**Population genetics of the school shark (*Galeorhinus galeus*)
in New Zealand, Australian and Chilean waters**

By

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This thesis is dedicated to the memory of my grandmother (Tally)

Abstract

The school shark (*Galeorhinus galeus*) is a coastal benthic-pelagic species that is highly migratory and has a widespread distribution in temperate waters. This species matures late, has a relatively low fecundity and is slow growing, which makes it vulnerable to overfishing. They are commercially fished throughout their distribution, and some global stocks have been under pressure because of poor management. In Australia, longline and gillnet fisheries targeted pregnant females and juveniles around Victorian and Tasmanian nursery grounds, resulting in loss of historical inshore nursery habitat. School shark tagging programmes have reported migration between Australian and New Zealand stocks, but preliminary genetic studies have suggested that there are slight genetic differences between the stocks. Currently, the Australian and New Zealand school shark fisheries are assessed and managed as separate stocks. However, the question of whether this species is comprised of a single population or multiple sub-populations in the South Pacific remains unresolved. Given the commercial importance of the school shark fisheries and the concern about stock levels on the regional and trans-Tasman scales, knowledge of stock structure is essential for effective management. The aim of this thesis research was to determine the levels of genetic diversity and population structure of *G. galeus* in New Zealand and Australia, and compare these to a population in Chile, using mitochondrial DNA (mtDNA) sequencing and microsatellite DNA markers.

The DNA sequence of an 893 base pair region of the mtDNA control region (CR) was determined using 475 school shark samples and nine microsatellite DNA loci were genotyped in 239 individuals. Analyses of the data revealed strong evidence of genetic differentiation between *G. galeus* populations in Australasia and Chile, suggesting restricted gene flow among populations in the western and eastern areas of the Pacific Ocean. The F_{ST} values ranged from 0.188 to 0.300 for CR mtDNA, and 0.195 to 0.247 for microsatellite DNA in *G. galeus*. However, there was no evidence of stock differentiation among New Zealand/Australian sample sites for either mtDNA or microsatellite DNA data. These results support the model of a single panmictic stock across the Tasman Sea. The similarity of the results obtained from the maternally inherited mtDNA and biparental inherited microsatellite loci did not support the suggestion of sex-biased dispersal of *G. galeus* in the New

Zealand/Australia region and it was concluded that females and males had similar patterns of dispersal.

Sharks can be either monogamous or polygamous, which is important when considering stock assessments and harvesting models. Multiple paternity has been reported in several shark species, however, the number of sires per litter varies considerably among species. An investigation of multiple paternity (MP) was conducted in *G. galeus* by assessing the levels of relatedness within progeny arrays using six polymorphic microsatellite DNA markers. Five “families” (mother and litters) were sampled from the North Island of New Zealand and a parentage analysis was conducted. The minimum number of males contributing to each progeny array was estimated by identifying the putative paternal alleles by allele counting and reconstructing multilocus genotypes method. The analysis showed the occurrence of genetic polyandry in *G. galeus*; two of five litters showing multiple sires involved in the progeny arrays (40%). The minimum number of sires per litter ranged from one to four. Although MP was only detected in two litters, this finding is consistent with the known reproductive characteristics of *G. galeus*. It can potentially store sperm for long periods of time and has a specific mating season when males and females typically mix on the edge of the continental shelf. Detecting MP within a litter has highlighted the importance of the post-copulatory selective processes in the *G. galeus* mating system, and this has implications for the management and conservation of genetic diversity.

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Chapter 1

1.2 General Introduction

1.2.1 Fisheries management and the stock concept

Fish are the only major human food item that is still harvested from the natural environment. Marine fishes have become of particular concern due to their high level of exploitation and their use in the aquaculture industry (FAO 2012). However, good management is essential to ensure the sustainable use of a natural biological resource in order to maintain the productivity of a stock (Musick & Bonfil 2005). Crucial to management is an accurate stock assessment because it is typically used to set the regulations and estimate the maximum sustainable yield of a fishery. A stock assessment model is based on a range of fisheries and biological data, including age structure and growth rates, fecundity, sex ratio, life history, mortality, migratory patterns and total biomass. The first step in the process of conducting a stock assessment is to define the harvested or managed unit of a fishery. A stock is most often defined based on geographic location, but natural populations can be spread over a wide range (Walker et al. 2008). It can be difficult to match the most appropriate biological unit of a fishery species to a stock assessment.

A fisheries stock assessment model is developed based upon the notion of an ideal “stock”. Several stock definitions have been discussed in Carvalho & Hauser 1994 and Grant & Waples 2000. In general “stock” refers to a group of organisms whose demographic and genetic trajectory is largely independent from other such groups (Waples 1998). Several approaches have been used to identify a “stock” and these can be classified as direct or indirect methods. Direct methods of egg and larval surveys, and tagging data (mark/recapture) have provided a useful understanding of the stock movements in many fish species (Kohler & Turner 2001). However, these methods are often logistically difficult to undertake, involving intensive surveys that yield relatively little data, and are unable to determine whether immigrants to a stock will survive and reproduce (Waples 1998). For this reason, it is considered important to use genetic markers, which provide an indirect estimation of the relative contribution of migration and/or dispersal among stocks (Waples 1998, Selkoe et al.

2008). When genetic data are combined with other approaches (the direct methods) patterns of genetic connectivity and levels of population structure are more likely to be determined.

The application of the stock concept has been a challenge for stock assessment purposes. This is because the ocean has been viewed as an environment that lacks dispersal barriers (Waples 1998, Ward 2000). However, complex patterns of genetic structure have been reported in many teleost (Ward & Elliot 2001) and elasmobranch species (Dudgeon et al. 2012). For instance, several coastal species exhibit low levels of gene flow between Australia and New Zealand, which suggests oceanographic and environmental conditions are acting as barriers to gene flow in the Tasman Sea (Ward & Elliot 2001). In addition, shark species through the eastern Pacific have different dispersal patterns: a) by stepping stones of islands in some populations connected by coastline in the scalloped hammerhead, *Sphyrna lewini* (Duncan et al. 2006); b) a complete separation is considered for some coastal species such as lemon shark, *Negaprion brevirostris* and sicklefin lemon shark, *N. acutidens* (Schultz et al. 2008), *Galeorhinus galeus* (Chabot & Allen 2009), and *S. zygaena* (Testerman et al. 2008) or no evidence of isolation of oceanic and pelagic species such as *S. lewini* (Duncan et al. 2006) and blacktip shark, *Carcharhinus limbatus* (Keeney & Heist 2006). These findings have provided an important understanding of the levels of genetic connectivity in several marine fishes, and emphasize the use of genetic methods for understanding dispersal patterns.

1.2.2 Molecular markers for fisheries management

The application of genetic markers to fisheries management has resulted in significant insights into the population ecology of exploited marine species. Genetic studies have demonstrated genetic differentiation among marine populations, over small to large geographic scales (Laikre et al. 2005, Dudgeon et al. 2012). The roles of genetic methods in fisheries science are, a) to determine the number of stocks and the level of genetic connectivity, and b) to determine the level of genetic variation in a stock so it can be managed to preserve its evolutionary resilience (Carvalho & Hauser 1994, Laikre et al. 2005). The evolutionary processes of mutation, natural selection, random genetic drift and migration influence genetic variation in a population. Genetically

isolated populations diverge from each other typically because the evolutionary processes of drift and selection operate independently in each group. However, two populations will be genetically similar even if there is a small amount of migration between them (Waples 1998). Genetic connectivity between populations is primarily a function of the number of migrants (mN_e , where m = migration rates and N_e = effective population size) and theoretically only one migrant per generation is needed between populations to homogenize genetic variation. In contrast, demographic connectivity between populations is a function of the proportion of migrants (m) exchanged. When there has been no migration between two stocks genetic markers will generally have the power to demonstrate this, suggesting that both stocks are also demographically uncoupled. In contrast, when even small amounts of migration occur between stocks genetic markers will typically show connectivity, but the demographic connectivity between stocks is less certain; they could or could not be demographically coupled.

Recent technological advances in molecular genetics have improved the resolution to understand the genetic connectivity among populations. Several types of molecular markers have been developed and utilized for fisheries management, which can be categorized as either protein-based markers (i.e. allozymes) or DNA-based markers. Allozyme electrophoresis was the first technique used to assess genetic stock structure in elasmobranchs (Smith 1986, MacDonald 1988, Heist et al. 1995, Ward & Gardner 1997, 1998). Subsequently, DNA techniques became available that used mitochondrial DNA (mtDNA) restriction fragment-length polymorphism (RFLP) (Heist et al. 1996a, b, Ward & Gardner 1997). For example, genetic analyses of allozymes and RFLP have shown that *G. galeus* is a single species worldwide and genetically different among ocean basins (Ward & Gardner 1997). On a smaller geographic scale, these studies showed only weak levels of differentiation between east coast of New Zealand and west Tasmania in Australian populations. However, the lack of genetic variation detected using these molecular markers and limited number of sample collection sites, makes this finding of limited use.

More recently, the polymerase chain reaction (PCR) amplification and direct sequencing of specific genome regions allowed more DNA loci to be sampled, which substantially improved the level of statistical resolution e.g. mtDNA control region and microsatellites (nuclear DNA/nDNA) (Heist 2005, Dudgeon et al. 2012, Portnoy & Heist 2012). A range of sample DNA qualities could be utilized by PCR-based

techniques making sample collecting and sharing much easier. Choosing DNA markers that were assumed to be neutral became important because the results were less likely to be confounded by any adaptive processes (selection).

Neutral molecular markers provided insight into the processes of migration (gene flow) and genetic drift. They are called “neutral” based on the neutral theory of evolution proposed by Motoo Kimura (1964). This theory states that polymorphisms of a population are selectively neutral, and their evolutionary fate is determined by genetic drift (random fluctuation of gene frequencies) (Kimura 1989). The rate at which one allele was substituted in a population (k) would have equal rates at which one mutation arose each generation (μ). In other words, the level of polymorphism is proportional to the mutation rate that arose each generation in a population. It means that random genetic drift and mutation play a significant role in evolution or in forming the genetic structure of the species (Kimura 1989).

The mtDNA was the first marker to be used in a genetic structure studies. The mtDNA is maternally inherited, and it has an elevated mutation rate. The mtDNA-genes are relatively easy to amplify due to conservative sequence regions (i.e. transfer ribonucleic acids, tRNAs), and a wide range of PCR primers are available. The mtDNA genome has a quarter the effective population size (N_e) when compared to nuclear gene regions, which increases its sensitivity to genetic drift and theoretically it should more often show differentiation between isolated populations (Nielsen et al. 2009). However, all genes on the mtDNA genome are completely linked and this molecule only represents the genetic patterns of a single locus, which limits its statistical power.

One of the most useful mtDNA regions for analysing stock structure in elasmobranchs is the “control region” (CR). It is usually the only non-coding region in the vertebrate mtDNA genome due to its role in replication and transcription of mtDNA molecules. The vertebrate CR is subdivided in three domains that differ from each other in base composition, rate and mode of evolution. The central domain of CR is relatively conserved, whereas the flanking regions are typically hypervariable (Saunders & Edwards 2000, Heist 2005). For this reason (fast rate of evolution), the whole CR is commonly used to determine levels of polymorphism and enable to estimate the current status of population structure of species. Many studies have used

the CR to assess genetic structure in shark species (Pardini et al. 2001, Keeney et al. 2005, Duncan et al. 2006, Stow et al. 2006, Lewallen et al. 2007, Ahonen et al. 2009, Chabot & Allen 2009, Dudgeon et al. 2009, Benavides et al. 2011, Karl et al. 2011, 2012). These have revealed different dispersal capabilities among species, which result in different levels of stock structure.

Microsatellites are now one of the most popular markers in population genetic studies. They are co-dominant DNA markers that are inherited in Mendelian terms. They are defined as Simple Sequence Repeat (SSRs) or Short Tandem Repeats (STRs) widely distributed through the chromosomes. Microsatellites consists of motifs of one to six nucleotides repeated several times e.g. TG₁₀, which means an array comprising the sequence TG repeated 10 times (Li et al. 2002, Guichoux et al. 2011). Most microsatellites are dinucleotide repeats and the length of those repetitive arrays varies among taxa. For example, fishes have the longest dinucleotide repeats (>20 repeat unit with a maximum of 87, Schug et al. 1998), whereas insect species varies considerably (between 16 for social wasp *Parachartergus coloboperus* and >30 for *Drosophila melanogaster*; Schug et al. 1998).

Despite of mechanisms of develop of microsatellite is not completely understood, it is commonly thought that evolves by replication slippage. This process involves a gain or reduction of one or more repeat unit (Levinson & Gutman 1987, Schlötterer & Tautz 1992, Ellegren 2004). For example the (TG)₁₀ mentioned in the previous paragraph might become and (TG)₈ or (TG)₁₂ in some individual's offspring. Several models have been proposed to explain and predict the mutation processes that affect microsatellite evolution (Estoup et al. 2002). The most adopted model is the stepwise mutation model (SMM). The model states that microsatellites mutations alters the length of a repetitive array though the gain or removal one unit (Kimura & Ohta 1978). The infinite allele model (IAM) assumes that mutations may create and infinite number of repeat with allelic states not presented in the allelic distribution (Kimura & Crow 1964). The two-phase model (TPM) is an extension of the SMM and it considers the probability for a microsatellite mutation to involve more than one unit (Di Rienzo et al. 1994).

Mutations cause a change in the overall length of the region containing the microsatellite locus, and consequently the quantification of alleles distinguishable by

molecular size (Li et al. 2002). The primary methodology used for detecting variation of microsatellite allele lengths is amplification of the microsatellite containing-region by PCR and using electrophoresis to determine the sizes of the PCR products. Fluorescent dye labelling technology has enabled microsatellites loci to be combined and analysed together whenever their allele sizes do not overlap, and if they do overlap different coloured fluorescent dyes can be used. This approach expands the scope of the genetic analysis to include multiplexing and pool-plexing different loci together, consequently reducing the cost and time (Guichoux et al. 2011).

Microsatellites markers are used as tools for a wide range of applications. They are applied for molecular forensics, parentage analysis, genetic structure and connectivity, and phylogenetic relationships (Guichoux et al. 2011). To date, several polymorphic loci have been developed in different species of elasmobranchs (Heist & Gold 1999, Pardini et al. 2000, Feldheim et al. 2001a, 2001b, Schrey & Heist 2002, Heist et al. 2003, Keeney & Heist 2003, Chapman et al. 2004, McCauley et al. 2004, Portnoy et al. 2006, DiBattista et al. 2008, Chabot & Nigenda 2011, Broomer & Stow 2010). Compared to mtDNA, microsatellites nDNA markers enable different levels of genetic resolution because they sample more independent points (regions) on the genome (Nielsen et al. 2009). In spite of microsatellite loci studies in shark showed high levels of genetic diversity compared with mtDNA studies, little evidence of genetic differentiation has been found i.e. within ocean basins (Dudgeon et al. 2012). However, any attempt to estimate structure of highly mobile marine species, such as sharks, will be challenging for any molecular markers. (Graves 1998, Heist et al. 2005) All these considerations should be taken into account to provide useful information about the stock structure in shark species.

1.2.3 Distribution and Biology of *Galeorhinus galeus*

The school shark (*Galeorhinus galeus*) belongs to the family Triakidae, and it has a widespread distribution in temperate waters of the eastern North Atlantic, western South Atlantic, north eastern and south eastern Pacific, South Africa, New Zealand and Southern Australia (Last & Stevens 2009). It occurs all around New Zealand, but its distribution in Australia is restricted to southern waters from Moreton Bay (southern Queensland) to Cape Leeuwin (Western Australia), including Lord Howe

Island and Tasmania (Last & Stevens 2009). A semi-pelagic species, it inhabits the continental shelf from nearshore to 660 m in depth (Anderson et al. 1998, Walker 1999, Hurst et al. 2000, West & Stevens 2001), and also occurs in the pelagic zone and offshore (Walker 1999, Bagley et al. 2000).

Galeorhinus galeus, as well as many other species of elasmobranchs, exhibit a low reproductive potential. This is characterised by low fecundity, slow growth, late sexual maturity, and high longevity (Olsen 1954, 1959, 1984, Grant et al. 1979, Francis & Mulligan 1998, Hurst et al. 1999). They are aplacental viviparous and their fecundity varies between 15-52 pups depending on the size and age of the mother (Olsen 1954, 1984, Walker 1999, Walker 2005). Sexual maturity occurs at an older age in females than males, with females reaching maturity at about 13-15+ years (124-140 cm total length, TL), whereas in males it is at about 8-17+ years (125-135 cm TL) (Olsen 1954, 1984, Francis & Mulligan 1998, Walker 2005). The longevity of New Zealand *G. galeus* is still uncertain, however the oldest known shark was estimated to be 25 years old (Francis & Mulligan 1998). By contrast, results from an Australian long-term tag recovery study (after 42 years), have suggested that *G. galeus* could attain an age of ~60 years (Walker 1999).

The reproduction of *G. galeus* has been well described through its distribution range. The reproductive cycle in female is characterised by a long ovarian and gestation period estimated to be three years (triennial) (Olsen 1984, Peres & Vooren 1991, Walker 2005). Therefore pregnant females give birth approximately every 3 years (Peres & Vooren 1991, Walker 2005). This means that *G. galeus* population will comprise females at three different reproductive stages: (a) about one-third of the population consist of sharks with resting oviducts (after parturition) and vitellogenesis in the ovary; (b) another one-third of the population will have large mature follicles, oviducal gland and uteri ready for ovulation; and (c) one-third will have full-term embryos in the oviducts (Peres & Vooren 1991, Walker 2005). This fact has biological significance because only one-third of the mature females will be supporting the annual recruitment of newborns.

Tagging data from larger *G. galeus* have been obtained in Australia and New Zealand waters. These studies have showed national or international migration patterns in and between Australian (Olsen 1953, 1954, Stanley 1988, Walker 1989,

Walker et al. 1997) and New Zealand waters (Paul 1988, Francis & Mulligan 1998, Hurst et al. 1999, Francis 2010). One tagged female released in the Great Australian Bight (Australia) was recaptured 3,500 km away, after 4.1 years, between the North and South Islands of New Zealand. It was released again and recaptured off Australia 2,600 km away, 2.8 years later (Walker et al. 2008). The maximum recorded distance travelled by a shark is 4940 km after 9.6 years (Hurst et al. 1999). The distance travelled increased significantly after the first year of liberty. Thirty percent of *G. galeus* tagged in New Zealand were recaptured outside the release Quota Management Area (QMA) within 1-2 years; after 2-5 years, 60% were recaptured outside the release QMA, and a significant proportion of sharks emigrated to Australian waters (23%) (Francis 2010). Due to relatively high emigration rates from New Zealand to Australian waters, Hurst et al. (1999) considered both populations to be the same stock unit. These findings suggest that there is trans-Tasman exchange of migrants, which presumably allows the physical mixing of the *G. galeus* population at a level that is significant enough to affect the stock structure (Figure 1.1).

Figure 1.1: Movements of New Zealand tagged school shark, *Galeorhinus galeus* to Australia adapted from Hurst et al. 1999.

The migration patterns of *G. galeus* in southern Australia waters can be inferred from long term tagging data (Figure 2.2). Pregnant females move into the nursery areas along the southern Australian coast. After parturition, females leave the nursery, whereas newborn sharks (0+ years) remain around local areas before moving

into deeper coastal waters during winter months (July-September) (Olsen 1954, 1984). The juveniles return to their former nurseries during the next spring or move to adjacent estuaries and bays. As a consequence, mixing of the juvenile population occurs among several nursery areas (Olsen 1954, 1984, Walker 1999). Some individuals (2+ years) showed random patterns of migration in open waters, where they mix with other stocks; for example, a male of 58.5 cm TL moved 1120 km in 544 days from Pittwater (Tasmania) to Kangaroo Island (South Australia) (Olsen 1954).

Figure 1.2: Movements of Australian school shark, *Galeorhinus galeus* within Australian waters adapted from Olsen 1984.

Adults tend to range more widely than juveniles. Adults migrate inshore in summer and offshore during winter, and also move in schools to the warmer northern waters of South Australia and New South Wales in winter before returning south in late spring (Olsen, 1954). The shark usually occurs in small schools segregated predominantly into individuals of the same sex and age (Last & Stevens, 2009). Conversely, mixing of males and females occurs in deep waters of the continental shelf, coinciding with the beginning of the mating season in May-June (Olsen 1984). In addition, aggregations of pregnant females in different stages of gestation have been found in waters of the Great Australian Bight (Walker 2000, Walker 2005, Walker et al. 2008). These findings regarding adult movement along with records from fishing surveys, suggest that mature female *G. galeus* remain in South

Australian waters during the gestation period. After this time, they start the reverse migration to the nursery areas in Victoria and Tasmania to give birth.

Pupping areas are typically harbours, shallow bays and sheltered coasts (Olsen 1954, 1984, Paul 1988, Paul & Sanders 2001). The location of the most important pupping areas in New Zealand is unknown. However, trawl surveys suggest they may be around the east and west coast of the North and South Island except northern of North Island and southern of South Island, and the central-south coast of the South Island (Hurst et al. 1999). By contrast, several nursery areas are well described in Victorian and Tasmanian waters i.e. Port Phillip, Port Sorell, St. Helen, Pittwater (Olsen 1954, 1959, 1984, Stevens & West 1997, TSSC 2009). However, due to the strong fishing pressures on *G. galeus* populations in southern Australian waters and the ongoing habitat degradation in inshore nursery areas, the abundance of newborns and juveniles has declined in coastal waters off Victoria and Tasmania.

1.2.4 New Zealand and Australasian *Galeorhinus galeus* Fisheries

Fishing effort on *G. galeus* populations has been well documented in the past (e.g. an apparent collapse in California, Stevens 2000). This species has been fished in Australia since the mid-1920s and in New Zealand since the early 1940s by separate commercial fisheries. Australian fisheries began in 1927 in the eastern Bass Strait and spread to eastern South Australia and Tasmania during the 1940s. During this time, demersal longlines were used to target large mature *G. galeus*, which increased mortality of sub-adults (Olsen 1959, Walker 1999, Stevens 2005). As a result, the catch rate in southern Australia declined by more than 50%. The fishing impact was reflected in the catches of pups as well e.g. an 80% decline in Port Phillip Bay, Victorian waters (Olsen 1984, 1959, Walker 1998, 1999). Indeed, as noted by Olsen (1984) Pittwater and Port Sorell (Tasmanian waters) also indicate a decline in catch rates (Stevens 2005).

In the early 1960s, the fishery grew rapidly, peaking at over 3756 t (Walker 1999) due to the demand for shark meat in Victoria. Gillnetting was implemented in 1964, and by the early 1970s, most of the catch was taken by this gear. However, demand for the fish declined due to concern about high levels of mercury in the flesh,

resulting in the banning of fishing for large *G. galeus*. As a result, fishing effort shifted to the gummy shark (*Mustelus antarcticus*). After the lifting of the ban on *G. galeus*, catches of that species increased again, peaking at ~2000 t in 1986 (Walker 1999, AFMA 2009). Currently, *G. galeus* are not targeted, but are landed as bycatch in Australian *M. antarcticus* fisheries (Stevens 2005, McLoughlin 2007).

Australian *G. galeus* fisheries have reduced the stock to less than 15% of initial biomass, raising serious concerns (AFMA 2009, 2010a, DSEWPaC 2012). The depletion of the stock has led to a progressive reduction in the TAC (total allowable catch) from 800 t in 2002 to 240 t in 2010 (AFMA 2010b). It has been listed under the “conservation dependent” category of the Environment Protection and Biodiversity Conservation Act 1999 (EPBC Act) which allows for the *G. galeus* shark rebuilding strategy plan (TSSC 2009). The rebuilding strategy consists of several management measures and responses designed to recover the population to a given target biomass within a biologically reasonable timeframe (AFMA 2009, TSSC 2009). The management measures include: limited entry, area closures, gear restrictions, and minimum legal-size limits (TSSC 2009). Despite these measures, there is little evidence of stock recovery and as recently as September 2010, additional measures were being considered, including catch restrictions of co-occurring target species and area closures (AFMA 2010b).

In contrast, the New Zealand *G. galeus* fishery is much larger than the Australian one, and it is currently considered to be more sustainable. The fishery started with Maori using the meat, oil, and skins during pre-European times; however there is no quantitative information about their catches (Francis 1998, MPI 2012). During the 1940s, factory companies processed the livers of *G. galeus*, however the catches were not recorded. After this, the New Zealand market started to export *G. galeus* meat to Australia. The landings increased to 300-600 t from 1957 to 1971, but the banning of the meat’s importation to Australia resulted in a reduction of the New Zealand fishery (Francis 1998, Paul & Sanders 2001). However, the fishery increased rapidly from 500 t to 5,600 t between 1979 and 1984 due to the re-opening of the Australian market. The New Zealand TAC was set at 2590 t, which was more than 50% of the average annual catch for the previous three years. Between 1995 and 1996, the total landings rose to 3387 t and the TAC (3106 t) was exceeded for the first time (Francis 1998). Since then, the average catch in New Zealand *G. galeus* fishery

has consistently exceeded 3000 t (MPI 2012). However, there is a concern about the sustainability of the fishery due to high TAC levels through recent decades. As a consequence, it has been proposed that fishing pressure on adults, especially large mature females, should be minimised to maintain the productivity of the fishery.

There are few recreational restrictions on the *G. galeus* fishery in New Zealand. The minimum mesh-sizes for gillnetting are 125 mm for Auckland Fisheries Management Area (AFMA) and 150 mm for Central, Southland and Challenger Fisheries Areas. The minimum dragnet mesh size is 100 mm for AFMA. Additionally, several general restrictions apply for commercial gillnets and longlines such as: limits on the length of gillnets, number of hooks per line, number of longlines, soak time, proportion of a bay or estuary that can be blocked by a gillnet, and areas that can be fished (Walker et al. 2006). Recreational daily bag limits of mixed species apply to *G. galeus* as well i.e. person can catch 20-30 fish for the northern, central and southern regions of the country (MPI 2012).

In conclusion, given the commercial importance of *G. galeus* stock and the concern about population levels (both at trans-Tasman and local scales), knowledge of stock structure is essential for the effective management of the fisheries in New Zealand and Australia. This is particularly important given the major depletion of stocks in Australia and their conservation status on the IUCN Red List as vulnerable in Australia and near threatened in New Zealand (Walker et al. 2006).

1.2.5 Goals of the thesis research

Studies of *G. galeus* using tagging and early genetic markers have supported the idea of large genetically homogeneous population in New Zealand and Australian waters. However, the question of whether this species comprises a single population or multiple sub-populations has not been properly resolved. The overall goal of this thesis research is to determine the levels of genetic variation and differentiation within and among *G. galeus* populations in New Zealand and Australia by using mtDNA and nDNA molecular markers. Samples from Chile have also been included in the analysis due to opportunity to cover the entire distribution of the genetic variation of the species within South Hemisphere (i.e. no New Zealand and Chilean samples were reported in Chabot & Allen 2009).

Three categories of genetic structure described in Laikre et al. (2005) will be tested in order to classify the genetic connectivity of *G. galeus*. The categories of genetic structure are 1) single panmictic stock, 2) isolation by distance, and 3) discrete stocks. The first category involves genetic flow that is so extensive that the entire population is not reproductively isolated, behaving as a single stock. The second category involves genetic variation more or less continuously over space. The gene flow among nearby individuals is larger than those separated by long distance, resulting in successive genetic change over geographic distance defined as “Isolation by distance” (IBD). The last category is characterised by a very small amount of migration between stocks, which could be sufficient to differentiate between closely geographic regions. Two hypotheses of population genetic structure were tested in this thesis research:

H_{01} : There is a single panmictic stock in the New Zealand and Australia region.

H_{A1} : There are separate populations in New Zealand and Australia due to isolation-by-distance and/or discrete differences.

If H_{01} is rejected, a further set of hypotheses were tested:

H_{02} : There are panmictic stocks within New Zealand and Australia.

H_{A2} : Discrete separate sub-populations occur within New Zealand and Australia due to isolation-by-distance and/or discrete differences.

Chapter 2 aims to describe and contrast the patterns of genetic connectivity inferred from the mtDNA control region of three shark species (*G. galeus*, *Squalus griffini* and *Sphyrna zygaena*) that are distributed in South Pacific Ocean. Two additional species were included (*S. griffini* and *S. zygaena*) in order make a comparison with the genetic structure of *G. galeus*, and therefore compare the population patterns over large and small comparable spatial distances. In Chapter 3, I examine the use of next generation sequencing technology (NGS) to develop microsatellite markers for *G. galeus*. Nineteen putative microsatellite loci were tested in three collection sites from Australasia region and Chilean ones as well, in order to determine their utility to test for genetic differentiation. Additionally, nine other microsatellite loci designed by Chabot & Nigenda (2011) were integrated as well to this Chapter. In Chapter 4, I studied the level of genetic variation inferred by microsatellite DNA markers for *G. galeus* for adult collections in order to test for spatial population structure. Coalescent-based methods were carried out to estimate migration rates inferred by mitochondrial DNA (CR) and microsatellite loci in order to assess population connectivity of this species across the Tasman Sea. In Chapter 5, I investigate evidence of multiple-paternity among embryos in *G. galeus* by assessing the levels of relatedness of progeny using microsatellite DNA markers. Five “families” (mothers and litters) were sampled and a parentage analysis was conducted using six microsatellite loci. Finally, Chapter 6 aims to integrate the information generated in the previous chapters in order to synthesise the implications of this research; the results for *G. galeus* are compared with other shark species, and possible future research directions are discussed.

Chapter 2

Contrasting the population genetic structures of the school shark (*Galeorhinus galeus*), northern spiny dogfish (*Squalus griffini*) and smooth hammerhead (*Sphyrna zygaena*) using mitochondrial DNA control region sequences

2.1 Abstract

The three species of shark that have similar distributions in the Tasman Sea and South Pacific Ocean were compared using mitochondrial DNA control region (CR) sequencing to understand their population genetic structures. DNA sequences between 900 and 600 base pair of the CR were determined from 475 *Galeorhinus galeus*, 145 *Squalus griffini* and 156 *Sphyrna zygaena* individuals. Relatively high levels of haplotype diversity (h) were found for *G. galeus* ($h = 0.75$) and *S. griffini* ($h = 0.70$), compared with *S. zygaena* populations ($h = 0.62$), but relatively low level of nucleotide diversity (π) were found for *G. galeus* ($\pi = 0.015$) and *S. griffini* ($\pi = 0.023$), in comparison with *S. zygaena* populations ($\pi = 0.024$). AMOVA analysis and pairwise genetic differences (Φ_{ST}) for *G. galeus* showed a significant level of genetic differentiation between a New Zealand/Australian population and the Chile population. Within the New Zealand and Australia region there was a lack of genetic differentiation, suggesting a single panmictic stock mediated by high levels of female gene flow. Evidence of two genetically distinct populations of the endemic New Zealand species *S. griffini* was found when comparing samples from the Kermadec Islands (~600 nautical miles northeast of Auckland), and a combination of samples from sites at Gisborne, Westland, and Chatham Rise (mainland stocks). For *S. zygaena*, a weak level of genetic structure was found across the South Pacific Ocean, but there was no evidence of genetic differentiation between sampling sites in New Zealand and Australia, and between Chile and Ecuador. The mitochondrial DNA control region data support the suggestion of joint management of the Australian and New Zealand *G. galeus* stocks, but further consideration should be given to managing the New Zealand *G. galeus* as two separate stocks. Similarly there is support for

considering New Zealand *S. griffini* as northern and southern stocks. In contrast to the other two species, *S. zygaena* stock structure should be more thoroughly investigated using other mitochondrial DNA markers and/or with a high level of resolution as microsatellites (sampling more independent regions on the genome).

2.2 Introduction

Population genetic markers are important tools for defining the reproductive units within a fishery (stocks) and monitoring the levels of genetic variation within an exploited species. Genetic data enable the identification of fish stocks (both spatially and temporally), providing an indirect estimate of the migration rates among fishery sites (Nielsen et al. 2009). However, genetic information has only slowly been integrated into stock assessment models and it is often used in combination with other approaches such as direct stock identification methods (e.g. tagging programmes) (Waples et al. 2008, Dichmont et al. 2011). Population genetic methods are used to identify groups of individuals within a species that share the same reproductive process, which can be used as the basis for defining management units (Laikre et al. 2005). Furthermore, understanding and managing the genetic constituents of a population is important for the long-term persistence and adaptability of a commercial species (Allendorf et al. 2008). Managing genetic diversity is particularly important for mitigating the risks of inbreeding and reduced adaptability that could be caused when stocks are fished down to small sizes (Frankham 2002).

The sustainability of shark fisheries has been a concern for the last three decades. Many species have become overexploited because of the high demand for meat and fins for human consumption (Musick & Bonfil 2005). Moreover shark species often have low management priority when resources are limited and basic fisheries data are lacking (Stevens et al. 2000). Effective fishery management relies on a stock assessment, which requires a good understanding of a range of biological parameters such as the number of reproductively independent units within a fishery and their structure (Carvalho & Hauser 1994, Grant & Waples 2000). Traditionally, large shark species were thought to be highly mobile and typically compressed one large and widely distributed population, whereas smaller species were thought to have a lower power of dispersal and might be comprised of several structured populations (Musick et al. 2004). However, tagging studies have shown that this generalization is not often true and many species migrate over reasonably large distances (Hurst et al. 1999, Kohler & Turner 2001, Walker et al. 2008).

Despite the high dispersal potential of many shark species, recent genetic studies have shown that coastally distributed species, particularly of the family

Carcharhinidae, lack significant amounts of gene flow across ocean basins (Duncan et al. 2006, Keeney & Heist 2006, Schultz et al. 2008, Chabot & Allen 2009, Portnoy et al. 2010). Some species have complex migration patterns and their dispersal pattern can vary significantly during their life cycle due to mating, pupping, and maturation behaviors (Hueter et al. 2005). Where known these life history parameters biological features are taken into account when attempting to identify stock structure.

Galeorhinus galeus is a commercially fished shark species that is widely distributed in temperate waters (Last & Stevens 2009). It occurs throughout New Zealand waters, but in Australia it is restricted to southern waters from Moreton Bay (southern Queensland) to Cape Leeuwin (Western Australia), including Lord Howe Island and Tasmania (Last & Stevens 2009). *Galeorhinus galeus* has been managed separately in New Zealand and Australia, and the average annual catch in New Zealand currently exceeds 3000 t, whereas in Australia it is limited to 240 t (AFMA 2010, MPI 2012). The Australian management authorities were concerned about the sustainability of the *G. galeus* fishery and they introduced a long-term fishing plan to rebuild the stock within a biologically reasonable timeframe (AFMA 2009). However, *G. galeus* stocks in Australia still remain unacceptably low. The limited reproductive potential and the trend of declining population size worldwide suggest that this species is overexploited and it has been listed as a globally *Vulnerable* species by the IUCN (Walker et al. 2006).

Tagging studies have shown that the Australasian *G. galeus* are long-lived and highly mobile within and between New Zealand and Australia (Olsen 1953, 1954, Stanley 1988, Paul 1988, Walker 1989, Francis & Mulligan 1998, Hurst et al. 1999, Walker et al. 2008, Francis 2010). For example, one female released in the Great Australian Bight was recaptured 3,500 km away (4.1 years later) between the North and South Islands of New Zealand. It was released again and recaptured off Australia 2,600 km away (2.8 years later; Walker et al. 2008). The maximum distance travelled was 4,940 km (over 9.6 years; Hurst et al. 1999). The overall findings of the tagging studies have been used to support the hypothesis of a single Australasian stock in the fisheries assessments of *G. galeus* (Punt et al. 2000, Walker et al. 2008).

The patterns of gene flow among *G. galeus* populations sampled in different ocean basins have been documented using Restriction Fragment Length

Polymorphisms (RFLPs) of the mtDNA and the direct DNA sequencing of the mtDNA control region (CR). These studies have shown a significant level of genetic differentiation among ocean basins (Ward & Gardner 1997, Chabot & Allen 2009). A weak level of differentiation was reported between the east coast of New Zealand and west Tasmania in Australian populations (Ward & Gardner 1997). These genetic markers did, however, have several limitations; the RFLP technique has a very limited ability to detect genetic variation, the sample sizes were uneven, and the sampling did not cover the entire distribution of the species within the region (e.g. no New Zealand samples were reported in Chabot & Allen 2009). Despite the drawbacks of the tagging and genetic studies, the results have been used to support a model of separate but genetically homogeneous stocks of *G. galeus* in New Zealand and Australia. The question of whether Australasian *G. galeus* comprises a single stock or multiple sub-stocks has not been properly resolved.

The northern spiny dogfish (*Squalus griffini*) is a New Zealand endemic benthonic shark species with a distribution that overlaps with *G. galeus* although more patchy in its distribution. *Squalus griffini* occurs along the continental margin off the west coasts of the North and South islands from about 34°S to 44°S. Off the east coast, it has been distributed southward to almost 45°S, although infrequently caught south of 38°S, with the exception of Chatham Rise between 42°S - 44°S, and 175°W - 179°W respectively (Anderson et al. 1998, Duffy & Last 2007). This species has also been recorded north of New Zealand on Wanganella Bank and Norfolk Ridge (to at least Norfolk Island), Kermadec Ridge (to at least Raoul Island, 29°S) and the Louisville Ridge. The distribution data suggest that the Kermadec Islands and Chatham Islands *S. griffini* populations are somewhat isolated from the mainland populations. However, the population genetic structure of this species has not been studied.

Squalus griffini is often caught as bycatch in non-directed fisheries. The total catch is small and it is not in the Quota Management System (QMA) in New Zealand. There is a scarcity of data available about commercial catches and very little information about life cycle and habitat ecology of *S. griffini*. The un-standardized annual catch-per-unit-effort (CPUE) suggests large parts of the distribution range of the species remain unfished or only lightly commercially fished (Duffy 2011). Research trawl surveys off the west coast of the South Island showed no particular

downward trends in relative biomass (Cavanagh & Lisney 2003), which put it in the *Least Concern* category of species by the IUCN (Duffy 2011). However, it is not known whether set net and bottom longlines fisheries may cause localised stock depletion. *Squalus griffini* is assumed to have low reproductive potential, comparable to the closely related *mitsukurii* species (Duffy 2011).

