Population structure, temporal stability and
seascape genetics of two endemic New Zealand

Pleuronectiformes,

Rhombosolea plebeia (sand flounder)

and

R. leporina (yellowbelly flounder)

by

Heather B. Constable

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Victoria University of Wellington
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This thesis was conducted under the supervision of

Dr. James Bell,

Prof. Jonathan Gardner

&

Dr. Peter Ritchie

Victoria University of Wellington

Wellington, New Zealand

Victoria
UNIVERSITY OF WELLINGTON

*Te Whare Wānanga
o te Ūpoko o te Ika a Māui*

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Abstract

New Zealand's coastal waters are an integral part of the social, economic and environmental heritage of this Pacific archipelago. Evolving in isolation for 82 million years under volatile tectonic action and volcanism, the marine biogeography of New Zealand is complex and diverse. Many hypotheses have been proposed to explain the subdivisions of biogeographic areas based on species distributions, habitat and population genetics. In this study, I test whether there is differentiation in coastal population connectivity between northern and southern provinces, the location of the break and what environmental factors may explain the patterns observed.

Sandy, soft bottom and estuarine ecosystems make up a large proportion of the coastline, but are not well-represented in population genetic studies in New Zealand and internationally. I chose *Rhombosolea leporina* (sand flounder) and *Rhombosolea plebeia* (yellowbelly flounder) as endemic, commercially and traditionally important inhabitants of the shallow coastal waters and estuaries to explore levels of gene flow among most of the marine biogeographic regions of the New Zealand mainland.

The goal of this thesis research was to (1) develop polymorphic DNA microsatellite markers and (2) investigate the population genetic patterns at multiple spatial scales. Although these species have a relatively long pelagic larval duration (PLD) of ~70 days, I found a significant level of population structure for both species. There was a pattern of isolation by distance and a north to south break in connectivity on the east coast for *R. plebeia*, but an east to west disjunction in *R. leporina*. There was no evidence of a north to south genetic break in *R. leporina*, however populations on the south east coast of the South Island were significantly differentiated in both species.

A test for temporal effects (3) of genetic variation was conducted to determine whether spatial patterns of differentiation were consistent across multiple sampling seasons and age classes. Aspects of the sweepstakes recruitment success (SRS) hypothesis were tested by examining differences in allele frequencies and levels of

genetic diversity as a function of time. The analyses found evidence of temporal stability between years and between juveniles and adults.

Lastly, (4) the coastal and estuarine environmental variables were modelled using information from two public GIS datasets and several measures of genetic differentiation. The aim of this chapter was to determine which environmental and geospatial factors showed a significant level of correlation with the spatial genetic patterns reported in the earlier studies. For *R. leporina*, latitude, sediment and current speeds were significantly correlated with the genetic estimates of F_{ST} , F'_{ST} and Jost's D . In *R. plebeia*, a correlation was found between latitude, longitude, sediment, current speeds, sea surface temperature and width of the estuary mouth. The results of the modelling study suggest avenues for further research using candidate genes, such as heat shock proteins and rhodopsin.

This was the first study of New Zealand pleuronectids using a multidisciplinary approach with microsatellite DNA markers, GIS, and an array of bioinformatics software to study coastal connectivity on multiple spatial and temporal scales. Significant genetic structuring was found among populations of animals that are potentially well connected through continuous sandy, soft bottom environments and a long PLD. Despite similar life histories and ecologies, the two species were quite divergent in that there was little cross amplification of markers, different patterns of genetic structure and separate outcomes from environmental modelling. These results suggest that managing several species under one management plan may be an oversimplification of the complexities of the population dynamics and evolutionary histories of these species. Conservation and management options for coastal fisheries and possible avenues for future research are proposed.

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This work is dedicated to my father, Bryan Constable, who inspired and encouraged me to pursue a life of curiosity, respect and service to the natural world.

“The Flounder”

Illustration by Günter Grass

“It has always been my private conviction that any man who puts his intelligence up against a fish and loses had it coming.”

-John Steinbeck

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Chapter 1. Introduction

The world's marine ecosystems are under enormous anthropogenic pressure from overfishing, pollution, debris, ocean acidification and invasive species (Scavia et al. 2002, Hewitt et al. 2004, Halpern et al. 2007). Over 70 percent of the world's fish stocks are fully or over exploited (Botsford et al. 1997) and the number continues to rise (Kappel 2005, Pauly et al. 2005, Food and Agriculture Organization of the United Nations, FAO 2012). Predatory fish are disproportionately affected, with as few as 10% of virgin stocks remaining (Myers and Worm 2003). These effects are becoming more apparent and are commonly featured in the mainstream media. It could be argued that in this point in history, we still only have a rudimentary understanding of marine ecosystems.

The study of marine population connectivity is crucial to our understanding of the replenishment of fisheries (Shaklee and Currens 2003) and how genetic diversity is maintained. These same mechanisms of exchange of genes are key to the potential recovery from a local disturbance (Hughes et al 2010) and effectiveness of marine protected area design (Palumbi 2003, Berumen et al. 2012). On a deeper time scale, maintenance of population structure and local adaptation is the basis for the

mechanisms species' evolution in the ocean (Palumbi 1994, Hilbish 1996, Rocha 2007).

