail within the General Discussion (Chapter 7).

Chapter 2 Genetic diversity and structure of wild *P. maxima* populations - Mitochondrial DNA[†]

2.1 Introduction

The Indo-Australian Archipelago is a globally significant reservoir of marine and coral reef biodiversity and is one of the world's most species-rich regions (Roberts *et al.*, 2002). Marking the junction between both the Pacific and Indian Oceans, and the tectonic convergence of the Australian and Eurasian continental plates, the region is characterised by geographic and oceanographic complexity (Gordon & Fine, 1996). As a possible reflection of these physical intricacies and their effect on the evolution and ecology of species, phylogenetic studies on marine and coral reef organisms of the Indo-Australian Archipelago have shown that this region is of unquestionable biogeographic and evolutionary importance (Barber *et al.*, 2000 ; Bellwood & Wainwright, 2002 ; Benzie, 1998 ; Briggs, 1999 ; Briggs, 2005). However, it is still uncertain whether broad scale mechanisms affecting population dynamics in this region are pervasive across a collective of species, or if these processes impact the population structure of each organism differently as a result of varying ecologies and life-histories (Lourie *et al.*, 2005).

The incorporation of molecular genetic analyses into population studies throughout the Indo-Australian Archipelago has revealed otherwise undetectable factors influencing population dynamics in this region. For species that are broadly distributed across both Indian and Pacific Ocean basins, the Sumatra-Java island chain of the Indo-Australian Archipelago recurrently delineates a sharp intra-specific genetic break (Lavery *et al.*, 1996 ; Williams & Benzie, 1998 ; Benzie, 1998). It is inferred that the Indo-Australian Archipelago has functioned as a restrictive barrier to gene flow over evolutionary timescales, particularly during historical periods of lowered sea level, driving a genetic divergence between Indian and Pacific Ocean populations.

[†] Manuscript: Lind C.E., Evans B.S., Elphinstone M.S., Taylor J.J.U., Jerry D.R. (in review) Phylogeography of a pearl oyster (*Pinctada maxima*) across the Indo-West Pacific: evidence of strong regional structure and population expansions but no phylogenetic breaks. Marine Biology

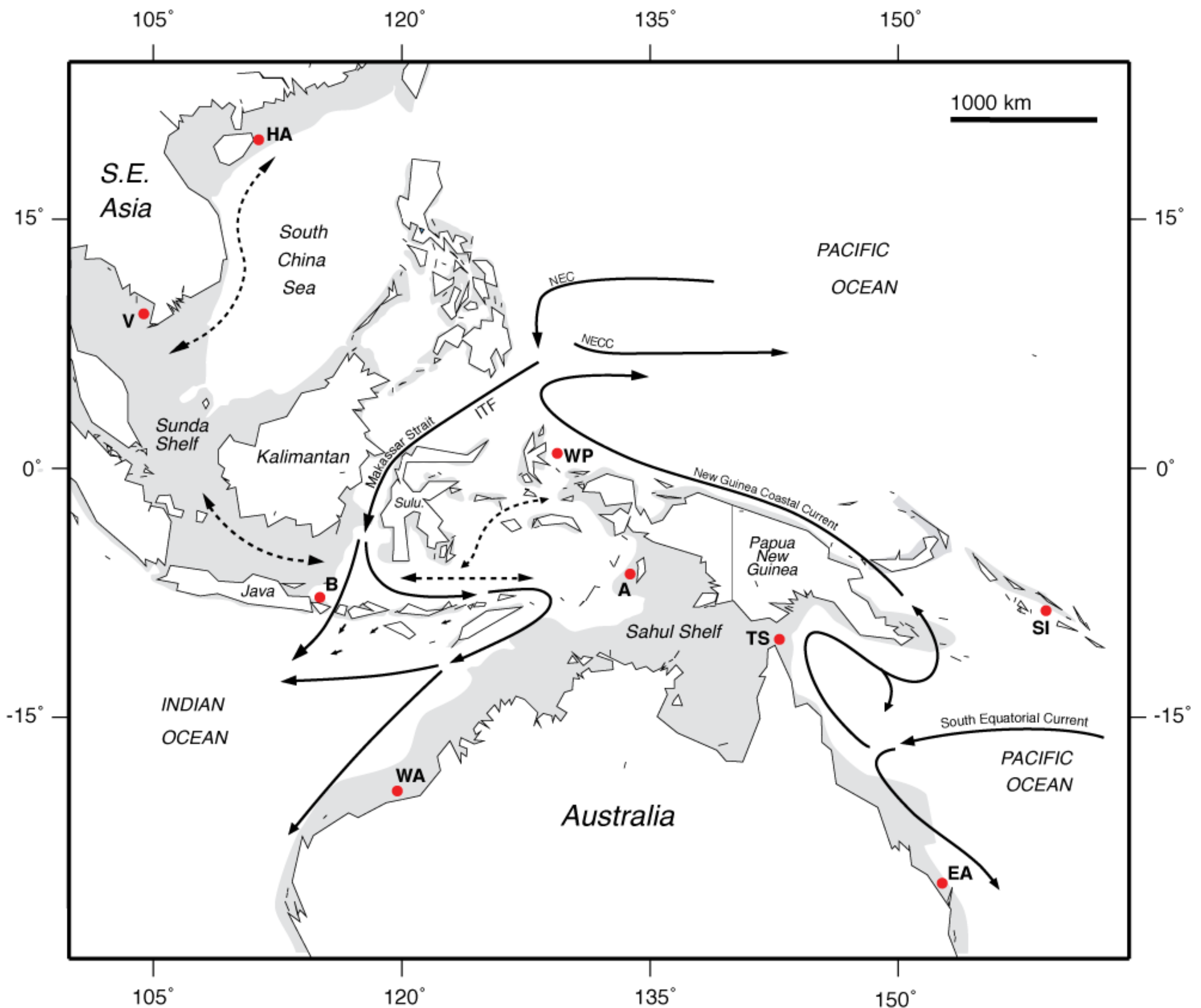


Figure 2.1 Map showing *Pinctada maxima* sampling locations throughout the Indo-Australian Archipelago. Shaded area indicates shoreline during Pleistocene low sea level stands (100m below present day level, following Voris, 2000). Black arrows indicate major ocean currents across the region, dotted arrows show seasonally reversing currents (simplified from Gordon & Fine, 1996 ; Wyrtki, 1961). ITF, Indonesian Throughflow Current; NEC, North equatorial current; NECC, North equatorial counter current; Sulu., Suluwesi; HA, Hainan Island; V, Vietnam; WP, West Papua; B, Bali; A, Aru; WA, Western Australia; TS, Torres Strait; EA, East Australia; SI, Solomon Islands.

For some species, however, the Indo-Australian Archipelago has not been so impervious to historical dispersal, with several studies indicating an absence of deep phylogenetic divergence between regions, implying regular genetic exchange between ocean basins (Uthicke & Benzie, 2003 ; Lessios *et al.*, 2003). Patterns of population differentiation across the Indo-Australian Archipelago are not only restricted to the axes of Indian/Pacific separation. During periods of

lowered sea levels (most recently during the Late Pleistocene, ~17 000 years ago), a complex bathymetry of expansive continental shelf, extended steep ridges and deep troughs within the archipelago itself has isolated ocean basins (e.g. Celebes and Sulu Seas) by exposing physical land barriers between populations that could once freely exchange migrants (see Voris, 2000). Several examples of sharp phylogenetic breaks have been observed between populations separated by small geographic distances, which have most likely been due to such separation of smaller ocean basins (Barber *et al.*, 2002 ; Lourie *et al.*, 2005 ; Timm & Kochzius, 2008). Population separation due to Pleistocene sea level fluctuations has also been cited as a major contributor to genetic disjunctions either side of the Torres Strait, where genetic exchange has been limited due to an exposed Sahul Shelf between northern Australia and Papua New Guinea (Chenoweth *et al.*, 1998 ; Gopurenko & Hughes, 2002 ; Lukoschek *et al*., 2007). In addition to the effects historical isolation and separation of oceanic basins may have had on the formation of genetic structuring, species within this region are also impacted by oceanographic factors, such as immense passage of water conveyed by the Indonesian Throughflow current (Figure 2.1), which moves water from the Pacific to Indian Oceans. Such factors can play a significant role in shaping population genetic patterns in the region by either facilitating or restricting passive larval transport between populations (Barber *et al.*, 2002 ; Kochzius & Nuryanto, 2008).

In addition to its evolutionary and ecological significance, the highly productive marine ecosystems of the Indo-Australian Archipelago also provide a vital economic resource to surrounding nations through small-scale fisheries and tourism (Burke *et al*., 2002). Unfortunately the high incidence of unsustainable commercial practices and environmental degradation has placed this region under considerable threat of over-exploitation (Burke *et al.*, 2002), resulting in the need to assign high priority to the implementation of conservation efforts and resource management practices throughout the region (Roberts *et al.*, 2002). For commercially exploited marine organisms, identifying and understanding factors influencing population dynamics is a critical step towards their appropriate management and sustainable utilisation; however, the evident complexity of population structuring throughout the Indo-Australian Archipelago requires continued investigation in order to better understand how historic and contemporary physical processes affect marine organisms in the region.

The silver-lipped pearl oyster, *Pinctada maxima,* is commercially valued for the production of 'South Sea' pearls and is widespread throughout the tropical and sub-tropical regions of the Indo-Australian Archipelago. An extended planktonic larval stage of 17 - 24 days (Rose & Baker, 1994) provides potential for *P. maxima* to broadly disperse via strong ocean currents, however, microsatellite DNA variation suggests dispersal and gene flow capabilities are limited, resulting in population genetic structuring throughout its distribution (Benzie & Smith-Keune, 2006 ; Lind *et al*., 2007). However, the phylogenetic history of *P. maxima* remains unknown. By adopting a phylogeographic approach, it is possible to ascertain whether the contemporary structure of *P. maxima* populations shown by microsatellites is reflective of a genetic footprint borne as a result of historical biogeographic influences, and is the primary focus of this study. Using sequence information from mitochondrial DNA, this study investigates population genetic aspects of *P. maxima* throughout a biogeographically complex and ecologically important region. Through the potential identification of genetically differentiated populations, this will allow a more informed management of a commercially significant resource throughout the Indo-Australian Archipelago, and further increase understanding of the mechanisms of historical biogeographic influences on the genetic structure of marine organisms in the region.

2.2 Materials and Methods

2.2.1 Tissue sampling

Tissue samples were taken from 367 *P. maxima* individuals from nine populations throughout the Indo-Australian Archipelago, spanning China, Vietnam, Indonesia, tropical Australia and the Solomon Islands (Figure 2.1). Local divers collected live oysters from naturally occurring oyster beds in 2004 and 2005, and foot, mantle or muscle tissue samples were excised and preserved in 70-80% ethanol.

2.2.2 DNA extraction, PCR conditions & haplotype detection

Preserved tissue was digested in a CTAB buffer with 20 mg ml^{-1} proteinase K for 1-3 hrs at 55°C, followed by a phenol:chloroform:isoamyl alcohol purification protocol to extract total genomic DNA (gDNA) (Sambrook *et al.*, 1989). Genomic DNA (gDNA) was quantified by

comparison to DNA concentration standards after agarose gel electrophoresis using the ImageJ 1.33 software package (Wayne Rasband, 2004) and resuspended in ddH_2O to a concentration of 5 ng μl^{-1}.

Individual *P. maxima* were assessed for genetic variability using heteroduplex / temperature gradient gel electrophoresis (TGGE) analysis (Campbell *et al.*, 1995 ; Elphinstone & Baverstock, 1997) to screen for sequence mutations of a 680-base pair region of the mitochondrial cytochrome oxidase subunit I (COI) gene (using primers COIeF: 5' ATA ATG ATA GGA GGR TTT GG 3' and a GC-clamped reverse primer, COIeR-GC: 5' CGC CCC GCC GCG CCC CGC GCC CGT CCC GCC GCC CCC GCC CGC TCG TGT RCT ACR TCC AT 3' (Arndt *et al.*, 1996)). The implementation of a GC-clamp to the reverse strand primer was to reduce complete denaturation of PCR fragments during TGGE, and can also improve the detection of single-base changes in DNA fragments across denaturing gradients (Sheffield *et al.*, 1989). Polymerase chain reactions (PCR) were conducted in 15 µl volumes with reagent concentrations of 1 x PCR buffer containing 1.5 mM $MgCl_2$ (QIAGEN), 0.2 mM each dNTP, 0.5 µM each primer, 0.03 U μl^{-1} Taq DNA polymerase (QIAGEN) and 0.2-0.4 ng μl^{-1} of DNA template. Thermocyler conditions were as follows: an initial denaturation step of 94 ^{o}C for 2 min, followed by 30 cycles of 94 ^{o}C for 1 min, 45 ^{o}C for 1 min, 72 ^{o}C for 1 min 30 secs; followed by a final elongation step of 72 ^{o}C for 5 min. Each individual was heteroduplexed (HD) with a reference sample (see below) and subjected to TGGE on 5% polyacrylamide gels (8 M urea, 1 x ME buffer) following the HD/TGGE procedures outlined by (Campbell *et al.*, 1995). A melt transition of ~48 ^{o}C was determined from a perpendicular gel with a temperature gradient of 20-60 ^{o}C (300 V, 1.5 hrs), and subsequent parallel gels were run across a temperature gradient of 28-58 ^{o}C (300 V, 2.5 hrs). Three candidate reference samples were tested on a subsample of 10 individuals to determine which reference could best resolve heteroduplex bands. Haplotype 1 was the best resolving reference and thus used for all subsequent HD/TGGE. To aid haplotype scoring accuracy, positive (i.e. reference-known haplotype HD) and negative (i.e. reference-reference HD) control samples were run on each gel. HD banding patterns were visualised through silver staining.

Representative samples (2-4 samples per haplotype) from each putative haplotype resolved by TGGE were amplified with COIeF and COIeR (no GC-clamp: 5' GCT CGT GTR CTA CRT CCA T 3') primers using the same PCR conditions as above. PCR products were then purified by centrifugation through Sephadex™ (Sigma-Aldrich) columns before being sequenced by Macrogen Inc. (Seoul, Korea). Where uncertainty in scoring TGGE haplotypes by eye was encountered (e.g. abnormal sample migration due to an air-bubble or imperfection in a gel), samples were re-amplified and sent for sequencing. In all instances of uncertain scoring, sequence data confirmed samples to be the same haplotype as indicated on the gel.

2.2.3 Statistical analyses

Sequence data were proofread and aligned by eye using SEQUENCHER v4.5 (Gene Codes Corporation) before being subjected to further phylogenetic analyses. Haplotype diversity and nucleotide diversity (Nei, 1987) were calculated for each population using ARLEQUIN v3.1 (Excoffier *et al.*, 2005). To test the selective neutrality of mutational differences observed across populations, and investigate for indications of rapid population expansions, Tajima's *D*-test statistic and Fu's *F*-test were generated using ARLEQUIN v3.1. Further investigation for genetic signals of population equilibrium was performed using Harpending's raggedness index (HRI), and the sum of squared deviations (SSD) between the observed and expected distribution of pairwise sequence mismatches, calculated using ARLEQUIN v3.1. A hierarchical analysis of molecular variance (AMOVA) was undertaken using ARLEQUIN v3.1 to determine whether spatial partitioning of genetic variation was present on a regional (Φ_{CT}) and/or population level (Φ_{ST}) (based on 1000 permutations). Pairwise Φ_{ST} values were also calculated between all populations, and the significance of population differentiation determined after 1000 permutations. Using pairwise Φ_{ST} values together with geographic location of each population, Monmonier's maximum difference algorithm (Monmonier, 1973) was implemented using BARRIER v2.2 (Manni *et al.*, 2004) to provide a computational geometric approach toward identifying putative genetic boundaries across the distribution of *P. maxima*. In association with spatial information defined by population locations, the algorithm defines a path of the greatest rate of change in a given distance measure (in this case pairwise Φ_{ST}) based on Delaunay triangulation and Voronoi tessellation (Manni *et al.*, 2004). A Mantel Test to determine the

statistical significance of the correlation of pairwise Φ_{ST} and the geographical distance between two populations was implemented with GENALEX v6 (Peakall & Smouse, 2006), where geographic distance between sample locations was measured as the shortest distance by water using GOOGLE EARTH (Google Inc.). According to likelihood ratio tests implemented in MODELTEST (Posada & Crandall, 1998), the sequence evolution model of (Tamura & Nei, 1993) with a predicted gamma distribution of 0.069 (TrN + G) best fitted the data set and was used in AMOVA and subsequent phylogenetic analyses. To investigate the phylogenetic relationship amongst haplotypes, minimum spanning trees based on (Tamura & Nei, 1993) genetic distance were generated in ARLEQUIN v3.1, and bootstrap maximum parsimony and neighbour-joining trees (based on 10 000 bootstrap replicates) were generated in PAUP* v4.0b10 (D. L. Swofford, Florida State University). A nested clade phylogeographical analysis (NCPA) was also conducted using GEODIS v2.5 (Posada *et al*., 2000) to explore geographic associations with haplotypes or clades of haplotypes. To perform NCPA, a parsimonious haplotype network was first created in ARLEQUIN v3.1 based on TrN + G distances with network ambiguities resolved following (Posada & Crandall, 2001); from which a nested cladogram was produced following the guidelines described in Templeton (1998). A permutational contingency test for non-random distribution of haplotypes/clades was conducted for each clade at each nesting level using population location as a categorical variable and permuted 1000 times. Since the contingency test is only concerned with categorical populations without distance information, it can be interpreted as an analogue to test an island model of genetic exchange (Templeton, 1998). In addition to contingency tests, a geographical distance test for each nesting clade was also implemented incorporating the relationship between clade distances (D_c), nested clade distances (D_n) and interior-tip clade distances (I-T), and interpreted following the inference key outlined in (Templeton, 2004). Since haplotype/clades located on a terminal point of a haplotype network are most likely to be younger than those located internally, NCPA also allows evolutionary and historical demographic inferences to be drawn based on the geographic distributions of internal and terminal (tip) clades.

2.3 Results

2.3.1 Genetic diversity and sequence phylogeny

From 367 individual pearl oyster samples TGGE and sequencing identified 47 haplotypes. The 635 bp mtDNA COI fragment analysed exhibited 45 variable sites (0.07%), of which 14 (0.02%) were parsimony informative. The most frequently observed haplotype was present in samples from every population except Solomon Islands, whilst the second most abundant haplotype was far more restricted in its distribution, observed predominantly throughout southeast-Asian locations (Figure 2.2). Populations from central Indonesia (i.e. Bali, West Papua) exhibited generally higher genetic diversity at both haplotype and nucleotide levels compared to other populations, with the exception of eastern Australia and Solomon Islands (Table 2.1). Samples from Solomon Islands showed greatest haplotype and nucleotide diversity, however, this result must be interpreted with caution given only five individuals were sampled from this location. A mean pairwise sequence divergence of 1.0% (maximum 1.8%) indicated that haplotypes were closely related. This was supported by phylogenetic analyses, with maximum parsimony and neighbour-joining trees giving low bootstrap support (<50%) for the presence of any deep phylogenetic lineages or divergence amongst sequences (data not shown).

2.3.2 Demographic history and population structure

Significantly negative values of Fu's F_s statistic were observed in the peripherally located populations of Hainan Island, Vietnam and eastern Australia, indicating historical demographic expansion events in these regions (Table 2.1). Tajima's D also indicated a significant deviation from selective neutrality in the Hainan Island population, however, this result can also be interpreted as a consequence of demographic changes (Table 2.1). A significantly negative Tajima's D statistic can also indicate population size expansion (by highlighting an excess of low frequency polymorphisms) (Tajima, 1989a, 1989b), which, in the case of the Hainan Island population, seems to be a likely explanation due to its location at the periphery of *P. maxima*'s natural distribution. A significant partitioning of genetic variation amongst populations was revealed by AMOVA when no regional grouping was inferred (Φ_{ST} = 0.372, P < 0.001); highlighting genetic structuring throughout the natural range of *P. maxima* on a population level.

Table 2.1 Genetic diversity statistics and tests of selective neutrality for *Pinctada maxima* populations across the Indo-Australian Archipelago. N_h, number of haplotypes; N_p, number of polymorphic sites per population; *h*, haplotypic diversity; π, nucleotide diversity; SSD, sum of squared differences of the mismatch distribution; HRI, Harpending's Raggedness index (Significance as indicated).

Population	Location	*n*	N_h	N_p	*h* ± S.E.	π ± S.E.	Fu's *F*	Tajima's D	SSD	HRI
1. Hainan Island	19.66N, 112.0E	36	9	8	0.51 ± 0.10	0.001 ± 0.001	-6.16**	-1.72*	0.002	0.073
2. Vietnam	9.08N, 105.25E	28	9	8	0.73 ± 0.08	0.002 ± 0.001	-3.97**	-1.03	0.013	0.101
3. West Papua	1.13N, 130.54E	61	13	17	0.80 ± 0.04	0.004 ± 0.002	-2.60	-0.86	0.012	0.038
4. Bali	8.32S, 114.92E	52	13	16	0.82 ± 0.04	0.004 ± 0.002	-3.08	-0.83	0.026	0.078
5. Aru	6.43S, 134.63E	55	8	13	0.74 ± 0.05	0.004 ± 0.002	1.41	0.30	0.072**	0.219**
6. Western Australia	19.29S, 119.75E	56	7	13	0.57 ± 0.07	0.003 ± 0.001	0.01	-1.13	0.440**	0.360
7. Torres Strait	10.97S, 143.18E	55	7	9	0.73 ± 0.04	0.003 ± 0.002	0.23	-0.13	0.029	0.105
8. Eastern Australia	24.20S, 152.84E	19	7	8	0.82 ± 0.06	0.002 ± 0.002	-2.37*	-0.94	0.006	0.089
9. Solomon Islands	8.30S, 158.6E	5	4	8	0.90 ± 0.16	0.006 ± 0.004	-0.26	-1.17	0.132	0.270

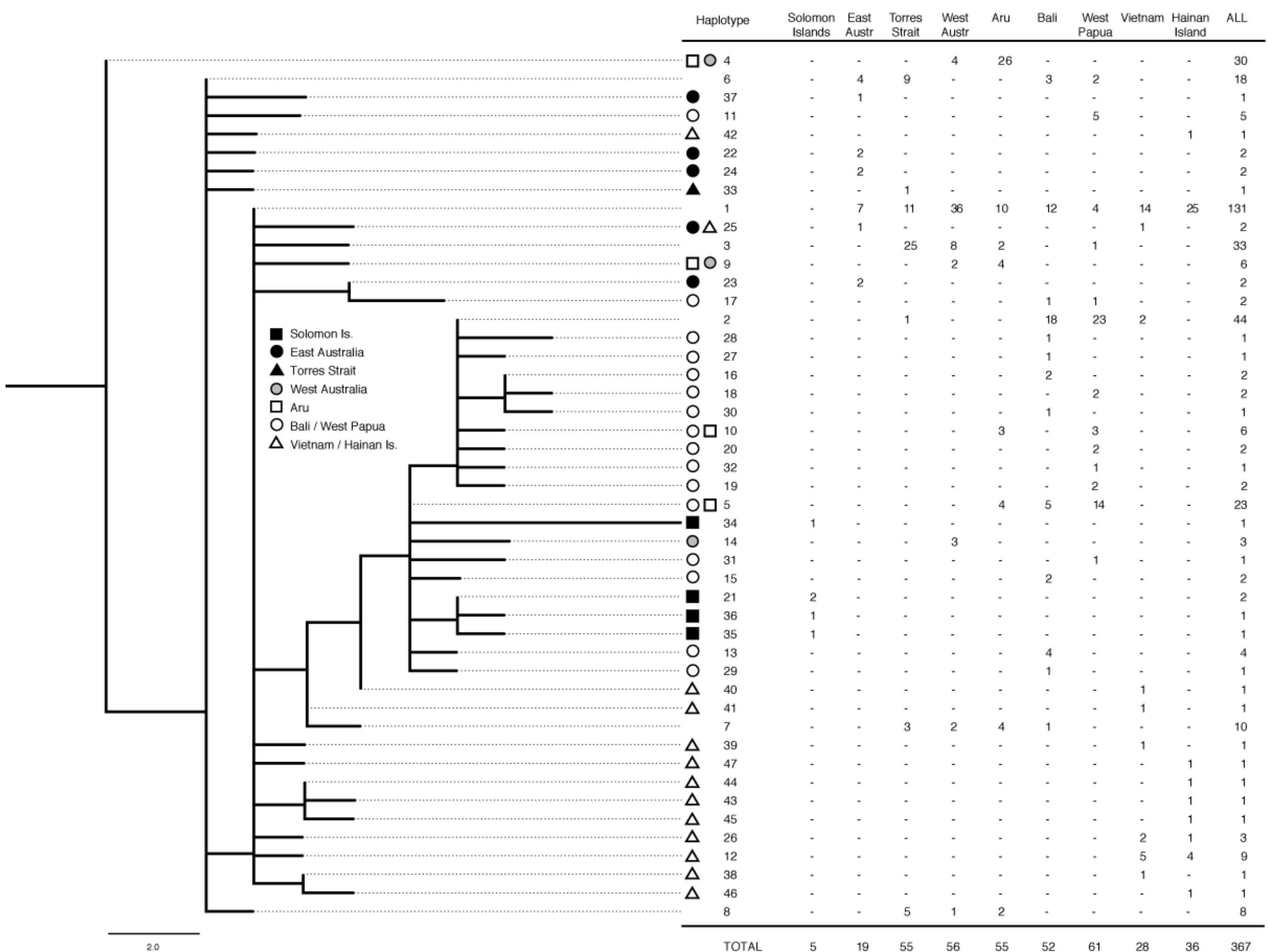
Table 2.2 Hierarchical analysis of molecular variance (AMOVA) to partition mtDNA COI gene variation amongst possible regional groupings of *Pinctada maxima* populations. HA, Hainan Island; V, Vietnam; WP, West Papua; B, Bali; WA, West Australia; A, Aru; TS, Torres Strait; EA, East Australia; SI, Solomon Islands.

Regional grouping	Amongst Population	Amongst Groups		
	Φ_{ST}	Φ_{CT}	% variation	*P*-value
[HA - V - WP - B - WA] [A - TS - EA - SI]	0.393***	0.072	7.23	0.30
[HA - V - WP - B - A - WA] [TS - EA - SI]	0.369***	-0.007	0.00	0.45
[HA - V - B] [WP - A - WA - TS - EA - SI]	0.329***	0.065	6.54	0.68
[HA - V] [WP - B] [WA - A] [TS - EA] [SI]	0.402***	0.338	33.87**	0.002
[HA - V] [WP - B - SI] [WA - A] [TS - EA]	0.403***	0.324	32.42***	<0.001
[HA - V] [WP - B - A] [WA] [TS - EA] [SI]	0.382***	0.081	8.10	0.27
[HA - V] [WP - B] [A - WA - TS - EA] [SI]	0.436***	0.362	36.22***	<0.001
[HA - V] [WP - B] [A] [WA] [TS - EA] [SI]	0.392***	0.360	36.02**	0.007
NO GROUPS	0.372***	-	-	<0.001

Table 2.3 Pairwise Φ_{ST} estimates between *Pinctada maxima* populations, based on the genetic distance of Tamura & Nei (1993) with gamma correction. Bold values indicate significant differences (*P* < 0.05 after false discovery rate correction (Benjamin & Hochberg, 1995)); underlined values become non-significant after correction.

Population	Hainan Island	Vietnam	West Papua	Bali	Aru	West Australia	Torres Strait	East Australia	Solomon Islands
1. Hainan Island	-								
2. Vietnam	0.035	-							
3. West Papua	**0.541**	**0.434**	-						
4. Bali	**0.484**	**0.363**	<u>0.011</u>	-					
5. Aru	**0.241**	**0.210**	**0.447**	**0.407**	-				
6. Western Australia	**0.059**	**0.061**	**0.474**	**0.410**	**0.157**	-			
7. Torres Strait	**0.271**	**0.256**	**0.538**	**0.492**	**0.241**	**0.110**	-		
8. Eastern Australia	**0.166**	**0.148**	**0.492**	**0.445**	**0.131**	**0.107**	**0.192**	-	
9. Solomon Islands	**0.812**	**0.684**	**0.283**	**0.312**	**0.547**	**0.683**	**0.719**	**0.693**	-

On a regional scale, however, hierarchical AMOVA showed that genetic structuring was more complex than simple broad-scale explanations of genetic differentiation (e.g. differentiation across Wallace's Line or Indo-Pacific separation). The partitioning of genetic variation was best explained when populations were grouped into regions coinciding with northern Asia, central Indonesia, and north-west Australia (including Aru) with eastern Australia (Table 2.2). The realisation of strong genetic structuring throughout the region was further confirmed when pairwise Φ_{ST} values amongst populations showed significant differentiation in all pairwise comparisons except between Hainan Island and Vietnam, and between Bali and West Papuan populations, indicating a high genetic exchange between these population pairs (Table 2.3).



Haplotype	Solomon Islands	East Austr	Torres Strait	West Austr	Aru	Bali	West Papua	Vietnam	Hainan Island	ALL
4	-	-	-	4	26	-	-	-	-	30
6	-	4	9	-	-	3	2	-	-	18
37	-	1	-	-	-	-	-	-	-	1
11	-	-	-	-	-	-	5	-	-	5
42	-	-	-	-	-	-	-	-	1	1
22	-	2	-	-	-	-	-	-	-	2
24	-	2	-	-	-	-	-	-	-	2
33	-	-	1	-	-	-	-	-	-	1
1	-	7	11	36	10	12	4	14	25	131
25	-	1	-	-	-	-	-	1	-	2
3	-	-	25	8	2	-	1	-	-	33
9	-	-	-	2	4	-	-	-	-	6
23	-	2	-	-	-	-	-	-	-	2
17	-	-	-	-	-	1	1	-	-	2
2	-	-	1	-	-	18	23	2	-	44
28	-	-	-	-	-	1	-	-	-	1
27	-	-	-	-	-	1	-	-	-	1
16	-	-	-	-	-	2	-	-	-	2
18	-	-	-	-	-	-	2	-	-	2
30	-	-	-	-	-	1	-	-	-	1
10	-	-	-	-	3	-	3	-	-	6
20	-	-	-	-	-	-	2	-	-	2
32	-	-	-	-	-	-	1	-	-	1
19	-	-	-	-	-	-	2	-	-	2
5	-	-	-	-	4	5	14	-	-	23
34	1	-	-	-	-	-	-	-	-	1
14	-	-	-	3	-	-	-	-	-	3
31	-	-	-	-	-	-	1	-	-	1
15	-	-	-	-	-	2	-	-	-	2
21	2	-	-	-	-	-	-	-	-	2
36	1	-	-	-	-	-	-	-	-	1
35	1	-	-	-	-	-	-	-	-	1
13	-	-	-	-	-	4	-	-	-	4
29	-	-	-	-	-	1	-	-	-	1
40	-	-	-	-	-	-	-	1	-	1
41	-	-	-	-	-	-	-	1	-	1
7	-	-	3	2	4	1	-	-	-	10
39	-	-	-	-	-	-	-	1	-	1
47	-	-	-	-	-	-	-	-	1	1
44	-	-	-	-	-	-	-	-	1	1
43	-	-	-	-	-	-	-	-	1	1
45	-	-	-	-	-	-	-	-	1	1
26	-	-	-	-	-	-	-	2	1	3
12	-	-	-	-	-	-	-	5	4	9
38	-	-	-	-	-	-	-	1	-	1
46	-	-	-	-	-	-	-	-	1	1
8	-	-	5	1	2	-	-	-	-	8
TOTAL	5	19	55	56	55	52	61	28	36	367

Figure 2.2 Haplotype frequencies and minimum spanning tree of nucleotide divergence between *Pinctada maxima* cytochrome oxidase I haplotypes, based on Tamura & Nei genetic distance (1993) with gamma correction. Coloured shapes indicate haplotypes found in 1-3 locations, highlighting patterns of regional haplotype distribution.

A correlation of geographic distance between populations and pairwise Φ_{ST} was not observed (Mantel's Test, $P = 0.515$) indicating an isolation-by-distance model of genetic differentiation was not applicable on a broad scale throughout the range of *P. maxima*, and that other restrictions/barriers to historical gene flow may be in effect across its distribution. The approximate geographic locations of several putative genetic barriers based on pairwise Φ_{ST} values were identified using Monmonier's algorithm, and are shown in Figure 2.3. Interestingly, the geographic area bound by putative barriers A and B bear a striking similarity to the path of the Indonesian Throughflow Current (Gordon & Fine, 1996) (Figure 2.1), which indicates that oceanographic factors may have a significant influence in shaping the genetic structure of this species.

