GENETIC EFFECTS OF PEARL CULTURE PRACTICES AND RECRUITMENT OF THE BLACK-LIPPED PEARL OYSTER *(PINCTADA MARGARITIFERA)* IN FRENCH POLYNESIA

by

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The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "GENETIC EFFECTS OF PEARL CULTURE PRACTICES AND RECRUITMENT OF THE BLACK-LIPPED PEARL OYSTER (*PINCTADA MARGARITIFERA*) IN FRENCH POLYNESIA" by Vicky Yaroshewski in partial fulfilment of the requirements for the degree of Master of Science.

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DEDICATION PAGE

This work is dedicated to my family and friends who offered me so much support and encouragement. Without them none of this would be possible. I would also like to dedicate my thesis to my father, Max Yaroshewski. Wishing you were here to share in this moment with me. I love you and miss you very much.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

French Polynesia relies solely on the collection of wild *Pinctada margaritifera* spat for pearl oyster culture. This was developed to help protect the wild populations from overexploitation, but it is feared that massive spat collection could lead to erosion of genetic diversity both in farmed and wild stocks.

Wild and farmed collections of *P. margaritifera* from four atolls in French Polynesia were genotyped at eight microsatellite loci to determine whether there was a loss of genetic diversity from the wild to adjacent farmed aggregations. The average allelic richness for wild samples was not significantly different from that seen for farmed samples, but there was a significant effect of atoll and locus. Pair-wise genetic differentiation (F_{ST}) was not significant between adjacent wild and farmed collections or across atolls. Overall there was no evidence for a loss of genetic variability in farmed oysters. Both farmed and wild individuals analyzed here were adults and could have originated from multiple spawning events in time and space. This could have masked genetic processes linked to recruitment happening at a finer scale. *P. margaritifera* demonstrates high recruitment variability, but the number of parents contributing to a successful cohort of juveniles recruited on collectors is unknown. Low effective number of breeders and variable recruitment are assumed to be responsible for the genetic patchiness that has been observed at a small spatial scale for this species and this could lead to a loss of genetic diversity in both the farmed and wild stocks.

The genetic diversity and family make-up of three groups of 1.5 year old oysters were assessed using 13 microsatellite markers. These individuals were harvested on collectors in three closely located sites of the Takapoto atoll. Higher recruitment density and higher allelic richness was observed in one zone compared to the other two. Significant genetic differentiation was also observed at a small spatial scale. Pair-wise F_{ST} estimates between collectors within zone were not significant, but were generally significant across zones. Estimates of effective population size and number of families present for these individuals were larger than expected and suggested that the numbers of parents contributing to the recruits on these collector lines were not limited. Similar results were obtained when assessing monthly cohorts of recruits collected in Takapoto over 5 months with 11 microsatellites. Levels of allelic richness were not significantly different among monthly cohorts, and were comparable to the levels observed in the adult samples above. Small but significant temporal genetic differentiation was observed between the monthly cohorts. Again, there was no evidence for low effective population size or for significant family structuring and it did not appear that a limited number of parents produced these temporal cohorts.

Patchy genetic structure was observed, but recruitment on collectors does not seem to be driven by a limited number of successful parents. It does not appear that the current pearl culture practices are negatively impacting the local farmed and wild stocks of *P. margaritifera* in French Polynesia by reducing their levels of genetic diversity.

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I would like to thank the many people who have helped during the completion of my thesis. My supervisor Christophe Herbinger for all his guidance and support, as well as affording me the opportunity to visit some of the sample locations in my study. Members of my committee, Paul Bentzen and Daniel Ruzzante provided much constructive comments on the thesis and assistance when needed. Paul Debes, Denis Roy, Philippe Fullsack and Kate Crosby offered a lot of statistical advice and help with my data analysis, for this I am really grateful. Many of the study site maps were created by Remi Daigle, thank you so much for you time and effort. Sarah Reid assisted with partial genotyping and data collection for the first chapter. Scholarship and research funding was provided by NSERC, Agence Nationale de Recherche (ANR), Le Service de la Perliculture (PRL), and by the A- scholarship through the Faculty of Graduate Studies at Dalhousie University. Emergency funding was provided by the Lett Fund at Dalhousie University, which greatly helped in a time of need. The people at the Centre Océanologique du Pacifique in Vairao, Ifremer, Tahiti and at the PRL station in Takapoto who worked very hard to obtain study samples for this research, and who were all very generous and accommodating during my field season, thank you. Finally, a special thanks to my family and friends for their support and keeping me sane. I sincerely and humbly thank all these people.

Chapter 1: Introduction

1.1. Features of the French Polynesian Archipelagos and Ocean Circulation

The region of French Polynesia falls within 5° to 30° South and 160° to 130° West (Martinez *et al.*, 2009) and is composed of five archipelagos, the Tuamotu range and number of atolls being the largest (Figure 1.1). The 77 atolls within Tuamotu have variable sizes, lagoon depths, and levels of exchange with the open ocean. Generally those with deep passes are larger (200-1600 km^2) and deeper (10-60 m) than those without passes (2-184 km^2 and 10 m average depth) (Rougerie, 1995). Ocean temperatures vary from 22-24°C in the south-east to more than 27°C in the north-west region of French Polynesia (Rougerie and Rancher, 1994). Due to local differences in evaporation, salinity varies across this area as well, generally above 36 psu in the Tuamotu Archipelago and east of Tahiti, while regions west of 155°W are less than 35.5 psu. Within the atolls the properties of the lagoon water can vary as well, especially with different levels of local water residence time, which is influenced by the number of passes and "hoa", shallow areas of water exchange between the open ocean and the lagoon (Rougerie, 1995). Local residence times can be on the order of weeks to months depending on the state of enclosure of the atolls. If ocean water can enter through the hoas, but cannot escape because of the lack of a deep pass, then residence time can be from months to many years. In this condition the lagoon becomes hypersaline (up to 41 psu in Takapoto) due to high levels of evaporation, losing as much as 0.5-1 m yr-1 of

freshwater, which is being replaced by equivalent amounts of oceanic water (36 psu) (Rougerie, 1995). Not only does the degree of enclosure influence residence time, but it also controls nutrient richness (chlorophyll concentration) and productivity of the lagoon. Open lagoons, such as Fakarava and Rangiroa, have low productivity and nutrient levels corresponding to an oligotrophic habitat. However, semi- and fully enclosed lagoons can have elevated phosphate and nitrate concentrations around ten times higher (or more) than the surrounding ocean, sometimes becoming eutrophic (Rougerie, 1995).

The tropical south Pacific is highly influenced by the trade winds, which create large scale ocean circulation and are also responsible for precipitation. The trade winds can be variable depending on shifts in the South Pacific Convergence Zone (SPCZ), which experiences seasonal and interannual changes, such as during periods of ENSO (El Niño-Southern Oscillation) (Martinez *et al.*, 2009). The SPCZ is an area where two types of trade winds converge, the southeastern and northeastern trade winds (Rougerie and Rancher, 1994). The effect of the SPCZ is strongest in the austral summer, producing an area of weak winds and heavy rainfall. Generally its presence in French Polynesia determines the strength of the rainy season, but it usually only affects the western portion of the Tuamotu Archipelago. The rest of the Tuamotus, especially the southeastern region, can be quite arid with low rainfall (as little as 1m yr^{-1}) and high evaporation (as much as 2m yr^{-1} or more) (Rougerie and Rancher, 1994).

At 0-20°S the Southern Equatorial Current (SEC) is a major westward flow of 10-20 cm s^{-1} average speed affecting northern Polynesia (Rougerie and Rancher, 1994). The velocities increase during the winter, with some intermittent currents occurring during summer months (Martinez *et al.*, 2009) (Figure 1.2). For example, the Subtropical

Countercurrent (STCC) flows eastward below 20°S, above the deeper westward flowing SEC, and the South Equatorial Countercurrent (SECC) forms between 9-16°S predominantly from early December to mid March. There is also a small Marquesas Countercurrent (MCC) which flows eastward around the Nuku Hiva and Hiva Oa islands at about 5 cm s^{-1}. It moves south of the islands during austral summer and north of the archipelago during winter (Martinez *et al.*, 2009). The exception is during ENSO events when no true MCC can be defined (Rougerie and Rancher, 1994).

During an ENSO event, which lasts about 12 to 18 months, the trade winds become weak or stop, and surface currents break down. French Polynesia, as far as central Tuamotu, can experience cyclones because westerly winds converge and increased ocean temperatures (>30°C) (Rancher and Rougerie, 1995; Rougerie and Rancher, 1994). It is also during these events that southern Polynesia experiences drought conditions, while the Marquesas Islands show increased precipitation, from an average of 1.2m during regular seasons to about 6m during the 1992 ENSO event (Rancher and Rougerie, 1995; Rougerie and Rancher, 1994). The ENSO events also further destabilize the current field in the tropical south Pacific so that circulation in this zone becomes mainly eastward flowing (Martinez *et al.*, 2009; Rancher and Rougerie, 1995; Rougerie and Rancher, 1994). The STCC strengthens, but the SECC is the dominant eastward current from about 10-22°S and at speeds of about 8 cm s^{-1}, while the SEC becomes weak and shallow, reduced to within 15-19°S.

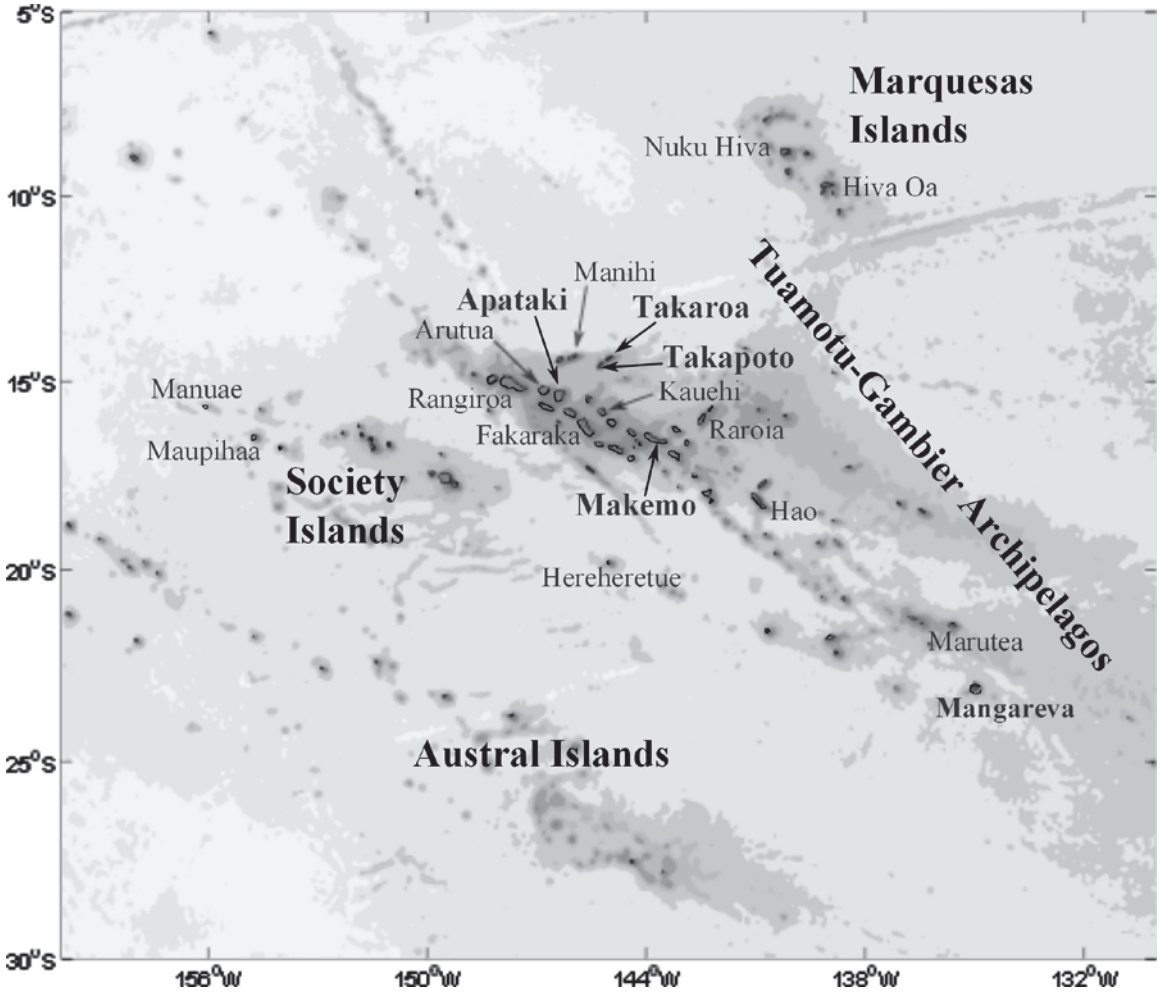

Figure 1.1. Map of the five archipelagos (Society, Tuamotu, Gambier, Marquesas, and Austral) from French Polynesia, along with the localization of some islands where *P. margaritifera* has been sampled for the current (bold) and previous (Arnaud- Haond *et al.*, 2008) studies.

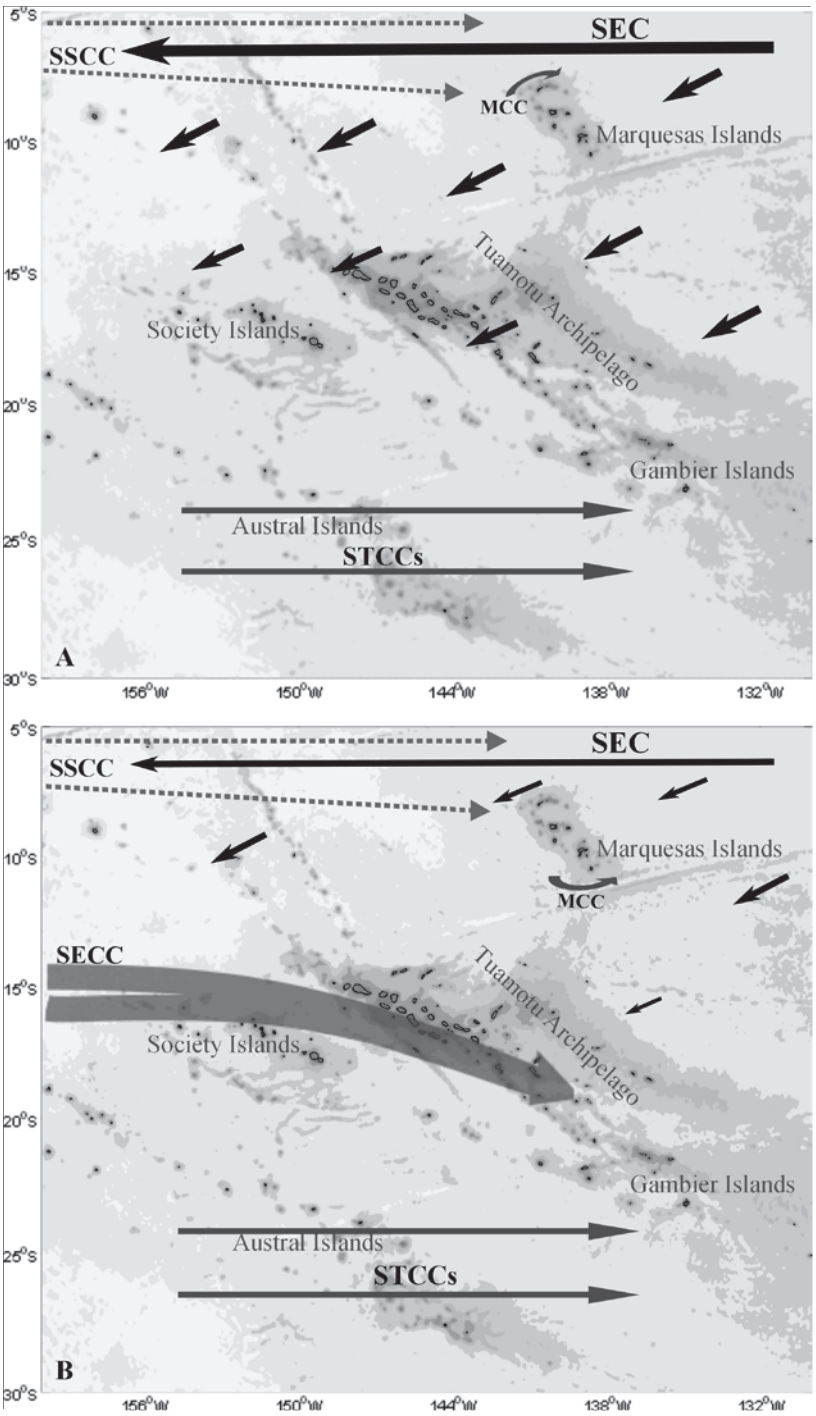

Figure 1.2. Summary of some of the main currents in French Polynesia during the winter (A; July, August, September) and summer (B; January, February, March). Thickness of the arrows represents the relative strength of the currents. Black arrows indicate the main westward flow of the South Equatorial Current (SEC), while the gray arrows show the eastward counter currents such as the Subtropical Countercurrent (STCC), Marquesas Countercurrent (MCC), Subsurface Countercurrent (SSCC), and the South Equatorial Countercurrent (SECC). The latter of these is only present during the summer months.

1.2. Growth and Reproduction of Pinctada margaritifera

The black-lipped pearl oyster (*Pinctada margaritifera*) is found throughout the Indian and Pacific Oceans and is particularly abundant in the oligotrophic lagoons of atolls in French Polynesia (Pouvreau *et al*., 2000a; Sims, 1992). Like many species of the genus *Pinctada*, *P. margaritifera* is a protandrous hermaphrodite with a sex ratio in the wild that tends to reach 1:1 as age increases (Gervais and Sims, 1992; Sims, 1993a), usually around the fourth or fifth year (Sims, 1993a). Under culture conditions there is generally a predominance of males. For example, in the Takapoto lagoon Pouvreau *et al.* (2000a) found that 75% of older oysters in culture were male after one year. Sex changes can occur (male to female or female to male), which can even be reversible under certain conditions (Gervais and Sims, 1992). Indeed, sex reversal from female to male has been repeatedly observed at the Centre Océanologique du Pacific (COP) (C. Herbinger, personal communication). Full maturity in *P. margaritifera* is reached at two years of age, which is later than most other smaller species of *Pinctada* where this is attained by the end of the first year at the latest (Gervais and Sims, 1992; Pouvreau *et al.*, 2000a). The black-lipped pearl oyster in French Polynesia breeds throughout the year, but with greater recruitment during the warmer months (from December to June in Takapoto) (Fairbairn, 2009). Strategies for spawning differ among locations. The breeding season of *P. margaritifera* in the Red Sea, for example, tends to be much more discrete (Saucedo and Monteforte, 1997), as are the spawning seasons for many other members of the Pteriidae family (Gervais and Sims, 1992). Also, *P. margaritifera* in Taiwan appear to have two spawning seasons, roughly in July and November (Hwang *et al.*, 2007).

Interestingly, Fairbairn (2009) found that there were monthly peaks in recruitment, which seemed to correspond to cycles surrounding the full moon (Mills *et al.*, 2009; Ubertini, 2009). This is a common phenomenon in nature and many species are known to synchronize movement, feeding, growth or reproduction around lunar cycles, such as sharks (Shepard *et al.*, 2006), reef fishes (Robertson *et al.*, 1990), and invertebrates (Skov *et al.*, 2005) including bivalves (Tammi *et al.*, 1996; Tebano and Paulay, 2001). *Pinctada margaritifera* demonstrates high fecundity, like many marine bivalves, as well as highly variable recruitment (Friedman *et al.*, 1998; Friedman and Bell, 1999; Oengpepa *et al.*, 2006). Pearl oysters are broadcast spawners, releasing large numbers of gametes into the water column where they are fertilized. The release of gametes in *P. margaritifera* appears somewhat synchronous in both sexes, such as has been observed in the Takapoto lagoon (Pouvreau *et al.*, 2000a). The life of planktonic larvae generally lasts about 28 days, but can be extended to four weeks (Sims, 1993). After this time a foot develops and the larvae prepare for settlement. Oyster larvae can shorten or lengthen the planktonic stage depending on availability of favourable conditions and suitable habitat. It is within these early stages that the larvae experience high mortality, which is initially due primarily to predation. It is thought that fast growth phases in juveniles provide protection against predators (Sims, 1993a). Indeed, the greatest initial growth rates are for early settlers and average about 100-120 mm in shell diameter (dorso-ventral height) within the first two years (Coeroli *et al.*, 1982, as cited by Sims, 1993a; Sims, 1993b). Based on growth experiments it took between 1.5 and 2 years for juveniles to reach a dorso-ventral height (DVH) of 100mm (*i.e.* the size at which time pearl oysters are suitable for seeding/grafting) (Table A1). Although, the rate of growth

was lower within the Takapoto lagoon and it may take longer than this to reach implantation size (Pouvreau *et al.*, 2000b; Pouvreau and Prasil, 2001). Growth rates decline after this time (Pouvreau *et al*., 2000b) (Table A1); this is logical given that the oysters have reached sexual maturity and would allocate great amounts of energy to reproduction. Average maximum shell diameter recorded after two year of age was about 140-170 mm (Gervais and Sims, 1992). Growth rates among individuals can vary considerably, as well as between geographical locations, especially if seasonal growth is exhibited like in Japan or Taiwan (Hwang *et al.*, 2007). In French Polynesia, growth rates are greater in *P. margaritifera* when cultured in lagoons or near shore waters of high islands and the open ocean as compared to the lagoons of atolls (Pouvreau and Prasil, 2001). Growth rates tend also to increase under experimental conditions when effects such as crowding and fouling are reduced, but this can vary when the oysters are held in different hatchery set-ups (Table A1) (Friedman and Southgate, 1999; Pit and Southgate, 2003a; Southgate and Beer, 1997). For example, Southgate and Beer (1997) found that 19 week-old spat that were held in plastic trays at a density of 100 juveniles had significantly greater DVH (40.48 mm ± 0.9 SE) compared to those held in pearl nets at the same density (34.28 mm ± 0.58 SE). In general, growth rates of *P. margaritifera* vary among individuals, as well as location and habitat (atoll *vs.* island and open ocean). The main commonality is that this species exhibits much greater growth rates as juveniles compared to those who have reached sexual maturity (*i.e.* those older than two years of age).

1.3. Economic Importance and Methods of Pearl Production

The pearl oyster is currently cultivated for its black pearls, but the mother of pearl (the "nacre" secreted by the mantle tissue) was traditionally used for ornamentation in French Polynesia. In the late 19th century, commercial fishing and diving for pearl oyster shells was developed for the buttons industry (Cabral, 1989). Natural pearls were by-products of this shell harvesting activity, but were very valuable. It did not take long before intense harvesting produced significant population declines. Overexploitation of this species caused depletion and complete exhaustion of wild pearl oyster stocks in several lagoons in French Polynesia by the 1950's and 60's (Intes, 1984, as cited by Cabral 1989). Since that time the harvesting of wild *P. margaritifera* has been prohibited. As an alternative with less potential to threaten the local wild stocks, the local Fishery Service (Service de la Pêche, SPE) encouraged some of the first attempts to produce black pearls using grafting (pearl seeding) techniques pioneered by Japanese researchers on the akoya pearl oyster (*Pinctada fucata*) (Gervais and Sims, 1992; Sims 1993 and references therein). In the wild, pearls are rare, occurring only in approximately one out of every 15,000 (C. Herbinger, personal communication). However, the use of the pearl grafting technique can potentially induce the formation of a pearl in thousands of farm-reared oysters at one time. To ensure that the newly developing pearl culture industry would not put harvesting pressure on the wild stocks yet again, efficient methods to collect wild spat (juveniles) were also developed. Wild

spat collection would allow the harvesting of large numbers of juveniles while leaving the wild adult stocks untouched.

The spat collection techniques have shown a lot of variation over the years, but currently it is common practice to use subsurface long-line systems (Pouvreau *et al*., 2000b). Spat collectors are generally composed of plastic sheets or shade-mesh, although numerous materials such as branches and oyster shells have been used in the past, whatever was locally available and cost effective (Southgate, 2008). These three-dimensional structures are typically suspended from floating structures (longlines or rafts) and deployed in lagoons with abundant numbers of larvae approaching the settlement stage (Southgate, 2008). After a three to four week planktonic stage, the pearl oyster larvae settle on the collectors and are then called spat (juveniles). The spat are left on the collectors for 6-12 months, upon which time they are harvested and transferred onto different 'ongrowing' systems such as downlines or lantern nets (Pouvreau *et al*., 2000b; Southgate, 2008). After another 9-12 months (when 2-2.5 years of age), the pearl oysters are selected for grafting. During this operation, known as grafting or seeding, a live tissue fragment (graft) from the mantle of a donor pearl oyster, along with a small inorganic bead or 'nucleus' (generally obtained from the shell of a freshwater mussel), is implanted into the gonad of a recipient pearl oyster (Arnaud-Haond *et al*., 2007). If the graft is not rejected, the implanted mantle tissue will grow and completely surround the nucleus, forming a 'pearl sac'. This mantle tissue will secrete layers of mother of pearl (nacre) around the nucleus. It takes approximately 18 months after implantation for the production of a pearl. After the pearl has been collected, the oysters are sometimes re-implanted to produce another pearl (Pouvreau *et al*., 2000b).

Compared to other pearl producing countries, French Polynesia is quite unique in its ability to successfully collect large numbers of juvenile pearl oysters that can be grafted and put into cultivation. This has been central to the rapid development of the industry. For more than 20 years, black pearl aquaculture has had an increasing influence in this country. Today, black pearl oyster culture for pearl production is the second most important economic activity in French Polynesia after tourism and is the largest export activity. For example, the production of pearls reached almost 6 metric tons in 2000, which had a value of $175 million US (Pouvreau *et al*., 2000a). The industry employs about 12% of the work force, and has seen the creation of around 1000 farms on 34 different atolls (Arnaud-Haond *et al.*, 2003b). Pearl production is especially important on isolated islands as it provides a source of income to maintain human populations and counteracts rural exodus. However, there was no initial consideration for the genetic consequences to wild pearl oyster populations that may be associated with the quick rise of the industry. In the past there have been extensive juvenile transfers among different islands because several atolls where pearl oysters are cultivated have poor local recruitment. The transfer of spat between distant islands is now prohibited, but transfer among closely located atolls still occurs today. It has been suggested that these activities have led to a genetic homogenization of the wild populations (Arnaud-Haond *et al*., 2004). Even in atolls where local recruitment was good and no inter-island juvenile transfer took place, it is not known whether the present method of collection of pearl oyster juveniles could be associated with a reduction of genetic diversity. This would occur primarily in the local farmed collections, which could then impact the local wild

stocks in subsequent generations. For these reasons it is important to assess the genetic impacts of past and present pearl oyster culture practices.

The primary objective of my thesis was to understand the genetic dimensions associated with juvenile recruitment on spat collectors and to assess whether large scale spat collection could be associated with changes in the genetic diversity of *Pinctada margaritifera*. This will be investigated at different spatial and temporal scales, using 8-13 microsatellite markers, which were developed by Herbinger et al. (2005) or by myself in the past year (unpublished data).

The first chapter compares the genetic diversity of four pairs of wild and farmed samples. This was a retrospective study where I re-analyzed samples that were collected and initially analyzed with four anonymous nuclear markers exhibiting limited variability (Arnaud-Haond *et al*., 2003b). This was done to evaluate whether the current method of spat collection was associated with a loss of genetic variability from the local wild adult stocks to the local farmed adult groups of pearl oysters in four different atolls in French Polynesia.

The second chapter is also a retrospective study, which involves the analysis of genetic differentiation and genetic diversity among young adults recruited at three sites within the Takapoto lagoon (Arnaud-Haond *et al*., 2002b). This also included the estimation of the number of breeders that contributed to oysters successfully recruited on collectors, which survived to be harvested one year later. This was accomplished using microsatellite-based sibship analysis.

Finally, the third chapter involves determining the level of genetic diversity and differentiation in juveniles obtained from spat collection in two different sites within the

Takapoto atoll over a complete year cycle. This analysis involved much finer spatial and temporal scales of recruitment as collectors were deployed every month and retrieved every two months. The purpose of this project was to break down overall recruitment into individual cohorts in order to better understand the mechanisms that may drive variation in the genetic composition of recruits on collectors, and to estimate the number of parents more accurately. This was also accomplished by analysis of genetic variability and differentiation of the cohorts, followed by microsatellite-based sibship analysis.

Chapter 2: Genetic diversity of farmed and adjacent wild stocks of the black pearl oyster (*Pinctada margaritifera*) in French Polynesia

2.1. Introduction

The black pearl oyster (*Pinctada margaritifera*) was initially harvested for its shell, which contained mother of pearl (nacre) to make jewelry, buttons and other trinkets (Cabral, 1989). While the nacreous shells are still used in this capacity, it is the black pearl that is highly sought and quite valuable. This by-product of the shell industry occurred rarely in nature. However, with pearl seeding techniques developed in the 1970's, thousands of pearls could be produced at one time. For this purpose, hundreds of thousands of oysters are cultured in farms across the South Pacific.

Presently, farms in French Polynesia rely almost entirely upon wild collected spat (Arnaud-Haond *et al.*, 2003b). Hatchery produced stocks are not used in any significant way, which is in contrast to pearl production in most other countries (Durand *et al.*, 1993; Lind *et al.*, 2009; Yu and Chu, 2006) or to many other types of mollusc culture (Benzie and Williams, 1996; English *et al.*, 2000; Hedgecock and Sly, 1990; Hedgecock *et al.*, 1992). The pearl culture industry in French Polynesia is therefore highly dependent on the genetic resources that are available from the local wild stocks. In order to properly manage the wild populations, it is essential to monitor the genetic variability that exists within black pearl oysters in the wild, and to assess potential impacts of pearl oyster culture operations. However, while numerous studies have concentrated on the impacts of hatchery propagation on the genetic variability of resulting stocks (*Pinctada fucata*

martensii: Wada, 1986a; *Pinctada fucata*: Yu and Chu, 2006; *P. maxima*: Lind *et al.*, 2009; and *P. margaritifera* in Japan: Durand *et al.*, 1993), there have been few studies that focused on the genetic variation in stocks obtained from wild spat collection (*P. margaritifera*: Arnaud-Haond *et al.*, 2003b; 2004; 2008). This is a significant gap in our knowledge and is particularly important because it is still unknown whether the genetic variation seen in the wild populations is adequately represented in farmed individuals harvested on the collector lines.