The smooth hammerhead shark (*Sphyrna zygaena*) is a circumglobally-distributed species, which occurs in coastal-pelagic and semi-oceanic environmental habitats. It is found in temperate and tropical waters from shallow (less than 20 m) to 200 m along the continental shelf. However, the full range of the species in tropical waters is not known due to its confusion with *Sphyrna lewini*. In the southwest Pacific it is distributed in Australia from southern New South Wales to central western Australia and New Zealand, including Lord Howe and Kermadec Islands, while in the southeastern Pacific it is distributed from Ecuador to Chile (Last and Stevens 2009). Tagging data suggest that juvenile and sub-adult *S. zygaena* make large-scale directional movements in South Africa (~300 km) in response to seasonal sea surface temperature changes (Diemer et al. 2011). A genetic survey of the mtDNA control region reported population subdivision among four geographic regions with little evidence of gene flow among the Atlantic, North Pacific, southeast Pacific and Indo-Pacific oceans (Testerman et al. 2008).

Sphyrna zygaena has been listed as a globally *Vulnerable* species by the IUCN (Casper et al. 2005) because of reports of declining population sizes and life history characteristics that make it susceptible to over-exploitation. In New South Wales (Australia), this species along with *S. lewini* and *S. mokarran*, is caught by a commercial line fishery and recreational game fishery. The majority of the hammerhead catch is comprised of *S. zygaena* and *S. lewini* (Scandol et al. 2008). The total commercial hammerhead landings peaked in 1993 at 15 t and in 1994 at 7 t and annual commercial catches have averaged ~3 t over the last five years (Casper et al. 2005). *Sphyrna zygaena* has also been reported in set net catches in the New South Wales beach protection programme (Krough 1994), which uses large mesh gillnets as a protective measure for beach users. In New South Wales, hammerheads (mostly *S. zygaena*) made up nearly 50% of the beach protection catch of 4,715 sharks in the period from 1972-73 to 1989-90 (Reid & Krough 1992, Casper et al. 2005). This species is an important bycatch species in the West Australian demersal gillnet

fishery, but the relatively low numbers caught have not caused a significant impact on population numbers.

In New Zealand waters, *S. zygaena* is reasonably abundant around the northern North Island and it is the most common shark species reported in aerial surveys conducted along the northwest coast (Casper et al. 2005, Francis 2010). Neonates and small juveniles are commonly found in shallow coastal waters of the northern North Island, but are absent further south. They are caught as bycatch in commercial gillnet fisheries for flatfish and taken by trawlers and Danish seiners. Juveniles caught in gillnets frequently die before they can be released and a lot are finned as well (Smith & Benson 2001). Adults are mainly taken by game-fishers as bycatch, the annual catch is small and many of the sharks are released alive (Casper et al. 2005, Francis 2010). Currently, *S. zygaena* is the only representative of *Sphyrna* in New Zealand waters, which make easy the identification and information from catches. However, species-specific information about catches is very limited in Australian waters because they are often grouped together under a single category (*Sphyrna* group).

In Ecuador and Chilean waters, *S. zygaena* is caught as bycatch by artisanal and industrial-fishing vessels using surface longlines and drift gillnets. In Ecuador, *S. zygaena* represents 11% of the total chondrichthyan landings, which typically contain high numbers of juveniles (Casper et al. 2005). In Chilean waters, there is no record of catches and/or landing for *S. zygaena*. However, the majority of bycatch are processed in Iquique and Caldera ports for its meat and fins (northern of Chile) (Lamilla et al. 2005). The meat is usually traded locally for human consumption, whereas the fins are dried and processed to be exported to the Asian market, mainly Hong Kong (Hernandez et al. 2008).

The aim of this Chapter was to determine the levels of genetic variation and differentiation using mtDNA CR sequences among populations of three shark species (*G. galeus*, *S. griffini* and *Sphyrna zygaena*), which are co-distributed in the southwestern and southeastern Pacific Oceans. Two additional species were included (*S. griffini* and *S. zygaena*) in order to make a comparison with *G. galeus* genetic structure and population patterns over similar spatial distances. The genetic results are used to identify the putative stock structure of these species. Furthermore, non-coalescent and coalescent approaches were used to test for non-

equilibrium population dynamics and make inferences about their historical genetic effective population sizes.

2.3 Methods

2.3.1 Sampling collection

Tissue samples of *G. galeus* were taken from individuals utilizing two different sampling approaches. The first method targeted adult sharks from three geographic regions: New Zealand, Australia and Chile. Samples were collected from four areas around New Zealand (NZ): East of North Island (ENI), Kaikoura (KAI), Chatham Rise (CHA) and Solander Islands (SOL) (Figure 2.1A); and six locations from Australia (AUS): Shoulder (SHO), Great Australian Bight (GAB), Eyre (EYR), King (KIN), Tasmania (TAS) and Frederick Bay (FRE) (Figure 2.1A). Due to geographic proximity, small sample size and lack of genetic differentiation, samples from SHO and GAB, and EYR and KIN were pooled together, respectively for analysis purposes. Thus SHO and GAB was named SOU, and EYR and KIN became POR (Figure 2.1A). Additionally, *G. galeus* fin samples were collected from a shark fin warehouse in Santiago (SAN) from Chile (CHI), which were previously used for an identification genetic study of shark species involved in the Chilean fin trade (Hernandez et al. 2009) (Figure 2.1B). Adult collections were coordinated with on board observers and fisheries biologists from the Ministry of Fisheries and National Institute of Water and Atmospheric Research (NIWA) in New Zealand, and Commonwealth Scientific and Industrial Research Organization (CSIRO), Department of Primary Industry, and Marine and Freshwater Fisheries Research Institute in Australia.

The second method targeted pups in New Zealand and Australian pupping areas. During this study, six locations were sampled in New Zealand: Kaipara Harbour (KAH), Manukau Harbour (MAN), Napier (NAP), Kapiti Coast (KAP), Mapua (MAP), and Golden Bay (GOL); and one in Tasmania: Upper Pittwater (UPW) (Figure 2.2). The surveys were coordinated with scientists from Department of Conservation (DOC), New Zealand National Aquarium-Napier, Mapua Aquarium-Nelson, Victoria University of Wellington from New Zealand, and CSIRO (for collections from Tasmanian waters). Captures were made by demersal longlines. Newborn young and juveniles were caught and released back into the water within a maximum period of two hours from capture. It is known from previous studies that

handled young *G. galeus* are highly active and in good condition up to two hours after capture on a longline (Stevens & West 1997).

Additionally, another group of samples were obtained from a previous *G. galeus* population genetic study, which utilized allozymes and RFLP-mtDNA (Ward & Gardner 1997). These samples were collected in 1993-1994 from global sampling locations and extensive sampling from New Zealand and Australian waters. Unfortunately, only samples from Tasmania (Australia) were recovered from the remaining frozen tissues stored at CSIRO. Since many of the samples were unlabelled or labelled improperly, it was only possible to identify fewer than half of the samples by location according to the dataset provided by Bob Ward (CSIRO). Therefore, samples of six nurseries areas from Tasmanian waters were used: Upper Pittwater (UPW), Blackman Bay (BLA), Norfolk (NOR), Ralph Bay (RAL), Isthmus Bay (ITH), and Swansea (SWA) (Figure 2.1).

Figure 2.1: Sampling sites, sample sizes (in parentheses), and geographic distribution (shaded area) of *G. galeus* collected from New Zealand (NZ), Australia (AUS), and Chile (CHI). **A.** and **B.** Sites where adult individuals were sampled. The locations are: ENI (east North Island, NZ), CHA (Chatham Rise, NZ), KAI (Kaikoura, NZ), SOL (Solander Island, NZ), SHO (Shoulder, AUS), GAB (Great Australian Bight, AUS), EYR (Eyre, AUS), KIN (King, AUS), TAS (west Tasmania, AUS), FRE (Frederich Bay, AUS), and SAN (Santiago, CHI). The SHO and GAB (SOU), and EYR and KIN (POR) were considered as one location in the analyses (see text details). **C.** Sites where pups were sampled across the Australasian region. The locations are: KAH (Kaipara Harbour), MAN (Manukau Harbour), NAP (Napier), KAP (Kapiti Coast), MAP (Mapua), GOL (Golden Bay), UPW (Upper Pittwater), STA (St. Helens), and SOR (Port Sorell). **D.** Individuals collected from Tasmanian waters during 1993-1994. The locations were: Upper Pittwater (UPW), Blackman Bay (BLA), Norfolk (NOR), Ralph Bay (RAL), Isthmus Bay (ITH), and Swansea (SWA). These locations, excluding SWA, were pooled as HOB (Hobart area) for descriptive genetic diversity, haplotypes distribution, and temporal AMOVA.

Squalus griffini tissues samples were taken from Kermadec Islands (KER) in the North Island, Gisborne (GIB), Westland (WES) and Chatham Rise (CHA) from the South Island in New Zealand (Figure 2.2A). Samples were collected during fishing surveys that were organized by scientists from DOC, Te Papa Fish Collection, and the NIWA. During the same time, samples of *S. zygaena* were obtained from Australia, New Zealand, Chile and Ecuador (Figure 2.2B). The samples were contributed by scientists of SARDI Aquatic Sciences from Adelaide, South Australia, Fish Collection of Australian Museum from Sydney, DOC from New Zealand, and a researcher at Universidad Nacional Autónoma de México from Ecuador. Chilean samples were obtained from dry fins sampled from two commercial warehouses of shark fins in Chile.

Figure 2.2: Sampling sites, sample sizes (in parentheses), and geographic distribution (shaded area) of *S. griffini* collected from New Zealand in GIB (Gisborne), WES (Westland), CHA (Chatham Rise), and KER (Kermadec Islands).

Figure 2.3: Sampling sites, sample sizes (in parentheses), and geographic distribution (shaded area) of *S. zygaena* collected from AUS (Australia), NZ (New Zealand), CHI (Chile), and ECU (Ecuador).

2.3.2 DNA Extraction, amplification and sequencing

Genomic DNA was extracted from tissue samples using phenol-chloroform protocols (Sambrook et al. 1989). The CR of the mtDNA was amplified and sequenced by using species-specific primers designed in Primer 3 (Rozen & Skaletsky 2000), implemented in Geneious Pro v5.5.2 (Biomatters Ltd.). Primers, approximate sizes of the amplified product and PCR conditions are detailed in Table 2.1. The resulting amplicons were sequenced from both forward and reverse directions during the initial data collection stage. Firstly, twenty sequences were sequences in both directions in order to evaluate the accuracy of forward and reverse sequences results. When DNA sequence methods were consistently only forward sequences were obtained for the remaining samples. PCR amplifications were carried out in 25 µl volumes containing 67 mM Tris-HCl Ph 8.8, 16 mM (NH₄)₂SO₄, 2.0 mM MgCl₂, 20 mM dNTPs, 1.5 mM MgCl₂, 10 µM of each primer, 0.4 units of BIOTAQ™ DNA Polymerase (Bioline) and 1 µl of DNA (20-40 ng/µl DNA concentration). PCR was performed in a TGradient Thermal Block (Biometra, Goettingen, Germany). Amplicons were visualised under UV-light after electrophoresis in a 1% agarose gel and staining with ethidium bromide. PCR products were purified using ExoSAP-IT kits and the DNA sequences were determined using an ABI 3730 Genetic Analyzer (Massey University). CR-mtDNA sequences were edited in Geneious Pro v5.5.2, and aligned using the Geneious Alignment option defined by default parameters (Drummond et al. 2011). In order to resolve any ambiguities and confirm the nucleotide calling, the chromatograms were thoroughly checked by eye. The CR-haplotype data from Chile were compared to other sequences reported from South American that were retrieved from Genbank (refer to Discussion for more details).

Table 2.1: Primer sequences and PCR conditions of CR for each shark species studied.

Species	Primer sequences	Size (bp)	PCR cycling conditions
<i>Galeorhinus galeus</i>	GGCR_F: 5'-CGAACCTAGCCCTTGCCCCAC-3' GGCR_R: 5'-ACGTCGGTCCTCGTTTATAGGGG-3'	893	95°15", 60°30", 70°60", 30 cycles
<i>Squalus griffinii</i>	SGCR_F 5'-AGCCCACATATCCTAATATACCA-3' SGCR_R 5'-GCGAATATTTCCACACGCA -3'	645	94°30", 63°30", 70°60", 35 cycles
<i>Sphyrna zygaena</i>	SPH_F 5'-ACCGGTTTTTGTACGTCAGT -3' SPH_R 5'-ACATTGGCCCTCGTTTATAGGGGT-3'	760	94°30", 63°30", 70°60", 35 cycles

2.3.3 Data analyses

2.3.3.1 Genetic diversity and population structure

Descriptive genetic diversity statistics were estimated as the number of segregation nucleotide sites (k), number of haplotypes (n), private haplotypes (ph , haplotypes restricted to a single sample and not found elsewhere), haplotype diversity (h , measure of the frequencies and number of haplotypes among individuals), and nucleotide diversity (π , average weighted sequence divergence between haplotypes) for each population using ARLEQUIN 3.1.1 (Excoffier et al. 2005). To determine whether the sampling efforts were sufficient because of uneven sample sizes, rarefaction curves were estimated using ANALYTIC RAREFACTION 1.3 (UGA Stratigraphy Lab website; <http://www.uga.edu/~strata/software/>). Rarefaction curves were constructed by plotting the number of samples sequenced against the expected number of mitochondrial haplotypes.

An analysis of the molecular variance (AMOVA; Excoffier et al. 1992) was performed in ARLEQUIN 3.1.1. AMOVA analysis estimates the variance components and Φ -statistics values partitioned at three levels of hierarchical subdivision: among groups (Φ_{CT}), within populations (Φ_{ST}), and the interaction of both (among+within, Φ_{SC}) for CR-mtDNA sequence. Hierarchical AMOVA was performed to evaluate the effects of temporal and spatial population subdivision. For *G. galeus*, a temporal analysis was carried out on NZ and AUS adult collections that were grouped based on years of sampling. Thus, samples from 2007-2010 (ENI, KAI, CHA, SOL, SOU, POR, TAS, and FRE), and also AUS samples from 1994 (SWA and HOB) were pooled together. Secondly, two spatial analyses were undertaken to test the hypothesis of panmixia among collections from: 1) NZ, AUS and CHI, and 2) NZ and AUS. For *S. griffini* a spatial population structure analysis were undertaken, where the hypothesis of panmixia was tested among collections from GIB-WES-CHA (pooled together) and KER. For *S. zygaena* a spatial analysis was carried among collections from NZ-AUS (pooled together) and CHI-ECU (pooled together). The genetic distance matrix for AMOVA was estimated by pairwise differences and the significance levels of the variance components and Φ -statistic values were tested by 20,000 nonparametric permutations (Excoffier et al. 1992).

Additionally, pairwise genetic differences (Φ_{ST}) were estimated as indicators of gene flow between all populations for each species. Conventional Φ_{ST} that were based upon haplotype frequencies were computed in ARLEQUIN 3.1.1 with 20,000 permutations. Datasets were analysed separately by sampling design, for adult and pup *G. galeus* collections. In order to minimise type I error (probability of incorrectly rejecting a true null hypothesis), sequential Bonferroni correction of the significant values was estimated for the pairwise Φ_{ST} due to relatively high numbers of simultaneous comparisons (Rice 1989). To test for isolation by distance (IBD), linearised Φ_{ST} values were plotted against the natural logarithm of geographic distance (kilometres) among sampling sites. This analysis was only carried out on *G. galeus* and *S. griffini* populations. Geographic distances were estimated by ruler option in Google Earth v.6.2.2 (Google Inc.). The distance calculations consist of the shortest path, by sea, between any two sampling locations. Significance was evaluated for each location using a Mantel test as implemented in ARLEQUIN 3.1.1 using 20,000 permutations.

Haplotype genealogies were estimated for all the CR-mtDNA sequences obtained in this study. The reconstruction of genealogies was performed using phylogenetic algorithms in order to estimate the relationship between haplotypes without ambiguities or unresolved connection (Salzburger et al. 2011). A phylogenetic reconstruction was estimated with a maximum likelihood (ML) approach in the PHYML 3.0 (Guindon et al. 2010). The substitution model was set according to the highest log-likelihood obtained with JMODEL 0.1.1 (Posada 2008). The Jukes & Cantor (JC69, 1969) was obtained as the most suitable model for *G. galeus*. Only the proportion of invariable sites ($p\text{-inv}$) was fixed (0.9030). The Hasegawa Kishino Yano (HYK + G, 1985) was preferred for *S. griffini* ($p\text{-inv}$ = 0.8260) and HYK for *S. zygaena*. The rest of the parameters in the substitution model, including tree searching and branch support were set as defaults in PHYML 3.0. The ML tree was loaded in HAPLOVIEWER software (Salzburger et al. 2011), where the haplotype genealogy was reconstructed and edited.

2.3.3.2 Mutation-drift and migration-drift equilibrium

Three different approaches were used to explore any signature of population expansion of the CR sequences for the shark species analysed: (a) neutrality test (D and F_s), (b) mismatch distribution analyses, and (c) Bayesian skyline plot (BSP). Computation of Tajima's D (Tajima 1989) and Fu's F_s (Fu 1997) statistics and their significance values were tested by coalescent simulations under the infinite-sites model in ARLEQUIN 3.1.1. For neutral markers D and F_s tests, significant negative values (less than $p = 0.02$) are expected in populations that have undergone recent population expansion. While, significant positive values are expected in populations that have recently experienced population contraction (Tajima 1989, Fu 1997). However, Fu's F_s test is more sensitive than other tests at detecting the signal of population expansion (Fu 1997, Ramos-Onsins & Rozas 2002). Mismatch distribution analyses were also performed to evaluate possible events of population expansion (Rogers & Harpending 1992). This analysis represents the distribution of pairwise haplotype differences calculated from DNA sequences. The shape of the distribution indicates different scenarios in population history, in particular being influenced by episodes of population expansion (Jobling et al. 2004). Thus, a population that undergoes expansion tends to have a unimodal and smooth distribution of haplotype differences, whereas a multimodal and rough distribution reflects long term stability (Rogers & Harpending 1992). The observed distribution was tested against models of constant population size and population growth-decline in DNASP 5.10 (Librado & Rozas 2009). In order to distinguish between these two types of distribution a Harpending's raggedness index (H_{ri} ; Harpending, 1994) and sum of squared deviations (SSD) were estimated to fit the sudden expansion model. Demographic parameters θ_0 , θ_1 and τ were estimated from a mismatch distribution. These parameters were estimated in order to examine the changes in population size, where θ_0 was the population size before expansion, θ_1 was the population size after the expansion, and τ was the age of the expansion expressed in unit of mutational time. The time (t) since population expansion occurred could then be estimated under the equation $\tau = 2\mu t$ (Rogers & Harpending 1992), where τ represents the age of population mutational time, and μ the mutation rates of the sequence. The μ was defined as $2\mu k$, where μ was mutation rates and k was number of nucleotide base pair of CR-mtDNA analysed. The CR mutation rate of the *G. galeus* is unknown; however,

the substitution rate of five species of sharks is already estimated (reported in Dudgeon et al. 2012). According to this, the minimum and maximum values of calibrated mutation rates of 4×10^{-9} and 2.15×10^{-9} substitution/sites/year were used to estimate μ (Duncan et al. 2006, Keeney & Heist 2006). Additionally, the effective population size (N_{ef}) was calculated using the estimates of co-ancestry coefficient (θ_s) obtained in ARLEQUIN 3.1.1. Thus the equation for $N_{ef} = \theta_s / 2\mu$ (μ calculation are described above) was used. The mutation rates to estimate N_{ef} were the same as used to estimate the time since population expansion occurred.

Additionally, a Bayesian skyline plot (BSP) approach was implemented in BEAST 1.7.2 (Drummond & Rambaut 2007). This technique permits the estimation of N_{ef} through time and it does not require a specific demographic model (e.g. constant size, exponential growth, etc.) prior to the analysis as mismatch distribution does (Drummond et al. 2005). The BSP model generates posterior distribution of the effective population sizes through time using Markov chain Monte Carlo (MCMC) sampling. The substitution model (HKY + I) was selected according to the result of JMODEL 0.1.1 by using the corrected Akaike Information Criterion (AICc). In order to assess evidence of significant substitution rate variation among lineages in the CR sequences of each shark species, a likelihood-ratio test (LRT) was performed in PAUP* 4.0b10 (Swofford 2000). LRT tests were estimated both with and without enforcing substitution rate constancy. The LRT scores were compared under a Chi square (X^2) test. A strict molecular clock approach assuming two different mutation rates of 4×10^{-9} and 2.15×10^{-9} substitution/sites/year was set (Duncan et al. 2006; Keeney & Heist 2006). The coalescent Bayesian skyline process was chosen as tree prior assuming 10 grouped coalescent intervals for New Zealand and Australian pooled sequences, and 5 grouped coalescent intervals for Chilean sequences. A piecewise-constant skyline model was set as well (Drummond et al. 2005). A Markov chain was run for 500 million generations, and the first 10% were discarded as burn-in. Inspection of results was conducted in Tracer 1.5.0 (Rambaut & Drummond 2009) for checking whether performed runs have reached the stationary (> 200) of the posterior probabilities and effective sampling size (ESS) for all parameters. Finally, the BSP was reconstructed and plotted in Tracer 1.5.0 as well.

2.4 Results

2.4.1 *Galeorhinus galeus*

An 893 bp DNA sequence of the 5' end of the CR was determined using DNA from 475 samples of *G. galeus*. The base composition of the aligned data set was 12.5% guanine, 33.1% adenine, 34.5% thymine, and 19.8% cytosine (32.3% GC content). A comparison of the sequences revealed 35 distinct haplotypes defined by 25 segregation sites, which were made up of 16 transitions and 11 transversions (GenBank Accession numbers KC118081-KC118115). The overall haplotype (h) and nucleotide diversity (π) were 0.746 ± 0.015 and 0.001 ± 0.001 , respectively. Haplotype and nucleotide diversity were very similar in NZ ($h = 0.735 \pm 0.032$, $\pi = 0.001 \pm 0.001$) and AUS ($h = 0.729 \pm 0.027$, $\pi = 0.001 \pm 0.001$; Table 2.2) for the adult collection. However, the CHI ($h = 0.800 \pm 0.089$, $\pi = 0.002 \pm 0.001$) area showed higher levels of diversity than NZ/AUS, but not significantly higher. Additionally, haplotypes and nucleotide diversities were slightly higher in the NZ pup collection ($h = 0.753 \pm 0.028$, $\pi = 0.004 \pm 0.002$), than AUS ones ($h = 0.713 \pm 0.038$, $\pi = 0.001 \pm 0.001$; Table 2.2). A rarefaction analysis showed that most of the populations (excluding Chatham Rise collections, CHA) did not reach a plateau when the cumulative allelic richness was plotted against the sequences analysed (Figure 2.3).

Table 2.2: Genetic variability from the control region sequences of *Galeorhinus galeus* for the open waters (OW) and pupping areas (PA). Sampling size (N), number of segregation sites (k), number of haplotypes (n), private haplotypes (ph), haplotype diversity (h), and nucleotide diversity (π).

Sampling	Region	Sites	Date	N	k	n	ph	h	π
Open water	NZ	ENI	2010	30	5	7	1	0.654±0.085	0.001±0.001
		KAI	2010	24	7	8	1	0.757±0.076	0.001±0.001
		CHA	2010	30	3	4	1	0.637±0.064	0.001±0.001
		SOL	2010	26	8	10	2	0.822±0.060	0.002±0.001
		Total		110	8	15	5	0.735±0.032	0.001±0.001
	AUS	SOU	2007	47	8	10	3	0.770±0.040	0.002±0.001
		POR	2007	34	5	6	0	0.672±0.070	0.001±0.002
		TAS	2008	28	5	6	3	0.733±0.057	0.002±0.001
		FRE	2009	24	4	6	0	0.746±0.061	0.001±0.001
		Total		133	8	15	6	0.729±0.027	0.001±0.001
	CHI	SAN	2006	10	3	4	4	0.800±0.089	0.002±0.001
	NZ/AUS/CHI Total			253	19	25	15	0.754±0.020	0.002±0.001
Pupping areas	NZ	KAH	2009	11	2	3	0	0.473±0.162	0.001±0.001
		MAN	2009	21	5	6	1	0.752±0.061	0.001±0.001
		NAP	2009	33	8	8	2	0.686±0.063	0.001±0.001
		KAP	2009	19	6	8	3	0.871±0.044	0.001±0.001
		MAP	2009	30	8	9	3	0.789±0.065	0.002±0.001
		GOL	2009	26	4	5	0	0.748±0.049	0.001±0.001
		Total		140	8	17	9	0.753±0.028	0.004±0.002
	AUS	UPW	2009	30	5	6	0	0.683±0.079	0.001±0.001
		SWA	1994	19	4	5	1	0.743±0.064	0.001±0.001
		HOB	1994	33	5	7	1	0.744±0.052	0.001±0.001
		Total		82	5	10	2	0.713±0.038	0.001±0.001
	NZ/AUS Total			222	13	21	4	0.738±0.023	0.001±0.001
	TOTAL			475	25	35	26	0.746±0.015	0.001±0.001

Figure 2.4: Haplotype rarefaction curve for the *G. galeus* CR sequences for each population sampled for the open waters sampling. Solid gray lines represent each population in **A.** New Zealand (ENI, KAI, CHA, SOL), **B.** Australia (SOU, POR, TAS, and FRE) and **C.** Chile. Dashed lines represent the 95% confidence intervals for rarefaction curves.

The hierarchical AMOVA analysis for the entire data set showed that the null hypothesis of global panmixia was rejected because of a significant level of genetic heterogeneity among collection sites ($\Phi_{ST} = 0.37$, $p < 0.001$; Table 2.2A). Results of the temporal AMOVA revealed non-significant temporal genetic differentiation among year groups for NZ and AUS collections from 1994 to 2010 ($\Phi_{ST} = 0.06$, $p = 0.15$). Significant spatial genetic differentiation was detected among regions i.e. NZ, AUS and CHI ($\Phi_{ST} = 0.50$, $p < 0.001$). Subsequently, a second spatial AMOVA was run for NZ and AUS collections (excluding CHI), which revealed non-significant differentiation across the Tasman Sea ($\Phi_{ST} = 0.01$, $p = 0.09$). Therefore, the result of the second AMOVA suggested that statistical significance of the spatial pattern (among groups) of the first AMOVA was influenced by the CHI population, while the bulk of variation observed ($> 90\%$, $p = 0.10$) was explained within populations from New Zealand and Australia. This result was supported for six pairwise Φ_{ST} values, which showed weak genetic differentiation within New Zealand (e.g. ENI-SOL, $p = 0.002$, Table 2.2B), and between New Zealand and Australia (e.g. SOL-POR, $p =$

0.007). However, they were non-significant after sequential Bonferroni correction of α . All Φ_{ST} values between New Zealand and Australia comparisons were very low and ranged from -0.024 (SOU-FRE) to 0.120 (ENI-SOL), whereas they showed the highest and most significant Φ_{ST} values in comparison with the Chilean population, ranging from 0.188 (SOL-SAN) and 0.300 (CHA-SAN). The sequences from the pup collections revealed non-significant pairwise Φ_{ST} values among all populations in New Zealand and Australia (Table 2.2C). All comparisons that involved the KAH site were significantly different from the other nurseries sampled in NZ, but these genetic differences were most likely caused by the small sample size of KAH. Lastly, the Mantel test indicated there was no significant relationship between genetic distance and geographic distance for all sites across the Tasman Sea adult's collections ($R^2 = 0.04$, $p = 0.38$).

Table 2.3: **A.** AMOVA among five temporal groups of *G. galeus* samples from Australasia region (NZ and AUS), among three spatial groups (NZ, AUS, and CHI), and two spatial groups (NZ and AUS). The d.f. is the degrees of freedom, SSD is the sum of squares, VC is variance components, and % of total variation. **B.** Pairwise Φ_{ST} values (below diagonal) and p values (above diagonal) between NZ, AUS and CHI populations from adult collections. The p values are indicated between brackets for AMOVA, and significant p values for the pairwise Φ_{ST} are represented by * ($\alpha = 0.05$), ** after sequential Bonferroni correction ($\alpha = 0.0014$). **C.** Pairwise Φ_{ST} values between NZ and AUS populations from pup collections. Significant p values are represented by * ($\alpha = 0.05$).

Structure tested	Source of variation	d.f	SSD	VC	% of total	Φ -statistics
Global panmixia	Among groups	8	82.95	0.35	37.38	$\Phi_{ST} = 0.37$ ($p < 0.001$)
	Within populations	244	144.09	0.59	62.62	
	Total	252	227.04	0.94		
Temporal (1994,2008,2009,2010)	Among years	3	3.39	0.01	1.37	$\Phi_{ST} = 0.06$ ($p = 0.15$)
	Among population within years	6	3.42	-0.01	-0.12	$\Phi_{CT} = 0.01$ ($p = 0.07$)
	Within populations	285	168.55	0.59	98.75	$\Phi_{SC} = 0.02$ ($p = 0.48$)
	Total	294	175.36	0.60		
Spatial (NZ,AUS,CHI)	Among regions	2	77.89	0.57	48.59	$\Phi_{ST} = 0.50$ ($p < 0.001$)
	Among population within regions	6	5.01	0.01	0.73	$\Phi_{CT} = 0.49$ ($p = 0.04$)
	Within populations	244	144.09	0.59	50.69	$\Phi_{SC} = 0.01$ ($p = 0.09$)
	Total	252	227.04	1.17		
Spatial (NZ and AUS)	Among Tasman Sea (TS)	1	0.84	-0.01	-0.02	$\Phi_{ST} = 0.01$ ($p = 0.09$)
	Among population within TS	6	5.01	0.01	1.45	$\Phi_{CT} = -0.01$ ($p = 0.52$)
	Within populations	235	137.69	0.59	98.59	$\Phi_{SC} = 0.01$ ($p = 0.10$)
	Total	242	143.59	0.59		

B

Sites	New Zealand			Australia				Chile	
	ENI	KAI	CHA	SOL	SOU	POR	TAS	FRE	SAN
ENI	-	0.395	0.298	0.002*	0.046*	0.544	0.170	0.125	$p < 0.001^{**}$
KAI	-0.001	-	0.633	0.048*	0.351	0.632	0.701	0.292	$p < 0.001^{**}$
CHA	0.006	-0.015	-	0.021*	0.293	0.788	0.736	0.305	$p < 0.001^{**}$
SOL	0.120	0.050	0.078	-	0.032*	0.007*	0.102	0.034*	$p < 0.001^{**}$
SOU	0.038	0.002	0.005	0.046	-	0.424	0.393	0.957	$p < 0.001^{**}$
POR	-0.008	-0.014	-0.021	0.090	-0.002	-	0.489	0.594	$p < 0.001^{**}$
TAS	0.019	-0.018	-0.020	0.031	-0.001	-0.007	-	0.314	$p < 0.001^{**}$
FRE	0.030	0.009	0.007	0.061	-0.024	-0.013	0.006	-	$p < 0.001^{**}$
SAN	0.290	0.225	0.299	0.188	0.218	0.278	0.239	0.231	-

C

Sites	KAH	MAN	NAP	KAP	MAP	GOL	UPW
KAH	-	0.034*	0.056	0.020*	0.111	0.029*	0.212
MAN	0.122	-	0.582	0.302	0.276	0.702	0.175
NAP	0.085	-0.014	-	0.056	0.163	0.544	0.275
KAP	0.121	0.070	0.051	-	0.245	0.270	0.067
MAP	0.044	0.009	0.017	0.011	-	0.281	0.362
GOL	0.121	-0.021	-0.009	0.011	0.008	-	0.143
UPW	0.025	0.022	0.007	0.045	-0.009	0.025	-

The haplotype genealogy was constructed for the entire dataset (475 CR sequences), which showed evidence of two different clades of CR sequences from the entire dataset (NZ, AUS and CHI) separated by 5 mutational steps (Figure 2.4A). New Zealand and Australian haplotypes indicated a star-shaped genealogy dominated by three common haplotypes (haplotypes 1, 3 and 4). They differed by a single substitution and no haplotypes were more than 3 substitutions different from the most frequent haplotypes. Nineteen of the thirty haplotypes were unique for NZ or AUS populations (NZ = 7, AUS = 12) and were found in a small number of CR sequences (≤ 3). Eleven haplotypes were shared between NZ and AUS, corresponding to 36.7% from the overall number of haplotypes found in both regions. The examination of the distribution of the haplotype frequencies also revealed that there were three most common haplotypes (Hap1, 3 and 4). These haplotypes were distributed in different proportions in the majority of the sampling locations. Haplotype 1 was the principal haplotype found at all sampling locations, with the exception of SOL. The rest of the haplotypes were distributed in lower frequencies (Figure 2.4B).

Mismatch distribution for the entire dataset deviated significantly from the expected distribution under the constant and growth-decline population model (Figure 2.5A), which can be seen in the graph as a poor fit of the observed pairwise differences (solid line) and constant expected distribution model (dashed line). This result was supported by the lack of statistical significance of the Tajima's D test ($p = 0.13$; Figure 2.5B). However, the growth-decline population model could not be rejected for NZ and AUS populations, when they were pooled together for the mismatch distribution (Figure 2.5A). Fu's F_s values were negative and highly significant in NZ ($p = 0.001$), and in AUS ($p = 0.001$) suggesting population expansion in the past. This result is enhanced by the trend towards significance showed by the sum of squares distribution (SSD) from AUS ($p = 0.05$). In contrast, none of the D values and H_r index was statistically significant. Additionally, the resulting mismatch model was characterised by expansion time parameter $\tau = 1.25$ in NZ and $\tau = 1.33$ in AUS, and population sizes parameters $\theta_0 = 0$ and $\theta_1 = 99.999$ (Figure 2.5B). Using the mutation rates (μ) reported for CR and the estimation of τ by population, the time since the last expansion for NZ and AUS populations was estimated to be between 175 ka to 346 ka ago for the slower and faster mutation rates, respectively. Additionally, the BSPs indicated that NZ and AUS lineages for the CR

Figure 2.6: **A.** Comparison between observed and expected mismatch distributions of pairwise differences sequences of *G. galeus* under a constant and growth-decline population model performed from adult collection. Solid lines represent the observed pairwise differences, dashed lines the expected distribution for constant population model and dotted lines for growth-decline model. Blue areas encompass 95% C.I. for observed data. **B.** Table of neutrality tests, mismatch distribution, co-ancestry coefficient and effective population sizes estimates from CR sequences for three regional populations. The p values are indicated between brackets for the statistics. Tajima test (D), Fu's test (F_s), Harpending's raggedness index (H_r), sum of squared distribution (SSD), population size before expansion (θ_0), population size after expansion (θ_1), age of population mutational time (τ), time since population expansion occurred (t) for mutation rates of 4×10^{-9} and 2.15×10^{-9} , respectively; coancestry coefficient (θ_S), and effective female population sizes for both mutation rates as well (N_{ef}).

Figure 2.7: Bayesian skyline plots (BSPs) of *G. galeus* sequences represent population size changes through time (years) for NZ and AUS populations and CHI separated, inferred with CR lineages and assuming mutation rates of 4×10^{-9} substitution/sites/year (Figure A and C) and 2.15×10^{-9} substitution/sites/year (Figure B and D). Blue areas encompass 95% highest posterior density (HPD).

2.4.2 *Squalus griffini*

A 645bp sequence of the 5' end of the CR was determined for 145 of *S. griffini*. The base composition of this fragment was on average 2% guanine, 34.4% adenine, 33% thymine, and 20.5% cytosine (33.5% GC content). A comparison of sequences revealed 14 haplotypes, which were defined by 11 segregation sites that comprised 7 transitions and 4 transversions (GenBank Accession numbers KC431810-KC431823). The overall haplotype (h) and nucleotide diversity (π) were 0.695 ± 0.023 and 0.002 ± 0.001 , respectively. Haplotype and nucleotide diversity were very similar for GIB, WES and KER sites ($\sim h = 0.65$, $\pi = 0.001$), but there was less diversity in the CHA site ($h = 0.49 \pm 0.058$, $\pi = 0.001 \pm 0.001$; Table 2.4). A rarefaction analysis revealed that most of the populations did not reach a plateau when the cumulate allelic richness was plotted against the sequences analysed (Figure 2.7).

Table 2.4: Genetic variability from the CR sequences for *S. griffini*. Sample size (N), number of segregation sites (k), number of haplotypes (n), private haplotype (ph), haplotypes diversity (h), and nucleotide diversity (π).

Sites	Date	N	k	n	ph	h	π
GIB	2004	3	1	2	0	0.667 ± 0.314	0.001 ± 0.001
WES	2005-2009	50	7	1	5	0.652 ± 0.050	0.002 ± 0.001
CHA	2007-2008	54	4	5	2	0.486 ± 0.058	0.001 ± 0.001
KER	2007-2008	38	8	7	4	0.627 ± 0.076	0.002 ± 0.001
TOTAL		145	11	14	11	0.695 ± 0.023	0.002 ± 0.001

Figure 2.8: Haplotype rarefaction curve for the *S. griffini* CR sequences for each population sampled (GIB, WES, CHA, and KER). Dashed lines represent the 95% confidence intervals for rarefaction curves.

The hierarchical AMOVA analysis for the entire data set showed that the null hypothesis of global panmixia was rejected due to significant level of genetic heterogeneity among collection sites ($\Phi_{ST} = 0.32$, $p < 0.001$; Figure 2.8A). This results suggested genetic differentiation among regions for GIB-WES-CHA pooled and KER. These results were consistent with the pairwise Φ_{ST} values, which showed genetic differentiation between most comparisons that involved KER-WES and KER-CHA ($p > 0.001$, Figure 2.8B). Conversely, there was non-genetic differentiation between KER-GIB ($p = 0.062$) because of small sample size for GIB ($N = 3$), but it is clear the differences in haplotype distribution in comparison with KER (Figure 2.8D). The Φ_{ST} values varies between -0.064 (GIB-CHA) and 0.384 (WES-KER). Mantel test indicated there was no significant relationship between genetic distance and geographic distance for the four sites sampled ($R^2 = 0.46$, $p = 0.29$).