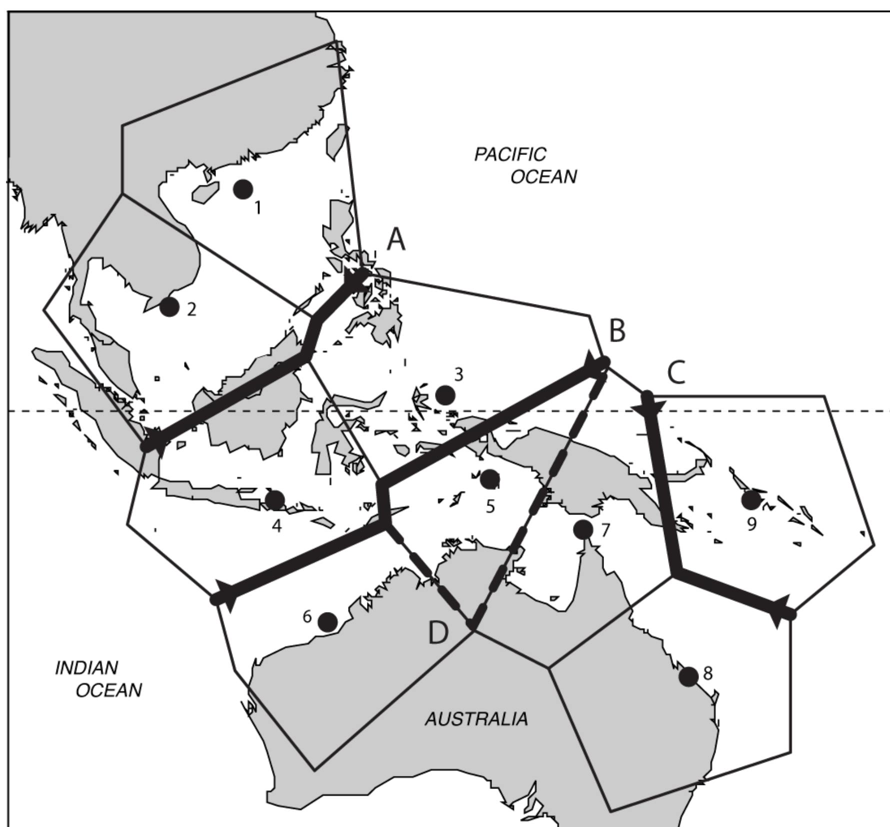


Figure 2.3 Location of the three most likely biogeographic barriers identified with Monmonier's algorithm overlayed on a map of the Indo-Australian Archipelago. Thick black lines indicate paths of the greatest rate of change in pairwise Φ_{ST} genetic distances (i.e. possible barriers), thin lines show Voronoi tessellation. 1, Hainan Island; 2, Vietnam; 3, West Papua; 4, Bali; 5, Aru; 6, Western Australia; 7, Torres Strait; 8, East Australia; 9, Solomon Islands.

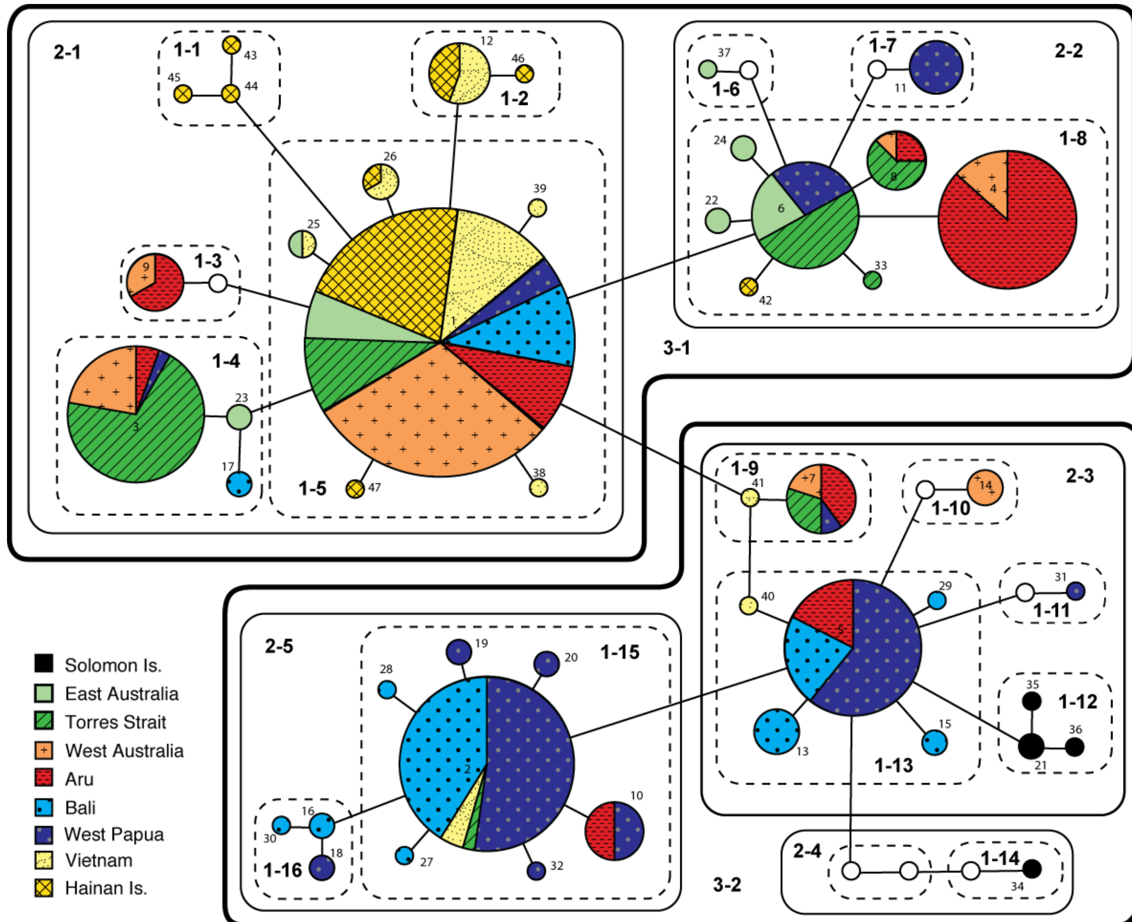


Figure 2.4 Nested cladogram of *Pinctada maxima* COI haplotype network. Circles represent different haplotypes, with relative size proportionate to its observed frequency across the Indo-Australian Archipelago. Each haplotype is separated by a single mutational change, with white circles indicating inferred haplotypes; colours indicate proportional distribution of a haplotype amongst each population. Dashed, thin and thick black borders indicate 1-step, 2-step and 3-step clades respectively, with clade names in bold within each border. Minor text labels indicate haplotype name.

2.3.3 Nested Clade Analyses

From the 47 haplotypes detected, a nested cladogram could be completely resolved by two, 3-step clades, with haplotypes evenly distributed between each 3-step clade (24 and 23 haplotypes for 3-1 and 3-2 respectively) (Figure 2.4). At the highest nesting level (total cladogram), a highly significant permutational contingency test (χ^2 = 170.8, P < 0.001) indicates a significantly non-random representation of each population across the two 3-step clades, and is visually evident in Figure 2.4. Contingency tests also revealed significant associations between population and nested clades within both 3-step nesting clades, and for several 2-step and 1-step nesting level clades (Table 2.4), highlighting the presence of a geographic association of haplotypes/clades at all nesting levels. Since each nesting level reflects varying

evolutionary time scales, contingency tests at each clade level may reveal long standing or relatively recent geographic associations with haplotypes/clades, however, the absence of geographic distance information allows no inferences to be made on potential historical factors (e.g. past fragmentation, range expansions) contributing to such genetic association patterns (Templeton, 1998). By incorporating geographic distances in association with the interior or tip position of clades (reflecting a clade's relative age), geographical distance tests allow such historical demographic inferences to be drawn. For *P. maxima*, several significant associations (P < 0.05) were identified through geographic distance tests (Table 2.4). At the highest level of inference clades 3-1 and 3-2 both showed significant outcomes, with patterns of geographic association reflecting a population demography of restricted gene flow with isolation-by-distance (Table 2.4) based on the most recent inference key (Nov 2005) provided with GEODIS v2.5 (Posada *et al.*, 2000) documentation. At lower nesting levels, patterns of past population fragmentation were identified in clade 1-4 and was largely driven by haplotypes sampled from Torres Strait, whilst a pattern of contiguous range expansions was identified within clade 1-5. Clades 1-4 and 1-5 were both nested within clade 2-1, and significant geographic associations in this nesting clade were indicative of patterns expected by long distance colonisation events and/or contiguous range expansions (Table 2.4). This outcome is most likely to be attributed to the close geographic proximity within tip clades of 2-1 when compared to broadly distributed haplotypes found in the interior clade 1-5. The high incidence of Hainan Island and Vietnam haplotypes (and to a lesser extent between Aru and Western Australia) in tip clades within 2-1 indicates a genetic signal of demographic expansions into northern Asian regions. Several other clades with significant geographic distance tests were also observed, showing patterns of restricted gene flow with isolation-by-distance within central Indonesian regions (1-15) and across northern/eastern Australia (2-2), plus some evidence of long distance dispersal events between Indonesia and Vietnam (clade 1-13) (Table 2.4).

Table 2.4 Nested Clade Phylogenetic Analysis of *Pinctada maxima* cytochrome oxidase I haplotypes. Black outlines demarcate geographic distance test results for each nesting clade; significance of permutational contingency tests is indicated by †† ($P < 0.01$) or † ($P < 0.05$) beside significant clades.

Haplotypes			1-step clades			2-step clades			3-step clades		
Clade	D_c	D_n	Clade	D_c	D_n	Clade	D_c	D_n	Clade	D_c	D_n
HA43	0	0									
HA44	0	0									
HA50	0	0	1-1	0^{*S}	3355						
12	1133	1074									
HA46	0	705									
I-T	1133	367	1-2	1132^{**S}	3391^{**L}						
9	0	0	1-3	1443^{**S}	2465^{**S}						
3	1645^{**S}	1757^{**S}	1-4††	1858^{**S}	2956						
17	1836	2424									
23	0	2688^{**L}									
I-T	-1655	896^{**L}									
Inf:	1-2-3-4-9NO: FR										
1	3130^{*S}	3147	1-5	3187	3134						
25	7355^{*L}	3985	I-T	1588^{**L}	134						
26	1269	3092	Inf:	1-2-3-5-6-13-14NO:LDC/CRE		2-1††	3197^{**L}	3081^{**L}			
HA36	0	3136									
38	0	3058									
39	0	3059									
I-T	815	-165^{*S}									
Inf:	1-2-11-12NO: CRE										
37	0	0	1-6	0	2769						
11	0	0	1-7	0^{*S}	1868						
4	801^{**S}	1522^{**S}	1-8††	1860	1876						
6	2213	1972^{**L}	I-T	1859^{*L}	-142						
8	1366	1436	Inf:	1-2-3-4NO: RGF-IBD		2-2†	1899^{**S}	2579^{**S}			
22	0	2729^{*L}				I-T	1298^{**L}	503^{**L}			
24	0	2729^{*L}				Inf:	1-2-3-4NO: RGF-IBD		3-1††	3014^{**L}	2818^{**L}
33	0	1321									
HA31	0	4503^{*L}									
I-T	1418^{**L}	293									
Inf:	1-2-3-5-6-7-8NO: Inc										
7	0	4479									
41	1693	2002									
I-T	1693	-2476	1-9	2250	2107						
14	0	0	1-10	0^{*S}	2185						
31	0	0	1-11	0	1441						
35	0	0	1-12	0^{**S}	3660^{**L}						
36	0	0									
21	0	0									
5	1145^{**S}	1312	1-13††	1445^{**S}	1810^{**S}						
13	0^{*S}	1270	I-T	1656^{**L}	-942^{**S}						
15	0	1228	Inf:	1-2-3-5-15-18: Inc		2-3††	2096^{**L}	1813^{**L}	3-2††	1809^{**S}	2245^{**S}
29	0	1207									
40	0	3010^{*L}									
I-T	1097^{*L}	113									
Inf:	1-2-3-5-6-7YES:RGF-LDD										
34	0	0	1-14	0	0	2-4	0	3675			
2	1487	1386^{*L}									
10	806^{*S}	1195									
19	0	867									
20	0	867									
27	0	1267									
28	0	1267									
32	0	859									
I-T	1115^{**L}	306									
Inf:	1-2-3-4NO: RGF-IBD		1-15	1432	1397	2-5	1424^{**S}	1529^{**S}			
16	0	1049	1-16	1377	1100	I-T	694^{**L}	250^{**L}			
18	0	1574	I-T	55	297	Inf:	1-2-3-4NO: RGF-IBD				
30	0	918									
I-T	0	-306									

D_c, Clade distance; D_n, Nested clade distance; I-T, interior-tip distances; Inf, chain of inference (following Templeton, 2004); IBD, isolation by distance; RGF, restricted gene flow; FR, allopatric fragmentation; CRE, contiguous range expansion; LDD, long distance dispersal; LDC, long distance colonisation; S, significantly small; L, significantly large; **, $P < 0.01$; *, $P < 0.05$;

2.4 Discussion

2.4.1 Genetic diversity and range-wide gene flow patterns

Based on frequency and sequence analyses of the mitochondrial COI gene, there exists strong regional genetic structure throughout the natural distribution of *P. maxima*. Moderate to high levels of genetic diversity were observed in *P. maxima* populations, which is within the range of variability seen across the Indo-Pacific in other marine species for the same gene region (Barber *et al.*, 2002 ; Benzie *et al.*, 2002 ; de Bruyn *et al.*, 2005 ; Gopurenko & Hughes, 2002 ; Kochzius & Nuryanto, 2008 ; Uthicke & Benzie, 2003). Mitochondrial diversity also followed patterns of diversity observed with microsatellite markers, in that *P. maxima* populations located near range peripheries generally tend to be less genetically diverse (Lind *et al.*, 2007). Previous studies have identified population structure in *P. maxima* using microsatellite DNA markers (Benzie & Smith-Keune, 2006 ; Lind *et al.*, 2007), although mtDNA data presented here indicates a much stronger genetic partitioning than previously realised. Nevertheless, this confirms that despite an extended planktonic larval stage (17-24 days) providing the potential to broadly disperse, gene flow throughout the distribution of *P. maxima* is restricted.

A stepping-stone, isolation by distance, model of gene flow implies that more geographically distant populations are less likely to exchange genetic migrants and are therefore more likely to exhibit genetic differences arising from the random processes of genetic drift (Kimura & Weiss, 1964). Such a significant correlation between genetic differentiation and geographic distance of populations (reflecting patterns of isolation-by-distance (IBD)) was not observed in *P. maxima* across the scale of its distribution. However, given that maternally inherited mtDNA is more prone to population perturbations as a consequence of its smaller effective size, the failure of IBD patterns from mtDNA may indicate a more significant imprint of complex oceanographic influences or historical factors such as biogeographic barriers and repeated population expansions/contractions on the genetic structure of *P. maxima*. Indeed, analysis of gene flow patterns through pairwise Φ_{ST} values show strong differentiation amongst some populations (e.g. Torres Strait vs Bali, West Papua; Hainan Island; Vietnam vs Solomon Islands) yet little

genetic difference is detected between others (e.g. Hainan and Vietnam; Bali and West Papua) (Table 2.3) suggesting a more complex pattern of regional genetic structure is present.

2.4.2 Influence of prevailing ocean currents on gene flow patterns

Given that *P. maxima* is a sessile benthic organism once its planktonic larvae have settled, passive transport via ocean currents is effectively the sole mechanism allowing gene flow across broad geographic regions; and therefore should play a significant role in the genetic structuring of this species. However, throughout Indo-Pacific marine systems the planktonic larval duration or dispersal capability of an organism has been shown to be an unreliable predictor of population connectivity and genetic structuring patterns (Barber *et al.*, 2002 ; Bay *et al.*, 2006 ; Ovenden *et al.*, 2004). Based on particle dispersion models across the northwest shelf of Australia, *P. maxima* larvae will passively travel up to 60 km from their origin of spawning, although are most likely to settle within ~30 km due to the prevailing ocean current and tidal conditions in this region (Condie *et al.*, 2006). This level of movement is perhaps less than expected given *P. maxima*'s extended planktonic larval phase of 17-24 days (Rose & Baker, 1994); however, it appears that single generation dispersal of this magnitude is still sufficiently large to maintain high gene flow over evolutionary time amongst populations spanning thousands of kilometres along the northwest Australian coastline (Benzie *et al.*, 2003 ; Benzie & Smith-Keune, 2006 ; Johnson & Joll, 1993).

A high connectivity between Hainan Island and Vietnam populations is suggested by hierarchical AMOVA and pairwise Φ_{ST} values, yet limited genetic exchange between more southern regions (Table 2.2). Strong ocean currents in the western South China Sea, particularly along the coastline of Vietnam (Wyrtki, 1961) (Figure 2.1), supports that a high connectivity via passive larval transport on ocean currents would be likely, and is a plausible explanation for the genetic similarity observed between Vietnam and Hainan Island. Additionally, with approximately 10 million m^3 s^{-1} of water flowing via the Indonesian Throughflow Current (ITF) from the Pacific Ocean towards the Indian Ocean, mostly through the Makassar Strait (Gordon & Fine, 1996 ; Gordon *et al.*, 2003), passive larval transport via the ITF provides an obvious predictor for high gene flow through the constricted Indonesian

seaways. Interestingly, Monmonior's algorithm predicts two possible genetic barriers in locations which isolate the South China Sea and surround an area of close resemblance to the path of the ITF (A and B, Figure 2.3), with the populations sampled within each of these areas (Hainan Island-Vietnam and Bali-West Papua respectively) showing a high genetic similarity (Table 2.3). Evidence for ITF mediated gene flow patterns has been observed in several other Indo-Pacific invertebrate and fish species (e.g.Kochzius & Nuryanto, 2008 ; Barber *et al.*, 2002 ; Timm & Kochzius, 2008) and is consistent with results presented here. The ITF's deflection away from Aru and Western Australia towards the Indian Ocean may also explain the genetic dissimilarity between these populations and those from central Indonesia. The role of the ITF is presenting as a significant factor in shaping population genetic patterns of marine species within Indonesia, and mtDNA evidence from this study suggests the ITF also has a prominent influence on the genetic structuring of *P. maxima*.

Genetic patterns observed in the Solomon Islands are also intriguing, indicating a large genetic divergence in populations from this region, with haplotypes more closely related to those found predominantly in West Papua / Bali (Figure 2.2 and Figure 2.4). It has been observed in two giant clams species (*Tridacna maxima* and *T. gigas*), that populations from the Solomon Islands are more genetically similar to populations from the Philippines than those from the Great Barrier Reef, Australia; and highlight the significance of historical dispersal patterns rather than present-day ocean circulation in the formation of genetic patterns across the west Pacific (Benzie & Williams, 1995 ; Benzie & Williams, 1997). Although data observed in this study is suggestive of a similar pattern occurring in *P. maxima*, such conclusions must be drawn with appropriate caution, given only five individuals were sampled from this region.

2.4.3 Genetic impact of Pleistocene ocean basin isolation and exposure of continental land masses

In addition to oceanographic influences, episodes of lowered sea level have contributed to phylogeographic patterns across the Indo-Australian Archipelago through the formation of physical land barriers across the region, blocking passages of gene flow and causing allopatric differentiation between previously (or presently) connected populations. During periods of

Pleistocene polar glaciation (most recently ~17 000 bp), lowered sea levels of up to 120 m below present day levels left the Sunda Shelf, Sahul Shelf and other shallow seafloor regions exposed, causing separation of ocean basins across the Indo-Malay region (Voris, 2000). Signatures of historical vicariance between ocean basins has persisted in several present day populations across the Indo-Malay region and northern Australia, where deep genetic divergence has been observed in marine invertebrates (Barber *et al.*, 2006 ; Barber *et al.*, 2002 ; Gopurenko & Hughes, 2002), and fish species (Lourie *et al.*, 2005 ; Timm & Kochzius, 2008 ; Chenoweth *et al.*, 1998). A historically isolated South China Sea basin caused by an exposed Sunda Shelf to the west and the Philippines to the east may have contributed to the genetic differences observed in *P. maxima* from this region compared to other populations across the Indo-Malay archipelago, yet sustained an adequate gene flow to maintain homogeneity between Hainan Island and Vietnam.

The expansive, shallow continental regions of the Sunda Shelf across the Gulf of Thailand, and the Sahul Shelf between Australia and Papua New Guinea (Figure 2.1) are also prominent features in marine biogeography within the Indo-Australian Archipelago, and have likely played a significant role in shaping population genetic patterns in *P. maxima*. Rapid re-colonisation of exposed land regions with rising sea levels (particularly the Sunda Shelf (Hanebuth *et al.*, 2000) could heighten genetic differentiation of populations in these regions through founder effects, and is considered a significant contributor to genetic patterns in several marine species throughout the Indo-Malay region (e.g. Arnaud *et al.*, 1999 ; Nelson *et al.*, 2000 ; Lourie *et al.*, 2005 ; Mahidol *et al.*, 2007). Genetic differentiation from (re)colonisation events are particularly relevant to broadcast spawning marine bivalves, where a small number of effective breeders can contribute large proportions of offspring within a generation with a 'sweepstakes'-like chance of reproductive success (Hedgecock, 1994 ; Hedrick, 2005), increasing the likelihood of genetic drift in small or newly colonised populations. In *P. maxima*, Fu's *F*-test and nested clade analysis (Table 2.1 & Table 2.4 respectively) indicate that genetic signatures of demographic and/or range expansions are present in the northern populations of Hainan Island and Vietnam, which is consistent with expectations based on Sunda Shelf re-colonisation. Historical population expansions within the Indo-Malay region have also been suggested from genetic

patterns in a tropical abalone species (*Haliotis asinina*, Imron *et al.*, 2007), as well as in mudcrabs (Gopurenko *et al*., 1999), and sea cucumbers (*Holothuria nobilis*, Uthicke & Benzie, 2003). It must be noted, however, that signatures of range expansions in *P. maxima* may also be simply due to the peripheral locations of these populations (especially eastern Australia and Hainan Island), which may have seen repeated population expansion and contraction in response to historical fluctuations in environmental conditions in range peripheries.

2.4.4 No deep phylogenetic divergence yet geographic haplotype associations

Phylogenetics studies have shown a strong influence of regional vicariance on the formation of divergent genetic clades across regions of the Indo-Australian Archipelago separated by only 100's of kilometres (Barber *et al.*, 2002 ; Kochzius & Nuryanto, 2008 ; Timm & Kochzius, 2008), with a realisation that genetic connectivity can be significantly restricted despite a potential to broadly disperse. In *P. maxima*, however, the presence of deep phylogenetic divergence was not detected. A lack of deep genetic divergence and the close relationship of haplotype sequences (1% mean divergence between 47 haplotypes) in *P. maxima* may suggest that its high dispersal potential is occasionally realised and long distance dispersal events have periodically occurred, as is inferred by nested clade analyses. Alternatively, phylogenetic patterns seen in *P. maxima* are also in agreement with patterns observed over much broader scales. It is often observed that populations from north-west Australia are more phylogenetically related to Asian/Pacific clades than those from other Indian Ocean regions (Benzie *et al.*, 2002 ; Bay *et al*., 2004 ; Uthicke & Benzie, 2003 ; Williams & Benzie, 1998), and is likely to also be the case in *P. maxima*.

Nested clade analyses of *P. maxima* show a significant association between haplotypes and geographic location, and further highlights a significant influence of historical population separation. This, along with the observation of several small clusters of closely related haplotypes found only in Hainan Island, Solomon Islands and to a lesser extent in Bali/West Papua (Figure 2.2 and Figure 2.4), could be indications that preliminary lineage sorting has occurred across the distribution of *P. maxima* (Avise, 1994).

Chapter 3 Genetic diversity and structure of wild *P. maxima* populations - Microsatellites[†]

3.1 Introduction

The importance of intra-specific genetic diversity in natural populations is well established (Crozier, 1997) and has been identified as a global priority for conservation. Not only is genetic diversity a driving force behind evolutionary adaptation and ultimately speciation, its role is fundamental in the ability of a species to persist when challenged by various environmental pressures (eg. disease outbreak, food shortage, climate change) (Allendorf & Luikart, 2007). A meta-analysis by Reed and Frankham (2003) of 34 studies of various organisms from vertebrate, invertebrate and plant taxa found significant positive correlations between genetic diversity and fitness (or components of fitness), highlighting the need to protect intra-specific genetic diversity.

However, the distribution of genetic variation is often non-uniform and partitioned throughout a species natural distribution. Factors such as dispersal capabilities, habitat availability and historic biogeographic influences can all significantly affect the partitioning of intra-specific genetic diversity, as can random factors such as genetic drift (Allendorf & Luikart, 2007). Populations toward the periphery of a species distribution are particularly prone to the latter, as the continual expansion and contraction of smaller populations can lead to multiple genetic bottlenecks, manifesting in the loss of potentially significant genetic variation (Lesica & Allendorf, 1995). Additionally, environmental extremes often encountered at range limits can intensify selective pressures, driving genetic divergence from more central populations (Garcia-Ramos & Kirkpatrick, 1997). It is commonly perceived that the genetic diversity of peripheral populations is lower when compared to centrally located populations, however, this is not always the case.

[†] Manuscript: Lind C.E., Evans B.S., Taylor J.J.U & Jerry D.R. 2007, Population genetics of a marine bivalve, *Pinctada maxima*, shows differentiation and reduced diversity at range limits. Molecular Ecology 16, 5193-5203

Clines in genetic diversity can be unrelated to a present-day range centre if uninhabitable environments or geographic features force unidirectional population expansion from historical refugia (e.g. Garner *et al*., 2004). Consequently, it can be postulated that the greatest genetic diversity may be found in regions having the most biogeographically and ecologically stable habitat. The conservation worth of peripheral populations is therefore uncertain, however, it is clear that continued investigation of the spatial distribution of genetic diversity could play a key role in further understanding this issue.

Bisecting the Indian and Pacific Ocean basins is the Indo-Australian Archipelago, a globally significant region of rich marine biodiversity. The evolutionary origins of such diversity are not well understood and have been regularly debated (see review by Bellwood & Wainwright, 2002), and in an attempt to better understand the mechanisms involved in maintaining or generating the rich species diversity in this region, researchers have employed molecular genetic techniques to target population dynamic aspects such as dispersal, gene flow and biogeographic influences in marine and coral reef species of the Indo-Australian Archipelago. These studies have revealed that dispersal is often complex and can contrast with a species' apparent dispersal potential (Barber *et al*., 2002); that gene flow amongst populations can be cryptic (Benzie, 1999a); and biogeographic influences are a significant cause of intra-specific genetic divergence; yet patterns can be non-concordant across different species (Lourie & Vincent, 2004 ; Barber *et al.*, 2002 ; Keenan, 1994). Combinations of these influences have culminated in sharp genetic breaks within the seemingly continuous distributions of multiple species across a range of taxa (Imron *et al.*, 2007 ; Barber *et al.*, 2006 ; Lourie *et al.*, 2005 ; Ovenden *et al*., 2004), the occurrence of which are commonly reported around central Indonesia. Although this region has been identified as an epicentre of faunal diversity for marine and coral reef species (Roberts *et al*., 2002), studies have indicated that the Indo-Australian Archipelago may also be a region of greater intra-specific genetic diversity. Earlier work investigating mitochondrial DNA variation in sea urchins in the Indo-West Pacific showed that nucleotide diversity decreases sharply as geographic distance from the Indo-Australian Archipelago increases (Palumbi *et al*., 1997), and a similar pattern of decreasing mtDNA diversity is also seen in the giant tiger prawn across the Indian Ocean, west of the Indo-

Australian Archipelago (Benzie *et al*., 2002). Throughout the Indo-Australian Archipelago, the implementation of conservation measures such as marine parks and protected areas are of high priority (Roberts *et al.*, 2002 ; Hughes *et al*., 2002), but to avoid the potentially detrimental long-term genetic consequences of inadvertently protecting areas of low genetic diversity it is necessary to identify broad-scale patterns of intra-specific diversity (Bell & Okamura, 2005). It may therefore be worthwhile targeting regions of high intra-specific diversity, however, presently multi-locus population studies across the Indo-Australian Archipelago incorporating sufficient sampling intensities and localities needed to identify broad scale patterns of genetic variation are limited. In this regard, the utilisation of genetic markers such as highly polymorphic microsatellites may be particularly useful in genetic diversity studies throughout this region.

The silver-lipped pearl oyster, *Pinctada maxima*, is a bivalve mollusc that is widespread throughout the tropical and sub-tropical regions of the Indo-Australian Archipelago (Figure 3.1) and is commercially valued for pearl production (Shirai, 1994). *P. maxima* is a broadcast spawning species with a planktonic larval period of 17-24 days, after which metamorphosis and settlement occurs, shortly followed by a sessile phase that persists for the remainder of its life cycle (Rose & Baker, 1994). Studies on the population dynamics of *P. maxima*, have been restricted to only a small portion of its global range and have focused within the major commercial aquaculture regions of northwest Australia and southern Indonesia. This has revealed genetic differentiation between Indonesian and Western Australian populations (Benzie & Smith-Keune, 2006), and is thought to be a consequence of the historical separation of the Pacific and Indian Ocean basins.

Unlike other Indo-Australian Archipelago marine species, whose widespread distributions can extend across the entire Indian or Pacific Oceans, *P. maxima*'s natural range is restricted only to the Indo-Australian Archipelago (Shirai, 1994). Since the extended planktonic larval phase of *P. maxima* provides a potential to broadly disperse, it is assumed that the distribution of this species is governed by the prevailing habitat and ecological vagaries unique to this region rather than its dispersal ability. *P. maxima* may therefore be more sensitive to historical disturbances affecting the homogeneity of genetic diversity throughout the Indo-Australian

Archipelago, presenting an opportune species to investigate and give potential insights into broad-scale patterns of intra-specific diversity in the region. This study used microsatellite DNA markers to assess the spatial partitioning of genetic variation and diversity in *P. maxima* as a model Indo-Australian Archipelago marine species, and investigates whether peripheral populations are likely to exhibit genetic differentiation and decreased diversity when compared to central Indo-Australian Archipelago populations.

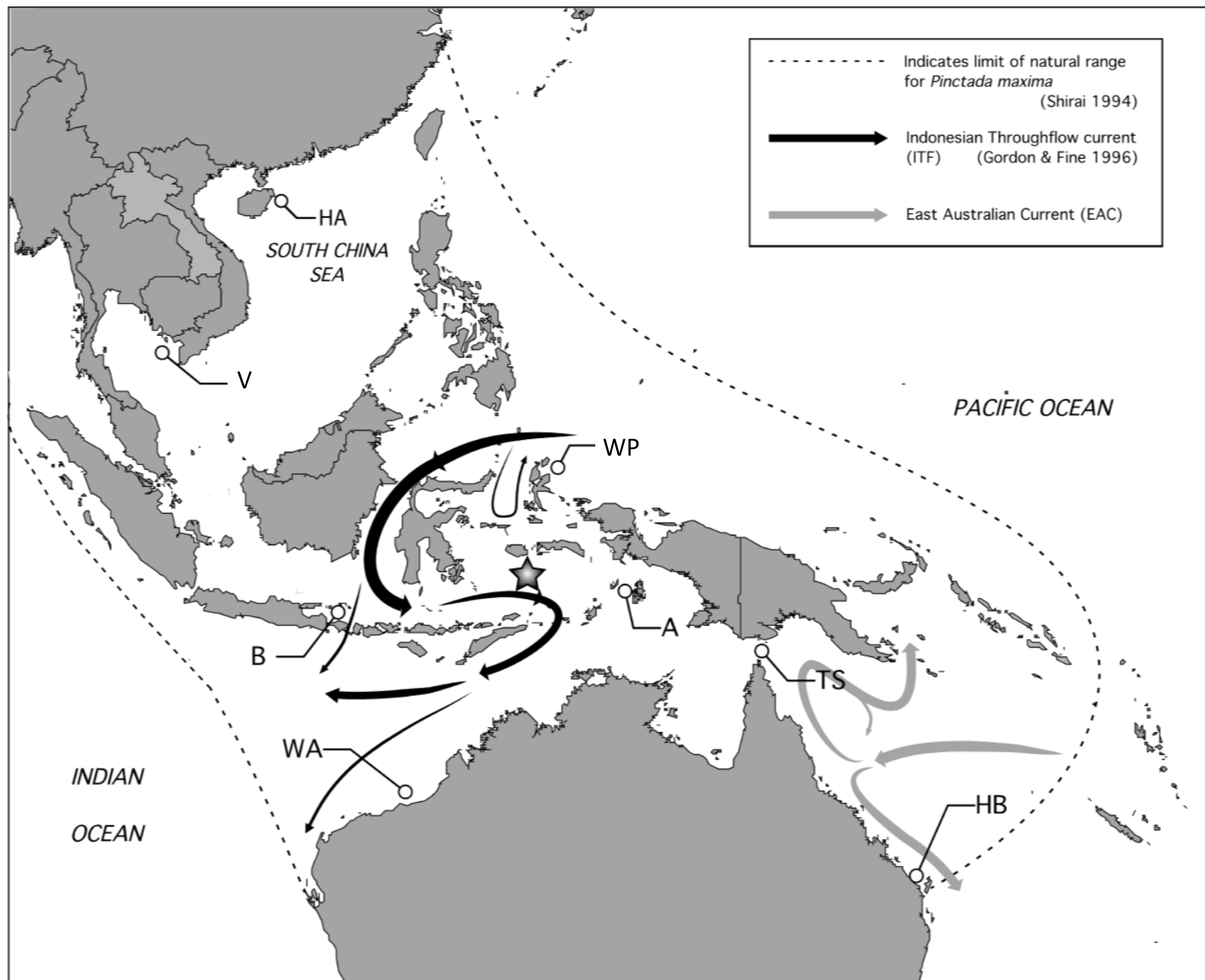


Figure 3.1 Sampling locations of *Pinctada maxima* throughout the Indo-Australian Archipelago. A = Aru; B = Bali; WP = West Papua; WA = Western Australia; V = Vietnam; TS = Torres Strait; EA = Eastern Australia and HA = Hainan Island. Star indicates approximate centre of present-day distribution. Thick and thin arrows indicate major and minor currents, respectively (not to scale).