During the relatively quick development of this industry, there was no initial thought as to the genetic consequences that could arise from raising farmed individuals in habitats contiguous with the wild populations, especially when the farmed stocks represent large numbers of potentially reproducing individuals (Arnaud-Haond *et al.*, 2003b). As an example, it was estimated that there were as many as 1.8 million farmed oysters in relation to about 4 million wild pearl oysters in the Takapoto lagoon in 1997 (Prou, 1999, as cited by Arnaud-Haond *et al.*, 2003b). This is particularly important because *P. margaritifera* shows much variation in abundance and recruitment (Arnaud-Haond *et al.*, 2002b; Fairbairn, 2009; Friedman *et al.*, 1998; Friedman and Bell, 1999; Oengpepa *et al.*, 2006). One of the factors leading to variability in recruitment is likely high variance in reproductive success. Indeed, many other species of bivalves have been shown to have highly variable reproductive success, resulting in low effective population sizes or low effective number of breeders, both in hatchery propagated stocks and in natural populations (Boudry *et al.*, 2002; Hedgecock, 1994a; Hedgecock, 2007; Hedgecock *et al.*, 1992; Hedgecock and Sly, 1990; Launey *et al.*, 2001). Accordingly, the question arises as to whether *P. margaritifera* also has cohorts of recruits that could

come from a low effective number of parents, especially those cohorts represented on collector lines. In such a scenario, those new recruits collected in very large numbers on the suspended lines are protected and removed from predation and other factors that may otherwise cause high juvenile mortality in the wild. If these farmed individuals arose from a limited pool of parents, they would have lower genetic variation compared to the wild individuals. Subsequently, they are farmed and allowed to grow to adulthood where they can reproduce and possibly create large amounts of larvae that will settle back into the wild, therefore potentially affecting the genetic diversity of the wild stocks. Over many generations, such negative impacts might amplify. If the new generation of recruits also comes from a limited number of parents, now with lower genetic diversity, the next cohort of new recruits on collector lines might demonstrate even lower genetic variance. In other words, we could see a significant loss of genetic diversity primarily in the farmed stocks, which could result in a loss of genetic diversity in the wild stocks over many generations (Arnaud-Haond *et al.*, 2003b). For these reasons it is important to have a good understanding of the genetic composition of wild and farmed *P. margaritifera*.

The answers to the above question have important implications for the conservation of wild stocks and, therefore, the long-term future of the pearl oyster aquaculture industry, given its dependence on wild spat. This importance is exemplified by the situation during 1980's when the pearl culture industry in French Polynesia was growing rapidly. In some atolls there was insufficient recruitment to meet demand of spat for the increasing number of farms. As a result, juveniles were translocated in large numbers between atolls of the Society, Tuamotu and Gambier Archipelagos. A recent analysis of current population structure showed genetic homogeneity between the wild

populations of *P. margaritifera* in the Society and Tuamotu-Gambier Archipelagos, the atolls of which are hundreds of kilometres apart (Figure 1.1). It is thought that the lack of genetic heterogeneity is consequence of the past translocations events (Arnaud-Haond *et al.*, 2002b; 2004). Further complications occurred in 1985, when many atolls experienced large scale mortality of farmed populations (Cabral, 1989). Earlier juvenile transfers might have been responsible by causing the spread of disease or lower resistance to local disease among the translocated individuals. For example, it is believed that commercial shipments of juveniles from the Takapoto lagoon lead to the spread of disease among French Polynesian atolls (Reed, 1985, as cited by Sims, 1993a). Spat transfer continued well into the 90's, such as in 1995 when 500,000 juveniles from Maupihaa (Society Islands) were transferred to Manihi, Rangiroa, Takapoto and Takaroa (northern Tuamotu Islands) (Arnaud-Haond *et al.*, 2004). This activity still occurs, but is now supposed to be mostly restricted to closely situated atolls within the northern Tuamotu Archipelago (Service de la Perliculture, 2009).

Overall, the full extent of the possible impacts of the pearl farming activities on the wild populations is still not well known. Along with the fear of loss of genetic diversity in the farmed stocks, it is thought that this industry has caused a genetic homogenization of once distinct wild populations. Because of this there has been increased recognition for the need to better understand and characterize the genetic resources of current pearl oyster stocks and to determine whether the pearl culture practices are negatively affecting the genetic diversity of farmed and local wild *P. margaritifera* stocks. This chapter compares the genetic diversity of four pairs of adjacent wild and farmed adult collections of *P. margaritifera* from the Tuamotu-

Gambier archipelago, as well as the level of genetic differentiation among these atoll populations, with the use of eight microsatellite markers that were recently developed for this species (Herbinger *et al.*, 2005). Most of these samples were previously analyzed with four anonymous nuclear markers that exhibited limited variability (Arnaud-Haond *et al*., 2003b). In the earlier study, the four farmed samples were characterized by a smaller number of alleles, as well as slightly smaller observed and unbiased heterozygosity compared to their adjacent wild populations (Arnaud-Haond *et al.*, 2003b). However, the differences were not significant and it was concluded that the current spat collection practices did not appear to lead to erosion of genetic diversity in the cultured populations. Nonetheless, the authors cautioned that the absence of stronger genetic differentiation between the wild and farmed samples might have been due to the limited power of four the markers used, warranting the use of more variable markers. In the present study, I will use twice as many hypervariable microsatellite markers, which should allow for a more powerful test to detect weak levels of genetic differentiation and/or weak erosion of genetic diversity, and to determine more definitely whether spat collection methods could lead to deterioration of the genetic resources in the local wild stocks.

2.2. Methods and Materials

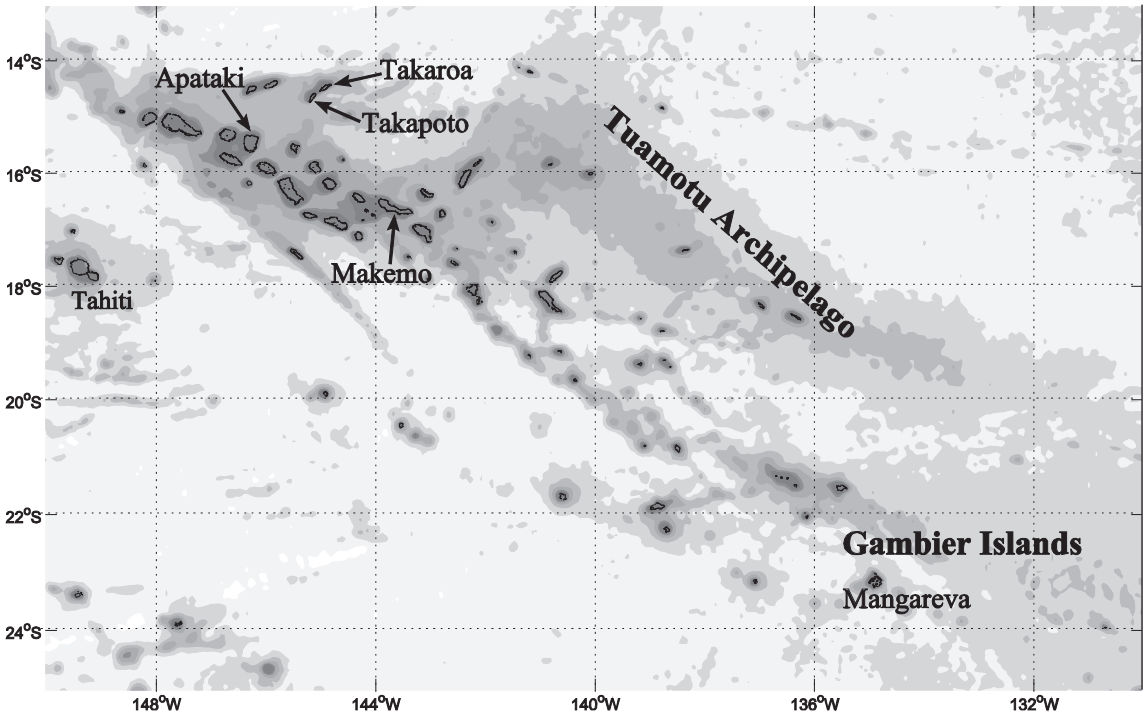

Figure 2.1. Map of the Tuamotu-Gambier Archipelagos and localization of the atolls from which *P. margaritifera* populations were sampled for this study.

2.2.1. Sampling

Two hundred and ninety-five adult pearl oyster samples from adjacent farmed and wild collections were obtained from four atolls in French Polynesia: Apataki (A), Takaroa (T), Makemo (MK), and Mangareva (M) (Arnaud-Haond *et al*., 2003b) (Figure 2.1). The sample sets from the Tuamotu-Gambier archipelago included the same as those collected and used in Arnaud-Haond *et al.* (2003b), but only 14 wild samples from the Apataki atoll were still available. I also analyzed one additional individual each from the Apataki-farmed and Takaroa- and Makemo-wild samples, as well as 27 from the

Mangareva-wild and 20 from the Mangareva-farmed collections (Table 2.1). These supplementary samples had been collected at the same time as the original samples analyzed in Arnaud-Haond (2003b), but were not included due to difficulties with DNA extraction and amplification using the original protocol (phenol-chloroform protocol). These samples were successfully processed with the protocol described below.

2.2.2. DNA Extraction and Amplification

The DNA from either mantle or adductor muscle tissue was extracted using the "glassmilk" protocol, outlined in Elphinstone *et al.* (2003). The extracted DNA was amplified through PCR at eight microsatellite loci following Herbinger *et al.* (2005), but with some modification (Table 2.2, see also Appendix, Table A2).

Pmarg37 generally amplified well at the original annealing temperature of 48°C; however, on occasion there were excess bands on the gel image. To improve this, the annealing temperature was raised to 50°C or 51°C, resulting in greater ease of identifying true alleles. Pmarg29 was not used, even though the motif (sequence repeat) suggested it was a tetranucleotide, the amplified region behaved like a mononucleotide microsatellite and was extremely difficult to score. Pmarg44 was excluded because it was found to amplify the same locus as Pmarg79.

Finally, after undergoing denaturing polyacrylamide gel electrophoresis on 6% polyacrylamide gel, the PCR products, which were labeled with HEX dye, were visualized using an FMBio II fluorescent imaging system (Hitachi Software Engineering).

2.2.3. Data Analysis

In order to test the dataset for the possible presence of null alleles and scoring errors, the program Micro-Checker (van Oosterhout *et al.*, 2004) was used for each locus. Microsatellite Toolkit and FSTAT (Goudet, 2001) were used to calculate observed heterozygosity (H_{obs}) and Nei's unbiased gene diversity (H_{nb}) or expected heterozygosity (Nei, 1987).

Decreased allelic richness (number of different alleles observed in a sample) is a good indicator of recent population bottleneck, as rare alleles are lost very rapidly (Herbinger *et al.*, 2003). However this measure is quite sensitive to sample size. To compare allelic richness among differing samples sizes, allelic richness can be corrected (i.e. adjusted to the smallest sample size) by resampling among larger samples. This was performed with FSTAT (Goudet, 2001). Evidence for systematic difference in corrected allelic richness between adjacent farmed and wild samples was tested by ANOVA for a block design without replication:

$$A_{ijk} = \mu + PopType_i + Atoll_j + Locus_k + \varepsilon_{ijk} \qquad (2.1)$$

where A_{ijk} = corrected allelic richness for population type i (farmed or wild), atoll j (4 atolls), locus k (8 loci, treated as a block), and ε_{ijk} = random error term associated with observation A_{ijk}. This analysis was performed twice, a first time with allelic richness corrected to the smallest sample size in each atoll separately and a second time with allelic richness corrected to the smallest sample size in all atolls. The first analysis

maximizes the chance of detecting systematic change in allelic richness among population type (wild or farmed) but at the cost of a biased estimation of the effect of atoll since the minimal sample size in each atoll would be quite different (from minimal n=12 in Apataki to minimal n = 42 in Mangareva). In contrast, the second analysis permits an unbiased estimation of the effect of atoll, but with possibly less power to detect changes in allelic richness between farmed and wild samples as richness is estimated on a corrected sample size of 12 for all populations.

Finally, FSTAT was used to estimate genetic differentiation (F_{ST}) between all pairs of populations using the estimator θ (Weir and Cockerham, 1984). The significance of these estimates was tested by 1000 random permutations of individuals between samples and by using Bonferroni correction for multiple tests (Rice, 1989).

2.2.4. F_{ST} Power Analysis

The statistical power and α error of my analysis, when testing genetic homogeneity (F_{ST}) among and within pearl oyster populations, were tested using the program POWSIM 4.0 (Ryman and Palm, 2006). This was done both under the conditions of the initial analysis (4 moderately variable loci, Arnaud-Haond *et al.*, 2003b) and with the current data set (8 hypervariable microsatellite loci). This simulation-based program estimates power and α error for chi-square and Fisher's exact tests with a null hypothesis of genetic homogeneity among populations being tested. At a predefined level of divergence (F_{ST}), the program creates a large number of simulated populations that have diverged from a common base population. The simulation is done through

random number generation under the Wright-Fisher model without migration or mutation (Ryman and Palm, 2006). It is assumed that the base population is infinitely large, having a specified number of loci that are independent and selectively neutral with defined allele frequencies. The base population is then divided into *s* subpopulations of equal effective population size (N_e) by randomly sampling $2N_e$ genes, and allowed to drift over *t* generations. This then gives the expected degree of divergence after generation *t*, $F_{ST} = 1-(1-1/2N_e)^t$ (Nei, 1987).

POWSIM was used to determine the power that was achieved at various sample sizes for both studies. In order to create the allele frequencies for the simulation, the observed number and frequency of the alleles were averaged across all populations for each dataset from Arnaud-Haond *et al.* (2003b) and the present study. These global allele frequencies were then inputted into the simulation model. Furthermore, simulations were run with a set combination of N_e and *t* to look at the degree of divergence that could be detected, to correspond to the recent anthropogenic homogenization (Arnaud-Haond *et al.*, 2004). Assuming that a reproductive generation is every 3-4 yrs, the number of generations (*t*) used in the simulation was 20 and N_e set at 5000. The sets of parameters would result in an expected F_{st} of 0.002. This value was chosen as it was quite close to the average pairwise F_{st} reported in the original analyses (average F_{ST} =0.0043) and the present study (average F_{ST} = 0.0027, see Table 2.4). Finally, the population sizes of each atoll were run with the collection numbers mentioned above, as well as with population sizes of 20, 40, 60, 80, and 100 for each atoll. For example, one run set the population sizes of all atolls at 20 individuals. This was done to help visualize the minimum sample

size required for optimal statistical power when using anonymous nuclear markers and microsatellite markers (Figure 2.4).

2.3. Results

2.3.1. Heterozygosity and Allelic Richness

Across all loci, the observed heterozygosity (H_{obs}) varied from 0.58 to 0.62 in wild samples and from 0.54 to 0.64 in farmed collections, while the expected heterozygosity (H_{nb}) ranged from 0.89 to 0.91 and from 0.87 to 0.89 in wild and farmed collections respectively. The H_{obs} values were comparable in each pair of wild and farmed collection per atoll. For Takaroa and Mangareva, there were small decreases in observed heterozygosity (H_{obs}) from the wild to the farmed samples, but for the Apataki and Makemo atolls the reverse was seen (Figure 2.2, Table 2.1). There was also a small decrease in the expected heterozygosity (H_{nb}) from the wild to adjacent farmed samples, while no such systematic pattern was seen with observed heterozygosity (Figure 2.2, Table 2.1). For all population pairs, there were heterozygote deficiencies observed when averaged across all loci.

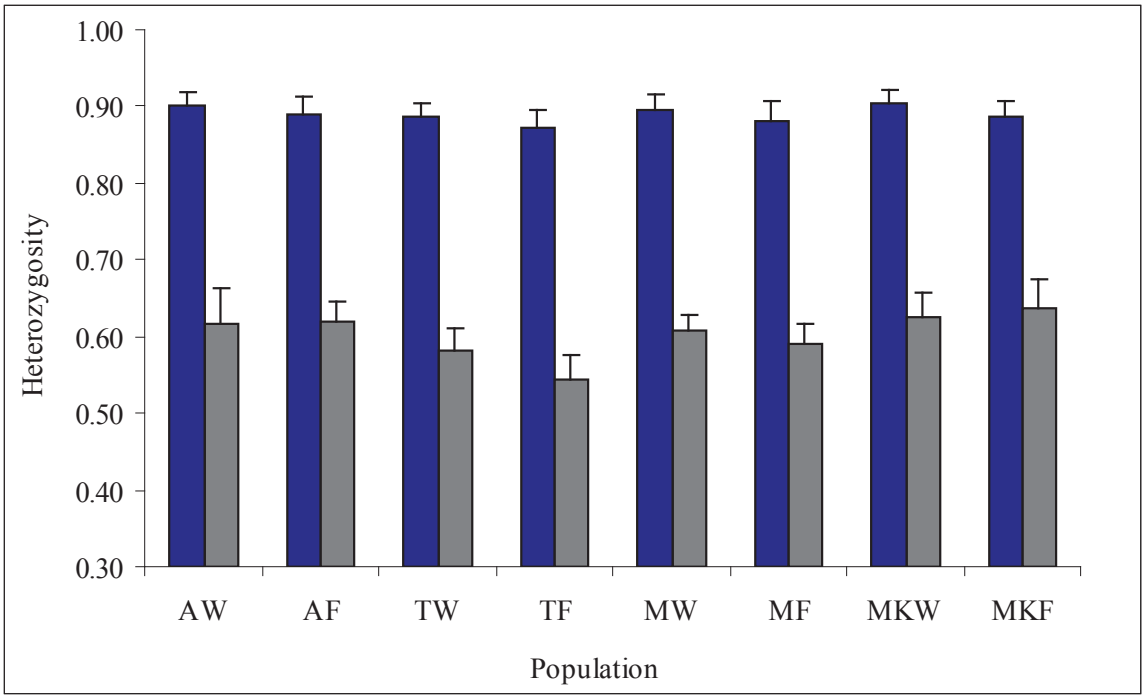

Figure 2.2. The expected heterozygosity (H_{nb}) (blue) and observed heterozygosity (H_{obs}) (grey) across all loci, for all four pairs of wild (W) and farmed (F) collections. Standard deviation bars included.

Table 2.1. The atolls (Apataki, Takaroa, Makemo and Mangareva) and archipelagos from where the sample sets were collected, the abbreviated name of wild (W) and farmed (F) collections, total sample size (*N*), unbiased expected heterozygosity (H_{nb}), observed heterozygosity (H_{obs}), and the mean number of alleles per locus (N alleles, original study) or corrected allelic richness (present study). The values from this study (using microsatellite markers) are compared to those observed in the study by Arnaud-Haond *et al.* (2003b), which used four anonymous nuclear markers.

		Arnaud-Haond *et al.* (2003b)				This Study			
Archipelago	Atoll	*N*	H_{nb}	H_{obs}	N alleles	*N*	H_{nb}	H_{obs}	Corrected Richness*
Tuamotu	A-W	29	0.34	0.29	3.75	14	0.90	0.62	10.99
	A-F	42	0.34	0.31	3.25	43	0.89	0.62	11.30
	T-W	39	0.37	0.35	4.00	40	0.89	0.58	10.62
	T-F	30	0.34	0.31	3.50	30	0.87	0.54	10.10
	MK-W	28	0.41	0.37	3.25	29	0.91	0.63	11.41
	MK-F	22	0.37	0.35	2.75	22	0.89	0.64	11.01
Gambier	M-W	40	0.37	0.32	3.75	67	0.90	0.61	11.41
	M-F	30	0.37	0.26	3.25	50	0.88	0.59	11.02

* = corrected for smallest sample size of 12

The microsatellite loci were highly variable, having overall total allele counts from nine alleles (Pmarg68) to 58 alleles (Pmarg45). Allelic richness averaged across all populations and corrected for a minimum overall sample size (n=12), varied from 6.91 (Pmarg68) to 15.35 (Pmarg45). This can be contrasted with the variability observed in the original study (Arnaud-Haond *et al.* 2003b) where allele counts varied from 2 to 4 in all locus-population combinations.

Within atoll, comparisons showed a small decrease in allelic richness from wild to farmed samples at most loci in three of the atoll lagoons (Makemo, Takaroa, and Mangareva), but the reverse was observed in Apataki (Figure 2.3). Overall, the wild collections had an allelic richness of 14.33 alleles, averaged over all loci and all four populations, while the farmed collections showed an average richness of 13.65 alleles. Despite the small decrease in allelic richness from wild to farmed samples, this difference was not significant. This was demonstrated in the ANOVA results which showed there was no significant effect of population type (wild *vs.* farmed) (P-value = 0.215) on allelic richness; however, there was a strong and significant effect of locus and atoll (Table 2.3, upper panel). As mentioned in the Material and Methods section, this analysis was performed to maximize the power of the comparisons between Wild and Farmed allelic richness, but at the cost of inflating the effect of atoll. When the analysis was repeated with allelic richness corrected to the smallest sample size over all populations (n = 12), the results were comparable (Table 2.3, lower panel). Not surprisingly, the effect of locus was still very strong and the effect of Wild versus Farmed was still non significant. As expected, the main difference between the two analysis methods was the effect of

atoll; it was much weaker in the analysis considering all atolls, but still significant (P-value = 0.028).

Table 2.2. The forward (F) and reverse (R) primer sequences, core repeat motif, and observed allele size range for each microsatellite locus used in the current study, designed for *Pinctada margaritifera* by Herbinger *et al.* (2005).

Locus	Primer sequences	Motif	Range (bp)
Pmarg2	F-GAT CCT ACG ATG ATT GCT TTG TC R-TGC AAC GTA TCA GGT TAT GTT TG	$(AC)_{20}$	169-251
Pmarg7	F-CGT CAG TGG GAG TCA AAT ATT CG R-AGG AAG GGC ATG TCA TAA GGA AC	$(GACA)_7$	156-188
Pmarg11	F-TCT GTC CGT CCA TCT AGC R-ACA ATG CAT ATC AAG TCA GC	$(GACA)_7GAAA(GACA)_3GAAA$ $(GACA)_2GAAA\ (GACA)_3$	164-258
Pmarg37	F-GTC AGG ATC TCC TTT ATC TC R-AGG AGA TAT GTC ATT GCT G	$(CA)_{15}$	137-217
Pmarg45	F-TCT GCCTGA CAA GTT ACG AAC R-ATA CAT TGA AGC TCG TCT CCT C	$(GACA)_9(GACG)_7(GACA)_9$	112-280
Pmarg68	F-GTT GCC TGT GAA ACA TAG TG R-CAG TTA TGG CTG TGG ACC	$(GACA)_2GATG(GACA)_5$	140-184
Pmarg77	F-GTT CAG CCA TTC TTG AGA AG R-TGA GTC AAT ATT TAG CTC GAA G	$(GACA)_{13}$	116-212
Pmarg79	F-AGT AAG TTG TAG CCA AAT ATG TGC R-GGA ATATCA AAC ACA GGT CAC TC	$(GACA)_7$	198-266

Table 2.3. Analysis of variance of allelic richness for all four pairs of populations. In the upper panel, the allelic richness was corrected to the smallest sample size in each of the four atolls (n=12 for Apataki, n=26 for Takaroa, n=16 for Makemo, and n=42 for Mangareva). In the lower panel, the allelic richness was corrected to overall smallest sample size (n=12).

Source	**DF**	**SS**	**MS**	**F**	**P**
Wild *vs*. Farmed	1	7.49	7.49	1.57	0.215
Locus	7	1298.04	185.43	38.94	0.000
Atoll	3	440.45	146.82	30.83	0.000
Error	52	247.63	4.76		
Total	63	1993.61			
Wild *vs*. Farmed	1	1.01	1.01	1.21	0.277
Locus	7	546.02	78.00	93.09	0.000
Atoll	3	8.26	2.75	3.28	0.028
Error	52	43.57	0.84		
Total	63	598.86			

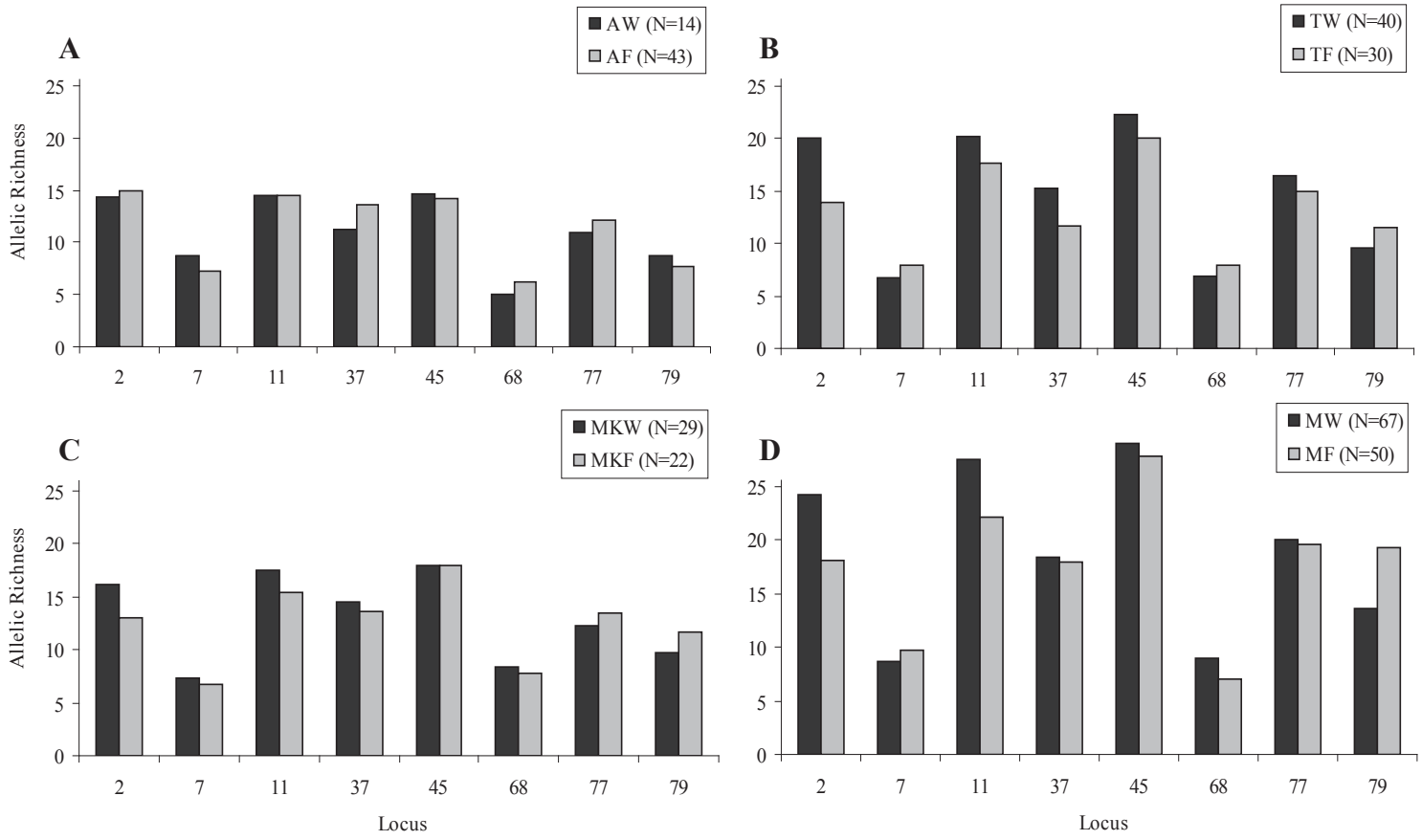

Figure 2.3. Allelic richness per locus for each pair of wild (W) and farmed (F) collections: A) Apataki; B) Takaroa; C) Makemo; and D) Mangareva. This is based on a minimum sample size of 12 individuals for Apataki, 26 individuals for Takaro, 16 individuals for Makemo, and 42 individuals for Mangareva.

2.3.2. Genetic Differentiation and Power Analysis

Finally, when looking at genetic differentiation, there were no significant estimates of F_{ST} either between wild and adjacent farmed samples or among collections of the different atolls (Table 2.4). This indicates very little genetic differentiation at a fairly large geographical scale and between population types.

Table 2.4. Pair-wise similarity matrix of F_{ST} values estimated among groups of *P. margaritifera* from the Tuamotu-Gambier archipelago, based on eight microsatellite markers (below the diagonal - this study) and four anonymous nuclear DNA markers (above the diagonal - Arnaud-Haond *et al.*, 2003b).

	A-W	A-F	T-W	T-F	MK-W	MK-F	M-W	M-F
Apataki Wild	-	0.000	0.005	0.000	0.000	0.000	0.006	0.000
Apataki Farmed	-0.0031	-	0.000	0.000	0.005	0.000	0.011	0.000
Takaroa Wild	-0.0070	0.0054	-	0.002	0.007	0.007	0.012	0.004
Takaroa Farmed	-0.0001	0.0046	0.0092	-	0.010	0.000	0.020	0.000
Makemo Wild	-0.0038	0.0012	-0.0030	0.0062	-	0.002	0.005	0.000
Makemo Farmed	0.0024	0.0052	0.0052	0.0127	-0.0014	-	**0.024**	0.000
Mangareva Wild	-0.0053	-0.0012	-0.0017	0.0034	-0.0037	0.0038	-	0.000
Mangareva Farmed	0.0000	0.0019	0.0051	0.0104	-0.0027	0.0000	-0.0044	-

Significant value after the 1000 permutation test is in bold, $\alpha = 0.05$. Note that negative F_{ST} values reported in this study indicate non-existent genetic differentiation, i.e. they are non different from 0.000.