Figure 2.9: Population structure results for *S. griffini* from four sites: GIB, WES, CHA, and KER. **A.** Table of AMOVA results among two groups: first group was composed of GIB, WES, and CHA; and second was KER. The p values are indicated between brackets for the statistics. The d.f. is the degrees of freedom, SSD is the sum of squares, VC is variance components, and % of total variation. **B.** Table of pairwise Φ_{ST} values (below diagonal) and p values (above diagonal) between populations. The asterisks (**) represent significant values at $p < 0.001$. **C.** Haplotype genealogy for 175 CR sequences. Circles represent the haplotypes with area being equivalent to frequency. The number of individuals is reflected inside the circles. Each line indicates one mutational step between haplotypes and small black circles indicates hypothetical missing haplotype. **D.** Haplotype frequencies of CR sequences where the pie charts represent 3 different haplotypes (Hap1, 2 and 5) and the green pie area reflects 11 private haplotypes pooled as one category called “pri-Hap”.

The star-shaped haplotype genealogy showed evidence of population differentiation that indicate that KER samples have different haplotypes frequencies than the rest of the collection sites (Figure 2.8C). This results are consistent with the the pairwise Φ_{ST} . Eleven of the fourteen haplotypes were unique to their collections site and they were found in a small number of CR sequences (≤ 2), while the other three haplotypes were shared among all collection sites. The haplotypes typically differed by a single substitution when compared with the less frequent haplotypes (≤ 2). The private Hap 6 is separated by at least two mutational steps from all other haplotypes (Hap11). The distribution of haplotype frequencies of CR was characterised by two common haplotypes (Hap 1 and 2) that were found in GIB, WES, and CHA (Figure 2.8D). However, those haplotypes were found in a small number of sequences (≤ 4) for KER, where haplotype 5 was the more frequently found.

Mismatch distribution for the entire dataset showed a good fit between the observed pairwise differences (solid line) and expected distribution model (dash line; Figure 2.9A). None of the D values, H_r and SSD values of population expansion were statistically significant. However, Fu's F_s values showed a significant departure from neutrality for the entire data and when GIB-WES-CHA sequences were pooled together. Mismatch model was also characterised by expansion time parameter $\tau = 0.81$ in GIB-WES-CHA and $\tau = 0.94$ in KER, and population sizes parameters $\theta_0 = 0$ and $\theta_1 = 99.999$ (Figure 2.9B). Using the mutation rates (μ) reported for CR and the estimation of τ by population, the time since the last expansion was estimated between 157 ka to 293 ka years ago for the slower and faster mutation rates for GIB-WES-CHA populations (Figure 2.9B). In addition, the BSPs result suggests that the GIB-WES-CHA population experience a relatively steady population expansion in the past. The CR of for these three populations showed an increase in the population size over ~ 30 or 60 ka years ago according to both different mutation rates (Figure 2.10A,B). This result is strongly supporting for the results of F_s values, which were able to detect genetic signature of sudden expansion through the time. However, the BSPs for KER indicated that the population size has been constant over the 70 or 125 ka years ago (Figure 2.10 C,D).

Figure 2.10: **A.** Comparison between observed and expected mismatch distributions of pairwise differences sequences of *S. griffini* under a constant and growth-decline population model. Solid lines represent the observed pairwise differences, dashed lines the expected distribution for constant population model and dotted lines for growth-decline model. Blue areas encompass 95% C.I. for observed data. **B.** Table of neutrality tests, mismatch distribution, co-ancestry coefficient and effective population sizes estimates from CR sequences for two regional populations: GIB-WES-CHA and KER. The p values are indicated between brackets for the statistics. Tajima test (D), Fu's test (F_s), Harpending's raggedness index (H_r), sum of squared distribution (SSD), population size before expansion (θ_0), population size after expansion (θ_1), age of population mutational time (τ), time since population expansion occurred (t) for mutation rates of 4×10^{-9} and 2.15×10^{-9} , respectively; coancestry coefficient (θ_s), and effective female population sizes for both mutation rates as well (N_{ef}).

Figure 2.11: Bayesian skyline plots (BSPs) of *S. griffini* sequences represent population size changes through time (years) for three pooled populations (GIB-WES-CHA) and KER inferred with CR lineages and assuming mutation rates of 4×10^{-9} substitution/sites/year (Figure A and C) and 2.15×10^{-9} substitution/sites/year (Figure B and D). Blue areas encompass 95% highest posterior density (HPD).

2.4.3 *Sphyrna zygaena*

A 760 bp sequence of the 5' end of the CR was determined using 156 of *Sphyrna zygaena* samples. The average base composition was 11.6% guanine, 31.2% adenine, 35.3% thymine, and 21.9% cytosine (32.3% GC content). Comparison of sequences showed 14 haplotypes that were characterised by 5 segregation sites, comprising 3 transitions and 1 transversion (GenBank Accession numbers KC431824-KC431828). The overall haplotype (h) and nucleotide diversity (π) were 0.615 ± 0.024 and 0.002 ± 0.001 , respectively. New Zealand and Australia showed the lowest haplotype and nucleotide diversity NZ and AUS (0), whereas Chile and Ecuador were more diverse (CHI: $h = 0.434 \pm 0.082$, $\pi = 0.001 \pm 0.001$; ECU: $h = 0.568 \pm 0.058$, $\pi = 0.002 \pm 0.001$; Table 2.5). A rarefaction analysis for CHI and ECU populations indicate that cumulative allelic richness by the number of sequences analysed reached a plateau (Figure 2.11). Because NZ and AUS populations were characterised by only one haplotype for the CR, the rarefaction curve was described as a flat line for both.

Table 2.5: Genetic variability from the CR sequences for *S. zygaena*. The sampling size (N), number of segregation sites (k), number of haplotypes (n), private haplotypes (ph), haplotypes diversity (h), and nucleotide diversity (π).

Sites	Date	N	k	n	ph	h	π
NZ	2008-2009-2010	49	0	1	0	0.000±0.000	0.000±0.000
AUS	2009-2010	26	0	1	0	0.000±0.000	0.000±0.000
CHI	2006-2011	46	4	4	1	0.434±0.082	0.001±0.001
ECU	2007-2008	35	4	4	1	0.568±0.058	0.002±0.001
TOTAL		156	5	5	2	0.615±0.024	0.002±0.001

Figure 2.12: Haplotype rarefaction curve for the *S. zygaena* CR sequences for each population sampled for NZ, AUS, CHI and ECU. Dashed lines represent the 95% confidence intervals for rarefaction curves.

The hierarchical AMOVA analysis for the entire data set showed that the null hypothesis of global panmixia was rejected due to a significant level of genetic heterogeneity among collection sites ($\Phi_{ST} = 0.73$, $p < 0.001$; Figure 2.12A). This results suggested genetic differentiation among groups (NZ-AUS pooled) and (CHI-ECU). These results were consistent with the pairwise Φ_{ST} values, which indicated that NZ and AUS were genetically differentiated from CHI and ECU (Figure 2.12B). The Φ_{ST} values varied between 0 (NZ-CHI) and 0.741 (NZ-CHI).

Figure 2.13: Population structure results for *S. zygaena* from four sites: NZ, AUS, CHI, and ECU. **A.** Table of AMOVA results among two groups: first group was composed of NZ and AUS, the second for CHI and ECU. The d.f. is the degrees of freedom, SSD is the sum of squares, VC is variance components, and % of total variation. **B.** Table of pairwise Φ_{ST} values (below diagonal) and p values (above diagonal) between populations. The asterisks (**) represent significant values at $p < 0.001$. **C.** Haplotype genealogy for 156 CR sequences. Circles represent the haplotypes with area being equivalent to frequency. The number of individuals is reflected inside the circles. Each line indicates one mutational step between haplotypes and small black circles indicates hypothetical missing haplotype. **D.** Haplotype frequencies of CR sequences where the pie charts represent different haplotypes (Hap1, 2 and 4) and the green pie chart reflect 2 private haplotypes pooled as one category called “pri-Hap”.

The haplotype genealogy showed evidence of population structure across the South Pacific Ocean based on the CR sequences (Figure 2.12C). Two of the five haplotypes were unique to sample sites and they were found at low frequency (≤ 0.01) in CHI and ECU. The haplotypes from CHI differed from each other by a single nucleotide substitution. The distribution of haplotype frequencies of CR was characterised by a single haplotype for NZ and AUS (Hap 4), while CHI and ECU were represented by similar haplotype frequencies (Hap 1, 2, and 4) where the Hap 2 represented more than 50% in both areas (Figure 2.12D), the rest was composed of Hap 1, 4 and two private haplotypes (one for CHI and another for ECU).

Mismatch distribution for the entire dataset and CHI-ECU deviated significantly from the constant expected distribution under the sudden expansion model, which are shown by the very poor fit of the observed pairwise differences (solid line) and expected distribution model (dashed line) (Figure 2.13A). This result was supported by the lack of statistical significance for both neutrality and demographic analysis (Figure 2.13B). Additionally, the resulting mismatch model was characterised by expansion time parameter $\tau = 0.758$ for CHI-ECU and population sizes parameters $\theta_0 = 0$ and $\theta_1 = 99.999$ (Figure 2.13B). Using the mutation rates (μ) reported for CR and the estimation of τ by population, the time since the last expansion for CHI-ECU populations was estimated between 125 ka to 232 ka ago for the slower and faster mutation rates, respectively. Additionally, the BSPs indicate that CHI-ECU lineages for the CR of *S. zygaena* showed a stable population size over ~ 60 or 125 ka ago according to both different mutation rates (Figure 2.14). This result is strongly supported by the results of D and H_r statistics, which were not able to detect genetic signature of sudden expansion through the time.

Figure 2.14: **A.** Comparison between observed and expected mismatch distributions of pairwise differences sequences of *S. zygaena* under a constant and growth-decline population. Solid lines represent the observed pairwise differences, dashes lines the expected distribution at constant population model and dot lines at growth-decline model. Blue areas encompass 95% C.I. for growth-decline model. **B.** Table of neutrality tests, mismatch distribution, co-ancestry coefficient and effective population sizes estimates from CR sequences for CHI-ECU and Entire dataset. The p values are indicated between brackets for the statistics. Tajima test (D), Fu's test (F_s), Harpending's raggedness index (H_r), sum of squared distribution (SSD), population size before expansion (θ_0), population size after expansion (θ_1), age of population mutational time (τ), time since population expansion occur (t) for mutation rates of 4×10^{-9} and 2.15×10^{-9} , respectively; coancestry coefficient (θ_s), and effective female population sizes for both mutation rates as well (N_{ef}).

Figure 2.15: Bayesian skyline plots (BSPs) of *S. zygaena* sequences represent population size changes through time (years) for to pooled populations (CHI-ECU) populations inferred with CR lineages and assuming mutation rates for mutation rates of 4×10^{-9} substitution/sites/year (Figure A) and 2.15×10^{-9} substitution/sites/year (Figure B). Blue areas encompass 95% highest posterior density (HPD).

2.5 Discussion

2.5.1 Genetic diversity

Several mtDNA gene regions have been used to study genetic diversity and structure within and among populations of elasmobranch species. Most of the studies use the CR due to its higher level of polymorphism in comparison to other mtDNA genes. However, regions such as *coI*, *nd2*, *nd4*, and *cytb* are also useful in molecular studies for providing a more detailed description of genetic diversity and different haplotype groups (Ward et al. 2007, Murray et al. 2008, Richards et al. 2009) or populations (Hauser 2009, Dudgeon et al. 2009, Ovenden et al. 2010, Schluessel et al. 2010, Veríssimo et al. 2010, Straube et al. 2011). Interestingly, most mtDNA gene regions are characterised by low haplotypic and nucleotide diversity, and only about 2% of the nucleotide positions are variable (Figure 2.1, 2.2 in Supplementary material, values were established across the sites studied). The mtDNA CR sequences from this study of *G. galeus*, *S. griffini*, and *S. zygaena* showed similar levels of variation to previous reports of CR sequences from *G. galeus* (Chabot & Allen 2009) and *S. zygaena* (Testerman et al. 2008).

The level of intraspecific genetic diversity reported for shark species generally varies with habitat differences. Those species from pelagic and oceanic environments more commonly move long distances, and there is often an exchange of migrants between populations, which means the two populations function as a single large and biologically unit. The strength of drift is reduced and the mutations accumulated over time remain stable, resulting in high levels of genetic diversity e.g. *Prionace glauca* and *Rhincodon typus* (see Group III in Supplementary Figure 2.1 and Figure 6.1). Conversely, low levels of haplotypic and nucleotide diversity are found in coastal, reef, and near shore species e.g. *Carcharhinus limbatus*, *Negaprion brevirostris*. The findings of this chapter may reflect the fact that *G. galeus* is a species that occupies both coastal and oceanic habitats. The present study found relatively high levels of haplotype diversity within *G. galeus* populations ($h = 0.75$), when compared with the two other species ($h = 0.70$, *S. griffini*; and $h = 0.62$, *S. zygaena*). The variation found in the *G. galeus* population was higher than the levels reported for other coastal species, including *Carcharias taurus* ($h = 0.72$, Ahonen et al. 2009), *Carcharhinus leucas* ($h = 0.51$, Kark et al. 2011) and *Negaprion acutidens* ($h = 0.28$, Schultz et al.

2008). Conversely, larger ocean-going species such as *Rhincodon typus* (Castro et al. 2007, Ramírez-Marcías et al. 2007) and *P. glauca* (Ovenden et al. 2009) showed higher levels of mtDNA diversity than *G. galeus*.

2.5.2 Genetic differentiation and population structure

The dispersal capabilities, environmental conditions and life-history strategy are the major factors that shape genetic population structuring of marine fishes. Thus, continuously distributed species e.g. *Thunnus albacares*, *Katsuwonus pelamis* (Ely et al. 2005), *Hoplostethus atlanticus* (Varela et al. 2012), *Isurus oxyrinchus* (Schrey & Heist 2003), *Rhincodon typus* (Castro et al. 2007), and *Squalus acanthias* (Veríssimo et al. 2010) displayed high level of genetic connectivity over large spatial scales (i.e. ocean basins). While, species such as *Xiphias gladius* (Rosel & Block 1996), *Makaira nigricans* (Bluonaccorsi et al. 2001), and *Prionace glauca* (Ovenden et al. 2009) exhibits high level of genetic connectivity among populations. In contrast, less vagile species with discontinuous distribution e.g. *Pomatomus saltatrix*, *Scromber japonicus* (Graves 1998), *Galeohrinus galeus* (Chabot & Allen 2009), and *Sphyrna lewini* (Duncan et al. 2006) shows comparatively greater intraspecific genetic differentiation across similar spatial scales. These findings have provided an important understanding of the levels of genetic connectivity in marine fishes i.e. bony fishes and shark species, and emphasize the use of genetics methods for understanding dispersal patterns.

One of the goals of this study was to examine the patterns of genetic population structuring of *G. galeus*, *S. griffini* and *S. zygaena*. Genetic connectivity was studied using CR sequence data over (a) large spatial scales across South Pacific Ocean (SPO) for *G. galeus* and *S. zygaena*, and (b) small spatial scales across Tasman Sea for *G. galeus* or within New Zealand waters for *S. griffini*, and along Eastern Pacific Ocean from Ecuador to Chile. At large spatial scales, the samples showed lack of genetic connectivity across SPO from either *G. galeus* or *S. zygaena*, suggesting that gene flow is highly restricted across SPO populations. These results are consistent with earlier genetic studies that used CR sequences data, which suggested that the Australian *G. galeus* samples were significantly different to South American (Chabot & Allen 2009), and New Zealand and Australian *S. zygaena* samples showed similar

patterns across SPO (Testerman et al. 2008). The biogeographic barrier between South America and Australasia is traditionally recognized as the “East Pacific Barrier (EPB)” (Dudgeon et al. 2012) and it extends approximately 7000 km along Pacific Ocean. The EPB affects population connectivity among several shark species, for example, *Carcharhinus brachyurus* (Benavides et al. 2011) showed a significant level of genetic differentiation between Australia/New Zealand and Peru. In contrast, species such as *Squalus acanthias* showed only a shallow, but statistically significant, level of genetic differentiation among collection site in New Zealand, Chile and Argentina (Veríssimo et al. 2010).

The haplotype diversity that was found in Chilean populations of *G. galeus* can be compare to the diversity reported from sites in Peru and Argentina by obtained the CR sequences from GenBank (Chabot & Allen 2009). A comparison of South American sequences showed that there was a group of similar haplotypes in the region (Figure 2.3 Supplementary material). However, increasing the sample sizes and improving the distribution of sample coverage is needed to obtain a complete understand of the population structure of South American *G. galeus*. The samples from Chile and Peru shared two haplotypes, suggesting that there is gene flow along the continental shelf between these sites. Similar to *G. galeus* populations across the Tasman Sea, the shared genetic diversity of *S. zygaena* along Ecuador and Chile coasts indicates that there is genetic connectivity between these regions. Furthermore, *S. lewini* showed little genetic structuring among populations connected along the coastline, suggesting that genetic connectivity is often promoted by continuous continental margins (Duncan et al. 2006).

At a smaller spatial scale, several coastal species exhibit high levels of exchange of haplotypes in common, displaying lack of population structuring. The result of this study showed that both *G. galeus* and *S. zygaena* have a high level of genetic connectivity within and between New Zealand and Australia, and along Chile and Ecuador. These results are consistent with previous allozymes and RFLP-mtDNA markers studies that samples sites throughout the Tasman Sea and that found a weak, but not significant, level of genetic differentiation between sites on the east coast of New Zealand and western Tasman in Australia (Ward & Gardner 1997). However, the conclusions that could be made from those types of genetic markers are limited because they typically had a low power of statistical resolution. The idea of a single

panmictic *G. galeus* stock in Australasia region is strongly supported by the result of intensive tagging studies, which documented relatively important levels of migration across Tasman Sea (Olsen 1953, 1954, Stanley 1988, Paul 1988, Walker 1989, Walker et al. 1997, Francis & Mulligan 1998, Hurst et al. 1999, Francis 2010). The lack of genetic differentiation throughout the Tasman Sea is correlated with major regional similarities in the life-history characters of *G. galeus* (e.g. size and age to reach sexual maturity), which suggests that the Tasman Sea (New Zealand or Australia) population has been evolving as a single evolutionary unit for a considerable period of time. Similar findings of a lack of genetic differentiation between New Zealand and Australia have been reported for other commercially important marine fishes, for example, the bony fish species *Nemadactylus macropterus*, *Rexea solandri*, *Macruronus novaezelandiae*, and *Hoplostethus atlanticus* (Ward & Elliot 2001, Varela et al. 2011), and the for shark species *Carcharhinus brachyurus* and *Carcharodon carcharias* (Benavides et al. 2011, Pardini et al. 2001).

In contrast, *S. griffini* shows a low level of genetic connectivity within New Zealand waters, suggesting limited gene flow between sites at the Kermadec Islands and sites around the coast of New Zealand (see pairwise Φ_{ST} values). All the haplotypes were shared among four collection sites; however, the differences observed between sites were mainly driven by variation in the frequency of the most common haplotype (Hap5) at the Kermadec Islands. Similar patterns of variation of the common haplotype frequencies were found in *Stegostoma fasciatum*, which showed a level of genetic differentiation along Queensland coast in Australia (Dudgeon et al. 2009).

Sedentary behaviour, disjunct distributions and/or sex-biased dispersal are characteristics that are most often associated with high levels of genetic differentiation among large marine fish populations (Palumbi 1994). Sex-biased dispersal is when individuals of one sex tend to stay or return to their natal site or group of breeders (philopatric) while individuals of the other sex are more prone to disperse away from their natal area (Hueter et al. 2005). One approach used to detect philopatric behaviour is to test for difference between the patterns of genetic structure detected using biparental inherited nuclear genome (e.g. microsatellites) and the pattern detected using maternally transmitted mtDNA. Higher F_{ST} values for mtDNA, but

lower F_{ST} values for nuclear markers are often taken to indicate a higher level of site fidelity in females than males (Hueter et al. 2005). Sex-specific patterns of population genetic variation have been observed in several species of sharks e.g. *Carcharodon carcharias* (Pardini et al. 2001), *Isurus oxyrinchus* (Schrey & Heist 2003), *Negaprion brevirostris* (Schultz et al. 2008), *Carcharhinus lumbeus* (Portnoy et al. 2010), *Carcharhinus leucas* (Karl et al. 2011). These studies demonstrated that females typically remained closer to their home territories, and males tend to roam over larger distances.

The use of biparentally inherited genetic markers e.g. microsatellite, single nucleotide polymorphic (SNPs), may provide more insight to understand whether genetic connectivity is reflecting the same dispersal pattern in both sexes or if there is a difference. Microsatellites are widely dispersed and its distribution cover independent regions along the chromosomes, with no known coding functions (unlike genes which code for specific proteins). The lack of coding constraints ensures that mutations accumulate more quickly than coding regions of the DNA. Future studies should consider (a) increasing the sample size and adding new collection sites, (b) employing nuclear markers such as microsatellites in conjunction with other mitochondrial markers (e.g. ND2 gene) to compare the pattern of population structure using different inheritance molecular markers, and (c) comparing genetic samples of newborn young and/or pups with microsatellite markers would be a valuable steps to understand particularly the genetic structuring of *G. galeus*.

2.5.3 Demographic History

Overfishing and habitat degradation are pressures that can reduce population size, which will increase the strength of genetic drift and cause genetic variation to be lost. The Tajima's D and Fu's F_s tests were used to investigate whether the DNA sequences conformed to a selectively neutral model of evolution, but these tests are also sensitive to changes in population size. The tests gave negative values for the populations data from the three shark species, which indicated that there was an excess of low frequency haplotypes. If it is assumed that natural selection has been a negligible force and the CR has been influenced primarily by genetic drift, then the Tajima's D and Fu's F_s results showed no genetic evidence for a declining population

size. However, demographically the population could have recently shrunk but the process of drift will require more time before the expected decline in genetic diversity is detectable. The test results are expected under the model of population connectivity mainly for *G. galeus* and *S. griffini* because of the high number of private haplotypes found in these populations. The observation that some populations such as *G. galeus* POR and FRE from Australia and *S. griffini* GIB from New Zealand did not have private haplotypes could be because the sample sizes at these sites were too small to detect all of the variation.

New coalescent and non-coalescent methods have led to some significant advances in the field of population genetics, of particular note are the methods used to infer the demographic history of a population from gene sequences (Drummond et al. 2005). Coalescent methods such as Bayesian skyline plots (BSPs) often provide more resolution than non-coalescent methods (e.g. Tajima's D and Fu's F_s and mismatch distribution) because they can account for how drift, mutation and migration influence a gene genealogy (Felsenstein 1992, Pybus et al. 2000). In this study both the coalescent and non-coalescent methods showed that *G. galeus* populations had a pattern of population size contraction followed by a period of expansion. This pattern was particularly obvious in the samples collected from sites in New Zealand and Australia. A similar pattern of population size change was observed in the data collected from *S. griffini* sample sites in Gisborne, Westland, and Chatham Rise. The mismatch distribution analysis also suggested that the populations have undergone a recent expansion. However, the time that has elapsed since these population expansions is slightly different for both species. The mean of the mismatch distribution for *G. galeus* was further to the right the y-axis than *S. griffini* population. The mismatch analysis suggested that the time since population expansion occurred for *G. galeus* was similar than *S. griffini* (175-326 ka and 157 to 293 ka ago, respectively). However, the BSPs estimate for the age since expansion suggested a more recent times ~ 60 or 125 ka years ago for *G. galeus* and 70 or 125 ka years ago for *S. griffini*.

The combination of high haplotype diversity, low nucleotide diversity and a star-shaped haplotype genealogy for the mtDNA data, suggests that *G. galeus* and *S. griffini* experienced a loss of genetic variation because of a population size reduction and then the population size began to recover, which meant more new mutations

persisted in the population as drift weakened. This pattern of genetic diversity (high h and low π) is very common in shark species and it has been reported for species such as *S. lewini* (Duncan et al. 2006), *Carcharhinus limbatus* (Kenney & Heist 2006), *Negaprion brevirostris* (Schultz et al. 2008), *Carcharhinus brachyurus* (Benavides et al. 2011), *Centroscymnus coelolepis* (Veríssimo et al. 2010) (see Figure 2.1 and 2.2 in Supplementary material). When the relationship between CR haplotype diversity and nucleotide diversity of 20 shark species was compared, three groups of species with different combinations of small and large values of h and π were observed. The first group was made up of nine species with low levels of both h and π (e.g. *Sphyrna lewini*, *S. zygaena*, *Carcharhinus leucas*), which might have been caused by reduction in population size or local extinctions, and found by recolonization (Grant & Waples 2000). The second group was characterised by large values of h and lower values of π , which is a pattern typically caused by a population expansion reducing the strength of drift and retaining more haplotypes when they arise (Rogers & Harpending 1992, Grant & Waples 2000). According to the present and previous CR studies (Chabot & Allen 2009), *G. galeus*, *S. griffini*, and *S. zygaena* from Ecuador and Chile fall into this second group ($h > 0.5$, $0.0002 \leq \pi \leq 0.06$), which is comprised of most of the sampled shark species (e.g. *S. lewini*, *Carcharhinus limbatus*, *Carcharhinus leucas*, *Carcharhinus brachyurus*, *Carcharodon carcharias*, and *Cetorhinus maximus*). The last group comprises species with a high h and moderate high π , which is a pattern consistent with a population that has been large and stable over a long period of time. This group is characterised by large highly mobile species that occur in continuous distributions rather than group I and II, which are composed of smaller shark species with discontinuous distributions (Graves 1998, Karl et al. 2011). Only five species *S. lewini*, *Squalus acanthias*, *Rhincodon typus*, *C. obscurus* and *Prionace glauca* showed this latter pattern.

2.5.4 Management implication

Population genetic studies are an important component of the science that underpins fisheries management decisions. When genetic markers show that fishery populations are genetically subdivided, the best explanation is that the differences have arisen because of restricted gene flow. Consequently, a set of stocks can be identified and

stock assessment models can be applied to each stock in conjunction with information about their recruitment, growth, mortality, etc. However, if genetic data suggest that a fishery only has one panmictic stock but there is other evidence to suggest that there might be two or more stocks then the genetic results should be looked at critically. A fishery could be comprised of multiple stocks, but the stocks have only appeared recently which means the pattern cannot be seen in the genetic data yet (need more time), or that the genetic markers did not have the level of resolution required to detect multiple stocks. In this study, the genetic data suggested that there were single stocks of *G. galeus* and *S. zygaena* that extended across the economic zones of New Zealand and Australia, and another stock along the continental margins for *G. galeus* from Peru and Chile and for *S. zygaena* from Ecuador and Chile. The tagging data is consistent with these suggested stock models and there is not any compelling evidence to contradict this conclusion.

New Zealand and Australian waters are important fishing grounds for many commercial marine fish e.g. *G. galeus*. Despite some evidence that the Tasman Sea can act as a barrier for gene flow for marine commercial fish, the majority of the species surveyed show a lack of any significant barrier to dispersal (Pardini et al. 2001, Ward & Elliot 2001, Benavides et al. 2011, Varela et al. 2012). However, despite of the complex migration patterns and population structuring of *G. galeus* described by intensive tagging programs and biological background of *G. galeus* in Australasia region, it is a difficult species for stock assessment and management purposes.

Although the mtDNA CR could not reject the null hypothesis of a single stock of *G. galeus* between New Zealand and Australia, this species has been separated into locally (New Zealand and Australia) manageable single stocks due to: (a) previous molecular markers showed low resolution to test genetic differentiation, (b) tagging data showed low rates of movements from Australia to New Zealand, supporting the hypothesis of two stocks, and (c) for administrative and logistic reasons *G. galeus* is considered as separate stocks. This is more precautionary for *G. galeus* in Australia because if the assumption is made of one single stock, there is no reason to reduce the fishing rates in Australia (Waples et al. 2008). It is uncertain whether these regional management units are adequate for *G. galeus*; moreover when the stock is depleted in Australia, and the fishery is considered sustainable in New Zealand. Additionally, it is

also concerning that the apparent sustainability of *G. galeus* biomass in New Zealand waters has been used to maintain of the high TAC levels through the last decade (>3000 t.). In Australian waters the historical nurseries areas have reduced and/or stopped to produce newborn young or pupping activity e.g. Port Phillip, Port Sorell, and St. Helen (Stevens & West 1997), and most of the productive nurseries are in New Zealand waters.

2.6 Appendices

Supplementary Table 2.1: Genetic diversity from the control region sequences among 20 shark species by each sampling locations reported to the date. Sampling locations, sampling size (N), haplotypes diversity (h), nucleotide diversity (π), effective female population sizes (N_{ef}) are detailed by each reference. ID is the reference number for each population in the supplementary figure.

Species	Sampling locations	N	h	π	N_{ef}	References	ID
<i>Carcharhinus limbatus</i>	Bulls Bay, South Carolina	34	0.37	0.0004	n/a	Keeney & Heist 2006	26
	Georgia	13	0.46	0.0004	n/a		31
	Pine Island, Sound Florida	45	0.79	0.0012	n/a		77
	Terra Ceia Bay, Florida	45	0.72	0.0011	n/a		63
	Yankeetown, Florida	45	0.80	0.0013	n/a		80
	Padre Island, Texas	49	0.81	0.0015	n/a		81
	Laguna Yalahau, Yucatan	60	0.79	0.0029	n/a.		76
	Belize city, Belize	13	0.68	0.0007	n/a		58
	Dangriga, Belize	19	0.53	0.0005	n/a		37
	North Carolina	17	0.68	0.0017	n/a		56
<i>C. leucas</i>	Gulf Coast of Florida	13	0.77	0.0014	n/a	Karl <i>et. al.</i> 2011	74
	Northern Gulf	23	0.25	0.0009	n/a		18
	Brazil	49	0.33	0.0006	n/a		21
	Australia	37	0.24	0.0003	n/a		17
<i>C. brachyurus</i>	Brazil	1	0	0	n/a	Benavides <i>et. al.</i> 2011	2
	Mexico	1	0	0	n/a		3
	Namibia	26	0.84	0.0020	n/a		83
	NZ	15	0	0	n/a		4
	Peru	13	0.76	0.0018	n/a		71
	South Africa	26	0.70	0.0014	n/a		60
	Spain	1	0	0	n/a		5
	Indonesia	8	0.86	0.0104	n/a		105
	West Australia	13	0.28	0.0016	n/a		20
	East Australia	7	0.67	0.0030	n/a		54
<i>C. sorrah</i>	Indonesia	8	0.71	0.0027	n/a	Ovenden <i>et. al.</i> 2009	62
	West Australia	8	0.25	0.0007	n/a		19
	East Australia	13	0.78	0.0032	n/a		75
	Gulf of Carpentaria	20	0.64	0.0054	n/a		106
<i>C. plumbeus</i>	Taiwan	46	0.90	0.0021	n/a	Portnoy <i>et. al.</i> 2010	84
	Hawaii	23	0.54	0.0016	n/a		41
	Eastern Australia	43	0.54	0.0022	n/a		42
	Western Australia	25	0.93	0.0022	n/a		87
	South Africa/Indian	15	0.87	0.0047	n/a		97
	Gulf of Mexico	23	0.95	0.0021	n/a		90
	Delaware Bay	52	0.91	0.0021	n/a		85
	Chesapeake Bay	55	0.93	0.0022	n/a		88
	Eastern shore of Virginia	47	0.91	0.0020	n/a		86
	Pacific Mexico	9	0	0	n/a		1
<i>Negaprion brevirostris</i>	Bahamas	30	0.54	0.0008	1.4×10^4	Schultz <i>et. al.</i> 2008	38
	Brazil	30	0.34	0.0004	2.6×10^4		25
	Guinea Bissau	11	0.62	0.0010	1.3×10^4		49
	Taiwan	5	0	0	n/a		10
<i>N. acutidens</i>	French Polynesia	16	0	0	n/a	Schultz <i>et. al.</i> 2008	11
	New Caledonia	2	1.00	0.0019	n/a		91
	Eastern Australia	10	0.33	0.0006	1.6×10^4		22
	Western Australia	25	0.60	0.0015	1.7×10^4		47
	NZ	49	0	0	n/a		12
<i>Sphyrna zygaena</i>	Australia	26	0	0	n/a	This study	13
	Chile	46	0.50	0.0010	n/a		35
	Ecuador	35	0.57	0.0010	n/a		45
	Indonesia	28	0.74	0.0111	n/a		110
<i>S. lewini</i>	West Australia	4	0.50	0.0163	n/a	Ovenden <i>et. al.</i> 2009	113
	East Australia	15	0.59	0.0002	n/a		44

Species	Sampling locations	N	h	π	N_{eff}	References	ID
<i>Sphyrna lewini</i>	Baja, Pacific	44	0.51	0.0009	$3.8 \times 10^6 - 1.1 \times 10^7$	Duncan <i>et. al.</i> 2006	36
	Pac Panama, Pacific	8	0.75	0.0019	$1 \times 10^6 - 3.1 \times 10^7$		71
	Hawai, Pacific	44	0.09	0.0002	550-1600		14
	Philippines, Pacific	15	0.67	0.0110	$1.1 \times 10^4 - 3.2 \times 10^4$		111
	Taiwan, Pacific	20	0.70	0.0073	$2.6 \times 10^6 - 7.8 \times 10^6$		108
	East Australia, Pacific	32	0.66	0.0057	$1.2 \times 10^4 - 3.5 \times 10^4$		107
	Thailand, Indian	2	0	0	n/a		8
	West Australia, Indian	26	0.68	0.0095	$3.4 \times 10^3 - 1.1 \times 10^4$		109
	Seychelles, Indian	12	0.17	0.0012	$8.1 \times 10^3 - 2.7 \times 10^4$		16
	South Africa, Indian	25	0.42	0.0046	$9 \times 10^3 - 3 \times 10^4$		30
	West Africa, Atlantic	6	0.33	0.0006	$1.5 \times 10^5 - 5 \times 10^5$		24
	Brazil, Atlantic	3	0.67	0.0024	n/a		55
	Atlantic Panama	2	1.00	0.0037	n/a		92
	Gulf of Mexico, Atlantic	16	0	0	n/a		9
	East Coast USA, Atlantic	16	0.53	0.0010	$1.8 \times 10^7 - 6 \times 10^6$		40
<i>Galeorhinus galeus</i>	Africa	16	0.70	0.0010	46.808	Chabot & Allen 2009	59
	Australia	50	0.76	0.0013	83.221		73
	California	26	0.73	0.0019	89.545		68
	Peru-Argentina	12	0.93	0.0022	63.680		89
	UK	12	0.79	0.0014	61.718		79
<i>G. galeus</i>	New Zealand (NZ)	110	0.74	0.0013	$2.13 \times 10^5 - 3.96 \times 10^5$	This study	66
	Australia	133	0.73	0.0013	$2.31 \times 10^5 - 4.3 \times 10^6$		65
	Chile	10	0.80	0.0016	$1.48 \times 10^5 - 2.76 \times 10^5$		82
<i>Ginglymostoma cirratum</i>	Brazilian Islands	37	0.43	0.0004	n/a	Karl <i>et. al.</i> 2012	28
	Natal, Brazil	15	0.56	0.0005	n/a		43
	Bimini, Bahamas	21	0.43	0.0004	n/a		27
	Florida, USA	37	0.16	0.0001	n/a		15
	Glover's reef, Belize	33	0.49	0.0004	n/a		32
<i>Carcharias taurus</i>	Eastern Australia	66	0	0	n/a	Ahonen <i>et. al.</i> 2009	6
	Western Australia	24	0.43	0.0021	n/a		29
	South Africa	77	0.61	0.0013	n/a		48
	Northwest Atlantic	12	0.53	0.0009	n/a		39
	Japan	9	0	0	n/a		7
	Brazil	6	0.33	0.0006	n/a		23
	California	59	0.79	0.0013	n/a		78
<i>Carcharodon carcharias</i>	Pacific	34	0.73	0.0013	n/a	Jorgensen <i>et. al.</i> 2010	78
<i>Cetorhinus maximus</i>	Atlantic	27	0.72	0.0014	n/a	Hoelzel <i>et. al.</i> 2006	67
<i>Prionace glauca</i>	Indonesia	19	0.89	0.0070	n/a	Ovenden <i>et. al.</i> 2009	64
	Mid-north Pacific	20	0.89	0.0072	n/a		102
	West Australia	4	1.00	0.0088	n/a		99
	East Australia	17	0.89	0.0078	n/a		104
	Global distribution	44	0.97	0.0072	n/a		100
<i>Squalus acanthias</i>						Hauser <i>et. al.</i> 2007	
<i>S. griffini</i>	Gisborne, NZ	3	0.67	0.0010	n/a	This study	57
	Chatham Rise, NZ	54	0.48	0.0009	n/a		33
	South Westland, NZ	50	0.65	0.0016	n/a		53
	Kermadec, NZ	38	0.63	0.0015	$3.68 \times 10^5 - 6.85 \times 10^5$		50
	Atlantic	19	0.93	0.0070	n/a		101
<i>Rhincodon typus</i>	Western Indian	18	0.95	0.0050	n/a	Castro <i>et. al.</i> 2007	95
	Eastern India	12	0.91	0.0040	n/a		96
	Northwest Pacific	12	1.00	0.0050	n/a		93
	Northeast Pacific	8	0.96	0.0060	n/a		94
	Bahia los Angeles	19	0.95	0.0200	n/a		115
<i>R. typus</i>	Bahia de la Paz	13	0.90	0.0200	n/a	Ramírez-Macias <i>et. al.</i> 2007	114
	Banco Gorda	4	0.50	0.0100	n/a		112
	Brazil	83	0.82	0.0048	n/a		98
<i>Rhizoprionodon lalandii</i>						Mendonça <i>et. al.</i> 2009	
<i>Stegostoma fasciatum</i>	Indo-West-Pacific	20	0.48	0.0009	n/a	Dudgeon <i>et. al.</i> 2009	34
<i>Centroscyllium coelelepis</i>	Ireland	45	0.64	0.0019	n/a	Verissimo <i>et. al.</i> 2010	52
	Portugal	41	0.57	0.0014	n/a		46
	Madeira	17	0.74	0.0020	n/a		70
	Azore	40	0.74	0.0022	n/a		69
	Mauritania	46	0.63	0.0017	n/a		51
	South Africa	22	0.69	0.0019	n/a		61

Supplementary Figure 2.1: Relationship between the haplotypes (h) and nucleotide diversity (π) of the CR sequences for 21 species of sharks. Only single values were coloured by species, however those species showing similar values were represented in black circles.