3.2 Materials & Methods

3.2.1 Tissue sampling

Local divers collected 404 adult oysters from naturally occurring beds between 2004-2006 from eight sites from Australia (Western Australia - 19.29S, 119.75E; Torres Strait - 10.97S, 143.18E and Eastern Australia - 24.20S, 152.85E), Indonesia (Bali - 8.32S, 114.92E; Aru - 6.43S, 134.63E and West Papua - 1.13N, 130.54E), Vietnam (9.08N, 105.25E) and China (Hainan Island - 19.66N, 11.20E), spanning a significant portion of the natural range of *P. maxima* (Figure 3.1). Biopsies were taken from the foot, mantle or adductor muscle tissue and preserved in 70-100% ethanol.

3.2.2 DNA extraction & microsatellite amplification

Preserved tissue was digested in a CTAB buffer with 20 mg ml^{-1} proteinase K for 1-3 hrs at 55°C, followed by a phenol:chloroform:isoamyl alcohol purification protocol to extract total genomic DNA (gDNA) (Sambrook *et al.*, 1989). gDNA was quantified by comparison to DNA concentration standards after agarose gel electrophoresis using the ImageJ 1.33 software package (Wayne Rasband, National Institute of Health, 2004) and resuspended in ddH_2O to a concentration of 5 ng/µl.

Table 3.1 Microsatellite markers and PCR conditions used in *P. maxima* population study

Locus Name	Fluorescent label	T_A (C°)	$MgCl_2$ conc. (mM)	Reference
JCUPm_1g8	FAM or HEX	55	3.5	(Evans *et al.*, 2006)
JCUPm_20e10	TET	55	3.0	(Evans *et al.*, 2006)
JCUPm_27d7	FAM	55	1.5	(Evans *et al.*, 2006)
JCUPm_27a1	FAM	55	1.5	(Evans *et al.*, 2006)
Pmx-16_41	FAM	58-50†	1.5	(Smith *et al.*, 2003)
Pmx-18_21	TET	58-50†	1.5	(Smith *et al.*, 2003)

† touch-down PCR cycle, T_A decreases by 2 C° every 5 cycles until 50 C°

Individual genotypes were obtained using six polymorphic microsatellite loci (Table 3.1) (Evans *et al.*, 2006 ; Smith *et al.*, 2003). PCR was conducted in 15 µl volumes using the following conditions: 1 x PCR buffer with 1.5 mM $MgCl_2$ (QIAGEN), 0.2 mM dNTPs, 0.3 U of *Taq* DNA polymerase (QIAGEN) and 5 ng of gDNA. PCR for loci *Pmx-18_21* and *Pmx-16_41* also

included 1 x Q-solution (QIAGEN) (as per Smith *et al.*, 2003). Fluorescent-labelled primer and $MgCl_2$ concentrations varied for each marker according to original published conditions. Thermocycler programs for all PCR (except for *Pmx-18_21* and *Pmx-16_41*) began with an initial denaturation step for 3 mins at 94°C followed by 35 cycles of 94°C for 45 sec, 55°C for 30 sec and 72°C for 45 sec, then a final extension step at 72°C for 5 mins. To reduce non-specific amplification the PCR cycling conditions for *Pmx-18_21* and *Pmx-16_41* used a touchdown program where annealing temperature was sequentially lowered from 58°C, 56°C, 54°C and 52°C (for 5 cycles each) followed by 50°C for 20 cycles (Smith *et al.*, 2003).

To remove residual salts, PCR products were purified using an ammonium acetate:ethanol precipitation protocol (Sambrook *et al.*, 1989). PCR products together with a Tamra-400 size standard underwent capillary electrophoresis on a MegaBACE auto-sequencer (Amersham Biosciences) and allele sizes were then calculated using the MegaBACE Fragment Profiler v1.2 software (Amersham Biosciences).

3.2.3 Statistical analyses

Allele frequencies, genetic diversity and allelic richness were calculated using software FSTAT 2.9.3.2 (Goudet, 1995) To eliminate bias due to differences in sample sizes, allelic richness was calculated using a rarefaction technique based on the smallest sample size (Leberg, 2002). Observed heterozygosity (Ho) and expected heterozygosity (He) under Hardy-Wienberg equilibrium (HWE) was estimated using ARLEQUIN 3.1 (Excoffier *et al.*, 2005). Significant differences between Ho and He, indicating a departure from Hardy-Weinberg proportions, were determined using ARLEQUIN.

F-statistics (Weir & Cockerham, 1984) were used to partition the proportion of variance due to within (F_{IS}) and among population (F_{ST}) genetic differences. Weighted estimates of global F_{IS} and F_{ST} (through AMOVA Excoffier *et al.*, 1992) and pairwise population F_{ST} comparisons were performed using ARLEQUIN 3.1 (Excoffier *et al.*, 2005), with the significance of pairwise F_{ST} values tested using a non-parametric Monte-Carlo approach (1000 permutations). To control the likelihood of Type I errors associated with multiple pairwise comparisons, the False Discovery

Rate correction method was applied to *P*-values (Benjamin & Hochberg, 1995) using software PAIRWISE MULTIPLE TESTS (M. W. Watkins 2005). To assist interpretation of population comparisons, pairwise F_{ST} measures were graphically represented through multidimensional scaling (MDS) using SPSS 14 (SPSS Inc.). In addition to traditional *F*-statistics, we adopted Bayesian methods of identifying genetic structure using BAPS 2.2 (Corander *et al.*, 2003) to group genetically homogeneous clusters of populations.

To determine if the pattern of genetic differentiation in this species conformed to an isolation by distance pattern of genetic differentiation a Mantel's Z-test was performed on matrices of pairwise F_{ST} values and population geographic distances using GENALEX 6 (Peakall & Smouse, 2006). Here, geographic distance between populations was measured as the shortest distance via water, using the distance calculator function in the computer package WORLD BOOK ATLAS 1.1 (World Book Inc, 2004). Important to note in this instance, is that distances measured in this manner may not reflect 'real' pathways, which are likely to be more complex.

3.3 Results

3.3.1 Genetic diversity

Estimators of genetic diversity varied across loci and population samples (Table 3.2). Levels of polymorphism among the microsatellite loci and populations analysed in this study were variable, with the overall number of alleles detected at a given locus (*A*) ranging from four alleles at the *JCUPm27_d7* locus, to 23 alleles at *Pmx18_21*. Allelic richness (R_S), which accounts for sample size biases, showed a distinct trend of fewer alleles per locus at peripheral populations (eg. Eastern Australia, Hainan Island) than at more central locations, with mean R_S ranging from 6.66 (Hainan Island) to 8.05 (Aru) (Table 3.2). Mean R_S of central populations (Aru, Bali and West Papua) was significantly greater than that of peripheral populations (Eastern Australia, Vietnam and Hainan Island) (*t* - test, P = 0.004). From the approximate midpoint of *P.maxima*'s

Table 3.2 Genetic diversity statistics of *Pinctada maxima* populations, showing total alleles (A); allelic richness (*R*s) (Leberg, 2002); observed heterozygosity (*H*o); expected heterozygosity equilibrium (*H*e); and inbreeding co-efficient (F_{IS}). Departure from Hardy-Weinberg expectations of heterozygosity (HWE) is indicated by bold type (*P* < 0.05). Underlined text indicates a different microsatellite locus.

	Hainan Island	Vietnam	West Papua	Bali	Aru	West Australia	Torres Strait	Eastern Australia	Global
n	46	30	62	55	67	60	60	24	404
JCUPm 1_g8									
Allele Range	213-239	217-247	213-249	213-241	213-245	213-251	217-245	213-241	213-251
A	9	12	15	13	16	15	10	8	19
Rs	8.423	10.952	10.974	11.769	12.263	7.688	11.271	7.625	8.169
Ho	0.795	0.863	0.882	0.861	0.879	0.860	0.775	0.813	0.841
He	0.783	0.857	0.868	0.849	0.874	0.868	0.789	0.780	0.834
F_{IS}	0.089	-0.005	0.031	-0.035	0.152	0.051	0.14	-0.078	0.043
JCUPm 20_e10									
Allele Range	158-170	158-186	158-178	158-178	158-194	158-190	158-190	158-182	158-194
A	4	5	6	6	10	7	7	6	10
Rs	3.917	4.89	4.382	4.638	6.919	6.286	4.898	6	5.966
Ho	0.639	0.632	0.653	0.588	0.657	0.596	0.736	0.657	0.645
He	0.512	0.618	0.648	0.649	0.782	0.695	0.692	0.706	0.663
F_{IS}	0.151	-0.109	0.046	-0.082	-0.045	-0.063	0.103	-0.239	-0.030
Pmx 16_41									
Allele Range	222-258	222-270	218-266	218-258	218-258	218-262	218-258	222-258	218-270
A	10	10	13	11	11	12	10	9	14
Rs	8.125	9.342	9.44	10.142	10.027	9.622	8.686	8.739	10.069
Ho	0.831	0.814	0.869	0.859	**0.89**	0.822	**0.886**	0.867	0.855
He	0.801	0.818	0.843	0.812	0.807	0.828	0.788	0.812	0.814
F_{IS}	0.033	0.059	0.035	-0.08	0.112	-0.091	0.154	0.099	0.040
Pmx 18_21									
Allele Range	95-131	95-123	91-159	91-155	87-139	97-143	99-139	99-131	87-159
A	13	11	14	16	18	14	14	12	23
Rs	10.794	9.994	12.075	10.863	11.507	11.23	9.479	11.634	11.745
Ho	0.861	0.860	0.851	0.876	0.848	0.827	0.881	0.843	0.856
He	0.853	0.841	0.857	0.880	0.892	0.857	0.858	0.872	0.864
F_{IS}	0.143	0.149	0.148	0.213	0.174	0.033	-0.041	-0.086	0.092
JCUPm 27_d7									
Allele Range	123-147	123-147	123-151	123-151	123-147	123-147	123-147	123-147	123-151
A	3	3	4	4	3	3	3	3	4
Rs	2.996	2.913	3.387	3.392	2.983	2.744	2.35	3	3.11
Ho	0.447	0.242	0.385	0.368	0.418	0.26	0.466	0.584	0.396
He	0.391	0.417	0.495	0.496	0.369	0.378	0.382	0.44	0.421
F_{IS}	0.291	0.728	0.278	0.336	0.08	0.231	-0.035	0.559	0.309
JCUPm 27_a1									
Allele Range	252-280	256-268	256-284	256-276	256-268	256-280	248-284	260-152	248-284
A	8	5	8	7	5	5	8	3	10
Rs	5.691	4.521	5.102	5.843	4.614	6.85	3.7	3	5.985
Ho	**0.549**	**0.275**	**0.521**	**0.43**	0.448	0.621	0.728	0.428	0.500
He	0.751	0.622	0.74	0.701	0.594	0.58	0.73	0.434	0.644
F_{IS}	-0.018	0.52	0.22	0.313	0.282	0.492	0.131	-0.016	0.241
Total									
Allele Range									
A	47	46	60	57	63	56	52	41	80
Rs	6.66	7.10	7.56	7.77	8.05	7.40	6.73	6.67	7.33
Ho	0.687	0.614	0.694	0.664	0.690	0.664	0.745	0.699	0.682
He	0.682	0.696	0.742	0.731	0.720	0.701	0.707	0.674	0.706
F_{IS}	0.106	0.115	0.105	0.075	0.123	0.082	0.081	0.028	0.089

distributional range (identified in Figure 3.1), regression analysis between mean population R_S and geographic distance from this midpoint showed a strong, significant, negative correlation ($R^2 = 0.71$, $P = 0.009$). This demonstrated that as the geographical distance of a population increased from the midpoint of the species' current range allelic richness correspondingly decreased (Figure 3.2a). This pattern is mirrored by expected heterozygosity measures (H_E), which show a decreasing trend towards peripheral locations (Table 3.2).

Mean observed heterozygosity (H_O), did not differ greatly amongst populations (mean H_O = 0.614-0.745) and H_O was comparable amongst populations for most loci (Table 3.2). There were, however, several significant deviations from Hardy-Weinberg expectations of heterozygosity when individual loci were analysed separately, but such departures were restricted to only two loci (*Pmx16_41* and *JCUPm27_a1*). Heterozygosity excesses were observed only in the Torres Strait and Aru populations at the *Pmx16_41* locus, whereas significant deficits were found in *JCUPm27_a1* for Hainan Island, Vietnam, West Papua and Bali (Table 3.2). A similar correlation to that based on allelic richness between *H*o and geographical distance was not found suggesting that this parameter was comparatively insensitive to detecting changes in genetic diversity among *P. maxima* populations. (Figure 3.2b).

Table 3.3 Analysis of molecular variance (AMOVA) describing the partitioning of genetic variation for eight *Pinctada maxima* populations.

Source of variation	DF	Est. Var.	%
F_{ST}			
Among populations	7	0.056	2.7 %
Among individuals within populations	396	0.163	7.9 %
Within populations	404	1.843	89.4 %
Total	807	2.302	100 %
R_{ST}			
Among populations	7	6.706	2.3 %
Among individuals within populations	396	35.215	12.2 %
Within populations	404	254.67	85.4 %
Total	807		100 %

3.3.2 Genetic structure analyses

Pinctada maxima exhibits significant partitioning of genetic variation across its present natural distribution and is genetically structured. Analysis of molecular variance (AMOVA) revealed that the proportion of the global genetic variation attributed to differences among populations was highly significant for both F_{ST} (based on allele frequencies) and R_{ST} (based on allele sizes) estimates (F_{ST} = 0.027; R_{ST} = 0.023, P < 0.001) (Table 3.3). When these estimates of genetic structuring are further examined on a pairwise population basis (Table 3.4) a regional pattern of population structure emerges. Pairwise R_{ST} values were largest between Torres Strait and Hainan Island (R_{ST} = 0.070, P < 0.001), whilst differences in genetic variance from allele frequencies were greatest between the Torres Strait and Vietnam populations (F_{ST} = 0.063, P < 0.001). F_{ST} values for the two northernmost populations, Hainan Island and Vietnam, were significantly different to all other populations (with the exception of Vietnam and Bali), as were the differences in the Torres Strait and Western Australian populations when compared to all other populations (Table 3.4). Comparatively, genetic differentiation amongst Indonesian populations (ie. West Papua, Bali and Aru) was low, although F_{ST} differences between Aru and West Papua were statistically significant (Table 3.4). Differences in allele sizes (ie. R_{ST}) amongst populations revealed fewer significant differences. However, estimates based on this parameter still showed a similar pattern of differentiation to that of F_{ST} values in Hainan Island, Torres Strait and Western Australian populations, which showed statistically significant differences to most other populations (Table 3.4). The pattern of genetic differentiation seen in pairwise F_{ST} and R_{ST} values is consistent with Bayesian cluster analysis, which identified three clusters within the global population with a marginal posterior probability of 0.999 (maximum probability of other partitions = 0.01). The pronounced differentiation of both Torres Strait and Western Australian populations is clearly seen when cluster analysis is presented graphically alongside a MDS representation of pairwise F_{ST} measures (Table 3.3).

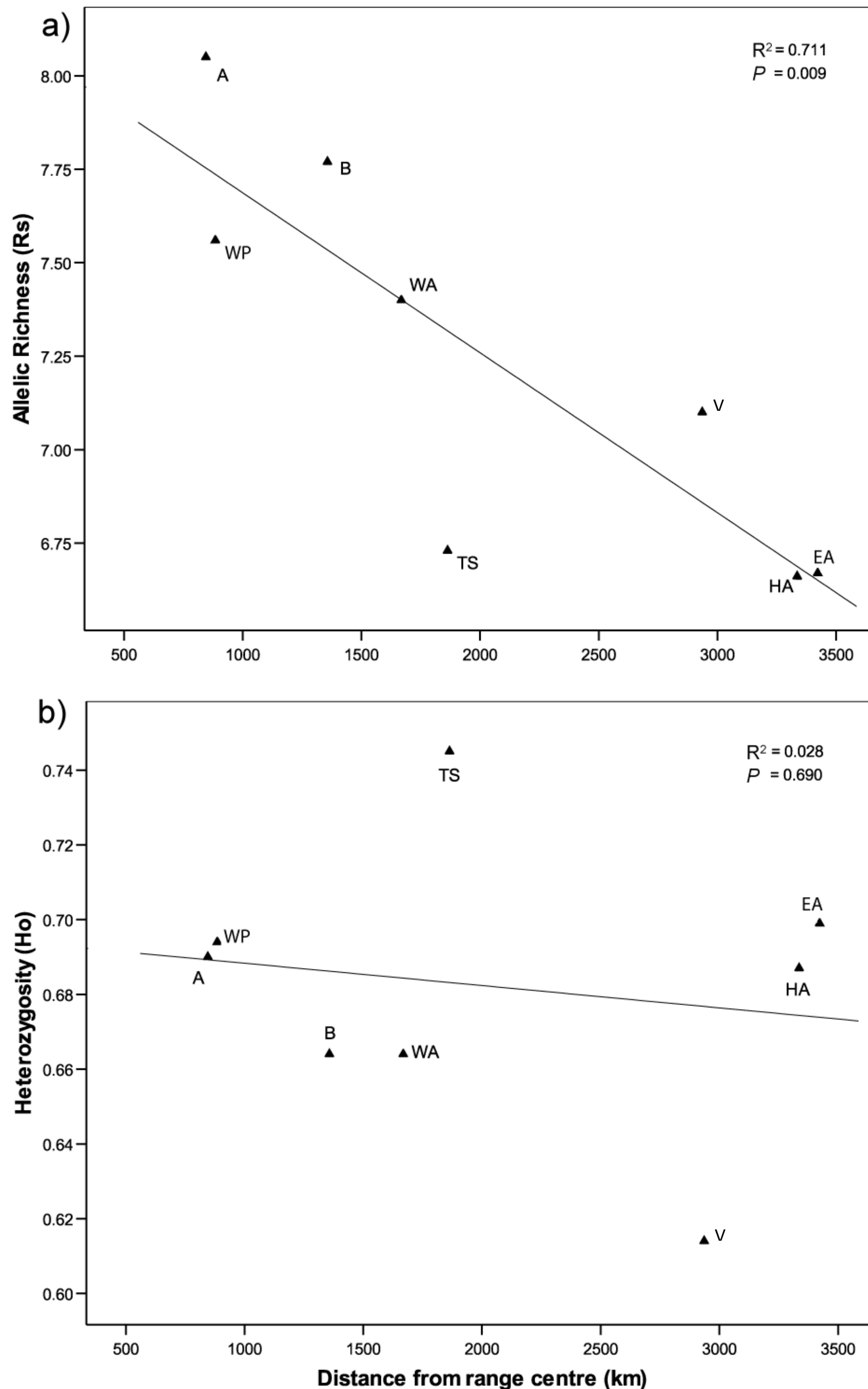


Figure 3.2 Regression analysis of *Pinctada maxima* populations, showing the correlation between geographic distance from the centre of its natural range and mean a) allelic richness (*R*s) and b) observed heterozygosity from six microsatellite loci. A = Aru; B = Bali; WP = West Papua; WA = Western Australia; V = Vietnam; TS = Torres Strait; EA = Eastern Australia and HA = Hainan Island.

Table 3.4 Pairwise F_{ST} (below diagonal) and R_{ST} (above diagonal) estimates of *Pinctada maxima* populations based on six microsatellite loci. Bold values indicate significant differences at $P < 0.05$ (corrected by False Discovery Rate method; (Benjamin & Hochberg, 1995); underlined values become non-significant after correction.

R_{ST} / F_{ST}	Hainan Island	Vietnam	West Papua	Bali	Aru	West Australia	Torres Strait	Eastern Australia
Hainan Island	-	**0.037**	**0.039**	**0.028**	**0.043**	**0.059**	**0.070**	**0.053**
Vietnam	**0.022**	-	0.022	0.009	0.000	**0.023**	**0.055**	0.017
West Papua	**0.015**	**0.016**	-	0.000	0.009	**0.027**	0.011	0.011
Bali	**0.019**	0.005	0.004	-	0.005	0.018	0.013	-0.004
Aru	**0.020**	**0.019**	**0.007**	0.003	-	0.011	**0.027**	0.006
West Australia	**0.043**	**0.042**	**0.039**	**0.036**	**0.032**	-	**0.050**	**0.051**
Torres Strait	**0.046**	**0.063**	**0.038**	**0.041**	**0.025**	**0.053**	-	-0.002
Eastern Australia	**0.033**	**0.033**	**0.016**	0.012	0.008	**0.052**	**0.025**	-

Figure 3.3 Multidimensional scaling plot of pairwise *Pinctada maxima* F_{ST} values. Points within dashed lines indicate groups identified through Bayesian cluster analysis ($P < 0.01$). A = Aru; B = Bali; WP = West Papua; WA = Western Australia; V = Vietnam; TS = Torres Strait; EA = Eastern Australia and HA = Hainan Island.

3.3.3 Population connectivity

An isolation by distance (IBD) explanation of genetic divergence between populations predicts as the geographical distance between two populations increases, a corresponding positive correlation in a genetic distance measure will be observed consequent of decreased gene flow or connectivity. When comparing the correlation between geographical and genetic distances

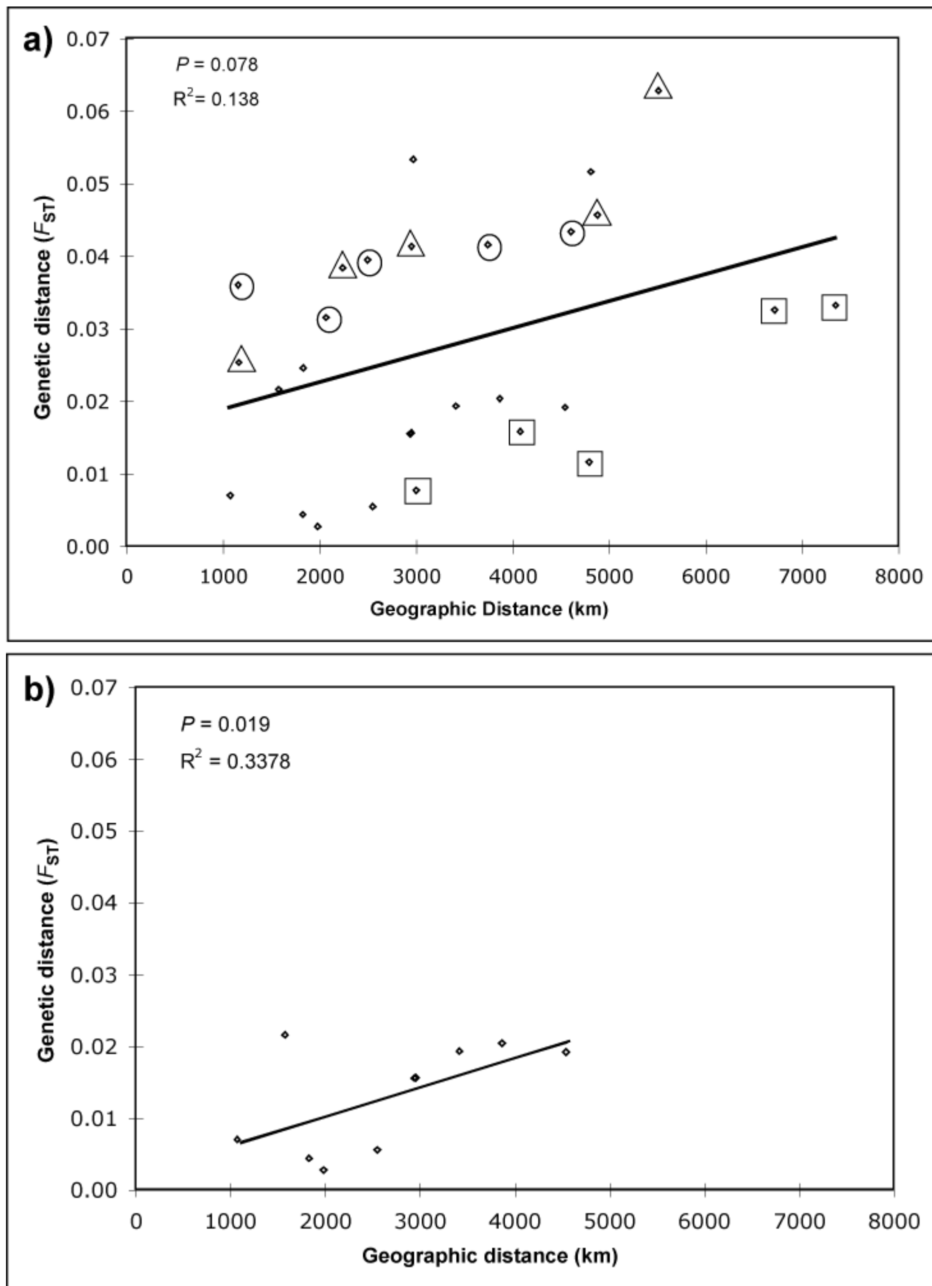


Figure 3.4 Pairwise geographic distance against F_{ST} values to assess Isolation by Distance amongst *Pinctada maxima* populations. A) All populations. Highlighted data points indicate separate comparisons of Asian populations with Torres Strait (triangle), Western Australia (circle) and Eastern Australia (square); B) Populations within Asia only i.e. Hainan Island, Vietnam, West Papua, Bali and Aru.

among all pearl oyster populations in this study no significant IBD effects were evident (Mantel's Z-test R^2 = 0.138, P = 0.078), indicating the presence of more complex patterns of gene flow at the global species range level (Figure 3.4a). However, when populations were grouped in accordance with present ocean current patterns and biogeographic zones (i.e. Southeast Asia, Northern Australia), further investigation highlighted that IBD is acting in this species at the regional scale. A significant pattern of IBD was found among Asian populations (R^2 = 0.338, P =

0.019) (Figure 3.4b), and is evident when Western Australia, Torres Strait and Eastern Australia populations are compared individually with Asian populations (Figure 3.4a).

3.4 Discussion

Major findings of this study show that *P. maxima* exhibits decreased genetic diversity towards its range limits, and its populations are genetically structured throughout its distribution. This demonstrates that, despite a relatively long planktonic larval phase (17-24 days) (Rose & Baker, 1994), genetic exchange between some populations is not sufficient to nullify phenomena that generate population differences, namely genetic drift and selection. This is supported by Benzie and Smith-Keune (2006), who also found genetic discrepancies between southern Indonesian and Northwest Australian populations of *P. maxima*, citing historical separation of Pacific and Indian Ocean basins as the dominant factor shaping population differentiation. They reported overall F_{ST} and R_{ST} values of 0.009 and 0.016 respectively, considerably lower than global estimates of genetic differentiation presented here ($F_{ST} = 0.027$, $P < 0.001$; $R_{ST} = 0.023$, $P < 0.001$). Based on our data, which examined populations on a broader spatial scale, *P. maxima* exhibits stronger genetic population structuring than previously reported. Similar patterns of population differentiation reflecting Indian and Pacific Ocean separation have also been observed for other marine species inhabiting the region (reviewed by Benzie, 1998). It is believed that during times of lowered sea levels a near impassable land barrier formed between Sumatra and Timor which had a significant effect on the population structure of multiple marine organisms in this region (Benzie, 1999b ; Duda & Palumbi, 1999 ; Lavery *et al*., 1996 ; Williams & Benzie, 1997). However, this phenomenon is not necessarily universal across all marine species (Lessios *et al*., 2003 ; Uthicke & Benzie, 2003). It is apparent that the patterns seen in *P. maxima* are congruent with historical Indian/Pacific separation; however, the clear distinction of both Western Australian and Asian populations from those in the Torres Strait indicates that there are additional influences shaping the population genetic structure of this species.

The breakdown of an isolation-by-distance (IBD) pattern of differentiation amongst populations on a global scale, but not on a regional scale, is a good indication that the presence of other

factors affecting the historical gene flow may be in operation between some regions of *P. maxima*'s range. It suggests that gene flow between Asian populations and those outside the region may still periodically occur, but that gene flow modulates over greater geographical distances and eventually reaches a level where the exchange of migrants is not strong enough to counter the effects of random genetic drift. This effect may be magnified by the presence of other oceanographic or biogeographic factors present within the species' distributional range. A biogeographic break across the Torres Strait and northern Australia has been previously documented, whereby periodic fluctuations in sea level have resulted in a land bridge connecting Australia and Papua New Guinea (Voris, 2000), forming a physical barrier to gene flow either side of the Torres Strait and Arafura Sea. This has been cited as the major factor responsible for genetic differences in several marine finfish (Keenan, 1994 ; Ovenden *et al*., 2002 ; Chenoweth *et al*., 1998) and invertebrate populations (Gopurenko & Hughes, 2002 ; Ward *et al*., 2006) across northern Australia. Significant R_{ST} and F_{ST} differences between Torres Strait and other populations (notably with Western Australia and Aru), and the Torres Strait 'cluster' reported here, show a genetic disjunction consistent with vicariant disturbance of population connectivity across the Torres Strait; however, the influence of oceanographic factors on population sub-division cannot be ruled out. The influence major ocean currents have on the definition of population genetic structure patterns has been highlighted previously for the sea scallop (Kenchington *et al*., 2006), a marine bivalve with similar life history traits to *P. maxima*, and could be a significant factor in this instance also. The deflection of the Indonesian Throughflow Current and the East Australian Current away from northern Australia (Figure 3.1) means the lack of major ocean currents passing through the Torres Strait may also provide limits to larval transport across this region even through periods of high sea level. The combination of historical vicariance and present day ocean currents across northern Australia is therefore a likely cause of the genetic break seen in *P. maxima* across the Torres Strait.

Since the processes by which genetic diversity accumulates (i.e. by either mutation or migration incursions) are slow, yet such diversity can be lost far more rapidly (via founder effects and genetic bottlenecks), significant changes or differences in genetic diversity most likely arise through loss rather than gain (Amos & Harwood, 1998). This seems a plausible explanation for

the significant differences in diversity seen here between peripheral populations which generally have fewer alleles than those located more centrally, particularly in northern populations whose location on the shallow Sunda Shelf have possibly seen multiple founder events due to recolonisation following glacial maxima sea level fluctuations (Hanebuth *et al*., 2000).

Whilst it cannot be discounted that the genetic differences in *P. maxima* may indeed be the consequences of natural selection operating at the environmental extremes of this species' range, an emerging pattern of intra-specific diversity consistent with these results is now presenting in the Indo-Australian Archipelago. It has been shown for several widespread marine species (Benzie *et al.*, 2002 ; Palumbi *et al.*, 1997), as well as in a mangrove species (Arnaud-Haond *et al*., 2006), that intra-specific diversity has a tendency to peak towards the centre of the Indo-Australian Archipelago. Several reasons could account for this. The complexity and abundance of habitat area available for tropical reef fishes and corals is invoked as a significant factor accounting for the exceptional biodiversity in the Indo-Australian Archipelago (Bellwood & Hughes, 2001). The larger populations that can therefore be sustained in this region are more likely to maintain greater genetic variation than smaller populations outside the region, where suitable habitat is sparser (Frankham, 1996). The presence of certain factors such as habitat variety, environmental stability, or the ability to sustain larger populations, may simply mean that within the Indo-Australian Archipelago genetic diversity is less likely to be lost over time due to catastrophic stochastic events, and subsequently is maintained at higher levels relative to less structurally diverse regions. It has been shown that genetic diversity peaks within a species range do not always coincide with a range centre (Garner *et al.*, 2004), or the putative region of species origin (Benzie *et al.*, 2002). Therefore, it may be more suitable to utilise ecological and demographic stability as potential predictors of genetic diversity maintenance. This explanation has also been put forward to account for the parallel species and genetic diversity gradients seen in the Pacific, which highlights the similarities in mechanisms causing their loss despite the fundamental differences in how each are generated (Palumbi, 1997). Given the emerging pattern of higher intra-specific genetic diversities within the Indo-Australian Archipelago, this aligns well with a recent hypothesis proposing the rich species diversity in the region is due to its function as a Centre-of-Survival, as opposed to the traditional Centre-of-Origin and Centre-

of-Accumulation hypotheses (Barber & Bellwood, 2005). It must be noted, however, that although this is a possible explanation for the patterns of genetic diversity seen in *P. maxima*, it is beyond the power of this study to determine which 'centre-of' hypothesis best explains the rich biodiversity in the Indo-Australian Archipelago, since technically only the centre has been examined (Briggs, 1999).