Marine dispersal and recruitment

Genetic patterns can elucidate demographic patterns of connectivity and allow broad biogeographic hypotheses to be tested (Ryan and Plumbi 2010, Riginos 2011). Such data can show distinct populations, evidence of clines of reduced gene flow with increased geographic distance, termed isolation by distance or panmixia (Laikre et al. 2005). Strong population structure has been found for marine species, for example, the California Grunion (*Leuresthes tenuis*) (Byrne et al. 2013) and the spiny sea star in the New Zealand's Fiordlands (*Coscinasterias muricata*) (Perrin et al. 2004). Based on the island model of dispersal, adjacent populations are more closely related than distant ones (Wright, 1943). This spatial pattern has been shown in the purple sea urchin *Stronglyocentrotus purpuratus* in the Indo-West Pacific (Palumbi 1997), the barnacle *Balanus glandula* along the California coast (Sotka 2004) and copper rockfish *Sebastes caurinus* along the West Coast of Canada and the United States (Buonaccorsi 2002).

Panmixia has been demonstrated over large geographic areas in many species, such as the European eel *Anguilla anguilla* (Palm et al. 2009), the Japanese eel *Anguilla japonica* (Han et al. 2010), the Indo-Pacific sleek unicorn fish *Naso hexacanthus* (Horne and Herwerden 2013) and the triplefin *Grahamina gymnota* (Hickey et al. 2009). In some cases, populations that were once thought to be panmictic were found to have structure when the subject was revisited using larger sample sizes or more sensitive genetic markers, as in the case of *Jasus edwardsii* (Thomas and Bell 2013) and *Perna canaliculus* (Wei et al. 2013).

Given that a large majority of marine animals have a pelagic dispersal stage followed by a demersal adult stage (Nybakken and Bertness 2005), the duration of the pelagic stage may indicate how connected populations are (Cowen and Sponagule 2009, Shanks 2003). Animals with a long pelagic larval duration (PLD), on the scale of months to years, have greater potential for being transported further away from their location of origin, hence connecting spatially separated populations demographically and genetically. It was once thought that PLD could be used as a

proxy for dispersal distance, hence animals with a shorter PLD probably have more genetic differentiation between populations than those that disperse further (Shanks 2003). Therefore, we would expect a positive relationship between pelagic larval duration and dispersal distance.

However, there are too many exceptions to this rule of thumb that render predictive ability of this hypothesis useless (Shanks 2009, Weersing and Toonen 2009). For example, the kelp rockfish (*Sebastes atrovirens*) are live-bearing fish that are strongly associated with kelp, yet no population genetic structure was found in this species over 800 km (Gilbert-Horvath et al. 2006). On the other end of the spectrum, the New Zealand rock lobster (*Jasus edwardsii*) has the longest known PLD of any marine species (> 2 years), yet has three distinct populations within New Zealand, over 1100km (Thomas and Bell 2013). In a review of New Zealand fauna, Ross (2009) noted larval duration had a positive correlation with population connectivity, however this pattern was highly variable in organisms with shorter larval durations (<10 days). Weersing and Toonen (2009) reviewed 300 peer reviewed studies and found a no relationship with PLD and genetic structure after direct developers were removed from the analysis.

Spatial and Temporal Effects

The distance that a larvae is able to disperse through the environment can certainly have an effect on the scale of spatial structure of populations. The connectivity of marine populations is not only strongly affected by biology (Scheltema 1986, Rocha et al. 2007), larval behaviour (Kingsford et al. 2002), and currents (Galindo et al. 2006, White et al. 2010), but also the spatial arrangement of suitable habitats for recruitment (Parsons 1996). During the pelagic stage, currents or upwelling could limit dispersal, change the course of transport, retain the particles in eddies or advect the larvae offshore.

The developing larvae must also find appropriate substrate and geography for settlement at the appropriate time in larval development. Therefore, although the marine environment appears to have few limitations to the movement of adults in a population, these geographic factors could have profound effects on the dispersal and settlement of larvae. Biogeography could be extremely important to the formation of

populations. These environmental effects could play a role in shaping genetic structure of many species simultaneously (Briggs & Bowen 2012, Riginos 2011). Biogeographic patterns across species with different life history characteristics could be especially interesting because these mechanisms hint at underlying mechanisms of stable physical population boundaries in the marine environment.

Examination of the genetic structure of adult populations may demonstrate patterns of differentiation in fish populations, but variation in dispersal and survival over time can lead to homogenization of genetic patterns (Waples 1987). The inherent variability of oceanography and climate may cause small scale patches of heterogeneity termed chaotic genetic patchiness (Johnson and Black 1984). This may be a result of small windows of opportunity for larval survival, which could result in a bottleneck effect during the pelagic stage, as evidenced in reduced genetic variation or temporal differences between cohorts, termed “sweepstakes recruitment” by Hedgecock from his work on the Pacific oyster *Crassostrea gigas* (1994). However, a study testing this hypothesis in the sea urchin, *Strongylocentrotus purpuratus* found no difference in genetic diversity between juveniles and adults or between cohorts over 7 years (Flowers 2002).