Supplementary Figure 2.2: Haplotype genealogy of *G. galeus* sequences from maximum likelihood (ML) tree of samples from present study (NZ, AUS, CHI) and a haplotype network adapted according to the one reported by Chabot and Allen (2009) for CR sequences from Peru (PER) and Argentina (ARG). Circles represent the haplotypes with area being equivalent to frequency. The number of individuals is reflected inside the circles. Each line indicates one mutational step between haplotypes. Haplotypes from PER and ARG were obtained from GenBank. Refer to Figure 2.4A for details of NZ/AUS haplotypes genealogy.

Chapter 3

The development of microsatellite DNA markers for school shark (*Galeorhinus galeus*)

3.1 Abstract

School sharks (*Galeorhinus galeus*) are common in New Zealand and Australia, but there has only been a limited amount of research into their genetic structure. This chapter reports the development and testing of 15 *G. galeus* microsatellite loci that were developed using next-generation sequencing methods and fluorescent-labelled primer genotyping (ABI 3730). Approximately 200 *G. galeus* samples collected from three locations were genotyped and three of the microsatellite loci were found to be polymorphic (ranging from 2-9 alleles). The observed heterozygosity was between 0.135 and 0.510 and the expected heterozygosity between 0.169 and 0.598, which was low when compared with most of elasmobranch species. The three polymorphic loci were combined with another previously reported seven loci (see Chapter 4 and Chapter 5), and used to describe genetic diversity, population structuring, effective population sizes and parentage of *G. galeus* populations in Australasia.

3.2 Introduction

DNA microsatellites are simple sequence repeats (SSRs), or highly repetitive DNA segments, of nuclear DNA. SSR often vary by the number of repetitive elements and can be highly variable within a population. SSR typically have a higher mutation rate than other areas of the nuclear genome because during replication the DNA polymerase enzyme can slip as it copies the repetitive element, which results in an insert or deletion event. Microsatellite alleles differ in size by multiples of the core repeat motif (2-6 bp) and they need to be distinguished using high resolution (O'Connell & Wright 1997, Guichoux et al. 2011). DNA microsatellites are a popular genetic marker for determining levels of variation within and between populations and for studying parentage. This marker type has been widely used in fisheries for the identification of genetic stock structure, determining effective population size, species identification, product provenance and fisheries surveillance, and monitoring breeding patterns in aquaculture (Guichoux et al. 2011, Dichmont et al. 2011, Dudgeon et al. 2012).

The major disadvantage of microsatellite DNA is that development can be expensive and time-consuming. Traditionally there were two methodological approaches taken for their development, either hybridization selection (Armour et al. 1994), or linker and ligation procedures (Hamilton et al. 1999). However, the advent of next-generation sequencing (NGS) technology revolutionized the development process because this method can rapidly produce hundreds of thousands of nuclear DNA sequences that can be quickly searched for repeat regions and primers flanking these regions designed immediately. This approach also enables all repeat types to be discovered and is not limited by the number of DNA probes that are available in the laboratory when screening a genomic library.

The most common NGS approach uses the GS-FLX Titanium (Roche 454 Life Science, USA, commonly named as 454) platform because it is the best system for generating the large average sequence read sizes (approximately 500 bp) that are needed to provide a reasonable number of primer design options either side of the repeat element. Fluorescent dye labelled genotyping technology (e.g. ABI 3730) has enabled multiplexing and pool-plexing of SSRs markers, which is when several loci are combined and analysed together whenever their allele sizes do not overlap if they

use that same coloured dye, but if the alleles sizes of loci do overlap then different coloured fluorescent dyes can be used to distinguish them (Schuelke 2000, Guichoux et al. 2011). This combined approach reduces the genotyping costs and analysis times.

The NGS approach can be described in four steps: (1) A sample of genomic DNA is first nebulized into small fragments. Each individual DNA strand is attached to a bead, and separated from all other strands into its own micro-PCR droplet (Emulsion PCR: a one tube oil-water mix that creates millions of separate droplets that contain all of the ingredients for micro-PCRs), then cycled to produce a large number of copies of each strand. The synthetic DNA sequences that form a cluster on each bead are read as light emitted during a pyrosequencing process. (2) The resultant DNA sequences are then searched and those containing a repetitive element are separated. (3) Those sequences initially selected are filtered. Each repeat-DNA type is identified and sequences that have repeats at the sequence ends (i.e. there is no room for a primer) are discarded. (4) The program Primer 3 is used to generate a range of primer options, the best primers (based on fragment size, small difference between primer melting temperatures, etc) are chosen, the oligonucleotides are synthesized and each locus is screened for polymorphism and whether they have technical problems (e.g. null alleles, ambiguities caused by stuttering, etc) (Abdelkrim et al. 2009, Guichoux et al. 2011).

The goal of this Chapter was to develop microsatellite markers for New Zealand and Australian *G. galeus* populations using the NGS technology (Roche 454). In Chapter 4 and 5 these new markers will be combined with previously published microsatellite markers and the panel of markers will be used to study the genetic variation of school sharks, describing the genetic diversity, assessing population genetic structuring, estimate population sizes and parentage study.

3.3 Methods

DNA used for the development of the microsatellite library was isolated from five blood samples obtained from two juveniles of school shark captured in Manukau Harbour, New Zealand. Genomic DNA was purified using a standard phenol-chloroform extraction protocol (Sambrook et al. 1989). The DNA quality and quantity was assessed using a NanoDrop spectrophotometer (model ND-1000) and visualised using agarose gel electrophoresis. The five best DNA extractions were sent to the Roche 454 sequencing service (Department of Anatomy and Structural Biology, Otago University). The DNA samples were prepared for the whole genome shotgun sequencing on the Roche Genome Sequencer FLX instrument utilizing the GS FLX Titanium Rapid Library Preparation Kit (Roche Applied Sciences, Indianapolis, USA) following the manufacturer's protocol. The library was quantified for DNA fragment size distribution and concentration (Agilent 2100 Bioanalyzer) and then processed with the GS FLX emulsion PCR. Sequencing was performed using 1/8th of an LR70 plate.

The resulting DNA sequence reads were converted into a single FASTA format file and screened for perfect and imperfect microsatellite motifs (di, tri, tetra) using the software QDD v1.0 (Meglécz et al. 2010). Primers for the identified microsatellite loci were designed using PRIMER3 (Rozen & Skaletsky 2000), which was embedded in QDD software and the default settings were used. In addition, MSATCOMMANDER v0.8.1 (Faircloth 2008) was used to identify the number of perfect microsatellites within the FASTA file. Fifteen loci were chosen and their primer pairs were synthesized. An M13F sequence (5'-TGTAACGACGGCCAGT) was added to the 5'-end of the forward primer so that a fluorescent label could be attached to the amplicons during the PCR process (for details about the M13 tailing method see Schuelke 2000). The primer sequences and PCR conditions for each locus are listed in Table 3.1.

Initially DNA isolated from between 5 and 15 samples from five different collection sites (four from New Zealand and one from Australia) were used to test for polymorphism and optimise the amplification of loci. Subsequently, 200 samples from New Zealand, Australia and Chile were screened using the markers to provide a more detailed characterization of each locus. Each microsatellite locus was PCR

amplified separately in 10 µl volumes containing 67 mM Tris-HCl pH 8.8, 16 mM (NH₄)₂SO₄, 2.0 mM MgCl₂, 0.05 µM of forward primer with an M-13 tail, 0.2 µM of reverse primer, 0.05 µM of a fluorescently labelled M-13 primer (either FAM, VIC, or PET, Invitrogen), 0.8 mM of each dNTP, 0.3 units of BIOTAQTM DNA Polymerase (Bioline), and 1 µl of template DNA (50-80 ng/µl DNA concentration). Thermal cycling was performed on a TGradient Thermal Block (Biometra, Goettingen, Germany). There was an initial denaturing step of 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 62°C for 45 s, and 72°C 45 s, and finally a series of 8 cycles of 94°C for 30 s, 53°C for 45 s, and 72°C for 45 s and a final extension of 72°C for 10 min. Multiple loci were pool-plexed into wells on a 96-well format plate based on the combinations of loci pairs recommended by an analysis conducted using MULTIPLEX MANAGER 1.0 program (Holleley & Geerts 2009). Capillary separation of the fluorescently labelled PCR-fragments was conducted on an ABI3730 Genetic Analyser (Massey University Genome Service). Allele sizes were determined using an internal lane standard LIZ 500 (ABI) and the Peak Scanner v1.0 software (Applied Biosystems).

The dataset was initially checked for the presence of null alleles, allele size shifts and scoring errors due to stuttering, using MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004). Genetic diversity estimates, such as the number of alleles per locus, observed (H_o), and expected (H_e) heterozygosities, tests for linkage disequilibrium (LD) between pairs of loci and deviation from Hardy Weinberg equilibrium (HWE), were calculated using ARLEQUIN 3.1.1 (Excoffier et al. 2005). The coefficient of inbreeding (F_{IS}) was estimated using GENEPOP 4.0 (Raymond & Rousset 1995). HWE significance was calculated using the exact test (Guo & Thompson, 1992) with a Markov chain of 10⁴ steps and dememorization of 10⁴ steps.

3.4 Results

A total of 46,602 DNA sequences were generated by the FLX instrument. The length of the reads ranged from 62 bp to 790 bp with a mean of 246.3 bp ± 40.7 bp (Figure 3.2). Overall, there were not many DNA sequences retrieved (relatively short reads ~250 bp), as expected with the technical improvements of the sequencing services nowadays (long reads ~500 bp). These limitations suggested that only a few

microsatellite loci within the DNA sequence file would be suitable for primer design because most of the repeats would have short (or no) flanking sequence regions.

A search of the sequences using MSATCOMMANDER found 3,082 sequences that contained perfect microsatellite-like repeat motifs; 2442 dinucleotide, 271 trinucleotide, 298 tetranucleotide, 71 pentanucleotide and one hexanucleotide.

Figure 3.1: Distribution of sequence length of 46,602 DNA readings obtained from NGS (Roche 454).

The search for acceptable microsatellite-containing sequence using QDD found 15 loci that were recommended for further assessment. Most of these 15 loci were dinucleotide-type repeats: five loci contained simple repeats (*Ggal1*, 2, 3, 5, 16), two loci contained compound simple repeat (*Ggal10*, 11), and seven loci contained imperfect repeats. One locus (*Ggal15*) contained a trinucleotide repeat (Table 3.1). The test of the 15 loci using over 200 DNA samples showed that only three loci were polymorphic (*Ggal8*, 14, 15) and the numbers of alleles range from 2-9 (see Table 3.1). The observed heterozygosity ranged between 0.135 for *Ggal8* and 0.510 for *Ggal15* and the expected heterozygosity between 0.169 for *Ggal8* and 0.598 for *Ggal15*. After correction for multiple tests, all three polymorphic loci deviated significantly from HWE because of heterozygote deficiency. A test of linkage disequilibrium showed that all pairs of loci were unlinked.

Table 3.1: Characteristics of 15 microsatellite loci for *G. galeus*. N = sample size screened for each, A = number of alleles, H_o = observed heterozygosity, H_e = expected heterozygosity, and F_{is} = inbreeding coefficient (negative values = heterozygote excess and positive values = heterozygote deficiency). NA means not-applicable.

Locus	Forward primer 5'-3'	Reverse primer 5'-3'	Motif	N	Size (bp)	A	H_o	H_e	F_{is}
<i>Ggal1</i>	CGAATGGCCTAATTCTGCTC	CCCGGGAACGTTTAGTTTTA	(TC) ₁₃	205	NA	0	NA	NA	NA
<i>Ggal2</i>	ACCCAGCCAGAGTCAGCACC	ACCTCTCTATCCCCTCGAAGATGC	(AG) ₁₂	212	117	1	0	0	0
<i>Ggal3</i>	TCAGTGAGAAAGCCAAAGAGC	CGATGCTTCACACTCTGTCC	(GA) ₉	205	194	1	0	0	0
<i>Ggal5</i>	TTCTTTCCTTCTCGGGTGTG	GGTAATGGCAAAAGGAGTGC	(AG) ₈	212	NA	0	NA	NA	NA
<i>Ggal6</i>	AGAGGGGAACTGAGACACCA	TACAATCGACAGGGCAACAA	(AG) ₄ AA(AG) ₈	217	NA	0	NA	NA	NA
<i>Ggal7</i>	CTGGCTGGTGTTCOAAGG	GCATAGTGGTGGTGTGCTGT	(TC) ₂ AT(CCT) ₂ CTT(CCT) ₅ (GCT) ₂	205	183	1	0	0	0
<i>Ggal8</i>	CGTGCTTGTGGCAAATTTTA	GCAGGAACAGCGAGTGATTA	(CT) ₂ CA(GT) ₂ (CT) ₆ CC(GT) ₂	217	121-123	3	0.135	0.169	0.203
<i>Ggal9</i>	AGAGAGGGAACTGAGACACCA	TACAATCGACAGGGCAACAA	(GA) ₅ AA(GA) ₇	217	NA	0	NA	NA	NA
<i>Ggal10</i>	ATTGGCAAAAGAAGCAATGG	GGCCTGTTCTGTGCTGTAT	(TG) ₆ (AG) ₃	212	154	1	0	0	0
<i>Ggal11</i>	AATTTGGTGGCAATTTGACG	CTGGGATCGAGGTCTGAAAG	(AG) ₇ (GA) ₂	217	NA	0	NA	NA	NA
<i>Ggal12</i>	ATGGGGTAAGGAATGGAAGG	AGCACCAAGTAAGGGCAGAA	(GA) ₂ TGA(AG) ₂ (GGAG) ₂	205	NA	0	NA	NA	NA
<i>Ggal13</i>	CCAAGGTTTGATTTGCCACT	CAGTTCCCGAGTCTGAGTCC	(CA) ₂ AA(CA) ₆	212	135	1	0	0	0
<i>Ggal14</i>	AGTCAAAGGATGCTCGCAGT	GGGCGATTATTTGGATGAGA	(AA) ₂ AC(AG) ₇	217	117-125	4	0.378	0.525	0.281
<i>Ggal15</i>	GTGACCTCCATCACC GACTT	GCCTTTGTGAAGAGCCTCAA	(CAA) ₈	205	132-159	9	0.510	0.598	0.147
<i>Ggal16</i>	TCACTCGAGTCCGTCCAAAT	CTGGGTGGACACTCAGGTTAG	(AG) ₇	217	NA	0	NA	NA	NA

3.5 Discussion

The present study reported only three microsatellite loci, and they had several limitations to identifying and developing microsatellites. These include: (a) the average read length was ~250, which is an important factor given that two-thirds of the microsatellite detected are too close to either fragment end to enable development of flanking PCR primers (Abdelkrim et al. 2009, Castoe et al. 2010, Csencsics et al. 2010, Lepais & Bacles 2010, Parchman et al. 2010, Guichoux et al. 2011, Gardner et al. 2011); (b) the number of repeats is very low for the majority of microsatellite loci tested, with the exception of *Ggal1* and *Ggal2* (13 and 12 repeats respectively). This situation is critical to define which sequences actually represent microsatellites. It is known that more repeats have higher mutation rates, which ensures detection of polymorphisms (Weber 1990, Heist & Gold 1999, Ellegren 2000, Petit et al. 2005, Kelkar et al. 2008, Guichoux et al. 2011, Gardner et al. 2011). The number of polymorphic loci, number of repeats, number of alleles and genetic diversity (heterozygosity) found in the present study were lower than the results reported in the majority of previous studies elasmobranch species, with some exceptions e.g. *Carcharhinus plumbeus* (Heist & Gold 1999). Nevertheless, the development of three new microsatellite loci for *G. galeus* using NGS provides a valuable tool for describing genetic diversity, genetic population structuring, effective population sizes and parentage studies when combined with other previously published microsatellite loci from this species.

Thirty two published studies (including the present study) have reported novel microsatellite loci involving 29 elasmobranch species (Table 3.1 in Supplementary Material). Most of these studies have been of shark i.e. 25 species (Selachii) and only a few have been from the rays and skates i.e. 4 species (Batoidea). The hybridization selection and linker and ligation methodologies have been the most commonly employed microsatellite isolation techniques reported. Only six studies obtained genomic libraries for microsatellite development using the NGS approach, these were for the species *Mustelus antarcticus* (Boomer & Stow 2010), *G. galeus* (Chabot & Nigenda 2011), *M. henlei* (Chabot 2012), *Manta alfredi* (Kashiwagi et al. 2012), *Isurus oxyrinchus* (Taguchi et al. 2013) and the present study.

Microsatellite DNA accounts for three percent of the human genome. Dinucleotide repeats are the most abundant, followed by mono- and tetranucleotides repeat and tetranucleotide repeats. When considering all the possible dinucleotide repeat, (CA)_n is the most common, followed by (AT)_n, (GA)_n, (GC)_n (Ellegren 2004). There are only four possible types of dinucleotide CA (or GT), GA (or CT), AT, and GC. Note that CA repeat is the AG repeat on the opposite strand, which means the only difference between these motifs is the direction that they were originally sequenced. The published literature on population genetic studies of sharks that used microsatellites loci comprised 14 studies (including the present study), which included 15 shark species (Table 3.2 in Supplementary Material). A total of 111 microsatellite loci have been reported, which consist of di-nucleotide repeat units (93.7%), tri- (0.9%), tetra- (4.5%), and hexa- (0.9%) (Figure 3.1 in Supplementary Material). Within the di-nucleotide, CA was the highest reported repeats class (72.1%), GA (18.9%), and the others were GC (1.8%), and TA (0.9%).

The majority of microsatellite loci are composed of di-nucleotide repeats in several classes i.e. Insecta, Teleostei, Mammalia and Plantae (Wang et al. 1994, Schug et al. 1998, Li et al. 2002, Perumal et al. 2008). This review of motif repeats in shark studies are consistent with other taxa, which indicated a high frequency of (CA)_n, followed by (AT)_n, (GA)_n, (GC)_n (Ellegren 2004). However, the most common motif types can also vary widely among taxa (Dieringer & Schlotterer 2003, Gardner et al. 2011). Those studies are based upon genome sequencing projects rather than small amount of microsatellites loci reported for population structure studies in shark species. Nevertheless, the first genomic sequencing projects are already on progress for *Raja erinacea*, *Squalus acanthias*, and *Rhincodon typus* (Dudgeon 2012). These studies will provide more insight about, for example, the distribution and frequency of microsatellites repeats in elasmobranch genomes. Despite of this drawback, nevertheless this information showed a clear trend comparable with other species.

3.6 Appendices

Supplementary Material

Supplementary Table 3.1: List of microsatellite loci reported in primer notes to date for elasmobranch species. Three methodology approaches were utilised to develop SSRs: hybridization selection (HS), linker and ligation procedures (LL), expressed sequence tags (ESTs), and Roche 454. The number of loci, number of repeat, observed heterozygosity (H_o), and expected heterozygosity (H_e) are reported. Number of repeats and alleles, and observed and expected heterozygosity are represented by ranges across loci.

Species	Methodology	N° loci	N° of repeats	A	H_o	H_e	Reference
<u>Selachii</u>							
<i>Carcharhinus plumbeus</i>	HS and LL	3	6-10	2-3	NR	0.07-0.54	Heist & Gold 1999
<i>C. plumbeus</i>	HS and LL	5	13-42	4-39	0.63-1	0.57-0.96	Portnoy <i>et. al.</i> 2006
<i>C. plumbeus</i>	HS and LL	5	16	12	NR	0.84	Portnoy <i>et. al.</i> 2007
<i>C. limbatus</i>	HS and LL	16	4-34	1-22	0-0.96	0-0.94	Keeney & Heist 2003
<i>C. tilstoni</i>	HS	5	4-28	4-24	0.63-1	0.57-0.96	Ovenden <i>et. al.</i> 2006
<i>C. sorrah</i>	HS	5	2-19	5-24	0.12-0.82	0.16-0.95	Ovenden <i>et. al.</i> 2006
<i>Negaprion brevirostris</i>	HS and LL	3	20-25	13-22	0.72-0.90	0.73-0.90	Feldheim <i>et. al.</i> 2001a
<i>N. brevirostris</i>	HS and LL	15	2-33	1-43	0-0.87	0-0.90	Feldheim <i>et. al.</i> 2001b
<i>N. brevirostris</i>	HS	5	11-18	9-23	0.69-0.87	0.66-0.88	DiBattista <i>et. al.</i> 2008
<i>Sphyrna tiburo</i>	HS	4	NR	6-35	0.51-0.87	0.55-0.69	Chapman <i>et. al.</i> 2004
<i>S. lewini</i>	HS and LL	15	NR	6-29	0.40-0.91	0.54-0.90	Nance <i>et. al.</i> 2009
<i>Mustelus antarcticus</i>	Roche 454	12	3-19	4-11	0.32-0.95	0.30-0.84	Boomer & Stow 2010
<i>M. henlei</i>	Roche 454	10	NR	2-10	0.24-0.89	0.23-0.86	Chabot 2012
<i>Galeorhinus galeus</i>	Roche 454	13	NR	3-12	0-0.86	0-0.87	Chabot & Nigenda 2011
<i>G. galeus</i>	Roche 454	15	1-13	1-9	0-0.51	0-0.60	This study
<i>Prionace glauca</i>	HS	10	2-27	3-35	0.24-0.91	0.27-0.95	Fitzpatrick <i>et. al.</i> 2011
<i>P. glauca</i>	HS and LL	8	2-16	2-14	0.08-0.78	0.08-0.94	Mendoça <i>et. al.</i> 2012
<i>Isurus oxyrinchus</i>	HS and LL	5	4-53	14->57	0.77->0.90	0.82-0.96	Schrey & Heist 2002
<i>I. oxyrinchus</i>	Roche 454	15	6-16	2-23	0.08-0.88	0.08-0.91	Taguchi <i>et. al.</i> 2013
<i>Ginglymostoma cirratum</i>	HS and LL	9	3-26	3-15	0.17-0.90	0.16-0.92	Heist <i>et. al.</i> 2003
<i>Carcharodon carcharias</i>	HS and LL	5	3-23	3-10	0.45-0.95	0.51-0.83	Pardini <i>et. al.</i> 2000
<i>Squalus acanthias</i>	HS and LL	8	2-11	2-9	0.37-0.84	0.51-0.82	McCauley <i>et. al.</i> 2004
<i>Stegostoma fasciatum</i>	HS	14	6-33	3-22	0.40-0.97	0.53-0.97	Dudgeon <i>et. al.</i> 2006
<i>Carcharias taurus</i>	HS and LL	8	4-32	21-30	0.29-1	0.28-0.92	Feldheim <i>et. al.</i> 2007
<i>Chiloscyllium plagiosum</i>	HS and LL	12	3-28	4-12	0.23-0.91	0.41-0.89	Ding <i>et. al.</i> 2009
<i>Rhincodon typus</i>	HS	9	4-22	2-17	0.20-0.83	0.21-0.86	Ramirez-Macias <i>et. al.</i> 2009
<i>Hexanchus griseus</i>	HS	14	NR	10-69	0.24-1	0.76-0.96	Larson <i>et. al.</i> 2009
<i>Scyliorhinus canicula</i>	ESTs	17	5-15	2-12	0.13-0.88	0.12-0.83	Griffiths <i>et. al.</i> 2011
<i>Centroscyllium coelepis</i>	HS and LL	10	6-35	3-29	0.60-0.98	0.59-0.96	Verissimo <i>et. al.</i> 2011
<i>Centroselachus crepidater</i>	HS and LL	13	3-30	3-7	0.18-0.95	0.25-0.82	Helyar <i>et. al.</i> 2011
<u>Batoidea</u>							
<i>Raja clavata</i>	HS	5	2-21	8-48	0.36-0.94	0.32-0.98	Chevolot <i>et. al.</i> 2005
<i>Aetobatus flagellum</i>	HS and LL	8	6-22	2-6	0.21-0.79	0.20-0.71	Yagushita & Yamaguchi 2009
<i>A. narinari</i>	HS and LL	10	6-40	2-19	0.35-0.97	0.33-0.95	Sellas <i>et. al.</i> 2011
<i>Manta alfredi</i>	Roche 454	10	6-18	2-10	0.22-0.87	0.20-0.80	Kashiwagi <i>et. al.</i> 2012

Supplementary Table 3.2: Genetic diversity from microsatellite loci reported to date for population genetic studies among 15 shark species by location. Sampling size (N), number of alleles (A), observed heterozygosity (H_o), and expected heterozygosity (H_e) are reported. For *Negaprion brevirostris* and *N. acutensis* the allele richness *i.e.* asterisk “*” is represented rather than number of alleles due they were not reported by each loci in Schultz et al. 2008. For *Squalus acanthias* the number of alleles is represented across eight loci, for this reason is only one value.

Species	Sampling locations	N	Locus	Motif	A	H_o	H_e	Reference
Selachii								
<i>Carcharhinus plumbeus</i>	mid-Atlantic Bight & Gulf of Mexico	71	<i>Cpl</i> -6	(TC) ₈	2	NR	0.23	Heist & Gold 1999
		71	<i>Cpl</i> -38	(CA) ₄ TA(CA) ₆	2	NR	0.07	
		71	<i>Cpl</i> -39	(TG) ₉	3	NR	0.54	
<i>Carcharhinus plumbeus</i>	Taiwan	48	<i>Cli</i> -12	(GT) ₉	11	0.77	0.73	Portnoy <i>et. al.</i> 2010
		48	<i>Cli</i> -103	(GA) ₅ (GA) ₁₄ (GA) ₇	15	0.88	0.88	
		48	<i>Cpl</i> -53	(TG) ₁₈	7	0.58	0.70	
		48	<i>Cpl</i> -90	(AC) ₂₄	22	0.98	0.94	
		48	<i>Cpl</i> -128	(CA) ₁₃ TA(CA) ₁₃	29	0.94	0.94	
		48	<i>Cpl</i> -132	(TG) ₁₆	18	0.92	0.90	
		48	<i>Cpl</i> -166	(GT) ₁₇	45	1	0.98	
		48	<i>Cpl</i> -169	(TG) ₄₂	31	1	0.96	
	Hawaii	23	<i>Cli</i> -12	(GT) ₉	3	0.39	0.40	
		23	<i>Cli</i> -103	(GA) ₅ (GA) ₁₄ (GA) ₇	9	0.87	0.84	
		23	<i>Cpl</i> -53	(TG) ₁₈	5	0.61	0.65	
		23	<i>Cpl</i> -90	(AC) ₂₄	10	0.91	0.75	
		23	<i>Cpl</i> -128	(CA) ₁₃ TA(CA) ₁₃	11	0.65	0.78	
		23	<i>Cpl</i> -132	(TG) ₁₆	8	0.83	0.79	
		23	<i>Cpl</i> -166	(GT) ₁₇	23	0.91	0.94	
		22	<i>Cpl</i> -169	(TG) ₄₂	14	0.95	0.85	
	eastern Australia	43	<i>Cli</i> -12	(GT) ₉	22	0.74	0.65	
		43	<i>Cli</i> -103	(GA) ₅ (GA) ₁₄ (GA) ₇	14	0.88	0.87	
		43	<i>Cpl</i> -53	(TG) ₁₈	6	0.67	0.64	
		43	<i>Cpl</i> -90	(AC) ₂₄	24	1	0.94	
		43	<i>Cpl</i> -128	(CA) ₁₃ TA(CA) ₁₃	19	0.81	0.91	
		43	<i>Cpl</i> -132	(TG) ₁₆	15	0.86	0.90	
		43	<i>Cpl</i> -166	(GT) ₁₇	42	0.98	0.98	
		43	<i>Cpl</i> -169	(TG) ₄₂	29	0.95	0.96	
	western Australia	30	<i>Cli</i> -12	(GT) ₉	8	0.73	0.73	
		30	<i>Cli</i> -103	(GA) ₅ (GA) ₁₄ (GA) ₇	15	0.87	0.91	
		30	<i>Cpl</i> -53	(TG) ₁₈	5	0.43	0.69	
		30	<i>Cpl</i> -90	(AC) ₂₄	23	1	0.95	
		30	<i>Cpl</i> -128	(CA) ₁₃ TA(CA) ₁₃	16	0.80	0.89	
		30	<i>Cpl</i> -132	(TG) ₁₆	17	0.90	0.91	
		30	<i>Cpl</i> -166	(GT) ₁₇	29	0.93	0.96	
		30	<i>Cpl</i> -169	(TG) ₄₂	25	0.97	0.96	
	South Africa & Indian Ocean	15	<i>Cli</i> -12	(GT) ₉	9	0.67	0.83	
		15	<i>Cli</i> -103	(GA) ₅ (GA) ₁₄ (GA) ₇	13	1	0.91	
		15	<i>Cpl</i> -53	(TG) ₁₈	6	0.67	0.72	
		15	<i>Cpl</i> -90	(AC) ₂₄	17	0.93	0.95	
		14	<i>Cpl</i> -128	(CA) ₁₃ TA(CA) ₁₃	12	0.79	0.92	
		15	<i>Cpl</i> -132	(TG) ₁₆	15	0.87	0.94	
		14	<i>Cpl</i> -166	(GT) ₁₇	18	0.93	0.96	
		15	<i>Cpl</i> -169	(TG) ₄₂	19	0.93	0.94	
	Gulf of Mexico	23	<i>Cli</i> -12	(GT) ₉	10	0.87	0.88	
		23	<i>Cli</i> -103	(GA) ₅ (GA) ₁₄ (GA) ₇	7	0.70	0.70	
		23	<i>Cpl</i> -53	(TG) ₁₈	4	0.57	0.60	
		23	<i>Cpl</i> -90	(AC) ₂₄	17	0.91	0.93	
		23	<i>Cpl</i> -128	(CA) ₁₃ TA(CA) ₁₃	10	0.78	0.81	
		23	<i>Cpl</i> -132	(TG) ₁₆	8	0.87	0.79	
		23	<i>Cpl</i> -166	(GT) ₁₇	24	0.96	0.97	
		15	<i>Cpl</i> -169	(TG) ₄₂	23	0.91	0.95	
	Delaware Bay	55	<i>Cli</i> -12	(GT) ₉	10	0.87	0.84	
		55	<i>Cli</i> -103	(GA) ₅ (GA) ₁₄ (GA) ₇	10	0.73	0.64	
		55	<i>Cpl</i> -53	(TG) ₁₈	4	0.47	0.54	
		55	<i>Cpl</i> -90	(AC) ₂₄	20	0.89	0.89	
		55	<i>Cpl</i> -128	(CA) ₁₃ TA(CA) ₁₃	18	0.85	0.85	
		55	<i>Cpl</i> -132	(TG) ₁₆	13	0.92	0.85	
		55	<i>Cpl</i> -166	(GT) ₁₇	47	1	0.98	
		55	<i>Cpl</i> -169	(TG) ₄₂	33	0.93	0.95	
	Chesapeake Bay	47	<i>Cli</i> -12	(GT) ₉	10	0.91	0.86	
		46	<i>Cli</i> -103	(GA) ₅ (GA) ₁₄ (GA) ₇	7	0.50	0.5	
		46	<i>Cpl</i> -53	(TG) ₁₈	3	0.50	0.54	
		47	<i>Cpl</i> -90	(AC) ₂₄	20	0.98	0.90	
		47	<i>Cpl</i> -128	(CA) ₁₃ TA(CA) ₁₃	14	0.87	0.87	
		47	<i>Cpl</i> -132	(TG) ₁₆	10	0.87	0.84	
		47	<i>Cpl</i> -166	(GT) ₁₇	36	0.94	0.97	
		47	<i>Cpl</i> -169	(TG) ₄₂	31	0.96	0.95	
	eastern shore of Virginia	52	<i>Cli</i> -12	(GT) ₉	12	0.90	0.84	
		52	<i>Cli</i> -103	(GA) ₅ (GA) ₁₄ (GA) ₇	8	0.67	0.67	
		52	<i>Cpl</i> -53	(TG) ₁₈	4	0.56	0.58	
		52	<i>Cpl</i> -90	(AC) ₂₄	21	0.92	0.92	
		52	<i>Cpl</i> -128	(CA) ₁₃ TA(CA) ₁₃	15	0.83	0.84	
		52	<i>Cpl</i> -132	(TG) ₁₆	10	0.79	0.83	
		52	<i>Cpl</i> -166	(GT) ₁₇	39	0.98	0.97	
		52	<i>Cpl</i> -169	(TG) ₄₂	34	0.96	0.96	

Species	Sampling locations	N	Locus	Motif	A	H _e	H _s	Reference
<i>Carcharhinus limbatus</i>	Bull Bay South Carolina	34	<i>Clf</i> -2	(GA) ₁₀	1	0	0	Keeney <i>et. al.</i> 2005
		34	<i>Clf</i> -7	(GT) ₂₀	24	0.91	0.96	
		34	<i>Clf</i> -12	(GT) ₉	2	0.06	0.06	
		34	<i>Clf</i> -13	(GT) ₁₉	18	0.91	0.89	
		34	<i>Clf</i> -55	(GT) ₁₀	7	0.38	0.38	
		34	<i>Clf</i> -100	(TG) ₄ (GT) ₁₀	4	0.15	0.14	
		34	<i>Clf</i> -107	(GT) ₁₄	6	0.77	0.73	
		34	<i>Clf</i> -108	(GT) ₁₂	7	0.68	0.75	
	Georgia	13	<i>Clf</i> -2	(GA) ₁₀	2	0.08	0.08	
		13	<i>Clf</i> -7	(GT) ₂₀	18	1	0.97	
		13	<i>Clf</i> -12	(GT) ₉	3	0.39	0.34	
		13	<i>Clf</i> -13	(GT) ₁₉	10	0.69	0.91	
		13	<i>Clf</i> -55	(GT) ₁₀	5	0.31	0.29	
		13	<i>Clf</i> -100	(TG) ₄ (GT) ₁₀	1	0	0	
		13	<i>Clf</i> -107	(GT) ₁₄	4	0.92	0.68	
		13	<i>Clf</i> -108	(GT) ₁₂	8	0.85	0.78	
	Pine Island Sound Florida	72	<i>Clf</i> -2	(GA) ₁₀	4	0.08	0.08	
		72	<i>Clf</i> -7	(GT) ₂₀	34	0.97	0.96	
		72	<i>Clf</i> -12	(GT) ₉	3	0.10	0.09	
		72	<i>Clf</i> -13	(GT) ₁₉	21	0.88	0.92	
		72	<i>Clf</i> -55	(GT) ₁₀	5	0.25	0.25	
		72	<i>Clf</i> -100	(TG) ₄ (GT) ₁₀	4	0.17	0.16	
		72	<i>Clf</i> -107	(GT) ₁₄	4	0.65	0.68	
		72	<i>Clf</i> -108	(GT) ₁₂	8	0.68	0.72	
	Terra Ceia Bay Florida	71	<i>Clf</i> -2	(GA) ₁₀	4	0.06	0.08	
		71	<i>Clf</i> -7	(GT) ₂₀	31	0.96	0.95	
		71	<i>Clf</i> -12	(GT) ₉	3	0.04	0.04	
		71	<i>Clf</i> -13	(GT) ₁₉	21	0.94	0.92	
		71	<i>Clf</i> -55	(GT) ₁₀	5	0.24	0.24	
		71	<i>Clf</i> -100	(TG) ₄ (GT) ₁₀	4	0.20	0.18	
		71	<i>Clf</i> -107	(GT) ₁₄	5	0.71	0.69	
		71	<i>Clf</i> -108	(GT) ₁₂	8	0.70	0.74	
	Yankeetown Florida	66	<i>Clf</i> -2	(GA) ₁₀	3	0.17	0.16	
		66	<i>Clf</i> -7	(GT) ₂₀	33	0.99	0.96	
		66	<i>Clf</i> -12	(GT) ₉	3	0.11	0.10	
		66	<i>Clf</i> -13	(GT) ₁₉	21	0.96	0.91	
		66	<i>Clf</i> -55	(GT) ₁₀	5	0.26	0.23	
		66	<i>Clf</i> -100	(TG) ₄ (GT) ₁₀	4	0.14	0.16	
		66	<i>Clf</i> -107	(GT) ₁₄	5	0.73	0.70	
		66	<i>Clf</i> -108	(GT) ₁₂	7	0.73	0.71	
	Padre Island Texas	50	<i>Clf</i> -2	(GA) ₁₀	3	0.12	0.12	
		50	<i>Clf</i> -7	(GT) ₂₀	29	0.92	0.95	
		50	<i>Clf</i> -12	(GT) ₉	3	0.12	0.12	
		50	<i>Clf</i> -13	(GT) ₁₉	17	0.96	0.91	
		50	<i>Clf</i> -55	(GT) ₁₀	7	0.28	0.27	
		50	<i>Clf</i> -100	(TG) ₄ (GT) ₁₀	4	0.12	0.15	
		50	<i>Clf</i> -107	(GT) ₁₄	5	0.66	0.68	
		50	<i>Clf</i> -108	(GT) ₁₂	7	0.76	0.74	
	Laguna Yalahau northern Yucatan	84	<i>Clf</i> -2	(GA) ₁₀	4	0.18	0.17	
		84	<i>Clf</i> -7	(GT) ₂₀	31	0.96	0.95	
		84	<i>Clf</i> -12	(GT) ₉	3	0.10	0.09	
		84	<i>Clf</i> -13	(GT) ₁₉	25	0.92	0.92	
		84	<i>Clf</i> -55	(GT) ₁₀	7	0.48	0.36	
		84	<i>Clf</i> -100	(TG) ₄ (GT) ₁₀	4	0.21	0.22	
		84	<i>Clf</i> -107	(GT) ₁₄	6	0.61	0.63	
		84	<i>Clf</i> -108	(GT) ₁₂	8	0.81	0.76	
	Belize City	10	<i>Clf</i> -2	(GA) ₁₀	3	0.50	0.43	
		10	<i>Clf</i> -7	(GT) ₂₀	10	1	0.93	
		10	<i>Clf</i> -12	(GT) ₉	1	0	0	
		10	<i>Clf</i> -13	(GT) ₁₉	9	1	0.91	
		10	<i>Clf</i> -55	(GT) ₁₀	2	0.40	0.53	
		10	<i>Clf</i> -100	(TG) ₄ (GT) ₁₀	3	0.40	0.36	
		10	<i>Clf</i> -107	(GT) ₁₄	4	0.70	0.74	
		10	<i>Clf</i> -108	(GT) ₁₂	3	0.50	0.48	
	Dangriga	19	<i>Clf</i> -2	(GA) ₁₀	3	0.17	0.26	
		19	<i>Clf</i> -7	(GT) ₂₀	20	1	0.95	
		19	<i>Clf</i> -12	(GT) ₉	3	0.11	0.10	
		19	<i>Clf</i> -13	(GT) ₁₉	17	0.95	0.94	
		19	<i>Clf</i> -55	(GT) ₁₀	4	0.32	0.47	
		19	<i>Clf</i> -100	(TG) ₄ (GT) ₁₀	4	0.47	0.48	
		19	<i>Clf</i> -107	(GT) ₁₄	5	0.63	0.67	
		19	<i>Clf</i> -108	(GT) ₁₂	5	0.63	0.59	
<i>Carcharhinus sorrah</i>	Indonesia	31	<i>LS</i> -15	(AC) ₂₀	2	0.03	0.03	Ovenden <i>et. al.</i> 2009
		25	<i>Cs</i> -5	(GT) ₁₉	10	0.76	0.79	
		47	<i>Cs</i> -12	(GT) ₁₉	5	0.47	0.55	
		42	<i>Clf</i> -100	(TG) ₄ (GT) ₁₀	4	0.05	0.07	
		28	<i>Cs</i> -8	(CA) ₂₈	24	0.86	0.94	
		53	<i>LS</i> -15	(AC) ₂₀	4	0.09	0.13	
	west Australia	51	<i>Cs</i> -5	(GT) ₁₉	17	0.92	0.89	
		51	<i>Cs</i> -12	(GT) ₁₉	7	0.61	0.57	
		46	<i>Clf</i> -100	(TG) ₄ (GT) ₁₀	8	0.50	0.45	
		40	<i>Cs</i> -8	(CA) ₂₈	27	0.98	0.95	
	east Australia	37	<i>LS</i> -15	(AC) ₂₀	2	0.08	0.08	
		37	<i>Cs</i> -5	(GT) ₁₉	10	0.89	0.83	
		36	<i>Cs</i> -12	(GT) ₁₉	7	0.61	0.57	
		34	<i>Clf</i> -100	(TG) ₄ (GT) ₁₀	4	0.32	0.36	
	Gulf of Carpentaria	29	<i>Cs</i> -8	(CA) ₂₈	24	0.86	0.93	
		57	<i>LS</i> -15	(AC) ₂₀	6	0.19	0.18	
		46	<i>Cs</i> -5	(GT) ₁₉	13	0.74	0.82	
		67	<i>Cs</i> -12	(GT) ₁₉	6	0.61	0.49	
		61	<i>Clf</i> -100	(TG) ₄ (GT) ₁₀	6	0.25	0.29	
		48	<i>Cs</i> -8	(CA) ₂₈	30	0.85	0.95	