This study shows greater genetic diversity in *P. maxima* populations from central Indonesia. Interestingly, this region is uniquely encapsulated by biogeographic breaks; with axes of population differentiation in multiple marine species identified towards its south (Barber *et al.*, 2000), west (Lourie & Vincent, 2004) and east (Keenan, 1994 ; Ovenden *et al.*, 2002). These patterns are reminiscent of historically isolated basins formed by Pleistocene fluctuations in sea level, with land bridges serving as barriers to gene flow amongst once (or presently) connected populations. During times of high sea level, however, subsequent admixture of divergent populations could see increased levels of genetic diversity at the interface of merging populations (Petit *et al.*, 2003), as perhaps seen in our data set which show the three Indonesian populations with limited genetic structure and evidence of high mixing. This phenomenon is also evident in an Indo-Pacific mollusc species (*Haliotis asinina*) with differing dispersal potential to *P. maxima*. This abalone species shows high genetic diversity and limited genetic structure within the Indo-Malay region, yet populations from both west and east Australian regions exhibit clear phylogenetic breaks and decreased genetic diversity in comparison (Imron *et al.*, 2007). Contrasting with this is the strong genetic structuring seen in *Haptosquilla pulchella* populations inhabiting the same region, believed to be caused by the separation of ocean basins during times of lowered sea levels (Barber *et al.*, 2002). The suggestion by (Imron *et al.*, 2007), however, that contiguous range expansion of *H. asinina* populations, perhaps from a Malaysian centre, could negate the retention of allopatric differences formed during Pleistocene isolation of ocean basins is in agreeance with population genetic patterns of *P. maxima* seen in the present study. Even if a particular biogeographic feature does not universally affect all species, the proximity of the aforementioned breaks around central Indonesia may therefore see a peak in intra-specific diversity levels here for multiple species, compared to more distantly located populations.

The occurrence of intra-specific genetic diversity hotspots have been reported in terrestrial flora, and show that environmental and geographic features can have an overriding influence on the dynamics of multiple species despite differing ecologies and dispersal potential (Petit *et al.*, 2003). The question of whether or not the Indo-Australian Archipelago may have a similar effect on the genetic diversity of multiple species has been given limited attention. This study provides additional evidence that regions within the Indo-Australian Archipelago may harbour greater levels of intra-specific genetic diversity. However, continued investigation on the patterns of intra-specific diversity using comprehensive sampling and appropriate genetic tools is necessary if it is to be determined whether the Indo-Australian Archipelago is indeed a region of increased genetic diversity maintenance. The incorporation of such genetic diversity information with the already abundant species richness data may provide a powerful tool for future conservation efforts within the Indo-Australian Archipelago and give additional clues towards identifying the underlying factors generating or maintaining such biodiversity in the region.

Chapter 4 Genetic diversity of wild vs. cultured *P. maxima* populations[†]

4.1 Introduction

Once the life-cycle of an aquaculture species has been closed and culture techniques refined, the well-controlled environment of a hatchery presents a perfect platform to implement selection programmes seeking improvement in commercially valuable traits (Hulata, 2001). A challenging problem for many aquaculture industries, however, is how best to avoid the loss of genetic diversity over ensuing generations, since in closed populations the reductive processes of genetic drift (e.g. founder effects, differential family survival, domestication selection) are intensified. In many cases, the significance of maintaining adequate levels of genetic variability within a population are often overlooked, or the practices leading to the greatest loss of genetic diversity are not understood and therefore procedures are not undertaken to prevent its reduction. Not only will genetic diversity within a population increase its ability to withstand environmental perturbations and disease outbreaks (Gamfeldt & Kallstrom, 2007), a sufficient level of genetic variability is essential in order to maintain a sustained response from long-term selection for commercially important traits (Davis & Hetzel, 2000). It therefore should be a major priority for aquaculture operations to not only capture, but also maintain, as much of the naturally occurring variation as possible within domesticated populations. Whether the overall production goal is for commercial or restocking purposes, avoiding the loss of valuable genetic variability poses a major stumbling block to aquaculture. The accumulation of genetic diversity in natural populations is very slow (1000's of years). However, if appropriate precautions aren't implemented throughout the culture process this diversity can be lost in as little as a single generation (Porta *et al.*, 2007 ; Jackson *et al.*, 2003). Founder effects, differential survival of progeny and non-random mating can all lead to low levels of genetic diversity, which can have a direct impact on the availability of favourable genes and increase the risk of inbreeding

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[†] Manuscript: Lind C.E., Evans B.S., Knauer J., Taylor J.J.U. & Jerry D.R. 2009 Decreased genetic diversity and a reduced effective population size in cultured silver-lipped oysters (*Pinctada maxima*). Aquaculture, 286 (1-2) 12-19.

depression in future generations (Hartl & Clark, 1989). Aquaculture practices, past and present, have consistently produced populations with significantly lower genetic diversity than their wild progenitor populations leaving substantial cause for concern (Sbordoni *et al.*, 1986 ; Sbordoni *et al.*, 1987 ; Eknath & Doyle, 1990 ; Withler, 1990 ; Benzie & Williams, 1996 ; Xu *et al.*, 2001 ; Alarcon *et al.*, 2004 ; Evans *et al.*, 2004a ; Lundrigan *et al.*, 2005). As a result, there is growing trepidation amongst aquaculturists regarding the genetic consequences of their farm or hatchery practices and the long-term success of their breeding programs (Campton, 2004 ; McAndrew, 2001).

One such aquaculture industry where there is concern regarding the retention of genetic diversity is that of “South Sea” pearl production. The commercial production of South Sea pearls (based on the culture of the silver-lip pearl oyster *Pinctada maxima*) began in the 1950’s and has subsequently grown into a significant aquaculture industry throughout northern Australia and southeast Asia (Southgate, 2007). Initially this industry was based around the seeding of either wild adult pearl oysters collected from naturally occurring oyster beds, or newly settled wild spat from field collectors. In the last 20 years, however, closure of this species’ life-cycle has produced a major shift away from the harvest of wild oysters towards the farming of hatchery-produced seedstocks. In Indonesia, the world’s largest producer by volume of South Sea pearls, effectively all pearl production is now based on hatchery-bred oysters, while in northern Australia hatchery produced oysters can contribute up to 50% of pearl production (Shor, 2007 ; Fletcher *et al.*, 2006).

The culture of *P. maxima* routinely involves hatchery practices such as mass-spawning, use of uneven broodstock sex ratios, communal rearing of different families and size grading. These practices have been shown to negatively impact levels of genetic variability and effective genetic sizes (N_e) in a variety of aquaculture species (e.g. Frost *et al.*, 2006 ; Taris *et al.*, 2006 ; Brown *et al.*, 2005 ; Sekino *et al.*, 2003), and may be affecting *P. maxima* populations in a similar fashion. Additionally, *P. maxima* is a highly fecund, broadcast spawning species (Rose & Baker, 1994), a combination of traits which, in hatcheries, can easily result in populations with low N_e due to the likelihood of a few individuals producing the majority of offspring in a given

generation (Fiumera *et al*., 2004). Broodstock that are subsequently chosen from such cohorts are also more likely to be closely related, which can lead to ongoing problems associated with inbreeding depression and reduce the response to selection significantly (Bentsen & Oleson, 2002).

Earlier studies on Japanese Akoya pearl oysters (*P. fucata martensii*) showed that selection based on commercial traits can erode genetic variability within cultured populations of this species (Wada, 1986). Similarly, (Durand *et al.*, 1993) found a reduction in allelic diversity in black-lip pearl oysters (*P. margaritifera*) after three generations of culture. However, a more recent investigation on Chinese *P. fucata* revealed no differences in the genetic diversity of hatchery populations compared to local wild populations, which may indicate an improvement in the culture practices in this species (Yu & Chu, 2006).

To date there has been no investigation into whether cultured *P. maxima* have suffered reductions in genetic diversity, compared to their wild counterparts. In this study, we compare the genetic properties of three wild and five hatchery-propagated *P. maxima* populations from Indonesia using polymorphic microsatellite DNA methods. We also explore the genetic consequences of two different spawning techniques employed in separate hatchery populations. This approach will provide for the first time, an insight into the effective genetic size and level of relatedness within cultured *P. maxima* populations and enable a better understanding of how efficient current culture practices are at capturing and maintaining genetic variability.

4.2 Materials and methods

4.2.1 Sample collection & hatchery procedures

Local divers collected wild *P. maxima* samples from naturally occurring beds at three locations across Indonesia (Bali - 8.32S, 114.92E; Aru - 6.43S, 134.63E and West Papua - 1.13N, 130.54E) between 2004 and 2005. Representative samples from five hatchery-bred cohorts (Mass-spawned; Controlled spawn A, B and C; and a Selected spawn) were taken in 2005 from two different commercial hatcheries in Indonesia. All broodstock from each different hatchery

group were sourced from one or more of the three wild Indonesian populations mentioned above. In the mass-spawned cohort, all broodstock oysters were placed in a single tank and cross-fertilization occurred randomly in the water column when males and females simultaneously released gametes. Controlled-spawn cohorts were propagated using a more manipulated spawning method, and permitted a better gauge of broodstock contributions. In this method, all broodstock were placed in a single tank to initiate gamete release as described previously; however, as each female commenced releasing eggs, it was removed, the mantle cavity rinsed and placed into a specially designed spawning tray that allowed the separate collection of eggs from individual females whilst still permitting fertilization from spawning males. Zygotes collected from each different female were then reared in separate tanks for 2 days, after which they were then counted and placed together in equal proportions in a larger tank and communally reared. This allowed the monitoring of any mass mortality or ‘crashes’ of particular maternal families that can often occur within this period, and also allows a direct estimate of female contributions for each cohort prior to communal rearing. The selected-spawn cohort was produced using the same technique as the controlled-spawn groups, however, broodstock for this group were specifically selected based on silver nacre colour - a commercially important trait. Broodstock for all hatchery groups were sourced directly from wild populations, except for those from the select-spawn group, which were predominantly F_1 hatchery-produced animals and some wild oysters (< 5). Tissue samples from all pearl oysters were taken from the foot, mantle or adductor muscle tissue, and preserved in 70-80% ethanol for later DNA analyses. Age of progeny from cultured groups ranged between 3-12 months of age, however, within a given sample all individuals were of the same age.

4.2.2 DNA extraction and microsatellite amplification

Preserved tissue was digested in a CTAB buffer with 20 mg ml^{-1} proteinase K for 1-3 hrs at 55°C, followed by a phenol:chloroform:isoamyl alcohol purification protocol to extract total genomic DNA (gDNA) (Sambrook *et al.*, 1989). gDNA was quantified by comparison to DNA concentration standards after agarose gel electrophoresis using the ImageJ 1.33 software package (Wayne Rasband NIH 2004) and resuspended in ddH_2O to a concentration of 5 ng μl^{-1}.

Individual genotypes were obtained using six polymorphic microsatellite loci (Table 4.1). PCR was conducted in 15 µl volumes using the following conditions: 1 x PCR buffer with 1.5 mM $MgCl_2$ (QIAGEN), 1 x Q solution (QIAGEN), 0.2 mM dNTPs, 0.3 U of Taq DNA polymerase (QIAGEN) and 5 ng of gDNA. The primers *Pmx-022*, *Pmx-16_23*, *Pmx-16_41*, *Pmx-18_21* (Smith *et al.*, 2003), *JCUPm-1g8* (Evans *et al.*, 2006) and *JCUPm-26h5* (Fwd- 5'TAGTCCTTTGCATATGACCTTGG 3'; Rev - 5'ATCGTGTTACAACCAAAGCGTTC 3') were used for genotyping and subsequent parentage analyses. PCR for *JCUPm-1g8* and *JCUPm-26h5* loci did not require Q solution. Fluorescent-labelled primer and $MgCl_2$ concentrations varied for each marker according to original published conditions (Table 4.1). Thermocycler programs for all PCR began with an initial denaturation step for 3 mins at 94°C followed by 35 cycles of 94°C for 45 sec, locus specific T_A for 30 sec (see Table 4.1) and 72°C for 45 sec, then a final extension step at 72°C for 5 mins. To reduce non-specific amplification the PCR cycling conditions for *Pmx-18_21*, *Pmx-16_23* and *Pmx-16_41* used a touchdown program where annealing temperature was sequentially lowered from 58°C, 56°C, 54°C and 52°C (for 5 cycles each) followed by 50°C for 15 cycles (Smith *et al.*, 2003).

Table 4.1 Microsatellite marker suite used for genetic diversity analyses of wild and cultured *P. maxima* populations

Locus Name	Fluorescent label	T_A (C°)	$MgCl_2$ conc. (mM)	Reference
Pmx-022	HEX	50	1.5	(Smith *et al.*, 2003)
Pmx-16_23	TET	58-50†	1.5	(Smith *et al.*, 2003)
Pmx-16_41	FAM	58-50†	1.5	(Smith *et al.*, 2003)
Pmx-18_21	TET	58-50†	1.5	(Smith *et al.*, 2003)
JCUPm-1g8	HEX	55	3.0	(Evans *et al.*, 2006)
JCUPm_26h5	FAM	50	3.0	Unpublished*

† Touch-down PCR cycle, TA decreases by 2 C° every 5 cycles until 50 C°

* Primer sequence: Fwd- 5' TAGTCCTTTGCATATGACCTTGG 3'
Rev - 5' ATCGTGTTACAACCAAAGCGTTC 3'

PCR products were purified using an ammonium acetate:ethanol precipitation protocol to remove residual salts, dinucleotides and primers (Sambrook *et al.*, 1989). PCR products underwent capillary electrophoresis together with a Tamra-400 size standard on a MegaBACE auto-sequencer (Amersham Biosciences) and allele sizes were then calculated using MegaBACE Fragment Profiler v1.2 software (Amersham Biosciences).

4.2.3 Statistical analyses

Allele frequencies, inbreeding co-efficient (F_{IS}) and allelic richness (R_S) (accounting for differences in sample size following Leberg, 2002) were calculated for all populations using software FSTAT 2.9.3.2 (Goudet, 1995). Observed and expected heterozygosities (Nei, 1987) per locus for each population were calculated using Arlequin v3 (Excoffier *et al.*, 2005). Moment-based effective population size (N_e) estimates based on heterozygote excess (Pudovkin *et al*., 1996) and linkage disequilibrium (Waples, 2006) were calculated for each population using NeEstimator v1.2 (Peel *et al*., 2004) and LDNe (Waples & Do, 2008) respectively. To gain an understanding of the genetic similarities amongst individuals in a population, mean pairwise relatedness estimate of each population were calculated using the methods of (Queller & Goodnight, 1989) and a maximum-likelihood relatedness estimator (Konovalov & Heg, 2008) available in KinGroup v.2 (Konovalov *et al*., 2004). To further investigate individual relationships within populations in the absence of broodstock genotype information, full-sib kin groups were reconstructed. However, since the construction of full or half-sib kin groups using pairwise relatedness estimators can give a high proportion of incongruous full-sib triads (Rodriguez-Ramilo *et al*., 2007), we implemented the methods of (Herbinger *et al*., 2006) to infer genealogical relationships amongst individuals in hatchery cultured populations using Pedigree v2.2 (online, http://herbinger.biology.dal.ca:5080/Pedigree.) This method uses a Markov Chain Monte Carlo approach to generate various partitions of individuals (i.e. kin groups) using co-dominant molecular data and is described in detail in (Butler *et al*., 2004 ; Smith *et al*., 2001).

4.3 Results

4.3.1 Genetic diversity statistics

Allele frequencies for each locus were calculated for all populations (see Appendix A). All populations showed polymorphism at each locus, however, consistently fewer alleles were found in hatchery-produced *P. maxima* populations than the three wild Indonesian populations (Table 4.2). Across all six loci, 98 different alleles were present within the three wild populations

Table 4.2 Genetic diversity statistics for wild and cultured populations of *P. maxima*, showing number of alleles (A), allelic richness (R_s), observed heterozygosity (H_o), expected heterozygosity (H_e) and Wright's fixation index (F_{IS}) for each microsatellite locus. Bold H_o values indicate significant departures ($P < 0.05$) from Hardy-Weinberg expectations.

	WILD				CULTURED					
	Aru	**Bali**	**West Papua**	**Total**	**Mass Spawn**	**Controlled A**	**Controlled B**	**Controlled C**	**Selected**	**Total**
n	67	55	62	184	75	89	92	92	94	442
Pmx-022										
Allele Range	147-181	141-181	145-177	141-181	145-175	145-177	141-179	141-177	155-179	141-179
A	16	18	15	19	11	13	14	11	11	17
R_s	15.5	17.9	14.6	16.0	10.8	12.1	13.1	9.6	10.6	11.2
H_o	0.94	0.81	0.90	0.88	**0.90**	**0.97**	**0.95**	**0.84**	**0.78**	0.89
H_e	0.90	0.89	0.89	0.90	0.84	0.87	0.88	0.79	0.77	0.83
F_{is}	-0.04	0.09	-0.01	0.01	-0.08	-0.11	-0.09	-0.06	-0.01	-0.07
Pmx-16_23										
Allele Range	230-274	230-274	230-266	230-274	230-254	230-252	230-264	230-264	230-254	230-264
A	17	15	15	18	9	10	11	9	10	14
R_s	16.2	15.0	14.7	15.3	8.9	9.1	10.7	8.9	9.5	9.4
H_o	**0.85**	0.87	0.93	0.88	**0.97**	**0.86**	**0.94**	**0.73**	**0.80**	0.86
H_e	0.90	0.90	0.91	0.90	0.86	0.85	0.80	0.77	0.83	0.82
F_{is}	0.05	0.03	-0.03	0.02	-0.14	-0.02	-0.18	0.05	0.04	-0.05
Pmx-16_41										
Allele Range	218-258	218-258	218-266	218-266	218-258	218-254	218-266	222-250	218-254	218-266
A	11	11	13	13	8	8	10	8	9	11
R_s	10.9	10.9	12.5	11.5	7.7	7.6	9.8	8.0	9.0	8.4
H_o	0.79	0.93	0.84	0.85	0.83	**0.83**	**0.89**	0.85	0.86	0.85
H_e	0.89	0.86	0.87	0.87	0.78	0.62	0.85	0.79	0.84	0.77
F_{is}	0.11	-0.08	0.04	0.02	-0.06	-0.34	-0.05	-0.08	-0.03	-0.11
Pmx-18_21										
Allele Range	87-139	91-155	91-159	87-159	95-131	99-127	91-127	95-127	95-127	91-137
A	18	16	14	21	7	10	11	10	7	13
R_s	16.5	15.9	13.5	15.3	6.9	8.9	10.5	9.8	6.7	8.6
H_o	**0.70**	**0.69**	**0.73**	0.71	**0.84**	0.67	0.83	**0.96**	**0.51**	0.76
H_e	0.85	0.88	0.85	0.86	0.80	0.58	0.81	0.83	0.61	0.73
F_{is}	0.17	0.21	0.15	0.18	-0.05	-0.15	-0.02	-0.15	0.17	-0.04
JCU Pm_1g8										
Allele Range	213-245	213-241	213-249	213-249	213-245	219-239	213-239	219-241	219-245	213-245
A	16	13	15	17	8	8	10	10	10	15
R_s	15.5	13.0	14.6	14.4	7.7	7.9	9.5	10.0	9.6	8.9
H_o	**0.75**	0.89	0.85	0.83	0.68	**0.84**	**0.86**	**0.92**	**0.74**	0.81
H_e	0.88	0.86	0.88	0.87	0.66	0.78	0.83	0.83	0.83	0.78
F_{is}	0.15	-0.04	0.03	0.05	-0.04	-0.08	-0.03	-0.11	0.10	-0.03
JCU Pm_26h5										
Allele Range	150-186	150-170	150-170	150-186	150-162	150-162	150-170	150-170	150-182	150-182
A	10	5	6	10	3	3	3	4	4	5
R_s	9.5	5.0	5.9	6.8	3.0	3.0	2.9	4.0	4.0	3.4
H_o	0.67	0.69	0.62	0.66	**0.59**	0.59	0.41	**0.60**	0.64	0.57
H_e	0.66	0.62	0.67	0.65	0.57	0.52	0.51	0.62	0.60	0.57
F_{is}	-0.02	-0.10	0.08	-0.01	-0.03	-0.12	0.20	0.02	-0.05	0.00
Total										
A	88	78	78	98	46	52	59	52	51	75
R_s	14.0 ± 1.2	12.9 ± 1.9	12.6 ± 1.4	13.2 ± 1.4	7.5 ± 1.1	8.1 ± 1.2	9.4 ± 1.4	8.4 ± 0.9	8.2 ± 1.0	8.3 ± 1.1
H_o	0.78 ± 0.04	0.81 ± 0.04	0.81 ± 0.05	0.80 ± 0.04	0.80 ± 0.06	0.79 ± 0.06	0.81 ± 0.08	0.82 ± 0.05	0.72 ± 0.05	0.79 ± 0.05
H_e	0.85 ± 0.04	0.83 ± 0.04	0.85 ± 0.04	0.84 ± 0.04	0.75 ± 0.05	0.70 ± 0.06	0.78 ± 0.05	0.77 ± 0.03	0.75 ± 0.04	0.75 ± 0.04
F_{is}	0.08	0.03	0.04	0.05	-0.07	-0.12	-0.04	-0.06	0.03	-0.05

samples, whereas a total of 75 alleles were found across all cultured populations, of which all were present in the wild samples. Mean allelic richness (R_s), a sample size bias corrected estimator of the number of alleles per locus, ranged from 12.6 ± 1.4 (Aru) to 14.0 ± 1.2 (West Papua) in the wild populations, whilst cultured populations exhibited considerably lower values ranging from 7.5 ± 1.1 (Mass spawned) to 9.4 ± 1.4 (Controlled B) (Table 4.2). In several instances, single locus R_s values of wild populations were more than double that of some cultured populations (e.g. Aru, Bali vs. Mass spawned and Selected populations at *Pmx-18_21*; Aru, West Papua vs. Controlled B at *JCUPm_26h5*), indicating a substantial loss of genetic variability at certain loci in some populations. Observed heterozygosity (H_o) did not differ markedly across populations overall, with mean H_o of all populations between 0.78 and 0.82, with the exception of the Selected population, having a slightly lower H_o of 0.72 ± 0.05 (Table 4.2). Expected heterozygosity (H_e) and F_{IS} values both highlight heterozygote deficiencies in wild populations, whilst heterozygote excesses are much more prevalent in the cultured populations. Deviations from Hardy-Weinberg expectations of heterozygosity were seen in both wild and cultured populations (Table 4.2). All cultured populations showed significant departures ($P < 0.05$) from HWE for *Pmx-022* and *Pmx-16_23* loci, and significant HWE deviations were also observed for *JCUPm-1g8* in all populations except the Mass Spawn group. Of the wild populations, departures were observed in Aru, Bali and West Papua populations for *Pmx-18_21*, and also in the Aru population for *Pmx-16_23* and *JCUPm-1g8* markers (Table 4.2).

4.3.2 Effective population sizes

Stark contrasts in effective population sizes (N_e) were seen between wild and cultured populations (Table 4.3). N_e of cultured populations ranged from 3.5 (Mass Spawn) to 9.2 (Controlled A) whilst wild population N_e's ranged from 109.6 (West Papua) to 423.4 (Bali) calculated by the Linkage Disequilibrium (LD) method (minimum allele frequency 0.05). N_e calculated using the Heterozygote Excess (HE) method returned somewhat more variable yet similar results, with the Mass Spawn population showing the lowest effective genetic size (5.9) and wild populations showing considerably larger N_e than cultured populations (Table 4.3). Given that some marine bivalves are reported to show heterozygote deficiencies in natural populations, and the potential occurrence of null alleles, HE-based methods are prone to bias.

However, the similarity in overall patterns of HE and LD based N_e estimates across populations indicate that potential biases such as null alleles and heterozygote deficiencies are unlikely to be problematic in this instance. Relatively narrow 95% confidence intervals, particularly in cultured populations, indicate that the comparatively new LD method (Waples, 2006) is a potentially reliable estimator of N_e in this scenario, where demographic information such as sex ratios, census sizes and number of offspring per male/female is limited.

Table 4.3 Effective population sizes (N_e) of wild and cultured *P. maxima* populations based on heterozygote excess (Pudovkin *et al.*, 1996) and linkage disequilibrium (including 95% confidence intervals) (Waples, 2006) methods.

Population	Broodstock used		N_e - Heterozygote Excess	N_e – Linkage Disequilibrium			
	M	F	N_e	Min. allele freq used*	N_e	95% CI Lower	95% CI Upper
Aru	n/a	n/a	∞	0.05	119.3	58.1	740.8
				0.02	157.7	77.6	1148.6
				0.01	223.0	108	3051.0
Bali	n/a	n/a	∞	0.05	432.4	88.2	∞
				0.02	263.8	87.8	∞
				0.01	1115.6	145.9	∞
West Papua	n/a	n/a	101.1	0.05	109.6	55.5	508.0
				0.02	123.6	69.6	362.6
				0.01	152.7	67.5	∞
Mass spawn	19	9	5.9	0.05	3.5	3.0	4.1
				0.02	3.9	3.3	6.0
				0.01	4.1	3.6	6.2
Controlled A	20	2	6.0	0.05	9.2	4.6	14.2
				0.02	9.8	6.8	13.3
				0.01	11.0	7.9	14.8
Controlled B	20	5	13.3	0.05	3.8	2.6	8.2
				0.02	6.0	3.4	9.5
				0.01	9.6	6.5	13.1
Controlled C	20	5	9.5	0.05	3.9	3.0	7.4
				0.02	7.0	4.1	9.2
				0.01	8.2	6.4	10.2
Select	> 15	14	67.5	0.05	7.1	3.5	11.6
				0.02	10.4	7.4	14
				0.01	11.9	8.7	15.7

4.3.3 Relatedness and relative contributions of full-sib groups

Mean pairwise relatedness co-efficients (R_{xy}), a measure of genetic similarity relative to the population mean, across all populations ranged from -0.115 ± 0.006 (Aru) to 0.287 ± 0.003 (Controlled A) (Figure 1). R_{xy} calculated through the methods of (Konovalov & Heg, 2008) returned lower mean estimates than from (Goodnight & Queller, 1999), however, the trends

seen across populations were consistent. For both relatedness co-efficients, the Controlled Spawn A group showed the highest mean R_{xy}, followed by the Mass Spawn, and Selected groups, whilst Controlled B and C groups showed the lowest. However, all cultured populations exhibited greater mean relatedness than the wild populations (Figure 4.1).

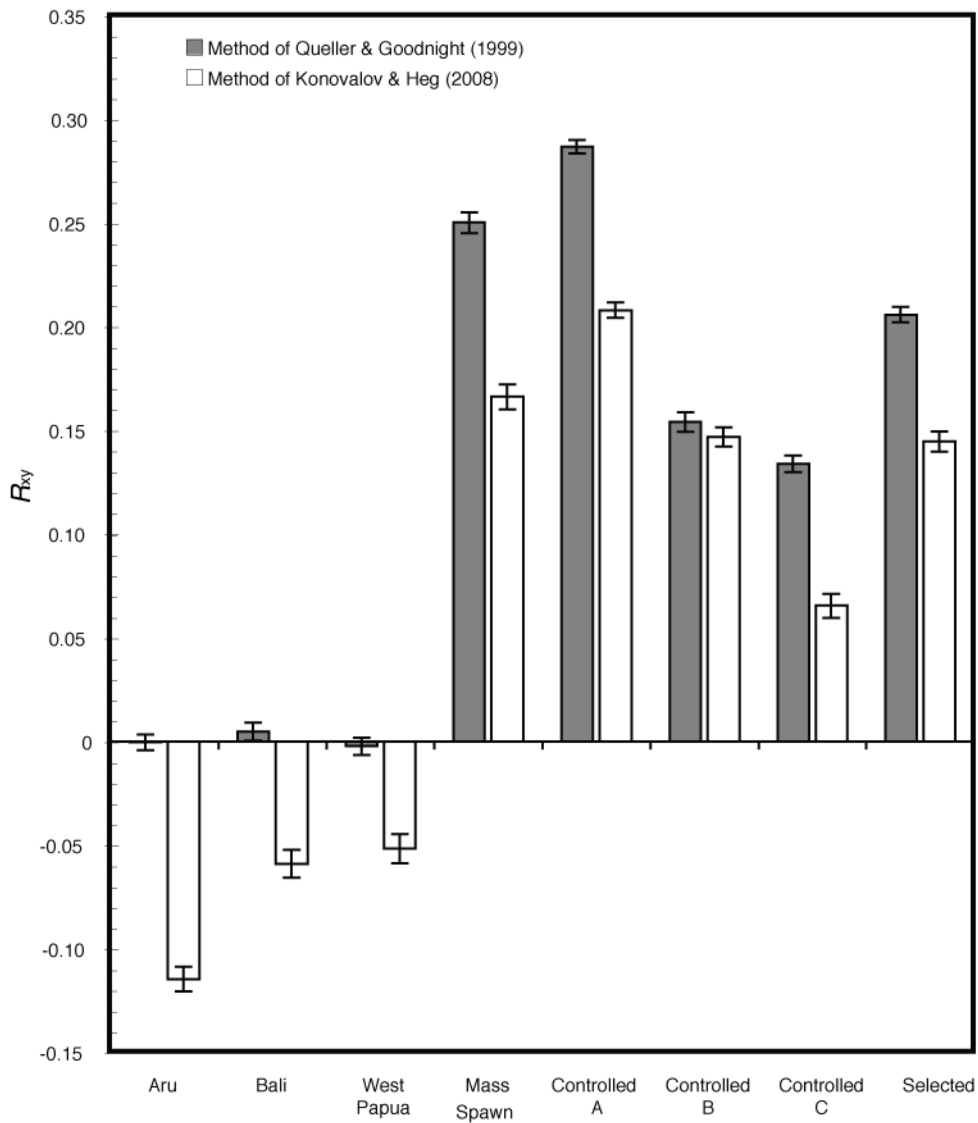

Figure 4.1 Mean relatedness values (R_{xy}) for cultured and wild pearl oyster populations. Grey bars show means (± SE) following the methods of Goodnight and Queller (1999), white bars show mean R_{xy} (± SE) following Konovalov and Heg (2008). Negative R_{xy} values are due to the unbiased estimation of relatedness and effectively represent an R_{xy} value of 0.

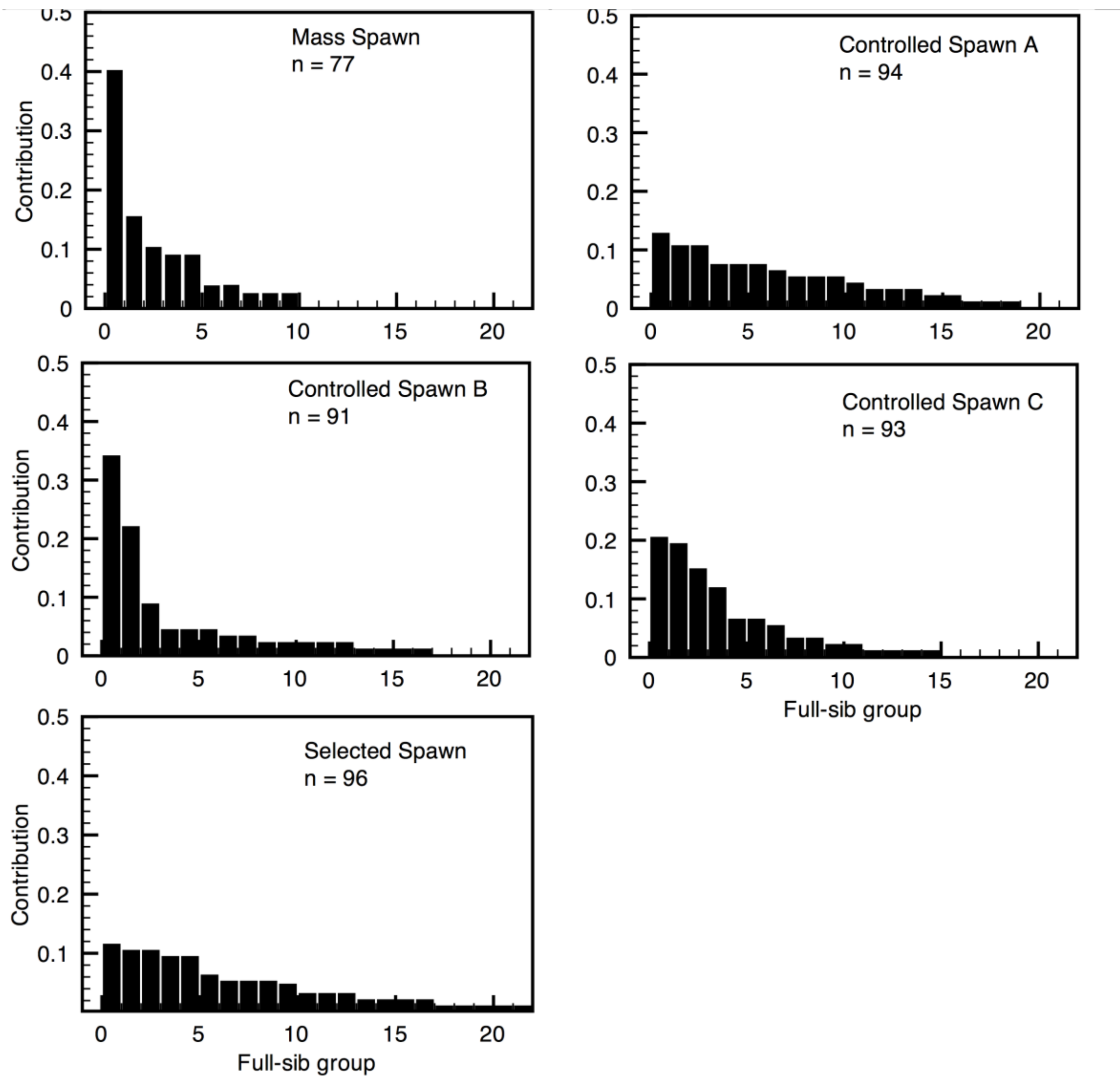

Figure 4.2 Relative contributions of full-sib groups for cultured populations of *P. maxima*, generated without pedigree information.