The simulation-based computer program POWSIM was used to evaluate power and α error for the F_{ST} analyses both in this study and that of Arnaud-Haond *et al.* (2003b). Figure 2.4 shows the results with the parameters set at $Ne = 5000$ and $t = 20$ for an expected $F_{ST} = 0.002$, a value close the average F_{ST} values observed in both studies (Table 2.4). The data set using anonymous nuclear markers maintained a realised α error close to the expected level ($\alpha = 0.05$) for testing the null hypothesis of genetic homogeneity, in both the chi-square and Fisher's exact tests. The data set using microsatellite marker loci also maintained a realised α error close to the expected level

for the chi-square test, but the α error from the Fisher's exact test was slightly inflated (as much as 0.081 in some simulations with low sample sizes). The best results for both types of markers were achieved when the sample size was higher. The same was true for the power (1-β). As expected, for any power level, fewer samples were required when using microsatellite marker loci than anonymous nuclear marker loci to detect weak levels of differentiation. It was observed, for example, that for an expected F_{ST} of 0.002, a sample size of about n = 32 and n = 100 was required to achieve a power of 80% when using microsatellite marker and anonymous nuclear marker loci respectively (Figure 2.4). The average sample size was about 33 in the Arnaud-Haond *et al.* (2003b) analysis and about 37 in the microsatellite analysis, with only two populations with sample sizes substantially lower than 32 in the last case. Within the parameter limits that were used for the simulations, it would seem that the original study had limited power while the newer analysis had quite adequate power to detect weak genetic differentiation among populations. Indeed, the probability of obtaining a significant result ($P < 0.05$) in contingency tests among populations was 0.9560 (chi-square) for microsatellite marker loci and 0.194 (chi-square) for anonymous nuclear marker loci with the observed sample sizes (Table 2.5). This would seem to indicate that the absence of genetic differentiation detected in the present study (Table 2.4) was not due in part to a limited power.

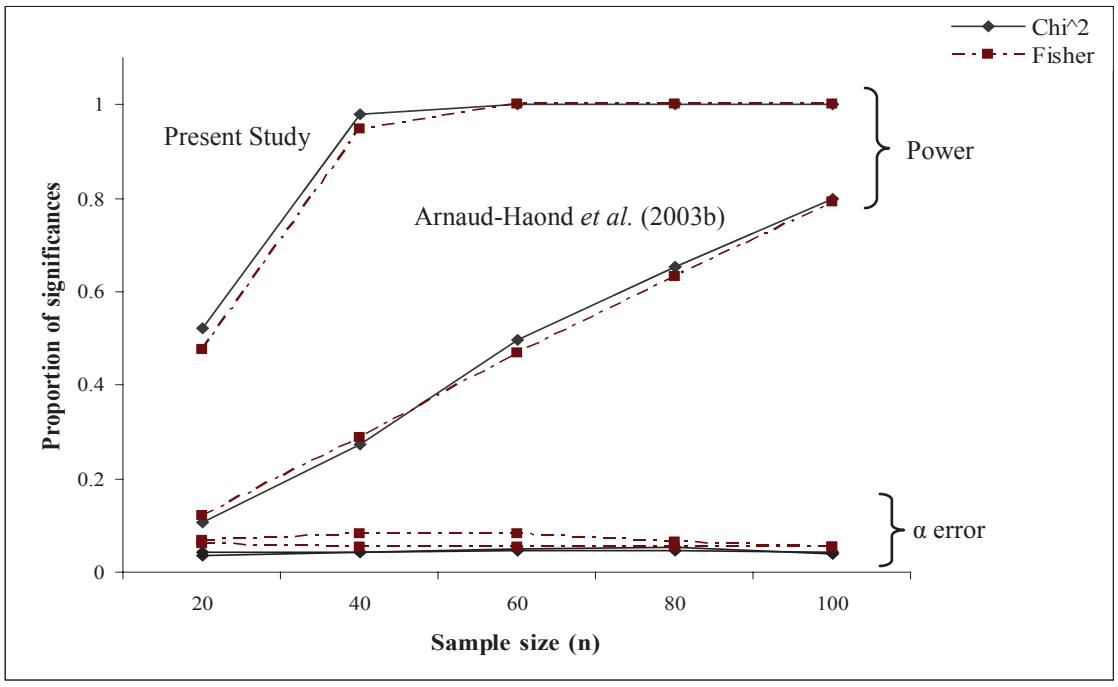

Figure 2.4. Simulated estimates (1000 runs) of power and α error when using eight microsatellite marker loci (present study) and four anonymous nuclear marker loci (Arnaud-Haond *et al.*, 2003b). This was sampling *n* individuals from each of eight populations with an expected divergence (F_{ST}) = 0.002, where N_e = 5000 and t = 20.

Table 2.5. Tests for power using global allele frequencies for microsatellite marker loci (upper panel, present study) and anonymous nuclear marker loci (lower panel, Arnaud-Haond *et al.*, 2003b) with observed population sizes. This shows the probability of obtaining a significant result ($P < 0.05$) in contingency tests among populations for two different combinations of t (number of generations to drift) and N_e (effective population size).

N_e	t	#Chi^2	Chi^2	#Fisher	Fisher_P	Aver F_{ST}	Expected F_{ST}
2000	20	500	1.000	500	1.000	0.0050	0.0050
5000	20	478	0.9560	464	0.9280	0.0020	0.0020
2000	20	292	0.584	282	0.564	0.0050	0.0050
5000	20	97	0.194	99	0.198	0.0020	0.0020

Number of runs/replicates = 500

2.4. Discussion

2.4.1. Heterozygosity

Significant heterozygote deficiencies were observed at all loci compared to expectations from Hardy-Weinberg equilibrium. While some studies have reported heterozygote excess in hatchery propagated stocks of bivalves (Gaffney *et al.*, 1996; Wada, 1986a), deficits in heterozygosity seem to be far more common and are well documented among marine bivalves (Beaumont, 1991; Colgan, 1987; David *et al.*, 1997b,c; Durand and Blanc, 1989; Evans *et al.*, 2006; Gaffney, 1990; Gaffney *et al.*, 1990; Gosling, 1989; Koehn and Gaffney, 1984; Mallet *et al.*, 1985; Toro and Vergara, 1995; Vadopalas *et al.*, 2004; Zouros and Foltz, 1984). However, the theories put forth to explain this phenomenon are also numerous, some of which include the Wahlund effect, inbreeding (David *et al.*, 1997b), overdominance (Koehn *et al.*, 1988), aneuploidy (Gaffney, 1990), genetic drift, selection (Beaumont, 1991; Toro and Vergara, 1995), and null alleles (Foltz, 1986; Lemer *et al.*, 2011; Vadopalas *et al.*, 2004; Yu *et al.*, 2008). More recently, one of the dominant theories to explain heterozygote deficits, especially with microsatellite loci, is the presence of null alleles. Null alleles (alleles that are present but not amplified, generally because a mutation(s) in the primer region(s) prevents proper annealing) are common artefacts of the PCR process and are frequently observed in many invertebrates, particularly molluscs. The presence of null alleles has been confirmed across different types of molecular markers such as allozyme loci (David *et al.*, 1997b,c; Foltz, 1986; Parker at al., 2003) and PCR-based loci (anonymous nuclear

markers: Arnaud-Haond *et al.* 2002a; microsatellites: Astanei *et al.*, 2005; Benzie and Smith-Keune, 2006; McGoldrick *et al.*, 2000; Yu *et al.*, 2008). In the present study, it is probable that the observed heterozygote deficiencies are due primarily to the presence of null alleles. The possible presence of null alleles was inferred using Micro-Checker, for seven of the eight loci. This has been confirmed by reconstructing pedigrees of known full-sib families (data not shown). Furthermore, a recent paper by Lemer *et al.* (2011) clearly demonstrated for *Pinctada margaritifera* samples, from 3 locations in French Polynesia, that null alleles were responsible for deviations from H-W equilibrium for three loci (Pmarg 45, 68 and 37). Using a three step process, with combinations of various primers for each of the three loci, initially significant heterozygote deficiencies were eliminated. Furthermore, once the null alleles were corrected all three populations appeared at HWE, indicating that the null alleles were responsible for the initial disequilibrium (Lemer *et al.*, 2011). This revealed that homozygote excess was caused by the presence of null alleles because of mutations at the primer binding sites, and is more than likely responsible for the observed null alleles in the present study. This not only has consequences for heterozygosity, but allelic richness and genetic differentiation as well, which will be discussed in the following sections.

2.4.2. Genetic Diversity in Wild and Farmed Collections

Arnaud-Haond *et al.* (2003b) found slight and non-significant decreases in the number of alleles as well as expected and observed heterozygosity between the wild and the adjacent farmed samples. They concluded that the current methods of spat collection

did not seem to lead to strong reduction in genetic variability in the farmed individuals. However, the authors cautioned that the study was based on the use of four anonymous nuclear markers with limited variability, which might be insufficient to detect slight loss of alleles. In particular, they noted that their farmed collections had systematically lower numbers of alleles, with an average loss of 0.5 alleles or 13.5% of the allelic richness present in the wild populations. The present study allowed for a much more powerful re-assessment of the level of allelic diversity in these samples. Average corrected allelic richness was 14.33 in the wild samples and 13.65 in the farmed groups. This average loss of 0.68 alleles represented about 4.8% of the allelic richness present in the wild samples, but was far from significant (Table 2.3, P-value = 0.215 and P-value = 0.277, upper and lower panel respectively). Effect of locus (treated here as a block) was very strong in both analyses. This was expected as the various loci used here are characterized by large inherent differences in allelic richness. For example, Pmarg7 and Pmarg68 clearly show lower allelic richness as compared to Pmarg11 and Pmarg45, and this is independent of atoll or population type (Figure 2.3). The effect of atoll was highly significant in the first analysis (Table 2.3, upper panel), a result driven in a large part by the different minimal sample sizes among the four atolls in that analysis. However, when using the same corrected size (12), the effect of atoll was weaker but still significant (Table 2.3, lower panel). This seems to be due to a lower average allelic richness in the Takaroa atoll (10.36 alleles) than in the other three atolls (Apataki: 11.15; Makemo: 11.21; Mangareva: 11.21). This represented a small difference (0.79-0.85 alleles), but it was significant because the lower richness of Takaroa was seen in both wild and farmed collections and fairly systematically for all loci (data not shown). In contrast, the equally

small average loss of allelic richness of 0.68 alleles between the wild and farmed groups was not significant because the trend was not systematic. In all population pairs, there were some loci which had higher allelic richness in the farmed individuals than in the wild individuals (Figure 2.3). Some differences among atolls could be observed as well, for example average loss of allelic richness from wild to farmed collections appeared to be pronounced in Takaroa, but nonexistent in Apataki (Figure 2.3). Overall, it would appear that the analyses performed here had sufficient power to detect a small loss of allelic richness, such as seen among atolls. The absence of significant loss of alleles associated with aggregation types (wild versus farmed) is thus probably not due to a lack of power. These data show little evidence for loss in genetic variability between the wild and adjacent farmed collections of *P. margaritifera*. These results are in agreement with the observations of Arnaud-Haond *et al.* (2003b), which were based on considerably less variable markers.

The presence of null alleles at most of these loci could have a bearing on these conclusions. If non-visible (null) alleles could have been properly detected, this might have resulted in different allelic richness in the various samples. Nonetheless, it is likely that the conclusions reached here are fairly robust to this problem. In an analysis of 166 individuals with 3 loci (Pmarg45, Pmarg68, and Pmarg37), Lemer *et al.* (2011) analyzed all individuals with multiple combinations of various primers for each locus. The authors detected the presence of null alleles and identified the size of the non-amplifying alleles. However, this only resulted in the discovery of one additional allele at one locus. All other null alleles were shown to be alleles that had previously been seen. These alleles were likely not identical by descent, since some had mutations in the priming site while

some did not. However, the presence of null alleles could only hide strong loss of allelic richness in the farmed groups compared to their paired wild samples if the wild groups were systematically characterized by a higher frequency of null alleles. Given that the loci used here are neutral and that the farmed individuals are only one generation removed from the wild oysters, such a scenario is not very plausible.

With notable exceptions of previous studies by Arnaud-Haond *et al.* (2002b, 2003b; 2004), very few studies have looked at the impacts of aquaculture techniques on genetic diversity of farmed stocks that were obtained solely from the collection of wild spat. Much of the literature has focused on genetic impacts of stocks that were propagated in hatcheries. In most instances there were significant decreases in genetic diversity of hatchery stocks compared to the wild stocks from which they originated. This was frequently characterized by reduced allelic richness in numerous hatchery produced species in relation to the wild populations (*Crassostrea gigas*: Appleyard and Ward, 2006; *Hyriopsis cumingii*: Li *et al.*, 2009; *Ostrea edulis*: Lallias *et al.*, 2010a; *Tridacna gigas*: Benzie and Williams, 1996), as well as a displayed loss of rare alleles and significantly altered allele frequencies (*Haliotis rufescens*: Gaffney *et al.*, 1996; *Crassostrea virginica*: Vrijenhoek *et al.*, 1990) for microsatellite and allozyme loci, and even reduced gene diversity in mitochondrial 16S rRNA and COI genes (*Chlamys nobilis*, Yuan *et al.*, 2009). In some cases, loss of genetic diversity can be noticeable in as little as one generation. Hatchery produced abalone in Australia (*Haliotis rubra*) and South Africa (*Haliotis midae*) showed decreases in the number of alleles per locus for the F1 progeny of wild caught broodstocks (Evans *et al.*, 2004). The cultured populations of silver-lipped pearl oyster (*Pinctada maxima*) from Indonesia showed reduced allelic

richness compared to the wild population and an increased mean pair-wise genetic relatedness (Lind *et al.*, 2009). For the black-lippped pearl oyster (*P. margaritifera*) in Japan, it was found that by the third generation of hatchery reared oysters there was as much as an 18% reduction in the number of alleles at allozyme loci and significant changes in allele frequencies with respect to the wild sample (Durand *et al.*, 1993).

While many studies followed this general trend of reduced genetic diversity in hatchery stocks, there were instances, depending on the species cultured and the particular hatchery and its practices, where this was not the case. Cultured populations of the Pacific oyster (*Crassostrea gigas*) in Australia, which were introduced to Tasmania, were compared to "naturalized" populations from the same region, as well as wild populations from Japan, where they originated (English *et al.*, 2000). Here the cultured oysters were derived from hatchery-produced spat, yet showed little reduction in genetic variation compared to the naturalized or Japanese stocks and there was only a small amount of differentiation among all the populations. Similarly, in China where the industry now relies entirely on spat propagated in hatcheries, the cultured stocks of the pearl oyster (*Pincatada fucata*) in China showed similar levels of genetic diversity compared to the wild groups (Yu and Chu, 2006). Interestingly there was significant genetic differentiation (G_{ST}) between and among most populations. This seems to indicate that the particular hatcheries from the above studies are being properly managed and that they are able to maintain genetic diversity within hatchery stocks, an important aspect of the long-term stability and sustainability of the industry.

In the case of *P. margaritifera* in French Polynesia, this study and that by Arnaud-Haond *et al.* (2003b) indicate that the current methods of spat collection associated with

pearl culture practices are not significantly reducing genetic diversity of adult farmed stocks in comparison to adjacent adult wild stocks.

2.4.3. Genetic Differentiation within the Tuamotu-Gambier Archipelagos

It was expected that with greater variability of the molecular marker used here, there would be higher resolution in detecting weaker levels of genetic differentiation. The POWSIM simulations indeed showed that microsatellite markers had greater power to detect weaker levels of genetic divergence, and that fewer samples were required to achieve a desired power of 80% or greater. In fact, for an expected F_{ST} of 0.002, a value very close to the average pair-wise F_{ST} observed both here and in Arnaud-Haond *et al.* (2003b), the simulations showed that the previous study had a very low power while the present study had adequate power given the available sample sizes. Yet the results obtained in this study did not reveal stronger differences in genetic diversity between population types or patterns of genetic structure as compared to the previous study (Arnaud-Haond et al, 2003b). Despite the use of much more variable molecular markers, the genetic differentiation detected in the present study was still quite low and non-significant between all pairs of populations. These results cannot simply be explained by a lack of power, although the original observations (Arnaud-Haond et al, 2003b) could have, therefore, highlighting the value of re-analysing this data set with more variable markers. Another indication that there was no real pattern of genetic differentiation among these populations is that there was no discernible agreement among both sets of markers (Table 2.4). In the study by Arnaud-Haond *et al.* (2003b), the highest and only

significant F_{ST} value was found between the Makemo farmed and the Mangareva wild collections, and the two other highest F_{ST} values also involved the Mangareva wild sample. With the microsatellite data, differentiation between the Mangareva wild samples and all others was very low or nil (Table 2.4). In contrast, the four highest F_{ST} values in the microsatellite data set involved the Takaroa farmed collection, but differentiation between this population and the other was very low with the anonymous nuclear marker data (Table 2.4). Taken together, these results show not only that there was no detectable genetic differentiation among farmed and wild collections in the four atolls, but that there was no discernible genetic differentiation between any sample pair despite the fact that the atolls are separated by hundreds of kilometres. Therefore, it does not appear that the absence of strong genetic differentiation in the previous study (Arnaud-Haond *et al.*, 2003b) was due to the limited power of the markers used.

The present results are different from findings of many studies looking at genetic divergence between wild and farmed populations, especially where the farmed stocks come from closed hatchery systems (Benzie and Williams, 1996; Durand *et al.*, 1993; Li *et al.*, 2009; Yu and Chu, 2006). In these closed-cycle systems, there was generally significant differentiation among wild and farmed populations, as well as within farmed stocks, just as loss of genetic diversity in farmed samples had been observed (see previous section). For the culture of black pearls in French Polynesia, the industry uses almost exclusively the spat of wild oysters caught on collector lines. The recruited *Pinctada margaritifera* is afterwards cultivated in an environment very close to and sometimes overlapping with the wild population. In such an open culture system, the farmed aggregations did not seem to undergo much genetic differentiation from the wild

groups, just as they did not seem to be subject to loss of genetic diversity. This is demonstrated in Table 2.4, which shows no significant differentiation between population types.

At a larger spatial scale, there was little genetic structure between atoll populations, despite the large geographical distances separating them. This is consistent with previous studies by Arnaud-Haond *et al.* (2004; 2008) that have found little genetic differentiation of wild *P margaritifera* stocks from the Tuamotu-Gambier and Society Archipelagos. This may reflect three non-mutually exclusive situations. The first explanation could involve connectivity at a large scale. *Pinctada margaritifera* is a species that exhibits high fecundity and a planktonic larval stage lasting about three to four weeks (Saucedo and Monteforte, 1997). In general, it is expected that marine species with greater larval dispersal capabilities will have high gene flow and be represented by populations with less genetic structure (Palumbi, 1996, and references therein). There are indeed numerous records of such a pattern being observed in natural populations of marine invertebrates (Ayre and Hughes, 2000; Banks *et al.*, 2007; Duda and Palumbi, 1999; McMillen-Jackson and Bert, 2004), including bivalves (Durand and Blanc, 1989; Yu and Chu, 2006). However, gene flow can be limited by oceanic currents and other barriers, which would result in significant genetic structuring despite some species' long-range dispersal abilities (Duda and Palumbi, 1999; Lind *et al.*, 2007; Yasuda *et al.*, 2009). For example, *Pinctada maxima* populations in Indonesia were significantly differentiated from those in Western Australia for which the authors proposed the possibility of little or no gene flow between them (Benzie and Smith-Keune, 2006; Lind *et al.*, 2007). These studies suggested that oceanographic features could act

as barriers and decrease genetic connectivity. In the case of *P. margaritifera*, this could explain significant genetic differentiation of the Marquesas Island populations compared to the other archipelagos, due to the South Equatorial Current and the Marquesas Counter-Current, which could act as barriers that effectively cut off larval supply from the Marquesas Islands to the Northern Tuamotu atolls (and vice versa) (Arnaud-Haond *et al.*, 2003b) (Figure 1.2). In another example, Benzie and Smith-Keune (2006) noticed that most Western Australian populations sampled could be considered all one stock, with the exception of the Exmouth Gulf sample, which was significantly different from the others. Yet, despite the fact that the F_{ST} values were statistically significant, there were still high levels of gene flow. Furthermore, significant genetic differentiation was observed among *P. margaritifera* populations in the Western Pacific [between the Great Barrier Reef and Pacific islands (Kiribati and the Cook Islands)], despite estimates of relatively high gene flow (number of migrants/generation $N_em = 5.0$) (Benzie and Ballment, 1994). However, this last example estimated gene flow using F_{ST} assuming the island model, which is based on many assumptions that are not realistic in natural populations (Whitlock and McCauley, 1999). It assumes for example the same rate and number of migrants among each population, which is also composed of equal numbers of individuals (Whitlock and McCauley, 1999). The likely presence of barriers to gene flow in the study by Benzie and Ballment (1994), as suggested by significant genetic structuring, would seem to indicate that the assumption of equal migration was violated. Therefore, using this method can result in significant biases in estimates of N_em. While it is very important to have a good understanding of ocean circulation and currents to better conceptualize larval transport and connectivity, it is more difficult and time

consuming in practice to acquire detailed surface and deep water current data. Such information for areas surrounding the atolls of French Polynesia comes from only a handful of studies (Martinez *et al.*, 2007; 2009 and references therein). Circulation and transport information is important for understanding larval movements within and surrounding the semi enclosed atolls of the Tuamotu Archipelago where most of the pearl farms are located (e.g. Ahe: Andréfouët *et al.*, 2006). Aside from knowledge of possible gene flow barriers, additional information on this aspect could cast some light on larval transport and connectivity within and between atolls.

The second possible explanation for the observed lack of genetic structuring (especially between Tuamotu and Gambier Archipelago populations pre and post translocation events) is that all local populations died during the last glaciation episode and the present populations were recolonized from a single (or a few) refugium (Arnaud-Haond *et al.*, 2008; Vermeij, 1987). Most atolls within French Polynesia are fairly shallow and only have maximum depths of about 60m (Rougerie, 1995). There is evidence that sea levels during the last glaciation period (~18,000 yr bp) fell to around 121m below current levels (Bard *et al.*, 1996; Fairbanks, 1989). Given this, populations of pearl oysters within the atolls would have become extirpated when the atolls dried out. Assuming some oyster populations survived along the reef fringes of higher islands, the atoll populations could have been recolonized from these refugia with postglacial rises in sea level (Paulay and Meyer, 2002; Vermeiji, 1987). Furthermore, with possibly large effective population sizes and potentially substantial gene flow among populations, the various local populations would not have had enough time to differentiate.

Finally, the current population structure, or absence thereof, could be mostly due to the large scale spat translocations (transfers) that have occurred since the 1980's with the rapid development of pearl culture in French Polynesia (Arnaud-Haond *et al.*, 2004). The authors in that last study had the opportunity to genotype wild populations of *P. margaritifera* from the early 1980's, before the occurrence of mass spat transfers associated with pearl oyster culture. They found significant genetic structuring, especially between populations from the Society, Marquesas and Tuamotu-Gambier archipelagos in the early samples, but noticed that the genetic differentiation between the Society and Tuamotu-Gambier archipelagos in the year 2000 samples was no longer statistically significant. They therefore proposed that the absence of strong population structure currently observed was primarily due to past and present spat translocations. Yet some questions remain: for example both new and old population samples from Mangareva showed no significant differentiation from the Tuamotu populations, even though there have been no recorded juvenile transfers to the Gambier archipelago (Arnaud-Haond *et al.*, 2004). All the early analyses (Arnaud-Haond *et al.*, 2003a, b; 2004; 2008) have been performed with the same three to four anonymous nuclear markers characterized by limited variability. As the present study shows for the four pairs of populations, the power to detect low genetic differentiation was potentially quite limiting with these early markers. A general re-analysis with the current set of microsatellite markers of these samples, complemented by a more thorough sampling of farmed and wild groups coming from locations with and without pearl farms, is probably warranted.

Overall, there were no significant F_{ST} comparisons among or within population types of *P. margaritifera*. This is in agreement with the previous study by Arnaud-Haond *et al.* (2003b), with the exception of the comparison between Mangareva Wild and Makemo Farmed where significant differentiation was observed. While the current pearl culture techniques do not appear to significantly reduce genetic diversity from wild to farmed collections, these results would suggest that low genetic differentiation at large spatial scales may be caused in part by anthropogenic interference, namely juvenile transfers. However, some caution should be taken when interpreting levels of genetic differentiation with the use of microsatellite loci because the presence of null alleles may be partly responsible for low observed gene divergence. After Lemer *et al.* (2011) corrected the genotypes of three *P. margaritifera* populations (Tepoto, Motutunga, and Tuanake) using redesigned primer pairs, they found significant genetic differentiation of the Tepoto population from the other two. Prior to these corrections no significant differentiation was found between these populations, suggesting that null alleles were responsible. Therefore, with the aid of redesigned primer pairs to re-amplify homozygotes and null individuals it might help to resolve issues of low levels of differentiation.

2.5. Conclusions

The present results show that in four atolls, adult farmed aggregations obtained from spat collection did not experience strong mechanism of genetic erosion compared to the local wild collections. Observed decreases in allelic richness at hypervariable

microsatellite loci are powerful indicators of recent bottlenecks. The present study appeared to have enough power to detect slight but significant differences in allelic richness among atolls, but not among population type. The change in corrected allelic richness from wild to farmed samples was not significant. Similarly, no significant genetic differentiation among farmed and wild collections was detected, and more generally no significant genetic structure was observed among the four pairs of samples. Such a result was not a possible consequence of low power as simulations showed that very substantial power was attained with the available sample sizes. Overall, the results presented here are in accordance with those from Arnaud-Haond *et al.* (2003b). These results allow stronger statements to be made about the lack of detectable loss of genetic diversity in or lack of genetic differentiation between adult farmed and wild oysters, because the original study was possibly characterized by low power while the present was not.

Even though the present results indicate that the current spat collection practices do not appear to produce negative genetic consequences, at least in the four pairs of populations studied, genetic analyses of recruitment at a finer scale appear warranted. The individual adults analysed here were most likely derived from a mixture of recruitment cohorts. Although specific information is limited, both wild and farmed adults were probably of various ages and may have been collected in various natural banks (wild) or collector lines (farmed). In other words, the adults analysed here were perhaps recruited at different times and locations, and each of the four farmed and wild samples could have represented a mixture of these individuals. Such a process might

have masked some of the genetic changes that would be associated with spat collection at a finer scale. These aspects are looked at in Chapters 3 and 4.

Chapter 3: Genetic variability and effective number of parents contributing to adults collected at three locations within the Takapoto Lagoon

3.1. Introduction

Little is known about the amount of genetic variation present in the wild populations of the black-lipped pearl oyster that is represented in spat on collectors because the number of parents that successfully produce collected spat is essentially unknown. For these reasons it is essential to better understand how recruitment processes could affect genetic diversity of the collected spat and ultimately the farmed adults, and maybe even the wild populations if the farmed individuals successfully reproduce. In order to do this, factors such as spatial distribution, recruitment, and effective number of parents are important to know. The black-lipped pearl oyster (*Pinctada margaritifera*), like many other marine invertebrates, has high fecundity (Pouvreau *et al.*, 2000b) and potentially wide dispersing planktonic larvae that can stay in the water column for about three weeks (Sims, 1993), similar to *Pinctada mazatlanica* (Saucedo and Monteforte, 1997). One might thus assume that the populations should be quite large and well mixed. However, there is possibility for large variation in the number of offspring successfully recruited to the next generation when the organisms show high mortality in early life stages, which is the case for this species (Friedman and Bell, 1999). If this is so, then the species' effective population size will be lower than the actual population size and one would expect lower genetic diversity of newly settled cohorts compared to the adult population (David *et al.*, 1997c; Hedgecock, 1994a; Li and Hedgecock, 1998). This

highly variable reproductive success has been compared to a "sweepstakes lottery", where there are few big winners and many losers. Due to varying oceanographic conditions that control the transport and mixing of gametes, only a few individuals may contribute their genes to a large proportion of new recruits. This could effectively create chaotic patchiness in the genetic composition of the recruits (Hedgecock, 1994a, b; Hedgecock *et al.*, 2007; Johnson and Black, 1982, 1984; Li and Hedgecock, 1998).

Indeed, such genetic heterogeneity has been commonly observed in marine invertebrates (David *et al.*, 1997c; Moberg and Burton, 2000; Nikiforov, 2000; Watts *et al.*, 1990) including the black pearl oyster (Arnaud-Haond *et al.*, 2008) and has been linked to variable reproductive success (David *et al.*, 1997a). High variation in reproductive success of *Crassostrea gigas* (Boudry *et al.*, 2002; Li and Hedgecock, 1998) and *Ostrea edulis* (Hedgecock *et al.*, 2007) has been shown to occur. Boudry *et al.* (2002) even found uneven parental contribution despite attempts to balance the contribution of gametes between males and females during controlled fertilization. Similarly, Lemay and Boulding (2009) found highly variable parental contribution in hatchery-spawned northern abalone (*Haliotis kamtschatkana*), where in one case a single male sired all offspring in a spawning event. In these examples, a key factor for observing such patterns of variable reproductive success may be high larval mortality. For *P. margaritifera*, it was demonstrated that there is genetic homogeneity among the wild populations over several hundred kilometres, but this was contrasted by small scale (less than 10 km) observations of significant heterogeneity (Arnaud-Haond *et al.*, 2002b; 2008). In the Takapoto lagoon, significant genetic differentiation among three wild beds was observed. At the same fine scales (<10 km), there was significant genetic

differentiation among young adults that had settled on collectors from three closely located lines. It was proposed that this could arise from high variation in the number of adults contributing to the next generation (Arnaud-Haond *et al.*, 2008), implying that reproductive success is quite variable and the effective number of parents could be relatively small (Hedgecock *et al.*, 2007).