Species	Sampling locations	N	Locus	Motif	A	H _a	H _e	Reference
<i>Carcharhinus obscurus</i>	Indonesia	22	<i>Clh</i> -108	(GT) ₁₂	4	0.73	0.69	Ovenden <i>et. al.</i> 2009
		18	<i>Cs</i> -2	(GT) ₂ (GC)(GT)(GC)(GT) ₁₁	14	0.56	0.88	
		17	<i>Cs</i> -6	(CA) ₆ AA(CA)	16	0.88	0.86	
		25	<i>LS</i> -24	(AC) ₁₂	3	0.44	0.52	
	west Australia	66	<i>Clh</i> -108	(GT) ₁₂	4	0.58	0.59	
		48	<i>Cs</i> -2	(GT) ₂ (GC)(GT)(GC)(GT) ₁₁	15	0.42	0.90	
		27	<i>Cs</i> -6	(CA) ₆ AA(CA)	16	0.89	0.88	
		59	<i>LS</i> -24	(AC) ₁₂	4	0.54	0.56	
	east Australia	5	<i>Clh</i> -108	(GT) ₁₂	4	0.80	0.70	
		4	<i>Cs</i> -2	(GT) ₂ (GC)(GT)(GC)(GT) ₁₁	4	0.50	0.72	
		5	<i>Cs</i> -6	(CA) ₆ AA(CA)	8	1	0.86	
		7	<i>LS</i> -24	(AC)	3	0.14	0.56	
<i>Carcharhinus leucas</i>	north Carolina	19	<i>Clh</i> -7	(GT) ₂₀	19	1	0.95	Kark <i>et. al.</i> 2011
		19	<i>Clh</i> -107	(GT) ₁₄	13	0.90	0.86	
		19	<i>Clh</i> -106	(GA) ₁₁	5	0.68	0.65	
		19	<i>Clh</i> -108	(TG) ₄ (GT) ₁₀	16	0.95	0.92	
	Gulf coast of Florida	19	<i>Clh</i> -112	(GT) ₄ (GT) ₁₄	23	0.95	0.97	
		35	<i>Clh</i> -7	(GT) ₂₀	31	0.94	0.96	
		34	<i>Clh</i> -107	(GT) ₁₄	15	0.91	0.87	
		35	<i>Clh</i> -106	(GA) ₁₁	4	0.37	0.43	
	northern Gulf	33	<i>Clh</i> -108	(GT) ₁₂	24	0.97	0.91	
		34	<i>Clh</i> -112	(GT) ₄ (GT) ₁₄	28	0.88	0.97	
		36	<i>Clh</i> -7	(GT) ₂₀	25	0.94	0.95	
		36	<i>Clh</i> -107	(GT) ₁₄	18	0.89	0.84	
	Brazil	33	<i>Clh</i> -106	(GA) ₁₁	5	0.49	0.42	
		27	<i>Clh</i> -108	(GT) ₁₂	15	0.93	0.90	
		28	<i>Clh</i> -112	(GT) ₄ (GT) ₁₄	28	0.71	0.97	
		54	<i>Clh</i> -7	(GT) ₁₄	28	0.91	0.95	
<i>Negaprion brevirostris</i>	middle and southern Atlantic Ocean	55	<i>Clh</i> -107	(GT) ₁₄	23	0.84	0.87	Feldheim <i>et. al.</i> 2001
		45	<i>Clh</i> -106	(GA) ₁₁	4	0.64	0.60	
		55	<i>Clh</i> -108	(GT) ₁₂	29	0.98	0.95	
		47	<i>Clh</i> -112	(GT) ₄ (GT) ₁₄	30	0.79	0.96	
	Bimini, Bahamas	545	<i>LS</i> -11	(AC) ₃₃	43	0.68	0.69	
		688	<i>LS</i> -15	(AC) ₂₀	30	0.79	0.80	
		673	<i>LS</i> -22	(AC) ₂₃	22	0.87	0.90	
		726	<i>LS</i> -30	(AC) ₂₅	19	0.73	0.75	
		30	<i>LS</i> -11	(AC) ₃₃	6*	0.59	NR	
		30	<i>LS</i> -15	(AC) ₂₀	9*	0.80	NR	
<i>N. brevirostris</i>	Brazil	30	<i>LS</i> -48	(AC) ₃₈	12*	0.95	NR	Schultz <i>et. al.</i> 2008
		30	<i>LS</i> -52	(AC) ₂₀	13*	0.95	NR	
		30	<i>LS</i> -54	(CT) ₁₀ (CA) ₈	3*	0.46	NR	
		30	<i>LS</i> -22	(AC) ₂₃	9*	0.89	NR	
		30	<i>LS</i> -30	(AC) ₂₅	6*	0.67	NR	
		30	<i>LS</i> -75	(TC) ₁₁ (AC) ₁₁ AG(AC) ₁₀	4*	0.68	NR	
		30	<i>LS</i> -82	(AC) ₂₅	8*	0.78	NR	
		30	<i>LS</i> -11	(AC) ₃₃	6*	0.59	NR	
		30	<i>LS</i> -15	(AC) ₂₀	6*	0.67	NR	
		30	<i>LS</i> -48	(AC) ₃₈	10*	0.92	NR	
		30	<i>LS</i> -52	(AC) ₂₀	11*	0.94	NR	
		30	<i>LS</i> -54	(CT) ₁₀ (CA) ₈	4*	0.57	NR	
	Guinea-Bissau	30	<i>LS</i> -22	(AC) ₂₃	8*	0.88	NR	
		30	<i>LS</i> -30	(AC) ₂₅	5*	0.75	NR	
		30	<i>LS</i> -75	(TC) ₁₁ (AC) ₁₁ AG(AC) ₁₀	4*	0.54	NR	
		30	<i>LS</i> -82	(AC) ₂₅	8*	0.76	NR	
		11	<i>LS</i> -11	(AC) ₃₃	1*	0	NR	
		11	<i>LS</i> -15	(AC) ₂₀	11*	0.93	NR	
		11	<i>LS</i> -48	(AC) ₃₈	11*	0.94	NR	
		11	<i>LS</i> -52	(AC) ₂₀	13*	0.97	NR	
		11	<i>LS</i> -54	(CT) ₁₀ (CA) ₈	1*	0	NR	
		11	<i>LS</i> -22	(AC) ₂₃	5*	0.61	NR	
		11	<i>LS</i> -30	(AC) ₂₅	7*	0.79	NR	
		11	<i>LS</i> -75	(TC) ₁₁ (AC) ₁₁ AG(AC) ₁₀	2*	0.09	NR	
	Mexico	11	<i>LS</i> -82	(AC) ₂₅	4*	0.41	NR	
		9	<i>LS</i> -11	(AC) ₃₃	3*	0.68	NR	
		9	<i>LS</i> -15	(AC) ₂₀	5*	0.71	NR	
		9	<i>LS</i> -48	(AC) ₃₈	11*	0.93	NR	
		9	<i>LS</i> -52	(AC) ₂₀	13*	0.96	NR	
		9	<i>LS</i> -54	(CT) ₁₀ (CA) ₈	3*	0.54	NR	
		9	<i>LS</i> -22	(AC) ₂₃	7*	0.82	NR	
		9	<i>LS</i> -30	(AC) ₂₅	8*	0.85	NR	
		9	<i>LS</i> -75	(TC) ₁₁ (AC) ₁₁ AG(AC) ₁₀	3*	0.57	NR	
		9	<i>LS</i> -82	(AC) ₂₅	11*	0.86	NR	
<i>N. acutidens</i>	western Australia	25	<i>LS</i> -11	(AC) ₃₃	3*	0.77	NR	Schultz <i>et. al.</i> 2008
		25	<i>LS</i> -15	(AC) ₂₀	3*	0.78	NR	
		25	<i>LS</i> -52	(AC) ₂₀	3*	0.81	NR	
		25	<i>LS</i> -54	(CT) ₁₀ (CA) ₈	1*	0	NR	
	eastern Australia	25	<i>LS</i> -75	(TC) ₁₁ (AC) ₁₁ AG(AC) ₁₀	3*	0.86	NR	
		25	<i>LS</i> -82	(AC) ₂₅	2*	0.41	NR	
		10	<i>LS</i> -11	(AC) ₃₃	3*	0.71	NR	
		10	<i>LS</i> -15	(AC) ₂₀	2*	0.58	NR	
	New Caledonia	10	<i>LS</i> -52	(AC) ₂₀	4*	0.92	NR	
		10	<i>LS</i> -54	(CT) ₁₀ (CA) ₈	2*	0.56	NR	
		10	<i>LS</i> -75	(TC) ₁₁ (AC) ₁₁ AG(AC) ₁₀	3*	0.82	NR	
		10	<i>LS</i> -82	(AC) ₂₅	2*	0.53	NR	
		2	<i>LS</i> -11	(AC) ₃₃	1*	0	NR	
		2	<i>LS</i> -15	(AC) ₂₀	2*	0.67	NR	
		2	<i>LS</i> -52	(AC) ₂₀	2*	0.67	NR	
		2	<i>LS</i> -54	(CT) ₁₀ (CA) ₈	1*	0	NR	
		2	<i>LS</i> -75	(TC) ₁₁ (AC) ₁₁ AG(AC) ₁₀	2*	0.50	NR	
		2	<i>LS</i> -82	(AC) ₂₅	2*	0.50	NR	

Species	Sampling locations	N	Locus	Motif	A	H _a	H _e	Reference
<i>N. acutidens</i>	French Polynesia	16	LS-11	(AC) ₃₃	2*	0.63	NR	Schultz <i>et. al.</i> 2008
		16	LS-15	(AC) ₂₀	2*	0.50	NR	
		16	LS-52	(AC) ₂₀	1*	0	NR	
		16	LS-54	(CT) ₁₀ (CA) ₈	2*	0.54	NR	
		16	LS-75	(TC) ₁₁ (AC) ₁₁ AG(AC) ₁₀	3*	0.70	NR	
		16	LS-82	(AC) ₂₅	2*	0.29	NR	
	Taiwan	15	LS-11	(AC) ₃₃	3*	0.76	NR	
		15	LS-15	(AC) ₂₀	3*	0.84	NR	
		15	LS-52	(AC) ₂₀	2*	0.38	NR	
		15	LS-54	(CT) ₁₀ (CA) ₈	2*	0.38	NR	
		15	LS-75	(TC) ₁₁ (AC) ₁₁ AG(AC) ₁₀	3*	0.89	NR	
		15	LS-82	(AC) ₂₅	2*	0.53	NR	
<i>Sphyrna lewini</i>	Indonesia	74	Ct-6	(CA) ₁₄	1	0	0	Ovenden <i>et. al.</i> 2009
		74	Ct-7	(GT) ₁₀ (GC)(GT)(GC)(GT)	6	0.66	0.71	
		82	Cli-100	(TG) ₄ (GT) ₁₀	14	0.82	0.87	
	east Australia	44	Ct-6	(CA) ₁₄	1	0	0	
		44	Ct-7	(GT) ₁₀ (GC)(GT)(GC)(GT) ₂	6	0.80	0.71	
	west Australia	44	Cli-100	(TG) ₄ (GT) ₁₀	10	0.80	0.85	
		42	Ct-6	(CA) ₁₄	2	0	0.17	
		31	Ct-7	(GT) ₁₀ (GC)(GT)(GC)(GT) ₂	7	0.61	0.72	
		40	Cli-100	(TG) ₄ (GT) ₁₀	11	0.75	0.87	
		41	Gc-4	(CA) ₁ (CAGA) ₅ (GT) ₉	3	0.34	0.36	
<i>Ginglymostoma cirratum</i>	Brazilian islands	39	Gc-7	(GT) ₇ TT(GT) ₅	6	0.69	0.66	Kark <i>et. al.</i> 2012
		41	Gc-10	(GT) ₂₆	20	0.83	0.92	
		40	Gc-11	(GT) ₁₂	3	0.30	0.27	
		42	Gc-18	(GT) ₉	3	0.31	0.36	
		38	Gc-25	(GAGACA) ₆ (GC) ₂ (GA) ₁₃	7	0.71	0.77	
		42	Gc-35	(GT) ₁₀	4	0.64	0.56	
		42	Gc-37	(GA) ₁₅	6	0.63	0.60	
	Rio Grande do Norte	15	Gc-4	(CA) ₁ (CAGA) ₅ (GT) ₉	3	0.47	0.48	
		14	Gc-7	(GT) ₇ TT(GT) ₅	5	0.50	0.78	
		14	Gc-10	(GT) ₂₆	9	0.71	0.91	
		15	Gc-11	(GT) ₁₂	3	0.20	0.19	
		14	Gc-18	(GT) ₉	3	0.64	0.54	
		14	Gc-25	(GAGACA) ₆ (GC) ₂ (GA) ₁₃	4	0.86	0.70	
		14	Gc-35	(GT) ₁₀	3	0.50	0.47	
		15	Gc-37	(GA) ₁₅	5	0.80	0.67	
	Bimini, Bahamas	23	Gc-4	(CA) ₁ (CAGA) ₅ (GT) ₉	3	0.35	0.31	
		23	Gc-7	(GT) ₇ TT(GT) ₅	4	0.78	0.74	
		23	Gc-10	(GT) ₂₆	16	0.91	0.94	
		23	Gc-11	(GT) ₁₂	3	0.52	0.60	
		23	Gc-18	(GT) ₉	3	0.48	0.44	
		23	Gc-25	(GAGACA) ₆ (GC) ₂ (GA) ₁₃	7	0.91	0.81	
		23	Gc-35	(GT) ₁₀	4	0.52	0.53	
		23	Gc-37	(GA) ₁₅	6	0.57	0.50	
	Big Pine Island	37	Gc-4	(CA) ₁ (CAGA) ₅ (GT) ₉	3	0.46	0.38	
		36	Gc-7	(GT) ₇ TT(GT) ₅	6	0.78	0.75	
		36	Gc-10	(GT) ₂₆	18	0.92	0.92	
		37	Gc-11	(GT) ₁₂	3	0.54	0.48	
		37	Gc-18	(GT) ₉	3	0.46	0.41	
		36	Gc-25	(GAGACA) ₆ (GC) ₂ (GA) ₁₃	9	0.64	0.78	
		37	Gc-35	(GT) ₁₀	4	0.57	0.45	
		35	Gc-37	(GA) ₁₅	6	0.43	0.44	
	Caribbean coast of Belize	34	Gc-4	(CA) ₁ (CAGA) ₅ (GT) ₉	3	0.41	0.37	
		35	Gc-7	(GT) ₇ TT(GT) ₅	6	0.71	0.77	
		35	Gc-10	(GT) ₂₆	17	0.91	0.94	
		35	Gc-11	(GT) ₁₂	3	0.37	0.35	
		35	Gc-18	(GT) ₉	3	0.26	0.42	
		35	Gc-25	(GAGACA) ₆ (GC) ₂ (GA) ₁₃	6	0.60	0.74	
		35	Gc-35	(GT) ₁₀	4	0.49	0.49	
		35	Gc-37	(GA) ₁₅	6	0.40	0.47	
<i>Prionace glauca</i>	Indonesia	26	Cs-2	(GT) ₂ (GC)(GT)(GC)(GT) ₁₁	15	0.81	0.83	Ovenden <i>et. al.</i> 2009
		33	Ct-4	(CA) ₇	5	0.55	0.63	
		30	Ct-6	(CA) ₁₄	6	0.40	0.57	
		31	Cli-107	(GT) ₁₄	2	0.26	0.31	
		28	Cli-100	(TG) ₄ (GT) ₁₀	5	0.36	0.37	
	mid north Pacific	20	Cs-2	(GT) ₂ (GC)(GT)(GC)(GT) ₁₁	17	0.95	0.88	
		20	Ct-4	(CA) ₇	5	0.75	0.67	
		20	Ct-6	(CA) ₁₄	6	0.55	0.62	
		20	Cli-107	(GT) ₁₄	3	0.15	0.19	
		3	Cli-100	(TG) ₄ (GT) ₁₀	2	0	0.44	
	west Australia	21	Cs-2	(GT) ₂ (GC)(GT)(GC)(GT) ₁₁	12	0.62	0.83	
		47	Ct-4	(CA) ₇	5	0.70	0.69	
		43	Ct-6	(CA) ₁₄	6	0.35	0.61	
		42	Cli-107	(GT) ₁₄	4	0.31	0.36	
		37	Cli-100	(TG) ₄ (GT) ₁₀	5	0.30	0.37	
	east Australia	16	Cs-2	(GT) ₂ (GC)(GT)(GC)(GT) ₁₁	16	0.88	0.87	
		18	Ct-4	(CA) ₇	5	0.56	0.64	
		17	Ct-6	(CA) ₁₄	5	0.41	0.66	
<i>Carcharodon carcharias</i>	New Zealand/Australia	14	Cli-107	(GT) ₁₄	3	0.29	0.36	Pardini <i>et. al.</i> 2000
		16	Cli-100	(TG) ₄ (GT) ₁₀	5	0.38	0.42	
		52	Ccar-1	(AC) ₂₂	6	0.62	NR	
		52	Ccar-6	(AC) ₁₇	4	0.70	NR	
		52	Ccar-9	(TG) ₂₃	15	0.35	NR	
	South Africa	52	Ccar-13	(TG) ₄ TT(TG) ₉ TT(TG) ₃ TTTT(TG) ₂₃	9	0.31	NR	
		52	Ccar-19	(TG) ₁₆ CG(TG) ₃	4	0.30	NR	
		43	Ccar-1	(AC) ₂₂	5	0.88	NR	
		43	Ccar-6	(AC) ₁₇	5	0.56	NR	
		43	Ccar-9	(TG) ₂₃	12	0.41	NR	
		43	Ccar-13	(TG) ₄ TT(TG) ₉ TT(TG) ₃ TTTT(TG) ₂₃	7	0.29	NR	
		43	Ccar-19	(TG) ₁₆ CG(TG) ₃	3	0.38	NR	

Species	Sampling locations	N	Locus	Motif	A	H _a	H _e	Reference	
<i>Carcharias taurus</i>	Brazil	5	<i>lox-12</i>	(GA) ₄ (GT) ₈	4	1	0.75	Ahonen <i>et. al.</i> 2009	
		5	<i>Ct-4</i>	(CA) ₇	3	0.40	0.38		
		5	<i>Ct-6</i>	(CA) ₁₄	5	1	0.78		
		5	<i>Cta-45-69</i>	(CTTT) ₁₀	4	1	0.68		
		5	<i>Cta-45-93</i>	(TAGA) ₁₆ (CAGA) ₇	5	1	0.85		
	5	<i>Cta-56-72</i>	(AG) ₁₂	2	0.60	0.45			
	eastern Australia	63	<i>lox-12</i>	(GA) ₄ (GT) ₈	5	0.70	0.65		
		63	<i>Ct-4</i>	(CA) ₇	5	0.68	0.67		
		63	<i>Ct-6</i>	(CA) ₁₄	5	0.51	0.53		
		63	<i>Cta-45-69</i>	(CTTT) ₁₀	12	0.62	0.58		
		63	<i>Cta-45-93</i>	(TAGA) ₁₆ (CAGA) ₇	8	0.81	0.77		
	63	<i>Cta-56-72</i>	(AG) ₁₂	5	0.49	0.47			
	northwest Atlantic	12	<i>lox-12</i>	(GA) ₄ (GT) ₈	1	0	0		
		12	<i>Ct-4</i>	(CA) ₇	3	0.67	0.56		
		12	<i>Ct-6</i>	(CA) ₁₄	2	0.08	0.08		
		12	<i>Cta-45-69</i>	(CTTT) ₁₀	11	0.75	0.92		
		12	<i>Cta-45-93</i>	(TAGA) ₁₆ (CAGA) ₇	5	0.55	0.74		
	12	<i>Cta-56-72</i>	(AG) ₁₂	1	0	0			
	South Africa	75	<i>lox-12</i>	(GA) ₄ (GT) ₈	8	0.70	0.72		
		75	<i>Ct-4</i>	(CA) ₇	6	0.68	0.65		
		75	<i>Ct-6</i>	(CA) ₁₄	8	0.64	0.69		
		75	<i>Cta-45-69</i>	(CTTT) ₁₀	10	0.75	0.78		
		75	<i>Cta-45-93</i>	(TAGA) ₁₆ (CAGA) ₇	11	0.85	0.80		
	75	<i>Cta-56-72</i>	(AG) ₁₂	6	0.55	0.48			
	western Australia	24	<i>lox-12</i>	(GA) ₄ (GT) ₈	5	0.63	0.80		
		24	<i>Ct-4</i>	(CA) ₇	6	0.88	0.75		
		24	<i>Ct-6</i>	(CA) ₁₄	4	0.75	0.69		
		24	<i>Cta-45-69</i>	(CTTT) ₁₀	10	0.72	0.83		
		24	<i>Cta-45-93</i>	(TAGA) ₁₆ (CAGA) ₇	8	0.88	0.86		
	24	<i>Cta-56-72</i>	(AG) ₁₂	4	0.64	0.50			
	Japan	7	<i>lox-12</i>	(GA) ₄ (GT) ₈	1	0	0		
		7	<i>Ct-4</i>	(CA) ₇	1	0	0		
		7	<i>Ct-6</i>	(CA) ₁₄	1	0	0		
		7	<i>Cta-45-69</i>	(CTTT) ₁₀	2	0.14	0.14		
		7	<i>Cta-45-93</i>	(TAGA) ₁₆ (CAGA) ₇	2	0.40	0.35		
	7	<i>Cta-56-72</i>	(AG) ₁₂	2	0.71	0.48			
	<i>Squalus acanthias</i>	Japan	49	8 loci pooled, <i>Df-u285</i> (CT) ₁₁ / <i>Df-t289</i> (TCC) ₇ / <i>Df-j451</i> (AC) ₁₀	6.25	0.56	0.56		Verissimo <i>et. al.</i> 2010
		Washington/Oregon	50	<i>Df-j445</i> (AC) ₁₀ / <i>Saca-gall</i> (TG) ₉ C(TG)/ <i>Saca-6396</i> (CA) ₁₉	6.88	0.55	0.55		
		California	49	<i>Saca-3853</i> (TC) ₉ GC(TC) ₂ / <i>Saca-3853</i> (TG) ₁₄	6.50	0.54	0.55		
		New Zealand	46	8 loci pooled, <i>Df-u285</i> (CT) ₁₁ / <i>Df-t289</i> (TCC) ₇ / <i>Df-j451</i> (AC) ₁₀	7	0.69	0.65		
Chile		18	<i>Df-j445</i> (AC) ₁₀ / <i>Saca-gall</i> (TG) ₉ C(TG)/ <i>Saca-6396</i> (CA) ₁₉	6.25	0.64	0.64			
Virginia		55	<i>Saca-3853</i> (TC) ₉ GC(TC) ₂ / <i>Saca-3853</i> (TG) ₁₄	8.25	0.65	0.65			
Massachusetts		48	8 loci pooled, <i>Df-u285</i> (CT) ₁₁ / <i>Df-t289</i> (TCC) ₇ / <i>Df-j451</i> (AC) ₁₀	7	0.62	0.62			
Ireland		56	<i>Df-j445</i> (AC) ₁₀ / <i>Saca-gall</i> (TG) ₉ C(TG)/ <i>Saca-6396</i> (CA) ₁₉	8	0.62	0.62			
Irish Sea		48	<i>Saca-3853</i> (TC) ₉ GC(TC) ₂ / <i>Saca-3853</i> (TG) ₁₄	7.38	0.63	0.64			
Argentina		48		7.63	0.64	0.64			
<i>Rhincodon typus</i>	Global distribution (Atlantic, Pacific and Indian ocean)	68	<i>Rtyp-1</i>	(TG) ₂ TC(TC) ₆ TC(TG) ₂ (TG) ₉ (TG) ₉ TC(TG) ₇	5	0.69	0.68	Schmidt <i>et. al.</i> 2009	
	68	<i>Rtyp-2</i>	(TG) ₂ TC(TG) ₁₂ TC(TG) ₂ TC(TG) ₈ CG(TG) ₂	7	0.73	0.74			
	68	<i>Rtyp-3</i>	(TG) ₁₄ ..(TG) ₂ ..(TG) ₄	4	0.80	0.55			
	68	<i>Rtyp-4</i>	(CT) ₃ ..TTTCTGT(CT) ₃ GTCT	4	0.47	0.40			
	68	<i>Rtyp-5</i>	(CA) ₂₀	7	0.85	0.87			
	68	<i>Rtyp-6</i>	(GA) ₄₁	34	0.57	1			
	68	<i>Rtyp-7</i>	(CA) ₁ TG(CA) ₃ TA(CA) ₄ (CT) ₁ CC(CA) ₁₉	8	0.74	0.83			
	68	<i>Rtyp-8</i>	(CA) ₁ TGT(GC) ₄ (CA) ₉ TACA	3	0.45	0.46			
<i>Galeorhinus galeus</i>	west of North Island, New Zealand	40	<i>Ggal-14</i>	(AA) ₁ AC(AG) ₇	3	0.43	0.46	This study	
		40	<i>Ggal-15</i>	(CAA) ₈	7	0.32	0.61		
		40	<i>Gg-20</i>	(TC) ₈	2	0.13	0.29		
		40	<i>Gg-15</i>	(GA) ₁₂	11	0.86	0.86		
		40	<i>Gg-22</i>	(GT) ₈	6	0.61	0.66		
		40	<i>Gg-23</i>	(AC) ₁₂	7	0.65	0.75		
		40	<i>Gg-12</i>	(TA) ₉	8	0.65	0.80		
	40	<i>Gg-7</i>	(AG) ₁₀	5	0.68	0.66			
	40	<i>Gg-11</i>	(TCCC) ₁₀	6	0.46	0.74			
	Chatham Rise, New Zealand	47	<i>Ggal-14</i>	(AA) ₁ AC(AG) ₇	3	0.36	0.49		
		47	<i>Ggal-15</i>	(CAA) ₈	6	0.55	0.60		
		47	<i>Gg-20</i>	(TC) ₈	2	0.11	0.15		
		47	<i>Gg-15</i>	(GA) ₁₂	10	0.76	0.86		
		47	<i>Gg-22</i>	(GT) ₈	7	0.58	0.69		
		47	<i>Gg-23</i>	(AC) ₁₂	8	0.60	0.80		
		47	<i>Gg-12</i>	(TA) ₉	9	0.80	0.79		
		47	<i>Gg-7</i>	(AG) ₁₀	6	0.65	0.64		
		47	<i>Gg-11</i>	(TCCC) ₁₀	6	0.73	0.58		
	Solander Island, New Zealand	30	<i>Ggal-14</i>	(AA) ₁ AC(AG) ₇	3	0.30	0.35		
		30	<i>Ggal-15</i>	(CAA) ₈	5	0.57	0.57		
		30	<i>Gg-20</i>	(TC) ₈	2	0.23	0.21		
		30	<i>Gg-15</i>	(GA) ₁₂	10	0.87	0.86		
		30	<i>Gg-22</i>	(GT) ₈	5	0.30	0.60		
		30	<i>Gg-23</i>	(AC) ₁₂	7	0.82	0.81		
		30	<i>Gg-12</i>	(TA) ₉	8	0.56	0.72		
		30	<i>Gg-7</i>	(AG) ₁₀	5	0.50	0.55		
		30	<i>Gg-11</i>	(TCCC) ₁₀	5	0.52	0.50		
		43	<i>Ggal-14</i>	(AA) ₁ AC(AG) ₇	3	0.33	0.48		
	Shoulder, Australia	43	<i>Ggal-15</i>	(CAA) ₈	4	0.49	0.55		
		43	<i>Gg-20</i>	(TC) ₈	3	0.29	0.32		
		43	<i>Gg-15</i>	(GA) ₁₂	9	0.83	0.87		
		43	<i>Gg-22</i>	(GT) ₈	4	0.44	0.65		
		43	<i>Gg-23</i>	(AC) ₁₂	10	0.73	0.75		
		43	<i>Gg-12</i>	(TA) ₉	11	0.95	0.84		
43		<i>Gg-7</i>	(AG) ₁₀	5	0.63	0.65			
43		<i>Gg-11</i>	(TCCC) ₁₀	5	0.70	0.64			

Species	Sampling locations	N	Locus	Motif	A	H _a	H _e	Reference
<i>Galeorhinus galeus</i>	Tasmania, Australia	39	<i>Ggal</i> -14	(AA) ₂ AC(AG) ₇	4	0.31	0.59	This study
		39	<i>Ggal</i> -15	(CAA) ₈	5	0.59	0.56	
		39	<i>Gg</i> -20	(TC) ₈	2	0.23	0.35	
		39	<i>Gg</i> -15	(GA) ₁₂	10	0.78	0.87	
		39	<i>Gg</i> -22	(GT) ₈	5	0.77	0.70	
		39	<i>Gg</i> -23	(AC) ₁₂	9	0.82	0.80	
		39	<i>Gg</i> -12	(TA) ₉	12	0.77	0.83	
		39	<i>Gg</i> -7	(AG) ₁₀	5	0.70	0.66	
		39	<i>Gg</i> -11	(TCCC) ₁₀	4	0.71	0.64	
		24	<i>Ggal</i> -14	(AA) ₂ AC(AG) ₇	2	0.13	0.38	
		24	<i>Ggal</i> -15	(CAA) ₈	3	0.50	0.49	
		24	<i>Gg</i> -20	(TC) ₈	2	0.21	0.19	
<i>Batoidea</i> <i>Raja clavata</i>	Frederich Bay, Australia	24	<i>Gg</i> -15	(GA) ₁₂	11	0.71	0.89	
		24	<i>Gg</i> -22	(GT) ₈	6	0.67	0.70	
		24	<i>Gg</i> -23	(AC) ₁₂	8	0.78	0.80	
		24	<i>Gg</i> -12	(TA) ₉	10	0.67	0.84	
		24	<i>Gg</i> -7	(AG) ₁₀	5	0.46	0.57	
		24	<i>Gg</i> -11	(TCCC) ₁₀	4	0.36	0.52	
	Santiago, Chile	16	<i>Ggal</i> -14	(AA) ₂ AC(AG) ₇	2	0.63	0.51	
		16	<i>Ggal</i> -15	(CAA) ₈	6	0.69	0.76	
		16	<i>Gg</i> -20	(TC) ₈	4	0.36	0.52	
		16	<i>Gg</i> -15	(GA) ₁₂	5	0.93	0.78	
		16	<i>Gg</i> -22	(GT) ₈	5	0.80	0.67	
		16	<i>Gg</i> -23	(AC) ₁₂	6	0.50	0.70	
		16	<i>Gg</i> -12	(TA) ₉	2	0.20	0.29	
		16	<i>Gg</i> -7	(AG) ₁₀	3	0.47	0.40	
		16	<i>Gg</i> -11	(TCCC) ₁₀	4	0.13	0.34	
	Blackwater Bay	20	<i>Rc</i> -B3	(GA) ₁₇ T(GA)	6	0.44	0.66	Chevolot et al. 2008
		20	<i>Rc</i> -B4	(GA) ₁₅ (GGA) ₅ (GA) ₂₁	17	0.91	0.94	
		20	<i>Rc</i> -B6	(GA) ₁₇ (GGGA) ₂ (GG) ₂ (GA) ₉ GGGAGTGG(AG) ₆	18	0.96	0.94	
		20	<i>Rc</i> -G2	(CT) ₁₆ GTG(CT) ₃	5	0.57	0.59	
		20	<i>Rc</i> -E9	(GA) ₁₄ (GGGA) ₂ (GAGGGA) ₂ GGG(AG) ₁₃	4	0.36	0.39	
	Conwy Bay	8	<i>Rc</i> -B3	(GA) ₁₇ T(GA)	4	0.80	0.71	
		8	<i>Rc</i> -B4	(GA) ₁₅ (GGA) ₅ (GA) ₂₁	11	0.90	0.94	
		8	<i>Rc</i> -B6	(GA) ₁₇ (GGGA) ₂ (GG) ₂ (GA) ₉ GGGAGTGG(AG) ₆	11	1.00	0.94	
		8	<i>Rc</i> -G2	(CT) ₁₆ GTG(CT) ₃	5	0.40	0.51	
	Kish Bank 1	8	<i>Rc</i> -E9	(GA) ₁₄ (GGGA) ₂ (GAGGGA) ₂ GGG(AG) ₁₃	3	0.44	0.45	
		17	<i>Rc</i> -B3	(GA) ₁₇ T(GA)	3	0.63	0.64	
		17	<i>Rc</i> -B4	(GA) ₁₅ (GGA) ₅ (GA) ₂₁	15	1.00	0.93	
		17	<i>Rc</i> -B6	(GA) ₁₇ (GGGA) ₂ (GG) ₂ (GA) ₉ GGGAGTGG(AG) ₆	18	0.79	0.94	
	Kish Bank 2	17	<i>Rc</i> -G2	(CT) ₁₆ GTG(CT) ₃	6	0.55	0.61	
		17	<i>Rc</i> -E9	(GA) ₁₄ (GGGA) ₂ (GAGGGA) ₂ GGG(AG) ₁₃	6	0.26	0.29	
		22	<i>Rc</i> -B3	(GA) ₁₇ T(GA)	4	0.79	0.74	
		22	<i>Rc</i> -B4	(GA) ₁₅ (GGA) ₅ (GA) ₂₁	19	1.00	0.94	
	Tuskaw Bay	22	<i>Rc</i> -B6	(GA) ₁₇ (GGGA) ₂ (GG) ₂ (GA) ₉ GGGAGTGG(AG) ₆	16	0.96	0.90	
		22	<i>Rc</i> -G2	(CT) ₁₆ GTG(CT) ₃	5	0.71	0.67	
		22	<i>Rc</i> -E9	(GA) ₁₄ (GGGA) ₂ (GAGGGA) ₂ GGG(AG) ₁₃	4	0.52	0.58	
		24	<i>Rc</i> -B3	(GA) ₁₇ T(GA)	5	0.60	0.66	
	ICES area VIIA	24	<i>Rc</i> -B4	(GA) ₁₅ (GGA) ₅ (GA) ₂₁	20	0.89	0.94	
		24	<i>Rc</i> -B6	(GA) ₁₇ (GGGA) ₂ (GG) ₂ (GA) ₉ GGGAGTGG(AG) ₆	24	0.92	0.94	
		24	<i>Rc</i> -G2	(CT) ₁₆ GTG(CT) ₃	4	0.56	0.58	
		24	<i>Rc</i> -E9	(GA) ₁₄ (GGGA) ₂ (GAGGGA) ₂ GGG(AG) ₁₃	7	0.52	0.50	
	Carmarthen Bay 03	43	<i>Rc</i> -B3	(GA) ₁₇ T(GA)	7	0.53	0.76	
		43	<i>Rc</i> -B4	(GA) ₁₅ (GGA) ₅ (GA) ₂₁	22	0.93	0.95	
		43	<i>Rc</i> -B6	(GA) ₁₇ (GGGA) ₂ (GG) ₂ (GA) ₉ GGGAGTGG(AG) ₆	25	1.00	0.94	
		43	<i>Rc</i> -G2	(CT) ₁₆ GTG(CT) ₃	7	0.56	0.58	
	Carmarthen Bay 04	43	<i>Rc</i> -E9	(GA) ₁₄ (GGGA) ₂ (GAGGGA) ₂ GGG(AG) ₁₃	5	0.42	0.45	
		14	<i>Rc</i> -B3	(GA) ₁₇ T(GA)	5	0.43	0.62	
		14	<i>Rc</i> -B4	(GA) ₁₅ (GGA) ₅ (GA) ₂₁	14	0.88	0.94	
		14	<i>Rc</i> -B6	(GA) ₁₇ (GGGA) ₂ (GG) ₂ (GA) ₉ GGGAGTGG(AG) ₆	21	0.94	0.94	
	Caernarfon Bay 03	14	<i>Rc</i> -G2	(CT) ₁₆ GTG(CT) ₃	4	0.63	0.64	
		14	<i>Rc</i> -E9	(GA) ₁₄ (GGGA) ₂ (GAGGGA) ₂ GGG(AG) ₁₃	3	0.44	0.50	
		22	<i>Rc</i> -B3	(GA) ₁₇ T(GA)	5	0.44	0.58	
		22	<i>Rc</i> -B4	(GA) ₁₅ (GGA) ₅ (GA) ₂₁	22	0.93	0.95	
	Caernarfon Bay 04	22	<i>Rc</i> -B6	(GA) ₁₇ (GGGA) ₂ (GG) ₂ (GA) ₉ GGGAGTGG(AG) ₆	21	0.94	0.90	
		22	<i>Rc</i> -G2	(CT) ₁₆ GTG(CT) ₃	5	0.54	0.64	
		22	<i>Rc</i> -E9	(GA) ₁₄ (GGGA) ₂ (GAGGGA) ₂ GGG(AG) ₁₃	6	0.61	0.62	
		17	<i>Rc</i> -B3	(GA) ₁₇ T(GA)	4	0.56	0.64	
	Tremadog Bay 03	17	<i>Rc</i> -B4	(GA) ₁₅ (GGA) ₅ (GA) ₂₁	20	0.94	0.96	
		17	<i>Rc</i> -B6	(GA) ₁₇ (GGGA) ₂ (GG) ₂ (GA) ₉ GGGAGTGG(AG) ₆	21	1.00	0.93	
		17	<i>Rc</i> -G2	(CT) ₁₆ GTG(CT) ₃	5	0.65	0.67	
		17	<i>Rc</i> -E9	(GA) ₁₄ (GGGA) ₂ (GAGGGA) ₂ GGG(AG) ₁₃	3	0.39	0.42	
		12	<i>Rc</i> -B3	(GA) ₁₇ T(GA)	3	0.60	0.63	
		12	<i>Rc</i> -B4	(GA) ₁₅ (GGA) ₅ (GA) ₂₁	15	0.94	0.93	
		12	<i>Rc</i> -B6	(GA) ₁₇ (GGGA) ₂ (GG) ₂ (GA) ₉ GGGAGTGG(AG) ₆	16	0.94	0.90	
		12	<i>Rc</i> -G2	(CT) ₁₆ GTG(CT) ₃	3	0.46	0.39	
		12	<i>Rc</i> -E9	(GA) ₁₄ (GGGA) ₂ (GAGGGA) ₂ GGG(AG) ₁₃	5	0.63	0.59	
		15	<i>Rc</i> -B3	(GA) ₁₇ T(GA)	5	0.53	0.60	
		15	<i>Rc</i> -B4	(GA) ₁₅ (GGA) ₅ (GA) ₂₁	17	0.83	0.95	
		15	<i>Rc</i> -B6	(GA) ₁₇ (GGGA) ₂ (GG) ₂ (GA) ₉ GGGAGTGG(AG) ₆	18	1.00	0.95	
		15	<i>Rc</i> -G2	(CT) ₁₆ GTG(CT) ₃	6	0.63	0.54	
		15	<i>Rc</i> -E9	(GA) ₁₄ (GGGA) ₂ (GAGGGA) ₂ GGG(AG) ₁₃	6	0.83	0.64

Supplementary Figure 3.1: Distribution of di-nucleotide, tri-, tetra-, and hexa-, repeats units identified in microsatellite population structure studies reported to the date for 15 shark species. The number in brackets is the number of microsatellite repeat for each motif. The *x*-axis is the number of repeat units for each repeat motif and the *y*-axis is the number of microsatellites identified.