The construction of putative full-sib partitions allowed deeper investigation into potential factors affecting genetic diversity within cultured pearl oyster populations, and revealed some cohorts were largely dominated by only one or two full-sib groups (Figure 4.2). One full-sib group from the Mass Spawned population comprised 40% of the entire cohort, and similarly in Controlled Spawn B, where 56% of the population was made up of only two full-sib partitions. By contrast, no full-sib group from both the Controlled Spawn A and Selected populations was larger than 13% of the whole group (Figure 4.2).

4.4 Discussion

This investigation has revealed genetic diversity loss and reductions in effective genetic size in hatchery-produced populations of *Pinctada maxima* when compared to their wild progenitors. This demonstrates that, despite a shifted priority towards the use of hatchery-produced animals, hatchery procedures for *P. maxima* appear inefficient at capturing available genetic variability and must be improved in order to maximise potential gains from future selective breeding programmes.

On a population level, a reduction in the number of alleles observed at microsatellite loci can be indicative of a loss of potentially important functional genetic variation in other regions of the genome, and its avoidance should therefore be considered a priority in aquaculture. Despite this, substantial reductions in the overall number of alleles, and allelic richness (29 – 44%) were observed in all cultured *P. maxima* populations when compared to wild populations (Table 4.2), indicating that the problem of genetic diversity loss has either been overlooked or the approaches towards ameliorating it are ineffective. Reductions in genetic diversity of this magnitude are noteworthy, given that throughout the natural range of *P. maxima*, a difference in microsatellite R_s of only 17% has been observed between populations as a result of natural variation (Lind *et al.*, 2007). By comparison, hatchery produced stocks of the closely related black-lipped pearl oyster (*P. margaritifera*) showed a 17-18% reduction in the number of allozyme alleles compared to a wild sample (Durand *et al.*, 1993). However, this occurrence is not unique to the culture of pearl oyster species, as the loss of genetic diversity is has also been shown in many other aquaculture species including finfish (Frost *et al.*, 2006 ; Porta *et al.*, 2006), crustaceans (Sbordoni *et al.*, 1986) and other molluscs (Li *et al.*, 2004 ; Evans *et al.*, 2004a ; Benzie & Williams, 1996). An important consideration that must also be mentioned when discussing diversity loss in bivalve culture is that of broodstock collection from the wild. It is a common practice to collect obtain wild spat from artificial collection substrates suspended in open ocean during the natural spawning cycle of pearl oysters. These wild spat are then reared under standard husbandry practices until sexually maturity is reached, upon which, individuals may then be chosen as candidate broodstock for hatchery production. It has been suggested, however, that this practice might induce a first loss of genetic diversity as a consequence of

preferential settlement of some individuals onto collectors (Arnaud-Haond *et. al* 2003). It is plausible that this may have also occurred in several cohorts assessed here; however, diversity losses from the collection of wild spat were not quantified in this study.

In closed culture systems, avoiding inbreeding is an important consideration. Breeding of related individuals particularly over multiple generations will increase the risk of encountering genotypes that are deleterious in a homozygous state leading to a situation of reduced overall fitness known as inbreeding depression. Generally, the closed culture of *P. maxima* showed limited evidence for an increase in homozygosity despite a considerable reduction in R_s (Table 4.2). This pattern has also been observed in hatchery-produced abalone (*Haliotis* sp.) (Mgaya *et al.*, 1995 ; Evans *et al.*, 2004a) and several finfish species (Norris *et al.*, 1999 ; Lundrigan *et al.*, 2005) further supporting the view that heterozygosity is not as susceptible to decline as allelic richness in the immediate term. After six generations of selection in the Japanese pearl oyster, *P. fucata*, little evidence was found for a reduction in heterozygosity (Wada, 1986). However, indications of reduced heterozygosity (although not large) are seen in the Selected Spawn group, an F_2 generation, suggesting that continued breeding/selection from such groups may require regular genetic monitoring to avoid further reductions in heterozygosity and consequently inbreeding depression. A significant point to consider within this theme is that some degree of inbreeding and/or loss of diversity over multiple generations is to be expected even in successful selection programmes. What must be highlighted, however, is the significance of controlled genetic management within cultured populations, and that unintentional increases in homozygosity should be minimised to avoid potential problems associated with inbreeding depression.

Perhaps a more pertinent outcome in the context of future inbreeding risks is the marked increases in mean genetic relatedness (R_{xy}) estimates amongst individuals within cohorts compared to their wild progenitor populations (Figure 4.1). Relatedness can be interpreted as the likelihood of recent coalescence for a pair of individuals relative to a reference population (Rousset, 2002), meaning a population with a greater mean of pairwise R_{xy} estimates will possess dyads having an overall greater chance of sharing genes identical-by-descent due to a

recent coalescence. This measure can be particularly useful in aquaculture, since the high fecundity exhibited by many target species combined with advances in larval rearing techniques can mean that relatively few breeders are able to produce large cohorts, resulting in populations with greater proportions of closely related individuals. In this study, cultured *P. maxima* populations showed mean R_{xy} considerably greater than wild populations (Figure 4.1), and suggest that the problem of utilising limited broodstock may be influential but not necessarily the sole cause for diversity loss. In the Controlled A group only two female broodstock were used, which gives a likely explanation for this group having the highest mean R_{xy}. However, contrasting to this in terms of number of female broodstock used is the Mass Spawn group, which also exhibits elevated R_{xy} values, indicating that the cause of elevated R_{xy} estimates may be due to other factors.

Whilst it is suggested that the decreased genetic variability in cultured *P. maxima* may simply be a result of using limited broodstock in each cohort, results also indicate that the addition of extra breeders may not necessarily solve the problem. Some populations, particularly the Mass Spawn and Control Spawn B groups, showed large skews in full-sib family representations (Figure 4.2), indicating that relatively few contributors can dominate cohorts and that the inclusion of further broodstock may not guarantee the desired impact on genetic diversity. In natural marine populations, (Hedgecock, 1994) hypothesised the large variance in reproductive success of individuals can be likened to that of a “sweepstakes” event due to a combined effect of high fecundity and the stochastic nature of larval viability. The practice of mass spawning, whereby all potential broodstock are placed in a single tank and fertilization occurs at random as gametes are released, is essentially a downscaled “sweepstakes” and presents as a logical culprit for the large skews in family representations in the Mass Spawn group investigated here (Figure 4.2). Its effect on diversity is detrimental, with the Mass Spawn group exhibiting the lowest allelic richness (R_s), high R_{xy} values and an effective population size (N_e) of only 3.5 (3.0 – 4.1 95% CI) despite using a total of 28 broodstock for spawning. Mass spawning practices have also been shown to give high and unpredictable variances in family sizes in cultured species such as Japanese flounder (*Paralichthys olivaceus*) (Sekino *et al.*, 2003), barramundi (*Lates calcarifer*) (Frost *et al.*, 2006) and in flat oysters (*Ostrea edulis*) (Launey *et al.*, 2001). In

these cases cohorts show a few families dominating with minor contributions from other broodstock regardless of the number of breeders used, severely impacting genetic diversity. This study shows similar results from the Mass Spawn group, verifying that this spawning approach is highly unpredictable and random in its outcomes. It is therefore recommended that the practice of mass spawning be avoided in pearl oyster hatcheries, particularly if maximising genetic diversity is a priority.

In an attempt to remove a degree of unpredictability associated with mass spawning of pearl oysters, controlled spawnings allowing eggs to be collected separately from each female were conducted through specially designed spawning tables. This allowed a better estimate of female contributions, however, male contributions remained uncertain. Generally higher N_e values were achieved with this approach, which is promising given that fewer female broodstock were used (Table 4.3); however, partitioning individuals into full-sib family groups show that large skews in family contributions are still possible (Figure 4.2). This outcome may be attributed to differential survival rates amongst different families, which has been shown to effect variance in reproductive success and subsequently genetic variability in cultured Pacific oysters (*Crassostrea gigas*) (Boudry *et al.*, 2002 ; Taris *et al.*, 2006).

4.5 Conclusion

This study shows a clear reduction of genetic diversity and effective population sizes in hatchery-produced *P. maxima* compared to their wild progenitor populations, which can occur in as little as a single generation. It is apparent that the practice of mass spawning, due to its unpredictable "sweepstakes" nature, is one of elevated risk in relation to genetic diversity losses and potential inbreeding in future generations. It may be possible to manage genetic diversity losses with further intervention at spawning, such as the separation of female contributions described here. However, this approach is not fail-safe. Cohorts produced using this approach still resulted in elevated relatedness values and variable family contributions. Further research must investigate the influence of differential survival rates amongst families in order to avoid large skews in family contributions and maximise effective population sizes. The impact of size

grading, which has not been investigated in this study, may additionally contribute to genetic diversity loss (Taris *et al.*, 2006 ; Frost *et al.*, 2006) and warrants future attention also.

Chapter 5 Differential family survival and its influence on N_e[†]

5.1 Introduction

The effective population size (N_e) of an aquaculture population is a prominent factor in its long-term success or failure. A high N_e indicates a higher number of contributing broodstock to a population, and can be a valuable gauge of how efficiently an aquaculture operation is capturing or maintaining genetic diversity. This is important, as sufficient levels of genetic diversity within aquaculture populations can provide stability in the face of environmental disturbances (Gamfeldt & Kallstrom, 2007) and will maximise the long-term response to selective breeding through genetic improvement programmes (Davis & Hetzel, 2000). Maintaining a high N_e will also reduce the risk of encountering problems associated with inbreeding, which have been well documented in aquaculture species (e.g Bierne *et al*., 1998 ; Eknath & Doyle, 1990 ; Evans *et al*., 2004b). N_e approximates the effective number of individuals genetically contributing to a population, and is maximised when random mating occurs between breeders with even sex ratios, with an equal contribution of offspring from each parent, no inbreeding and a constant population size across generations (Wright, 1931). However, these conditions are regularly breached in aquaculture. As a result, the ratio of N_e to the actual number of breeders used (N) is usually substantially less than 1 (Hedgecock *et al*., 1992), indicating a significant proportion of potentially valuable genetic variation is not being passed to subsequent generations. In addition to this, populations having small N_e are much more prone to the influences of random genetic drift between generations (Hartl, 2000).

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Culture practices contributing to decreased N_e have been reported in many commercial aquaculture populations (eg. Indian carps (Eknath & Doyle, 1990); *Penaeus japonicus* (Sbordoni *et al.*, 1986); *Ostrea edulis* (Saavedra, 1997); *Sparus aurata* (Brown *et al.*, 2005); *Lates calcarifer* (Frost *et al.*, 2006); *Crassostrea gigas* (Appleyard & Ward, 2006); *Salmo trutta* (Aho *et al.*, 2006)). In the majority of cases, the manifestation of low N_e in aquaculture populations is a cumulative result of several factors. Practices such as mass spawning of broodstock, using uneven sex ratios of broodstock, and indiscriminate size grading or culling will all contribute to lower N_e (Sekino *et al.*, 2003 ; Taris *et al.*, 2006) and must therefore be avoided in order to maximise the genetic potential of an aquaculture population.

Conceptually, these practices appear relatively simple to remedy, however, its practical execution is somewhat more challenging. Many aquaculture operations are still unable to create separate full-sib families because of a limited control of reproduction in many species. For example, barramundi (*Lates calcarifer*) and Atlantic cod (*Gadus mohrua*) broodstock (amongst many others) require social stimuli for gamete release, demanding mass-spawning scenarios (Frost *et al.*, 2006 ; Herlin *et al.*, 2008). The developing status of other aquaculture industries means that even when full-sib families can be produced, a lack of infrastructure, particularly tanks, may limit the number of families or lines that can be carried through a hatchery cycle. In this situation culturists are left with no option but to communally rear families. A particular consequence of these practices is that individual broodstock contributions are typically unknown to the culturist, and in many cases show large variance (e.g Brown *et al.*, 2005). Since a large variance in family sizes can have a particularly severe influence on the reduction of N_e (Kimura & Crow, 1963), this predicament is highly relevant to the maintenance of genetic variation in aquaculture populations.

Attempts to reduce discrepancies in broodstock contributions and family sizes in communally reared cohorts have not been overwhelmingly successful. Equalising sperm contributions prior to fertilization has still resulted in variable (and often highly skewed) male contributions in several aquaculture species, most likely because of differences in sperm potency (Gaffney *et al.*, 1993 ; Withler & Beacham, 1994 ; Selvamani *et al.*, 2001). This has also been observed in

common carp (*Cyprinus carpio*), where a large component of variance in the proportion of larvae sired by different males remains unexplained, despite investigating and controlling for multiple sperm quality parameters (Kaspar *et al*., 2007). In addition to variances in fertilization due to sperm competition, differences in the subsequent survivorship of individual families can further exaggerate differential family sizes. Differential survival leading to skewed family contributions has been shown to be a significant contributor towards reduced N_e in Pacific oysters (*C. gigas*) (Boudry *et al.*, 2002) and barramundi (*L. calcarifer*) (Frost *et al.*, 2006), and could be due to varying broodstock condition prior to spawning, or a combination of genetic factors leading to dissimilar survival rates amongst families. Overcoming large variance in family sizes therefore presents a significant obstacle towards maximising N_e of communally reared aquaculture populations.

The silver-lipped pearl oyster, *Pinctada maxima,* is a commercially significant aquaculture species farmed throughout Southeast Asia and northern Australia, and is an attractive candidate for genetic selection programmes to improve commercially important traits involved in the production of “South Sea” pearls (Evans *et al*., 2007a). It has been shown, however, that culture practices in *P. maxima* can lead to skewed family sizes reducing the N_e of cultured populations and therefore limiting the availability of potentially valuable genetic variation that could be exploited by selection (Lind *et al.*, 2009). In theory, large family size variance may be overcome by equalizing family contributions (thereby improving N_e), but empirical testing of this has been limited. It is not certain whether equalizing contributions is a viable option for diversity maintenance, or will variable survival rates subsequent to equalization negate any effect this has on improving N_e? In order to further understand this conundrum and improve hatchery production, the effects of differential family survival on N_e of cultured *P. maxima* was investigated. Using microsatellite DNA markers to carry out parentage analyses, this study tracks the survival of individual *P. maxima* families that are communally reared in a commercial pearling operation to reveal the extent that differential survival is occurring and contributing to family size variations. Secondly, we investigate whether the simple practice of equalising maternal family sizes prior to communal rearing decreases family size variance and results in an improvement of N_e compared to stocking families in their naturally produced proportions.

5.2 Materials and methods

5.2.1 Tissue sampling, spawning and communal rearing procedures

To investigate whether different *P. maxima* families exhibit varying survival rates during communal rearing following normal industry practices, two pearl oyster cohorts from a commercial hatchery were produced and sampled over time for subsequent DNA parentage analyses. These two cohorts were produced using a total of 11 females (5-6 per cohort) and 11-12 males (from a spawning pool of >150 broodstock). Unlike edible oysters (e.g. *Crassostrea* spp.), sacrificial strip-spawning (which allows the creation of deliberate and controlled family crosses) is difficult and not widely practiced in pearl oyster culture, and it is believed that male-mediated chemical response cues and thermal manipulation are still necessary for adequate female gamete release (Southgate, 2008). Spawnings in this study were done using specially designed, compartmentalised, spawning tables that allowed the separate collection of female gametes whilst permitting fertilization from potentially all 12 males (verified through DNA parentage analyses, CEL *unpublished data*). As a consequence of this 'semi-controlled' approach, initial female contributions can be quantified through direct counting, however, initial male contributions cannot. Fertilized eggs from each female were collected and reared separately in 400L tanks for two days post fertilization, and then communally pooled together in 5000 L tanks for further rearing following a standard commercial protocol until larval settlement. Two 5000 L tanks were used, each containing pooled larvae from 5 (Cohort A) or 6 (Cohort B) different females (i.e. from different 400 L tanks). Maternal families were stocked without specific attention given to stocking proportions; rather, the priority was to ensure each 5000 L tank contained equal numbers of larvae stocked at equal density (5 larvae mL^{-1}). Stocking density of the 400 L and 5000 L tanks was enumerated by counting the average number of larvae in several homogenous (through aeration) 20 mL samples from each tank using a stereomicroscope (3x counts per tank), with the 5000 L tanks adjusted to 5 larvae mL^{-1} using excess larvae from larger families. Spat collectors (square metal frames with 18-20 individual lengths of rope across), containing settled pearl oysters, were removed from the 5000 L tanks at 30 days and transferred onto longlines suspended in open ocean, enabling pearl oysters to filter-feed on naturally occurring plankton. To ensure no bias was incurred by the possibility of

faster or slower settling families, no spat collectors were removed from larval tanks until all spat had settled. Pearl oysters were regularly "thinned out" (through separation, not culling) to prevent overcrowding and maintain an optimal rearing density. Juvenile oysters were transferred from the rope spat collectors into 32-pocket panels (2 individuals per pocket) at 72 days old (time of initial tissue sampling) and are then transferred to 16-pocket panels (one oyster per pocket) by 12 months of age. To optimise feeding efficiency through the reduction of biofouling build-up, animals were cleaned at regular intervals using a high-pressure water gun, as per standard industry practice. Throughout this paper we refer to these cohorts as the *non-experimental* cohorts.

To assess temporal changes in relative contributions of different pearl oyster families through DNA parentage analyses, tissue samples were obtained from all adult broodstock (via foot biopsy) and from 150-200 randomly selected individuals from each cohort when pearl oysters were 72 days and 18 months of age (non-experimental cohorts) and preserved in 70-80% ethanol. To ensure random sampling of juveniles, two set ropes (containing 5-10 juveniles on each) were taken from each settlement panel used. Biopsies were taken from the foot tissue of 18-month-old individuals, however, the small size of 72 day old pearl oysters meant non-lethal tissue sampling was impractical and individuals sampled at this time were sacrificed.

An experiment was also created to determine whether equalising maternal contributions prior to communal rearing would result in less variable family sizes, and ultimately greater N_e, compared to stocking at their naturally produced proportions. For this component, spawning and larval rearing was conducted as described previously; however in this instance using 5 female and 11 male broodstock. Maternal family sizes at day 2 were enumerated microscopically by counting the average number of larvae in a homogenous (through aeration) 20 mL sample from each 400 L maternal family tank (3x counts per tank). From these calculations, an initial maternal family size could be estimated. After two days of separate rearing in 400 L tanks, the five maternal families were counted and divided into six 5000L tanks according to two stocking treatments (i.e. 3 replicates per treatment) – equalised maternal family sizes (E) and natural family size (N), based on maternal family size at 2 days post fertilization. Overall tank survival was recorded

regularly for each replicate, and 200 juvenile pearl oysters were sacrificed from each tank at 60 days old and preserved in 70-80% ethanol for DNA analyses. All tanks 5000 L were stocked with an initial stocking density of 5 larvae ml^{-1} and were subjected to identical feeding regimens and husbandry conditions. These cohorts are referred to as the experimental cohorts/oysters.

5.2.2 DNA extraction and microsatellite genotyping

DNA was extracted from preserved tissues using a simple digest preparation developed during this study through modification of a lysate/PCR buffer protocol (Sambrook *et al.*, 1989). Approximately 1 mm^2 sized pieces of tissue were digested at 55°C for 3-4 hours in 100 µl of digestion buffer containing 670 mM Tris-HCl pH 8.0, 166 mM NH_4SO_4, 0.2 % Tween-20 ® (Sigma-Aldrich), 0.2 % IGEPAL® CA-630 (Sigma-Aldrich) and 1 µg μl^{-1} Proteinase K. Immediately after digestion, samples were subjected to a 5 min incubation at 95°C to deactivate Proteinase K and then frozen overnight at -20°C before use. These two steps were considered essential for optimal results. Prior to use for PCR, thawed DNA preparations were vortexed briefly, then centrifuged for 1 min at 1000 g to pellet undigested cellular debris.

Table 5.1 Microsatellite marker suite and PCR conditions used for parentage assignment of *P. maxima*

Locus name	Flouresent label	$MgCl_2$ conc. (mM)	T_A (C°)
JCUPm-26h5	FAM	3.0	50
JCUPm-1g8	HEX	3.0	55
Pmx-022	HEX	1.5	50
Pmx-16_23	TET	1.5	58-50†
Pmx-16_41	FAM	1.5	58-50†
Pmx-18_21	TET	1.5	58-50†
Pmx-16_05	TET	3.0	52-45†
Pmx_008	FAM	3.0	50

† Touch-down PCR cycle, T_A decreases by 2 C° every 5 cycles until lower limit

Six polymorphic microsatellite loci were amplified for each sample using the primer pairs *Pmx-022*, *Pmx-16_23*, *Pmx-16_41*, *Pmx-18_21* (Smith *et al.*, 2003), *JCUPm-1g8* (Evans *et al.*, 2006) and *JCUPm-26h5* (Fwd- 5'TAGTCCTTTGCATATGACCTTGG 3'; Rev - 5'ATCGTGTTACAACCAAAGCGTTC 3'). Where these six markers were unable to provide unambiguous pedigree assignment, two additional markers (*Pmx-16_05 and Pmx-008,* (Smith *et al.*, 2003)) were amplified and utilised for parentage determination. PCR was conducted in 15 µl volumes, containing 1 x PCR buffer without $MgCl_2$ (BIOLINE), 1 x Q solution (QIAGEN) (not required for *JCUPm-1g8* and *JCUPm-26h5*), 0.2 mM dNTPs, 1.5 - 3.0 mM $MgCl_2$ (marker

dependent, see Table 1), 0.027 U μl^{-1} of BIOTAQ DNA polymerase (BIOLINE) and 0.5 μl DNA preparation. Thermocycler programs for all PCR began with an initial denaturation step of 3 mins at 94°C followed by 35 cycles of 94°C for 45 sec, locus specific T_A for 30 sec (Table 5.1) and 72°C for 45 sec; then a final extension step at 72°C for 5 mins. To reduce non-specific amplification the PCR cycling conditions for *Pmx-18_21*, *Pmx-16_23* and *Pmx-16_41* used a touchdown program where annealing temperature was sequentially lowered from 58°C, 56°C, 54°C and 52°C (for 5 cycles each) followed by 50°C for 15 cycles (Smith *et al.*, 2003). *Pmx-16_05* was also amplified using a similar touchdown program, with 52°C and 45°C the upper and lower $T_{A.}$ To allow co-loading and simultaneous electrophoresis post-PCR, forward primers were fluorescently labelled with FAM, TET or HEX dyes.

To remove residual salts, primers and dinucleotides, PCR products were purified using an ammonium acetate:ethanol precipitation protocol (Sambrook *et al.*, 1989). Purified PCR products underwent capillary electrophoresis together with a Tamra-400 size standard on a MegaBACE auto-sequencer (Amersham Biosciences) and allele sizes were then calculated using MegaBACE Fragment Profiler v1.2 software (Amersham Biosciences).

5.2.3 Statistical analyses and pedigree assignment

Allele frequencies and allelic richness (R_s) (following Leberg, 2002) were calculated using FSTAT 2.9.3.2 (Goudet, 1995). Observed and expected heterozygosities (Nei, 1987) were calculated using Arlequin v3 (Excoffier *et al.*, 2005). Unambiguous assignment of offspring to a parent pair was performed using the exclusion based methods implemented in FAP v3.6 (Taggart, 2007), allowing no greater than two allelic mismatches per offspring/parent pair combination and a zero base pair allele size tolerance. N_e was calculated (although in these cases can also be interpreted as equivalent to the effective number of breeders, commonly referred to as N_b) using a demographic approach accounting for variance in male and female broodstock contribution and family size (Lande & Barrowclough, 1987), and is expressed as:

$$N_e = \frac{4\, N_{e(f)} \,.\, N_{e(m)}}{N_{e(f)} + N_{e(m)}}$$

Where,

$$N_{e(f)} = \frac{N_f K_f - 1}{[K_f + (V_f / K_f) - 1]}$$

and

$$N_{e(m)} = \frac{N_m K_m - 1}{[K_m + (V_m / K_m) - 1]}$$

K_m and K_f are the mean number of offspring per male and female broodstock, and V_m and V_f are the variance of the number of offspring per male and female. N_f and N_m are the number of female and male broodstock used respectively. The rate of inbreeding (ΔF) was also calculated according to:

$$\Delta F = \frac{1}{2 (N_e)}$$

For the two non-experimental cohorts, survival between day 72 and 18 months was calculated for each family using:

$$S = 100 \times \frac{P_{18} \times C_{18ij}}{P_{72} \times C_{72ij}}$$

Where P equals the total population size at time 72 days or 18 months, and C is the relative contribution of a given family arising from the i th female and j th male at 72 days and 18 months (as approximated from parentage analyses). Owing to the lack of replication within non-experimental cohorts and the susceptibility of survival to be substantially over/under estimated in families with very small relative contributions, family survival measurements were not subjected to any further statistical analyses. As an analogue to survival, the more robust relative contribution data was used, given that under a null hypothesis of there being no differences in survival over time we will also see no changes in relative contributions. Therefore we can appropriately reject the null hypothesis if significant changes in relative contributions are

observed. Consequently, pseudo-probability testing of the χ^2 statistic (α = 0.05) using CHIRXC (Zaykin & Pudovkin, 1993) was implemented to test the statistical significance of changes in overall male or female contributions, and the relative contribution of full-sib families over time.

Two-tailed t-tests (α = 0.05) were used to compare N_e between experimental treatment groups, i.e. to test whether equalising maternal family sizes prior to communal rearing significantly affects N_e in hatchery pearl oyster populations when compared to stocking with naturally produced family proportions.

5.3 Results

5.3.1 Parentage assignment

With six microsatellite markers, 96.0% of genotyped offspring from Cohort A and 93.1% of offspring from Cohort B could be assigned to a single parental pair (Table 5.2). Initial assignment success using the same six microsatellite markers was not as high within the experimentally manipulated groups, with assignment success of 84.1% and 87.7% for Equalised and Natural stocking treatments respectively. To increase the number of progeny assigned in these cohorts, post-hoc genotyping of remaining unassigned individuals using two additional markers (*Pmx-008* and *Pmx-1605*) was performed, however, this improved assignments to a single parental pair only marginally (88.6% and 89.7% for Equalised and Natural stocking treatments, respectively, Table 5.2). Unassigned individuals from these groups were unambiguously assigned to a male parent; however, FAP could not discriminate between two females (Dam 1 and Dam 5) as the maternal parent in all instances. To ensure the non-inclusion of unassigned individuals would not influence experimental outcomes, N_e was calculated for the scenarios where unassigned progeny were either all from Dam 1 or all from Dam 5 (under the assumption that the actual value would fall somewhere in between). Assignment of these individuals to either Dam 1 or Dam 5 had no bearing on the statistical significance of experimental results (α = 0.05)(data not shown), and therefore was deemed acceptable to ignore unassigned individuals for the analyses of the experiment. For both non-

experimental and experimental groups, only unambiguously assigned individuals were used in analyses.

Table 5.2 Parentage assignment success of *P. maxima* using six microsatellite markers. Where low assignment occurred, two additional markers were included, as indicated.

Cohort / Treatment	Number of individuals genotyped	Number unambiguously assigned	No match	% assigned unambiguously
Cohort A	371	356	3	96.0%
Cohort B	332	309	0	93.1%
Equalised Treatment	420	353	0	84.1%
8 loci	420	369	0	88.6% *
Natural Treatment	448	393	0	87.7%
8 loci	448	402	0	89.7% *

Table 5.3 Genetic diversity statistics and effective population sizes (N_e) of two commercial *P. maxima* cohorts and two replicated experimental treatments (Equalised and Natural maternal family sizes). R_s, allelic richness; H_o, H_e, observed and expected heterozygosity;, VarC$_{m/f}$, variance in male and female broodstock contributions; ΔF inbreeding co-efficients; *N* is number of broodstock. Values are mean ± standard error (where appropriate).

Group	*n*	Age	N_f	N_m	R_s	H_e	H_o	VarC$_f$	VarC$_m$	N_e	N_e/N	ΔF
Cohort A	175	72d	5	12	8.61 ± 0.97	0.77 ± 0.03	0.81 ± 0.06	87.3	125.6	8.7	0.51	0.058
	175	18mth			8.82 ± 1.06	0.79 ± 0.03	0.82 ± 0.03	225.8	101.4	7.8	0.46	0.064
Cohort B	158	72d	6	11	8.67 ± 1.24	0.79 ± 0.04	0.87 ± 0.05	329.5	200.4	6.1	0.36	0.083
	150	18mth			8.75 ± 1.21	0.78 ± 0.04	0.84 ± 0.04	187.2	131.6	7.9	0.47	0.063
Equalised		60d	5	11	7.76 ± 0.20	0.73 ± 0.02	0.70 ± 0.02	100.9 ± 39.3	176.9 ± 19.3	7.2 ± 0.3	0.45 ± 0.02	0.070 ± 0.003
r1	142											
r2	159											
r3	68											
Natural		60d	5	11	8.34 ± 0.13	0.72 ± 0.01	0.70 ± 0.01	421.1 ± 50.8	199.5 ± 17.6	5.6 ± 0.1	0.35 ± 0.01	0.089 ± 0.002
r1	114											
r2	164											
r3	124											

5.3.2 Genetic diversity statistics

High levels of genetic diversity were seen in all populations. For the non-experimental cohorts, allelic richness (R_s), observed heterozygosity (H_o) and expected heterozygosity (H_e) all showed similar values between groups, and did not vary greatly over time (Table 5.3). Between the two experimental stocking treatments, heterozygosity measures were practically unchanged,

however, R_s was observed to be lower (although not statistically significant) in pearl oysters from the Equalised maternal size treatment (Table 5.3).

Table 5.4 Contributions of dams and sires of two *P. maxima* cohorts at 72 days and 18 months old, determined by DNA parentage analyses. Values to the left the dash (-) indicate number of individuals assigned to parents at 72 days, to the right side are individuals assigned at 18 months.

	Dams - Cohort A							Dams - Cohort B							
	A	B	C	D	E	Total d72	Total 18mths	U	V	X	W	Y	Z	Total d72	Total 18mths
Sires															
1	14-9	9-15	1-3	0-1	2-0	26	28	33-18	19-8	2-0	4-1	1-1		59	28
2		1-0	0-1	11-2		12	3		48-47		3-2	5-7	0-1	56	57
3	39-25	1-0		24-10		64	35	4-6			1-1			5	7
4			34-52			34	52							0	0
5		20-18	1-0			21	18				0-1	0-1		0	2
6		10-27	1-2	1-1		12	30				5-2	0-1	1-1	6	4
7		0-2		0-1	0-1	0	4			7-2	6-18	2-4	0-3	15	27
9					1-0	1	0	3-7	1-0	0-1	1-2	0-1		5	11
8					1-1	1	1		8-3	0-1				8	4
10			0-2		3-0	3	2		2-0		0-1	0-1		2	2
11			0-1		1-0	1	1				0-3		1-0	1	3
12	0-1					0	1			0-1	1-4			1	5
Total d72	53	41	37	36	8	175		40	78	9	21	8	2	158	
Total 18mths	35	62	61	15	2		175	31	58	5	35	16	5		150

5.3.3 Broodstock contributions, survival and N_e

5.3.3.1 Non-experimental oysters

Cohort A comprised 21 full-sib families of the potential 60 (5 x 12) families that could have been formed at spawning, with relative family contributions at day 72 ranging from 22.0% to 0.6% of the total group; and at the same age in Cohort B, 23 families (of the possible 72 (6 x 12) families) were recognized ranging from 30.1% to 0.6% in relative contributions. Contributions of each sire and dam, and the full-sib family contributions at 72 days and 18 months are seen in Table 5.4. Between 72 days and 18 months, overall survival was 13.9% and 15.6% for Cohort A and B respectively (Table 5.5). At 18 months, however, a significant shift in overall male and female contributions (Table 5.4), and full-sib family contributions were observed in both Cohort A and Cohort B (pseudo-probability, $P < 0.001$), demonstrating that some pearl oyster families exhibit higher survivorship than others. This is supported by individual family survival calculations, which ranged from 49.5% to 2.5% (Table 5.5). As a result, some families showed

substantial increases in proportional contributions (e.g. Family C04 (i.e. dam C x sire 4), increased from 19.2% to 29.0%) while others similarly decreased (e.g. Family U01, from 20.7% to 12.0%) (Table 5.5).