The New Zealand Environment

With high levels of endemism, New Zealand is a global hotspot for biodiversity (Myers 2000). New Zealand’s mainland (here referring to the North Island, South Island and Stewart Island) waters border on the southern-most edge of sub-tropic waters and span 13 degrees of latitude to the sub-polar regions. The oceanography consists of a complex current system of gyres, upwelling and river plumes, overlaid upon unique geological history (Wallis and Trewick 2009). Genetic differentiation between northern and southern species assemblages and populations has been documented in many species (Apte and Gardner 2002, Ayers & Waters 2005, Ross et al. 2009, Gardner et al. 2010, Will et al. 2011)

There are several hypotheses to support the divisions of biogeographic regions around New Zealand. In two recent comprehensive reviews of population genetic studies of coastal New Zealand taxa, 20 studies out of 58 (Gardner 2010) show a north and south split corresponding with to 41° S and 42° S (Ross et al. 2009,

Gardner et al. 2010). Shears (2008) used presence/ absence data for 151 species of algae and macroinvertebrates to evaluate six biogeographic classification hypotheses, and proposed 2 major biogeographic provinces north and south of Cook Strait and 11 regions where communities were significantly differentiated. Walls (2009) looked at habitat type and location to propose a similar biogeographic map with 9 regions around the mainland. There is high correlation between all of these hypotheses (Figure 1.1); all three noting a break around the Cook Strait, which marks a major change in species distributions and assemblages, indicating the possibility of a significant barrier to gene flow. The exact location and what is causing the break is unknown. It may be the result of the strong D'Urville Current through the Cook Strait or upwelling around the north end of the South Island.

Figure 1.1. Hypothesized biogeographic breaks around the New Zealand mainland. Black lines represent boundaries proposed by Walls (2009) based on a near shore classification system of habitats and ecosystems. Shears et al. (2008) regions are outlined in red and are based on species assemblages of algae and invertebrates. Gardner et al. (2010) break at the north of the South Island is based on a summary of 58 population genetic studies across multiple taxa and spatial coverage.

The overall view of biogeography in New Zealand has been skewed by the types of organisms studied, the environments examined, and the lack of resolution from the genetic markers used. While many population genetics studies in New Zealand mainland waters have focused on rocky intertidal species, very few have focused on soft sediment, sandy bottom and estuarine species, which represent a major gap in the overall patterns of coastal connectivity (Gardner et al. 2010). Estuaries tend to lie at the interface between human use and the natural environment and are important nursery grounds for a number of species (Little 2000) and vary widely in geomorphology, hydrodynamic processes and catchment geology (Hume et al. 2007). Information about connectivity among estuaries and between estuaries and sandy coastal areas is lacking (Francis et al. 2005). Biogeographic boundaries can serve as a proxy for spatial and temporal genetic boundaries, as they apply to numerous species over multiple studies. Gardner et al. (2010) also found that there are relatively few ichthyological studies from New Zealand.

The Study Species

A representative of sandy bottom estuary and coastal communities is the flatfish genus *Rhombosolea*, Subfamily *Rhombosoleinae* (Richardson 1843). *Rhombosolea* consists of four recognised species in New Zealand, *R. plebeia* (Sand Flounder), *R. leporina* (Yellow Belly Flounder), *R. retiaria* (Black Flounder) and *R. tapirina* (Greenback Flounder). The Subfamily Rhomosoleinea was first recognized by Regan (1910) within the suborder Pleuronectidae. Cooper and Chapleau (1988) suggested the status of the *Rhombosoleinae* to be raised to Family, therefore excluded from a full revision of the Plueronectidae. Chapleau (1993) found that the Family Pleuronectidae may not be monophyletic, with characters in *Rhombosoleinae* being pleisomorphic to many plueronectids. To date, *Rhombosoleinae* remains a Family (Nelson 2006) and the relationships therein have not been examined on a genus or species level.

Focusing on two closely related species *Rhombosolea plebeia* (sand flounder) and *R. leporina* (yellowbelly flounder) that are distributed throughout New Zealand with similar life history traits, provides experimental controls for certain biological

factors. Adults of both species are distributed throughout mainland New Zealand (Ayling and Cox 1982) and juveniles are two of the most abundant species in estuary systems (Morrison et al. 2002). This genus is valuable for commercial and customary fisheries, with landings of 3,275,000 kg in 2010 (Clements and Associates 2012). The PLD of these species, as measured as time from fertilization to metamorphosis, has never been measured. The sister species, *Rhombosolea tapirina*, is of interest for aquaculture, so more information is available on this species than others in the genus. For *R. tapirina*, the PLD last on average 69 days (Crawford 1984), which may also vary widely with temperature (Chambers and Leggett 1992). Depending on current speeds, a pelagic stage of several months, potential dispersal could be on the order of thousands of kilometres (Palumbi 2003, Shanks 2003).

Dispersal of *Rhombosolea* is most likely to occur during the pelagic larval stage as adult movements are somewhat restricted on the scale of the entire country. Tagging studies of adult *Rhombosolea leporina* and *R. plebeia* in Hauraki Gulf, Canterbury Bight and Banks Peninsula revealed that offshore movement occurred only during spawning events (Colman 1974, Colman 1978), but have been rarely been recorded moving 200 kilometres (Colman 1978). Colman (1976) also found variation in the number of fin ray counts in sand flounder, with higher numbers in the west of the North Island and lowest numbers in the southeast of the South Island, concluding there were 5 discrete regions or populations, however it has been widely reported that number of vertebrae, fin and dorsal rays are strongly affected by temperature (Hubbs 1922, Barlow 1961, Kinoshita et al. 2000) rather than genetic variation.