Implications of a sweepstakes reproductive success include lower genetic diversity within and greater population structuring between different cohorts of recruits compared to that within and among adult populations (Hedgecock *et al.*, 2007). A further possibility would be that of a much smaller effective population size (*Ne*). Here it is defined as the number of breeding individuals in an ideal population that undergoes the same amount of genetic drift (or rate of random genetic change) as the actual population from one generation to another (Wright, 1931). For marine invertebrates, the effective population size (N_e) tends to be smaller than the actual population size (N) in natural populations (Boudry *et al.*, 2002; Hedgecock, 1994a, b) and hatchery stocks (Hedgecock and Sly, 1990; Hedgecock *et al.*, 1992; Lind *et al.*, 2009). Using experimental outbreeding crosses of *C. gigas*, Boudry *et al.* (2002) observed a reduction of effective population sizes over time. Given this, one would also expect the effective number of parents or breeders (N_b) to be smaller than the census size (N). The effective number of breeders is similar to N_e, except that it deals with processes occurring within a single cohort, in populations with overlapping generations. Indeed, lower N_b to N has been observed in both natural (Hedgecock *et al.*, 2007; Arnaud-Haond *et al.*, 2008) and hatchery-propagated (Hedgecock *et al.*, 1992; Launey *et al.*, 2001) populations of marine bivalves, and marine gastropods (Gaffney *et al.*, 1996). Hedgecock *et al.* (2007) found

that flat oyster (*Ostrea edulis*) juveniles sampled had only 60% of the allelic diversity compared to the parents. They also estimated the effective number of parents to be only 10-20 adults. Similarly, different hatchery populations of *O. edulis* from Quiberon Bay had a reduction in the number of alleles compared to the founding wild population, as well as a low effective size of 3-20 individuals (Launey *et al.*, 2001).

This brings back the question as to whether cohorts of *P. margaritifera* recruits, and in particular those on collector lines, could come from a low effective number of parents. Within the pearl culture industry in French Polynesia, the answer to this could have important consequences. Pearl culture involves the rearing of large numbers of farmed pearl oysters within a single lagoon. Although these oysters occupy a different position in the water column, they are generally in close proximity to and sometimes overlapping with the habitat of wild individuals. If per chance the new farmed recruits, collected in very large numbers on suspended lines, arose from a limited pool of parents then they would have lower genetic variation compared to the wild individuals. In the farmed locations these individuals are protected and removed from predation and other factors that may otherwise cause high juvenile mortality in the wild. Some of the major predators of *P. margaritifera* include fish (pufferfish and triggerfish: Coeroli *et al.*, 1984), as well as gastropods (*Cymatium spp*.) and portunid crabs (Beer and Southgate, 2008; Friedman and Southgate, 1999). If these oysters are allowed to grow to adulthood where they can reproduce and possibly create large amounts of larvae that will settle back into the wild, then this could potentially affect the genetic variability of the wild stocks. Over many generations, negative genetic effects would be amplified. Each new generation of farmed recruits could come from a limited number of wild breeders,

demonstrating lower genetic diversity. If these individuals produce spat that recruit to the wild populations, decreasing its genetic variability, the following cohort of new recruits on collector lines might demonstrate even lower genetic variance and so on. In other words, a significant loss of genetic diversity would be observed primarily in the farmed stocks, which could result in a loss of genetic diversity in the wild stocks over many generations (Arnaud-Haond *et al.*, 2003b).

This chapter examines whether *P. margaritifera* young adults collected on suspended lines resulted from a sweepstakes type reproductive success with low effective number of breeders and strong genetic differentiation at a small geographic scale. Levels of genetic diversity and differentiation between young adults collected at three different zones within the Takapoto Atoll lagoon were compared using 13 microsatellite loci. These samples were previously analyzed by Arnaud-Haond *et al.* (2002b; 2008), who used the same four anonymous nuclear markers as described in Arnaud-Haond *et al.* (2002a). These previous studies observed weak but significant differentiation of Zone C oysters compared to the other two sites, as well as estimated low effective population size (N_e) for all three collector zones. In the present study, the use of three times as many molecular markers exhibiting greater variability should allow us to detect weak levels of genetic differentiation, and to determine if this species demonstrates patterns of a sweepstakes reproductive success and limited numbers of parents contributing to new cohorts of recruits. While the first chapter did no find any significant differences in allelic richness between the wild and farmed collections over large geographic ranges, the analysis of more small scale differences in recruitment is warranted. The individuals analyzed in chapter 1 were wild and farmed adults, which represented different age

classes and possibly different wild beds and farm locations within each atoll. They thus comprised different cohorts recruited at different times and locations, which may have masked genetic processes happening at a smaller scale linked to recruitment. The analysis in the present chapter will further help to determine more definitely whether spat collection methods could lead to deterioration of the genetic resources in the local wild stocks. A better understanding of processes such as reproductive success, recruitment, and effective number of parents contributing to collected spat in atolls of French Polynesia is essential for management and conservation efforts, and beneficial for the long term sustainability of the pearl industry. This could help develop appropriate protocols to maximize the amount of genetic variability of wild populations that is present among recruits on collectors and in farmed individuals, and minimize potential genetic impacts on the natural wild resources.

3.2. Methods and Materials

3.2.1. Marker Complementation and Design of New Microsatellite Markers

Due to high frequency of null alleles, as well as single base-pair polymorphisms at certain loci, it was difficult to score those loci accurately, and departures from Hardy-Weinberg Equilibrium were common. Attempts were made to redesign the existing eight primer pairs, in the hope that by moving the priming region the mutation would be avoided and amplification of more alleles would be achieved. Unfortunately, with the

exception of the redesigned Pmarg44 primer set, none of the redesigned primers yielded products that were easily scored or amplified alleles that were previously null. Fortunately, among the original primers, Pmarg44 and Pmarg79 were found to amplify the same region, but with primer regions slightly overlapping. The result was that Pmarg44 consistently amplified products that were 8 bases shorter than those of Pmarg79 and did not experience single base shifts. Interestingly, extra heterozygotes (amplification of an extra/null allele) were sometimes observed for a particular individual at the Pmarg79 locus. Therefore, Pmarg79 was used to complement Pmarg44 to recover more truly heterozygous individuals that appeared as pseudo homozygotes because of the presence of null alleles. At the same time this provided consistency and verification of scores at this locus.

A similar approach was used by Lemer *et al*. (2011) for three populations with Pmarg37, Pmarg45, and Pmarg68. They showed that it was not possible to find a single primer pair that would eliminate null alleles for a given locus, but by combining the information from two or even three sets of primers for the same microsatellite they were able to recover most of the information and eliminate apparent deviation from Hardy-Weinberg equilibrium.

Table 3.1. The primer sequences for the newly designed EST microsatellite markers for *P. margaritifera*, along with optimal annealing temperature (T°C), repeat motif, observed base pair (bp) range and number of alleles, as well as unbiased gene diversity (H_{nb}) and observed heterozygosity (H_{obs}) for the three collector zone populations within the Takapoto lagoon.

Locus and Primer Sequence	T (°C)	Motif	Range (bp)	No. of alleles	H_{nb}	H_{obs}
Pmarg012 F-CCACCAATGAATTGGAGTGGA* R- GGTCAACAACAACCAAAGTCAACA	58	$(TGTC)_5$	189-322	9	0.561	0.559
Pmarg158 F-TGGTGTTCAGTCTCATCATGCTT R-TTGTTGTCCTGCAAAGGGTTC*	55	$(GACA)_5(CA)_5$	154-192	16	0.724	0.604
Pmarg258 F-GTGAGACGGAATCACGGACA* R-TGCACTGTTTTCAGTGTCAACG	55	$(GACA)_6GGCA(GACA)_7$	152-238	27	0.463	0.450
Pmarg279 F-TCCTGTACCAGCAACTGGAGAA R-CAGACGCCCGACAAAATGAT*	55	$(TCTG)_8$	168-240	16	0.740	0.398
Pmarg442 F-TCCCCATCCATCTGCTTGTC* R-TGGGCCATTGAAGAGGTAACA	55	$(CATC)_6C(GTCT)5$	214-246	16	0.821	0.516

* primer sequence with M13 tail added

In order to have maximum power to detect genetic differentiation and family structuring of the samples in this analysis, five new tetranucleotide microsatellite markers were also designed. These markers were developed from expressed sequence tags (ESTs) for this species; whereas, the original markers which were developed from genomic clones with an enrichment process (Herbinger *et al.*, 2006). The EST sequence information was provided by Caroline Joubert (Centre Océanologique du Pacifique, IFREMER, Vairao, French Polynesia). It was hoped that by targeting microsatellite loci found in ESTs, the prevalence of null alleles and base pair shifts would be reduced. A total of 224 sequences were screened for microsatellite motifs using Simple Sequence Repeat Identification Tool (SSRIT: Temnykh *et al.*, 2001), restricting search parameters to a maximum motif-length group of tetramers with a minimum number of five repeats. Of these, 166 sequences were found to contain di- and tetra-nucleotide microsatellite repeats, which were then input into Perfect Microsatellite Repeat Finder (http://sgdp.iop.-kcl.ac.uk/nikammar/repeatfinder.html). This allowed for better visualization of the motif within the sequence so that manual editing could occur and sequences for primer design could be chosen. Following this, primers were designed around the motifs within 26 sequences with the aid of the program Primer3 (Rozen and Skaletsky, 2000). After primer testing, optimization and try-out, only five tetra-nucleotide markers remained that were used as additional loci for this chapter analysis. The primer information and optimal PCR amplification conditions are summarized in Table 3.1.

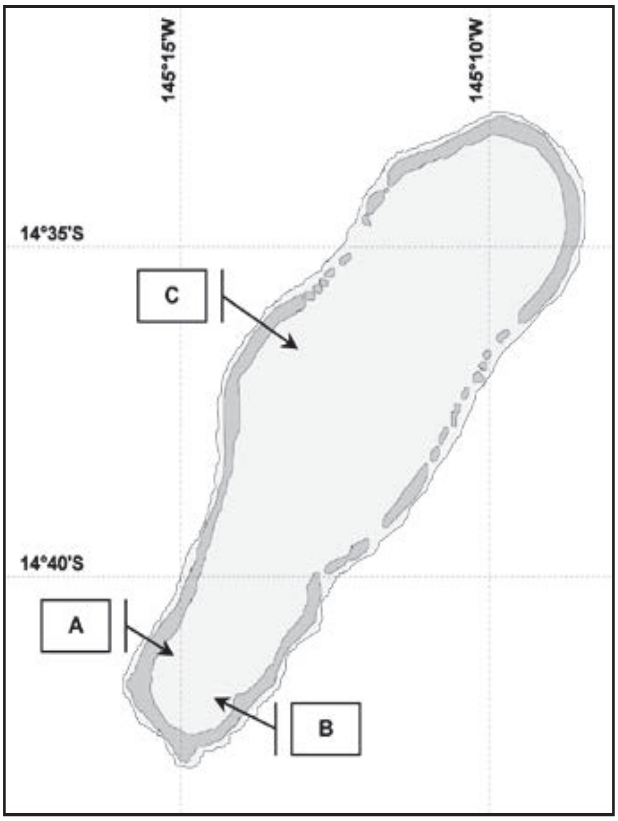

Figure 3.1. The three collector zones (A, B, C) within the Takapoto lagoon where young adult *P. margaritifera* were collected and analysed by Arnaud-Haond *et al*. (2002b, 2008), as well as in the present study. The figure was reprinted from by Arnaud-Haond *et al.* (2002b).

3.2.2. Sampling

Three groups of 10-15 months old oysters (n = 281) were obtained from three commercial collector lines deployed in separate locations in the Takapoto atoll lagoon in 2002 (Figure 3.1). The distance between collector Zones A and B was less than 2km, while a distance of about 10km separated Zones B and C. Zone "A" had a high density of oysters on the collectors, Zone "C" had a low density of oysters, and the adult collector density in Zone "B" was intermediate. These individuals were previously analyzed using four anonymous nuclear markers (Arnaud-Haond *et al.*, 2002b; 2008).

3.2.3. DNA Extraction and Amplification

The DNA was extracted using the glassmilk protocol (Elphinstone *et al.*, 2003). Next, the DNA was amplified through PCR at the eight original microsatellite loci, with the five additional newly designed primer sets to help maximize the power to detect genetic differentiation. Loci Pmarg11, Pmarg45, Pmarg77, and Pmarg79 were highly variable, but accurate allele size determination on the FMBio II fluorescent imaging system was difficult due to large allele ranges, significant gaps between alleles, limited stuttering, and the presence of single base polymorphisms. For these reasons, these four primers were moved to a capillary sequencing machine (Beckman CEQ 8000) instead of the fluorescent imaging platform.

3.2.4. Data Analysis

The raw data from the CEQ platform were binned in order to normalize the variation in the automated scoring process. For example, two alleles scored as 177.32 and 178.10 bases in length could be the same real size (178 bases), but could have been wrongly binned depending on bin sizes. Due to different migration rates of the alleles through the gel in the capillaries, it was normal to see ranges in scores up to one base difference in either direction. This problem was exacerbated by the presence of real single base polymorphisms, which is why it was necessary that these upper and lower limits be properly defined prior to data analysis. The raw CEQ data were binned using the MsatAllele 1.01 R Package (Alberto, 2009). This allele binning program was quite

comprehensive, flexible, and it allowed the visualization of the allele bins, which also helped with corrections.

For each locus, the dataset was tested for possible null alleles and scoring errors using Micro-Checker (van Oosterhout *et al.*, 2004). Hardy-Weinberg Equilibrium was tested among the newly designed loci using Arlequin 3.5.1.2 (Excoffier, 2010), running the simulations for 10000 permutations. This was important to ensure that there were no inherent problems with these new markers (such as excess homozygotes which could lower the power to detect weaker differentiation). Genetic diversity within and among individuals at the three collector sites was compared with Microsatellite Toolkit (Park, 2001) and FSTAT (Goudet, 2001). Genetic diversity was estimated based on levels of observed (H_{obs}) and expected (H_{nb}, Nei's unbiased gene diversity) heterozygosity (Nei, 1987), as well as allelic richness. Furthermore, the distribution of genetic variation among the three collector populations was measured with an Analysis of Molecular Variance (AMOVA) (GenAlEx 6.41: Peakall and Smouse, 2006), based on pair-wise differences. The significance of these estimates was tested by 10000 random permutations. Also, evidence for systematic difference in allelic richness between young adult populations on collectors within the three sampling zones was tested by two-way ANOVA for a block design, without replication. This was done in two ways, the first with Collector and locus as a block, and the second with Zone and locus as a block.

In order to see if there was genetic differentiation at a small spatial scale among collectors or zones, the various samples were compared using estimates of pair-wise F_{ST} (GenAlEx), based on the estimator θ (Weir and Cockerham, 1984). Analyses were repeated in FSTAT and Arlequin to verify the consistency within the results output. The

significance of these estimates was tested by 9999 random permutations of individuals between samples and by using Bonferroni correction for multiple tests. To assist in visualization of the potential 'population' differentiation within the Takapoto lagoon and to verify that collectors were grouped in an appropriate manner representative of the actual population structure for the above analysis, Structure 2.3.3 (Pritchard *et al.*, 2000) was run. This model-based clustering method infers population structure based on genotype data and assigns individuals to a population, and can identify migrants and admixed individuals.

Finally, the effective population size (N_e) and the number of breeders that contributed to the individuals on the collectors at the three sites were estimated. First, N_e was estimated using calculations of linkage disequilibrium, a method that was employed by the program LDNE (Waples and Do, 2008). Second, the numbers of progenitors of collector samples was estimated by reconstructing full-sib and half-sib relationships using Pedigree 2.2 (described in Butler *et al.*, 2004; Herbinger *et al.*, 2006; Smith *et al.*, 2001). In order to reconstruct sibship or kin groups, this program uses a Markov Chain Monte Carlo (MCMC) approach with co-dominant markers to generate partitions of individuals. The analysis was run with full-sib constraint, in which case the full-sib family group genotypes follow strict Mendelian rules, and without constraint, in which case individuals are partitioned into kin groups. These kin groups are composed of mixed related individuals such as full-sibs and half-sibs, with one advantage being that the analysis is quite robust to the presence of genotype errors (Herbinger *et al.*, 2006).

3.3. Results

3.3.1. Heterozygosity, Hardy-Weinberg Equilibrium and Genetic Diversity

FSTAT and Microsatellite Toolkit were used to measure allelic richness and heterozygosity. There were considerable decreases in heterozygosity from expected to observed values (Table 3.2). Unbiased gene diversity (H_{nb}) and observed heterozygosity were lowest in Zone C individuals, and ranged from 0.77 (Collector 1) to 0.81 (Collector 4) and from 0.56 (Collector 1) to 0.62 (Collector 2), respectively.

Table 3.2. The unbiased expected heterozygosity (H_{nb}) as well as observed heterozygosity (H_{obs}) per collector within the three zones in the Takapoto Lagoon, averaged over all 13 loci, but not corrected for smallest sample size.

Collector Zone	Collector Number	Sample Size	H_{nb}	H_{obs}
Zone C	Collector 1	37	0.77	0.56
Zone B	Collector 2	52	0.81	0.62
	Collector 3	41	0.78	0.61
Zone A	Collector 4	50	0.81	0.57
	Collector 5	50	0.81	0.57
	Collector 6	50	0.80	0.57

Furthermore, it was determined that most loci were not in Hardy-Weinberg equilibrium (HWE) for most populations, except for Pmarg37, Pmarg012, and Pmarg258. However, this changed depending on the collector "population" being sampled. For example, loci Pmarg44 and Pmarg158 were in HWE for the collectors of zones B and C, but not A. Estimates of HWE were also done in GenAlEx, with similar conclusions. The loci most often in HWE were Pmarg37, 44, 012, and 258. This varied somewhat

depending on the collector sample being analyzed. In general, these four markers seem to be less prone to departures from HWE, as they also had lower incidence of null alleles (as estimated by Micro-Checker) (Table 3.3).

Gene diversity was high, with the total number of different alleles ranging from 9 (Pmarg012) to 38 (Pmarg45). The mean number of alleles was highest for Zone A collector samples and lowest for Zone C collector samples (MNA in Table 3.4). Using allelic richness (standardized for lowest sample size n = 30), individuals from Zone A (Collectors 4,5,6) had higher values compared to the other two collector zones for most loci, except for Collector 2 (Zone B), which also had high allelic richness (Table 3.4). In a two-way ANOVA of richness with factors Collector and Locus (as a block), a significant effect of collector was detected ($F = 4.30$ with 5 & 60 df, P-value = 0.002) and not surprisingly a very strong effect of locus ($F = 124.6$ with 12 & 60 df, P-value < 0.001). Subsequent multiple comparison revealed that Collectors 4 and 5 (Zone A) had significantly higher allelic richness than Collector 3 (Zone B) and Collector 4 had nearly significantly (P-value = 0.056) higher richness than Collector 1 (Zone C). With the same analysis performed by Zone, with the collectors pooled, there was a significant effect of Zone ($F = 4.33$ with 2 & 63 df, P-value = 0.0017) and of course of locus again. Zone A had significantly higher allelic richness than Zone B and nearly significantly higher richness than Zone C (P-value = 0.075), but allelic richness was not different between Zone B and Zone C.

Table 3.3. The estimated null allele frequencies per locus for each *Pinctada margaritifera* collector population (Zones A, B, C), obtained using the methods by van Oosterhout (see Ousterhout *et al.*, 2004). Bold values indicate loci which have been estimated to have the presence of null alleles.

	Zone C	**Zone B**		**Zone A**			
	Collector 1	Collector 2	Collector 3	Collector 4	Collector 5	Collector 6	**Mean**
Pmarg2	**0.2516***	**0.2849***	**0.2424***	**0.2451***	**0.2393***	**0.2296***	0.2488
Pmarg7	**0.0960***	**0.1458***	**0.1019**	**0.1872***	**0.1726***	**0.1048***	0.1347
Pmarg11	**0.1614***	**0.0877***	**0.1197***	**0.1698***	**0.0691***	**0.1888***	0.1328
Pmarg37	0.0483	-0.0636	0.0201*	0.0024	0.0046	0.0409*	0.0194
Pmarg44	0.0062	-0.0313	-0.0746	-0.0064*	**0.0702***	0.0290	0.0176
Pmarg45	**0.2077***	**0.1135***	**0.1687***	**0.1174**	**0.1232***	**0.1169***	0.1412
Pmarg68	**0.2323***	**0.2048***	**0.2260***	**0.2438***	**0.2624***	**0.2228***	0.2320
Pmarg77	**0.2335***	**0.2473***	**0.1427***	**0.1553***	**0.1518***	**0.2162***	0.1911
Pmarg012	-0.0382	0.0112	-0.0483	-0.0004	-0.0648	0.0766	0.0146
Pmarg158	-0.0013*	**0.0936***	0.0670*	0.0523*	**0.1577***	0.0644*	0.0725
Pmarg258	-0.1911	0.0616	-0.2032	0.0634*	-0.0533	0.0675	0.0321
Pmarg279	**0.2698***	**0.2293***	**0.0866***	**0.2291***	**0.2745***	**0.1996***	0.2148
Pmarg442	0.0625	0.0197	**0.1844***	**0.2696***	**0.2883***	**0.2285***	0.1755

Negative values produced by the estimation method indicate null allele frequencies that were essentially zero.
* = Population samples that were not in HWE for that locus.

Table 3.4. Allelic richness per locus and collector site, standardized for smallest sample size of 30, average allelic richness over all loci, as well as the mean number of alleles (MNA) per collector, with standard deviation. The total sample size for each collector population is shown in brackets.

	Zone C	Zone B		Zone A			
Locus	Coll. 1 (37)	Coll. 2 (52)	Coll. 3 (41)	Coll. 4 (50)	Coll. 5 (50)	Coll. 6 (50)	Overall
Pmarg2	20.17	20.13	16.62	18.84	20.85	19.38	20.45
Pmarg7	10.55	9.69	8.81	11.43	8.44	10.86	12.15
Pmarg37	15.00	13.63	16.11	17.20	16.47	16.53	16.00
Pmarg44	11.24	12.26	11.31	10.44	14.73	10.55	12.09
Pmarg68	5.86	8.33	6.69	8.54	6.55	8.68	8.30
Pmarg11	21.62	22.45	22.96	24.25	23.62	20.52	23.41
Pmarg45	18.21	22.29	18.21	23.03	20.38	21.02	21.52
Pmarg77	17.48	17.59	15.43	18.54	19.34	16.09	18.61
Pmarg012	4.87	6.55	5.62	6.62	5.49	6.60	6.04
Pmarg158	7.81	8.93	7.38	9.48	9.66	7.24	8.68
Pmarg258	10.39	12.17	9.11	11.70	11.00	12.61	11.23
Pmarg279	9.92	9.66	10.59	11.40	8.29	9.67	10.75
Pmarg442	10.00	9.07	6.97	9.29	10.42	8.97	9.18
Average	12.55	13.23	11.99	13.90	13.48	12.98	
MNA	13.15 ± 5.74	15.54 ± 6.51	13.00 ± 6.01	15.85 ± 6.89	15.38 ± 7.01	14.62 ± 5.87	

3.3.2. Genetic differentiation

Genetic differentiation between all collector samples was estimated using GenAlEx. The results from Pair-wise matrices of F_{ST} values were constructed in two ways for GenAlEx: the first was using all collector sites as six separate 'populations'; and the other by combining collector samples from the same zones giving three 'populations'. Combining the collector samples also increased the sample number as well as the power of the analysis. All pair-wise F_{ST} values from the microsatellite data were quite small; however, most comparisons between collectors were significant (Table 3.5). Similar results were obtained with FSTAT and Arlequin. The collector from Zone C was significantly different from one of the Zone B collectors and all three Zone A collectors, and two Zone B collectors were significantly different from two of the Zone A collectors. There was no significant differentiation among the two collectors from Zone B or among the three collectors from Zone A. This shows that within zone, the different collectors seem to be samples from the same population of recruits. On the other hand, the great majority of the pair-wise F_{ST} comparisons among collectors from different zones are significant, despite being quite small. Another way to look at this is that there were 15 comparisons among collectors. Four comparisons were among collectors within the same zone and all four were non-significant. The other eleven comparisons were among collectors across zones and eight of these comparisons were significant. This indicates small but significant differentiation among the three zones. Indeed when all the collectors within Zone B and Zone A were pooled, the three F_{ST} comparisons across zones were significant (Table 3.6).

Table 3.5. Pair-wise F_{ST} matrix of pearl oyster samples from three collector zones in the Takapoto lagoon, based on 13 microsatellite loci. F_{ST} values from the present study are below the diagonal, while those from Arnaud-Haond *et al.* (2002b) are shown above the diagonal.

	Collector Zone					
	Zone C	Zone B		Zone A		
	Collector 1	Collector 2	Collector 3	Collector 4	Collector 5	Collector 6
Collector 1	-	**0.023**	**0.023**	0.015	**0.039**	**0.025**
Collector 2	0.0018	-	0.007	0.009	-0.001	0.004
Collector 3	**0.0115**	0.0032	-	0.004	0.013	0.004
Collector 4	**0.0075**	0.0005	0.0043	-	0.003	0.008
Collector 5	**0.0120**	**0.0077**	**0.0079**	0.0002	-	0.007
Collector 6	**0.0153**	**0.0079**	**0.0065**	0.0012	0.0020	-

Level of significance $\alpha = 0.05$. Significance testing [9999 permutations present study, 1000 permutations from Arnaud-Haond *et al.* (2002b)]. Values in Bold below the diagonal are significant after Bonferroni correction.
New threshold after Bonferroni correction: 0.003333333

Table 3.6. Pair-wise F_{ST} matrix of pearl oyster samples from three collector zones in the Takapoto lagoon, based on 13 microsatellite loci. The F_{ST} values are below the diagonal, while the corresponding probability values are shown above the diagonal.

	Collector Zone		
	Zone C	Zone B	Zone A
Zone C	-	**0.0023**	**0.0001**
Zone B	**0.0053**	-	**0.0001**
Zone A	**0.0110**	**0.0046**	-

Level of significance $\alpha = 0.05$. Significance tests (9999 permutations).
Those values in bold are significant after Bonferroni correction, new threshold: 0.016667.

These results were also borne out by the GenAlEx AMOVAs. This was repeated in Arlequin with similar results. In all cases it was found that the greatest proportion of the variation was seen within individuals (68%), but there was still a large proportion of variation among individuals (31%). Although very little of the variance was distributed among the three locations (Table 3.7), the little variation that was seen (only 1%) was still significant. The large source of variance within individuals was more than likely due an effect of locus since each locus was quite variable in the number of alleles observed.

This seems to be in agreement with the low but significant levels of population differentiation that were seen in the pair-wise F_{ST} comparisons.

Table 3.7. Summary AMOVA table for the proportion of genetic variance distributed among the collector populations within the Takapoto Lagoon. The data were separated and analyzed as three populations – each collector zone was considered a "population".

Source	DF	SS	MS	Est. Var.	%
Among Pops	2	24.871	12.435	0.033	1%
Among Indiv.	277	1935.834	6.989	1.679	31%
Within Indiv.	280	1016.500	3.630	3.630	68%
Total	559	2977.205		5.343	100%

Overall these results were consistent with the observations about genetic diversity (section 2.3.1). The recruits in the three zones appear to represent three "cohorts" which were weakly, but significantly differentiated and were characterized by different levels of genetic diversity.

3.3.3. Structure Analysis

This dataset was also run through Structure 2.3.3 (Pritchard *et al.*, 2000) to see if collectors/zones would be grouped in three clusters. The previous analyses showed small but significant differentiation between Zone A, B and C. However, it would appear that Structure was not able to recover any of the small differences that were observed among zones. There was no evidence of any separation of the three zones along any supposed clusters (Figure 3.2). Also, when looking at the Ln likelihood [Ln P(D)] estimates, the most consistent and lowest values were those for K = 1 or 2 (Table 3.8). The different Ln

P(D) values obtained for K = 1 were close to one another, but substantial variation was observed for K = 2, indicating that the program had a difficult time finding the same "peak". This program appeared to be unable to detect the very small levels of population differentiation seen in the F_{ST} seen in the analysis above, and suggested that we might be looking at one panmictic population or maybe two.