Table 5.5 Estimated survival rates of the largest full-sib *P. maxima* families from two commercial cohorts between 72 days and 18 months of age. Family codes are generated using alphabetical identifiers for different females and numerical identifiers for different male broodstock (eg. Family A03 = female A x male 03). Number of offspring per family is calculated from Relative Contribution x Total Number of Offspring.

Cohort	Family	Relative contribution Day 72	Number of offspring Day 72	Relative contribution 18 months	Number of offspring 18 months	Estimated survival %
A	A03	0.22	5443	0.14	479	8.8
	C04	0.19	4745	0.29	997	21.0
	D03	0.14	3350	0.06	192	5.7
	B05	0.11	2791	0.10	345	12.4
	A01	0.08	1954	0.05	173	8.8
	D02	0.06	1535	0.01	38	2.5
	B06	0.06	1396	0.15	518	37.1
	B01	0.05	1256	0.08	288	22.9
A	Other* (13 families)	0.09	2233	0.12	403	18.0
A	**Total**	**1.00**	**24704**	**1.00**	**3433**	**13.9**
B	V02	0.30	6666	0.31	1078	16.2
	U01	0.21	4583	0.12	413	9.0
	V01	0.12	2638	0.05	183	7.0
	V08	0.05	1111	0.02	69	6.2
	X07	0.04	972	0.01	46	4.7
	W07	0.04	833	0.12	413	49.5
	Y02	0.03	694	0.05	161	23.1
	W06	0.03	694	0.01	46	6.6
B	Other* (15 families)	0.18	3888	0.30	1032	26.5
B	**Total**	**1.00**	**22080**	**1.00**	**3440**	**15.6**

* Individual family data was still used (i.e. not pooled) when statistically assessing shifts in family contributions. Families are pooled in this table for the purpose of abbreviation

N_e at 18 months was nearly identical in both groups. Between 72 days and 18 months, however, N_e in Cohort A decreased from 8.7 to 7.8; whilst Cohort B showed an increase in N_e (from 6.1 to 7.9) over the same time period (Table 5.3). In both these cohorts, the pattern of increasing or decreasing N_e corresponds with changes in the variance of male or female broodstock contributions (Table 5.3) and is most likely explained by differential survival rates of individual families over time.

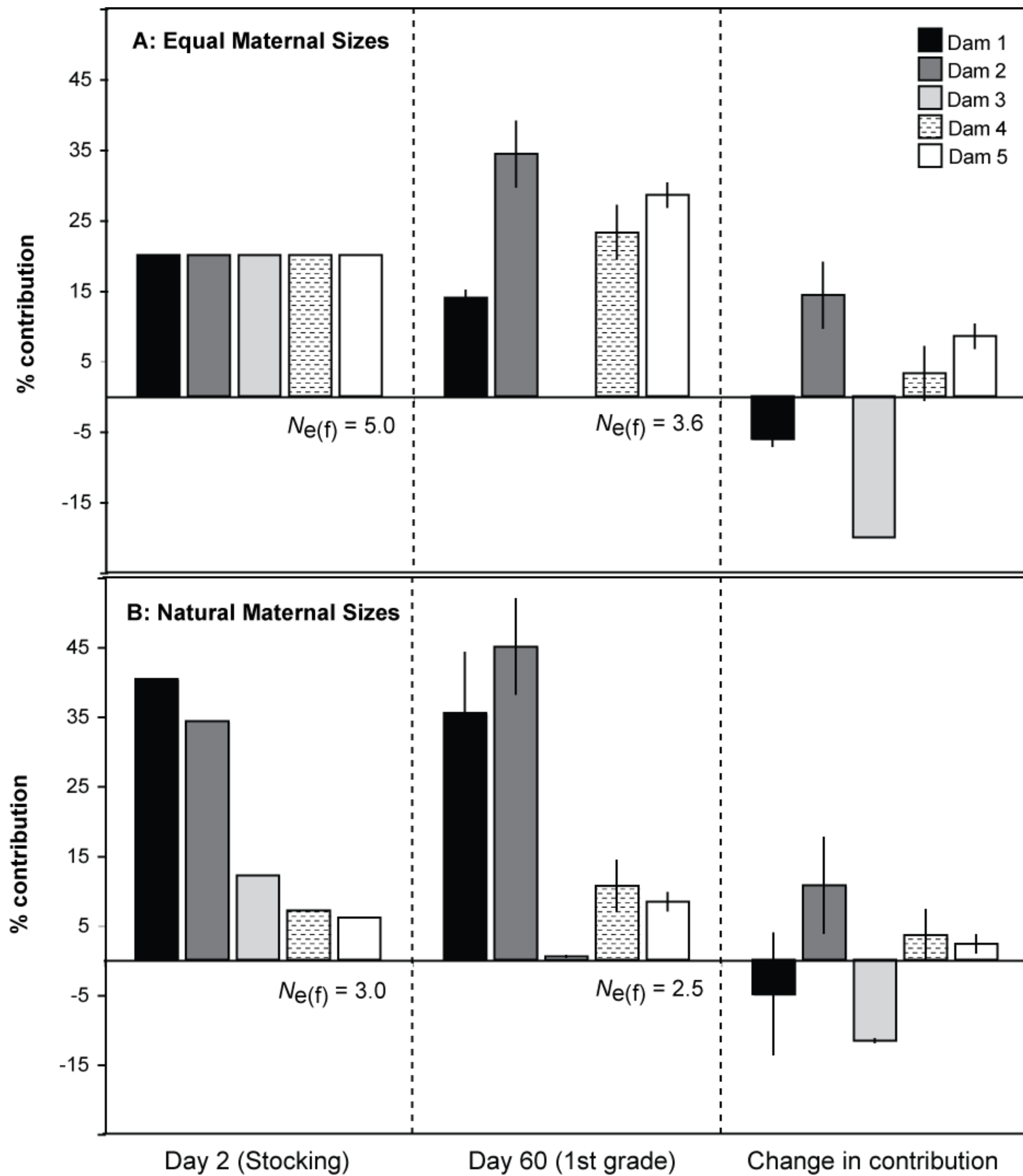


Figure 5.1 Relative maternal contributions of *P. maxima* at day 2 and day 60 from two experimental stocking treatments A) Equal maternal sizes and B) Natural maternal sizes (*n* = 3 per treatment), and the change in contribution over time (determined from % contribution d60 – % contribution d2). Female effective size ($N_{e(f)}$) is indicated at each sampling point. Error bars indicate standard error of the mean.

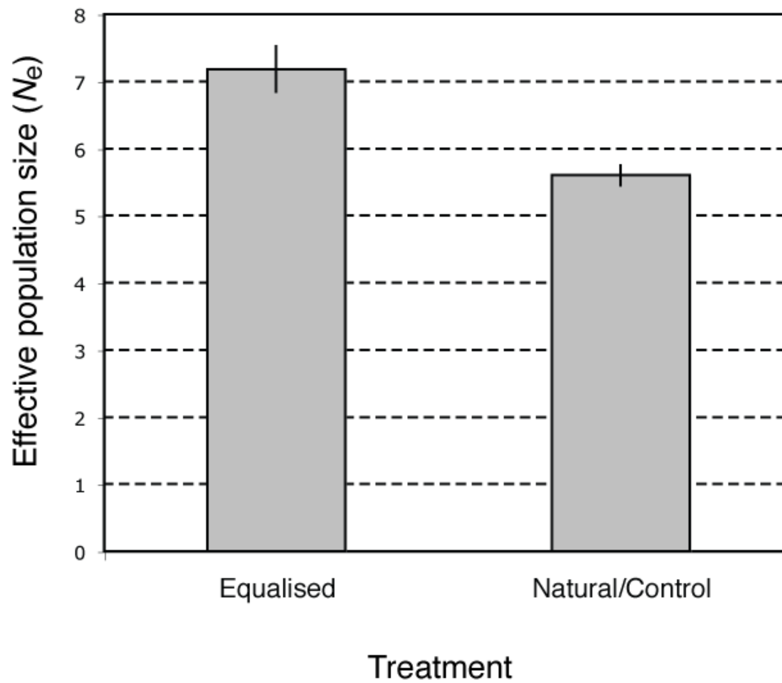


Figure 5.2 Differences in mean effective population size (N_e) of *P. maxima* age 60 days, from two experimental stocking treatments - Equalised maternal family sizes and Natural maternal family sizes (*n* = 3 per treatment, *P* = 0.013). Error bars indicate standard error of the mean.

5.3.3.2 Experimental oysters

At stocking, relative contributions in the Natural family size treatment were recorded at 40.2%, 34.0%, 12.7%, 7.0% and 6.1% for Dam 1, 2, 3, 4 & 5 respectively (Figure 5.1). Changes in relative maternal contributions between stocking and day 60 showed similar patterns in Equalised and Natural stocking treatments. For example, for both treatments, progeny from Dam 2 increased in relative proportion whilst offspring from Dam 3 were virtually unviable by day 60 (Figure 5.1). Along with the performance of the remaining maternal families, this highlights the subtle yet pertinent point that relative survival rates are reproducible within families but can still show a considerable variance between them. Equalising maternal family sizes prior to communal rearing, however, gave a substantially lower variance in maternal contributions compared to rearing with natural maternal sizes (Table 5.3). Consequently, a significantly greater mean N_e after 60 days (P = 0.013) from the equalised treatment was observed when compared to oysters reared at their naturally produced proportions and is evident in Figure 5.2. Pearl oysters reared after initially equalising maternal families had mean N_e of 7.2 ± 0.3 after 60 days, whereas mean N_e of oysters reared without family size

manipulations was 5.6 ± 0.1 (Table 5.3). Accounting only for female contributions, $N_{e(f)}$ of the Equalised treatment decreased from 5.0 at stocking to 3.5 ± 0.2, and was significantly greater at day 60 (P = 0.008) than $N_{e(f)}$ of the Natural family size treatment, which decreased from 3.0 at stocking to 2.4 ± 0.1.

5.4 Discussion

The major finding of this study is that significant differences in the survivorship of *P. maxima* families exist when exposed to the same environmental rearing conditions (i.e. communally reared, P < 0.001). Consequently, culturists are faced with the actuality that relative family contributions at stocking are an unsatisfactory indicator of contributions at a future date, such as at the age of spawning. From a genetic management perspective, this presents a challenging scenario since estimation of N_e and inbreeding rates based on contributions at an early stage may become unreliable and inaccurate as time progresses, unless regular genetic monitoring is implemented. What is important to note in this study, is that the actual change in family contributions (and therefore N_e) is not necessarily the emphasis, but more so importance of realizing that unless stocks are monitored this change will occur unrecognized.

As a consequence of potentially different family survival rates, variance in family size (unlike other factors that will influence N_e, such as uneven broodstock sex ratios) is prone to change over time within a given generation. This is particularly relevant in aquaculture where N_e is relatively small and fluctuations may be proportionately large. Within two commercially produced cohorts, we observed shifts in individual family representation of 8-10% in several instances over a period of approximately 15 months (Figure 5.1), attributed primarily to variable survival rates amongst families (Table 5.5). The subsequent effect on family size variance, particularly female variance in this case, between 72 days and 18 months of age shows interesting although not altogether unexpected results. While variable survival in Cohort A caused female reproductive variance to increase and, therefore, N_e to decrease over time, the opposite is seen in Cohort B (Table 5.3), highlighting an unpredictable influence of differential survival on family size variance. Additionally, the observation that male variance is less affected by survival over time compared to females is likely to be due to the use of fewer females than

males in this instance, and reiterates the importance of utilising sufficient broodstock numbers to protect N_e fluctuations.

Previous efforts to reduce family size variance in aquaculture populations have been less than encouraging. Manipulations such as equalising sperm contributions prior to fertilization can still result in variable male contributions due to various competitive factors, such as sperm motility and velocity differences amongst different males (Gaffney *et al.*, 1993 ; Boudry *et al.*, 2002 ; Wedekind *et al*., 2007 ; Withler & Beacham, 1994). However, all variation around male contributions cannot be attributed to these factors alone (Kaspar *et al.*, 2007 ; Linhart *et al*., 2005) and fertilization rates may be dependent on the compatibility of certain male/female genetic polymorphisms (e.g. Palumbi, 1999). On the other hand, attempts to reduce family size variance through manipulations post-fertilization have been hampered by evidence that offspring viability can have no significant relationship with initial fertilization success (Evans *et al*., 2007b). We show that equalising family sizes can significantly improve N_e if compared to stocking at natural proportions (Figure 5.1), however this outcome must be interpreted carefully. When comparing survival of maternal families, it is evident that variation in survival rates is present, but importantly, we find that the negative influence of the worst performing maternal family is indeed exaggerated by equalising family sizes (Figure 5.1). Consequently, a greater proportional decrease in $N_{e(f)}$ (and most likely overall N_e as well) is observed in the equalised treatment compared with no family size manipulations (Figure 5.1). In this study, we can only infer survival rate as a classifier for "poor" or "strong" performing families, however, this subsequently begs the question of whether poor survival is correlated with other performance traits at later life-stages? Although it has been shown in Pacific oysters (*C. gigas*) that family survival does not correlate with family growth performance (Degremont *et al*., 2007), in pearl culture one must also place considerable importance on the overall health of the cultured stock, as each individual is subjected to a minor surgical procedure in order to implant a pearl nucleus ("seeding"), allowing a pearl to form. For example, if poor family survival is indicative of a generally weak or susceptible family, a correlation between poor survival and other important traits such as poor pearl quality or mortality post-seeding may also be possible. Therefore, despite a significant improvement of N_e, the combination of possibly exaggerating the

representation of poor performing families (with a potential correlation with other traits) plus the lack of adequate predictors for these inferior performers means the practice of equalising families may not be a consistent method for improving N_e at this point. It is, however, important to consider the breeding objectives of a hatchery operation when considering genetic diversity management strategies such as suggested here. In cases where maintaining genetic diversity within a population is the priority (e.g. wild stock enhancement programs), it may be acceptable to sacrifice some degree of performance 'superiority' in order to capture maximum genetic diversity.

Factors such as broodstock conditioning, gonadal development and broodstock diet have been shown to affect offspring viability in bivalve aquaculture (Lannan, 1980 ; Muranaka & Lannan, 1984 ; Martínez *et al*., 2000 ; but see Cannuel & Beninger, 2005), however, it is important to note that variations in survival due to such non-genetic factors cannot be additively improved through selective breeding programmes. Alternatively, understanding genetic factors that may influence survival rates of different families could provide additional insights towards maximising N_e to greater effect. A significant additive genetic component to the variability of survival in bivalves has been reported, resulting in differences in family survival and significant heritability estimates (e.g. Evans & Langdon, 2006 ; Degremont *et al.*, 2007). This indicates that once the pedigree history of individual breeders is determined and survival heritability estimates are known, shifts in family contributions of communally reared families could potentially be predicted, permitting a better awareness of potential N_e fluctuations. Genetic interactions between parental genotypes could also play an important role in maximising offspring viability and reducing family size variability (Nordeide, 2007). For example, throughout the genome of another marine bivalve, the Pacific oyster (*Crassostrea gigas*), a large number of lethal, sublethal or mildly deleterious recessive mutations are believed to be present, and when occurring in a homozygous state, are predicted to significantly affect growth and survival in natural and cultured populations (Launey & Hedgecock, 2001). In this study, it is plausible that such genetic influences are in effect giving the observation of high variability in family survival, particularly when comparing within half-sib groups (i.e. families sharing one common parent), suggesting that the viability of offspring from a given broodstock may differ depending on the

genotype of its mate. Such interaction has been observed in sea urchins (*Heliocidaris erythrogramma*), where dam x sire interactions had a significant effect on embryo viability despite the absence of separate male or female effects (Evans *et al.*, 2007b), whilst in Atlantic cod (*Gadus morhua*), the choice of an 'optimal mate' can increase survival by as much as 74% above average (Rudolfsen *et al*., 2005). Although this study is restricted in its ability to isolate specific sources of variation related to differential family survival rates, this line of investigation could provide a potentially valuable tool in the genetic management of cultured pearl oysters and should be explored further.

5.5 Conclusion

This study demonstrates that for *P. maxima* culture, equalising maternal family sizes prior to communal rearing can give a greater N_e after 60 days by reducing family size variance. However this result must be interpreted with caution. It is still uncertain what factors underlie the differential survival rates of individual families. Without knowledge of underlying factors affecting the viability of particular families, and the correlations this may have with other commercially significant traits, it is possible to unintentionally magnify negative influences of "poor" performing families through family size equalisation. This study provides strong evidence for variable survival rates amongst different pearl oyster families, and shows that N_e is likely to fluctuate within a given generation. Due to the uncertainty of identifying superior or poor surviving families, the practice of equalising family sizes in order to maximise N_e may only become consistently beneficial once further progress is made towards understanding and then reducing variation in family survival rates and its potential correlation with other commercially significant traits.

Chapter 6 Influence of family-based growth on diversity maintenance

6.1 Introduction

Managing the level of inbreeding and avoiding the loss of genetic variation are significant factors in the long-term viability of closed populations. Low genetic diversity can restrict a population's ability to withstand fluctuating external pressures (eg. climate, disease, food availability), whilst continued inbreeding can lead to a state of reduced overall fitness, known as inbreeding depression (Allendorf & Luikart, 2007).

Aquaculture can substantially reduce genetic variability within domesticated populations (Alarcon *et al.*, 2004 ; Benzie & Williams, 1996 ; Eknath & Doyle, 1990 ; Evans *et al.*, 2004a ; Lundrigan *et al.*, 2005 ; Sbordoni *et al.*, 1986 ; Sbordoni *et al.*, 1987 ; Withler, 1990 ; Xu *et al.*, 2001); however, the necessity for actively maintaining high genetic diversity within these populations has traditionally been given limited attention. This can be attributed to the vast majority of cultured species having had little more than 3-4 generations of domestication beyond wild-caught progenitors, where the effects of inbreeding or low diversity have often not yet become manifest. Despite this, the need for conscientious genetic management in aquaculture is accelerating (Campton, 2004 ; McAndrew, 2001). Improved husbandry techniques, advances in reproductive biology, and the growing availability of molecular genetic tools have provided the potential for many aquaculture operations to implement and benefit substantially from advanced selective breeding programs (Davis & Hetzel, 2000). In these instances, the importance of maintaining high genetic variability within selected populations is central to the long-term success of such breeding programs (Davis & Hetzel, 2000). A sustained response to selection is dependent on the presence of sufficient genetic diversity within a population, and high levels of diversity will also allow continued selection if breeding objectives shift over time. Since genetic improvement by selective breeding is based on additive gains over multiple generations, significant attention must therefore be given towards avoiding intensive rearing practices that can substantially erode diversity.

Genetic diversity losses due to aquaculture can be grouped into two categories – a) between-generation and b) within-generation reductions. Between-generation reductions could be considered to result from practices that fail to pass adequate diversity onto the next generation, such as utilizing too few broodstock, or the use of mass-spawnings techniques where broodstock contributions are unknown and often highly skewed towards only a few individuals (e.g. Sekino *et al.*, 2003). Within-generation reductions are therefore those that occur within a given generation, and may arise from indiscriminate grading/culling of 'inferior' animals (Taris *et al.*, 2006), or when variable survival rates lead to the undetectable drop-out of individual families that are communally reared (e.g. Frost *et al.*, 2006). Between-generational influences tend to be particularly severe on diversity bottlenecks and increase future inbreeding risks due to the relative ease of producing large cohorts of closely related individuals from very few broodstock, whereas within-generation reductions tend to be proportionately lower. Nevertheless, the cumulative nature of diversity loss in aquaculture governs that all unintentional reductions of genetic variation - be it large or small - should be carefully restricted in order to achieve maximum responses to long-term selective breeding.

Variation in growth traits amongst different families within an aquaculture population can potentially contribute to a loss of genetic variation from both between and within-generation reductions. If communally reared families exhibit disparate growth rates, husbandry practices such as culling of smaller individuals may remove smaller families disproportionately. Additionally, if broodstock selection from a closed population is based solely on superior size without knowledge of individual pedigree, diversity is potentially compromised and the risk of inbreeding is increased by the selection of only fast-growing individuals, which could be from only a few (or even the same) fast-growing families. It has been shown in several commercial aquaculture species that practices such as grading/culling (Frost *et al.*, 2006 ; Taris *et al.*, 2006) and mass-selection (Appleyard & Ward, 2006 ; Bentsen & Oleson, 2002 ; De Donato *et al.*, 2005 ; Romana-Eguia *et al.*, 2005) can contribute to diversity loss and increase inbreeding. It is important, therefore, that aquaculturists comprehensively understand the implications of

selection methods and rearing practices on the maintenance of genetic variability within cultured populations in order to uphold sustainable selective breeding programs.

Valued for its utility in the production of high value "South Sea" pearls, the silver-lipped pearl oyster, *Pinctada maxima*, is widely cultured throughout Southeast Asia and northern Australia and has been recently identified as an attractive candidate for genetic improvement through selective breeding. Although husbandry techniques are well established in *P. maxima*, it has been shown that intensive rearing can substantially reduce the effective population size and genetic diversity of cultured *P. maxima* populations, and it is recommended that before selection can commence, the effect of culture practices on maintaining diversity should be further investigated (Lind *et al.*, 2009). Using microsatellite DNA parentage determination, this study investigates the significance of variable growth amongst communally reared *P. maxima* families, and investigates how aquaculture practices associated with growth variation may affect the long-term maintenance of genetic diversity within cultured populations. In particular, we address whether size grading will significantly partition genetic variation within a cohort, and whether culling smaller individuals influences the effective number of breeders (N_b) within a commercial cohort.

6.2 Materials and Methods

6.2.1 Spawning and rearing procedures

A single hatchery-propagated cohort was produced using 23 female and 35 male broodstock pearl oysters, from a pool of >150 candidate broodstock collected from wild populations in Indonesia. To allow parentage analyses, a small tissue sample was taken from the foot tissue of all potential broodstock, and preserved in 70-80% ethanol. Since single-family crosses are difficult to produce and separately rear for *P. maxima* (J.J.U. Taylor, *pers. comm.*), spawnings were conducted in specially designed tables that allow separate collection of female gametes whilst permitting fertilization from potentially all male broodstock. Fertilized eggs from each female were reared separately in 400L tanks for two days post fertilization, after which they were pooled into 5000L tanks for further rearing following a standard commercial rearing protocol until settlement. Between 2-5 maternal families were communally stocked into a total of

five 5000L tanks, each at a stocking density of 5 larvae mL^{-1}. After 30 days, spat collectors containing settled oysters were removed from the 5000L tanks and transferred onto longlines suspended in the open ocean, which allowed pearl oysters to filter feed on naturally occurring plankton. To facilitate an optimal feeding efficiency, pearl oysters were periodically separated and transferred into individually pocketed panels containing 64, 16 or 8 individuals per panel, depending on their size. As per standard industry practice, all panels were subjected to regular cleaning to remove biofouling build-up using a high-pressure water gun.

6.2.2 Oyster measurements and tissue sampling

To determine whether pearl oyster families exhibited different growth characteristics a representative sample of 650 oysters were sampled from the cohort at 18 months of age and dorso-ventral shell height (SH) measured (i.e. maximum external shell size measured perpendicular to the shell hinge). To enable *a posteriori* parentage assignment, foot tissue samples were also taken from each oyster at this time and preserved in 70-80% ethanol for later analyses.

6.2.3 DNA extraction, microsatellite genotyping and pedigree assignment

DNA extractions were performed following the method adopted in Chapter 5. Approximately 1 mm^2 sized pieces of tissue were digested at 55°C for 3-4 hours in 100 µl of digestion buffer containing 670 mM Tris-HCl pH 8.0, 166 mM NH_4SO_4, 0.2 % Tween-20 ® (Sigma-Aldrich), 0.2 % IGEPAL® CA-630 (Sigma-Aldrich) and 1 µg $µl^{-1}$ Proteinase K. Immediately after digestion, samples were subjected to a 5 min incubation at 95°C to deactivate Proteinase K and then frozen overnight at -20°C before use. These two steps were considered essential for optimal results. Prior to use for PCR, thawed DNA preparations were vortexed briefly, then centrifuged for 1 min at 1000 g to pellet undigested cellular debris.

Six polymorphic microsatellite loci were amplified for each sample using the primer pairs *Pmx-022*, *Pmx-16_23*, *Pmx-16_41*, *Pmx-18_21* (Smith *et al.*, 2003), *JCUPm-1g8* (Evans *et al.*, 2006) and *JCUPm-26h5* (Fwd- 5'TAGTCCTTTGCATATGACCTTGG 3'; Rev - 5'ATCGTGTTACAACCAAAGCGTTC 3'). PCR was conducted in 15 µl volumes, containing 1 x

PCR buffer without $MgCl_2$ (BIOLINE), 1 x Q solution (QIAGEN) (not required for *JCUPm-1g8* and *JCUPm-26h5*), 0.2 mM dNTPs, 1.5 - 3.0 mM $MgCl_2$ (marker dependent, Table 6.1), 0.027 U μl^{-1} of BIOTAQ DNA polymerase (BIOLINE) and 0.5 µl DNA preparation. Forward and reverse primer concentrations varied for each marker (Table 6.1). Thermocycler programs for all PCR began with an initial denaturation step of 3 mins at 94°C followed by 35 cycles of 94°C for 45 sec, locus specific T_A for 30 sec (Table 6.1) and 72°C for 45 sec; then a final extension step at 72°C for 5 mins. To reduce non-specific amplification the PCR cycling conditions for *Pmx-18_21*, *Pmx-16_23* and *Pmx-16_41* used a touchdown program where annealing temperature was sequentially lowered from 58°C, 56°C, 54°C and 52°C (for 5 cycles each) followed by 50°C for 15 cycles (Smith *et al.*, 2003). To allow co-loading and simultaneous electrophoresis post-PCR, forward primers were labelled with FAM, TET or HEX fluorescent dyes.

Table 6.1 Microsatellite marker suite and PCR conditions used for parentage assignment of *P. maxima*

Locus name	Flouresent label	$MgCl_2$ conc. (mM)	T_A (C°)
JCUPm-26h5	FAM	3.0	50
JCUPm-1g8	HEX	3.0	55
Pmx-022	HEX	1.5	50
Pmx-16_23	TET	1.5	58-50†
Pmx-16_41	FAM	1.5	58-50†
Pmx-18_21	TET	1.5	58-50†

† Touch-down PCR cycle, T_A decreases by 2 C° every 5 cycles until lower limit

To remove residual salts, primers and dinucleotides, PCR products were purified using an ammonium acetate:ethanol precipitation protocol (Sambrook *et al.*, 1989). Purified PCR products underwent capillary electrophoresis together with a Tamra-400 size standard on a MegaBACE auto-sequencer (Amersham Biosciences) and allele sizes were then calculated using MegaBACE Fragment Profiler v1.2 software (Amersham Biosciences).

Unambiguous assignment of offspring to a parent pair was performed using the exclusion based methods implemented in FAP v3.6 (Taggart, 2007), allowing no greater than two allelic mismatches per offspring/parent pair combination and a zero base pair allele size tolerance.

6.2.4 Statistical analyses

6.2.4.1 Phenotypic analysis

To determine whether the effect of different male or female broodstock will influence shell height of individual pearl oysters at 18 months, Kruskal-Wallis analysis of variance on ranks (alpha = 0.05) were performed using STATISTICA 8.0 (StatSoft Inc.).

6.2.4.2 Size grading, genetic diversity analysis and effective number of breeders (N_b)

To emulate an actual size grading, 18 month old pearl oysters were categorised into 10 mm size classes based on dorso-ventral shell height (SH). Based on this categorisation, the potential impact of various culling intensities (removal of individuals under 70, 80, 90, 100, 110 and 120 mm) on the allelic richness (R_s), number of families and the effective number of breeders (N_b) of the population were investigated. Allelic richness was calculated using FSTAT v2.9.3.2 (Goudet, 1995), and the effective number of breeders (N_b) (Hedrick *et al.*, 1995) was estimated as follows:

$$N_b = \frac{4\,(N_{em} \,.\, N_{ef})}{N_{em} + N_{ef}}$$

Where N_{em} and N_{ef} are the effective number of male and female breeders respectively, defined by:

$$N_{em} = \frac{N_m \,.\, k_m - 1}{k_m - 1 + V_{km} / k_m}$$

and

$$N_{ef} = \frac{N_f \,.\, k_f - 1}{k_f - 1 + V_{kf} / k_f}$$

N_f / N_m are the number of female/male broodstock used, k_m/k_f is the mean number of progeny per male/female broodstock and V_{km}/V_{kf} is the variance in the number of progeny per male/female broodstock (Lande & Barrowclough, 1987). The inbreeding co-efficient, or rate of inbreeding (ΔF) was calculated for each size class by $\Delta F = 1/(2N_b)$

It must be noted, that although it is highly unlikely that culturists would cull populations to a minimum SH of 100, 110 or 120 mm, these classes effectively represent what may be observed if only larger individuals were selected for use as potential broodstock (i.e. a mass-selection scenario).

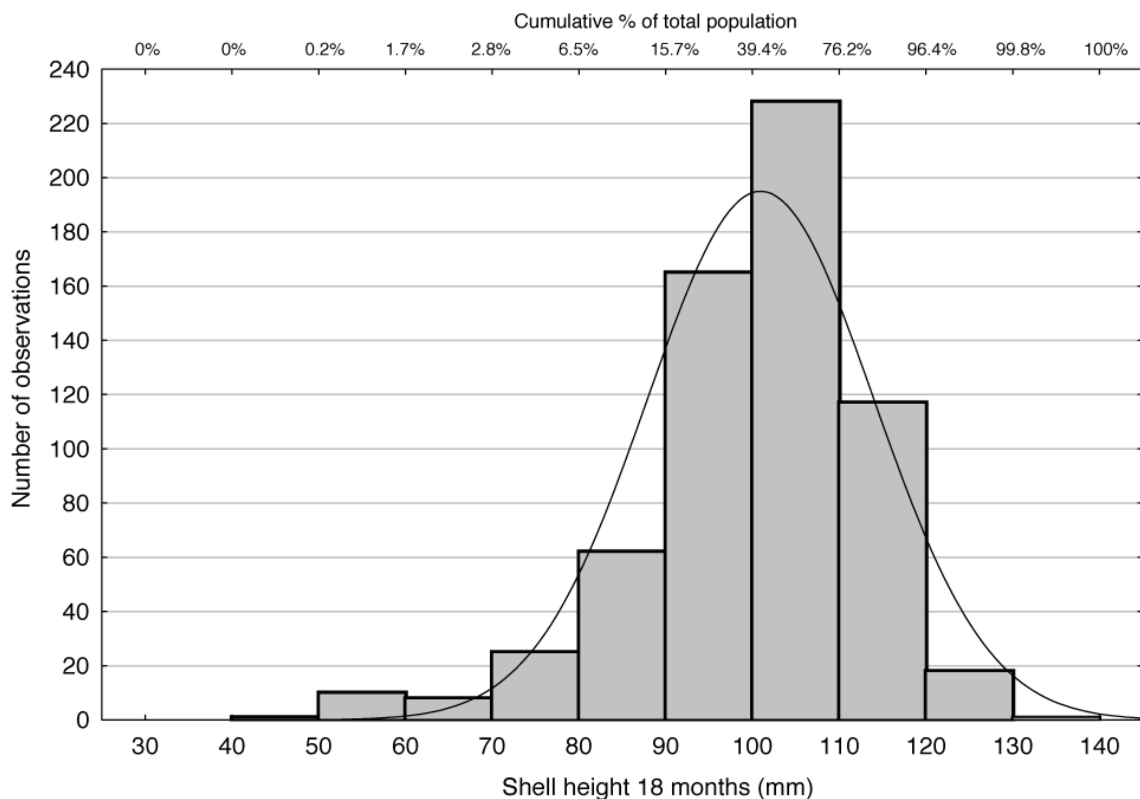


Figure 6.1 Frequency distribution of 18-month-old *P. maxima* amongst various dorso-ventral shell height size ranges (mm), approximated by a normal distribution (black line) (mean = 100.5 mm, std dev = 13.0).

6.3 Results.

6.3.1 Parentage assignment

Based on exclusion methods, 635 pearl oysters could be unambiguously assigned to a single parent pair using FAP (97.7% assignment success). Unassigned individuals were not included in subsequent analyses, and given their small number, removal from the dataset was assumed

to be of minimal consequence. Parentage assignment detected 17 females and 26 male broodstock contributing offspring to the population, from 33 full-sib families. Not all sires fertilized eggs from each dam, with many sires only fertilizing eggs from a single dam; however, several paternal and maternal half-sib relationships were formed through the fertilization of eggs from multiple dams by some males (Table 6.2).