Chapter 4

Population structure of the school shark (*Galeorhinus galeus*) in New Zealand, Australian and Chilean waters based on microsatellite DNA markers

4.1 Abstract

DNA microsatellite markers were used to determine the genetic population structure of the school shark, *Galeorhinus galeus* in Australia, New Zealand and Chile. In total of 239 individuals were genotyped using nine microsatellite loci, which were collected from three sites in New Zealand, three in Australia, and one site in Chile. The number of alleles ranged from 2 for the *Gg20* locus to 12 for the *Gg12* locus, and the expected heterozygosities ranged from 0.145 for *Gg20* to 0.886 for *Gg15*. The analyses of the microsatellites revealed a significant level of differentiation between New Zealand/Australia and Chile. In contrast, there was no evidence of differentiation between sites in New Zealand and sites in Australia. These results, in combination with mtDNA data (see Chapter 2), appear to be consistent with a single panmictic stock in New Zealand and Australian waters. The similarity of the results obtained from the maternally inherited mtDNA and biparental inherited microsatellite loci did not support the suggestion of sex-biased dispersal of *G. galeus* in the New Zealand/Australia region and it was concluded that females and males had similar patterns of dispersal.

4.2 Introduction

Understanding of patterns of connectivity is important for effective management of a fishery. Despite the commercial importance of several elasmobranch species and concerns about the sustainability of stocks, there have been few studies of stock structure (see details in Heist et al. 2005 and Dudgeon et al. 2012). Most stock connectivity studies have used direct methods (e.g. mark-recapture and other tagging data) to quantify survival and migration rates and determine the patterns of dispersal (Hurst et al. 1999, Kohler & Turner 2001). However, many of the direct methods can be logistically complicated, because they often require labour-intensive surveys that yield relatively limited data sets, and provide little information about reproductive success (Waples 1998). A combination of direct and indirect methods (e.g. genetic markers) may provide a better understanding of the patterns of population connectivity and structure. Over the last two decades, significant progress has been made to genetic methods used to identify fisheries stocks, estimate effective population size, and identify species for catch and trading surveillance (Dichmont et al. 2011, Dudgeon et al. 2012).

The level of intraspecific genetic connectivity is thought to be positively correlated with dispersal ability. Dispersal patterns tend to be associated with habitat preference (e.g. coastal, pelagic or benthonic), size and reproductive behaviour. In general terms, species of large sharks are considered highly mobile and disperse widely compared to smaller species, which do not tend to move large distances (Musick et al. 2004, Dudgeon et al. 2012). For example, high levels of gene flow have been reported for *Isurus oxyrinchus* (Schrey & Heist 2003), *Cetorhinus maximus* (Hoezel et al. 2000), and *Rhincodon typus* (Castro et al. 2007), which are large oceanic species with extensive migratory patterns. In contrast, species less vagile, demersal or benthonic have higher levels of intraspecific genetic differentiation e.g. *Raja clavata* (Chevolot et al. 2006), *Sphyrna lewini* (Duncan et al. 2006), *Galeorhinus galeus* (Chabot & Allen 2009), and *Carcharhinus brachyurus* (Benavidez et al. 2011).

A variety of molecular marker types have been used to determine the genetic stock structure of elasmobranchs (Heist 1999, Dudgeon et al. 2012). Early studies were often limited by lack of genetic variability detected using allozyme loci (Smith

1986, MacDonald 1988, Heist et al. 1995, Ward & Gardner 1997, 1998) and mitochondrial DNA (mtDNA) restriction fragment-length polymorphism (RFLP) (Heist et al. 1996a, b, Ward & Gardner 1997, 1998). In recent years mtDNA sequencing and microsatellite DNA markers have been widely used. Microsatellite DNA have been most popular because they are often highly variable, which means they have the statistical power to resolve patterns of gene flow. Microsatellite loci are typically selectively neutral genetic markers, unless they are linked to a gene under selection. The processes of migration, genetic drift and mutation determine allele frequencies in a population (Heist 1999, Dudgeon et al. 2012).

High levels of genetic differentiation in large marine fish populations have been associated with sedentary behaviour, disjunct distributions and/or sex-biased dispersal (Palumbi 1994). The most common pattern of sex-biased dispersal is when one sex is philopatric (i.e. tends to stay or return to its natal site or group to breed) while the other sex is more prone to disperse away from its natal area (Hueter et al. 2005). Different levels of differentiation between the sexes can be detected by comparing the patterns of variation detected using biparentally inherited nuclear markers and the maternally inherited mtDNA. High levels of differentiation at mtDNA and low levels of differentiation at nuclear DNA loci, is often interpreted as resulting from high site fidelity by reproducing females and widely dispersed reproducing males (Hueter et al. 2005). Sex-biased dispersal has been reported in several shark species based on the results of tagging, tracking, and genetic markers (Hueter et al. 2005, Heist 2009). Contrasting patterns of mtDNA and microsatellite loci variation in shark species such as *Carcharodon carcharias* (Pardini et al. 2001), *Isurus oxyrinchus* (Schrey & Heist 2003), *Negaprion brevirostris* (Schultz et al. 2008), *Carcharhinus plumbeus* (Portnoy et al. 2010), and *Carcharhinus leucas* (Karl et al. 2011) have been interpreted as sex-biased dispersal. These studies demonstrated that females typically remained closer to their home territories, when contrasted to males in the population, which roamed over larger distances. If female *G. galeus* are philopatric to their nursery area and females have formed separate sub-populations, then heavy fishing or habitat destruction could threaten genetically isolated nursery areas and there is a risk of sub-population collapse. Testing for different dispersal behaviour between the sexes is important for the management and conservation of shark fisheries.

Galeorhinus galeus is a highly mobile species which is widely distributed throughout the temperate waters of New Zealand and Australia. It is characterized by low reproductive potential (i.e. slow-growth, late sexual maturity) that make it highly susceptible to overfishing and habitat degradation. For example, the *G. galeus* stock in California was collapsed by fisheries (Ripley 1946, Stevens et al. 2001). In Australia during the 1970s, 84% of the mature biomass was fished out, resulting in an 80% decline of pups from Port Phillip Bay, Victorian waters (Walker 1998, 1999, Stevens et al. 2001). Habitat degradation in the area such as Geelong and demersal trawling has also put pressure on important nursery areas for *G. galeus*. Similar patterns were described for Port Sorell and St Helens in Tasmanian waters (Stevens 2005). The limit reproductive potential and the trend of declining population size, result in this species being listed as *Vulnerable* by the IUCN (Walker et al. 2006).

The high level of gene flow based on the control region (CR) of mtDNA between New Zealand and Australia (see Chapter 2) and the previously published tagging results of *G. galeus* (Hurst et al. 1999, Walker 2008, Francis 2010), are consistent with the notion of a single panmictic population. This result suggests the lack of any obvious oceanic barriers to migration between New Zealand and Australia. However, the dispersal behaviour of the species also needs to be tested to determine whether there is a cryptic type of population differentiation caused by sex-biased dispersal. The aim of this research was to compare the patterns of variation at nuclear DNA microsatellite loci with mtDNA in *G. galeus* populations sampled in New Zealand, Australia and Chile in order to describe population connectivity.

4.3 Methods

4.3.1 Sample collections

Samples from adult *G. galeus* were collected from NIWA (National Institute of Water and Atmospheric Research) fisheries surveys and by observers on longline commercial fishing vessels (see Chapter 2 for the collection details). Samples were taken from seven sites within New Zealand (NZ), Australia (AUS) and Chile (CHI). The samples were taken from three NZ sites; east of North Island (ENI), Chatham Rise (CHA) and Solander Islands (SOL) (see Figure 4.1A), three AUS sites; Shoulder (SHO), Tasmania (TAS), and Frederick Bay (FRE) (see Figure 4.1A), and Santiago (SAN) site in CHI (see Figure 4.1B). The sample sizes for each location are listed on Figure 4.1 and Table 4.2.

Figure 4.1: Sampling sites, sample sizes (in parentheses), and geographic distribution (shaded area) of adult sharks of *G. galeus* collected from **A.** New Zealand, Australia, and **B.** Chile. The sites are: ENI (east of North Island), CHA (Chatham Rise), SOL (Solander Island), SHO (Shoulder), TAS (West Tasmania), FRE (Frederich Bay), and SAN (Santiago).

4.3.2 PCR Amplification and Genotyping

Total genomic DNA was isolated from tissue samples using a standard phenol-chloroform extraction protocol (Sambrook et al. 1989). All samples were genotyped for a total of nine microsatellite loci. Two loci (*Ggal14* and *Ggal15*) were taken from the microsatellite DNA markers that were isolated and developed and reported in Chapter 3. The other seven loci (*Gg20*, *Gg15*, *Gg22*, *Gg23*, *Gg12*, *Gg07*, and *Gg11*) were taken from Chabot & Nigenda (2011) and were chosen for their ease of amplification and genotyping, and for their high allelic diversity. Microsatellite loci were PCR amplified separately in 10 µl volumes containing 67 mM Tris-HCL pH 8.8, 16 mM (NH₄)₂SO₄, 2.0 mM MgCl₂, 0.05 µM of forward primer labelled with M-13 tail, 0.2 µM of reverse primer, 0.05 µM of fluorescently labelled M-13 primers (FAM, VIC, or PET Invitrogen), 0.8 mM of each dNTPs, 0.3 units of BIOTAQTM DNA Polymerase (Bioline), and 1 µl of template DNA (50-80 ng/µl DNA concentration). Thermal cycling was performed on a TGradient Thermal Block (Biometra, Goettingen, Germany). Primer pairs and PCR conditions for each locus are detailed in Table 4.1. PCR products from multiple loci were pool-plexed into a single 96-plate well (one set with four loci and another with five loci) based on the combinations recommended by the MULTIPLEX MANAGER 1.0 program (Holleley & Geerts 2009). Capillary separation of the fluorescently labelled PCR-fragments was conducted on an ABI3730 Genetic Analyser (Massey University Genome Service). The allele sizes were scored using the AUTOBIN program (<http://www4.bordeaux-aquitaine.inra.fr/biogeco/Ressources/Logiciels/Autobin>), which uses an Excel macro to sort and plot the relevant gaps between allele sizes (Guichoux et al. 2011). The alleles were coded as three digit genotypes and the individuals contained missing data at no more than three loci.

Table 4.1: Primer sequences and PCR conditions of nine microsatellite loci of *G. galeus*.

Locus and primer sequences	Repeat	Size (bp)	First cycling conditions	Second cycling conditions
<i>Ggal</i> 14F 5'-AGTCAAAGGATGCTCGCAGT-3' <i>Ggal</i> 14R 5'-GGGCGATTATTTGGATGAGA-3'	(AA) ₂ AC(AG) ₇	117-125	94°30", 62°45", 72°45", 30 cycles	94°30", 53°45", 72°45", 8 cycles
<i>Ggal</i> 15F 5'-GTGACCTCCATCACCGACTT-3' <i>Ggal</i> 15R 5'-GCCTTTGTGAAGAGCCTCAA-3'	(CAA) ₈	132-159	94°30", 62°45", 72°45", 30 cycles	94°30", 53°45", 72°45", 8 cycles
<i>Gg</i> 20F 5'-GACCAAGGGTCATCCAGAC-3' <i>Gg</i> 20R 5'-TCAGCTTGGGCAATTCCAG-3'	(TC) ₈	197-207	94°30", 59°90", 72°60", 25 cycles	94°30", 53°90", 72°60", 20 cycles
<i>Gg</i> 15F 5'-GGCTGAATGGTTTCCCAGC-3' <i>Gg</i> 15R 5'-GCCTCCAACCTAGCATAGCC-3'	(GA) ₁₂	152-170	94°30", 59°90", 72°60", 25 cycles	94°30", 53°90", 72°60", 20cycles
<i>Gg</i> 22F 5'-TCCTGGGATGGCAACTTCG-3' <i>Gg</i> 22R 5'-AGGCCACCCAACCTATCCTG-3'	(GT) ₈	242-256	94°30", 59°90", 72°60", 25 cycles	94°30", 53°90", 72°60", 20 cycles
<i>Gg</i> 23F 5'-ACAGACCACAGGGCATGG-3' <i>Gg</i> 23R 5'-TGCAGAGCAGGCTAGATGG-3'	(AC) ₁₂	246-350	94°30", 59°90", 72°60", 25 cycles	94°30", 53°90", 72°60", 20 cycles
<i>Gg</i> 12F 5'-TGTCAAACACCATCGCAGG-3' <i>Gg</i> 12R 5'-TGCTCTGAAGTCTACAAGAATGG-3'	(TA) ₉	284-310	94°30", 59°90", 72°60", 25 cycles	94°30", 53°90", 72°60", 20 cycles
<i>Gg</i> 7F 5'-CTGTGGAACAACTCCAGC-3' <i>Gg</i> 7R 5'-AGCTGGTCGAGGTGAATGC-3'	(AG) ₁₀	298-312	94°30", 59°90", 72°60", 25 cycles	94°30", 53°90", 72°60", 20 cycles
<i>Gg</i> 11F 5'-AAGTTGCACGTTTCCCAGC-3' <i>Gg</i> 11R 5'-TACTGCAGGACCGGTTTCC-3'	(TCCC) ₄	334-354	94°30", 59°90", 72°60", 25 cycles	94°30", 53°90", 72°60", 20 cycles

4.3.3 Data analyses

4.3.3.1 Genetic diversity

The dataset was initially checked for the presence of null alleles, allele size shifts and scoring errors due to stuttering, using the software MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004). Population genetic diversity estimates such as the number of alleles per locus, observed (H_o) and expected (H_e) heterozygosities and coefficient of inbreeding (F_{IS}) were calculated in ARLEQUIN 3.1.1 (Excoffier et al. 2005). Allelic richness, allele frequencies, and F_{IS} across loci were estimated in FSAT 2.9.3.2 (Goudet 1995). Allele frequency distributions at each population and loci were generated using GENALEX 6.41 (Peakall & Smouse 2006). Deviation from Hardy Weinberg equilibrium (HWE) was tested across loci by population, and tests for pairwise Linkage Disequilibrium (LD) between all pair of allele across loci among all populations were calculated in ARLEQUIN 3.1.1. HWE significance was calculated using the exact test (Guo & Thompson, 1992) with a Markov chain of 10^6 steps and 10^5 dememorization steps. Significance values for LD were calculated using a likelihood-ratio test (LRT) of 20,000 permutations (Slatkin & Excoffier, 1996). Sequential Bonferroni corrections (Rice 1989) were applied when appropriate.

A coalescent simulation approach was implemented in LOSITAN (Antao et al. 2008) to discover outlier loci. The distribution of loci was simulated based on F_{ST} values and heterozygosity (H_e) using the method of Beaumont & Nichols (1996). Outlier simulations of 10^4 steps were conducted using a ‘neutral mean F_{ST} ’ and then by ‘forced mean F_{ST} ’, which improves the reliability of the mean F_{ST} (Antao et al. 2008). Simulations were run using NZ and AUS samples. Samples from CHI were excluded due to small sample size. The overall F_{ST} of 0.027, as expected for neutral markers, was used to estimate the upper and lower F_{ST} limits at the 0.99% confidence intervals. Both the stepwise (SMM) and the infinite allele (IAM) mutation models were used in the analysis (Luikart & Cornuet 1998)

In order to determine whether sufficient sample size was obtained to accurately measure allelic diversity within populations, allele discovery curves were constructed for the two most polymorphic loci using the “PopGenKit” package (Rioux Paquette 2011) in R 2.15 (Ihaka & Gentleman 1996; R Development Core Team 2011). The function computes the number of sampled alleles for a given constant

increase in sample size by jackknife resampling at 1,000 replicates. The results were used to plot allele discovery curves within each population.

4.3.3.2 Population structure

Genetic diversity within and among populations and ocean basins (defined as South Pacific Ocean and Tasman Sea) was estimated using an analysis of molecular variance (AMOVA, Excoffier et al. 1992) implemented in ARLEQUIN 3.1.1 (Excoffier et al. 1995). Spatial analysis was undertaken to test the hypothesis of panmixia among sites (NZ, AUS and CHI). The variance components and Φ -statistic values were partitioned at three levels of hierarchical subdivision: among ocean basins, within populations, and the interaction of both (among + within). The genetic distance matrix for AMOVA was estimated by pairwise differences and the significance levels of the variance components and Φ -statistics values were tested by 20,000 nonparametric permutations (Excoffier et al. 1995).

Levels of genetic differentiation among populations were estimated by four approaches. a) Testing for differences of allele frequencies distribution between all pairs of populations was conducted in GENEPOP 4.0.10 (Raymond & Rousset 1995). Unbiased P values were computed for each population pair at each locus using an Exact G test (Goudet et al. 1996), and P values for each population pair across all loci were also calculated by Fisher's exact probability test (Raymond & Rousset 1995). Markov chain parameters consist of a number of 10^4 dememorization steps, with 10^3 batches and 10^4 iterations per batch. b) Estimating the Wright's F_{ST} (θ estimator, Weir & Cockerham (1984) was computing in FSTAT 2.9.3.2 (Goudet, 1995). The significance values for θ were calculated by a 1,000 replicate bootstrap procedure over all loci using 99% confidence intervals. A sequential Bonferroni correction was used to minimise the chances of a type I error of significance over multiple tests (Rice 1989). c) Estimating the Slatkin's R_{ST} (Slatkin 1995) that considers the square size differences between alleles and it is more appropriate for microsatellite loci that evolve via step-wise mutations. The R_{ST} estimator was obtained using ARLEQUIN 3.1.1 (Excoffier et al. 1995). (d) Calculating the D_{ST} estimator (Jost 2008) using SMOGD 1.2.5 (Crawford 2010) with 1,000 bootstrap iterations. The approximate harmonic mean (H) was calculated from the mean and variance across loci (SMOGD

website www.ngcrawford.com/django/jost/). This statistic is more robust than F_{ST} due to the bias introduced by differences in diversity among populations, and also more appropriate for highly polymorphic markers (Jost 2008).

POWSIM 4.1 (Ryman & Palm 2006) was used to assess the statistical power of the sampled loci under different combinations of samples sizes, number of loci and alleles, and allele frequencies. The statistical power and alpha values were assessed using the Chi-squared and Fisher's exact tests under a specified level of population divergence (F_{ST}). Two set of simulations with 20,000 replications were run assuming a) a base population with the average observed allele frequencies for the microsatellite loci, and b) a division of seven populations with the sample sizes used in the present study (see number of sharks by population, Table 4.2). Different combinations of N_{ef} (effective population size) and t (generations of drift) were simulated (N_{ef}/t) corresponding to 500/50 and 2000/205 respectively.

A test for a pattern of genetic isolation-by-distance (IBD) was conducted using linearised F_{ST} values that were plotted against the natural logarithm of geographic distance (kilometres) between all possible pairs of NZ and AUS sites. Geographic distances were estimated by the ruler option in Google Earth v.6.2.2 (Google Inc.). The distance calculations consist of the shortest path, by sea, between every pair of sample sites. Significance was evaluated for the set of pairwise combinations using a Mantel's test implemented in ARLEQUIN 3.1.1 using 20,000 permutations.

4.3.3.3 *Relationship among populations and Bayesian clustering*

The genetic relationships among populations were examined using a Principal Component Analysis (PCA) based upon the pairwise F_{ST} values using the program PCA-GEN 1.2.1 (Goudet 1999). Simulations of 15,000 randomizations were performed to evaluate the reliability of the results using two schemes: a) all sampling sites, and b) only the NZ and AUS sites (excluding SAN, Chile). The genetic differences among all individuals from each population were also displayed by Factorial Component Analysis (FCA) using GENETIX 4.05 (Belkhir 2000). Additionally, a cluster analysis was used to investigate the genetic relationships among populations using POPULATIONS 1.2.32 (Langella 2002). The genetic

distances among populations were estimated using D_a (genetic distance; Nei et al., 1983) and the neighbor-joining algorithm was used to construct a dendrogram with the resulting distance matrix with and without 1,000 bootstrapped replicates of the dataset.

A Bayesian approach was used to test for population genetic structure using STRUCTURE 2.3.3 (Pritchard et al. 2000, Falush et al. 2003). This program analyses allele frequencies across multiple loci and assigns multi-locus genotypes or individuals to a 'population' cluster (K = the number of genetically distinct clusters) by minimizing Hardy-Weinberg and linkage disequilibrium in the genotype data. A Markov chain Monte Carlo (MCMC) simulation was run for 10^7 iterations with a burn-in period of 400,000 steps (Falush et al. 2003). Multiple runs were performed to evaluate the reliability of the results under admixture and non-admixture and locprior models because it performs better than other models for detecting genetic structure even in situations of low levels of genetic divergence or a limited number of loci (Hubisz et al. 2009). Two ranges of K values were tested depending on the number of population included in the analysis: (1) using all sample sites (K ranged from 1 to 7) and (2) only the NZ and AUS sample sites (K ranged from 1 to 6). STRUCTURE HARVESTER 0.6.92 (Dent & Bridgett 2012) was used to estimate the optimum number of K using the Log probability of data ($L(K)$; Pritchard et al. 2000). However, if the migration rates are not equal between populations, K may be incorrectly estimated. Thus, Delta K estimator (ΔK ; Evanno et al. 2005) was estimated. Delta K was more accurate than Log probability in estimating the true K because it is based on the rate of change in the log probability of data between successive K -values. Individuals were assumed to have been correctly assigned to a population when their q value (i.e. its posterior probability of belonging to original population) was at least 80% for the population (Pritchard et al. 2000).

4.3.3.4 *Mutation-drift and migration-drift equilibrium*

Three approaches were used to investigate whether there was evidence of a recent population size contraction. First, BOTTLENECK 1.2.02 (Pyri et al. 1999) was used to test for an excess of heterozygotes. Expected heterozygosity (H_e) was estimated from the observed alleles (k) at each locus and a distribution of heterozygosity was

generated based on the simulated coalescing of nine loci under the Stepwise Mutational Model (SMM), Infinite Alleles Model (IAM), and the Two-Phase Model (TPM). The interpretation of results focused on the TPM of mutation as it is recommended for microsatellite loci due to a better fit with observed allele frequency data than the IAM and SMM (Di Rienzo et al. 1994, Piry et al. 1999). The TPM option was set at 95% single-step mutations and 5% multiple-step mutations (Cornuet & Luikart 1996, Piry et al. 1999). The average expected equilibrium heterozygosity (H_{eq}) for each locus was compared to Hardy-Weinberg heterozygosity (H_e) and used to assess whether there was a heterozygote excess or deficit at each locus using a Sign test, standardized differences test, and Wilcoxon's test based on 1000 replications. The second approach used BOTTLENECK 1.2.02 to test for a mode shift of the allele frequency distribution from an L-shaped distribution that develops when the population is in a mutation-drift balance. Thirdly, a population size contraction signature was tested using *M*-RATIO method developed by Garza & Williamson (2001). This approach calculates *M*, which is the ratio of total number of alleles (*k*) compared to the range of allele sizes (*r*). The program M_P_VAL 100.0 (Garza & Williamson 2001) was used to calculate *M*-RATIO (*M*) for each population and contrast the observed values of *M* to a distribution of *M* values calculated from simulated populations assumed to be at mutation-drift equilibrium. The critical values of *M* were determined by simulations described in Garza & Williamson 2001. The simulations require three input parameters (θ , P_g , Δ_g). The parameter theta, $\theta = 4 N_{ef}\mu$, where N_{ef} = effective population size and μ = mutation rate. Calculation of effective population size was obtained in ARLEQUIN 3.1.1 for each population. Theta (θ) was estimated from the expected homozygosity (Hom_E). Assuming that the populations are in mutation-drift equilibrium, $\theta = (1 - Hom_E) / Hom_E$, where $Hom_E = 1 - H_E$, and H_E is the expected heterozygosity. The mutation rate (μ) was set at 1×10^{-3} mutations/generation/locus (Weber & Wong 1993, DeWoody & Avise 2000). For both parameters the percentage of mutations larger than a single step ($P_g = 0.10$) and the mean size of mutations larger than a single step ($\Delta_g = 3.5$) were chosen following the recommendation of Garza & Williamson (2001). Each set of simulations was run for 10,000 iterations.

4.4 Results

4.4.1 Genetic diversity

Two hundred and thirty nine *G. galeus* samples from New Zealand, Australia and Chilean sites were genotyped for nine microsatellite loci. Complete genotypes for all loci were obtained for 197 individuals and 42 individuals had missing data of up to three loci. No null alleles, allele size shifts, or error scoring were detected using MICRO-CHECKER 2.2.3 (at the 95% confidence interval). The summary statistics for genetic diversity at nine microsatellite loci are presented in Table 4.2. The least polymorphic loci were *Gg20* and *Ggal14*, which had between 2 and 3 alleles for NZ and AUS sites, and the most polymorphic loci were *Gg15* and *Gg12* for NZ and AUS, which had between 9-11 and 8-12 alleles respectively. When allelic richness was averaged (R_s) across all loci it was greatest in the NZ and AUS sites (4.657-5.050), when compared to the CHI site (3.984). The observed heterozygosity (H_o) ranged between 0.111 (CHA, NZ) for *Gg20*, to 0.863 (SOL, NZ) for *Gg15*. The expected heterozygosity (H_e) varied between 0.145 (CHA, NZ) for *Gg20*, to 0.886 (FRE, AUS) for *Gg15*.

After correction for multiple tests, only four of sixty three values showed significant departures from HWE ($P < 0.0008$) across nine microsatellite loci (Table 4.2). There was a significant level of heterozygote deficiency at the *Ggal 14* in TAS (AUS), *Gg15* in the CHA (NZ), *Gg22* in the FRE (AUS) and ENI (NZ) populations, as well as at the *Gg11* in the SOL (NZ) and SHO (AUS) populations (Table 4.2). These results support the hypothesis of the presence of null alleles at these loci. In addition to heterozygote deficiencies, only the *Gg22* and *Gg11* loci showed a significant level of LD ($P < 0.002$) (data not shown).

In addition, the allele frequency distributions showed that each locus had one relatively common allele that was shared among all populations (Figure 4.2, 4.3, 4.4). Private alleles were found at seven loci (*Ggal14*, *Ggal15*, *Gg20*, *Gg22*, *Gg12*, *Gg07*, and *Gg11*), and these were typically at low frequencies. All allele discovery curves reached a plateau when the cumulative allelic richness was plotted against the individuals genotyped (Figure 4.5). The loci *Gg15* and *Gg12* were found to be the most polymorphic for NZ and AUS, while the loci *Ggal15* and *Gg23* were for Chile.

Table 4.2: Summary of nine microsatellite loci for each *G. galeus* population. Population in New Zealand (NZ), Australia (AUS) and Chile (CHI) are as follows: ENI = east of North Island, CHA = Chatham Rise, SOL = Solander Islands, SHO = Shoulder, TAS = West Tasmania, FRE = Frederick Bay, and SAN = Santiago. Parameters are as follows: n , sample sizes; R_s , allelic richness, H_o , observed microsatellite heterozygosity; H_e , expected microsatellite heterozygosity; F_{is} , microsatellite inbreeding coefficient [negative values = heterozygote excess and positive values = heterozygote deficiency; AAL = Average across loci; values marked with * deviate from Hardy-Weinberg expectations nominally after sequential Bonferroni correction ($\alpha = 0.05/63 = 0.0008$).

Population	Parameter	Ggal14	Ggal15	Gg20	Gg15	Gg22	Gg23	Gg12	Gg7	Gg11	AAL
ENI (NZ) $n = 40$	Alleles	3	7	2	11	6	7	8	5	6	6
	R_s	2.547	4.524	1.998	8.510	4.480	6.037	7.155	4.459	5.133	4.983
	H_o	0.425	0.316	0.132	0.861	0.605	0.649	0.649	0.675	0.462	0.530
	H_e	0.460	0.610	0.287	0.863	0.659	0.746	0.799	0.664	0.743	0.648
	F_{is}	0.076	0.486*	0.545	0.002	0.082	0.132	0.190	-0.017	0.382*	0.183
CHA (NZ) $n = 47$	Alleles	3	6	2	10	7	8	9	6	6	6
	R_s	2.479	4.192	1.917	8.221	4.308	6.437	7.477	4.694	4.290	4.891
	H_o	0.362	0.553	0.111	0.761	0.575	0.600	0.804	0.652	0.733	0.572
	H_e	0.488	0.596	0.145	0.859	0.686	0.800	0.787	0.636	0.580	0.619
	F_{is}	0.260	0.072	0.236	0.115	0.164	0.252	-0.022	-0.025	-0.269	0.077
SOL (NZ) $n = 30$	Alleles	3	5	2	10	5	7	8	5	5	6
	R_s	2.433	3.942	1.986	8.414	3.840	6.379	6.993	3.646	4.276	4.657
	H_o	0.300	0.567	0.233	0.867	0.300	0.821	0.556	0.500	0.517	0.518
	H_e	0.352	0.574	0.210	0.861	0.604	0.806	0.716	0.554	0.499	0.575
	F_{is}	0.150	0.012	-0.115	-0.007	0.508*	-0.020	0.228	0.099	-0.037	0.101
SHO (AUS) $n = 43$	Alleles	3	4	3	9	4	10	11	5	5	6
	R_s	2.900	3.073	2.308	8.067	3.515	7.304	7.881	4.565	3.814	4.825
	H_o	0.333	0.489	0.286	0.833	0.442	0.731	0.950	0.628	0.697	0.597
	H_e	0.478	0.552	0.316	0.867	0.650	0.746	0.838	0.647	0.639	0.645
	F_{is}	0.305	0.117	0.098	0.039	0.322*	0.019	-0.135	0.030	-0.093	0.060
TAS (AUS) $n = 39$	Alleles	4	5	2	10	5	9	12	5	4	6
	R_s	3.301	3.477	2.000	8.618	4.117	7.096	8.659	4.618	3.566	5.050
	H_o	0.306	0.590	0.231	0.778	0.769	0.821	0.769	0.692	0.711	0.618
	H_e	0.589	0.562	0.345	0.871	0.700	0.792	0.833	0.662	0.638	0.664
	F_{is}	0.485*	-0.050	0.335	0.108	-0.101	-0.037	0.077	-0.046	-0.116	0.070
FRE (AUS) $n = 24$	Alleles	2	3	2	11	6	8	10	5	4	6
	R_s	1.911	2.542	1.985	9.491	4.625	7.350	8.362	4.321	3.777	4.929
	H_o	0.125	0.500	0.208	0.708	0.667	0.783	0.667	0.458	0.364	0.498
	H_e	0.120	0.494	0.191	0.886	0.707	0.800	0.840	0.565	0.519	0.569
	F_{is}	-0.046	-0.013	-0.095	0.204	0.058	0.022	0.209	0.192	0.304*	0.127
SAN (CHI) $n = 16$	Alleles	2	6	4	5	5	6	2	3	4	4
	R_s	2.000	6.000	3.857	4.867	4.733	5.624	2.000	2.999	3.778	3.984
	H_o	0.625	0.692	0.357	0.933	0.800	0.500	0.200	0.467	0.125	0.493
	H_e	0.508	0.757	0.521	0.779	0.671	0.704	0.287	0.398	0.337	0.553
	F_{is}	-0.240	0.089	0.323	-0.206	-0.200	0.296	0.311	-0.181	0.636	0.113
Total	# Sharks	235	234	232	229	236	229	228	235	232	232

Figure 4.2: Allelic frequency distribution for the microsatellite loci *Ggal14*, *Ggal15*, and *Gg20* across all populations of *G. galeus*. Allele sizes are given in base pairs of PCR products. The letter "p" represents private alleles found in one population.

Figure 4.3: Allelic frequency distribution for the microsatellite loci *Gg15*, *Gg22*, and *Gg23* across all populations of *G. galeus*. Allele sizes are given in base pairs of PCR products. The letter “p” represents private alleles found in one population.

Figure 4.4: Allelic frequency distribution for the microsatellite loci *Gg12*, *Gg07*, and *Gg11* across all populations of *G. galeus*. Allele sizes are given in base pairs of PCR products. The letter “p” represents private alleles found in one population.

Figure 4.5: Allele discovery curves for the most polymorphic loci. **A.** *Gg15* (NZ and AUS) and *Ggal15* (CHI) and **B.** *Gg12* (NZ and AUS) and *Gg23* (CHI).

4.4.2 Population structure

The AMOVA analysis showed evidence of population differentiation with significant components of variance within and between ocean basins ($F_{CT} = 0.05$, % $V = 5.45$, $P = 0.0419$ and $F_{SC} = 0.01$, % $V = 0.79$, $P = 0.0025$, Table 4.3A). However, more than 90% of the partitioned genetic variation was found within populations ($F_{ST} = 0.06$, % $V = 93.76$, $P < 0.0001$). The second spatial AMOVA was run for NZ and AUS (excluding CHI), which revealed non-significant differentiation among groups across Tasman Sea ($\Phi_{CT} = 0.0005$, $p = 0.39$). The Exact tests of allele frequency distributions indicated regional differentiation between NZ/AUS and CHI at most of the loci ($P < 0.001$; Table 4.3). Significant differences were detected between NZ and AUS that were driven by one or two loci (see superscript numbers; Table 4.3A). The *Ggal14* locus at the FRE site was significantly different from all other sample sites because of an absence of the 125 bp allele from the FRE site (see allelic distribution Figure 4.2F). The *Ggal14* locus was also an outlier in the LOSITAN simulation. This locus was removed from the subsequent analyses.

The pairwise F_{ST} analysis showed a significant level of genetic differentiation between collection sites in NZ/AUS, compared with CHI. However, there was no significant genetic differentiation among sample sites within and between the NZ and AUS regions (F_{ST} values below diagonal table, Table 4.3B). A similar pattern was seen in values of D_{ST} , with relatively higher values than F_{ST} when CHI was compared to all other sites and lower values of D_{ST} when comparison was made within and between NZ and AUS (Table 4.3B). Considering just the NZ and AUS sample sites, the Mantel test did not show a significant relationship between genetic and geographic distance ($R^2 = 0.008$ $P = 0.463$).

The POWSIN analysis indicated that the eight loci from all seven sample sites showed that the samples sizes were capable of detecting a true F_{ST} value, estimated to be as low as 0.05 with a probability of 100% (both Fisher's exact test and chi-square, those values are not shown) for the two different combination of N_{ef}/t simulated.

Table 4.3: A. AMOVA results for global panmixia, across ocean basins (New Zealand, Australia, and Chile) based on eight microsatellite loci. The asterisk (*) represents significant values at $P < 0.05$, and ** significant values at $P < 0.001$. The SSD is the sum of squares, VC is variance components, and % V is the percentage of total variation. **B.** Below the diagonal are the pairwise F_{ST} values (Weir & Cockerham 1984), in parentheses the R_{ST} values (Slatkin 1995), and in italics the D_{ST} values (Jost 2008) for eight microsatellite loci (excluding *Ggal14*). Above the diagonal is the number of loci, name of loci (superscript), and P values for Exact G tests from nine microsatellite loci (including *Ggal14*) between New Zealand, Australia and Chile populations. Significant P values for the pairwise F_{ST} and Exact tests are represented by * $P < 0.001$.