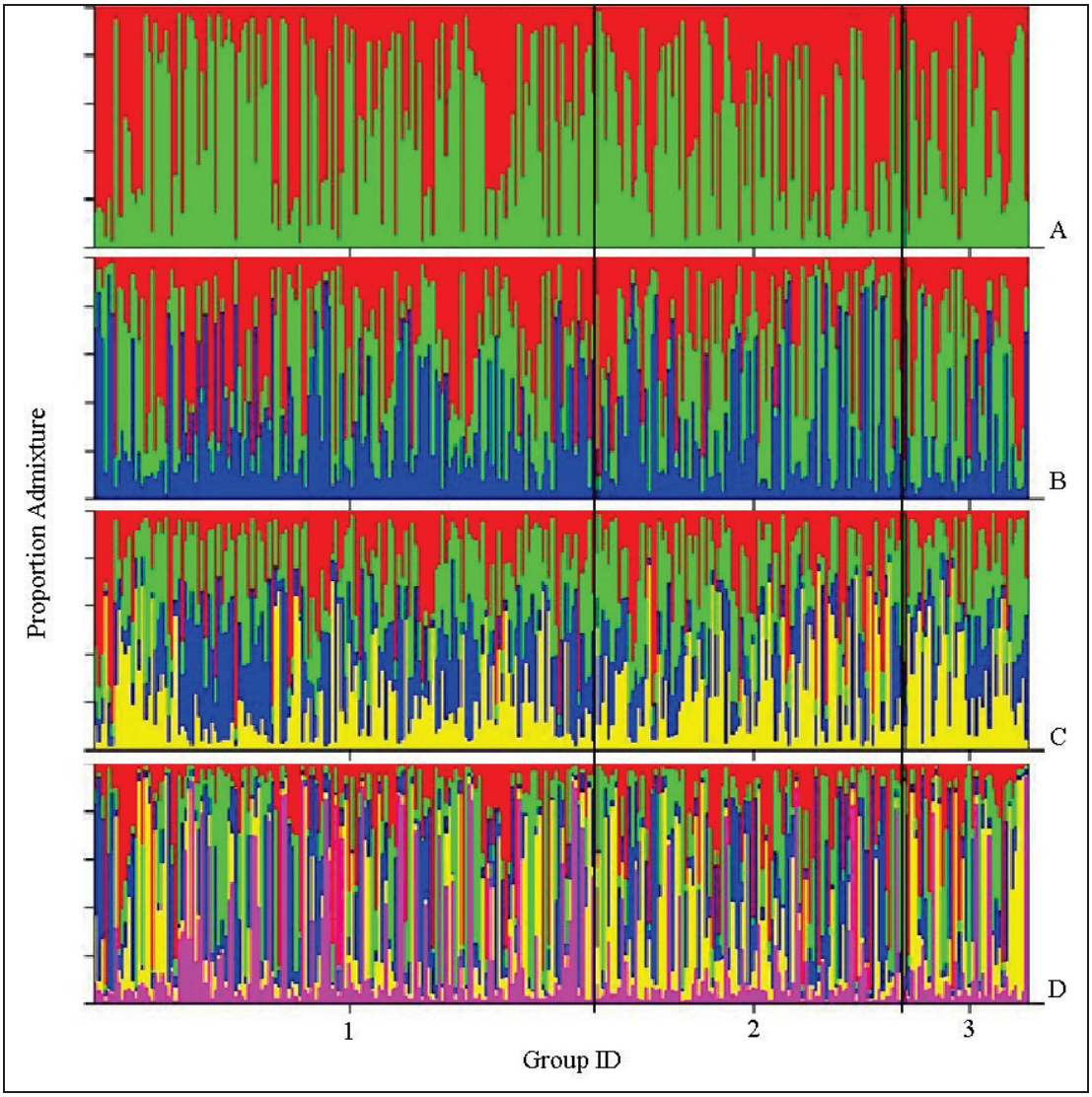

Figure 3.2. Parameter space exploration estimating the number of genetically based populations or clusters (K), based on 13 microsatellite loci in pearl oysters. A, B, C and D correspond to K=2, K=3, K=4 and K=5 clusters, respectively. Group 1 corresponds to all collector samples from Zone A, Group 2 represents Zone B, and Group 3 represents Zone C individuals.

Table 3.8. The ln likelihood [Ln P(D)] values for each proposed number of populations, K, at each run for the individuals from the three collector sites in the Takapoto Lagoon, based on Structure analysis.

	Run 1	Run 2	Run 3
K = 1	-15662.9	-15663.7	-15662.4
K = 2	-15769.7	-15931.1	-15575.9
K = 3	-15374.4	-15418.0	-15460.7
K = 4	-15450.9	-15386.2	-15305.5
K = 5	-15200.3	-15149.9	-15183.3

- 100000 iterations were performed for each K.

3.3.4. Family Pedigree Reconstruction and the Number of Breeders

Effective population size (N_e) was estimated using linkage disequilibrium in the program LDNE (Waples and Do, 2008). This was first done by separating the populations by collector zones (A, B, and C) and saving each as a different file. Each population was analyzed separately within the program. Furthermore, estimates of N_e were taken at different thresholds for lowest allele frequency permitted in the analysis (10%, 5%, 2%, and 1%). All estimates of N_e were large and had an upper confidence interval limit of infinity. For Collector Zone A, the lowest estimate of N_e was 847 (211.9 - ∞, 95% CI) using only allele frequencies of 10% or higher (Table 3.9). The LDNE program gave large negative numbers in most other cases, which indicates that there was insufficient information in the sample for LDNE to correctly estimate N_e. Other than the case of Zone A (150 individuals pooled) with a lowest allele frequency of 10%, the only positive estimates were for Zone B (93 individuals pooled). In these cases LDNE was able to return estimates of N_e = 3450.6 (850.5 - ∞, 95% CI) and N_e = 6321.2 (750.4 - ∞, 95% CI) and with lowest allele frequencies of 1 and 2% respectively (Table 3.9). The program LDNE was poorly able to produce credible results and the few positive

estimates of N_e were quite large, much larger than those estimated for the same collection zone populations in previous studies (Arnaud-Haond *et al.*, 2002b, 2008).

Table 3.9. Estimates of effective population size (N_e) based on calculations of linkage disequilibrium from the program LDNE. The 95% confidence interval is included, along with N_e estimated at different lowest levels of allele frequencies permitted in the analysis (1%, 2%, 5%, and 10%).

Collection Zone	**Lowest Allele Frequency**	**Ne (95% CI)**
Zone A (N = 150)	0.1	847 (211.9 - ∞)
	0.05	-2332 (1014.1 - ∞)
	0.02	-11669.4 (1596.7 - ∞)
	0.01	-2363.1 (13456.9 - ∞)
Zone B (N = 93)	0.1	-381 (625.2 - ∞)
	0.05	-1322.9 (717.4 - ∞)
	0.02	6321.2 (750.4 - ∞)
	0.01	3450.6 (850.5 - ∞)
Zone C (N = 37)	0.1	-83.1 (10294.6 - ∞)
	0.05	-209.3 (1140.2 - ∞)
	0.02	-465.8 (936.7 - ∞)
	0.01	-245.5 (-614.3 - ∞)

Significance tests (10000 permutations).

Another method used to see if the cohorts recruited on the collectors might have originated from a small number of parents was the reconstruction of sib-ship relationships. Overall, the pedigree analysis did not reveal any significant family or kin structuring. Group sizes were very small (1 to 3) and the number and size of the groups did not change very much without a full-constraint on the simulation (Figure 3.3). The oysters that were found on the collectors in each zone did not appear to be related to one another, and they did not appear to have originated from a limited number of parents.

This might indicate that the number of parents at the head of the cohorts was relatively large. It could also be that the young adults which had been collected were in fact a mixture of many different cohorts and any family signature that might have been present in cohorts taken separately could have been masked.

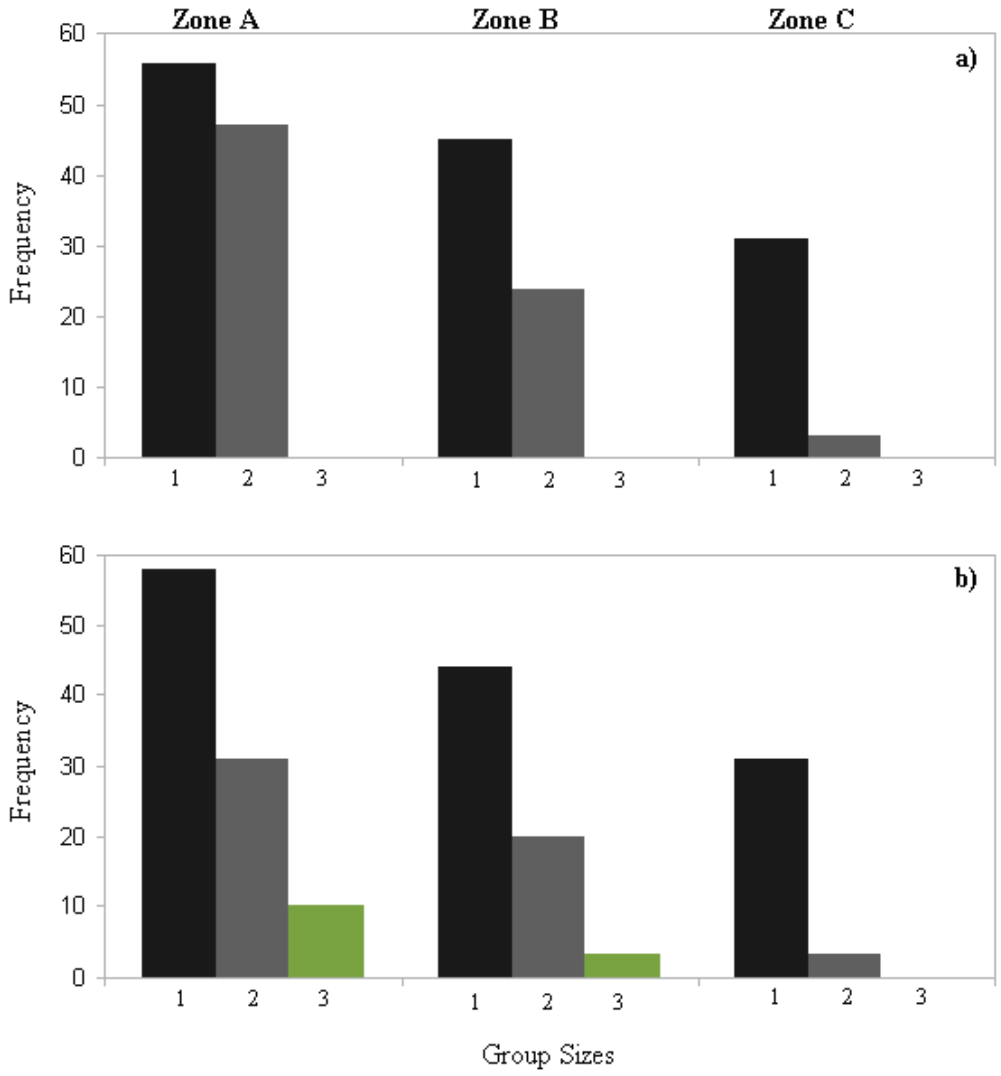

Figure 3.3. Frequency distribution of family group sizes from the Pedigree analysis for three collector zones (A, B, C) within the Takapoto Lagoon. All collectors were pooled within sampling zone. The family group sizes are shown for the simulation a) with full-sib constraint and b) without full-sib constraint.

3.4. Discussion

3.4.1. Distribution of Genetic Diversity at a Small Spatial Scale

An excess in homozygotes was observed once again, which was responsible for the deviations from HWE (Table 3.2). Deviations from HWE can be caused by different processes, but here again they were most likely caused by the presence of null alleles at high frequencies (Table 3.3). Lemer *et al*. (2011) showed that strong, significant departures from HW expectation in 3 populations at 3 loci (Pmarg37, Pmarg68, Pmarg45) were eliminated when multiple primer sets were used in combination to reduce the presence of null alleles. This is much like what was done here with Pmarg44 and Pmarg79 to help recover some of the non-amplified alleles. This primer complementation helped amplify 15 extra alleles, increasing the number of heterozygous individuals. It should be noted that no new alleles were amplified, all had been previously observed; however, in a couple instances it was a rare allele that was amplified.

Of the original loci, Pmarg37 and Pmarg44 were closest to HWE in this and the previous chapter, probably due to the fact that they had lower incidence of null alleles (Table 3.3). The newly developed loci were less variable than most of the old loci and three of these new loci also appeared to be less prone to null alleles (Table 3.3). Lastly, there were variations among populations as to whether certain loci experienced HWE and were estimated to have higher prevalence of null alleles. These differences could be due to simple chance, especially due to sampling if the sample size is not very large, or it

could be due to the particular origin (i.e. parents with or without null alleles) of the various recruits on collectors. Arnaud-Haond *et al*. (2002b) also observed significant departures from Hardy-Weinberg equilibrium in five of the six collectors due to heterozygote deficits using 4 anonymous markers. It was thought that the primary reason for these deficits was PCR artefacts such as null alleles (Arnaud-Haond *et al.*, 2003a).

At a small geographic scale, there were indications that there could be significant differences in the amount of genetic diversity seen among *P. margaritifera* recruits. The collectors from Zone A were found to have higher gene diversity compared to the other two collector zones. These observations were in general agreement with Arnaud-Haond *et al.* (2008), although in her case it was mostly apparent that the Zone C collector was less variable compared to the collectors in Zone A and B. A potential explanation is that the number of parents that contributed to collector Zone A recruits was higher than Zone B or C. Also, Zone C and to a lesser extent Zone B were characterized by having lower recruitment densities, while Zone A had the highest. This indicates that the cohorts that recruited at locations B and C might have been less numerous in the water column and/or did not encounter favorable local conditions for settling and surviving as compared to Zone A. The fact that both density and allelic diversity were higher in Zone A might be an indirect indication that the recruits in Zone A could have originated from a larger number of parents. The results from this chapter showed that there can be differences in genetic diversity at a small geographic scale.

These ideas are in accordance with Hedgecock's theory of a "sweepstakes-chance" reproductive success. Marine bivalve species such as pearl oysters tend to live in large populations, have high fecundity, and have extended planktonic larval stages.

Under these conditions there would be potential for great larval dispersal and, as a result, we would expect to see genetic homogeneity, even at large distances (David *et al.*, 1997c). However, like many marine invertebrates, bivalves also display high mortality in early developmental stages (Friedman and Bell, 1999; Pit and Southgate, 2003), which creates the potential for large variances in reproductive success (Li and Hedgecock, 1998). Indeed *Pinctada margaritifera* has displayed large variation in recruitment (Fairbairn, 2009; Friedman *et al.*, 1998). In such circumstances one would suspect that relatively few individuals may be successfully reproducing or rather there is a low effective population size (N_e) or number of breeders (N_b) at the source of cohorts (Hedgecock, 1994a, b). The successful reproduction of few parents as a result of chance situations causing high mortality is known as "sweepstakes reproduction" and could lead to genetic heterogeneity or "genetic patchiness" (Arnaud-Haond *et al.*, 2008; David *et al.*, 1997a; Flowers *et al.*, 2002; Hedgecock, 1994a). One of the genetic signatures of high variance in reproductive success includes lower genetic diversity in newly recruited cohorts compared to the adult population. These "patches" represent different spawning events from potentially different sets of individuals and different numbers contributing to reproduction, which vary depending on environmental changes (oceanographic conditions). This type of reproduction is likely responsible for observations of high genetic variation at a small spatial scale (between cohorts or within), but little overall differentiation across larger spatial scales (between populations and atolls, see chapter 2). Similar patterns described as the "chaotic" distribution of genetic diversity have been observed in fishes (Hogan *et al.*, 2010; Pujolar *et al.*, 2011), invertebrates (Ayre and Hughes, 2000; Banks *et al.*, 2007; McMillen-Jackson and Bert, 2004) and bivalves

(Kenchington *et al.*, 2006; Lallias *et al.*, 2010b; Li and Hedgecock, 1998; Taris *et al.*, 2009), including *P. margaritifera* (Arnaud-Haond *et al.*, 2008).

Finally, there lies another possible explanation for the observation of differences in genetic diversity at a small spatial scale within the Takapoto Lagoon. It may be that the recruits in Zone C (and in B to a lesser extent) were the result of one or a few spawning events (one or a few cohorts), whereas the recruits in Zone A represented a mixture of various cohorts that may have settled at different times on the same collectors, grew together and were then indistinguishable when they were harvested 10-15 months or so later. In other words, the higher density and higher genetic diversity observed in Zone A may simply reflect settlement of more cohorts than in Zone B and C, but each cohort was similar in terms of intrinsic diversity and estimated number of breeders.

The differences in genetic diversity observed here between Zones B/C and Zone A within the Takapoto Lagoon were also more pronounced than what had been seen in the second chapter. The previous chapter showed that Takaroa had slightly lower genetic diversity than the other atolls. This shows that there could potentially be substantial genetic variation at very small geographic scales.

3.4.2. Spatial Genetic Heterogeneity

In general it is thought that marine species with widely dispersing planktonic larvae tend to be genetically homogeneous over broad geographical ranges (Hedgecock, 2009). This tends to hold true for *P. margaritifera*, particularly in French Polynesia (Arnaud-Haond *et al.*, 2004, 2008; Benzie and Ballment, 1994). However, despite

genetic homogeneity at broad spatial scales, there can be variances in allelic frequencies or patchiness occurring among samples only short distances apart (sometimes equaling the amount of genetic variation on the order of 100's to 1000's of kilometres) (Arnaud-Haond *et al.*, 2008). This is no exception for the black-lipped pearl oyster, which is highly fecund and appears to display large variances in reproductive success. The present study showed that there was no significant differentiation among collectors within the same collection zone (Table 3.5), implying that the individuals that settled a few meters apart probably came from the same population of larvae. However, there was weak but significant genetic differentiation (estimated by pair-wise F_{ST}) between the recruits across the three collection zones (Table 3.5, 3.6), all less than 10 km apart. They were also characterized by different levels of genetic diversity (estimated by allelic richness). The analyses in this chapter were in general agreement with previous studies based on a limited number of less variable markers (Arnaud-Haond *et al.,* 2002b, 2008). With a larger number of hypervariable markers, a stronger and clearer pattern of genetic differentiation emerged where nearly all pair-wise F_{ST} comparisons across zones were significant even after Bonferroni correction (8 out of 11 comparisons), while only the collector from Zone C was significantly different from other collectors in the previous studies (4 out of 11 comparisons).

Interestingly, the Structure analysis failed to reveal any clear pattern of population structure. Even though the best Ln likelihood [Ln P(D)] was seen for a number of clusters of K =2, much variation was seen among different runs, which indicates that the program was not finding a clear stable distribution in clusters. Nearly as good a Ln likelihood was seen for a cluster of K = 1. Figure 3.2 clearly showed that for any number of clusters

from 2 to 5, the individuals from Zones A, B and C were not allocated as expected among Zones. This is most probably a consequence of the high level of null alleles at most loci. Structure operates by allocating the individuals into a given number of clusters in a way that will minimize deviation from Hardy-Weinberg expectations within each cluster. In other words, the assumption is that the overall sample is constituted of different subpopulations each in H-W equilibrium, and the overall sample exhibits deviations from H-W expectation because of Wahlund effects. However, if most of the loci have a high frequency of null alleles, the program will be unable to find a way to allocate individuals to eliminate H-W deviation in each of the cluster. Null alleles are also known to create problems for accurate estimation of pair-wise F_{ST} (Chapuis and Estoup, 2007), but it would appear that the F_{ST} analysis was more robust to these null allele problems that the Structure analysis.

These results would be consistent with expectations given that *P. margaritifera* is a species that likely displays a sweepstakes recruitment pattern, especially considering indirect evidence of high levels of early stage mortality (Friedman and Bell, 1999; Pit and Southgate, 2003). Individuals that recruit in a specific location (here the collectors in the 3 zones) may have been produced by different sets of parents leading to fine scale genetic structure. Fine-grained genetic structure or patchiness has also been observed for a number of other marine organisms (*crustaceans*: Marino *et al.*, 2010; Silva *et al.*, 2009; *fish*: Hogan *et al.*, 2010; Selkoe *et al.*, 2006; *molluscs*: Casu *et al.*, 2005; David *et al.*, 1997c; Gilg and Hilbish, 2003; Taris *et al.*, 2009; Todd *et al.*, 1998), where unpredictable ("chaotic") changes in genetic structure occurred spatially and/or temporally. Interestingly, a study comparing nine population samples of the nudibranch *Adalaria*

proxima over a 26.18 km range in Scotland, Todd *et al.* (1998) observed similar levels of differentiation compared to populations sampled over a 1600 km range, as well as an inverse relationship between geographic distance and genetic differentiation. Small-scale genetic heterogeneity was also observed in the bivalve *Gemma gemma* along the coast of Virginia and Maine (Casu *et al.*, 2005). Here there was significant differentiation between individuals and sample patches, but low levels of genetic diversity between patch locations. The greatest proportion of the genetic variation was found within patches (61%) (10 metres), followed by among patches within localities (37%) (100 metres apart), and then between localities (2%) (between Virginia and Maine). For the oyster *Pinctada maxima* throughout the Indo-Australian Archipelagos, 89.4% of the genetic variation was distributed within individuals, 7.9% among individuals within populations and 2.7% among populations (Lind *et al.*, 2007). This is similar to the AMOVA results from the present study where 68% of the genetic variance was distributed within individual oysters, 31% among individuals within zones, and 1% between populations (collector zones). Although the Lind *et al.* (2007) study was concerned with a different species and similar markers (microsatellites), it is striking that similar (low but significant) levels of genetic variation were distributed between "populations" located a few kilometres apart and across the Indo-Australian archipelago.

As mentioned above, it is important to note that the individuals sampled at each location here were young adults (10-15 months old), which could have been a combination of recruits spawned at different times and different combinations of genitors. In effect the true genetic signal could be masked. A much finer spatio-temporal analysis of recruitment is therefore warranted. The study by David *et al.* (1997c) analysed

temporal cohorts of the surf clam *Spisula ovalis* along the European Atlantic coasts. While F_{ST} estimates computed were low, there was significant genetic differentiation in time (among cohorts, $P<0.001$), space (among sites, $P = 0.004$), and both (among groups, $P<0.001$). Therefore, cohort analysis of *P. margaritifera* should be undertaken to better reflect the true dynamics of recruitment.

3.4.3. Estimated Number of Breeders Contributing to Recruits on Collectors

There lies great potential in species that have high fecundity and high larval mortality to have great variance in reproductive success. For most species, regardless of life history details, effective population sizes (N_e) tend to be lower than actual population sizes (N). However, population genetic theory also states that this is particularly true for organisms that experience variable recruitment (Hedgecock, 1994a, b). Observations of low N_e/N ratios have been frequently observed in populations of marine species, with reductions of as much as 10^2 to 10^5 (Flowers *et al.*, 2002; Hedgecock, 1994a). Similarly, many studies have reported low effective number of breeders (N_b). In particular this seems very common among bivalves (Hedgecock *et al.*, 1992), both in the wild and in hatchery propagated stocks. Not surprisingly, many studies have focused on economically important species such as the European flat oyster (*Ostrea edulis*) (Hedgecock *et al.*, 2007; Lallias *et al.*, 2010b; Launey *et al.*, 2001; Taris *et al.*, 2009) and the Pacific oyster (*Crassostrea gigas*) (Appleyard and Ward, 2006; Boudry *et al.*, 2002, Hedgecock, 1994a; Hedgecock and Sly, 1990). In these instances, both species were estimated to have a limited pool of parents as the source of cohorts in wild and hatchery

stocks, and parental contributions were uneven and highly variable. For example, in Quiberon Bay (Brittany, France) male *O. edulis* individuals contributing to wild progeny assays ranged from two to more than forty among brooding females, and parental contribution was skewed towards certain males (some contributing 50-100% to a progeny assay) (Lallias *et al.*, 2010b). The authors also found the effective number of breeders to be generally below 25 for all temporal cohorts collected in a hatchery. This was similar to estimates of the effective number of breeders for naturally spawned populations in the western Mediterranean Sea, where N_b was calculated to be from 10-20 adults (Hedgecock *et al.*, 2007).

The pearl oyster *Pinctada martensii* is another species used in aquaculture. In a closed broodstock population, selected over six generations, N_e was estimated to be between 30 and 40 in three of the four selection lines (Hedgecock *et al.*, 1992). The estimates of N_b were even smaller, which ranged from 15 to 21 in all four selected lines. The effective population size for 16 hatchery shellfish broodstocks was estimated; all were found to be less than 100, and 13 were less than 50. These estimates are insufficient for the long-term maintenance of genetic diversity within stocks (Hedgecock *et al.*, 2002). For the black-lipped pearl oyster *Pinctada margaritifera*, estimates of N_e from previous studies have been equally low. Using the same collector samples from within the Takapoto Atoll lagoon as the present study, Arnaud-Haond *et al.* (2002b) estimated an average effective number of genitors of 10 for five of the six collectors, with the exception of collector 4, which was about 22. The effective population size (N_e) for these individuals was estimated again by Arnaud-Haond *et al.* (2008) using the same method of variances in allelic frequency (Waples, 1989) with similar results. However

there were some variations depending on the putative "mother" population used in the estimates. For example, an N_e of 9-93 for all collectors was estimated if the 'Takapoto 2' wild population was the origin of the recruits, from 5-136 if the mother population was a pool of three Takapoto wild beds, and from 22-∞ if a pool of all the natural lying beds in the Tuamotu Archipelago was used. Overall, these previous studies implied that *P. margaritifera* have a limited number of parents contributing to new recruits.

However, analyses from the present study presented contrasting results. The current study used two methods to estimate if a limited number of parents might have been producing the individuals sampled on the collectors. The LDNE program gave large negative numbers in most cases, which indicates that there was insufficient information in the sample for LDNE to correctly estimate N_e. In the case of Zone A (150 individuals) with a lowest allele frequency of 10%, LDNE was able to return a positive estimate of N_e = 847 with an upper confidence limit of infinity (Table 3.9). This may indicate that the microsatellite dataset here was not suited to LDNE, as these loci were highly variable and showed substantial null allele frequencies. It might be easier to get a more accurate estimate of N_e by using genetic markers that are more conserved. In the second case, Pedigree was unable to detect any significant family structuring either under a strict full-sib constraint or without any constraints (kin group) (Figure 3.3). The latter type of analysis is fairly robust to the presence of scoring errors and null alleles. The absence of detectable kin group structure (Figure 3.3) indicates that there was limited evidence that the number of parents that contributed to the recruited individuals was restricted. Every recruit appeared to belong to a separate family with two distinct parents. This may indicate that the individuals on the collector lines were a mixture of

many different cohorts and/or the number of parents at the head of the cohorts was relatively large. This analysis shows that the number of breeders producing recruits on collectors in the Takapoto Lagoon may not be as limited as previously thought.

3.5. Conclusion

Under the theory of a sweepstakes reproductive success one would expect an unequal distribution of genetic variability within the population (Arnaud-Haond *et al.*, 2003b, 2008). If a species displays patchy genetic composition over time and space, there is potential for reduced genetic diversity in recruits compared to the adults. Assuming a low N_b, this could be amplified on collector lines when a large number of spat successfully settle and survive in a favourable environment. If a pearl oyster farm were supplied with large numbers of spat collected from a specific local area over a specific period of time, from a line of collectors for example, the genetic composition of that farmed aggregation could be quite different, and in particular might have lower genetic diversity compared to the wild oysters. In many lagoons, the farmed stocks represent a large biomass of potentially reproducing individuals (sometimes numbering in the millions). If these farmed individuals produce juveniles that successfully recruit back to the wild stocks, this could affect future wild stocks. Repeated over numerous generations, this could lead to a serious loss of genetic diversity in the wild populations (Arnaud-Haond *et al.*, 2003b, 2008). Several observations here were in agreement in the expectation of chaotic genetic patchiness, driven by sweepstake reproductive success. Significant differences in genetic diversity (estimated by corrected allelic richness) were

observed among the three collector zones, where the zone that showed the highest recruitment density also showed higher diversity. There were significant levels of differentiation among collector zones (at a small spatial scale of <10km, within a single lagoon) where no significant genetic structure was observed at a large spatial scale (100's km, across the Tuamotu-Gambier Archipelagos in Chapter 2). Using a large number of hypervariable markers, these results confirmed with increased precision the small spatial scale genetic patchiness that had been observed with four less variable markers (Arnaud-Haond *et al*., 2008). There was however a crucial difference. The sweepstake reproductive success assumes that the genetic patchiness is being driven by the fact that each group of recruits has been produced by a different set of parents and that these parental sets are quite small. Indeed if the different sets of parents were large random samples from the adult population, one would not expect much genetic differentiation among the progeny produced by these different sets. Arnaud-Haond *et al*. (2008) did in fact estimate a small range of genitors that could have contributed to these collector samples, although the actual number varied substantially depending upon the particular population that was used as the "mother" population in their method of variance approach. Here a more direct approach was used to reconstruct possible sibship relations among the recruits try to see if these had been indeed produced by a limited number of parents. No evidence was found that suggested the number of parents contributing to a cohort was limited. The method used here (Pedigree 2.2) has been utilized successfully to reconstruct family structure both among wild (e.g. Herbinger *et al*. 2006) and hatchery individuals (e.g. Lemay and Boulding 2009; Trippel *et al*., 2009). With 13 microsatellite markers and particularly under kinship grouping, this approach is very robust and

powerful. The statistical testing approach used here showed that there was essentially no evidence of a limited pool of parents at the source of the different collector recruits.

The individuals on the collector lines analysed here were young adults (about 10-15 mths old). Since the breeding cycle of *P. margaritifera* is almost continuous throughout the year (Fairbairn, 2009), it is possible that the individuals on the collector lines were combinations of different cohorts that were bred at different times. If this were the case, then the true family signal could have been masked. To try to elucidate the fine-scale recruitment of the pearl oyster populations within the French Polynesian lagoons, analyses of the genetic composition within juveniles and parental contributions to new recruits were therefore warranted. This would also help address another important effect of sweepstakes reproductive success, which would be the expectation to see lower genetic diversity in recruits compared to the adult population. In order to better test this, temporal cohorts from one site in the Takapoto lagoon in the following chapter will be compared to the overall genetic variation seen in the pooled Takapoto collector populations.

Chapter 4: Spat settlement at a fine spatio-temporal scale within the Takapoto Atoll lagoon

4.1. Introduction

For numerous shellfish aquaculture species, significant decreases have been found for genetic diversity in hatchery stocks compared to the wild stocks from which they originated (e.g. Hedgecock and Sly, 1990, Hedgecock *et al.*, 1992; Launey *et al.*, 2001). Even in natural populations, low effective number of breeders or low effective population size compared to census size have been observed or implied (Arnaud-Haond *et al.*, 2008; Boudry *et al.*, 2002; Hedgecock, 1994; Hedgecock *et al.*, 2007). As a result, it is feared that the pearl culture industry in French Polynesia may cause negative genetic effects in wild pearl oyster (*Pinctada margaritifera*) populations. If the farmed individuals, which are grown in a habitat contiguous with the wild population, have high densities and low genetic variability and are allowed to reproduce and settle back in the wild, after many generations we could see decreased genetic diversity in the wild stocks (Arnaud-Haond *et al.*, 2003b). This could occur if a limited number of parents are at the head of cohorts collected on suspension lines and used as the farmed stocks.