Table 6.2 Relative contribution of progeny by different *P. maxima* dams and sires at 18 months of age

Number of progeny	**Dam**																	
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**Total**
Sire																		
1	24	7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	31
2	.	8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	8
3	.	.	15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	15
4	.	.	.	.	10	.	.	.	.	.	.	.	.	.	.	.	.	10
5	.	.	.	.	.	.	.	.	14	.	.	.	.	.	.	.	.	14
6	.	.	.	.	10	.	.	.	.	.	.	.	.	.	.	.	.	10
7	.	.	.	.	24	.	.	.	.	.	.	.	.	.	.	.	.	24
8	.	.	.	44	.	.	.	.	.	.	.	.	.	.	8	.	.	52
9	.	.	.	.	.	33	.	.	.	.	.	.	.	.	.	.	.	33
10	.	.	.	.	8	.	.	.	.	.	.	.	.	.	.	.	.	8
11	.	.	.	.	.	.	.	42	.	.	.	.	.	.	.	.	.	42
12	.	.	.	.	19	.	.	.	.	.	.	.	.	.	.	.	.	19
13	.	.	.	.	21	.	.	.	.	.	.	.	.	.	.	.	.	21
14	.	.	.	.	.	45	.	.	.	.	.	.	.	.	.	.	.	45
15	.	.	.	.	.	.	.	.	.	.	.	18	.	.	.	.	.	18
16	.	.	.	.	.	.	.	30	.	.	.	.	.	.	.	.	.	30
17	.	.	.	7	.	.	9	.	.	17	.	14	.	.	.	.	.	47
18	.	.	.	.	.	.	26	.	.	6	.	.	.	.	.	13	.	45
19	.	.	.	.	.	.	.	.	.	.	55	.	.	.	.	.	.	55
20	.	.	.	.	.	.	.	.	.	.	.	27	.	.	.	.	.	27
21	.	.	.	.	.	.	.	.	.	.	.	.	18	.	.	.	.	18
22	.	.	.	.	.	.	.	.	33	.	.	.	.	.	.	.	.	33
23	.	.	.	.	.	.	.	.	.	.	.	.	.	15	.	.	.	15
24	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	3
25	.	.	.	.	.	.	.	8	.	.	.	.	.	.	.	.	.	8
26	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	4	4
Total	24	18	15	51	92	78	35	80	47	23	55	59	18	15	8	13	4	635

6.3.2 Shell height size distribution at 18 months

At 18 months, oyster SH ranged between 45 - 131 mm (mean = 100.5 mm; std dev. = 13.0). The greatest number of individuals fell within the <100<110 mm size class, with the frequency distribution across remaining classes approximating normal (Figure 6.1). The majority of full-sib families had a mean SH between 90 -110 mm, however, mean SH of several families were smaller than this size range (eg. Family 1, mean SH ± S.E = 72.8 ± 2.1 mm), indicating the potential for some pearl oyster families to be more affected by practices such as culling than other families within the population (

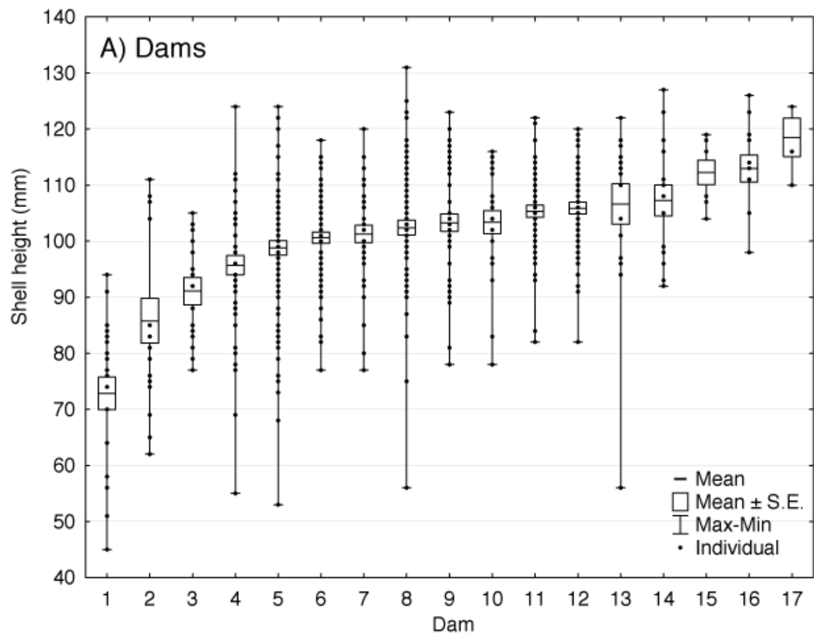
Figure 6.2). Analysis of mean SH of offspring from different sires or dams showed a significant influence of an individual's parent on this particular growth trait. Indeed, Kruskal-Wallis tests showed highly significant differences in mean SH of offspring from different dams ($H = 152.5$, df = 16, $P < 0.001$), and also amongst offspring from different sires ($H = 167.7$, df = 25, $P < 0.001$). The size distributions of offspring from each dam and sire is illustrated in Figure 6.2.

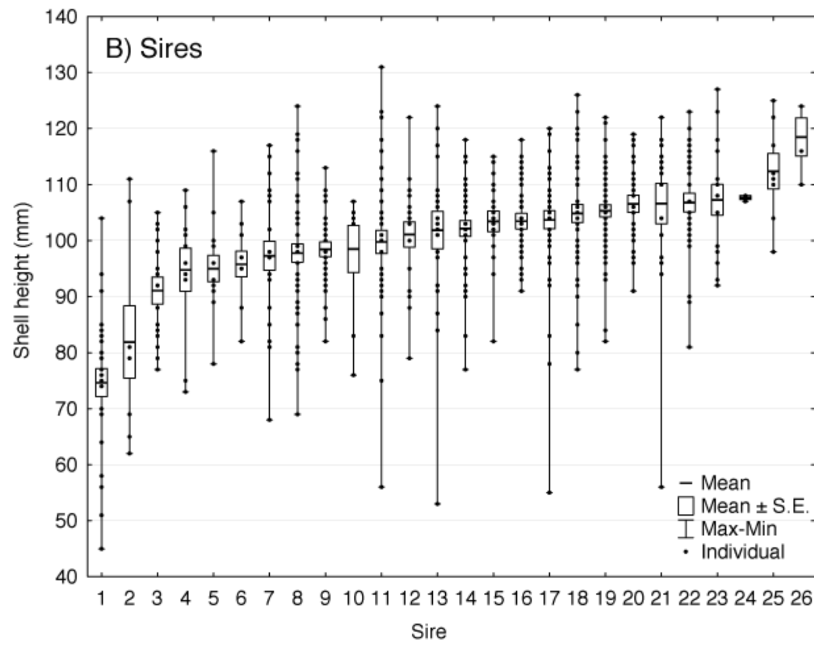
6.3.3 Effect of 'mock' culling / size selection

The removal of pearl oysters with SH smaller than 70, 80 or 90 mm had little effect on the number of contributing dams and sires or the number of alleles and allelic richness of the remaining population (Table 6.3). However, the effective number of breeders (N_b) and the inversely related rate of inbreeding (ΔF) were both influenced by the removal of smaller individuals from the population. When oysters smaller than 90 mm were removed compared to the total population, N_b decreased by 8.1% from 28.1 to 25.8 whilst ΔF increased from 1.78% to 1.94% (Table 6.3). When comparing oysters from the larger size grades - which may be representative of what may occur if future broodstock are selected only on superior size attributes without knowledge of pedigree - a sharp decline in the number of alleles and number of contributing dams and sires was observed (Table 6.3).

Table 6.3 Genetic variability and number of dams, sires and full-sib families contributing to various size ranges of a *P. maxima* population. N_b = effective number of breeders

Population size range	% of total population	No. sires	No. dams	Number of families	Number of alleles	Allelic Richness	N_b	Rate of inbreeding
ALL	100	26	17	33	78	8.7	28.1	1.78%
> 70 mm	97.2	26	17	33	78	8.7	27.5	1.81%
> 80 mm	93.5	26	17	33	78	8.6	26.9	1.86%
> 90 mm	84.3	26	17	33	78	8.5	25.8	1.94%
> 100 mm	60.6	26	16	32	74	8.5	25.8	1.94%
> 110 mm	23.8	20	15	26	69	8.5	25.5	1.96%
> 120 mm	3.6	12	11	12	58	8.7	30.2	1.65%


A) Dams
Shell height (mm)
140
130
120
110
100
90
80
70
60
50
40
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Dam
Mean
Mean ± S.E.
Max-Min
Individual


B) Sires
Shell height (mm)
140
130
120
110
100
90
80
70
60
50
40
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
Sire
Mean
Mean ± S.E.
Max-Min
Individual

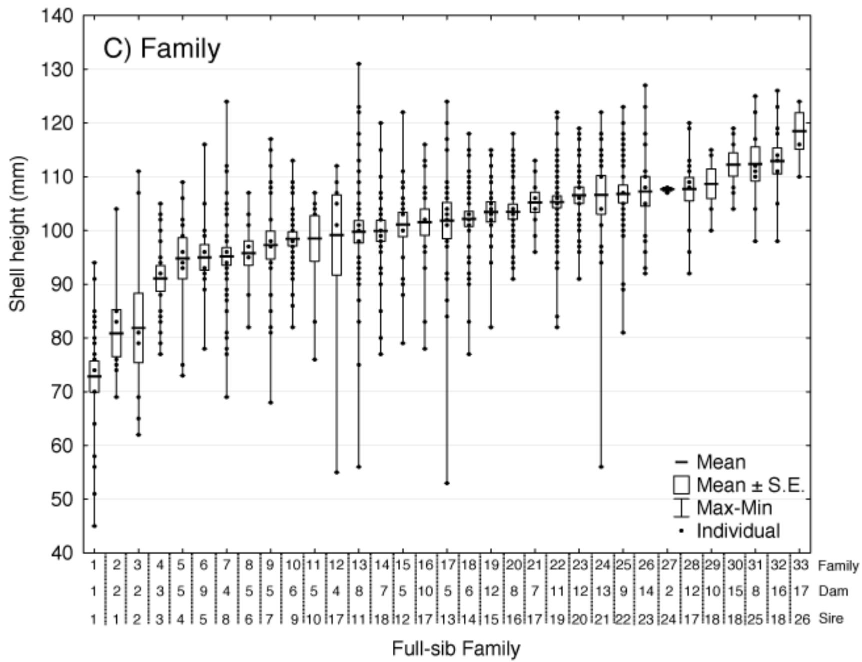

C) Family
Shell height (mm)
140
130
120
110
100
90
80
70
60
50
40
Mean
Mean ± S.E.
Max-Min
Individual
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 Family
1 2 2 3 5 9 4 5 5 6 5 4 8 7 5 10 5 6 12 8 7 11 12 13 9 14 2 12 10 15 8 16 17 Dam
1 1 2 3 4 5 8 6 7 9 10 17 11 18 12 17 13 14 15 16 17 19 20 21 22 23 24 17 18 18 25 18 26 Sire
Full-sib Family

Figure 6.2 (previous page) Dorso-ventral shell height (mm) distribution of individual *P. maxima* from different A) Dams; B) Sires and C) Full-sib families. Dams, sires and families are named based on mean shell height rank from smallest (1) to largest.

To further illustrate whether some pearl oyster families were removed/selected disproportionately to others when based on SH alone, a histogram was generated showing the percentage of each full-sib family (ranked from smallest to largest) affected by differing culling intensities (Figure 6.3). This clearly shows that culling individuals smaller than only 80 mm (representing the smallest 6.5 % of the population) can affect some families more than others (eg. Families 1, 2 & 3) (Figure 6.3A). Similarly, differences in mean SH amongst families means that if selecting only larger individuals (e.g. <110 mm) over 20% of all families are not represented at all (Figure 6.3D).

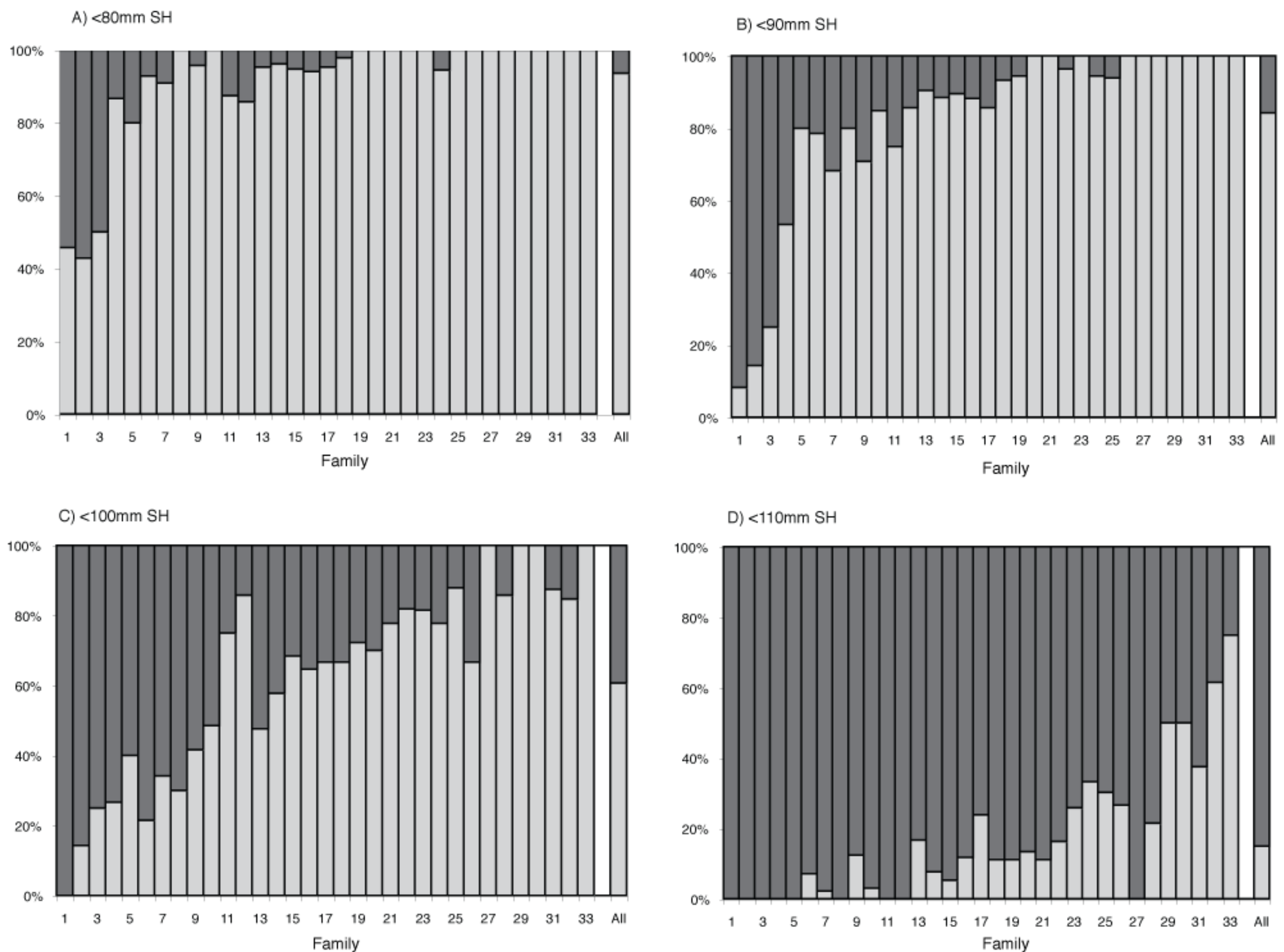


Figure 6.3 Percentage of individuals from different full-sib families affected by various size range cut-offs. Dark segments indicate percentage of a family with shell height A) < 80 mm; B) < 90 mm; C) < 100 mm and D) < 110 mm. Families are ranked from smallest (1) to largest (33) based on mean shell height.

6.4 Discussion

The desire for fast growth is central to almost all aquaculture operations; however, if optimal maintenance of genetic diversity is to be achieved, understanding the dynamics of growth variation amongst families within cultured populations is necessary. This study shows that in *P. maxima*, offspring from different male or female broodstock can have, on average, significantly different dorso-ventral shell heights after 18 months of communal rearing (P < 0.001). As a consequence, if management practices such as indiscriminate culling of smaller individuals are employed during *P. maxima* culture, the disproportionate removal of smaller families may see a loss or reduction of potentially valuable genetic variation. Conversely, the selection of only larger sized pearl oysters (for use as future broodstock) will favour the selection of families with larger mean size, increasing the likelihood of encountering genetic bottlenecks in future generations.

Although differences in mean family shell height are observed, the removal of smaller individuals at increasing culling intensities (up to 100 mm) did not affect genetic diversity indicators such as the raw number of alleles or the allelic richness (mean number of alleles per locus corrected for biases due to sample size (Leberg, 2002)) in the remaining population (Table 6.3). A broad range in individual shell height measurements within many families may explain this result, which sees most families distributed across several size grades and a number of families containing individuals that are considerably smaller than their other full-siblings(e.g. Families 5, 12, 13, 17 and 24,

Figure 6.2. Perhaps a better indicator of how culling affects the genetic composition of the population is seen through fluctuations in N_b, given its sensitivity to changes in the relative contributions of male and female broodstock (more specifically, the variance of progeny per dam or sire (Lande & Barrowclough, 1987)). The reduction in N_b as culling intensity increases can therefore be attributed to a shift in relative family contributions as a consequence of the disproportionate removal of individuals from smaller families (Figure 6.3).

Despite many examples of genetic studies investigating family-based variation in growth traits of communally reared aquaculture species (e.g. Herbinger *et al*., 1999 ; Coman *et al*., 2002 ;

Coman *et al*., 2004 ; Jerry *et al*., 2006), the influence of growth related culling practices on genetic diversity maintenance within aquaculture populations has been given limited attention. Whilst culling of 'inferior' individuals is seen as a method to improve aquaculture productivity by selecting stronger individuals for culture (LeBlanc *et al*., 2005), it has been shown that this practice can have negative impacts on retaining genetic variation within closed aquaculture populations (Taris *et al.*, 2006). Within *P. maxima*, however, only a minor reduction in molecular variation was observed after 'virtually' culling almost 40% of a population that contained progeny from 26 male and 17 female broodstock. Instead, results presented here are more similar to that observed in hatchery produced barramundi (*Lates calcarifer*) (Frost *et al.*, 2006), where the risk of genetic diversity loss from culling is increased by a non-uniform representation of families across different size grades - caused when on average some families grow faster or slower than others.

As eluded to earlier, potential genetic diversity losses from growth-related culture practices are not only restricted to the removal of smaller individuals. For culturists intending to improve growth, the most common approach to selective breeding is mass-selection, whereby candidate broodstock are selected primarily on the basis of superior phenotypic variation. Mass selection methods can potentially elicit a strong response to selection; however, a lack of pedigree information can also lead to increased levels of diversity loss due to inbreeding (Bentsen & Oleson, 2002). From this study, selection of *P. maxima* individuals > 120 mm shell height (~ 4% of total population) for the potential use as future broodstock, would see at least a 25% reduction in the number of alleles available to following generations and represents progeny from only 12 of the total 33 families; hence posing a considerable risk to future genetic diversity loss and increased levels of inbreeding. Implementing a lower shell height cut-off would be a better long-term strategy for maintaining diversity, and is evident by a much greater proportion of alleles and number of families detected in the >100 mm and >110 mm size classes. In hatchery populations of the closely related Japanese pearl oyster (*P. fucata martensii*), selection based on phenotypic superiority in growth and shell traits resulted in a significant reduction in protein isozyme variability after six generations, although based on these data, little evidence for inbreeding was observed (Wada, 1986). In a later study, however, it was shown

that the effects of inbreeding in selected pearl oyster lines can have a significant negative effect on growth and mortality (Wada & Komaru, 1994) and is likely to affect cultured *P. maxima* populations in a similar fashion. In most cases inbreeding is an unintentional consequence of breeding programs utilising broodstock of unknown pedigree, and has been demonstrated to influence several other aquaculture species negatively, including, amongst others, edible oysters (Bierne *et al.*, 1998 ; Evans *et al.*, 2004b), Indian carps (Eknath & Doyle, 1990) and penaeid shrimps (Bierne *et al*., 2000 ; Keys *et al*., 2004).

This study suggests that the influence of male or female broodstock on the growth of *P. maxima* (inferred from shell height variation) can play a significant role in the maintenance of genetic variability in closed aquacultured populations, particularly if communally reared families are subjected to indiscriminate (with respect to pedigree) culling and mass selection approaches. Although the risks of diversity losses due to culling are comparatively minor compared to the genetic implications of selecting potential breeders based solely on superior growth characteristics, the preservation of genetic diversity - be it large or small - should be of high priority if a sustained response to selective breeding efforts and minimal inbreeding is to be achieved (McAndrew, 2001). It is therefore recommended that to ensure the maintenance of genetic variation and long-term sustainability of future *P. maxima* selection programs, culturists should employ the use of molecular tools for parentage assignment of candidate broodstock to avoid mating related individuals, or implement structured breeding designs (e.g. Gjerde *et al*., 1996 ; Bentsen & Oleson, 2002 ; Dupont-Nivet *et al*., 2006 ; Li *et al*., 2008) intended to conserve genetic variability whilst maximising genetic response to selection.

Chapter 7 General Discussion

7.1 Characterisation of natural *Pinctada maxima* populations

Given the underlying foundation of any selective breeding program is based on the genetic content harboured in its founding broodstock, it is important to understand the genetic properties of wild populations from which broodstock are initially sourced. Concurrent selective breeding programs of the same species targeting the same commercially significant traits may have vastly different outcomes if the founding broodstock are selected from genetically distinct stocks. If aquaculture operations incorporate several geographically distant sites such as has been practiced by the Industry Partner of this research, which operates three sites within Indonesia separated by up to 2000 km, potential genetic differentiation amongst local stocks and adaptation to local environments may hamper or confound selective breeding progress if broodstock from different sites are treated as genetically alike. The natural range of *P. maxima* spans the tropical and sub-tropical oceans of the Indo-West Pacific, a region of biogeographical and ecological complexity where genetic structuring has been observed in many other marine species (see Benzie, 1998 ; Bellwood & Wainwright, 2002). If *P. maxima* populations are affected in a similar fashion, it is therefore pertinent to identify natural population structure of potential broodstock sources. To address this key issue, Chapters 2 and 3 of this thesis encompass the largest genetic survey of wild *P. maxima* populations to date, spanning the majority of its natural distribution and providing baseline genetic information for natural populations of potential commercial significance.

Indeed, this research shows that despite its potential to broadly disperse, wild *P. maxima* populations show genetic structuring across its natural distribution. It is also observed, however, that high genetic connectivity (and therefore genetic similarity) is realised across broad-scale distances (< 1000 km) in some regions, such as the mainland coastal areas of the South China Sea and along ocean-current pathways in central Indonesia (Chapter 3). These outcomes confirm and expand significantly on earlier genetic investigations of *P. maxima* populations, which indicated lower levels of genetic differentiation along the northwest Australian coast and

small regions of Indonesia (Johnson & Joll, 1993 ; Benzie *et al.*, 2003 ; Benzie & Smith-Keune, 2006).

From an aquaculture perspective, knowledge of genetic differentiation amongst *P. maxima* populations is valuable. If broodstock from genetically distinct, inbred populations are crossed in some cases we may observe an increase in commercially significant traits (e.g. growth, survival), particularly in bivalve molluscs, due to heterosis effects, known as hybrid vigour (Launey & Hedgecock, 2001). Such populations could potentially be targeted for intentional crossbreeding as a strategy to 'kick-start' a breeding program. By contrast, crossbreeding amongst broodstock from genetically divergent populations may also result in outbreeding depression, which can cause a reduced overall fitness in offspring of subsequent generations due to the dissolution of co-evolved genes that are of functional or adaptive significance (Lynch, 1991). Of particular interest for the Industry Partner is genetic differentiation identified amongst local populations surrounding their three Indonesian culture sites. Although no deeply divergent genetic lineages were detected amongst these populations, and indeed across the broader distribution of *P. maxima*, significant shifts in gene frequencies indicate genetic isolation between some populations has occurred for a period long enough to develop genetic structure and therefore possible phenotypic differences to different environments. This is significant when commencing a selective breeding program using stock from different regions, and the potential impacts this may have on selection efforts in *P. maxima* is a worthy target for future research.

Also of significance when considering the natural genetic structure of aquaculture species is the potential impacts that translocation of cultured animals may have on the wild populations surrounding culture sites. If an aquaculture operation comprises several farm sites located across a broad geographical range, the movement of farmed stock from one site to another may have detrimental impacts on wild populations in the event that farmed animals escape from culture facilities and interbreed with wild populations. It is important to preserve the genetic structure of wild populations, as genetically differentiated populations may contain unique genetic variants having adaptive significance to local environments. However, many years of stock translocations for aquaculture can homogenize genetic structure that may have been

present before animal transfers had occurred (Arnaud-Haond *et al.* 2004). Chapters 2 and 3 show that strong genetic structure is present across the natural distribution of *P. maxima,* indicating that continued transfers of stock from genetically distinct populations must proceed with an associated monitoring of wild populations to ensure that genetic homogenization does not occur as a result of culture practices. The outcomes from this thesis have significantly improved understanding on the patterns and influences on genetic structuring in *P. maxima* providing a solid basis for future investigations, which are discussed further in section 7.3.

In addition to characterising structure patterns of *P. maxima* populations, it is also important to identify distributional patterns in genetic variation. A sustained response to selective breeding is dependent on the presence of sufficient genetic diversity within the population, and in most selection programs this exploitable genetic variation is initially gained from wild-sourced broodstock. It is therefore logical to identify populations that may harbour higher levels of variation and allow a greater opportunity to maximise diversity within the founding broodstock. This thesis provides a valuable broad-scale picture on the patterns of genetic diversity across the natural range of *P. maxima* and shows that wild *P. maxima* populations generally show a high level of genetic diversity, having a distinct trend of reduced diversity at its range limits with central Indonesian populations showing significantly greater genetic variation (Chapter 2 and 3).

That greater levels of genetic diversity were observed in *P. maxima* populations located towards central Indonesia bodes well for pearl culture operations in this region, such as the Industry Partner. However, it does not preclude populations situated outside this region from being potentially valuable as a source of broodstock for genetic improvement programs. In several instances, populations (particularly those near the range periphery) were genetically differentiated and showed multiple unique genetic variants within individuals that were not detected elsewhere. When coupled with the considerable differences in environmental conditions across the distribution of *P. maxima*, genetic data indicate that such populations could potentially harbour individuals with commercially significant genetic characteristics (e.g. thermal tolerance, disease resistance genes) that could be exploited through selection.

By encompassing such a broad sampling area, the population genetics investigations of this thesis have enabled a significant step forward in understanding the extent of genetic structuring and baseline levels of genetic diversity in wild *P. maxima.*

7.2 Maintenance of genetic diversity in cultured *P. maxima* populations

A critical concept in selective breeding is that the pathway to genetic improvement is based on additive genetic gains, achieved through strategic matings over many successive generations of a closed population (Gjedrem, 2005). And given that a sustained response to selection is reliant on genetic variation within a population, it important that diversity is maintained within successive cultured populations and unintentional loss of valuable genetic resources due to culture practices are minimised. In addition, retaining genetic diversity within a population will greatly reduce the risk of encountering deleterious influences due to inbreeding (Bentsen & Oleson, 2002 ; Lynch, 1991). Outcomes of Chapters 4, 5 and 6 of this thesis highlight that if specific attention is not given towards retaining genetic diversity within cultured *P. maxima* populations, a significant loss of genetic resources and an increased likelihood of inbreeding will occur.

Amongst the growing documentation and evidence that aquaculture practices are generally very poor at maintaining diversity (e.g. Eknath & Doyle, 1990 ; Durand *et al.*, 1993 ; Benzie & Williams, 1996 ; Norris *et al.*, 1999 ; Brown *et al.*, 2005 ; Frost *et al.*, 2006), it is not surprising that cultured *P. maxima* populations investigated in this thesis exhibit substantially lower genetic variability that their wild progenitor populations (Chapter 4). More pertinent, however, is the realisation that in order to efficiently maintain genetic diversity over successive generations, the focus must turn to identifying and understanding how the relative impacts of specific culture practices have been in contributing to diversity losses.

Throughout an aquaculture production cycle there are several factors that may independently contribute to loss of genetic diversity. Importantly, these independent factors are cumulative in how each affects total diversity loss and, in combination, can have a rapid impact on closed populations. Results presented here show clearly that in *P. maxima* culture, husbandry

practices including spawning methods (Chapter 4), broodstock selection (i.e. broodstock numbers, sex ratios, trait-based selection without pedigree information - Chapter 4 and 6), family stocking manipulations (Chapter 5) and size grading (Chapter 6) may all influence the maintenance of genetic diversity. As a consequence, effective population sizes (N_e) of cultured populations were only 3.5 - 7.1 despite the use of 20 - 30 broodstock and in some instances a reduction in molecular diversity of > 40% in a single generation was observed when compared to wild progenitor populations. It is also revealed that a significant contributor to low N_e in cultured populations can be attributed to highly variable broodstock contributions, particularly in mass-spawned populations (although controlled spawns did not fare much better). Further to this, it was found that equalisation of family sizes prior to communal rearing would still result in variable family contributions due to differences in family survival rates. These findings are consistent with that observed in many other aquaculture species (Aho *et al.*, 2006 ; Alarcon *et al.*, 2004 ; Benzie & Williams, 1996 ; Boudry *et al.*, 2002 ; Brown *et al.*, 2005 ; Evans *et al.*, 2004a ; Frost *et al.*, 2006 ; Herlin *et al.*, 2008 ; Li *et al.*, 2004 ; Li *et al.*, 2007 ; Norris *et al.*, 1999 ; Porta *et al.*, 2007 ; Saavedra, 1997 ; Sekino *et al.*, 2003), indicating that the consequences of many of these practices are not restricted to *P. maxima* culture, but are problematic across aquaculture operations in general.

In order to utilise this growing body of information towards reducing the loss of diversity in *P. maxima* culture, and indeed the broader aquaculture field, a strategy to prioritise which processes and practices are most influential in diversity maintenance would be beneficial. In this case, I suggest grouping factors or practices contributing to diversity losses into two categories - 1) Between-generational losses and 2) Within-generational losses. Between-generation losses, caused by practices that fail to pass adequate diversity from one generation to the next, can occur when utilizing too few, or genetically related broodstock, or the use of spawnings techniques (such as mass-spawning) where broodstock contributions are unknown and often can be highly skewed towards only a few individuals (e.g. Sekino *et al.*, 2003 ; Herlin *et al.*, 2008 ; Fessehaye *et al.*, 2006). Within-generation losses may arise from indiscriminate grading/culling of smaller/‘inferior’ animals (Taris *et al.*, 2006), or when variable survival rates lead to the undetectable drop-out of individual families that are communally reared (e.g. Frost *et*

al., 2006). Through the investigation of hatchery-propagated populations in this thesis, it is observed that both between-generation losses due to mass-spawning (Chapter 4) and within-generation losses due to variable family-sizes, and variable family survival and growth rates (Chapters 5 and 6) can influence retention of genetic diversity maintenance in *P. maxima*. It is clear, however, that in *P. maxima* genetic diversity losses are more greatly affected by between-generational influences, whereas within-generation reductions tend to be proportionately lower. Whilst the cumulative nature of diversity loss in aquaculture governs that all unintentional reductions of genetic variation - be it large or small - should be carefully restricted in order for maximum response to long-term selective breeding and avoiding inbreeding, a prioritisation towards avoiding between-generational losses should provide a substantial improvement in genetic diversity maintenance in closed populations.

7.3 Future directions and concluding remarks

Genetic surveys in Chapters 2 and 3 of this thesis have provided significant advances in understanding the distribution of genetic diversity and factors influencing population structure in *P. maxima*, spanning a major portion of its geographic range. Throughout the natural range of *P. maxima* it is shown that several genetically distinct populations are present; however, as with many population genetic studies, the distinction of where one 'population' ends and another begins is not easily determined, especially where sampling covers broad geographical distances. Using the outcomes of this work as an initial framework, fisheries management or wild broodstock collection strategies could benefit further from studies targeting fine-scale population dynamics of *P. maxima* and is a worthy topic for future investigation. Additionally, it is possible that other genetically distinct populations are present in *P. maxima* that were not sampled by this research and could be uncovered with further population genetics studies.

In light of detecting significant genetic structure and the identification of genetically differentiated *P. maxima* populations, the next logical question in regard to aquaculture and genetic improvement programs is whether such genetic differences translate into phenotypic variation and can this variation be exploited by future selective breeding programs. Recent investigations have indicated that pearl oysters from different regions within Indonesia exhibit varying growth

rates when reared in similar environments (Kvingedal, 2009). Such findings indicate that genetic differences amongst pearl oyster populations may have meaningful implications when commercially utilised and, as such, future research on this topic is encouraged.