A

Structure tested	Source of variation	SSD	VC	% of total	F-statistics
Global panmixia	Among group	63.54	0.12	4.59	$F_{ST}=0.05$ ($p<0.0001$)**
	Within populations	1,170.45	2.57	95.41	
NZ, AUS, CHI	Among ocean basins	46.34	0.15	5.48	$F_{CT}=0.05$ ($p<0.0001$)**
	Among population within ocean	17.20	0.02	0.89	$F_{SC}=0.01$ ($p=0.0012$)*
	Within population	1,170.45	2.57	93.63	$F_{ST}=0.06$ ($p<0.0001$)**
	Total	1,233.99	2.74		
NZ, AUS	Among Tasman Sea (TS)	4.69	0.01	0.05	$F_{CT}=0.0005$ ($p=0.390$)
	Among population within TS	17.2	0.02	0.92	$F_{SC}=0.01$ ($p=0.001$)*
	Within population	1,106.86	2.59	99.03	$F_{ST}=0.01$ ($p<0.0002$)*
	Total	1,128.75	2.62		

B

Sites	New Zealand			Australia		Chile	
	ENI	CHA	SOL	SHO	TAS	FRE	SAN
ENI		2 ^{22,1}	2 ^{7,11}	1 ¹¹	1 ¹¹	2 ^{14,11*}	8 ^{15,20,15,22,23,12,7,11*}
CHA	0.014(0.065) 0.004		1 ²²	1 ²²	0	1 ^{14*}	6 ^{15,20,15,23,12,7*}
SOL	0.015(0.044) 0.011	0.010(0.020) 0.002		0	2 ^{22,1}	1 ^{14*}	7 ^{15,20,15,22,23,12,7*}
SOU	0.003(0.081) 0.001	0.010(0.059) 0.006	0.011(-0.010) 0.011		0	1 ^{14*}	7 ^{15,20,15,22,23,12,7*}
TAS	0.009(0.157*) 0.003	0.009(0.038) 0.002	0.005(0.051) 0.004	0.005(0.025) 0.001		1 ^{14*}	7 ^{15,20,15,23,12,7,11*}
FRE	0.075(0.020) 0.001	0.064(-0.011) 0.002	0.105(-0.001) 0.005	0.076(-0.015) -0.001	0.048(0.032) 0.001		5 ^{15,20,15,23,12*}
SAN	0.217*(0.326*) 0.320	0.205*(0.439*) 0.200	0.230*(0.427*) 0.250	0.204*(0.365*) 0.283	0.195*(0.382*) 0.271	0.247*(0.433*) 0.216	

4.4.3 Relationship among populations and Bayesian clustering

The PCA also showed that the CHI site was different from all of the NZ and AUS sites (PCA 1: $F_{ST} = 0.037$, 67.84% of total inertia, $P < 0.001$, Figure 4.6A). When the SAN sample site (CHI) was excluded, the PCA analysis of the NZ and AUS sites suggested some genetic differentiation, but the axis separation of these groups was not significant (Figure 4.6B). The FCA showed a similar result at the individual level instead of population (PCA). FCA showed a few individuals from the ENI (NZ) site outside of the main cluster of individuals from the other NZ and AUS sites (see Figure 4.7). The neighbor-joining dendrogram showed an almost star-shaped phylogeny with very little support for any grouping within and between NZ and AUS sites (Figure 4.8).

The STRUCTURE analysis distinguished three distinct clusters with the NZ, AUS, and CHI sample collections using the $L(K)$ approach for admixture and non-admixture models (the highest for $K = 3$; Figure 4.9). Two of the clusters were found in NZ and AUS: cluster one was comprised of ENI and cluster two was comprised of CHA, SOL, SHO, TAS, and FRE. A distinct third cluster was formed by the SAN samples (Figure 4.9). Most of the individuals grouped in cluster two and three were assigned to the same cluster (shown in gray and black bar graphs in Figure 4.9), while individuals collected from ENI were assigned to two groups of individuals, one was assigned to other sites in NZ and AUS (shown in white bar graphs) and another assigned to the same cluster (gray bar graphs). The majority of the individuals were correctly assigned with 80% or greater probability from their sites. However, the cluster one (ENI) revealed only four (10%, admixture model) and nine (22.5%, non-admixture model) individuals assigned to other groups different than the rest of NZ and AUS individuals. In addition, the best number of clusters was not clear because the $L(K)$ values never reached a plateau after $K = 3$, a criterion described in Pritchard et al. (2000). The ΔK approach described by Evanno et al. (2005) is considered to be a better method for approximating the true K , and this approach showed that the best K was two distinct clusters (the highest ΔK for $K = 2$; Figure 4.10), corresponding to a single genetic cluster including all sites in NZ and AUS and another different cluster with CHI.

Figure 4.6: 2D Plot of the first two principal components axes (PCA) of genotype frequencies for eight microsatellite loci (excluding *Gg14*) from ENI (east-North Island), CHA (Chatham Rise), SOL (Solander Island) in New Zealand (black circles); SHO (Shoulder), TAS (West Tasmania), and FRE (Frederich Bay) in Australia (gray circles), and SAN (Santiago) in Chile. Figure **A** represents all collection sites and **B** all sites excluding SAN.

Figure 4.7: 3D Plot of the analyses of principal components (AFC) of genotype frequencies for eight microsatellite loci (excluding *Gg14*). Circles represent each individual and different colours are the populations analyzed: ENI (east-North Island), CHA (Chatham Rise), SOL (Solander Island) in New Zealand; SHO (Shoulder), TAS (West Tasmania), and FRE (Frederick Bay) in Australia; and SAN (Santiago) in Chile.

Figure 4.8: Radial neighbor-joining dendrogram for eight microsatellite loci (excluding *Gg14*) screened from seven populations of *G. galeus* based on the Nei et al.'s, (1983) *Da*. Numbers are the bootstrap support indices of loci.

Figure 4.9: Bar plot of Bayesian clustering analysis from *G. galeus* for eight microsatellite loci (excluding *Gg14*) (239 individuals) performed using STRUCTURE. Each bar represents proportional probability of assignment to each genetic cluster. Sampling sites were organized by region: New Zealand (ENI, CHA, SOL), Australia (SHO, TAS, FRE), and Chile (SAN). **A.** Admixture and allele frequencies correlated model for $K = 3$. **B.** Non-admixture and allele frequencies correlated for $K = 3$. Black, grey, and white components of each bar represent the proportion in each of the three clusters.

Figure 4.10: Identification of the number of genetic clusters (K) of best fit observed for eight microsatellite loci under “delta K ” (ΔK) and “log probability of data” (Mean of $\text{LnP} \pm 1$) approaches. **A.** Admixture and allele frequencies correlated model for K ranging from 1-7. **B.** Non-admixture and allele frequencies correlated for K ranging from 1-7.

4.4.4 Mutation-drift and migration-drift equilibrium

The BOTTLENECK analyses did not show any evidence of the genetic effects of a contraction of population size of NZ, AUS and CHI. All sites were in equilibrium under the SMM and TPM mutational models, but the majority of populations were not in mutation-drift equilibrium under the IAM (Table 4.3). The allele frequency distribution showed that the population was in a mutation-drift balance and there was no evidence of genetic population size contraction (Figure 4.11; Table 4.4). In addition, M -ratio values for each population were higher than the critical value ($M < 0.68$) recommended by Garza & Williamson (2001) (see M values in Table 4.4).

Table 4.4: Results from the BOTTLENECK tests and M -RATIO analysis of eight microsatellite loci (excluding *Gg14*) for each population. Significant P values for Wilcoxon's test are represented with the asterisks.

Populations	BOTTLENECK			Allele frequency distribution	<i>M</i> -RATIO		
	Wilcoxon's test				θ	N_{ef}	M
	IAM	SMM	TPM				
ENI	0.006*	0.998	0.973	Normal L-shaped	1.77	442.50	0.75
CHA	0.037*	0.998	0.996	Normal L-shaped	1.66	415.00	0.71
SOL	0.098	0.990	0.986	Normal L-shaped	1.59	397.50	0.79
SHO	0.010*	0.875	0.875	Normal L-shaped	1.72	430.00	0.77
TAS	0.002*	0.809	0.727	Normal L-shaped	1.78	445.00	0.71
FRE	0.064	0.105	0.076	Normal L-shaped	1.63	407.50	0.70
SAN	0.010*	0.875	0.875	Normal L-shaped	1.53	382.50	0.77

Figure 4.11: Distribution of allelic frequencies sizes (X-axis) and the proportion of alleles (Y-axis) for nine microsatellite loci for each population. Black bars represent the proportion of alleles observed in each allele frequency classes.

4.5 Discussion

4.5.1 Genetic diversity

Microsatellite DNA markers that have been applied to population genetic studies in elasmobranch species have shown high levels of genetic diversity. A comparison with other species showed that *Galeorhinus galeus* had a relatively low level of genetic diversity i.e. an average of 5.71 alleles (A), and 0.61 expected heterozygosity averaged (H_e) across all loci (Table 4.1A in Supplementary material). However, shark species such as *Negaprion brevirostris*, *Carcharhinus leucas*, *Carcharhinus plumbeus* and *Isurus oxyrinchus* displayed the highest level of genetic diversity in comparison with the rest of elasmobranch species i.e. $A = 16.82-28.50$ and $H_e = 0.79-0.87$ (Schrey & Heist 2003, Feldheim et al. 2001, Schultz et al. 2008, Portnoy et al. 2010, Karl et al. 2011). The findings of these studies were based on highly polymorphic microsatellite loci. The higher number of repeat motifs (>20 repeats) and perfect (uninterrupted) motif sequences have produced a data set with more distinguishable alleles and consequently increased the level of genetic resolution.

Genetic diversity indexes are generally low in elasmobranch species ($A = 10.39$ and $H_e = 0.63$) in comparison with other marine fishes such as Actinopterygii i.e. ray-finned species that have higher number of alleles per locus ($A = 19.90$) and higher heterozygosities ($H_e = 0.77$). However, genetic diversity in elasmobranchs is relatively similar to Actinopterygii freshwater and anadromous fish species (Table 4.1B and C in Supplementary material). The differences in levels of genetic diversity between groups of fishes might reflect differences in their average effective population sizes. Most Actinopterygii marine species have external fertilization with high fecundity, producing thousands of viable eggs which lead to large and continuous populations. Subsequently, free-swimming adults are also good dispersers and contribute to genetic connectivity among subpopulations. In comparison with Actinopterygii marine species, elasmobranchs show lower potential for dispersal during their early life stages. For example, species such as *G. galeus* do not migrate long-distance in open water after 2+ years (Olsen 1954, Hurst et al. 1999, Francis 2010). In addition, the low genetic diversity of elasmobranchs may reflect their K -selected strategies such as extremely low fecundity (15-52 pups for *G. galeus*), long reproductive cycles (triennial for *G. galeus*), and late sexual maturity (8-17+ years for *G. galeus*) (Olsen 1954, 1984, Peres & Vooren 1991, Francis & Mulligan 1998, Walker 2005). These characteristics give elasmobranch species low reproductive potential in comparison with Actinopterygii marine species.

Although the levels of genetic diversity were low in *G. galeus* when compared to other elasmobranch species, the allelic richness (R_s) and expected heterozygosity (H_e) were different among populations. There were different levels of genetic diversity in New Zealand/Australia compared with Chile for several loci i.e. *Ggal14*, *Gg20*, *Gg12*, *Gg7*, and *Gg11*. This finding was consistent with the patterns of allele distributions, for example, 11 alleles were not present for loci *Gg12* in Chile when compared to the New Zealand/Australian sample. The absence of alleles in one of the populations indicates a longstanding barrier to gene flow and independent demographic histories in populations on each side of the South Pacific Ocean (SPO). This pattern is consistent with the results of the mtDNA CR study presented in Chapter 2, which revealed that the level of genetic diversity (i.e. haplotype and nucleotide diversity) were higher in Chile than New Zealand/Australia.

4.5.2 Genetic differentiation and population structure

Marine populations of elasmobranch species are thought to have high levels of genetic connectivity because of their high capacity for dispersal and the lack of biogeographic barriers or distance between habitats. However, life history traits and behaviour biology of some species can also limit gene flow. Elasmobranch species often have low reproductive potential, and also differential patterns of female and male migration. Females of some species show site fidelity or philopatry returning to a home area, birthplace or another locality during part of their life cycles, whereas males roam and disperse across their whole geographic distribution (Hueter et al. 2005). This observation has been supported by tagging/tracking migration studies and contrasting the degree of significant population structure between mtDNA (maternally inherited) and lack of structuring in microsatellites (biparentally inherited). Sex-biased philopatry has been reported for *Negaprion brevirostris* (Feldheim et al. 2002), *Carcharhinus limbatus* (Keeney et al. 2005), *Carcharodon carcharias* (Pardini et al. 2001, Blower et al. 2012), *Carcharhinus plumbeus* (Portnoy et al. 2010), and *Carcharhinus leucas* (Karl et al. 2011, Tillet et al. In review). The level of gene flow is typically determined by different behavioural and biological characteristics of species, which can affect the strength of genetic drift and natural selection (Palumbi 1994, Graves 1998, Dudgeon et al. 2012).

In contrast to the results of the mtDNA markers in elasmobranch species, microsatellite loci have shown evidence of genetic differentiation within ocean basins. High

level of population structure are found in highly vagile shark species have been found in samples from Atlantic and Pacific Oceans in *Isurus oxyrinchus* (Schrey & Heist 2003), Pacific and Atlantic in *Cetorhinus maximus* (Hoelzel et al. 2006), Indo-Pacific Ocean in *Negaprion acutidens* (Schultz et al. 2008), Pacific and Indian Oceans in *Rhincodon typus* (Schmidt et al. 2009), and across SPO for *Squalus acanthias* (Veríssimo et al. 2010). Conversely, higher levels of genetic connectivity were found for less vagile shark species that were coastal and benthonic with more restricted geographic distribution. For example, *Stegostoma fasciatum* exhibited population genetic differences between northern Australia and southeast Asia (Dudgeon et al. 2008), *Carcharhinus leucas* between northern coast of Brazil and gulf coast of Florida (Karl et al. 2011), *Carcharias taurus* between Brazil and South Africa (Ahonen et al. 2009), and *Raja clavata* between the Mediterranean basin, the Azores and the European continental shelf (Chevolot et al. 2006). At a small spatial scale, microsatellite markers have demonstrated that juveniles of *Carcharodon carcharias* are philopatric suggesting the presence of discrete nursery areas on the eastern and southwestern Australian coastlines (Blower et al. 2012). The genetic studies have provided a better understanding of the connectivity of elasmobranch species and their dispersal patterns and the traditional view about barriers (biological and/or biogeographic) to gene flow have been revised in this Chapter and Chapter 2.

At a large spatial scale, the results revealed a lack of genetic connectivity of *G. galeus* across SPO between populations of New Zealand/Australia and Chile. However, there was a high level of gene flow across the Tasman Sea between New Zealand and Australian populations. The sampling of *G. galeus* conducted for this study provided a better coverage of the North Island and South Island in New Zealand and along the Southern Shark Fishery in Australia i.e. including South Australian, Victorian and Tasmanian waters (McLoughlin 2007). The results indicated that *G. galeus* in New Zealand and Australia represent a single panmictic population. This finding was consistent with the results from the mtDNA CR that were reported in Chapter 2. Both the nuclear and mtDNA markers suggested that there is a substantial level of gene flow among sites in New Zealand and Australian waters, which is a conclusion also supported by extensive tagging data that indicate high migration rate across Tasman Sea (Hurst et al. 1999, Walker et al. 2008, Francis 2010). The similar patterns found for these markers, which have difference patterns of inheritance (i.e. nuclear is biparentally inherited and mtDNA is maternally inherited) suggest that there is no evidence of sex-biased dispersal. Female *G. galeus* are not expected to be philopatric to a specific reproductive

territory. However, it may also be possible that the mtDNA does not have enough resolution power to detect a pattern of philopatry in *G. galeus*.

4.5.3 Mutation-drift equilibrium

A reduction in population size can lead to the loss of genetic variation. It also can increase rates of inbreeding, the fixation of deleterious alleles, and reduce the adaptive potential of populations (Frankham et al. 2002, Allendorf & Luikart 2007). Population genetic theory predicts that rare alleles will be more readily affected by drift than more frequent alleles (Nei et al. 1975, Hedrick 2000). The presence of rare alleles in the New Zealand, Australia and Chile *G. galeus* populations suggest that the possible population size contraction caused by exploitation has had no appreciable effect on the pattern of genetic variation. This finding could be because not enough time has elapsed, but after a few more generations the loss of rare alleles could be a detectable pattern. The finding was also consistent with results of the mtDNA reported in Chapter 2. It is possible that the Australasian *G. galeus* populations are at mutational-drift equilibrium, even though they have experienced high levels of exploitation.

4.5.4 Management implication

It is important to note that population genetic findings reflect the long-term evolutionary processes of a species and their populations. In other words, genetic markers detect patterns of gene flow that have become established over thousands of years. Consequently, they are not often able to detect more recent changes in population size and migration/recruitment patterns typically caused by overfishing, habitat degradation or other anthropogenic disturbance. Nevertheless, if populations were declining then contemporary levels of migration might also be in decline. Species such as *G. galeus* are highly susceptible to overfishing and habitat degradation due to their low reproductive potential (e.g. low fecundity). Even when molecular markers have revealed historical migration patterns of species, it is important to investigate the extent of the movement and interbreeding between populations. This information is fundamental to identifying the number of populations, and the genetic connectivity among populations of vulnerable and commercially fished elasmobranch species.

This study reports for the first time the patterns of population genetic variation for *G. galeus* in New Zealand, Australia and Chile based on microsatellite loci. There was evidence of genetic differentiation across SPO between New Zealand/Australia and Chile, but no evidence of differentiation between New Zealand and Australia. The Trans-Tasman connectivity was consistent with previous tagging studies that suggested high migration rates across the Tasman Sea (Hurst et al. 1999, Walker et al. 2008, Francis 2010). The genetic data demonstrate that the migrants were reproductively successful and there were enough of them to genetically homogenise the Australasian stock (Waples 1998). *Galeorhinus galeus* is a fished species that is able to cross geographic and international management boundaries. This finding supports the suggestion that there should be a co-ordinated Australian and New Zealand management perspective on fishery. However administrative and logistic considerations in New Zealand and Australia have meant that this species is assessed and managed as separate stocks in each country, even though studies have now shown that they are most likely a single panmictic stock. It is probably time to re-examine the management of *G. galeus* and consider a single-stock management approach. The current TAC (total allowable catch) levels in New Zealand waters should be re-evaluated because the stock is considered “sustainable” (MPI 2012) in New Zealand but “overfished” in Australian.

In conclusion, this study demonstrated that *G. galeus* populations are genetically discrete population across SPO i.e. South Hemisphere (New Zealand, Australia and Chile). The use of CR of mtDNA (see Chapter 2) and microsatellite loci (the present Chapter) in three locations are fairly consistent with the thought that *G. galeus* is composed of a single panmictic stock in New Zealand and Australia. However, further genetic studies may provide a more comprehensive understanding of the patterns of genetic connectivity of *G. galeus* in the Australasia region, and investigate the possibility of temporal variation using samples of neonates, pups, juveniles and females from widely separated nursery areas. This approach would provide valuable and better information about the degree of site fidelity or philopatry in *G. galeus*.

4.6 Appendices

Supplementary Material

Supplementary Table 4.1: A. Genetic diversity at microsatellite loci reported to date for population genetic studies among 16 Elasmobranch species. Number of populations studied (N pop), number of loci (N loci), number of individuals screened across loci (N ind), number of alleles averaged across all loci (A), and expected heterozygosity averaged across all loci (H_e) are reported. Note for *Carcharodon carcharias* only observed heterozygosity (H_o) was reported in Pardini et al. (2001). **B.** Genetic diversity at microsatellite loci in teleost freshwater, teleost anadromous, teleost marine, and elasmobranch species. **C.** Graphical representation of Table B for four different groups of species.

A						
Species	N pop	N loci	N ind	A	H_e	References
<u>Selachii</u>						
<i>Carcharhinus plumbeus</i>	2	3	71	2.33(0.47)	0.28(0.19)	Heist & Gold 1999
<i>C. plumbeus</i>	9	8	333-336	16.82(10.31)	0.83(0.13)	Portnoy et. al. 2010
<i>C. limbatus</i>	9	8	419	8.67(8.53)	0.51(0.33)	Keeney et. al. 2005
<i>C. sorrah</i>	4	5	140-204	10.80(8.54)	0.54(0.32)	Ovenden et. al. 2009
<i>C. obscurus</i>	3	4	48-98	7.92(5.53)	0.73(0.14)	Ovenden et. al. 2009
<i>C. leucas</i>	4	5	124-145	19.15(9.00)	0.85(0.17)	Kark et. al. 2011
<i>Negaprion brevirostris</i>	3	4	2632	28.50(9.29)	0.79(0.08)	Feldheim et. al. 2001
<i>N. brevirostris</i>	4	9	80	19.80(NR)	0.81(NR)	Schultz et. al. 2008
<i>N. acutidens</i>	5	6	68	10.50(NR)	0.67(NR)	Schultz et. al. 2008
<i>Sphyrna lewini</i>	3	3	149-168	6.44(4.35)	0.54(0.35)	Ovenden et. al. 2009
<i>Ginglymostoma cirratum</i>	3	3	145-151	5.83(4.33)	0.58(0.20)	Kark et. al. 2012
<i>Prionace glauca</i>	4	5	64-118	6.60(4.43)	0.57(0.20)	Ovenden et. al. 2009
<i>Isurus oxyrinchus</i>	8	4	421-430	25.25(12.32)	0.87(0.05)	Schrey & Heist 2003
<i>Carcharodon carcharias</i>	2	5	95	7.00(3.69)	0.48(0.19)	Pardini et. al. 2001
<i>Carcharias taurus</i>	6	6	186	5.03(3.10)	0.54(0.28)	Ahonen et. al. 2009
<i>Squalus acanthias</i>	9	8	419	7.11(3.10)	0.61(0.04)	Verissimo et. al. 2011
<i>Rhincodon typus</i>	10	8	68	9.00(9.59)	0.69(0.20)	Schmidt et. al. 2009
<i>Galeorhinus galeus</i>	7	9	228-235	5.71(2.72)	0.61(0.19)	This study
<u>Batoidea</u>						
<i>Raja clavata</i>	11	5	214	9.72(7.00)	0.72(0.19)	Chevolut et. al. 2008
B						
Group of species	N loci	N sp	N ind	A	H_e	References
<u>Actinopterygii</u>						
Teleost freshwater	75	13	7755	9.10(6.10)	0.54(0.25)	DeWoody & Avise 2000
Teleost anadromous	43	7	5393	10.80(7.20)	0.68(0.12)	DeWoody & Avise 2000
Teleost marine	66	12	6005	19.90(6.60)	0.77(0.19)	DeWoody & Avise 2000
<u>Chondrichthyes</u>						
Elasmobranchs	86	17	6137	11.17(6.10)	0.64(0.19)	All cited in Table A

Chapter 5

Paternity assessment in school shark *Galeorhinus galeus* in New Zealand waters

5.1. Abstract

Sharks can be either monogamous or polygamous, which is important when considering stock assessments and harvesting models. Multiple paternity has been reported in several shark species, however, the number of sires per litter varies considerably among species. The aim of this study was to determine whether there was multiple paternity (MP) in *G. galeus* by assessing the levels of relatedness of progeny arrays using six polymorphic microsatellite DNA markers. Five “families” (mother and litters) were sampled from the North Island of New Zealand and a parentage analysis was conducted. The minimum number of males contributing to each progeny array was estimated by identifying the putative paternal alleles by manual allele counting and reconstructing multilocus genotypes method. The analysis showed the occurrence of genetic polyandry in *G. galeus*. Two of five litters showing multiple sires involved in the progeny arrays (40%). The minimum number of sires per litter ranged from one to four. Although MP was only detected in a two litters, this finding is consistent with the known reproductive characteristics of *G. galeus*. Females can potentially store sperm for long periods of time and the species has a specific mating season when males and females typically mix on the edge of continental shelf. Detecting MP within a litter has highlighted the importance of the post-copulatory selective processes in the *G. galeus* mating system, and this has implications for fishery management and conservation.

5.2 Introduction

Elasmobranch species have a range of reproductive modes such as oviparity, aplacental viviparity and placental viviparity (Wourms 1977, Wourms & Demski 1993, Grogan & Lund 2004). Within this group, species showed difference in ovarian cycles, gestation periods, and mating systems, but generally they exhibit a low reproductive potential e.g. slow growth rate, late age of sexual maturation, low fecundity. The *K*-selected features make elasmobranch vulnerable to fishing exploitation, caused by a reduction in reproductive capacity and effective population size (Smith et al. 1998, Stevens et al. 2000, Myers & Worm 2003, Dichmont et al. 2011). Fisheries managers and scientists have developed strategies to ensure the sustainable use of biological resource and maintain the productivity of a stock (Musick & Bonfil 2005). Crucial to management is a robust stock assessment (demographic and behaviour), which should include information about reproductive cycles and mating system (Rowe & Hutchings 2003).

Sharks litters can be either monandrous or polyandrous. Depending of the mating system, species can be affected in term of genetic variability and interbreeding, even varying the ability for adaptation within population (Sugg & Cheseer 1994, Frankham 2005). For instance, species that the number of males contributing to the gene pool is small, they lead to have very small effective population sizes and consequently make them vulnerable to overfishing. It is known that both mating strategies (i.e. monogamy and polyandry) have several benefit and cost. Thus, mating with multiple males may expose females to higher risks from sexually transmitted diseases, predation, copulation brutality, or other time or energy expenses associated with the mating process (Avise et al. 2002, Byrne & Avise 2012, Fitzpatrick et al. 2012). However, these risks could be outweighed by the benefits increased female fitness, for example as insurance against male sterility, offspring with more diverse genotypes, and inbreeding avoidance (Avise et al. 2002, Frankham 2005) These theoretical considerations suggest that promiscuity in elasmobranch can be genetically advantageous for females and males.

Molecular markers have been used to study mating systems and sexual selection. Fishes species have showed a diverse range of breeding behaviors and they have been a useful group for testing theories regarding mating systems and reproductive strategies using genetic markers e.g. microsatellite loci, in conjunction with behavior observations (Avise et al. 2002, Fitzpatrick et al. 2012). These finding have demonstrated that it is common for

females to copulate with multiple males (polyandry) and offspring are a mixture of full and half-sibs (Zeh & Zeh 2003). The evidence of polyandry in females of shark species has increased, and multiple mating is common reproductive strategy (see review in Byrne & Avise 2012, Fitzpatrick et al. 2012). Mating systems in elasmobranchs has been distinguished using: a) Direct behavioral observation of multiple mating in field described for *Ginglymostoma cirratum* (Carrier et al. 1994, Pratt & Carrier 2001), *Triaenodon obesus* (Whitney et al. 2004), *Manta birostris* (Yano et al. 1999), and *Dasyatis americana* (Chapman et al. 2003); and observation of captive sharks e.g. *Scyliorhinus rotifer* (Castro et al. 1988) and *Carcharias taurus* (Gordon 1993); and b) Genetic studies that assessed multiple paternity (MP) of offspring i.e. microsatellites loci are commonly used in several species such as: *Negaprion brevirostris* (Feldheim et al. 2001a, 2004, DiBattista et al. 2008), *Ginglymostoma cirratum* (Saville et al. 2002, Heist et al. 2011), *Sphyrna tiburo* (Chapman et al. 2004), *Carcharhinus altimus* and *C. galapagensis* (Daly-Engel et al. 2006), *Carcharhinus plumbeus* (Daly-Engel et al. 2006, 2007, Portnoy et al. 2007), *Squalus acanthias* (Lage et al. 2008, , Veríssimo et al. 2011), *Squalus mitsukurii* (Daly-Engel et al. 2010), *Rhincodon typus* (Schmidt et al. 2010), *Mustelus henlei* (Byrne & Avise 2012), and *Scyliorhinus canicula* (Griffiths et al. 2012), and one skate species, *Raja clavata* (Chevolot et al. 2007).

Microsatellite markers are used for genetic parentage analyses because they have a Mendelian pattern of inheritance (Avise 2002, Chapman et al. 2004). Basic analytical methods and sophisticated statistical approaches have been used for parentage assessment. Subtracting the more easily identified maternal alleles allows the paternal alleles to be identified, because the mother can usually be sampled at the same time as the offspring. Identifying the parental alleles in progeny has been used to determine the number of sires within the progeny array of a mother. Based on this notion, several reconstructions multilocus genotypes methods have been developed (e.g. GERUD software), which simulate increase the knowledge about mating system in several taxa such as birds, mammals, reptiles and elasmobranch species.

The school shark (*Galeorhinus galeus*) is a mobile and semipelagic/demersal shark species found globally in temperate waters of the eastern North Atlantic, western South Atlantic, north eastern and south eastern Pacific, South Africa, New Zealand and Southern Australia (Last & Stevens 2009). Commercially fished Australasia region where it is considered different stock across Tasman Sea (between New Zealand and Australia) for management purposes, but according to the results described in Chapter 2 (mtDNA) and 4

(microsatellite loci), *G. galeus* populations are composed of a single stock unit. Several stocks of *G. galeus* are in significant decline, prompting the species to be listed as vulnerable by the IUCN Red List of Threatened Species (Walker et al. 2006). In the Southwest Atlantic the *G. galeus* population is subject to intensive fishing such as: a) in Brazil and Uruguay, the CPUE has declined almost to zero; b) In Argentina, the CPUE for the trawler fleet has declined by around 80% during the past decade (Walker et al. 2006). In Australia, *G. galeus* fisheries have reduced the stock to less than 15% of initial biomass, raising serious concerns (AFMA 2009, 2010a, DSEWPaC 2012). The TAC dropped (total allowable catch) from 800 t in 2002 to 240 t in 2010 (AFMA 2010b). Proper management is critical because the low reproductive potential of *G. galeus* makes it particularly vulnerable to overexploitation.

Galeorhinus galeus, have internal fertilization and aplacental viviparity. Fecundity varies between 15-52 pups depending on the size and age of the mother (Olsen 1954, 1984, Walker 2005). Sexual maturity occurs later in females compared to males. Females reach maturity at about 13-15+ years (124-140 cm total length, TL), whereas in males it is at about 8-17+ years (125-135 cm TL) (Olsen 1954, 1984, Francis & Mulligan 1998, Walker 2005). The reproductive cycle in females is characterised by a long ovarian and gestation period estimated to be three years (triennial) (Olsen 1984, Peres & Vooren 1991, Walker 2005). Females give birth approximately every 3 years (Peres & Vooren 1991, Walker 2005). This means that *G. galeus* population will comprise females at three different reproductive stages: a) individuals with resting oviducts (after parturition) and vitellogenesis in the ovary, b) individuals with large mature follicles, oviducal gland and uteri ready for ovulation, and c) others with full-term embryos in the oviducts (Peres & Vooren 1991, Walker 2005). These portions mean that on average one-third of the mature females in a population will be producing the annual recruitment of newborns.

An understanding of *G. galeus* mating system is a fundamental requirement for any long-term, effective fishery management because the level of shark exploitation worldwide exceeds their reproductive capacity (Manire & Gruber 1990, Stevens et al. 2000, Baum et al. 2003, Myers & Worm 2003). Mating system information is important because reproductive strategies, such as polyandry, could make the population demographics more sensitive to fishing-induced mortality (Sugg & Cheseer 1994, Martinez et al. 2000, Chapman et al. 2004).

The goal of this chapter was to determine the pattern of parentage of five *G. galeus* families (mums and embryos) sample from North Island of New Zealand. The family groups were screened using six highly polymorphic microsatellite loci (see details in Chapter 4).

5.3 Methods

5.3.1 Sampling collection

Five *G. galeus* mother-litters groups (hereafter called a *Family*) were collected from two breeding grounds (Kaipara and Manukau harbours) located in the North Island of New Zealand. Four gravid females were captured using rod lines from Kaipara harbour and one female from Manukau harbour over two summer seasons in 2009 and 2012 (see details in Figure 5.1). Fins clips were taken from the mother and whole embryos were frozen and then transferred to the laboratory. The total length (TL) of each individual was recorded. In order to obtain reasonable sample sizes for the population genetic data of *G. galeus* (i.e. allele diversity), the tissues samples that were reported in Chapters II and IV from east of North Island (ENI, $n = 40$) were pooled with the genotyping data obtained from five pregnant females (KP, $n = 4$; MK, $n = 1$).

Figure 5.1: **A.** Collection location of *G. galeus* mother-litters groups (solid circles) of two putative breeding grounds on Kaipara (KP) and Manukau (MK) harbours, and east of North Island (ENI) samples used for population genetic analysis in this study. Numbers in brackets are the sample sizes. **B.** Table represents *G. galeus* gravid females (named as Mum) analysed in this study. Total length (TL) is showed in cm and LS represents litter size.

5.3.2 DNA Extraction and Genotyping

Genomic DNA was extracted from tissue samples using a standard phenol-chloroform protocol (Sambrook et al. 1989). Extracted DNA was checked for quality and quantified using a NanoDrop spectrophotometer (model ND-1000), and all samples were diluted to a standardised concentration of 50 ng/μL. All the individuals were genotyped using six microsatellite loci *Gg15*, *Gg22*, *Gg23*, *Gg12*, *Gg7*, and *Gg11*. All loci (*Gg22*, *Gg23*, *Gg12*, *Gg7*, and *Gg11*) were taken from Chabot & Nigenda (2011). These loci had a high level of allelic diversity, which is needed to detect MP in the pup arrays. All details about the microsatellite loci (i.e. primer sequences and PCR conditions) have been presented in Chapter 3 and 4. Microsatellite loci were PCR amplified separately in 10 μl volumes containing 67 mM Tris-HCL pH 8.8, 16 mM (NH₄)₂SO₄, 2.0 mM MgCl₂, 0.05 μM of forward primer labelled with M-13 tail, 0.2 μM of reverse primer, 0.05 μM of fluorescently labelled M-13 primers (FAM, VIC, or PET Invitrogen), 0.8 mM of each dNTPs, 0.3 units of BIOTAQTM DNA Polymerase (Bioline), and 1 μl of template DNA (50-80 ng/μl DNA concentration). Thermal cycling was performed on a TGradient Thermal Block (Biometra, Goettingen, Germany). Primer pairs and PCR conditions for each locus can be found in Table 4.1 (Chapter 4). PCR products from multiple loci were pool-plexed into a single 96-plate well (one set of products was comprised of four loci and another had five loci) based on the combinations recommended by the MULTIPLEX MANAGER 1.0 program (Holleley & Geerts 2009). Capillary separation of the fluorescently labelled PCR-fragments was conducted on an ABI3730 Genetic Analyser (Massey University Genome Service). The allele sizes were scored using the AUTOBIN program (<http://www4.bordeaux-aquitaine.inra.fr/biogeco/Ressources/Logiciels/Autobin>), which uses an Excel macro to sort and plot the relevant gaps between allele sizes (Guichoux et al. 2011). Whenever a new allele was detected in a mother and their offspring it was genotyped for second time to confirm the presence of a new allele. Although, it was not common to detect new alleles (9.4% of all sharks screened) the data quality was checked to avoid genotyping errors.

5.3.3 Statistical analyses

The microsatellite genotyping data of *G. galeus* collected from East of North Island (ENI, $n = 40$) were pooled with the genotyping data obtained from pregnant females resulting in 45 individual used in this study. MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004) was used to check for scoring errors and the presence of null alleles. Population genetic diversity indexes e.g. number of alleles per locus (A), observed (H_o) and expected (H_e) heterozygosities, exact tests for departures from Hardy-Weinberg equilibrium (HWE), and tests for pairwise Linkage Disequilibrium (LD) between loci were calculated in ARLEQUIN 3.1.1 (Excoffier et al. 2005). HWE significance was calculated using the exact test (Guo & Thompson, 1992) with a Markov chain of 10^6 steps and 10^5 dememorization steps. Significance values for LD were calculated using a likelihood-ratio test (LRT) of 20,000 permutations (Slatkin and Excoffier, 1996). Sequential Bonferroni corrections (Rice 1989) were applied when appropriate. Allele frequency distributions at each loci and progeny arrays and ENI population were generated using GENALEX 6.41 (Peakall & Smouse 2006).

The probability to detect and quantify MP within an offspring depend in the polymorphism of the microsatellite loci used in the study, the litter, the number of putative fathers and their corresponding reproductive success (Neff & Pitcher 2002). In order to compare the results of MP with other shark species, such as *Negaprion brevirostris*, *Ginglymostoma cirratum*, *Sphyrna tiburo*, *Squalus acanthias* and *Scyliorhinus canicula* (Feldheim et al. 2001, 2002, Chapman et al. 2004, Lage et al. 2008, Griffiths et al. 2012) two programs were used for the *G. galeus* multilocus genotypes data i.e. PRDM (Probability to detect multiple mating, Neff & Pitcher 2002) and GERUD 2.0 (Jones 2005). PRDM was used to estimate the power of detecting MP at six microsatellite loci. PRDM calculates the probability of detecting a multiple mating (fertilization) using the allele frequency distribution of each locus for ENI population and five pregnant females. Following similar simulations used in Chapman et al. 2004 and Byrne & Avise 2002, six scenarios for MP in *G. galeus* were tested: a) two males with equal breeding success (50% each), b) two males with unequal breeding success (66.7% and 33.3%), c) three males with equal breeding success (33.3% each), d) three males with unequal success (57%, 28.5% and 14.5%), e) four males with equal success (25% each), f) four males with unequal breeding success (50%, 27%, 14% and 7%). Because the probability to detect MP mating is also in function of the number of embryos analysed, PRDM simulation were ran with litter size ranging from 15 to 52 which is

the variation of the fecundity for *G. galeus* in Australasia region (Olsen 1954, 1984, Walker 2005) and it included the number of litters observed in this study (ranged from 23 to 34).