The theory of sweepstakes-chance reproductive success has been increasingly tested in order to explain observations of low effective populations sizes or numbers of breeders (Hedgecock, 1994a). Under this theory it is suggested that there is a very high variance of reproductive success and most individuals will fail to reproduce due to a chance mismatch of spawning activity with favourable oceanographic/habitat conditions.

If however, individuals are characterized by very high fecundity, a small number of individuals can replace a population and the effective population size could be substantially reduced compared to the expected Poisson distribution of an ideal population (Hedgecock, 1994a; b). A clue that can suggest high variance in reproductive success is genetic patchiness at different spatial and temporal scales, which has frequently been described as "chaotic", as first coined by Johnson and Black (1984). Often when this exists, populations that are only a few kilometres apart can display greater genetic differentiation than those separated by hundreds of kilometres (Hedgecock, 1994a, b; Johnson and Black, 1982; Watts *et al.*, 1990). Genetic patchiness has been observed in *P. margaritifera* at different spatial scales (Arnaud-Haond *et al.*, 2008) and has been confirmed in the present thesis (see Chapter 3). More genetic differentiation was observed within the Takapoto lagoon between collector samples, separated by less than 10km, than was observed among the wild and farmed samples separated by hundreds of kilometres. These results were consistent with chaotic (patchy) genetic composition such that there was greater genetic heterogeneity between "annual" cohorts than that observed among the adult spawning populations, which was associated with the sweepstake recruitment hypothesis (Hedgecock *et al.,* 2007). However, no family structure was uncovered among the various oysters harvested in the collectors (Chapter 3). It appeared that every individual had distinct parents and no evidence of a limited number of parents was apparent. This was a surprising result as it seemed that the observed patchy genetic pattern was not driven by the successful reproduction of a limited number of parents as implied by the sweepstake reproductive success theory.

It is important to note that the oysters that were analyzed in the previous chapter were 10-15 month old adults. Because this species is known to exhibit high variability in recruitment (Friedman *et al.*, 1998; Friedman and Bell, 1999; Oengpepa *et al.*, 2006) and reproduces throughout the year (Fairbairn, 2009), each collector sample could have then represented a mixture of various cohorts recruited in the same location (collector), but at different times during that period. This could have masked the true recruitment pattern in terms of number of contributing parents and its associated genetic signature. A fine-scale temporal analysis of juvenile settlement on collectors is necessary to get a better picture of the variation in reproductive success and recruitment of the black-lipped pearl oyster. This would allow the assessment of whether large cohorts of new juveniles recruited at the same time and place were produced by the successful spawning of a few parents.

The present chapter examines three general consequences expected of the theory of sweepstakes-chance reproductive success. This hypothesis predicts 1) lower genetic diversity in newly recruited cohorts compared to the general adult population, 2) significant genetic differentiation among temporal cohorts of new recruits (Hedgecock, 1994a) and 3) limited number of adults contributing to a cohort. This was accomplished by determining the level of genetic diversity and differentiation in juveniles obtained from monthly spat collection in two different sites within the Takapoto atoll over a five month period of high settlement. The final step involved estimating the number of breeders that appeared to have produced the juveniles recruited on collector lines each month, which was done using microsatellite-based sibship analysis. The analysis incorporated much finer spatial and temporal scales of recruitment compared to the previous chapter. Collectors were deployed every month and retrieved every two

months, ensuring that the juveniles analyzed were no more than two months old. The purpose of this project was to break down overall recruitment into identifiable individual cohorts in order to better understand the mechanisms that may drive variation in the genetic composition of recruits on collectors.

4.2. Methods and Materials

4.2.1. Sampling Location and Collections

In November 2006, two commercial lines of collectors were installed in the Takapoto Lagoon, in two closely located stations (~ 1km apart) in the southern part of the lagoon between Zones A and B from the previous chapter (Figure 3.1). A series of temporal collectors were deployed on these lines over a year cycle, from December 2006 to December 2007. Two replicate collectors were deployed every month at both sites and retrieved after two months. Once the juvenile pearl oysters were removed from the collectors, they were scanned, counted and organized into size classes, and stored in 90% ethanol. Size frequency distributions of the settled spat were generated for each collector, site, and month (Fairbairn, 2009, see Figure A1 in Appendix). This information was used to identify patterns (peaks) in recruitment, and in particular, identify well-represented cohorts of individuals that probably settled at the same time. Each individual spat retrieved from the temporal collector was at least ~2-3 weeks old (post settlement). The younger, smaller individuals (<1.5 mm) could not be identified

reliably among the many spat of other related species and were excluded. The recruits were no more than two months old given that the collectors were retrieved after two months in situ. There is some evidence that *P. margaritifera* larvae settle preferentially around a full moon (Mills *et al.*, 2009; Ubertini, 2009). This indicates that each bimonthly collector sample should include two cohorts at the most. One cohort would include numerous small individuals about three weeks old that settled around the immediately preceding full moon. The second potential cohort would be constituted of fewer (due to intervening mortality), but larger individuals that would have settled around 2 full moons ago and would be about 7 weeks old. Such a pattern can be seen in the size distributions of the spat collected from February to June (Figure A1 in Appendix). There is generally a fairly large peak of small individuals (~2 to ~ 6 mm), particularly obvious in April and June. Larger individuals (~ 6 to ~ 18 mm) are also generally observed, but in much smaller abundance. The newly recruited individuals in the five months of February to June were analyzed in the present study. As observed by Fairbairn (2009), recruitment from July to December was much more reduced and the numbers were too low to be characterized genetically. To further ensure that single cohorts were analyzed as much as possible, only the small individuals (2 to ~ 5 mm) were selected. Twenty-five individuals were randomly selected in this size range from each replicate collector, for a minimum of 50 juveniles per station and 100 per month. The only exception was the month of February, where all 82 small juveniles available were selected due to lower recruitment in that month.

4.2.2. DNA Extraction and Amplification

The DNA from the individuals of the identified cohorts was extracted using the glassmilk protocol (Elphinstone *et al.*, 2003). This method was chosen because it performed better than the DNEasy kits (Qiagen), given that there were small amounts of highly pigmented tissue used. Heavy pigmentation was present because the entire individual was used in the tissue digestion process. The 489 juveniles were genotyped at 11 loci (the original eight with the addition of three newly designed loci Pmarg012, Pmarg158, and Pmarg258) in order to characterize their genetic profiles and to assess the number of contributing parents. All loci were imaged on the Hitachi FMBio II fluorescent imaging system (Hitachi Software Engineering). As in the previous chapter, the locus Pmarg79 was used to complement Pmarg44 to maximize the number of heterozygous individuals by recovering non amplifying alleles (null alleles). Lastly, two data sets were generated, the first one being the raw data set with every allele scored as accurately as possible, including alleles showing a single base-pair shift. In the second data set, all alleles identified as subject to a base pair shift were binned back with the more common alleles.

4.2.3. Data Analysis

The dataset was tested for scoring errors and presence of null alleles per locus using Micro-Checker (van Oosterhout *et al.*, 2004). Genetic diversity within and among the monthly spat samples was assessed using allelic richness, corrected for smallest

sample size, as well as observed heterozygosity (H_{obs}) and Nei's unbiased gene diversity (H_{nb}) (FSTAT: Goudet, 2001). The proportion of genetic diversity among the monthly spat populations was measured using an Analysis of Molecular Variance (AMOVA) (Arlequin 3.5.1.2: Excoffier, 2010). This hierarchical AMOVA had several levels: among months, among stations within month, among individuals, and within individuals. The significance of these estimates was tested by 9999 random permutations. Furthermore, a three-way ANOVA of allelic richness was constructed with factors Station, Month and Locus (as a block), without replication. This was to test the effect of station and month within the temporal recruitment cohorts. Also, a two-way ANOVA without replication for a block design was performed to test for systematic difference in allelic richness between the monthly juvenile cohorts and the young adult populations (Collector Zones A, B, and C, Chapter 3).

Genetic differentiation among and within monthly cohorts was calculated in two different ways. The first method used pair-wise F_{ST} estimates based on Weir and Cockerham's (1984) θ estimator using the program GenAlEx 6.41 (Peakall and Smouse, 2006). The significance of these estimates was tested by 9999 random permutations of individuals between samples and by using Bonferroni correction for multiple tests (Rice, 1989). The tests were repeated using FSTAT to verify the consistency between the results outputs. The second method to test for genetic structuring between the monthly recruits was based on changes in allele frequencies or more specifically the distribution of alleles in the various sample groups. This was performed using Genepop 4.0.10 (Raymond and Rousset, 1995; Rousset, 2008). Pair-wise tests were computed for each population pair and locus and were based on Markov chain (MC) algorithms which

estimated exact P-values without bias (Guo and Thompson, 1992). P-values were then adjusted with Bonferroni correction. The null hypothesis (H_o) for these tests of genic/allelic differentiation was that "alleles are drawn from the same distribution in all populations". These analyses were performed first with separate replicate collectors and then with the replicate collectors pooled.

The potential importance of variance in reproductive success for fine scale recruitment was assessed by estimating the effective population size (N_e), as well as the potential number of breeders that may have contributed to a cohort seen on the collectors. Calculations of N_e were performed in the program NeEstimator (Peel *et al.*, 2004), which is based on the method developed by Waples (1989). This is based on the equation:

$$Ne = \frac{t}{2(F - \frac{1}{S})} \tag{4.1}$$

where t is the number of generations, F is the variance in allele frequencies, and S is the sample size. The estimation takes into account changes in allele frequencies in a population sampled at two different times, here between adults and juveniles. This method required two samples, the first being a putative parental source and the second being the sample to be tested, in this case the monthly cohorts of juveniles. The genotypes for all young adults within the Takapoto lagoon from collection Zones A, B, and C (Chapter 3) were pooled and used as the putative genetic source of genitors. Since the individuals from this reference "parent" population were sampled in 2002 and the monthly collector samples were sampled in 2007, we considered here one to two generations maximum separating these cohorts. It is important to note that this method is

highly sensitive to the estimated number of generations separating the two samples (equation 4.1).

Finally, an estimation of the potential number of breeders that may have produced the different cohorts was attempted using the reconstruction of full- and half-sib relationships with the aid of Pedigree 2.2 (Herbinger, 2005) and Colony 2.0 (Wang and Santure, 2009). Using the genotypes from multiple loci, possible parentage can be assigned and sibling relationships inferred among individuals based on maximum likelihood. The resulting family structuring with Pedigree 2.2 (if any) was also analysed with the resampling technique described in Herbinger *et al*. (2006) to determine if the detected sibship or kin groupings could be artefactual or real.

4.3. Results

4.3.1. Genetic Differentiation among Juvenile Cohorts

The tests for genetic structuring between monthly cohorts were first performed by comparing pair-wise F_{ST} estimates between all sampling replicates within station separately, in order to verify that there were no significant differences between collectors sampled within the same month at the same location (Table 4.1, see also Table A3 in Appendix). Of all ten comparisons between replicates, only one month (February) had a small but significant difference between the two replicates at station 2. However, this was no longer significant after Bonferroni correction. Therefore, it was assumed that there were generally no significant differences between replicates within stations for a

given month. This would be logically expected as since the two replicates were only 1 to 2 metres apart. Subsequent analyses were done by pooling both collector replicates within a single monthly station to increase sample size and power.

Table 4.1. Pair-wise F_{ST} matrix of pearl oyster juveniles from collectors within the Takapoto lagoon over five months, based on 11 microsatellite loci. Only the F_{ST} values between collector replicates (A and B) within the same station (1 or 2) are shown.

	Feb 1B	Mar 1B	Apr 1B	May 1B	Jun 1B	Feb 2B	Mar 2B	Apr 2B	May 2B	Jun 2B
Feb 1A	0.0095									
Mar 1A		-0.0017								
Apr 1A			-0.0001							
May 1A				0.0038						
Jun 1A					-0.0030					
Feb 2A						**0.0038**				
Mar 2A							0.0025			
Apr 2A								-0.0033		
May 2A									-0.0012	
June2A										-0.0031

Probability values based on 9999 permutations; value in bold is significant at $\alpha = 0.05$, but not after Bonferroni correction.

Considering sampling stations separate (replicates pooled), 16 of the 45 comparisons were significantly different at a P-value 0.05 (Table 4.2). After corrections for multiple comparisons, three remained significantly different: April 1 to June 1, April 1 to February 2 and February 2 to April 2. Comparisons among stations within month were not significant except again for February. If replicates and stations within month were pooled, eight of the 10 comparisons were significant at a P-value of 0.05 (Table 4.3) with three comparisons still significant after Bonferroni correction (those between the months of April and February, April and June, as well as May and June). To sum up, very little differentiation was seen between replicates or between stations within month (except in February), but significant temporal genetic structuring was seen. Despite the

pairwise F_{ST} levels being quite small, juvenile cohorts in February, April and June appeared significantly differentiated from one another. Notably, April and June were the two months characterized by the highest recruitment peaks (see Figure A1 in Appendix).

Genetic structure within and among monthly cohorts was also tested using comparisons in allele frequencies (as implemented in Genepop). Once again, looking at the ten comparisons between replicates within the same month and station, only the comparison in February (Station 2) was significant (see Appendix, Table A4). With replicates pooled, differences among stations within month were not significant except again in February. However, the majority of the comparisons across month either within station or across stations were significant, even after Bonferroni corrections (28 out 40 comparisons, Table 4.4). When replicates and stations were pooled for a given month, all ten comparisons among months were significant (Table 4.5). Therefore, the overall picture based on genic/allelic differences was very similar to that seen with pair-wise F_{ST}, although the former tests are more powerful and as expected, more comparisons were significantly different than in the previous analyses. In February, genetic differentiation was seen at a small spatial scale (between the two stations) or even surprisingly at a very small spatial scale (between the two replicates in Station 2). For every other month, no genetic differentiation was observed between replicates or between stations, but significant differences were seen among every monthly sample.

Table 4.2. Pair-wise F_{ST} matrix of pearl oyster juveniles from two sampling stations within the Takapoto lagoon over five months, based on 11 microsatellite loci. Pink shows the 5 comparisons across stations within the same month, light turquoise signifies the 10 comparisons across months within station 1, light green denotes the 10 comparisons across months within station 2, and yellow represents the 20 comparisons across station and across months.

Month/ Station	Feb 1	Mar 1	Apr 1	May 1	Jun 1	Feb 2	Mar 2	Apr 2	May 2	Jun 2
Feb 1	-									
Mar 1	0.0037*	-								
Apr 1	0.0038*	0.0010	-							
May 1	0.0000	0.0028	0.0034*	-						
Jun 1	0.0059*	0.0018	**0.0063**	0.0051*	-					
Feb 2	0.0044*	0.0054*	**0.0103**	0.0063*	0.0007	-				
Mar 2	0.0000	0.0000	0.0051*	0.0000	0.0001	0.0014	-			
Apr 2	0.0023	0.0012	0.0000	0.0000	0.0011	**0.0083**	0.0000	-		
May 2	0.0000	0.0007	0.0023	0.0000	0.0016	0.0045*	0.0001	0.0000	-	
Jun 2	0.0028	0.0001	0.0028*	0.0035*	0.0000	0.0018	0.0028*	0.0004	0.0017	-

Probability values based on 9999 permutations. * = significant only at $\alpha = 0.05$. Values in bold are significant after Bonferroni correction.

Table 4.3. Pair-wise F_{ST} matrix of pearl oyster juveniles from collectors within the Takapoto lagoon over five months, based on 11 microsatellite loci. F_{ST} values are below the diagonal, while probability values based on 9999 permutations are above the diagonal.

Monthly Cohort	Feb	Mar	Apr	May	Jun
Feb	-	0.0219	**0.0001**	0.2525	0.0174
Mar	0.0020*	-	0.0054	0.0524	0.0197
Apr	**0.0049***	0.0025*	-	0.0242	**0.0016**
May	0.0005	0.0014	0.0018*	-	**0.0009**
Jun	0.0021*	0.0018*	**0.0031***	**0.0034***	-

* = significant at $\alpha = 0.05$. Values in bold were also significant after Bonferroni correction for multiple comparisons

Table 4.4. The P-value from tests of genic differentiation (exact G test) for each population pair (according to sampling station and month) across all loci. Pink shows the 5 comparisons across stations within the same month, light turquoise signifies the 10 comparisons across months within station 1, light green denotes the 10 comparisons across months within station 2, and yellow represents the 20 comparisons across station and across months.

Cohort	Feb 1	Mar 1	Apr 1	May 1	Jun 1	Feb 2	Mar 2	Apr 2	May 2	Jun 2
Feb 1	-									
Mar 1	**0.00032**	-								
Apr 1	**≪0.00000**	**0.00084**	-							
May 1	0.10591	**0.00080**	0.01676	-						
Jun 1	**0.00050**	**0.00017**	**≪0.00000**	**≪0.00000**	-					
Feb 2	**0.00009**	**0.00022**	**0.00002**	**0.00014**	0.00145	-				
Mar 2	**0.00094**	0.01572	0.00357	0.00124	**0.00018**	**0.00044**	-			
Apr 2	**0.00023**	0.01227	0.00968	0.05187	0.00352	**0.00000**	0.01534	-		
May 2	**0.00007**	**0.00028**	**≪0.0000**	0.00429	**0.00025**	**0.00021**	**0.00001**	**0.00000**	-	
Jun 2	**0.00021**	0.00484	**0.00000**	**0.00027**	0.00197	0.01651	**0.00015**	0.00113*	**≪0.00000**	-

Monthly CohortComparisons with "highly sig" mean the chi-square value was infinity. Values in bold are P-values below the critical value at the threshold of 0.00111 (after correction for multiple comparisons).

Table 4.5. The P-value from tests of genic differentiation (exact G test) for each population pair (according to sampling month) across all loci. All replicates and stations within a month were pooled.

Monthly Cohort	February	March	April	May	June
February	0				
March	**0.000001**	0			
April	**0.000021**	**0.000038**	0		
May	**0.000654**	**0.000000**	**0.000002**	0	
June	**0.001091**	**0.000000**	**0.000002**	**0.00000**	0

Values in bold are P-values below the critical value at the threshold of 0.005 (after correction for multiple comparisons).

The AMOVA results generated by Arlequin were in good agreement with the results of the F_{ST} comparisons (Table 4.6). The greatest proportion of variation was seen within individuals (83.8%), and there was a considerable amount of variation among individuals (16.2%) as well. Very little of the variance was accounted for across months (0.2%), but it was significant. Variance among stations within month was not significant.

The F_{ST} analyses were also performed with the second data set where the alleles that showed single base-pair shifts were binned to size classes corresponding to the regular common alleles. The detected pattern of genetic differentiation was nearly identical to that described above, indicating that the conclusions of genetic divergence between temporal cohorts were robust to the presence of these scoring difficulties.

Table 4.6. Summary AMOVA tables for the proportion of genetic variance distributed among the monthly juvenile cohorts of *Pinctada margaritifera* within the Takapoto lagoon. The analysis was performed with all replicates and stations grouped within months. Significance tests: 10,000 permutations.

Source of Variation	**DF**	**SS**	**Var. Comp.**	**%**	**P-value**
Among months	4	16.61	0.00596 Va	0.21	0.0065
Among stations within month	15	44.93	- 0.00530 Vb	-0.19	0.8912
Among individuals	469	1525.53	0.45305 Vc	16.18	0.0000
Within individuals	489	1147.50	2.34663 Vd	83.80	0.0000
Total	977	2734.57	2.80033		

4.3.2. Genetic Diversity Within and Among Monthly Cohorts

Based on results from the pair-wise F_{ST} estimates, all replicates within station were pooled for further analysis, but stations remained separate. Neither observed heterozygosity (H_{obs}) nor expected heterozygosity (H_{nb}) were very variable from one spat cohort to the next (Figure 4.1). Averaged over all loci, H_{nb} ranged from 0.81 ± 0.06 SD (February) to 0.82 ± 0.05 SD (June), while H_{obs} varied from 0.59 ± 0.02 SD (April) to 0.63 ± 0.02 SD (June). As in previous Chapters, there were considerable decreases from expected (H_{nb}) to observed heterozygosity (H_{obs}) and significant deviations from H-W expectations were generally seen in every sample at most loci (Table 4.7). These heterozygote deficits were again most probably due to the presence of null alleles. Micro-Checker suggested null alleles were likely present at high frequency at all loci, except Pmarg37, 44, 012, and 258 (and to a lesser extent Pmarg158), which were much less prone to nulls (Table 4.7). These are the same loci that were characterized by lower estimated null allele frequencies in the two previous chapters. Overall, the levels of observed and expected heterozygosities were similar to those seen for the three adult collection zone samples within Takapoto from the previous chapter.

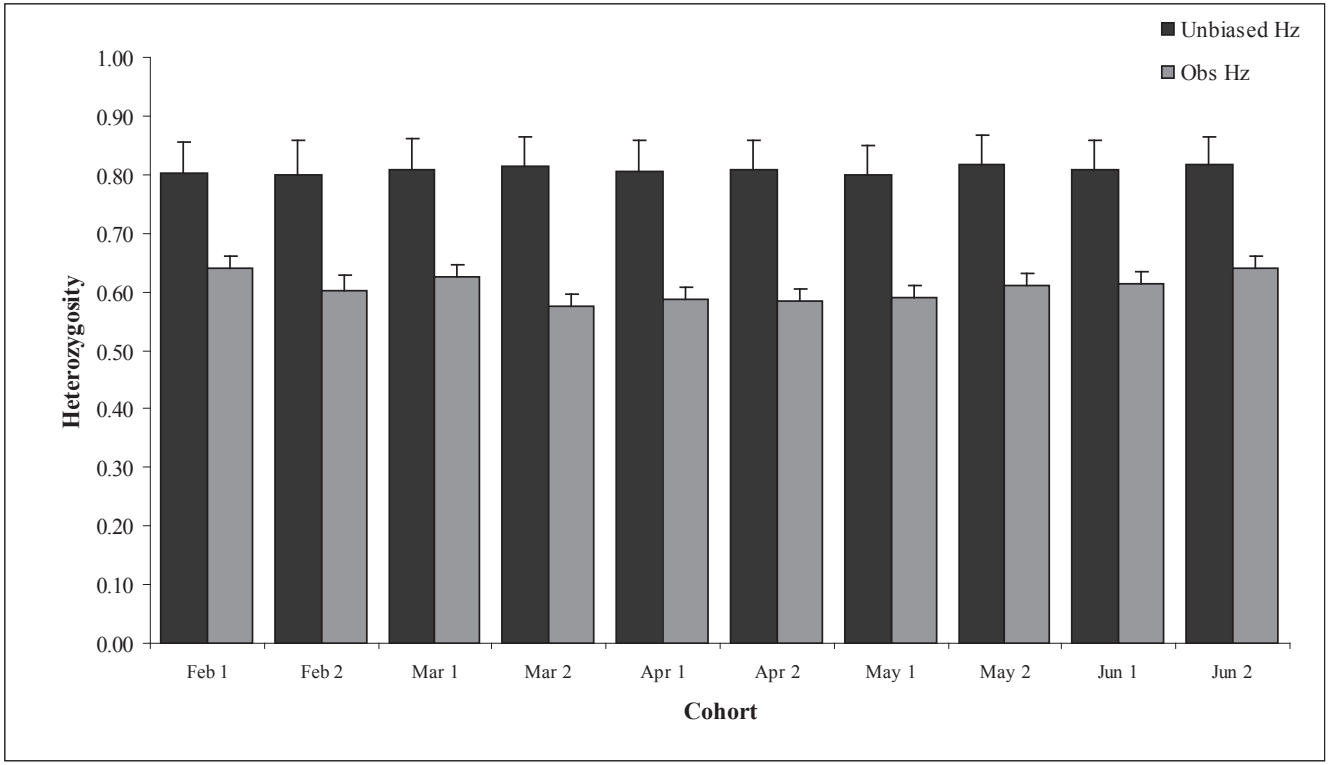

Figure 4.1. The unbiased gene diversity (H_{nb}) as well as observed heterozygosity (H_{obs}) averaged over all loci for each monthly cohort of pearl oyster juveniles within the Takapoto lagoon, separated by station (1 and 2). Standard deviation bars are included.

Table 4.7. The estimated null allele frequencies per locus for each monthly juvenile cohort (separated by sampling station 1 and 2) of *Pinctada margaritifera*, obtained using the methods by van Oosterhout (see van Oosterhout *et al.*, 2004). Bold values indicate loci which have been estimated to have the presence of null alleles.

	Feb 1	Feb 2	Mar 1	Mar 2	Apr 1	Apr 2	May 1	May 2	June 1	June 2	Mean
Pmarg2	**0.2109***	**0.2976***	**0.2649***	**0.2386***	**0.2858***	**0.2290***	**0.2262***	**0.2585***	**0.2550***	**0.2487***	0.2508
Pmarg7	**0.1301***	**0.1150**	**0.1162***	**0.0890**	**0.1010***	**0.1126***	**0.1082***	**0.1443***	**0.1037***	**0.1277**	0.1131
Pmarg11	**0.1380***	**0.1811***	0.0099*	**0.1306***	**0.1343***	0.0336	**0.0751***	**0.1753***	0.0220	**0.1098***	0.0863
Pmarg37	0.0259	-0.0051	0.0323	**0.0991***	0.0228	**0.0891**	**0.0997***	**0.0733**	**0.0801**	0.0018	0.0524
Pmarg44	0.0180	0.0589	-0.0064	**0.0513**	0.0304*	0.0513	0.0560	0.0171	0.0552	0.0354	0.0374
Pmarg45	**0.0618***	**0.1062**	**0.0822***	**0.0987***	**0.1158***	**0.1928***	**0.0582***	**0.1041***	**0.0831***	**0.0422**	0.0981
Pmarg68	**0.0905***	**0.2048***	**0.2751***	**0.2719***	**0.2851***	**0.2657***	**0.2330***	**0.1802***	**0.2692***	**0.1632***	0.2429
Pmarg77	**0.1982***	**0.2101***	**0.2054***	**0.2915***	**0.2079***	**0.1955***	**0.2187***	**0.1796***	**0.1584***	**0.2294***	0.2108
Pmarg012	-0.0441	-0.1593	-0.0069	-0.0246*	-0.0804*	0.0462	0.1040	-0.0418	**0.1015***	-0.1190	0.0252
Pmarg158	**0.0922**	-0.0062*	**0.1330***	**0.1515***	**0.1268**	**0.1795***	0.0579	**0.1179**	0.0745	**0.1693**	0.1103
Pmarg258	0.0507	0.0315	-0.0188	0.0473	0.0321	-0.0082	**0.1013***	-0.0084	**-0.0184**	0.0050*	0.0268

Negative values produced by the estimation method indicate null allele frequencies that are essentially zero.
* = Significant departures from H-W equilibrium observed for that locus and population.

The loci were highly variable with total numbers of alleles per locus ranging from 10 (Pmarg012) to 54 (Pmarg45). The monthly spat cohorts, with replicate collectors pooled within given station/month, demonstrated highly variable allelic diversity with a mean number of alleles in the order of 15-17 and allelic richness around 13-14 (Figure 4.2). Across all loci, genetic diversity was quite comparable for each monthly cohort (Figure 4.2, Table 4.9). In a three-way ANOVA of richness with factors Station, Month and Locus (as a block), a strongly significant effect of locus was not surprisingly detected ($F = 231.6$ with 10 & 94 df, $P < 0.001$), but neither the effect of station nor the effect of month was significant. Hence it appeared that despite the fact that cohorts appeared to be differentiated to some extent at least on a temporal scale, there were no significant differences in terms of genetic diversity (estimated by corrected allelic richness).

In general, looking at the mean number of alleles, as well as the allelic richness per locus (Figure 4.2, Table 4.8), the juvenile cohorts showed very similar levels of genetic diversity compared to the adult samples. Average standardized allelic richness ranged from 13.4 (May Station 1) to 14.5 (February Station 2). This was quite similar to that seen among the adult populations from Chapter 3 (Zones A, B, C), which ranged from 13 (Collector 1) to 14.6 alleles (Collector 4). When comparing the corrected allelic richness across all 11 loci for the ten monthly stations to that of the 6 collectors of the previous chapter (Table 4.8), with a two-way ANOVA, no significant differences were detected for young recruits in 2007 versus the 2002 young adults, but again a very significant effect of locus was detected.

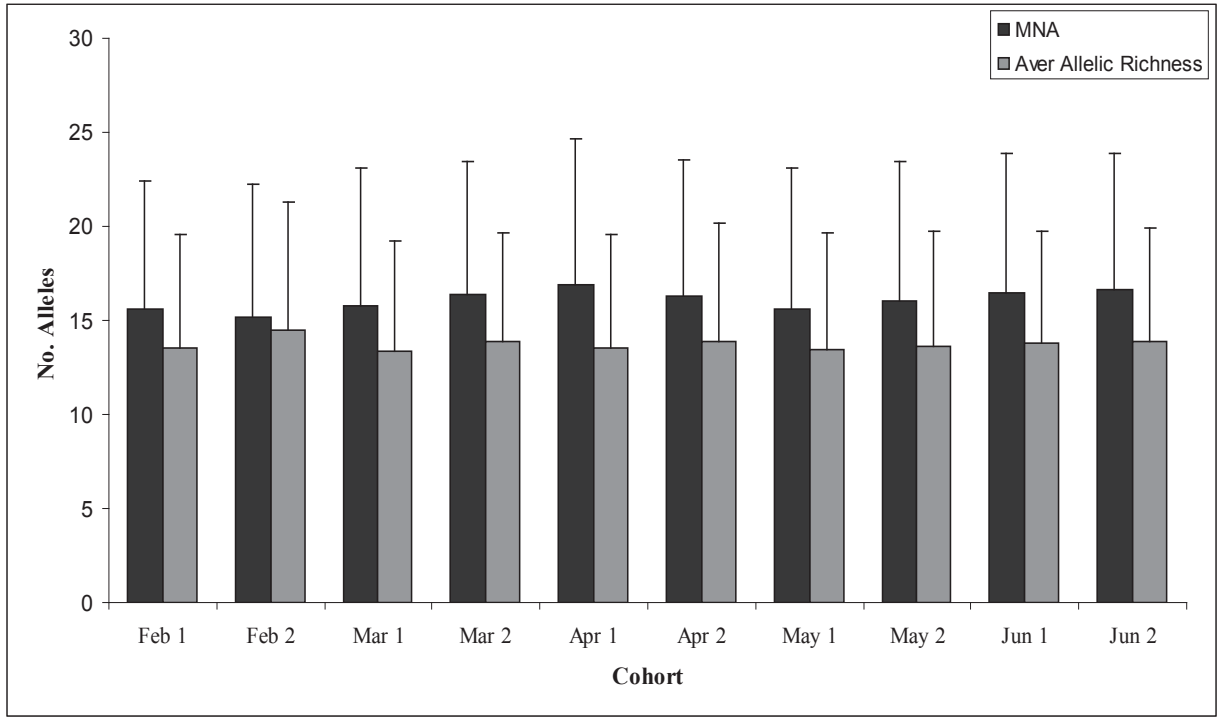

Figure 4.2. The mean number of alleles (MNA) and allelic richness of *P. margaritifera* juveniles per station (1 and 2) and month, averaged across all loci. Standard deviation bars included.