An overall message from this thesis is that culture practices need to be modified in order to improve genetic diversity maintenance in closed *P. maxima* populations. However, the solution to avoiding diversity loss within aquaculture populations, including *P. maxima*, is not as simple as it may seem. Indeed, several factors must still be addressed if diversity loss and inbreeding are to be minimised within cultured stocks. Whilst the use of small numbers of broodstock is an obvious contributor to reduced genetic variability, it is demonstrated in Chapter 4 that by merely introducing additional broodstock into a spawning pool, this may not be sufficient to increase diversity levels if the spawning procedures utilised are inefficient at capturing available genetic resources. In *P. maxima* culture, achieving single broodstock pair matings to create separate full-sib families is currently commercially unfeasible due to an unreliable success-rate in stimulating broodstock pairs to spawn in isolation (J.J.U. Taylor, *pers. comm.*). This issue is a clear candidate for future research efforts. Sacrificial strip-spawning of mature broodstock is also not favoured, as broodstock are deemed too valuable; whilst cryopreservation of pearl oyster gametes for repeated future use is still in experimental stages and not yet ready for commercial development (Acosta-Salmón *et al*., 2007 ; Choi & Chang, 2003). The current best solution is to create maternal half-sib families, a practice however, which still provide a significant impediment to accurately monitoring genetic contributions of individual broodstock, particularly males. Nevertheless, if maternal pedigree records can be maintained by avoiding communal rearing of maternal families, for example, it will provide a significant reduction in the risk of unintentionally mating related individuals (thereby reducing inbreeding) in future generations.

Further exploring factors that influence differential family survival rates could not only provide significant improvements in commercial outputs if survival is increased, but may also contribute to reducing the highly variable family sizes that occur within cultured populations and enable the maintenance of higher effective population sizes. If less variation in family survival rates can be

achieved, the practice of equalising family contributions investigated in Chapter 5 may become more reliable and useful towards improving N_e in cultured *P. maxima*.

In conclusion, the body of work entailed in this thesis has provided significant advances in understanding of factors affecting genetic diversity in both wild and cultured *P. maxima* populations. The outcomes of this have created a sound foundation from which selective breeding programs can be initiated and has highlighted important culture practices that must be improved (or avoided) in order to capture and retain genetic diversity within closed populations. In addition, this research has demonstrated for the first time the utility of DNA pedigree analyses for genetic diversity studies in *P. maxima*, and should provide a stimulus for ongoing research towards genetic improvement in this species.

Chapter 8 References

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Chapter 9 Appendix A

mtDNA cytochrome oxidase subunit 1 (COI) haplotype sequences (635bp) from Chapter 3:

1

TTTGGGAATTGGCTTATTCCCCTTCTGTTGGGTGGGCCGGATATGCATTTTCCTCGCTTGAATAATTTTAGG
TTTTGGGTTCTCCCTTGGGCTTTAGATTTGGCAATTATATCAGTTTTTACTGAGGGCGGATCTGGTACTGGT
TGGACGCTTTATCCACCCCTTTCTACCTATATCTATATGGGCAAGAGCGTAGATTTGACAATTTTTTCTCTC
CATTTGGCTGGGGTTGGTTCCATCTTTGGTTCTATTAACTTTATTGTCACAGTTCGTGCCATTAAGTTGACT
GACCTTCATTTGCTGTCGATGTTCCCTATTAGAATGTTGGTTACAGGCCACTTGCTGGTAGTTGCTTTGCCA
GTGTTAGCTGGTGGTTTGACTATGTTGCTCACTGATCGGCATTTTAACACGAGGTTTTTTTATCCGATTGGT
GGTGGTGATCCTGTTTTGTTTCAACATTTGTTTTGGTTTTTCGGTCACCCGGAGGTGTATGTGCTCATTTTG
CCTGGGTTTGGGCTAATTTCTCAGGTTGTTATGCAAAATGCTATGAAAAAACAGGTTTTTGGGAGTTTGGGG
ATGATGTACGCCATGATTGGGATTGGGTTTTTAGGGTTTATTGTGTGGGGCCATCACAT

2

TTTGGGAATTGGCTTATTCCCCTTCTGTTGGGTGGGCCGGATATGCATTTTCCTCGCTTGAATAATTTTAGG
TTTTGGGTCCTCCCTTGGGCTTTAGATTTGGCAATTATATCAGTTTTTACTGAGGGCGGATCTGGTACTGGT
TGGACGCTTTATCCACCCCTCTCTACCTATATTTATATGGGCAAGAGCGTAGATTTGACAATTTTTTCTCTC
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GACCTTCATTTGCTGTCGATGTTCCCTATTAGAATGTTGGTTACAGGCCACTTGCTGGTAGTTGCTTTGCCA
GTGTTAGCTGGTGGTTTGACTATGTTGCTTACTGATCGGCATTTTAACACGAGGTTTTTTTATCCGATTGGT
GGTGGTGATCCTGTTTTGTTTCAACATTTGTTTTGGTTTTTCGGTCACCCGGAGGTGTATGTGCTCATTTTG
CCTGGGTTTGGGCTAATTTCTCAGGTTGTTATGCAAAATGCTATGAAAAAACAGGTTTTTGGGAGTTTGGGG
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3

TTTGGGAATTGGCTTATTCCCCTTCTGTTGGGTGGGCCGGATATGCATTTTCCTCGCTTGAATAATTTTAGG
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GTGTTAGCTGGTGGTTTGACTATGTTGCTCACTGATCGGCATTTTAACACGAGGTTTTTTTATCCGATTGGT
GGTGGTGATCCTGTTTTGTTTCAACATTTGTTTTGGTTTTTCGGTCACCCGGAGGTGTATGTGCTCATTTTG
CCTGGGTTTGGGCTAATTTCTCAGGTTGTTATGCAAAATGCTATGAAAAAGCAGGTTTTTGGGAGTTTGGGG
ATGATGTACGCCATGATTGGGATTGGGTTTTTAGGGTTTATTGTGTGGGGCCATCACAT

4

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GGTGGTGATCCTGTTTTGTTTCAACATTTGTTTTGGTTTTTCGGTCACCCGGAGGTGTATGTGCTCATTTTG
CCTGGGTTTGGGCTAATTTCTCAGGTTGTTATGCAAAATGCTATGAAAAAACAGGTTTTTGGGAGTTTGGGG
ATGATGTACGCCATGATTGGGATCGGGTTTTTAGGGTTTATTGTGTGGGGCCATCACAT

5

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GTGTTAGCTGGTGGTTTGACTATGTTGCTTACTGATCGGCATTTTAACACGAGGTTTTTTTATCCGATTGGT
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CCTGGGTTTGGGCTAATTTCTCAGGTTGTTATGCAAAATGCTATGAAAAAACAGGTTTTTGGGAGTTTGGGG
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6

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GTGTTAGCTGGTGGTTTGACTATGTTGCTCACTGATCGGCATTTTAACACGAGGTTTTTTTATCCGATTGGT
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CCTGGGTTTGGGCTAATTTCTCAGGTTGTTATGCAAAATGCTATGAAAAAACAGGTTTTTGGGAGTTTGGGG
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7

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CCTGGGTTTGGGCTAATTTCTCAGGTTGTTATGCAAAATGCTATGAAAAAGCAGGTTTTTGGGAGTTTGGGG
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8

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9

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10

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11

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12

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13

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14

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15

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16

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17

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18

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19

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20

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21

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22

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23

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24

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25

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26

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27

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28

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29

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30

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31

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32

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33

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34

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35

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36

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37

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38

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39

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TTTTGGGTTCTCCCTTGGGCTTTAGATTTGGCAATTATATCAGTTTTTACTGAGGGCGGATCTGGTACTGGT
TGGACGCTTTACCCACCCCTTTCTACCTATATCTATATGGGCAAGAGCGTAGATTTGACAATTTTTTCTCTC
CATTTGGCTGGGGTTGGTTCCATCTTTGGTTCTATTAACTTTATTGTCACAGTTCGTGCCATTAAGTTGACT
GACCTTCATTTGCTGTCGATGTTCCCTATTAGAATGTTGGTTACAGGCCACTTGCTGGTAGTTGCTTTGCCA
GTGTTAGCTGGTGGTTTGACTATGTTGCTCACTGATCGGCATTTTAACACGAGGTTTTTTTATCCGATTGGT
GGTGGTGATCCTGTTTTGTTTCAACATTTGTTTTGGTTTTTCGGTCACCCGGAGGTGTATGTGCTCATTTTG
CCTGGGTTTGGGCTAATTTCTCAGGTTGTTATGCAAAATGCTATGAAAAAACAGGTTTTTGGGAGTTTGGGG
ATGATGTACGCCATGATTGGGATTGGGTTTTTAGGGT??????????????????????

40

TTTGGGAATTGGCTTATTCCCCTTCTGTTGGGTGGGCCGGATATGCATTTTCCTCGCTTGAATAATTTTAGG
TTTTGGGTCCTCCCTTGGGCTTTAGATTTGGCAATTATATCAGTTTTTACTGAGGGCGGATCTGGTACTGGT
TGGACGCTTTATCCACCCCTTTCTACCTATATTTATATGGGCAAGAGCGTAGATTTGACAATTTTTTCTCTC
CATTTGGCTGGGGTTGGTTCCATCTTTGGTTCTATTAACTTTATTGTCACAGTTCGTGCCATTAAGTTGACT
GACCTTCATTTGCTGTCGATGTTCCCTATTAGAATGTTGGTTACAGGCCACTTGCTGGTAGTTGCTTTGCCA
GTGTTAGCTGGTGGTTTGACTATGTTGCTCACTGATCGGCATTTTAACACGAGGTTTTTTTATCCGATTGGT
GGTGGTGATCCTGTTTTGTTTCAACATTTGTTTTGGTTTTTCGGTCACCCGGAGGTGTATGTGCTCATTTTG
CCTGGGTTTGGGCTAATTTCTCAGGTTGTTATGCAAAATGCTATGAAAAAACAGGTTTTTGGGAGTTTGGGG
ATGATGTACGCCATGATTGGGATTGGGTTTTTAGGGT??????????????????????

41

??GCATTTTCCTCGCTTGAATAATTTTAGG
TTTTGGGTTCTCCCTTGGGCTTTAGATTTGGCAATTATATCAGTTTTTACTGAGGGCGGATCTGGTACTGGT
TGGACGCTTTATCCACCCCTTTCTACCTATATTTATATGGGCAAGAGCGTAGATTTGACAATTTTTTCTCTC
CATTTGGCTGGGGTTGGTTCCATCTTTGGTTCTATTAACTTTATTGTCACAGTTCGTGCCATTAAGTTGACT
GACCTTCATTTGCTGTCGATGTTCCCTATTAGAATGTTGGTTACAGGCCACTTGCTGGTAGTTGCTTTGCCA
GTGTTAGCTGGTGGTTTGACTATGTTGCTCACTGATCGGCATTTTAACACGAGGTTTTTTTATCCGATTGGT
GGTGGTGATCCTGTTTTGTTTCAACATTTGTTTTGGTTTTTCGGTCACCCGGAGGTGTATGTGCTCATTTTG
CCTGGGTTTGGGCTAATTTCTCAGGTTGTTATGCAAAATGCTATGAAAAAACAGGTTTTTGGGAGTTTGGGG
ATGATGTACGCCATGATTGGGATTGGGTTTTTAGGGT??????????????????????

42

TTTGGGAATTGGCTTATTCCCCTTCTGTTGGGTGGGCCGGATATGCATTTTCCTCGCTTGAATAATTTTAGG
TTTTGGGTTCTCCCTTGGGCTTTAGATTTGGCAATTATATCAGTTTTTACTGAGGGTGGATCTGGTACTGGT
TGGACGCTTTATCCACCCCTTTCTACCTATATCTATATGGGCAAGAGCGTAGATTTGACAATTTTTTCTCTC
CATTTGGCTGGGGTTGGTTCCATCTTTGGTTCTATTAACTTTATTGTTACAGTTCGTGCCATTAAGTTGACT
GACCTTCATTTGCTGTCGATGTTCCCTATTAGAATGTTGGTTACAGGCCACTTGCTGGTAGTTGCTTTGCCA
GTGTTAGCTGGTGGTTTGACTATGTTGCTCACTGATCGGCATTTTAACACGAGGTTTTTTTATCCGATTGGT
GGTGGTGATCCTGTTTTGTTTCAACATTTGTTTTGGTTTTTCGGTCACCCGGAGGTGTATGTGCTCATTTTG
CCTGGGTTTGGGCTAATTTCTCAGGTTGTTATGCAAAATGCTATGAAAAAACAGGTTTTTGGGAGTTTGGGG
ATGATGTACGCCATGATT??

43

TTTGGGAATTGGCTTATTCCCCTTCTGTTGGGTGGGCCGGATATGCATTTTCCTCGCTTGAATAATTTTAGG
TTTTGGGTTCTCCCTTGGGCTTTAGATTTGGCAATTATATCAGTTTTTACTGAGGGCGGATCTGGTACTGGT
TGGACGCTTTATCCACCCCTTTCTACCTATATCTATATGGGCAAGAGCGTAGATTTGACAATTTTTTCTCTC
CATTTGGCTGGGGTTGGTTCCATCTTTGGTTCTATTAACTTTATTGTTACAGTTCGTGCCATCAAGTTGACT
GACCTTCATTTGCTGTCGATGTTCCCTATTAGAATGTTGGTTACAGGCCACTTGCTGGTAGTTGCTTTGCCA
GTGTTAGCTGGTGGTTTGACTATGTTGCTCACTGATCGGCATTTTAACACGAGGTTTTTTTATCCGATTGGT
GGTGGTGATCCTGTTTTGTTTCAACATTTGTTTTGGTTTTTCGGTCACCCGGAGGTGTATGTGCTCATTTTG
CCTGGGTTTGGGCTAATTTCTCAGGTTGTTATGCAAAATGCTATGAAAAAACAGGTTTTTGGGAGTTTGGGG
ATGATGTACGCCATGATT??

44

TTTGGGAATTGGCTTATTCCCCTTCTGTTGGGTGGGCCGGATATGCATTTTCCTCGCTTGAATAATTTTAGG
TTTTGGGTTCTCCCTTGGGCTTTAGATTTGGCAATTATATCAGTTTTTACTGAGGGCGGATCTGGTACTGGT
TGGACGCTTTATCCACCCCTTTCTACCTATATCTATATGGGCAAGAGCGTAGATTTGACAATTTTTTCTCTC
CATTTGGCTGGGGTTGGTTCCATCTTTGGTTCTATTAACTTTATTGTCACAGTTCGTGCCATCAAGTTGACT
GACCTTCATTTGCTGTCGATGTTCCCTATTAGAATGTTGGTTACAGGCCACTTGCTGGTAGTTGCTTTGCCA
GTGTTAGCTGGTGGTTTGACTATGTTGCTCACTGATCGGCATTTTAACACGAGGTTTTTTTATCCGATTGGT
GGTGGTGATCCTGTTTTGTTTCAACATTTGTTTTGGTTTTTCGGTCACCCGGAGGTGTATGTGCTCATTTTG
CCTGGGTTTGGGCTAATTTCTCAGGTTGTTATGCAAAATGCTATGAAAAAACAGGTTTTTGGGAGTTTGGGG
ATGATGTACGCCATGATT??

45

TTTGGGAATTGGCTTATTCCCCTTCTGTTGGGTGGGCCGGATATGCATTTTCCTCGCTTGAATAATTTTAGG
TTTTGGGTTCTCCCTTGGGCTTTAGATTTGGCAATTATATCAGTTTTTACTGAGGGCGGATCTGGTACTGGT
TGGACGCTTTATCCACCCCTTTCTACCTATATCTATATGGGCAAGAGCGTAGATTTGACAATTTTTTCTCTC
CATTTGGCTGGGGTTGGTTCCATCTTTGGTTCTATTAACTTTATTGTCACAGTTCGTGCCATCAAGTTGACT
GACCTTCATTTGCTGTCGATGTTCCCTATTAGAATGTTGGTTACAGGCCACTTGCTGGTAGTTGCTTTGCCA
GTGTTAGCTGGTGGTTTGACTATGTTGCTCACTGATCGGCATTTTAACACGAGGTTTTTTTATCCGATTGGT
GGTGGTGATCCTGTTTTGTTTCAACATTTGTTTTGGTTTTTCGGTCACCCGGAGGTGTATGTGCTCATTTTG
CCTGGGTTTGGGATAATTTCTCAGGTTGTTATGCAAAATGCTATGAAAAAACAGGTTTTTGGGAGTTTGGGG
ATGATGTACGCCATGATT??

46

TTTGGGAATTGGCTTATTCCCCTTCTGTTGGGTGGGCCGGATATGCATTTTCCTCGCTTGAATAATTTTAGG
TTTTGGGTTCTCCCTTGGGCTTTAGATTTGGCAATTATATCAGTTTTTACTGAGGGCGGATCTGGTACTGGT
TGGACGCTTTATCCACCCCTTTCTACCTATATCTATATGGGCAAGAGCGTAGATTTGACAATTTTTTCTCTC
CATTTGGCTGGGGTTGGTTCCATCTTTGGTTCTATTAACTTTATTGTCACAGTTCGTGCCATTAAGTTGACT
GACCTTCATTTGCTGTCGATGTTCCCTATTAGAATGTTGGTTACAGGCCACTTGCTGGTAGTTGCTTTGCCA
GTGTTAGCTGGTGGTTTGACTATGTTGCTTACTGATCGGCATTTTAACACGAGGTTTTTTTATCCGATTGGT
GGTGGTGATCCTGTTTTGTTTCAACATTTGTTTTGGTTCTTCGGTCACCCGGAGGTGTATGTGCTCATTTTG
CCTGGGTTTGGGCTAATTTCTCAGGTTGTTATGCAAAATGCTATGAAAAAACAGGTTTTTGGGAGTTTGGGG
ATGATGTACGCCATGATT??

47

TTTGGGAATTGGCTTATTCCCCTTCTGTTGGGTGGGCCGGATATGCATTTTCCTCGCTTGAATAATTTTAGG
TTTTGGGTTCTCCCTTGGGCTTTAGATTTGGCAATTATATCAGTTTTTACTGAGGGCGGATCTGGTACTGGT
TGGACGCTTTATCCACCCCTTTCTACCTATATCTATATGGGCAAGAGCGTAGATTTGACAATTTTTTCTCTC
CATTTGGCTGGGGTTGGTTCCATCTTTGGTTCTATTAACTTTATTGTCACAGTTCGTGCCATTAAGTTGACT
GACCTTCATTTGCTGTCGATGTTCCCTATTAGAATGTTGGTTACAGGCCACTTGCTGGTAGTTGCTTTGCCA
GTGTTAGCTGGTGGTTTGACTATGTTGCTCACTGATCGGCATTTTAACACGAGGTTTTTTTATCCGATTGGT
GGTGGTGATCCTGTTTTGTTTCAACATTTGTTTTGGTTTTTCGGTCACCCGGAGGTGTATGTGCTCATTTTG
CCTGGGTTTGGGCTAATTTCTCAGGTTGTTATGCAAAATGCTATGAAAAAACAGGTTTTTGGGAGTTTGGGG
ATGATGTATGCCATGATT??

Table 9.1 Microsatellite allele frequencies for *P. maxima* populations from Chapter 2 and Chapter 4

		CHAPTER 2				***CHAPTER 4***								
Locus	**Allele**	**Hainan Island**	**Vietnam**	**West Australia**	**East Australia**	**Torres Strait**	**Aru**	**Bali**	**West Papua**	**Mass Spawned**	**Controlled A**	**Controlled B**	**Controlled C**	**Selected**
JCUPm_20e10	*n*	46	30	60	21	59	67	55	61	0	0	0	0	0
	158	0.446	0.233	0.425	0.262	0.305	0.284	0.273	0.23	-	-	-	-	-
	162	0.043	0.05	0.042	0	0.017	0.022	0.009	0.025	-	-	-	-	-
	166	0.12	0.133	0.017	0.024	0.068	0.082	0.118	0.221	-	-	-	-	-
	170	0.391	0.55	0.475	0.524	0.39	0.507	0.573	0.5	-	-	-	-	-
	174	0	0	0	0.024	0.085	0.007	0.009	0.016	-	-	-	-	-
	178	0	0	0.025	0.071	0	0.03	0.018	0.008	-	-	-	-	-
	182	0	0	0.008	0.095	0.11	0.03	0	0	-	-	-	-	-
	186	0	0.033	0	0	0	0.015	0	0	-	-	-	-	-
	190	0	0	0.008	0	0.025	0.015	0	0	-	-	-	-	-
	194	0	0	0	0	0	0.007	0	0	-	-	-	-	-
JCUPm_ 27d7	*n*	44	30	60	23	58	65	53	61	0	0	0	0	0
	123	0.193	0.1	0.008	0.13	0.026	0.077	0.085	0.033	-	-	-	-	-
	143	0.716	0.867	0.85	0.565	0.664	0.738	0.783	0.762	-	-	-	-	-
	147	0.091	0.033	0.142	0.304	0.31	0.185	0.123	0.189	-	-	-	-	-
	151	0	0	0	0	0	0	0.009	0.016	-	-	-	-	-
JCUPm_27a1	*n*	43	30	60	23	60	62	54	59	0	0	0	0	0
	248	0	0	0	0	0.017	0	0	0	-	-	-	-	-
	252	0.023	0	0	0	0	0	0	0	-	-	-	-	-

JCUPm_27a1	256	0.012	0.067	0.35	0	0.058	0.032	0.028	0.025	-	-	-	-	-
(cont.)	260	0.64	0.85	0.492	0.739	0.458	0.726	0.741	0.669	-	-	-	-	-
	262	0.012	0.033	0.008	0.109	0.208	0.04	0.019	0.034	-	-	-	-	-
	264	0.093	0.033	0.142	0.152	0.092	0.153	0.148	0.169	-	-	-	-	-
	268	0.198	0.017	0	0	0.075	0.048	0.046	0.017	-	-	-	-	-
	272	0	0	0	0	0	0	0.009	0	-	-	-	-	-
	276	0.012	0	0	0	0	0	0.009	0.008	-	-	-	-	-
	280	0.012	0	0.008	0	0.083	0	0	0.068	-	-	-	-	-
	284	0	0	0	0	0.008	0	0	0.008	-	-	-	-	-
Pmx-1641	*n*	46	30	58	23	60	67	55	62	75	92	89	93	94
	218	0	0	0.009	0	0.067	0.097	0.009	0.024	0.02	0.152	0.045	0	0.074
	222	0.141	0.067	0.06	0.174	0.183	0.142	0.091	0.089	0.047	0.038	0.101	0.091	0.287
	226	0.207	0.167	0.276	0.022	0.075	0.119	0.091	0.113	0	0	0.011	0.022	0.032
	230	0.228	0.333	0.259	0.196	0.158	0.104	0.2	0.161	0.287	0.054	0.208	0.296	0.122
	234	0.228	0.217	0.052	0.109	0.067	0.157	0.255	0.234	0.307	0.092	0.253	0.312	0.059
	238	0.011	0.033	0.147	0.174	0.067	0.097	0.1	0.153	0.087	0.587	0.107	0.108	0.043
	242	0.043	0.033	0.078	0.13	0.1	0.149	0.091	0.081	0	0.043	0	0.075	0
	246	0.033	0.067	0.086	0.152	0.133	0.022	0.073	0.056	0.007	0.005	0.028	0.048	0.165
	250	0.087	0.033	0.009	0.022	0	0.045	0.055	0.048	0.18	0	0.051	0.048	0.043
	254	0.011	0.033	0.009	0	0.025	0.052	0.027	0.008	0	0.027	0.051	0	0.176
	258	0.011	0	0.009	0.022	0.125	0.015	0.009	0.008	0.067	0	0	0	0
	262	0	0	0.009	0	0	0	0	0.016	0	0	0	0	0
	266	0	0	0	0	0	0	0	0.008	0	0	0.146	0	0
	270	0	0.017	0	0	0	0	0	0	0	0	0	0	0
Pmx-1821	*n*	46	30	60	23	60	67	55	62	76	93	88	93	91
	87	0	0	0	0	0	0.022	0	0	0	0	0	0	0
	91	0	0	0	0	0	0.007	0.018	0.008	0	0	0.034	0	0
	95	0.087	0.067	0	0	0	0.007	0.027	0.016	0.151	0	0.006	0.134	0.049

Pmx-1821	97	0	0	0.008	0	0	0.007	0	0	0	0	0	0	0
(cont.)	99	0.043	0.067	0.058	0.065	0.042	0.075	0.127	0.081	0.197	0.043	0.142	0.129	0
	101	0.011	0.033	0	0.109	0.05	0.015	0.009	0.032	0.099	0.005	0.045	0	0
	103	0.207	0.217	0.275	0.261	0.233	0.269	0.255	0.25	0.336	0.629	0.159	0.263	0.571
	104	0	0	0	0	0.1	0	0	0	0	0	0	0	0
	105	0.043	0.167	0.075	0.043	0.025	0.037	0.073	0.065	0.099	0.097	0.057	0.016	0.077
	107	0.261	0.233	0.183	0.283	0.075	0.224	0.155	0.25	0.013	0.048	0.358	0.237	0.242
	109	0.109	0.067	0.042	0.022	0.008	0.022	0.036	0.089	0	0.108	0	0.048	0.011
	111	0.087	0.1	0.233	0.065	0.1	0.142	0.091	0.04	0	0.032	0.051	0.016	0.038
	115	0.043	0.017	0.067	0.043	0.133	0.06	0.082	0.073	0	0.011	0.028	0.097	0
	119	0.065	0.017	0.008	0.022	0.142	0.06	0.055	0.056	0	0.022	0.102	0.032	0
	123	0.022	0.017	0.017	0.043	0.05	0.022	0.027	0.008	0	0	0	0	0
	127	0.011	0	0.008	0.022	0.008	0.007	0.009	0.024	0	0.005	0.017	0.027	0.011
	131	0.011	0	0	0.022	0.025	0.007	0	0	0.105	0	0	0	0
	135	0	0	0.008	0	0	0.007	0	0	0	0	0	0	0
	139	0	0	0.008	0	0.008	0.007	0.009	0	0	0	0	0	0
	143	0	0	0.008	0	0	0	0	0	0	0	0	0	0
	151	0	0	0	0	0	0	0.009	0	0	0	0	0	0
	155	0	0	0	0	0	0	0.018	0	0	0	0	0	0
	159	0	0	0	0	0	0	0	0.008	0	0	0	0	0
JCUPm_1g8	*n*	40	30	60	24	60	67	55	62	75	93	83	92	78
	213	0.025	0	0.017	0.021	0	0.022	0.027	0.016	0.167	0	0.024	0	0
	215	0	0	0	0	0	0.007	0	0.016	0	0	0	0	0
	217	0	0.017	0.017	0.104	0.058	0.007	0.027	0.04	0	0	0.181	0	0
	219	0.375	0.2	0.092	0.188	0.117	0.097	0.155	0.234	0.547	0.398	0.024	0.12	0.103
	221	0.075	0.067	0.025	0	0.275	0.112	0.073	0.073	0.053	0.016	0.127	0.092	0.231
	223	0.075	0.133	0.183	0.208	0.067	0.112	0.227	0.081	0.1	0.075	0.024	0.337	0.295
	225	0.1	0.083	0.183	0	0	0.037	0.045	0.105	0	0.113	0.247	0.022	0.058
	227	0.213	0.267	0.242	0.313	0.358	0.254	0.218	0.153	0.08	0.075	0.193	0.136	0.064
	229	0.075	0.067	0.083	0.125	0.075	0.134	0.109	0.129	0	0	0.163	0.092	0

JCUPm_1g8	231	0.038	0.05	0.042	0	0.008	0.022	0	0.048	0	0	0.006	0.027	0
(cont.)	233	0	0	0	0	0.008	0.022	0.027	0.024	0	0.032	0	0.049	0.019
	235	0	0.017	0.033	0.021	0	0.03	0.018	0	0	0	0	0.065	0.006
	237	0	0	0.033	0	0.025	0.03	0.018	0.016	0	0.113	0	0	0.032
	239	0.025	0.017	0.008	0	0	0.075	0.027	0.048	0.027	0.177	0.012	0	0.115
	241	0	0.05	0.017	0.021	0	0.015	0.027	0.008	0.02	0	0	0.06	0
	245	0	0	0	0	0.008	0.022	0	0	0.007	0	0	0	0.077
	247	0	0.033	0.017	0	0	0	0	0	0	0	0	0	0
	249	0	0	0	0	0	0	0	0.008	0	0	0	0	0
	251	0	0	0.008	0	0	0	0	0	0	0	0	0	0
Pmx-022	*n*	0	0	0	0	0	62	54	61	62	92	81	93	81
	141	-	-	-	-	-	0	0.009	0	0	0	0.012	0.054	0
	145	-	-	-	-	-	0	0.019	0.008	0.024	0.076	0	0	0
	147	-	-	-	-	-	0.008	0.009	0	0	0	0	0	0
	151	-	-	-	-	-	0.008	0.009	0.008	0	0.005	0.012	0	0
	153	-	-	-	-	-	0	0.009	0.041	0	0.087	0.068	0	0
	155	-	-	-	-	-	0.04	0.065	0.041	0.218	0.022	0	0.005	0.019
	157	-	-	-	-	-	0.04	0.231	0.221	0.153	0.212	0.006	0.21	0.037
	159	-	-	-	-	-	0.073	0.056	0.016	0.016	0.038	0.08	0	0.049
	161	-	-	-	-	-	0.073	0.083	0.066	0.024	0.005	0.191	0.108	0.049
	163	-	-	-	-	-	0.185	0.083	0.09	0.008	0.033	0.198	0.108	0.13
	165	-	-	-	-	-	0.161	0.167	0.131	0.113	0.212	0.093	0.355	0.389
	167	-	-	-	-	-	0.048	0.083	0.074	0.234	0	0.031	0.005	0
	169	-	-	-	-	-	0.073	0.056	0.131	0	0.06	0.154	0.005	0.006
	171	-	-	-	-	-	0.097	0.028	0.057	0.161	0.065	0.062	0.016	0.031
	173	-	-	-	-	-	0.097	0.046	0.074	0.016	0.06	0.062	0.118	0.235
	175	-	-	-	-	-	0.056	0.028	0.008	0.032	0	0.006	0	0.025
	177	-	-	-	-	-	0.016	0.009	0.033	0	0.125	0	0.016	0
	179	-	-	-	-	-	0.016	0	0	0	0	0.025	0	0.031
	181	-	-	-	-	-	0.008	0.009	0	0	0	0	0	0

Pmx-1623	*n*	0	0	0	0	0	60	53	59	77	94	81	90	96
	230	-	-	-	-	-	0.15	0.151	0.119	0.195	0.138	0.037	0.094	0.031
	232	-	-	-	-	-	0.167	0.132	0.144	0.058	0.128	0.105	0.411	0.25
	234	-	-	-	-	-	0.092	0.151	0.11	0.045	0.271	0.247	0.05	0.005
	236	-	-	-	-	-	0.067	0.075	0.059	0	0.069	0.068	0.022	0
	238	-	-	-	-	-	0.108	0.132	0.136	0.208	0.144	0.08	0.139	0.094
	240	-	-	-	-	-	0.15	0.132	0.136	0.136	0.106	0.346	0.083	0.271
	242	-	-	-	-	-	0.092	0.047	0.068	0	0.005	0.049	0	0.047
	244	-	-	-	-	-	0.05	0.057	0.034	0	0.037	0	0.144	0
	246	-	-	-	-	-	0.008	0.047	0.059	0.11	0	0.012	0.039	0.068
	248	-	-	-	-	-	0.008	0.009	0.008	0	0.005	0.025	0	0
	250	-	-	-	-	-	0.05	0.009	0.051	0.078	0	0	0	0.057
	252	-	-	-	-	-	0.008	0	0.008	0.156	0.096	0.019	0	0.099
	254	-	-	-	-	-	0.017	0.019	0.042	0.013	0	0	0	0.078
	260	-	-	-	-	-	0.008	0.009	0.017	0	0	0	0	0
	262	-	-	-	-	-	0.008	0	0	0	0	0	0	0
	264	-	-	-	-	-	0.008	0.019	0	0	0	0.012	0.017	0
	266	-	-	-	-	-	0	0	0.008	0	0	0	0	0
	274	-	-	-	-	-	0.008	0.009	0	0	0	0	0	0
JCUPm_26h5	*n*	0	0	0	0	0	64	54	60	75	94	87	91	88
	150	-	-	-	-	-	0.281	0.306	0.267	0.42	0.112	0.54	0.346	0.557
	154	-	-	-	-	-	0.031	0.009	0.033	0	0	0	0	0
	158	-	-	-	-	-	0.07	0.148	0.192	0.5	0.255	0	0.121	0.136
	162	-	-	-	-	-	0.508	0.519	0.475	0.08	0.633	0.443	0.5	0.261
	166	-	-	-	-	-	0.008	0	0.025	0	0	0	0	0
	170	-	-	-	-	-	0.039	0.019	0.008	0	0	0.017	0.033	0
	174	-	-	-	-	-	0.031	0	0	0	0	0	0	0
	178	-	-	-	-	-	0.008	0	0	0	0	0	0	0
	182	-	-	-	-	-	0.016	0	0	0	0	0	0	0.045
	186	-	-	-	-	-	0.008	0	0	0	0	0	0	0