Colman (1972) studied several hundred samples of *Rhombosolea* from the Hauraki Gulf and found that male sand and yellowbelly flounder were sexually mature at 12 cm in *R. plebeia* and 15 cm in *R. leporina*, due to the presences of milky fluid in the testes. Fifty percent of female *R. plebeia* were found to be mature at 18 cm and 95% at 22.9cm. Fifty percent of *R. leporina* were mature at 27cm. Colman points out that at the legal limit of 25 cm, only 15% of *R. leporina* females are sexually mature, later confirmed by Mutoro (2001), which is still the current minimum catch size (Ministry of Primary Industries, MPI 2013a). He also points out that the current legal limit size may allow for males to spawn more than once in their lifetime, but females would be subject to heavy fishing pressure before their second

spawning season (Colman 1985). Therefore, animals might only spawn once during their lifetime and abundance is closely linked to year class strength (Colman 1985).

The current state of knowledge about fishery stocks is based on Colman's research, and there have been no further studies about the connectivity between populations since the nineteen seventies (MPI 2013a). Therefore, the population dynamics and stock structure of these animals are poorly understood according to the Ministry of Fisheries plenary documents, which currently manages a complex of 8 flatfish species as one (MPI 2013a).

Animals with similar pelagic larval durations of 9 to 10 weeks show genetic differentiation around New Zealand. For example, the echinoderm *Patiriella regularis* was surveyed country wide and genotyped at the mtDNA control regions, showing a North to South differentiation across the Cook Strait (Waters and Roy 2004, Ayers and Waters 2005). The mollusc *Siphonaria raulensis* was surveyed around the North Island and Kermadecs using RAPDs and showed differentiation between and among regions (Wood and Gardner 2007). In a Rhombosolea congeneric *Rhombosolea tapirina*, the greenback flounder, there is evidence of limited gene flow as evidenced by allozyme between Victoria and Tasmania populations, which is a distance of 300 km across the Bass Strait (van den Eden et al. 2000). Therefore, there is likely to be population structure in Rhombosolea over the scale of this study.

Population Structure and Local Adaptation

Larval flatfish spawned offshore must find their way back into estuaries, and some have specific habitat requirements, including temperature, sediment size and preferred prey (Bailey et al. 2005). However, most larval flatfish have poor swimming ability compared to other larval fish (Fukuhara 1988). In the process of recruitment, oceanographic events may dictate which estuaries juveniles are able to recruit to, explaining variation between cohorts. Conversely, if adults display philopatry, family structure may be evident in juveniles within estuaries, resulting in limited gene flow between them, which could lead to local adaptation.

Larmuseau et al. (2009) demonstrated local adaptation in the rhodopsin gene across different photic environments in the demersal sand goby, which corresponded

to population differentiation revealed by microsatellite analysis. Hemmer-Hansen et al. (2007) observed adaptive divergence in the heat shock gene Hsc70 which correlated with environmental conditions in European flounder. Larval *R. tapirina* showed improved feeding ability in turbid versus clear water across many prey densities (Shaw et al. 2006), suggesting variation in survival ability in different environments. Evidence of adaptive variation across the species range may be found in relation to different habitats.

The first component to investigating adaptive variation is to show a relationship between genetic variation and environmental variation. Genetic variation in population studies is measured using neutral markers, such as microsatellites. This is an intentional choice, as coding markers with a specific function could be highly conserved and not variable enough to detect differences between individuals. Coding genes may also be under selection. To get a population wide picture of genetic variation, many genes must be used. Several coding genes may be under different kinds of selection, resulting in a schizophrenic view of evolutionary forces. Thus, with neutral dna markers, we must rely only on correlation between genetic variation and environmental variables.

One method to elucidate the effect of the environment on genetic structure is to overlay pair-wise comparisons of genetic distance with biologically relevant environmental variables to look for correlations between population structure and environment (Manel et al. 2003, Morris and Ball 2006, Sork and Waits 2010), which may indicate local adaptation. The New Zealand Marine Environmental Classification (Snelder et al. 2005) describes a suite of environmental variables, such as sea surface temperature, depth, solar radiation, current speeds, substrate type that can be used for habitat analysis. The New Zealand Estuarine Ecology Classification System is specific to estuaries with variables concerning freshwater flow and the morphology of the estuaries (Hume et al. 2007). If there are breaks or clines in genetic diversity, these may correspond to heterogeneity in the marine environment.

Microsatellite markers are high resolution, rapidly evolving segments of DNA that allow fine-scale measurement of genetic heterogeneity and variation in populations (Avice 2004). A comprehensive molecular data set of species-specific markers can reveal breaks in genetic connectivity, identify clines in genetic distance

over geographic distance, provide evidence of hybridization, determine family relatedness, levels of genetic diversity and other population health indicators (Allendorf and Luikart 2007).

Aims of This Research

The purpose of this thesis is to elucidate patterns of population structure in two closely related species of endemic flatfish from New Zealand. I (1) developed polymorphic microsatellite markers from 454 sequence data. Using these markers, I (2) investigated population genetic patterns on multiple spatial scales by (a) describing the population genetic structure of *R. plebeia* and *R. leporina*, (b) tested for potential barriers (biogeographic, contemporary oceanographic, previously unidentified) to dispersal, and (c) compared the spatial genetic patterns of *R. plebeia* and *R. leporina*. To (3) test for temporal effects on population structure, (a) microsatellite allele frequencies were compared across age classes to determine whether there was inter-annual variation across years; between juveniles and adults collected in adjacent localities; and between pulses of recruits. The effects of genotyping a possibly non-random sample of juveniles (b) were investigated by comparing levels of relatedness within juveniles compared to adults and within cohorts. To test an aspect of the SRS hypothesis (c) levels of genetic diversity between adults and juveniles samples were compared. Lastly, (4) I used coastal and estuarine environmental variables with measures of genetic differentiation to determine which environmental and geospatial factors might be correlated with the spatial genetic patterns observed.