Table 4.8. Total sample sizes (in brackets) and allelic richness of monthly juvenile cohorts, corrected for smallest sample size of 28 individuals, per locus and sampling station (1 and 2). Also, allelic richness of the three adult collection zones (A, B, and C) within Takapoto, corrected for smallest sample size of 30 individuals, is shown with all collectors for a sample zone pooled and separated. Mean number of alleles (MNA) and standard deviation across all loci are included. Sample size of each population shown in brackets.

Cohort	Pmarg2	Pmarg7	Pmarg11	Pmarg37	Pmarg44	Pmarg45	Pmarg68	Pmarg77	Pmarg012	Pmarg158	Pmarg258	Aver.
Feb 1 (48)	17.1	10.0	19.7	17.3	12.4	24.0	8.5	17.6	4.2	7.9	10.6	13.6
Feb 2 (34)	18.8	8.6	22.3	16.4	13.2	27.4	7.9	19.0	5.8	9.5	10.6	14.5
Mar 1 (50)	18.1	9.9	20.6	14.9	14.1	22.8	8.5	16.4	4.7	6.9	10.1	13.4
Mar 2 (50)	19.0	9.5	18.5	17.6	12.6	23.8	9.5	18.5	6.4	7.1	10.3	13.9
Apr 1 (56)	19.8	10.2	19.2	14.4	12.7	25.2	7.8	16.1	6.3	7.3	10.3	13.6
Apr 2 (50)	20.3	11.0	22.4	16.8	11.6	20.6	9.3	19.5	5.5	5.2	10.5	13.9
May 1 (50)	18.9	8.3	22.1	16.1	10.5	19.7	7.6	20.6	4.6	6.8	12.3	13.4
May 2 (50)	18.3	8.5	23.0	16.3	10.2	22.3	9.4	17.4	5.2	8.0	11.2	13.6
Jun 1 (50)	17.7	9.5	21.6	15.7	12.1	24.2	8.5	17.6	5.4	8.2	11.0	13.8
Jun 2 (51)	17.5	8.6	19.4	17.3	13.4	23.0	8.6	20.6	5.2	7.3	12.1	13.9
Zone C (37)	20.2	10.5	21.6	15.0	11.2	18.2	5.9	17.5	4.9	7.8	10.4	13.0
Zone B (93)	19.4	9.3	22.6	15.3	12.2	21.6	7.4	17.2	6.4	8.3	10.9	13.7
Zone A (150)	20.2	11.1	23.5	16.7	12.0	21.8	8.2	19.1	6.1	9.0	11.6	14.5
Coll. 1 (37)	20.2	10.6	21.6	15.0	11.2	18.2	5.9	17.5	4.9	7.8	10.4	13.0
Coll. 2 (52)	20.1	9.7	22.5	13.6	12.3	22.3	8.3	17.6	6.6	8.9	12.2	14.0
Coll. 3 (41)	16.6	8.8	23.0	16.1	11.3	18.2	6.7	15.4	5.6	7.4	9.1	12.6
Coll. 4 (50)	18.8	11.4	24.3	17.2	10.4	23.0	8.5	18.5	6.6	9.5	11.7	14.6
Coll. 5 (50)	20.9	8.4	23.6	16.5	14.7	20.4	6.6	19.3	5.5	9.7	11.0	14.2
Coll. 6 (50)	19.4	10.9	20.5	16.5	10.6	21.0	8.7	16.1	6.6	7.2	12.6	13.6

Table 4.9. Allelic richness, corrected for smallest sample size of 66, per locus and monthly cohort. For each month, the individuals from each replicate and both stations were pooled.

Monthly Cohort	Pmarg2	Pmarg7	Pmarg11	Pmarg37	Pmarg44	Pmarg45	Pmarg68	Pmarg77	Pmarg012	Pmarg158	Pmarg258	Aver.
February	23.67	11.55	28.36	23.43	17.04	34.82	9.90	24.00	6.76	11.54	16.60	14.04
March	22.06	10.80	25.64	21.46	18.10	32.57	9.76	23.18	7.24	9.12	16.80	13.63
April	25.72	13.72	29.51	19.99	16.38	31.62	10.58	22.62	7.44	8.69	16.28	13.73
May	24.89	10.23	31.87	21.10	13.17	27.02	9.70	23.32	5.71	9.29	17.14	13.52
June	24.71	11.71	26.39	21.58	17.81	34.11	9.69	23.27	6.59	11.31	17.57	13.84
Overall	25.64	12.08	29.72	22.08	16.65	33.32	10.57	23.97	7.02	10.47	16.81	

4.3.3. Estimation of Effective Populations Size and Number of Genitors

The effective population size of genitors at the source of the monthly cohorts was estimated based on variances in allelic frequencies using NeEstimator. The young adults (10-15 month old) harvested in 2002 from the three collection zones within Takapoto were used as a surrogate putative source population. As the monthly juvenile cohorts were sampled in 2007, we assumed at most about one to two generations separating the two populations. If these populations were separated by one generation, N_e was estimated to be about three quarters the juvenile sample sizes (Table 4.10). The average N_e ranged from 55.6 (42.0-74.6, 95% CI) for June, to 75.3 (54.9-106.3, 95% CI) for April. If two generations separated the two sets of samples instead of one, the estimated effective population size doubled, as expected from equation 4.1 (page 91). With a 5-6 year interval separating the settlement/recruitment of the two samples, a number of intervening generations between 1 or 2 appeared credible, which indicated that the estimated N_e at the head of the juvenile cohorts were probably on the order of 100. This was substantially larger than that estimated for the number of genitors at the source of adult collector zone populations (Arnaud-Haond *et al.*, 2002b; 2008). With these new estimates it appears that the effective population size may not be as limited as previously thought.

Estimation of the number of parents that may have generated the various cohorts was also attempted using the reconstruction of full and half sibling relationships with the aid of Pedigree 2.2 and Colony 2.0. For each of the five monthly samples (replicates and stations pooled), the Pedigree analyses revealed many small groupings of individuals

(one to three) with and without a full sibling constraint imposed on the MCMC runs. The typical distribution of reconstructed sibship size was similar to that seen in the analyses of collector samples in the previous chapter (see Figure 3.3). The Pedigree simulation tool was used to assess whether any of the reconstructed partitions in kin groups might be real rather than artefactual. These partitions were generally not significant except for the partition in March ($p < 0.01$). This was driven by a single family of two, with an extremely high cohesion score. However, upon close examination of this family it was discovered that the two tissue sample were contiguous on the extraction plate and had nearly identical genotypes at eleven loci. It appears likely that these two individuals were actually the same because of an extraction mistake, and the significance of this family of two is probably spurious.

Table 4.10. Estimation of effective population size (N_e) of possible genitors at the source of cohorts on collector lines collected in monthly intervals within the Takapoto Lagoon. The average effective population size and the 95% confidence interval (indicated in brackets) are shown, considering different numbers of generations separating the reference population sample (collected in 2002) and the monthly cohorts (collected in 2007).

Month collected	*n*	1 generation	2 generations
February	82	68.1 (49.1, 98.1)	136.3 (98.2, 196.2)
March	100	65.5 (48.3, 90.8)	131.0 (96.6, 181.5)
April	106	75.3 (54.9, 106.3)	150.7 (109.9, 212.6)
May	100	68.3 (50.1, 95.7)	136.6 (100.1, 191.4)
June	101	55.6 (42.0, 74.6)	111.3 (84.1, 149.2)

To further verify if the absence of detected sibship structure might be somehow a consequence of the high rate of null alleles and the probable substantial level of scoring errors affecting Pedigree's accuracy, two series of simulations were performed (P.

Fullsack, unpublished data). Using the data from the March sample as a baseline, a large number of fictitious parents was generated. Each allele at every locus for every parent was then replaced with a probability of 20% by a null allele. One hundred and fifty full-sib families of size 10 or 20 were then constructed by transmitting the parental alleles to offspring following the laws of Mendel, but retransforming the genotypes of offspring that inherited a null allele into apparent homozygous genotypes. Pedigree was then run on the synthetic offspring database without full-sib constraints and managed to reconstruct accurately the family structure despite the high rate of null alleles at each locus. The same approach was used in a second set of simulations where each synthetic offspring allele was shifted plus or minus two bases with a probability of 10%. Again Pedigree (without full-sib constraints) managed to accurately reconstruct the structure despite a very high rate of scoring errors.

These results were also verified using Colony 2.0, in which genetic structure was tested in a similar way, but incorporating the type of mating system (polygamous for both sexes) and null allele frequency estimates into the simulations. While there seemed to be somewhat more structure here (many groups again, but slightly larger numbers of individuals grouped together), these groups did not show high fidelity when multiple runs were performed. This was indicated by the fact that most of the individuals were shuffled around and placed in different groups depending on the simulation, meaning that the Colony program created a different solution each run. This is generally an indication that the likelihood landscape was fairly flat and that the full and half sibling groups were not real.

Overall, these results suggested that there was no evidence of any family structuring within monthly cohorts. Each individual was likely its own family and there were just as many parent pairs as recruits sampled. This is in agreement as well with the large estimated N_e produced here. Therefore, it did not appear that a limited number of parents contributed to the juvenile cohorts recruited to collector lines within each sample month, contrary to expectations based on previous studies (Arnaud-Haond *et al.*, 2002b; 2008) and even more generally to expectations under the sweepstakes reproductive success.

4.4. Discussion

4.4.1. Genetic Consequences of Sweepstakes Reproductive Success

For marine species exhibiting high fecundity, coupled with high larval mortality due to stochastic oceanographic conditions, it is thought that large variances in reproductive success could occur (Hedgecock, 1994; Flowers *et al.*, 2002). If only a low numbers of individuals from a spawning event manage to produce progeny that are successfully recruited, then the genetic signature of one cohort to the next may change. This could result in patterns of genetic patchiness or heterogeneity frequently referred to as "chaotic". Indeed this phenomenon has been observed for many marine fishes and invertebrates (David *et al.*, 1997c; Hogan *et al.*, 2010; Johnson and Black, 1982, 1984; Li and Hedgecock, 1998; Marino *et al.*, 2010; Moberg and Burton, 2000; Pujolar *et al.*,

2011; Selkoe *et al.*, 2006, 2010; Silva *et al.*, 2009; Taris *et al.*, 2009), including *Pinctada margaritifera* at different spatial scales (Arnaud-Haond *et al.*, 2003b, 2004, 2008; also see Chapter 3). Furthermore, if sweepstakes reproduction is occurring, then it is predicted that certain genetic signals would be seen in cohorts of new recruits (Hedgecock, 1994a, b; Hedgecock *et al.*, 2007).

The first prediction the authors proposed was that the genetic diversity of the newly settled recruits should be less than that of the adult population. There are numerous studies comparing genetic diversity within and among temporal cohorts; however, few among these compared genetic diversity in newly settled cohorts to adult progenitors. Within these few studies, results varied depending on the sampling strategy and timing. For example, juvenile European flat oysters (*Ostrea edulis*) collected in the western Mediterranean Sea (near Sète, France) had about 40% lower allelic diversity than the adult sample, even though the sample of juveniles was much larger (Hedgecock *et al.*, 2007). Similarly, temporal cohorts of *O. edulis* larvae in Quiberon Bay (Brittany, France) displayed significantly lower allelic richness than in the adult populations under both natural and experimental conditions (Lallias *et al.*, 2010b). However, an earlier study by Taris *et al.* (2009) showed that cohorts of *O. edulis* from the same sampling area in Quiberon Bay did not display reduced allelic richness compared to adults. Lallias and colleagues proposed that the sampling window (15 days) was too wide in this previous study and that there were several cohorts integrated into one, hiding any true genetic differentiation. More recently, Hogan *et al.* (2010) found no significant differences in genetic diversity when comparing adult and juvenile damselfish (*Stegastes partitus*) sampled along the Mesoamerican barrier reef system. This species is interesting in that

adults are sedentary and defend small feeding territories, but the authors also noted that they have a unimodal lunar spawning cycle, and pelagic larval duration of about 24-40 days, not unlike *P. margaritifera*. So perhaps the juveniles sampled in this case represented a mixture of different cohorts or an admixture of spawning events from different locations.

The data from this chapter were in agreement with the results from Taris *et al.* (2009) and Hogan *et al.* (2010) and did not meet the first prediction of the sweepstakes reproduction theory. Within a single month, there was a lot of genetic diversity as estimated by corrected allelic richness in recruits the Takapoto lagoon. Even the February cohorts from both stations exhibited high variability despite having the lowest number of recruits. Indeed there were no significant differences for corrected allelic richness among the two stations or among the five months. More importantly, there was no significant difference in corrected allelic richness between the newly recruited juveniles here and the young adults (Zones A, B, C) (Table 4.8). The monthly cohort data did not suggest that new cohorts of recruits had significantly decreased genetic diversity compared to putative adult progenitors. Therefore, the first prediction of the theory of sweepstakes-chance reproductive success was not upheld by the data presented in this chapter. Of course, one difficulty here is that the young adult sample (Zones A, B, C) was only a surrogate for the adult progenitors. Even though these individuals were sampled in several locations, they were not all the same age and probably originated from different settled cohorts. As such, they may not be a fair, representative sample of the hypothetical adult progenitor population. Indeed getting such a representative sample may be illusory or very difficult at best. As noted by Arnaud-Haond *et al*. (2008) a

rigorous and probably exhaustive sampling strategy would be required to try to obtain a potentially unbiased sample of a putative parental population in a species exhibiting chaotic spatial structure like *P. margaritifera*.

The second prediction of the theory of sweepstakes reproductive success is that temporal cohorts of juveniles should be genetically differentiated (Hedgecock, 1994a, b). If different sets of adults contribute to each cohort and few individuals are reproductively successful due to unfavourable oceanographic conditions, this would be expected to result in random genetic drift. Unlike the limited examples of studies dealing with the first prediction as cited above, there have been frequent descriptions of genetic differentiation among temporal cohorts of new recruits or larvae in marine fishes (Hogan at al., 2010; Ruzzante *et al.*, 1996; Selkoe *et al.*, 2006), and invertebrates such as crustaceans (Johnson and Wernham, 1999; Marino *et al.*, 2010), echinoderms (Moberg and Burton, 2000; Watts et al, 1990), gastropods (Johnson and Black 1982; 1984) and even coral (Brazeau *et al.*, 2011). There is in particular abundant literature comparing genetic composition in juveniles or larvae for numerous bivalve species such as *Spisula ovalis* (David *et al.*, 1997a, c), *Ostrea edulis* (Hedgecock *et al.*, 2007; Lallias *et al.*, 2010b; Taris *et al.*, 2009), *Mytilus spp.* (Gilg and Hilbish, 2003a, b; Nikiforov, 2000) and *Crassostrea gigas* (Hedgecock, 1994a; Li and Hedgecock, 1998). The studies that are more notable are those with significant genetic structure among temporal cohorts, as well as those sampled from populations that are semi or completely isolated from neighbouring populations (e.g. Li and Hedgecock, 1998). This is because the population under study should be free from the influences of migration when temporal genetic

analyses are performed, as gene flow could cause changes in allele frequencies (Hedgecock, 1994b). This means that significant differences observed in gene frequencies among cohorts would not have been due to gene flow. Many of the aforementioned studies suggested that patterns of genetic differentiation among larvae or new recruits may have been due to the contribution of different groups of relatively small numbers of spawning adults, which could have resulted from a sweepstakes reproduction or highly variable reproductive success.

P. margaritifera within some atolls of French Polynesia are relatively isolated from other atoll populations. This is especially true of Takapoto, which has virtually no exchange with the open ocean. This atoll has not open passes and water exchange with the surrounding Pacific ocean occurs through a couple of "hoa", small, shallow surface channels where water circulates mostly as a result of wave action (Rougerie, 1995). Results from the current study showed small but significant temporal genetic structure among monthly cohorts using pair-wise F_{ST} estimates (Table 4.2, 4.3), and even more structure when comparing differences in allelic frequencies (Table 4.4, 4.5). Therefore, this pattern was likely not due to gene flow from a neighbouring population. More limited differentiation was seen on a spatial scale between the two collector lines. Such differentiation was only apparent in February. In the previous chapter, young adults from collectors at Zone A were significantly differentiated from those at Zones C and B, which meant that despite close proximity of these sites (<10kms) there was potential for restricted gene flow between these two regions within the atoll. It should be noted however that the differences between Zone A and Zones B and C in the previous chapter might have been driven by temporal processes in addition to spatial ones. As the

individuals had been harvested after 10-15 months in situ, each collector may have included cohorts recruited at different time. Indeed recruit density and allelic diversity was much higher in Zone A than Zone C possibly indicating multiple cohorts in Zone A. Spatial patterns of genetic differentiation may also have been produced by changes in dispersal patterns between patches/populations with different genetic structure (Flowers *et al.*, 2002). The latter situation is unlikely since the water currents within the lagoon are wind generated and are fairly constant, except during periods of strong winds or storm activity. In general the water masses in the southern part of the atoll where the two collector lines had been deployed should be expected to be well mixed during the period when maximum recruitment had been seen, except possibly in February.

For each collector only a small subset of individuals (25) were randomly sampled from a large number of recruits (see size frequency distributions in Fairbairn, 2009), except for February where all individuals were genotyped because of lower recruitment within that month. This month in particular was peculiar, which showed the only significant F_{ST} comparisons between collectors within station as well as between stations within month (Tables 4.1, 4.2, 4.4, see also Tables A3 and A4 in Appendix). It is plausible that recruitment was lower in February because of increased mortality coupled with fewer individuals spawning during the last two months and possibly less favourable habitat conditions. During the austral summer, absence of strong winds and swells along with strong precipitation cause stronger stratification of the water column (Rougerie, 1994). Within closed atolls such as Takapoto, lagoon water temperature can increase to 30°C or higher with correlated decreases in dissolved oxygen, as well as elevated salinity levels of about 41 psu (practical salinity units, similar to ppt) (Rougerie, 1994). These

habitat extremes can be stressful to corals and benthic fauna where they are present in high densities (Rougerie, 1994). It is possible that water stratification and more limiting environmental conditions might have been responsible for both lower recruitment and small scale spatial genetic differentiation that month.

In any case, there was clear genetic differentiation over time in spat cohorts of *P. margaritifera* within the Takapoto lagoon. Limited spatial differentiation was observed in February. Significant differentiation among young adult populations on collectors was also observed in three different zones, which as explained above may also have been driven in part by mixing of temporally differentiated cohorts. These observations were in agreement with the second prediction of a sweepstakes recruitment hypothesis stating that temporal cohorts should be differentiated (Hedgecock, 1994a, b). However, the observations of high levels of genetic diversity in all cohorts and the low levels of differentiation did not appear to suggest that these could be the result of the recruitment from a limited number of parents as expected under highly variable reproductive success.

4.4.2. The Number of Breeders Contributing to Temporal Cohorts

In the case of many marine invertebrates and fish with high fecundity and high larval mortality, there is the potential for considerable variance in reproductive success. Such a scenario could mean that only a small number of breeders successfully reproduce, while the majority fails to produce offspring (Hedgecock, 1994a). This has been described as something similar to a "sweepstakes lottery", where the few progenitors that succeed in reproducing could essentially replace the entire population (Hedgecock *et al.*,

2007). An important aspect of the theory of sweepstakes-chance reproductive success is that there are actually low effective numbers of breeders at the head of cohorts. This is then responsible for both the prediction of lower genetic diversity in cohorts compared to the adult population as a whole and for temporal (and spatial) genetic differentiation among cohorts. This last aspect would be driven by genetic drift as each cohort would originate from a limited number of parents. Indeed low effective population size (N_e) or effective number of progenitors (N_b) has frequently been observed in many marine populations (Boudry *et al.*, 2002; Hedgecock *et al.*, 2007; Lallias *et al.*, 2010b), but in particular within hatchery stocks of shellfish (Appleyard and Ward, 2006; Gaffney et al, 1996; Hedgecock and Sly, 1990; Hedgecock *et al.*, 1992; Launey *et al.*, 2001; Lind *et al.*, 2009). Four selected lines of the pearl oyster *Pinctada martensii* showed low estimates of number of breeders, as well as temporal variance effective population size (N_k) over six generations (Wada, 1986b, as cited by Hedgecock *et al.*, 1992). The number of breeders ranged from 15 to 21 in the selected lines, which each originated from 280 progenitors. Similarly, using the method of variance in allelic frequencies, there were low estimates of N_e for six collectors (the same Zone A, B, C collectors analysed in the previous chapter) of *P. margaritifera* within Takapoto (Arnaud-Haond *et al.*, 2008). That study used different samples as a putative genitor source, either a) one of three wild beds found in Takapoto, b) a pool of these three beds, or c) a pool of other natural laying beds in different atoll lagoons of the Tuamotu Archipelago. N_e for each collector varied according to the parental source, but was generally low in the range of 10 to 30 when using single Takapoto beds or the pool of beds, but larger (~40 to ~130) when using the Tuamotu pool. As is generally the case, these estimates had large error variance. In the

present study, the effective population size was also estimated using variance in allelic frequencies and by using the pooled genetic resources of the three Takapoto collection zones as the putative reference population. The N_e estimates for the monthly collector juveniles were substantially larger than those for the adults from the previous study (Table 4.10 and Table 5 in Arnaud-Haond *et al.*, 2008), particularly when that last study used only Takapoto samples as the putative progenitor source as was done here. In addition, sibship reconstruction among the young spat in the temporal collectors here did not yield any evidence that any of the young recruits were related. This implies that the estimated number of broodstock that generated these recruits was not limited and was higher than that observed for other bivalve species from hatchery and wild stocks (*Ostrea edulis*: Hedgecock *et al.*, 2007; Lallias *et al.*, 2010b; Launey *et al.*, 2001; *Crassostrea gigas*: Appleyard and Ward, 2006; Boudry *et al.*, 2002; Hedgecock and Sly, 1990; see also Hedgecock *et al.*, 1992 and references therein). In cultured silver-lipped pearl oysters (*Pinctada maxima*), it was observed that a single full-sib group comprised 40% of a mass-spawned cohort, while two full-sib groups made up 56% of a controlled-spawn population (Lind *et al.*, 2009). Test crosses of the Pacific oyster (*C. gigas*) sampled from the French Atlantic coast revealed not only low effective population sizes, but decreasing N_e with sampling time (Boudry *et al.*, 2002). Using pedigree analysis the authors found that N_e decreased by 62% and 32% in the two crosses performed, over 90 days. Even with attempts to balance gametic contributions, the authors noticed there were still uneven parental contributions between males and females.

Unlike many examples from previous studies, it did not appear that a limited number of parents were at the head of each monthly cohort. This may also explain why

no evidence of decreased genetic diversity was apparent in these cohorts. Nonetheless, temporal (and limited spatial) genetic heterogeneity was apparent among these cohorts, but it did not appear to have been driven by a limited number of different breeders producing the various cohorts. Even though there have been documented cases of highly variable recruitment for this species (Friedman *et al.*, 1998; Friedman and Bell, 1999; Oengpepa *et al.*, 2006), as well as observed genetic patchiness here (Chapter 3, present chapter) and in previous studies (e.g. Arnaud-Haond *et al*., 2008), the fine scale temporal analysis of genetic signatures in new recruits did not support the theory of sweepstakes-chance reproductive success in *P. margaritifera* within the Takapoto lagoon.

There have been several hypotheses put forward to explain patterns of chaotic genetic patchiness (Larson and Julian, 1999). In addition to sweepstakes chance-matching, these hypotheses include pre- and post-settlement selection, and variable sources of larvae. Pre- and post-settlement selection would imply that the microsatellite markers used in the present study and the anonymous nuclear markers used in the previous studies are somehow strongly linked to some fitness components. Launey and Hedgecock (2001) showed in several inbred hatchery-produced families of Japanese oysters that strong segregation distortion was quite apparent in 2 and 3 month old spat, but that the segregation ratios were not distorted when the larvae were very young. This was implied to reflect selection against deleterious mutations at fitness genes closely linked to the 19 microsatellite markers they used. Similar observations have been made for European flat oysters, which included more generally the implication of significant genetic load for bivalves (Bierne *et al.*, 1988; Boudry *et al.*, 2002; Launey and Hedgecock 2001). It may be that similar pre- and post-settlement selection is also

happening in *P. margaritifera*, which would lead to the patterns of temporal genetic differentiation that were observed. Lastly, these observations may also reflect variable sources of larvae that could have been produced by large numbers of genetically differentiated patches of individuals spawning at a different time. Considering the extended larval planktonic phase of this species, and despite obvious hydrodynamic mechanisms for mixing larvae of different origin in the lagoon, this implies that genetically differentiated larval "clouds" would still settle at different times and/or locations therefore leading to "chaotic genetic patchiness".

4.5. Conclusion

A recent worry within the pearl industry is that farmed oysters issued from massive spat collection and raised in the same environment as the wild individuals would have lower genetic diversity than the wild oysters given high variance in reproductive success and possible low effective number of breeders. Over many generations the reproduction of large numbers of farmed individuals with reduced genetic variability could eventually impact the wild population's genetic variability. Because of observed cases of high variability in recruitment, as well as spatial genetic heterogeneity, especially at smaller spatial scales, it was expected that *Pinctada margaritifera* would display low effective number of breeders at the source of recruits. Under the hypothesis of a sweepstakes reproductive success, juvenile cohorts were expected to have lower genetic diversity compared to the adult progenitors, but also to be genetically differentiated from each other in time. While there was significant genetic structure

between the monthly cohorts of juveniles, there was no evidence that these recruits had lower genetic diversity compared to the adult population. Furthermore, there was no significant family structure apparent from the pedigree analysis and estimates of effective number of breeders was quite high. Therefore, it would seem that the juvenile cohorts were not the result of a limited number of successful parents. While recruitment may be variable, it did not appear to drive the patterns of genetic patchiness observed for this species at least within the Takapoto lagoon.

Chapter 5: Conclusion

In many instances reported in the literature, there have been significant negative genetic effects associated with hatchery propagation and farming (aquaculture). The most significant consequences are large losses of genetic diversity within the farmed stocks compared to the original wild progenitors, as well as significantly lower effective population sizes. In turn, concerns have been raised about the possible negative impacts that such domesticated populations could have on the wild genetic resources if there was a conduit for gene flow between wild and cultured populations. Several documented cases of wild-domestic genetic interactions in fish, especially salmonids (Herbinger et al., 2003 and references therein), have made it apparent that careful monitoring of these potential impacts and further research in other species of fish and shellfish are needed.

In French Polynesia, overharvesting of the wild oysters for the button trade in the twentieth century caused significant population declines and crashes in several atoll lagoons by the 1950's and 60's (Intes, 1984, as cited by Cabral, 1989). This finally led to the closure of the fisheries. When pearl oyster farming activities started in the late seventies, regulations were put in place to protect the wild resources. Paramount among these was the continued interdiction of wild adult oyster harvesting, which led to the development of efficient spat collection techniques to procure the necessary individuals for culture. Present pearl production in French Polynesia is entirely based on the massive collection of spat in the lagoons of about 15 atolls and islands, and wild beds are entirely protected from harvesting in all the archipelagos. Despite this success, renewed concerns about possible negative impacts of the black pearl industry on the local wild populations

of *Pinctada margaritifera* have been expressed, mostly in the area of genetic impacts on the wild resource. During the phase of rapid expansion of the pearl industry, spat translocation among atolls and even archipelagos regularly took place. These practices are thought to be the primary cause of the genetic homogeneity that has been observed across the Tuamotu-Gambier Archipelagos (Arnaud-Haond *et al.*, 2004). Spat translocation is now more tightly regulated, although it still happens, and present concerns are now centered on whether the aquaculture practices may lead to loss of genetic variability in the wild stocks.

Presently the farms could potentially cause decreased genetic diversity in local wild populations over many generations if the farmed individuals, represented on collectors, came from a limited number of breeders and if these cultured individuals produce spat that will recruit back into the wild populations. Indeed, under the sweepstakes reproductive success hypothesis, which seems to be common in marine bivalves, one could see low effective numbers of breeders contributing to cohorts of new recruits. In this scenario, possible genetic signatures would include 1) comparable or greater genetic structuring at small spatial scales compared to broader spatial scales, 2) significant differentiation among temporal cohorts of recruits, as well as 3) reduced genetic diversity in new recruits compared to the parent pool from which they originated (Hedgecock, 1994a, b). The present thesis was concerned with gaining a better understanding of recruitment processes involved in spat collection and its potential genetic consequences.