Two approaches were used for parentage analysis were carried out. Firstly, manual allele counting by constructing a locus genotype for each embryo in a spreadsheet data set. The observed maternal allele is known from genetic or behavioral evidence and paternally is in question. For each offspring at each locus, the maternal allele is inferred by subtraction for each locus in order to obtain its paternally derived alleles. The analysis is conducted by manual inspection of the dataset. The criterion to establish the occurrence of MP of a litter for both approaches was determined for the presence of more than two paternal alleles across at least two loci (Avisé et al. 2002, Chapman *et al.* 2004, Lage et al. 2008). Secondly, the program GERUD 2.0 (Jones 2005) was used to reconstruct the parental multilocus genotypes from progeny arrays. This new version uses an exhaustive search algorithm to determine the minimum number of parents necessary to explain a given half-sib progeny array with one or neither parental known. This search involves three steps: a) establish the paternal alleles for each offspring by subtraction (approach is explained above); b) determine all combinations of alleles observed in the progeny array that yield paternal genotypes consistent with the array; and c) find the better combinations of paternal genotypes that sired the progeny array. In order to have some idea which solution is most probably, they were ranked by likelihood of a particular combination of fathers. Probability scores base on Mendelian segregation were estimated for the fifty combination of father with highest probabilities. Additionally, GERUD2.0 was used to calculate the expected exclusion probabilities based on the allele frequency of the population ENI. This calculation provides an indication of the power of the microsatellite loci used for parentage assessment.

5.4 Results

There were no scoring problems or null alleles detected at the six microsatellite loci. The six microsatellite loci showed relatively high allelic diversity in the population sampled (ENI, $n = 40$ and pregnant females, $n = 5$). The number of alleles found range from 5 alleles for *Gg7* to 11 alleles for *Gg15* (Table 5.1). Five of the six microsatellite loci screened in this study were found to be in HWE. Only loci *Gg11* exhibited significant departures from HWE ($P < 0.001$) due to its deficit of heterozygotes observed. Pairwise combination for *Gg23* and *Gg7* loci showed a significant level of LD ($P < 0.001$). Exclusion probabilities from GERUD for each locus identified the majority of microsatellites were informative markers for parentage assessment with high values of combined (all loci) exclusion probability (>0.94).

Table 5.1: Allelic frequency distribution and genetic diversities at six microsatellite loci for 45 *G. galeus* sharks from New Zealand waters i.e. East of North Island (ENI) and mums collected in the present study. Number of sharks scored (N_i), Number of alleles (A), observed heterozygosity (H_o), expected heterozygosity (H_e), and the probabilities of Hardy Weinberg Equilibrium (P_{HWE}). The asterisk (*) means deviate from Hardy-Weinberg expectations nominally after sequential Bonferroni correction ($\alpha = 0.05/6 = 0.008$). Exclusion probabilities (Ep) estimated in GERUD is presented by each locus and across loci.

<i>Gg15</i>		<i>Gg22</i>		<i>Gg23</i>		<i>Gg12</i>		<i>Gg7</i>		<i>Gg11</i>	
152	0.037	240	0.023	264	0.202	284	0.083	296	0.022	334	0.136
154	0.061	242	0.488	266	0.155	286	0.036	298	0.200	350	0.455
156	0.024	244	0.012	268	0.083	288	0.357	308	0.156	354	0.159
158	0.098	246	0.023	270	0.381	290	0.226	310	0.533	358	0.011
160	0.122	248	0.163	272	0.071	292	0.119	312	0.089	374	0.045
162	0.159	250	0.279	274	0.060	294	0.083			378	0.193
164	0.024	260	0.012	276	0.036	296	0.036				
168	0.244			278	0.012	298	0.048				
170	0.183					310	0.012				
172	0.012										
174	0.037										
N_i	41	43		42		42		45		44	
A	11	7		8		9		5		6	
H_o	0.829	0.651		0.690		0.643		0.667		0.432	
H_e	0.860	0.663		0.782		0.798		0.650		0.718	
P_{HWE}	0.355	0.175		0.409		0.125		0.029		0.001*	
											<i>All loci</i>
Ep	0.871	0.580		0.772		0.795		0.592		0.675	1.000

In general, the microsatellite loci provided a good level of power of detecting MP for the majority of the families (ranged from 99.2% to 100%, Table 5.2). The probability to detect multiple mating increased with the litter size and the number of fathers. Considering this result and that litters studied comprised more than 20 embryos, the use of this six microsatellite loci allow to this study to make reliable inferences on the mating system of *G. galeus* in New Zealand waters. Exclusion probabilities from GERUD for each locus (Table 5.1) identify that the six microsatellite loci are informative to detect MP solution when more than one unique combination of fathers can explain the data.

Table 5.2: Probability of detecting MP (PRDM) values for six microsatellite loci used assuming six mating schemes and specific litter size for each *Family* and overall families.

Mating schemes (hypothesized paternal skew)	Litter size						
	15	23	25	29	34	40	52
2 males (50:50)	0.997	0.998	0.999	0.999	0.999	0.999	0.999
2 males (66.7:33.3)	0.992	0.998	0.998	0.999	0.999	0.999	0.999
3 males (33.3:33.3:33.3)	1.000	1.000	1.000	1.000	1.000	1.000	1.000
3 males (57:28.5:14.5)	0.999	1.000	1.000	1.000	1.000	1.000	1.000
4 males (25:25:25:25)	1.000	1.000	1.000	1.000	1.000	1.000	1.000
4 males (50:27:14:7)	1.000	1.000	1.000	1.000	1.000	1.000	1.000

A minimum estimation of three sires and two sires were found in the *Family1* and *Family2* based on the simple allele counting for the construction multilocus genotype (Table 5.3). All the rest of *Families* were considered to be single paternity genotypes. Despite of *Family2* exhibited evidence of at least two sires at locus *Gg22*, the offspring are considered to be product of single paternity genotypes due to it was supported by one locus rather than all loci showing this pattern (e.g. *Family1*). Using the six microsatellite loci to estimate the minimum number of sires using GERUD resulted in genetic polyandry found in two of five progeny arrays (40%, Table 5.4)

Table 5.3: *Galeorhinus galeus Families* (Mum = mother and Emb = embryo) genotypes consistent with MP. Samples were genotyped using all loci, but only the relevant genotypes are represented below. Paternal alleles are shown in bold by subtraction from the maternal alleles.

Sample	<i>Gg15</i>	Sample	<i>Gg22</i>	Sample	<i>Gg23</i>	Sample	<i>Gg12</i>	Sample	<i>Gg7</i>	Sample	<i>Gg11</i>
<i>Family1</i>											
Mum1	170, 170	Mum1	242, 248	Mum1	272, 278	Mum1	288, 288	Mum1	310, 310	Mum1	350, 350
Emb1	170 , 170	Emb2	248, 250	Emb2	264 , 272	Emb13	288, 298	Emb2	310, 312	Emb2	350 , 350
Emb12	154 , 170	Emb3	242 , 242	Emb3	266 , 278			Emb3	310 , 310	Emb3	350, 354
		Emb27	248, 256	Emb4	278, 350					Emb4	334 , 350
				Emb15	272 , 272						
				Emb26	268 , 272						
<i>Family2</i>											
Mum2	152, 170	Mum2	244, 250	Mum2	266, 272	Mum2	290, 290	Mum2	310, 312	Mum2	350, 350
Emb37	162 , 170	Emb37	250 , 250	Emb37	272 , 272	Emb2	290 , 290	Emb37	312 , 312	Emb37	350 , 350
Emb40	152, 158	Emb39	250, 256					Emb40	298 , 310	Emb39	346 , 350
		Emb53	244, 248								
<i>Family3</i>											
Mum3	168, 168	Mum3	242, 250	Mum3	270, 272	Mum3	284, 290	Mum3	310, 312	Mum3	350, 350
Emb72	168 , 168	Emb72	250 , 250	Emb37	270 , 270	Emb72	284 , 284	Emb72	310 , 310	Emb72	350 , 354
Emb73	154 , 168	Emb73	242 , 242			Emb73	284, 300				
<i>Family4</i>											
Mum4	152, 170	Mum4	242, 250	Mum4	264, 264	Mum4	288, 294	Mum4	310, 312	Mum4	334, 354
Emb96	162 , 170	Emb99	242 , 242	Emb97	264, 270	Emb72	288, 290	Emb97	310 , 310	Emb96	350 , 354
Emb97	152, 160			Emb103	264 , 264			Emb99	312 , 312		
<i>Family5</i>											
Mum5	158, 168	Mum5	242, 250	Mum5	268, 272	Mum5	290, 310	Mum5	298, 310	Mum5	350, 350
Emb122	154 , 158	Emb122	242 , 242	Emb122	272, 274	Emb122	288 , 310	Emb122	298 , 298	Emb96	350 , 350
Emb123	168, 170	Emb123	242, 248			Emb127	290 , 290	Emb123	298, 308		

Table 5.4: Summary of analysed *Families* (mums and offspring) of *G. galeus* genotypes consistent with MP inferred in GERUD2.0. The number of sires (# sires) were estimated by allele counting (AC) using a multilocus genotypes (see details Table 5.3), and GERUD. The number of solution (# solutions), number of progeny (# progeny) and the probability score (*PS*) were obtained from GERUD as well.

<i>Families</i>	Number of offspring genotyped	Allele counting	GERUD	
		Number of sires	Number of sires	Paternal skew
<i>Family1</i>	34	3	4	27/3/2/2
<i>Family2</i>	34	2	2	19/15
<i>Family3</i>	23	1	1	23
<i>Family4</i>	25	1	1	25
<i>Family5</i>	29	1	1	29

5.5 Discussion

This study provides the first evidence of multiple paternity (MP) in *G. galeus*. In comparison to the previous studies that assessed paternity in elasmobranch i.e. 15 species in 18 studies, this study showed one of the lowest sample size of litter analyzed (five litters), but one of the largest average per litter size (22.8) across live-bearing shark species, compensating the likelihood to detect MP (Table 5.1 in Supplementary material). Most of the elasmobranch species have shown evidence of MP with exception of *Carcharhinus galapagensis* (Daly-Engel et al. 2006) and *Rhincodon typus* (Schmidt et al. 2010), which the number of litters and number of progeny per litter were quite low in comparison other studies i.e. 1 litter by each species, *C. galapagensis* 7 embryos and *R. typus* 29 embryos analysed. The observed range of 1-4 or 1-2 sires per offspring found in *G. galeus* is consistent with the same values among other genetically surveyed elasmobranch species. Parentage analyses demonstrated that female *G. galeus* showed evidence of polyandrous mating with different males during copulation events. In the present study, it was found that both methods used to detect MP i.e. allele counting vs. GERUD generated similar maternal and paternal locus genotypes reconstruction. Despite the fact that the number of litters sampled was low (five litters), the large number of embryos by offspring (22.8 average litter size; see Table 5.1 in Supplementary material) allowed detection of MP in at least two (40%) of five litters of *G. galeus*. There were multiple sired by four males for *Family1*, and sired by two males for *Family 2*. The occurrence of MP in *Family1* was strongly supported by four loci (*Gg 23*, *Gg12*, *Gg11*, and *Gg22*) across the same individuals genotyped e.g. Emb2, Emb3, and Emb4 (see details Table 5.3). In contrast, the MP detected in *Family2* was only distinguished by the *Gg22* locus. While this may be questionable, the statistical power to detect MP in *Family2* was high (83-100%, Table 5.2) and simulations ran in GERUD, giving a good support for the suggestion that the paternal contribution was accurately detected. The result also suggested that polyandry and monandry are occurring with roughly equal frequency in *G. galeus* population in New Zealand waters where this species is comprised by single panmictic population (results Chapter 2 and 4).

Sexual segregation is a common behavioral characteristic among vertebrate species e.g. elasmobranch. Segregation patterns in elasmobranch may results differences between sexes as consequence of differential habitat use or different life-history stages (Wearmouth et al. 2012). Elasmobranch species are usually segregated according to age, sex, and reproductive stage (Grogan & Lund 2004). For example, they can use different habitat that

might vary in the prey availability e.g. *Triakis semifasciata* (Carlisle & Starr 2009), *Negaprion brevirostris* (Morrissey & Gruber 1993), *Carcharhinus limbatus* (Heupel & Hueter 2002); and temperature e.g. *Schroederichthys chilensis* (Fariña & Ojeda), *Carcharodon carcharias* (Robbins 2007), *Carcharias taurus* (Bansemer & Bennett 2009), *Urobatis halleri* (Jirik & Lowe 2012), and also *G. galeus* (Walker et al. 2008), where pregnant females at most stages of gestation are observed during the gestation period in warm waters of the Great Australian Bight and Eastern region of the south coast of Western Australia.

Tagging data and fishing surveys have provided evidence that movement of *G. galeus* in southern Australia is related to the reproductive cycles of this species. Pregnant females with large follicles in the ovary and several eggs without macroscopically visible embryos in the uterus are considered to be ovulating (Walker 2005). This can develop over period of several months during June-January. Overlapping with this time, it is also known that adult *G. galeus* in both sexes migrate to edge of the continental shelf for mating purposes during colder months i.e. July-September in South Australian and New South Wales waters (Olsen 1954, 1989, Walker 2005). Under these observations, it is possible that copulation in *G. galeus* population in Australia/New Zealand may occur a few months before the ovulation. For instance, copulation behaviour within *G. galeus* population is thought to occur five months before ovulation in western south Atlantic (Peres & Vooren 1991). Consequently, they most likely have a mechanism to maintain and prolong sperm storage for subsequently fertilization.

In many animals females have the ability to store sperm in their reproductive tract. It is thought that the sperm storage has evolved because the optimal copulation time did not match with the optimal fertilization time or birthing habitat location (Birkhead & Moller 1993). In elasmobranch species, the nidamental gland is the structure that provides the storage of sperm before the ovulation and enhances the opportunities for sperm competition and probability of successful fertilization (Pratt 1993, Pratt & Tanaka 1994, Maruska et al. 1996, Fishelson & Baranes 1998, Hamlett et al. 1998, Conrath & Musick 2002, Storrie et al. 2008, Galíndez et al. 2010). The function of this structure for sperm storage has also been reported for *G. galeus* non-pregnant female (mature ovary) from (Peres & Vooren 1991), and confirmed from smears of another non-pregnant female (immature ovary) from New Zealand waters (personal observation, see Figure 5.2 in Supplementary material). Under this scenario, it is not difficult to imagine that fertilization during repeated ovulations could be achieved by multiple males, and consequently multiple sired can be detected in the offspring. In fact, most

of the elasmobranch species surveyed to the date exhibits levels of MP in the progenies analysed. Only two shark species have showed single paternity i.e. *Carcharhinus galapagensis* and *Rhincodon typus* (see % litter with MP Table 5.1 in Supplementary material).

The length of reproductive cycle varies widely among elasmobranch species. *Dasyatis* species and *Urobatis jamaicensis* may have two or three reproductive cycles in a year, where species such *Chlamydoselachus anguineus* and *G. galeus* can have 3 years or longer. Females of the school shark *G. galeus* have a well-defined triennial parturition frequency (Peres & Vooren 1991, Walker 2005). It means that one third of the mature females give birth every year and implies that the gestation period in *G. galeus* is highly synchronized with all phases from ovulation to birth (Peres & Vooren 1991, Walker 2005). Therefore, all gravid females should typically contain embryos in the same stage of develop. Consequently, it would be expected that there is synchrony in the mean total length (TL) of embryos, resulting two different groups: one with eggs present in uteri without macroscopically visible embryos present (0 mm TL, $n = 54$ pregnant females), and b) embryos macroscopically visible (approximately 150-300 mm TL, $n = 65$ pregnant females) observed at a specific time. This observation is consistent with the mean embryo length observed in the present study that were obtained at different years but at same season i.e. later spring (November 2009 for Mum3) to summer (February 2009 for Mum1, 2 and January 2012 for Mum 4 and 5). The synchrony of the five *G. galeus* females for both pregnant conditions is illustrated in the Figure 5.1 in Supplementary material. This graph is shown that the mean TL of embryos from four females (Group 1) was consistent with pregnant condition expected, and it is also consistent with second pregnant condition of one female with embryos at full-term and prepared for parturition (Group2). Nevertheless, macroscopically visible embryos of *G. galeus* are present the 12 month period from February to January with most attain a size of more than 300 mm TL at full term (Walker 2005). Within shark species it is common that a population exhibits synchronous growth of embryos over a period of ~12 months, particularly among inhabiting water < 300m such as *Mustelus lenticulatus* (Francis & Mace 1980), *Mustelus mento* (Smale & Compagno 1997), *Mustelus manazo* (Yamaguchi et al. 1997), *Mustelus canis* (Conrath & Musick 2002), *Mustelus antarcticus* (Walker et al. 2007), and also previously reported for *Galeorhinus galeus* (Walker 2005).

There is a positive relationship between uterine fecundity or number of in utero embryos and maternal body length (Musick & Bonfil 2005). It is though that as a female

becomes larger this increment results in a larger space in the body cavity to maintain embryos. This linear positive relationship is reported in population of *Sphyrna lewini* (Chen et al. 1988), *Squalus acanthias* (Hanchet 1988), *G. galeus* (Olsen 1984, Peres & Vooren 1991, Walker 2005). However, the linear regression values have generally low r^2 , so it may not be a very accurate predictor of fecundity. Nevertheless, because there is a tendency that smallest pregnant females carried fewer embryos (TL =144.5cm; 17 embryos) than a largest females (TL = 159cm; 41 embryos), it is possible that MP might be driven by increasing fecundity. This understanding is based on the MP observed from two *G. galeus* females, which were the largest females sampled here (163.2 and 157.8cm TL). However, further investigation and larger sampling size of pregnant females are needed to test the difference in litter size between monandrous (SP) and polyandrous litters (MP).

In summary, this study has shown that MP is common in the aplacental viviparous shark species *G. galeus* in New Zealand waters. This finding was consistent with many aspects of its reproductive biology, included prolonged breeding season and potential for sperm storage. The MP results may be a consequence of coactive mating behavior by males facilitated by a high male encounter rates experienced during breeding aggregations. However, future investigations should use larger sample size to more thoroughly understand this component of mating system in *G. galeus* population in New Zealand and Australian waters.

5.6 Appendices

Supplementary material

Supplementary Table 5.1: Summarize of the genetic mating systems studies reported to the date in elasmobranch species classified by reproductive strategy (RS), which are indicated as oviparity (O), aplacental viviparity (AV), and placental viviparity (PV). The number of microsatellite loci (No. of msat), number of litter (No. litter analysed), average number of litter size (Average litter size), number of progeny (No. progeny analysed), range of sires per litter, and percentage of litter with MP (% litter with MP) are reported for each species. The asterisk indicates the only study that use MHC II α locus instead of microsatellites loci.

Species	RS	No. of msat	No. litter analysed	Average litter size	No. progeny analysed	Range of sires per litter	% litter with MP	Reference
<u>Selachii</u>								
<i>Carcharhinus altimus</i>	PV	8	1	9	9	2	100	Daly-Engel <i>et. al.</i> 2006
<i>Carcharhinus galapagensis</i>	PV	8	1	7	7	1	0	Daly-Engel <i>et. al.</i> 2006
<i>Carcharhinus plumbeus</i>	PV	8	1	7	7	2	100	Daly-Engel <i>et. al.</i> 2006
<i>C. plumbeus</i>	PV	6	20	5.5	110	1-2	40	Daly-Engel <i>et. al.</i> 2007
<i>C. plumbeus</i>	PV	5	20	9.4	187	1-4	85	Portnoy <i>et. al.</i> 2007
<i>Galeorhinus galeus</i>	AV	6	5	22.8	145	1-4	40	This study
<i>Ginglymostoma cirratum</i>	AV	1*	1	1	32	4	100	Saville <i>et. al.</i> 2002
<i>G. cirratum</i>	AV	12	3	33.3	100	5-7	100	Heist <i>et. al.</i> 2011
<i>Mustelus henlei</i>	PV	4	14	13.2	185	1-3	93	Byrne & Avise 2011
<i>M. antarcticus</i>	AV	8	29	11.3	328	1-4	24	Boomer <i>et. al.</i> 2013
<i>M. lentiginatus</i>	AV	8	19	3.9	74	1-3	13	Boomer <i>et. al.</i> 2013
<i>Negaprion brevirostris</i>	PV	3	1	1	11	3	100	Feldheim <i>et. al.</i> 2001a
<i>N. brevirostris</i>	PV	9	97	7.3	707	1-4	87	Feldheim <i>et. al.</i> 2004
<i>N. brevirostris</i>	PV	11	85	4.8	408	1-4	81	DiBattista <i>et. al.</i> 2008
<i>Rhincodon typus</i>	AV	9	1	1	29	1	0	Schmidt <i>et. al.</i> 2010
<i>Scyliorhinus canicula</i>	O	12	13	11.5	150	1-4	92	Griffiths <i>et. al.</i> 2012
<i>Sphyrna tiburo</i>	AV	4	22	8.5	188	1-3	19	Chapman <i>et. al.</i> 2004
<i>Squalus acanthias</i>	AV	7	10	5	50	1-2	30	Lage <i>et. al.</i> 2008
<i>S. acanthias</i>	AV	7	29	4-11	NR	1-2	17	Verissimo <i>et. al.</i> 2011
<i>S. mitsukurii</i>	AV	8	27	6.6	178	1-2	11	Daly-Engel <i>et. al.</i> 2010
<u>Batoidea</u>								
<i>Raja clavata</i>	O	5	4	43.3	173	4-6	100	Chevolot <i>et. al.</i> 2007

Supplementary Figure 5.1: Plot of the five progeny analysed based on the mean embryo total length (TL) of embryos collected from pregnant females.

Supplementary Figure 5.2: Smears of nidamental gland of *G. galeus* no gravid with immature ovary. Both figures are the same but they are pointed different spermatozoa. The white dash line represents the orientation of each spermatozoa, and arrows indicate the head and tail of the spermatozoa.

Chapter 6

General Discussion

6.1. Genetic diversity and demographic history

Population genetics has provided important insights for fisheries science, by understanding levels of genetic variation and the boundaries of reproductive units. Population genetic analyses have two main goals: to determine the level of genetic variation and the amount of differentiation among sample sites. Neutral molecular markers, such as mitochondrial DNA (mtDNA) and microsatellite DNA (msatDNA), are typically used because they can be applied across a range of species and the evolutionary mechanisms that influence these genomic regions are relatively well understood. The DNA sequence variation that is detected using mtDNA enables gene genealogies to be estimated and phylogeographic methods to be applied. Various mtDNA regions have been used for marine fish studies such as COI, ATPase, ND2, ND4, CytB, and CR. Most of the studies have concentrated in the CR because it often shows high levels of polymorphism compared to other mtDNA genes (Dudgeon et al. 2012). However, there are exceptions this generalisation about higher levels of diversity; other gene regions such as ND2 in *Squalus acanthias* (Veríssimo et al. 2010, O'Brien et al. 2013); and ND2, ATPase and COI in *Stegostoma fasciatum* (Dudgeon et al. 2009) can be more variable. In contrast, msatDNA markers show differences in the size of alleles because of differences in the number of repeat units of alleles. Genealogical methods are not directly applicable to microsatellite DNA markers, but they can have more statistical power because each locus is usually an independent sample of variation in a genomic region. Microsatellites have had some success when studies have attempted to amplify the same (or similar) loci in closely related species (Guichoux et al. 2011, Dudgeon et al. 2012). However, most population genetic studies of elasmobranch are based on species-specific microsatellite loci that were designed for each species (see list Table 3.1 in Supplementary Material, Chapter 3).

The patterns of genetic diversity that have been detected using mtDNA and msatDNA marks have shown similar result across different marine species. The findings were similar between marine teleost species (Actinopterygii) and Elasmobranch species (sharks and skates). These fish studies reported levels of variation based on methods that involved RFLP-mtDNA, DNA sequencing of mtDNA regions (i.e. COI, CytB, and CR; Figure 6.1), and

msatDNA (Figure 6.2). The results reported in Chapters 2 and 4, showed that the CR mtDNA and msatDNA had high levels of genetic diversity in *G. galeus* in comparison the other marine fish species. Based on the estimates of haplotype and nucleotide diversity for CR mtDNA, the genetic variation found in *G. galeus* ($h = 0.79$, $\pi = 0.0014$) was higher than coastal shark species such *Ginglymostoma. cirratum* ($h = 0.16$, $\pi = 0.0001$; Karl et al. 2012), *Carcharhinus leucas* ($h = 0.33$, $\pi = 0.0006$; Karl et al. 2011), and *Negaprion brevirostris* ($h = 0.34$, $\pi = 0.0004$; Schultz et al. 2008). However, this was lower than pelagic species such as *Lamna ditropis* ($h = 0.99$, $\pi = 0.007$; O'Brien et al. 2013) and *Rhincodon typus* ($h = 0.95$, $\pi = 0.02$; Ramírez-Marcías et al. 2007). In addition, similar patterns of genetic diversity in *G. galeus* were observed for the expected heterozygosity and allele richness ($H_e = 0.61$, $R_S = 5.71$) that were estimated using the microsatellite loci. The levels of genetic variation reported for *G. galeus* were higher than reported for *Carcharhinus plumbeus* ($H_e = 0.28$, $R_S = 2.33$; Heist & Gold 1999), *Carcharodon carcharias* ($H_e = 0.48$, $R_S = 7.00$; Pardini et al. 2001), *G. cirratum* ($H_e = 0.58$, $R_S = 5.83$; Karl et al. 2002), but lower than *Rhincodon typus* ($H_e = 0.69$, $R_S = 9.00$; Schmidt et al. 2009), *N. brevirostris* ($H_e = 0.79$, $R_S = 28.50$; Feldheim et al. 2001), *C. leucas* ($H_e = 0.85$, $R_S = 19.15$; Karl et al. 2001), *Isurus oxyrinchus* ($H_e = 0.87$, $R_S = 25.25$; Schrey & Heist 2003). Genetic diversity values were shown in Table 2.1 for CR mtDNA (Chapter 2) and Table 3.1 for msatDNA (Chapter 3) in Supplementary Material.

Interestingly, the differences between the genetic diversity values observed in a same species were extremely different when comparing both molecular marker (mtDNA and msatDNA). For instance, lower variability for CR mtDNA is found for *Carcharhinus leucas*, *Negaprion brevirostris* ($h = 0.33$, $\pi = 0.001$ and $h = 0.34$, $\pi = 0.001$, respectively), whereas higher expected heterozygosity and allele richness were observed in both species ($H_e = 0.85$, $R_S = 19.15$; $H_e = 0.79$, $R_S = 28.50$, respectively) (see Figure 6.1 and 6.2). The microsatellite loci screened in these species (*C. leucas* and *N. brevirostris*), and including *Isurus oxyrinchus* were characterised by higher number of motif repeat e.g. (TG)₁₉, (GT)₂₀, (AC)₃₃ that were highly polymorphic with a large allelic range (Schrey & Heist 2003, Feldheim et al. 2001, Karl et al. 2011). This feature and the uninterrupted motif microsatellite enable more alleles to be distinguished and consequently improved the estimates of genetic diversity, resulting in different magnitude of genetic diversity when several loci are compared. This may also be attributed to differences in the modes of inheritance, where mtDNA is maternally inherited and nDNA is biparentally inherited.

The estimates of genetic diversity also contains information about past population history. The mtDNA (e.g. CR, ND2, and CytB) and nDNA (microsatellite loci) markers were used to elucidate the demographic history of populations and compared to other species. Because mtDNA data is made up of discrete DNA sequence states and the molecule does not undergo recombination, it can be used to reconstruct major demographic events in recent population history (Heist 2005, Nielsen et al. 2009). For instance, a comparison can be made between the haplotype and nucleotide diversity estimated from CR mtDNA data and compared among shark species (see details Chapter 2, Table 2.1 and Figure 2.1 in Supplementary material). Three historical demographic categories can be described and these indicate the state of a population population size after the impacted in the contraction, if one occurred in recent history. Mitochondrial DNA analyses also allows for some insight into how distribution, habitat and behavior of species might have shaped the genetic patterns. The global glaciation events that occurred over the last 1.8 millions years are often suggested to have been a major influence on recent population history (Karl et al. 2011, O'Brien et al. 2013).

The Pleistocene was characterized by repeated glacial cycles separated by short interglacial periods (Lamberck et al. 2002). During glacial periods, marine systems experinced changes in temperature and sea levels were lower e.g. largest drop ~ 130 m (Clark et al. 2009), which resulted in the reduction of coastal habitats and most likely large disruptions to the genetic connectivity among populations that was established during the interglacial periods. (Herbet et al. 2010) Phylogeographic studies using mtDNA have inferred much about the survival of population fragments in glacial refugia followed by post-glacial the expansion and secondary contact of populations and re-colonization of habitats (Hewitt 2004). The restriction of small populations to glacial refugia may have often led to high level of genetic differentiation and post-glacial secondary contact of these populations can be detected in modern population whenever a sample contains a mix divergent mtDNA lineages. For example, a recent phylogeographic analysis identified three distinct clades within populations *Carcharhinus amboinensis*, which was probably the result of isolation during the Pleistocene and continued restricted gene flow across contemporary northern Australia (Tillet et al. 2012). Two clades were found in *Raja clavata* from European populations suggesting that the British waters were a site of secondary contact after of repeated colonization from at least two ice-age refugia i.e. Iberian Peninsula and Azores (Chevolot et al. 2006). For *G. galeus*, the analyses of genetic data suggested that *G. galeus* in New Zealand/Australia

population have historically been undergoing a period of expansion well before the post-glacial warming and did not go through any severe population contraction during recent Pleistocene history.

Figure 6.1: Relationship between the haplotypes (h) and nucleotide diversity (π) surveyed using RFLP-mtDNA, *COI*, *CytB* and *CR* of several population of teleost marine species (black circles) reported in Grant & Waples 2000 and Varela 2012, and for population the CR sequences for 23 shark species (grey circles). The shark species labelled represents a particular population with high sample size (>30).

Figure 6.2: Relationship between the expected heterozygosity averaged and number of alleles surveyed using microsatellite loci of Teleost marine species (black circles) reported in DeWoody & Avise 2000 and Varela 2012, and for 16 elasmobranch species (grey circles). The values of number of alleles and expected heterozygosity were obtained from Table 4.2 in Supplementary Material. The dash line showed the expected relationship of heterozygosity to the effective number of alleles reported in Figure 2 according to Crow & Kimura (1970) in DeWoody & Avise 2000.

6.2. Genetic differentiation and population structure

Population genetic studies are usually implemented to assess the level of genetic connectivity among populations. Migrants moving and being reproductively successful with the residents at the new location determine level of population differentiation. Differences in gene frequencies among locations can be used indirectly to estimate patterns of gene flow and therefore the population structure of the species. For instance, the finding of genetic connectivity is more common in marine species than freshwater species because of a lack of barriers to migration in marine systems. A general conclusion is that population structure is particularly weak in highly mobile pelagic species (Waples 1998). The amount of genetic differentiation among populations is driven by population size, dispersal power, and the distance between the habitats occupied by different groups of individuals; genetic divergence is more often found in species that have limited amounts of suitable habitat that has a patchy distribution. Coastal shark species generally showed genetic differentiation across the large (but open) distances of oceans, but little or weak genetic differentiation along continental shelves.

Several examples of population genetic connectivity in shark species are discussed in Chapter 2 and 3 for CR mtDNA and nDNA (microsatellites). Based on that review of the literature, *G. galeus* was found to be a good example: populations from New Zealand and Australia are genetically connected across the Tasman Sea (see results Chapter 2 for mtDNA and Chapter 4 for nDNA). These results suggested that the *G. galeus* population has few barriers to reproductively successful migration, which was a finding consistent with their description of movements based on tagging studies (Olsen 1989, Hurst et al. 1999, Walker et al. 2008). However, New Zealand/Australian *G. galeus* populations appear to be separated across South Pacific Ocean and if there is any trans-Pacific migration it does not result in reproductively successful migrants. It is important to note that the molecular markers have detected patterns of gene flow that have become established over long periods of time (thousands of years). If that pattern has recently changed (e.g. within the last 20-30 years) then it would be hard to detect without very large sample sizes or until the evolutionary process of migration led to more widespread genetic homogenization. Nevertheless, these results have given an important understanding of the level of population structure or genetic connectivity (opposite patterns) in *G. galeus*.

6.3 Fisheries management

Strategies of management of living marine resource are strongly needed to ascertain the long-term maintenance of fisheries. A principal challenge for fisheries managers includes the development of strategies and decisions based on robust scientific evidence. The understanding of the life history and reproductive parameters are important for determining the best fisheries management response to a stock. Studies of stock structure are based on the notion of an ideal biological unit ('the stock') are usually determined using direct or indirect methods for detecting individual movements. Direct methods like surveys of egg and larva abundance, and tagging data (mark/recapture) provide are useful for understanding the movements in individual sharks (Kohler & Turner 2001). However, these methods are unable to determine whether immigrants to a stock will survive and reproduce. For this reason, it is important the use of genetic markers that provide an indirect estimation of the relative reproductive contribution of migrants among stocks areas.

Molecular genetic approaches provide a powerful tool for addressing questions of genetic structure and connectivity of marine fishes. The use of these techniques have increased rapidly and given significant insight into fisheries stock structure. These studies are important because it is possible to identify stock boundaries, determine the spatial dimension of the stock (genetic connectivity), and describe the level of genetic diversity. Genetic diversity is important to manage because over the long-term is preserves evolutionary resilience, and provide the diversity, via gene flow, to other depleted stock (Carvalho & Hauser 1994, Laikre et al. 2005). However, determining whether two stocks are from a single panmictic or from two separate stocks is not always easy and the results may not provide much assistance for management purposes.

Several fisheries management issues are often raised from genetic structure studies. When genetic differentiation between stocks has been detected there is a higher degree of certainty that both units are reproductively isolated. A difference can be easily detected using only a small sample size e.g. *G. galeus* samples from Chile ($N = 16$) was sufficient to detect genetic differentiation from New Zealand/Australia (Chapter II, Figure 5-20 individuals per population). This finding will be useful information for fishery managers. However, if no genetic differences are detected, it could be because either there is only one stock or there are two stocks that the genetic markers failed to detect. The uncertainty of this outcome is more to use for management purposes. There was uncertainty because: a) the type of genetic

marker used may not have enough resolution to detect a difference when they are two separate stocks; b) the genetic difference between populations could have arisen only recently, which means the pattern cannot yet be seen in the genetic (Group II and III, Figure 6.3); or c) only a small amount of gene flow is necessary to homogenize genetic variation among populations, especially among large populations (e.g. marine species) where drift is weak and differentiation can be relatively low. Only a small numbers of migrants ($n < 10$, Waples 1998) are need to be reproduce successful after moving between New Zealand and Australia populations. At this point, the level of gene flow at different rates i.e. 1%, 5%, 10%, 20%, 50% can be enough to show non-genetic differentiation (Group II, III, IV, Figure 6.3). However, those migration rates could have different consequences for stock assessment model. Consequently, fisheries genetics results have a degree of uncertain and may not be useful for fishery management purposes in some circumstances.

A lack of genetic differentiation at presumably neutral DNA markers such CR mtDNA and microsatellite loci, does not constitute proof of no genetic differentiation. The idea of a large and homogeneous panmictic stock between New Zealand and Australia is well supported for other sources of scientific information. The results of this thesis research were consistent with earlier genetic studies based on allozyme and RFLP-mtDNA and tagging data. However, The New Zealand and Australian *G. galeus* stock have so far been managed separately and assessment models used have estimates of spatial structure of the population derived from catch rates and tag /recapture data, the age-specific growth characteristics, pupping and recruitment processes, and the gear type employed in the fishery (Punt et al. 2000, 2005, MPI 2012). *G. galeus* is an example of a commercially species that straddles political and/or management boundaries with not joint management plan. Because administrative and logistic reasons school sharks in Australasia are considered as separate stocks. This is suggested to be more precautionary for *G. galeus* in Australia because if the assumption is made of one single stock, there is no reason to reduce the fishing rates in Australia (Waples et al. 2008). It is uncertain whether the regional management units are adequate for *G. galeus*; moreover the stock is depleted in Australia but the fishery is considered sustainable in New Zealand. Additionally, it is also concerning that the apparent sustainability of *G. galeus* biomass in New Zealand waters has been used to maintain of the high TAC levels through the last decade (>3000 t.). In Australian waters the historical nurseries areas have reduced and/or stopped to produce newborn young or pupping activity

e.g. Port Phillip, Port Sorell, and St. Helen (Stevens & West 1997), and most of the productive nurseries are in New Zealand waters.

Figure 6.3: Stages of population structure process for *G. galeus* in New Zealand and Australia **I.** Single panmictic population of *G. galeus* in New Zealand and Australia; **II.** and **III.** More or less genetic variation start appear recently between New Zealand and Australia; **IV.** and **V.** New Zealand and Australia comprise of two genetically different stocks. Blue colour means the relative amount of gene flow.

6.4 Recommendation for *Galeorhinus galeus* management

The new information presented in this thesis study is intended to inform management of *G. galeus* in the Australasian region. Following are four management considerations:

1. Genetic diversity ensures the evolutionary potential (ability to adapt to environmental changes) of the population and may buffer the shark population against climate change impacts. Stock sizes should be kept at current levels or higher to maintain the genetic diversity and genetic structure of New Zealand/Australian *G. galeus*. However, in New Zealand *G. galeus* stock size seem high and stable where in Australia the stock size is low and in collapse. Even though they are biologically one single stock - intermixing across geographical and political boundaries - the fishery status of the species is quite different in both countries, even in different part of each country. Therefore, they may need to be managed separately. For example, there are eight *G. galeus* management units (QMAs) in New Zealand that have separated TAC (total allowance catch). Even though, it is know that this species is a single stock in New Zealand, the TAC are allocated differentially in order to prevent any local depletion of *G. galeus* (e.g. protect aggregations of breeding females in Kaipara Harbour). In addition, treating New Zealand /Australia as a single stock is not practical because there would be no reason why to reduce the TAC in Australia. Consequently, different management approaches are needed.
2. The genetic information of a single biological stock should be integrated into the stock assessment models.
3. High-resolution genomic approaches (i.e. single nucleotide polymorphism, SNPs) should be considered for future genetic studies because they are able to detect gene flow and adaptive genetic variation. Genomics will enable functional differences (adaptive loci) of groups of individuals within the Australia/New Zealand stock to be detected. A detailed picture of the stock structure might enable a more precise set of area-specific management decisions.
4. Genetic monitoring of the New Zealand/Australian stocks should be conducted and emphasis should be put on sampling of neonates, pups, and juveniles. This approach will enable the genetic connectivity of different life stages to be

determined and the degree of site fidelity to be properly defined.

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