This study fills major gaps in our knowledge about connectivity among soft and sandy bottom habitats and estuaries. This is the first population genetics study of *Rhombosolea plebeia* using microsatellites, and the first genetics study of *R. leporina*. The temporal part of this research explores an often overlooked aspect of population genetic studies. The final chapter contributes to a new and growing body of research on seascape genetics.

Chapter 2. Characterization of 26 microsatellite loci for the genus *Rhombosolea* from 454 sequences using M13 and touchdown PCR

Abstract

To facilitate the study of coastal connectivity in two species of endemic New Zealand flounders, 26 microsatellite loci were identified and characterized from 454 sequence data using a combination of bioinformatics tools and cost effective lab techniques. For sand flounder (*Rhombosolea plebeia*), 12 microsatellite loci were developed with an average of 12.7 alleles per locus ranging from four to 35. For yellowbelly flounder (*Rhombosolea leporina*), 15 microsatellite loci were developed with an average of 9.7 alleles per locus ranging from three to 21. There was little cross-amplification between species, with the exception of one penta-repeat marker.

Introduction

Fisheries managers require scientific information in a timely manner to make informed decisions, something that is especially challenging when studying populations and stock structure. Marine species generally have large population sizes, widespread dispersal capabilities and high levels of genetic diversity that require sensitive tools to detect low levels of differentiation (Waples 1998). With the aid of next generation sequencing, I was able to quickly develop informative and cost-effective polymorphic microsatellite markers to study genetic diversity and population connectivity (Avisé 2004) of two commercially and customarily important species of New Zealand flounder, the sand flounder (*Rhombosolea plebeia*) and the yellowbelly flounder (*Rhombosolea leporina*).

Even though next generation sequencing is much faster and ultimately cost-effective compared to traditional methods of developing microsatellites (Abdelkrim et al. 2009), the initial price of sequencing can still be prohibitive: \$1,100 USD for 4.1 Mb for 1/16th of a plate on a Roche 454 Genome Sequencer FLX (Abdelkrim et al. 2009) to \$950 USD for 892.7 Mb on an IonTorrent PGMTM platform (Luo et al. 2012).

There are many bioinformatic tools available to help to advance marker identification and primer development through the initial expensive laboratory testing stage directly to final stages of development (Gardner et al. 2011). The software package QDD1 (Megléczy et al. 2010) simplifies the workflow between Clustal W2 (Larkin et al. 2007) for sequence alignment, Primer3 (Rozen and Skaletsky 2000) for primer design, and BLAST 2.2.18 (Altschul et al. 1997) for matching redundant sequences. Primer3 designs primers for a default optimal melting temperature of 60° (range between 57°C and 63°C) and provides primer penalties based on optimum annealing temperature, sequence length, likelihood of binding to the template and other parameters that allow the user to prioritize primers for testing. The software Geneious R6.1 and the associated Phobos Tandem Repeat Finder (Drummond et al. 2010) allow the user to 'test' these primer pairs theoretically *in silico* by matching them to the sequence data and scanning for

potential problems prior to investing in oligos and reagents. Geneious also has a primer design tool that can be used for comparison with primers from QDD/Primer3 and identification of partial repeats.

The 'touchdown' thermal cycling protocol allows for fast and effective screening of primer sets because it uses a range of annealing temperatures. This approach quickly ascertains which primer pairs amplify specific sequences without having to optimize the annealing temperature or PCR recipe (Hecker and Roux 1996). By starting with a high annealing temperature, which produces a high level of primer specificity for the targeted locus, then lowering subsequent annealing temperatures, the priming specificity is decreased which in turn increases the output of PCR products with fewer non-specific products to act as non-target PCR templates. In this case, a touchdown protocol was used throughout the study, providing ample and clear product with little nonspecific amplification.

Lastly, the use of the M13 protocol enables a single set of fluorescently labeled markers to be more efficiently used by an entire research group (Schuelke 2000). Since Applied Biosystems (ABI) owns the patent on fluorescent dyes NED, VIC and PET, only these dyes are compatible with their widely used ABI 3730 DNA Genetic Analyzer. A single directly labeled forward primer can cost as much as \$160.00 USD for the smallest amount available (Life Technologies), which is the same price as a fluorescently labeled M13 sequence but the latter can be used in conjunction with a range of PCR primers. The M13 primers each labeled with one of the four ABI compatible dyes can be PCR-amplified as a multiplex of loci depending on their sizes, or pooled together after separated PCRs (pool-plexed), and analyzed together to reduce the cost of genotyping, which can be the most prohibitive cost of a study. A combination of each of these methods can accelerate primer development, saving time and money.

Methods

DNA was extracted from the fin and muscle tissues using a phenol-chloroform protocol modified from Barker (2005), and stored in TE buffer at -20°C. The purified DNA was quantified using an Implen NanoPhotometer™ Pearl and the yields were an average of 1000 nanograms of DNA per microliter. The five best DNA samples

were prepared and run on 1/8th of a plate on the Genome Sequencer FLX (GS-FLX) System (Roche, Penzberg, Germany) according to the recommendations of the operator (New Zealand Genomics Limited). The software QDD1 (Meglec et al. 2010) was used to search the file of DNA sequences for unique sequences with a minimum of five copies of perfect microsatellite motifs using the default settings.