The results from this study provide evidence that the current spat collection practices from the pearl farming industry do not appear to be negatively affecting the

wild populations within the atoll lagoons. Within the four atolls of Apataki, Takaroa, Makemo and Mangareva (Figure 2.1), there were no significant decreases in genetic diversity from local wild to famed populations of *P. margaritifera* (Chapter 2). No significant genetic differentiation was seen between wild and farmed collections or among the four atoll populations across Tuamotu and Gambier, despite being separated by hundreds of kilometres (Chapter 2). In contrast, at small spatial scales within the Takapoto lagoon (<10kms) young adults from three collector sites showed low but significant genetic structuring. Using anonymous nuclear markers, the individuals from the Zone C collectors appeared to be differentiated from the other two sites (Arnaud-Haond *et al.*, 2002b, 2008), while Zone A oysters were significantly differentiated from the other two sites using microsatellite markers (Chapter 3). These results demonstrated that there was more genetic structure at a small spatial scale compared to what was observed at a much broader geographic scale. Furthermore at even smaller spatial and temporal scales within Takapoto, it was shown that there were small but significant differences between five temporal cohorts of *P. margaritifera* juveniles (Chapter 4, Tables 4.3, 4.5). In this respect, the first two genetic signatures of sweepstakes reproduction were observed, with good evidence of genetic patchiness at small spatial and temporal scales.

However, no significant decreases in genetic diversity in the juvenile cohorts compared to the pool of possible adults were found (Chapter 4). As such, the third genetic signature of Hedgecock's sweepstakes hypothesis was not observed. In fact, estimates of effective population size (N_e) of the parental pool that generated the temporal cohorts were higher than previously estimated (Chapter 4). Additionally, there

was no evidence that the young adults collected in Zones A, B, and C (Chapter 3) and the juvenile cohorts (monthly collections, Chapter 4) were the result of a limited number of parents, despite expectations from previous studies (Arnaud-Haond *et al.*, 2008; Hedgecock *et al.*, 1992; 2007; Lallias *et al.*, 2010b). Overall, genetic patchiness at small spatial and temporal scales was observed. However, these patterns did not appear to be driven by the successful reproduction of a limited number of parents as implied by the sweepstakes-chance hypothesis, at least within the Takapoto lagoon.

One possible explanation for the fine scale genetic patterns may be that pre- and post- settlement selection acting on fitness genes linked to the markers may be generating the observed small scale patchy structure. Another possible explanation may be that larval groups that settled and recruited at different times and locations may have originated from large and genetically differentiated parental patches. This explanation would require a mechanism to prevent various larval groups that were spawned at the same time to be thoroughly mixed and homogenized during their long planktonic phase. However, it may be conceptually easier to explain genetic differentiation on a temporal basis. The resulting genetic patchiness in temporal cohorts of recruits may have been produced by large groups of different individuals spawning at different times. If a different "patch" or set of patches spawns every month, and these are large numbers of oysters, then the possible genetic pool available for settlement each month would be relatively large, but would also show potentially different genetic signature.

Overall, there does not appear to be reduced genetic diversity in farmed oysters compared to local wild populations and there does not appear to be limited numbers of parents contributing to cohorts recruited to collector lines. Under these circumstances it

does not appear that the current methods of spat collection are negatively impacting the local wild populations. However, more fine scale analysis in other atolls with oyster culture would be warranted as environmental conditions and recruitment within each atoll lagoon may vary.

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APPENDIX

Table A1. Growth rate measurements of *P. margaritifera* based on mean dorsoventral height (DVH, mm), geographic location of study, age, and type of system in which the oysters were held during growth experiments. The average growth rate (mm yr^{-1}) is also given where possible.

Location	Growth Type	Age	Mean DVH (mm)	Study
Pioneer Bay, Australia	Nursery Culture	41 days	1.4 (± 0.1 SE)	Beer and Southgate (2008)
		4 weeks‡	3.9 (± 0.2 SE)	
		10 weeks‡	20.5 (± 0.6 SE)	
French Polynesia	Hatchery Culture	1 month	0.2 - 0.3	Coeroli *et al.* (1984)
		2 months	2.0 - 3.0	
		3 months	8.0 - 10	
		6 months	40 - 50	
		12 months	70 - 80	
Solomon Islands	Wild Spat, suspended in culture	initial sizes	8.3 - 51.5	Friedman and Southgate (1999)
		w/in 3 months	20.4 - 24.8**	
		w/in 5 months	30.7 - 36.5**	
Taiwan	Wild Spat	initial sizes	20.1-146.7	Hwang *et al.* (2007)
	initial size < 50mm	4-5 months	50 - 60	
	initial size > 50mm	1 year	85 - 105	
Pohnpei Lagoon, Federated States of Micronesia	Nursery Culture	6 months	39.8 (± 6.4 SE)	Ito *et al.* (2004)
		9 months	55.5 (± 7.1 SE)	
		12 months	83.9 (± 7.6 SE)	
Queensland, Australia	Nursery Culture			Pit and Southgate (2003a)
	uncleaned trays	16 weeks	16.2 (± 1.0 SE)	
	cleaned every 4 weeks	16 weeks	19.4 (± 1.2 SE)	
	cleaned every 8 weeks	16 weeks	21.2 (± 0.8 SE)	
Queensland, Australia	Nursery Culture			Pit and Southgate (2003b)
	runts (<5mm)	4 months	24.6 (± 0.4 SE)	
	normal growers (5-10mm)	4 months	32.3 (± 0.4 SE)	
	fast growers (10mm)	4 months	35.6 (± 0.4 SE)	
Takapoto Lagoon, French Polynesia	Suspended Culture			Pouvreau *et al.* (2000b)
	year class 1	2 years	76.5 (± 10.5 SD); 32.7 †	
	year class 2	3 years	113.3 (± 6.7 SD); 15.6 †	
	year class 3	4 years	126.8 (± 8.2 SD); 8.1 †	

Location	Growth Type	Age	Mean DVH (mm)	Study
French Polynesia	Suspended Culture	21-26 months	100*	Pouvreau and Prasil (2001)
		3 years	119 - 135 (± 6.9 SD); 19.7 - 31.8 †	
Cook Islands	Suspended Culture	1 year	~80 mm	Sims (1993b)
		2 years	~118mm	
		4 years	~122mm	
		5 years	~131mm	
Australia	Nursery Culture	43 days	1.37 (± 0.1 SE)	Southgate and Beer (1997)
	(density treatments)	106 days	11.2 (± 2.7 SE)	
	plastic trays 10	19 weeks	37.39 (± 1.47 SE)	
	50	19 weeks	35.70 (± 0.66 SE)	
	100	19 weeks	40.48 (± 0.91 SE)	
	pearl nets 20	19 weeks	39.22 (± 0.65 SE)	
	50	19 weeks	37.30 (± 0.41 SE)	
	100	19 weeks	34.28 (± 0.58 SE)	
	150	19 weeks	30.63 (± 0.55 SE)	
	200	19 weeks	29.77 (± 0.58 SE)	

* = the ideal size of oysters to be implanted with a nucleus for pearl production. Pouvreau and Prasil (2001) reported it took about 21-26 months for the oysters in their study to reach this size.
** = shell increment changes (mm) in the initial oysters after three and five months.
† = average growth rate (mm yr^{-1}) for the age group specified; ‡ = growth rate after settlement.

Table A2. The annealing temperature, observed size range of alleles, and optimal amplification conditions for the primers used. For all loci the final volume was 10 µL. (10x PCR buffer [New England Biolabs, 10 mM KCl, 10 mM (NH4)2SO4, 20 mM Tris-HCl, 0.1% Triton X-100, pH 8.8 at 25°C], Taq DNA polymerase [New England Biolabs])

Locus	T (°C)	Range (bp)	dNTP (0.2 mM)	10x PCR Buffer †	$MgSO_4$	Forward/Reverse primers	Taq DNA polymerase
Pmarg2	52	169-251	1 µL	1 µL	2 mM	0.3 µM (0.3 µL)	1 U
Pmarg7	52	156-188	1 µL	1 µL	1.5 mM	0.2 µM (0.2 µL)	0.5 U
Pmarg11	50	164-258*	1 µL	1 µL	1.5 mM	0.2 µM (0.2 µL)	1 U
Pmarg37	48*	137-217*	1 µL	1 µL	1.5 mM	0.2 µM (0.2 µL)	0.5 U
Pmarg45	50	112-280*	1 µL	1 µL	1.5 mM	0.2 µM (0.2 µL)	0.5 U
Pmarg68	50	140-184	1 µL	1 µL	1.5 mM	0.2 µM (0.2 µL)	0.5 U
Pmarg77	50	116-212*	1 µL	1 µL	2 mM	0.3 µM (0.3 µL)	1 U
Pmarg79	50	198-266	1 µL	1 µL	2 mM	0.3 µM (0.3 µL)	1 U

* = modified from Herbinger *et al.* (2005); † = $MgSO_4$ included in PCR buffer mixture.

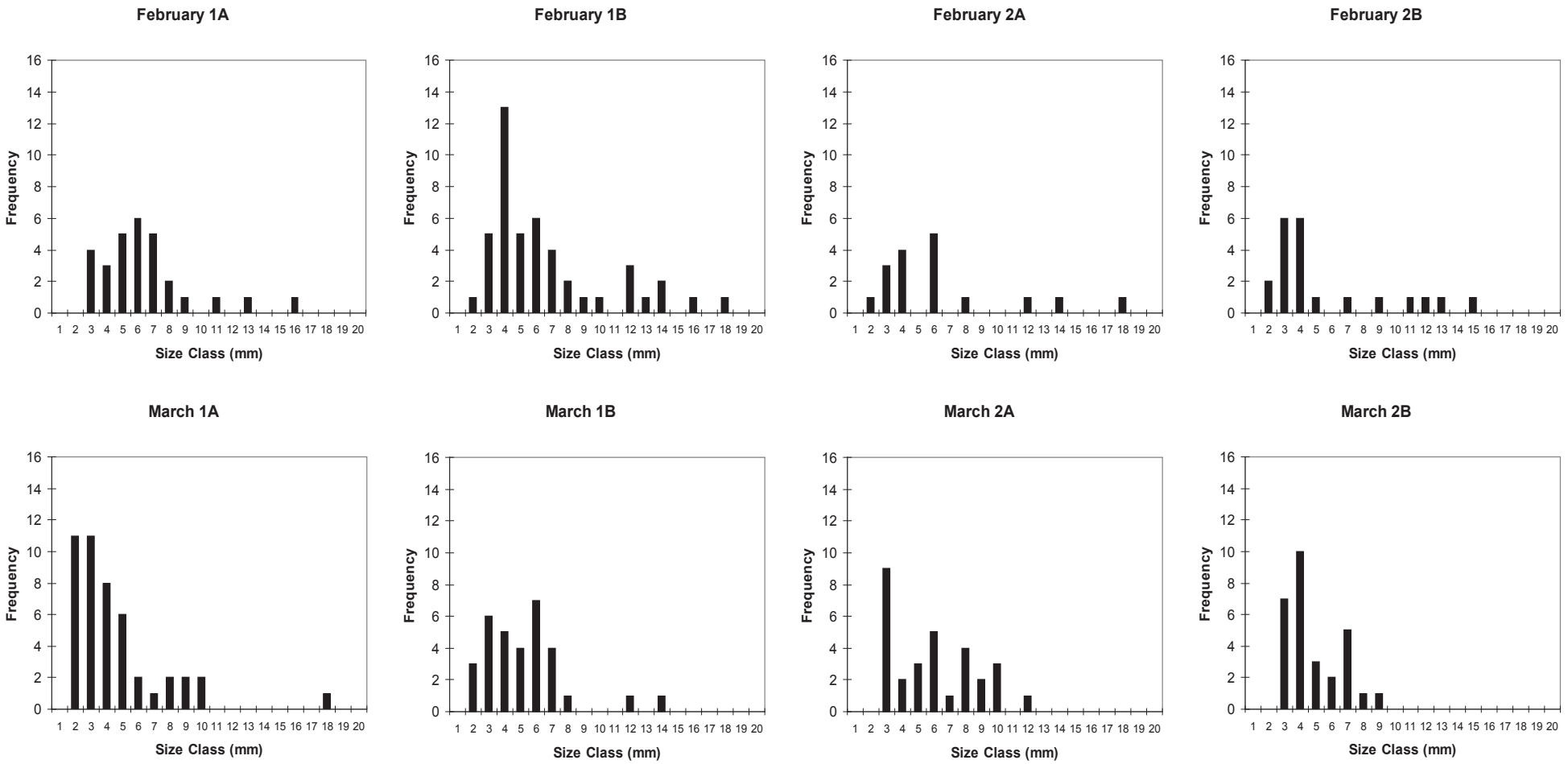

Figure A1. Size frequency distributions of *Pinctada margaritifera* spat per station (1 and 2) and replicate (A and B) collector, from February to June of 2007. Reprinted from Fairbairn (2009).

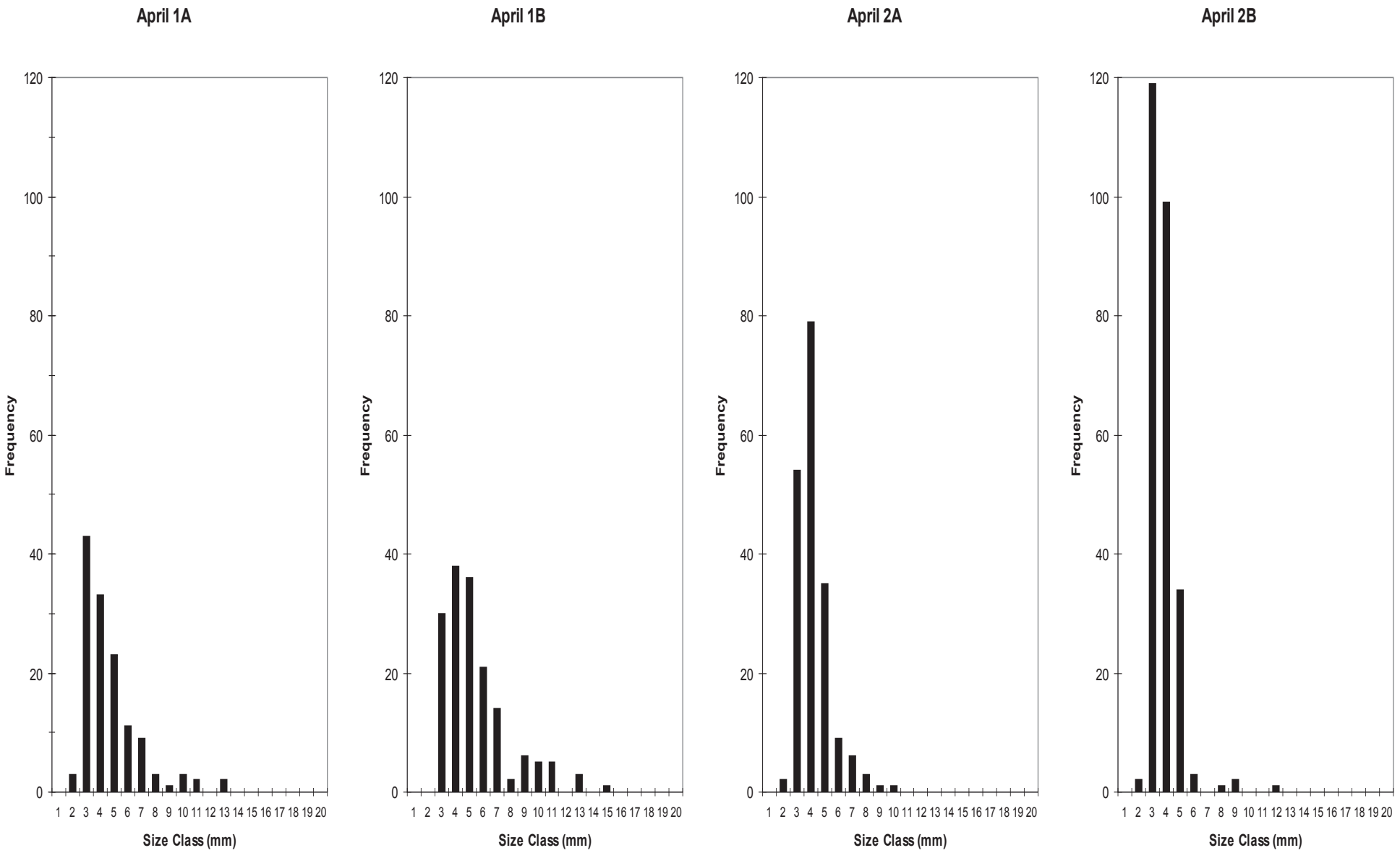

Figure 1A continued.

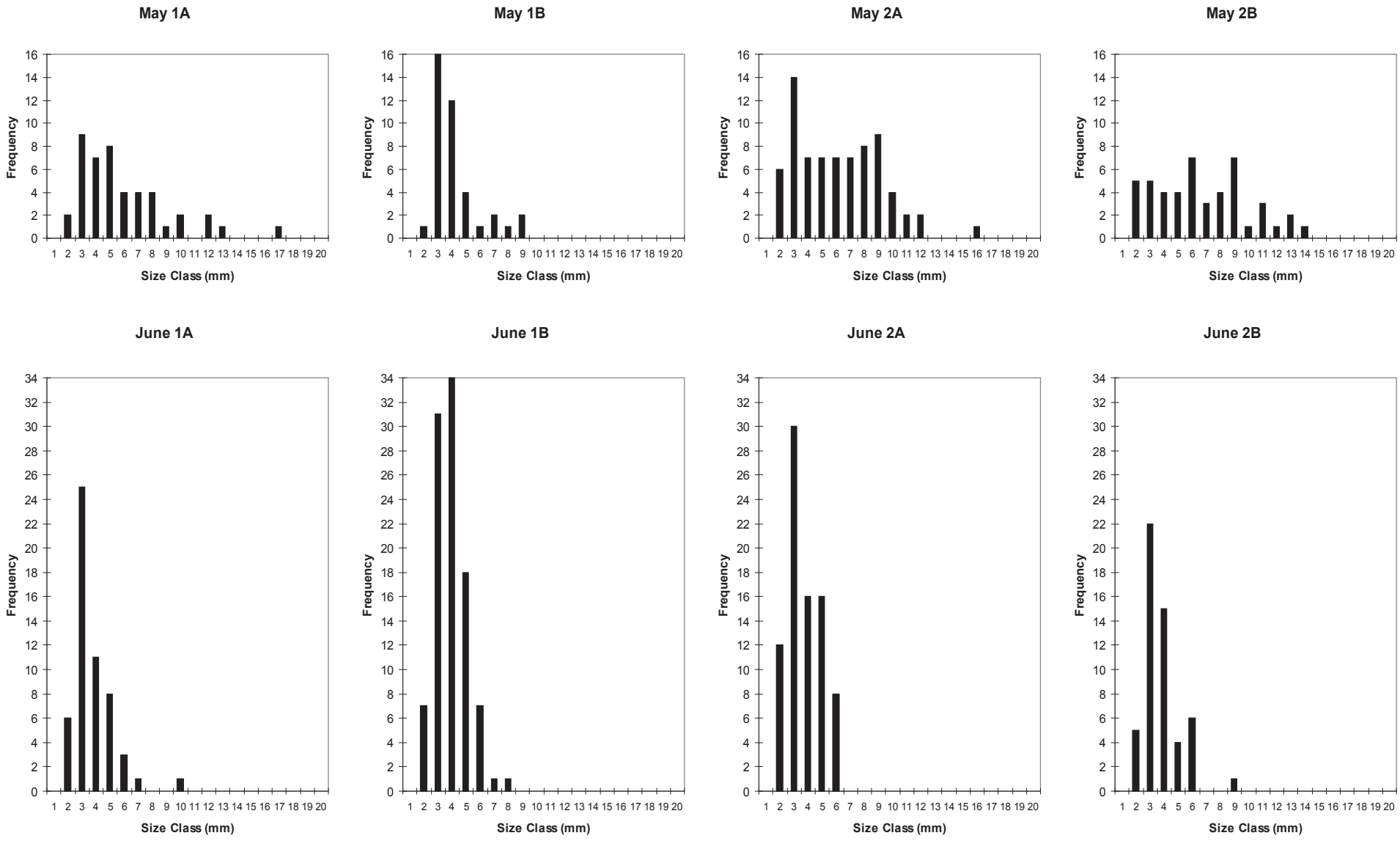

Figure A1 continued.

Table A3. Pair-wise F_{ST} matrix of pearl oyster juveniles from collectors at two sampling stations within the Takapoto lagoon over five months, based on 11 microsatellite loci. All replicates and stations are separate. F_{ST} values are below the diagonal, while probability values based on 3800 permutations are above the diagonal. Bold values are those significant at $\alpha = 0.05$. None were significant after correction for multiples comparisons.

	Feb1A	Feb1B	Feb2A	Feb2B	Mar1A	Mar1B	Mar2A	Mar2B	Apr1A	Apr1B	Apr2A	Apr 2B	May1A	May1B	May2A	May2B	Jun1A	Jun1B	Jun2A	Jun2B
Feb 1A	0	0.8661	0.5124	0.6832	0.9818	0.7126	0.9824	0.4045	0.6016	0.6142	0.9958	0.6250	0.9905	0.9855	0.8511	0.8958	0.6124	0.7313	0.4068	0.8213
Feb 1B	-0.0095	0	0.0016	0.2158	0.0471	0.0008	0.8626	0.0558	0.1697	0.0668	0.1640	0.0697	0.3645	0.2524	0.2508	0.0771	0.0095	0.5758	0.0334	0.2071
Feb 2A	0.0008	**0.0105**	0	0.0300	0.0732	0.0811	0.1634	0.0261	0.0047	0.0068	0.0413	0.0900	0.0832	0.0111	0.0047	0.2337	0.0279	0.1450	0.1576	0.1155
Feb 2B	-0.0021	0.0034	**0.0038**	0	0.0961	0.1197	0.7482	0.1482	0.4608	0.2942	0.1342	0.5271	0.0866	0.2966	0.2829	0.3961	0.6368	0.2526	0.2918	0.3858
Mar 1A	-0.0135	-0.0010	0.0091	0.0009	0	0.1047	0.8276	0.2411	0.8068	0.5734	0.4134	0.4311	0.2974	0.2582	0.3216	0.6463	0.0626	0.3347	0.2250	0.1713
Mar 1B	-0.0053	**0.0040**	0.0062	0.0005	-0.0017	0	0.6613	0.2479	0.1163	0.0155	0.1782	0.2121	0.0747	0.3526	0.0961	0.0634	0.0458	0.0545	0.0268	0.0800
Mar 2A	-0.0086	-0.0004	0.0091	-0.0018	-0.0035	-0.0040	0	0.4195	0.8540	0.2432	0.9800	0.9071	0.8045	0.9337	0.7350	0.8968	0.7074	0.8968	0.5134	0.1937
Mar 2B	-0.0040	0.0038	**0.0056**	0.0013	-0.0046	-0.0007	0.0025	0	0.2655	0.1529	0.1029	0.3829	0.1145	0.0261	0.0187	0.0471	0.0368	0.2192	0.0040	0.1818
Apr 1A	-0.0011	0.0007	**0.0193**	0.0061	-0.0049	0.0037	0.0038	0.0025	0	0.5316	0.6421	0.5482	0.3353	0.4184	0.7305	0.2579	0.4018	0.8113	0.0313	0.2966
Apr 1B	-0.0085	0.0007	**0.0099**	-0.0019	-0.0048	0.0003	0.0033	0.0003	-0.0001	0	0.1482	0.5097	0.6666	0.4232	0.0090	0.0521	0.0182	0.0692	0.0926	0.0953
Apr 2A	-0.0094	-0.0029	**0.0102**	0.0032	-0.0021	0.0013	-0.0024	-0.0021	-0.0037	0.0007	0	0.6200	0.7416	0.6361	0.4650	0.5066	0.9790	0.7958	0.0666	0.9034
Apr 2B	-0.0051	0.0020	0.0141	0.0016	-0.0033	0.0001	-0.0048	0.0004	-0.0021	-0.0028	-0.0033	0	0.7990	0.6432	0.1250	0.4292	0.3500	0.3000	0.0287	0.2708
May 1A	-0.0121	-0.0041	0.0072	0.0037	-0.0014	0.0028	-0.0002	0.0027	0.0017	-0.0050	-0.0041	-0.0046	0	0.3500	0.1979	0.3890	0.4505	0.0761	0.0845	0.6524
May 1B	-0.0083	0.0019	**0.0194**	0.0035	0.0012	0.0027	-0.0061	**0.0089**	0.0050	0.0033	0.0011	-0.0009	0.0038	0	0.6645	0.4705	0.1161	0.3287	0.0105	0.3158
May 2A	-0.0048	-0.0004	**0.0143**	0.0043	-0.0042	0.0028	0.0008	**0.0023**	-0.0046	0.0020	-0.0024	-0.0008	-0.0020	0.0028	0	0.6487	0.1553	0.6300	0.0055	0.1013
May 2B	-0.0095	-0.0016	0.0024	0.0018	-0.0044	0.0008	-0.0015	**0.0032**	0.0050	0.0020	-0.0002	0.0001	-0.0038	0.0046	-0.0012	0	0.1253	0.7637	0.2097	0.3037
Jun 1A	0.0005	**0.0082**	**0.0001**	-0.0045	0.0034	**0.0021**	0.0028	**0.0035**	0.0057	0.0057	-0.0033	0.0018	0.0007	0.0112	0.0041	0.0024	0	0.7347	0.1163	0.3971
Jun 1B	-0.0061	-0.0027	0.0012	0.0027	-0.0034	**0.0018**	-0.0027	-0.0024	0.0004	0.0004	-0.0050	0.0004	0.0033	0.0056	-0.0035	-0.0040	-0.0030	0	0.1547	0.2871
Jun 2A	-0.0047	**0.0028**	-0.0007	-0.0022	-0.0050	**0.0035**	0.0027	**0.0035**	0.0039	0.0010	0.0016	0.0018	-0.0008	**0.0133**	**0.0034**	0.0003	-0.0049	-0.0038	0	0.1576
Jun 2B	-0.0068	-0.0003	0.0042	0.0004	-0.0019	0.0022	0.0037	-0.0009	0.0015	0.0011	-0.0054	-0.0025	-0.0044	0.0055	0.0016	-0.0041	-0.0008	-0.0011	-0.0031	0

Indicative adjusted nominal level (5%) for multiple comparisons is: 0.000263; for just the comparisons between replicates within station and month it is: 0.005 .

Table A4. The P-value from tests of genic differentiation for each population pair across all loci (exact G test). Purple shows 10 comparisons between replicates within the same monthly station, pink shows the 20 comparisons between replicates across stations within the same month, light turquoise signifies the 40 comparisons across months within station 1, light green denotes the 40 comparisons across months within station 2, and yellow represents the 80 comparisons across station and across months.

Cohort	Feb1A	Feb1B	Mar1A	Mar1B	Apr1A	Apr1B	May1A	May1B	Jun1A	Jun1B	Feb2A	Feb2B	Mar2A	Mar2B	Apr2A	Apr2B	May2A	May2B	Jun 2A	Jun 2B
Feb1A	-																			
Feb1B	0.5221	-																		
Mar1A	0.8273	0.0013	-																	
Mar1B	0.1685	**0.0000**	0.0051	-																
Apr1A	0.0136	*	0.1857	**0.0000**	-															
Apr1B	0.1034	**0.0002**	0.0228	**0.0001**	0.0036	-														
May1A	0.4509	0.0033	0.0094	0.0015	0.0030	0.1483	-													
May1B	0.5015	0.0256	0.0066	0.0074	0.0158	0.0140	0.0132	-												
Jun1A	0.0132	**0.0000**	0.0004	0.0005	0.0023	**0.0000**	0.0128	*	-											
Jun1B	0.1130	0.0848	0.0139	**0.0002**	0.0189	0.0003	**0.0000**	0.0038	0.0182	-										
Feb2A	0.0072	**0.0000**	**0.0002**	**0.0002**	**0.0000**	**0.0000**	0.0004	*	**0.0002**	**0.0002**	-									
Feb2B	0.0338	0.0021	0.0003	**0.0002**	0.0005	0.0056	*	0.0021	0.0173	0.0020	**0.0001**	-								
Mar2A	0.4940	0.1469	0.0559	0.0225	0.0384	0.0012	0.0459	0.1857	0.0326	0.1440	**0.0001**	0.0223	-							
Mar2B	0.0076	*	0.0109	0.0003	0.0008	0.0004	*	**0.0000**	**0.0000**	0.0004	*	**0.0000**	0.0012	-						
Apr2A	0.4500	0.0074	0.0289	0.0056	0.0354	0.0007	0.0904	0.0564	0.2513	0.0814	**0.0000**	**0.0001**	0.2251	0.0011	-					
Apr2B	0.0387	0.0003	0.0394	0.0019	0.0625	0.0129	0.0978	0.0492	0.0035	*	**0.0001**	0.0024	0.1088	0.0003	0.1295	-				
May2A	0.1836	0.0028	0.0032	0.0005	0.0382	**0.0000**	0.0022	0.0759	**0.0001**	0.0354	**0.0000**	0.0020	0.0397	**0.0000**	0.0073	**0.0002**	-			
May2B	0.1909	0.0004	0.0755	0.0006	0.0004	**0.0001**	0.0180	0.0029	0.0009	0.0618	0.0019	0.0038	0.1352	*	0.0118	0.0022	0.0644	-		
Jun2A	0.0417	**0.0001**	0.0197	**0.0000**	**0.0000**	0.0003	**0.0002**	**0.0000**	0.0061	0.0019	**0.0000**	0.0010	0.0040	**0.0000**	0.0004	**0.0001**	*	0.0003	-	
Jun2B	0.1141	0.0004	0.0072	0.0038	0.0008	**0.0003**	0.1129	0.0094	0.0148	0.0008	0.0010	0.0018	0.0013	0.0034	0.2957	0.0107	*	0.0086	0.0018	-

* = "highly significant" P-value and means the chi-square value was infinity. Values in bold are P-values below the critical value at the threshold of 0.000263 (after correction for multiple comparisons) after 1000 iterations. Sample sizes for each collector were as follows: February, 17, 31, 19, 15; March, 25 for each collector; April, 31, 25, 25, 25; May, 25 for each collector; and June, 25, 25, 25, and 26.