Using the DNA sequence data and the primer pairs identified by QDD, each pair of primers was mapped onto its complementary sequence using Geneious 5.3 (Drummond et al. 2010) to determine the distance of the primer binding sites from the repeat motif, and if the DNA sequence contained imperfect repeats, other partial repeat sequences, or potential mutation sites within the primer binding region. Higher priority was given to the development of microsatellites that contained tetra and tri repeat units rather than di repeats because genotyping of these higher repeat motifs are easier to score.

Oligos were ordered with a M13 sequence (5'-TGTAACGACGGCCAGT-3') attached to the 5' end of the forward primer sequence. The PCRs were conducted in 15 µl volumes containing 10 mM Tris-HCl, (pH 9.0), 50 mM KCl, 0.2 mM dNTPs, 0.6 mg BSA, 1.5 pmol M13 primer, 1.5 pmol forward primer, 3.0 pmol reverse primer, 0.75 mM MgCl₂, 0.6 U DNA *Taq* Polymerase (Fisher Biotech) and 100 ng genomic DNA. Non-fluorescently labeled M13 sequences were used in the initial and testing round of PCR on four flatfish samples using two touchdown PCR protocols.

Thermal cycling was conducted on an Applied Biosystems GeneAmp 2700 PCR System using a combination of a touchdown and M13 protocol at two different temperature regimes; 65°C-55°C touchdown and 55°C-45°C touchdown. Touchdown protocols were adapted from Rahman et al. (2000) by adding 15 cycles of 53°C to anneal the M13 fluorescent primers to the 5' end (adapted from Schuelke 2000). The 65°C-55°C touchdown program was as follows: 95°C 3 min, 20 cycles of 30 s each at 95°C (denaturation), 65°C (annealing) with stepwise lowering of annealing temperature by 0.5° C each cycle, 72°C (elongation); followed by 15 cycles of 30 s each at 95°C, 55°C and 72°C; 20 cycles of 30 s each at 95°C, 53°C and 72°C to anneal fluorescent the M13 sequence; and lastly, a final extension of 72°C for 15 minutes. The 55°C-45°C touchdown program was as follows: 95°C 3 min, 20 cycles of 30 s each at 95°C (denaturation), 55°C (annealing) with stepwise lowering of annealing

temperature by 0.5° C each cycle, 72°C (elongation); followed by 15 cycles of 30 s each at 95°C, 45°C and 72°C; 20 cycles of 30 s each 95°C , 53°C and 72°C to anneal fluorescent M13 sequence; and lastly a final extension of 72°C for 15 minutes.

PCR products were electrophoresed in a 3% agarose gel with Hyperladder I (Bioline) to check for clean amplification at the expected size range and polymorphism across individuals. The primer pairs that showed evidence of polymorphism were then used to PCR-amplify loci (with the fluorescently labeled M13 sequence) using the DNA of 24 individuals collected from the same population. The PCR products were pooled according to size and colour of M13 tag and analyzed on an ABI 3730 Applied Biosystems Genetic Analyzer. Allele size calling was performed using GeneMarker 2.2.0 (SoftGenetics).

Fifty *R. plebeia* and 48 *R. leporina* were genotyped for 12 and 14 loci, respectively (summary genetic statistics are in Table 2.1). Allelic richness (Ar) was calculated in Fstat 2.3.9.2 based on a minimum of 41 individuals for *R. plebeia* and 44 for *R. leporina*, expected heterozygosity (H_E), observed heterozygosity (H_O), p-values of two-tailed tests for deviations from Hardy-Weinberg Equilibrium (P_{HWE}) and Weir and Cockerham (F_{IS}) were calculated in GenePop on the Web version 4.0.10 (<http://genepop.curtin.edu.au/>). The two-tailed HWE tests were performed using Markov chain parameters of 1000 dememorizations, 100 batches and 1000 iterations per batch.

Results

For *R. plebeia*, the results of 1/8th of a plate run on the Roche 454 sequencer yielded 130,127 DNA sequences with an average length of 550 bp. QDD1 identified 946 perfect microsatellite motif sequences each with a primer pair penalty of less than one. These primers were then mapped onto the original sequences using the “Test with Saved Primers” option in Geneious to evaluate 220 primer pairs. Based on the Geneious results, 52 primer pairs were tested, of which, 12 loci (one penta-, one tetra-, 9 tri- and 1 di-repeats) gave consistent results and were polymorphic.

Due to an upgrade by Roche to the FLX+ system, the 1/8th of a plate of *R. leporina* DNA had to be run multiple times to get satisfactory sequence fragment lengths (Heger 2012). Three separate runs of the FLX+ system yielded: 121,863 with

an average length of 193 bp, 108,171 with an average length of 229 bp, and 21,694 sequences with an average length of 313 bp. A fourth run on a GS Jnr platform yielded 130,757 fragments with an average length of 335 bp. These four runs were combined to form a file of 382,485 DNA sequences with an average length of 296 bp. The combination of these data files was legitimate since they were all sequenced from the same individual and QDD identifies and eliminates redundant sequences. The QDD analysis identified 2,643 perfect microsatellite sequences with a primer pair penalty less than one. Of these, 50 primer pairs were tested, giving 14 loci (five tetra-, 6 tri- and two di-repeats) that gave consistent results and were polymorphic. One *R. plebeia* primer cross-amplified well enough to be used (Rhpl 5), however the other loci did not amplify or were monomorphic in the other species.

Table 2.1. Microsatellite primers developed for *R. plebeia* and *R. leporina*

Locus ID	Repeat Motif	Primer Sequence Forward and Reverse 5' to 3'	Touchdown Annealing Temp	Size Range
<i>R. plebeia</i>				
Rhpl 5	ACAGC	TCTAATTGGCTGCTTCCACC TTGAAATTGGGGACTTCAGG	65-55	114-124
Rhpl 11	TGCG	TTCTTCTTGGTCACGTGCAG CACAGTCTGGTCGGAAAACA	65-55	209-251
Rhpl 25	TCA	TTCTGCTCCTTCTGCCGTAT ACCTTACGACAAGGCACCTG	55-45	127-136
Rhpl 57	ACC	CAGTGCATTTCAATCATGGG GACACTTGGCTGGATTGGTT	55-45	234-250
Rhpl 65	ATT	CAACGTGAGTGCTCCTCGTA GATGAGGGCACAGGAGGTAA	65-55	181-208
Rhpl 79	TG	CAACTCCGGGTTTTGAGTGT GAGCTCGGTATTGGCTGAAG	55-44	121-123
Rhpl 108	CAG	GTGGCAACGTCCAAGAATTT AGTAGGGAGTGCTGGGAACC	65-55	161-167
Rhpl 124	GGA	ATCTGTGCGTGACTCGTTTG GCTAAGATGTCCCTGGACCC	55-45	146-179
Rhpl 131	GAA	GAGCAGCTGAGTCTGGACCT GTGAAGCTGGACCTGTTGGT	65-55	189-207
Rhpl 133	CCT	GACTTCTCCCGTCTTCATGC TACAACTTGCCCTGCAACAC	55-45	134-144
Rhpl 136	TAT	GTGGAGGTTACGGATCACT GTTTCCCGCTGAAGTACCAG	65-55	159-165
Rhpl 145	TAG	TTGCCGATCCTTACTTTTGC CCATAGGCTGTAGCGTGTCC	55-45	173-219

<i>R. leporina</i>				
Rhle 1	GAACTT	TGTATGCTGTACATACAAAATAGACAA TTATATTTTCTTAATGCCTCAAGCTG	55-45	72-90
Rhle 10	ATTC	GTCCTCCTGTGGTTGCCTAC GCAGAAGGTCGCTAATAGGAA	55-45	125-183
Rhle 13	CAAT	GGTACACCTCGCCATCCC ATCTTCTCCTGAACTCCGCA	55-45	88-135
Rhle 15	TACT	TGTGAACATTTGGAGCAAGG CAGCAATAAGGTTTAACACGAAA	65-55	124-132
Rhle 19	AGG	TGTGACTGTGTCTGTGCGAA CTTAGATTTCTGCAAGGGCG	65-55	87-108
Rhle 20	GGA	TGGTTTAATTGCCCGTAAA TCTTAGCAGGACCAGCCACT	65-55	161-202
Rhle 21	TAAA	CGCAAACCTAAGTGAGAGCA TTGTGCAGATTCACGCTTTC	65-55	145-157
Rhle 22	ATT	TGGCTGTGTCATCATTGGTT TGA CTGGAGTTGCAGGTGAT	65-55	90-124
Rhle 23	CAT	TGCATGTGGTATGGTGAACA AGCAGTAAGCCCTGGACAGA	55-45	116-180
Rhle 29	TGT	TTCCAAAGACTGCACACTGC GTGTTACGGGGTGACATGGT	65-55	235-285
Rhle 37	TG	AGTTGTCAATTTCCCTGTGC AACACCCACGTGAGGAACTC	65-55	80-104
Rhle 40	AC	CCTAGCAGGGAGACAGATCG TGGTTGCGAGTTTGACAGTAG	65-55	141-153
Rhle 47	GGGT	AATTTCCAGGTGCCTGTGTC ACCATCCAAGACAGCTCCAT	65-55	102-118
Rhle 49	AAC	GGATCGATACACTCTGGCGT ATGCAACGTGTTGATTAGCG	65-55	103-132

For *R. plebeia* (Table 2.2) the average number of alleles was 12.7 alleles per locus, ranging from four to 35. Expected heterozygosity (H_e) ranged from 0.201 to 0.834 (mean $0.525 \pm \text{SD } 0.202$). Observed heterozygosity (H_o) ranged from 0.061 to 0.600 (mean $0.420 \pm \text{SD } 0.665$). Significant deviations ($P < 0.05$) from Hardy Weinberg Equilibrium (P_{HWE}) were only due to heterozygous deficiencies occurring at 5 loci (Rhpl 25, Rhpl 65, Rhpl 79, Rhpl 124 and Rhpl 145). Only Rhm 5, a penta-repeat, amplified DNA from *R. leporina* and there were three alleles.

For *R. leporina* (Table 2.2) there was an average of 9.7 alleles per locus ranging from three to 21. H_e ranged from 0.073 to 0.854 (mean of $0.559 \pm \text{SD of } 0.024$), whilst H_o ranged from 0.113 to 0.808 (mean of $0.535 \pm \text{SD of } 0.249$).

Significant deviations ($P < 0.05$) from HWE were only due to heterozygous deficiencies that occurred at 5 loci (Rhle 19, Rhle 20, Rhle 22, Rhle 23 and Rhle 37).

Table 2.2. Characterization of 12 microsatellite loci for *R. plebeia* and 15 microsatellite loci for *R. leporina*. Number of alleles (N_a), Allelic richness (A_r), expected heterozygosity (H_e), observed heterozygosity (H_o) and Weir and Cockerham (F_{IS}), p-values of two-tailed tests for deviations from Hardy-Weinberg Equilibrium (P_{HWE})

<i>R. plebeia</i>						
Locus ID	N_a	A_r	H_o	H_e	F_{IS}	P_{HWE}
Rhpl 5	6	3.539	0.530	0.551	0.080	0.543
Rhpl 11	19	5.141	0.628	0.717	0.011	0.403
Rhpl 25	6	3.032	0.068	0.264	0.267	0.018*
Rhpl 57	20	5.070	0.514	0.537	0.027	0.401
Rhpl 65	15	6.557	0.714	0.778	0.591	0.000*
Rhpl 79	4	3.019	0.309	0.486	0.357	0.004*
Rhpl 108	5	2.484	0.369	0.404	0.046	0.272
Rhpl 124	22	7.742	0.735	0.829	0.287	0.018*
Rhpl 131	7	3.939	0.551	0.583	0.007	0.698
Rhpl 133	6	3.422	0.367	0.392	-0.093	1.000
Rhpl 136	8	3.041	0.232	0.241	-0.126	1.000
Rhpl 145	35	10.761	0.625	0.874	0.325	0.000*
<i>R. leporina</i>						
Rhle 1	4	3.956	0.351	0.450	0.175	0.116
Rhpl 5	4	2.000	0.120	0.122	-0.082	1.000
Rhle 10	14	10.872	0.771	0.815	0.039	0.323
Rhle 13	12	5.929	0.644	0.665	0.042	0.394
Rhle 15	3	2.000	0.113	0.665	-0.057	0.395
Rhle 19	8	5.997	0.614	0.698	0.150	0.049*
Rhle 20	13	9.929	0.795	0.854	0.128	0.038*
Rhle 21	4	3.933	0.710	0.073	-0.036	1.000
Rhle 22	11	9.000	0.230	0.394	0.345	0.0004*
Rhle 23	21	16.667	0.704	0.394	0.351	0.0001*
Rhle 29	17	13.486	0.808	0.820	0.051	0.281
Rhle 37	12	7.936	0.587	0.773	0.233	0.0029*
Rhle 40	7	4.000	0.626	0.682	-0.023	0.657
Rhle 47	5	3.978	0.193	0.220	0.106	0.315
Rhle 49	11	7.993	0.754	0.756	-0.006	0.602

Discussion

Combining 454 sequence data, M13 labeled fluorescent markers and touchdown PCR protocol produces fast and cost-effective DNA microsatellite development and screening. The next generation sequencing approach yields an enormous amount of data - over 100,000 sequences produced by the 454 – so that QDD1 and Geneious were used to prioritize and scan potential primers to a manageable number before moving into the laboratory phase. A touchdown PCR protocol allows for fast and effective screening of primers with little optimization. Using a universal M13 primer can save thousands of dollars, depending on the number of primers that are being screened. As the cost of partial genome sequencing drops, these methods will become more feasible for small research budgets.

Cross amplification of microsatellite sequences is common in pleuronectids (Hoarau et al. 2002), and tends to work best for species that are closely related. Rico (et al. 2002) successfully cross-amplified loci in fish species as divergent as 470 mya, but no Pleuronectids were included in the analysis. As there is no existing phylogeny of the 20 species within the subfamily Rhombosoleidae, we do not know how divergent the members within are. Regan (1910) elevated the subfamily Rhombosoleina within the family Pleuronectidae, which Sakamoto (1984) maintained in subsequent revisions of the Pleuronectidae. Chapleau and Keast (1988) and Chapleau (1993) suggested that Rhombosoleina be elevated to family level, which was accepted and maintained by Nelson (2006). In their most recent analysis, Cooper and Chapleau (1998) excluded Rhombosoleinae from their analysis, so the systematic status, and therefore evolutionary history, remains unresolved. The level of divergence between these species may have important consequences on how well genetic markers cross-amplify, as well as their evolutionary and adaptive divergence.

Here we have developed the first microsatellite markers for the genus *Rhombosolea*, for the purposes of examining levels of genetic connectivity among coastal populations in New Zealand. The genus *Rhombosolea* has been studied in New Zealand and Australia for over 100 years as an important member of New Zealand marine ecosystems and fisheries, but there have been few genetic studies to

date. *Rhombosolea plebeia* has been studied in the Wellington Harbour using allozymes to examine the relationship between length and heterozygosity (Smith and Francis 1984, Smith 1987) and the relationship between genetic diversity and feeding specialist among Teleosts (Smith and Fujio 1982). Allozymes have also been used to study genetic variation and the population connectivity of the greenback flounder *Rhombosolea tapirina* in southeast Australia (van den Eden et al. 2000, Ward and Elliot 2001). These newly developed high resolution polymorphic microsatellite markers presented here can be used to investigate a vast array of relevant marine ecology.

Chapter 3. Spatial population structure in two sister species of endemic New Zealand flatfish (*Rhombosolea plebeia* and *Rhombosolea leporina*)

Abstract

Sandy, soft bottom and estuarine ecosystems make up a significant proportion many coastal environments, but research on these habitats are not well-represented in population genetic studies in New Zealand or internationally. I chose *Rhombosolea leporina* and *Rhombosolea plebeia* as endemic, commercially and traditionally important species in the shallow coastal waters and estuaries to: 1) describe the population genetics these two taxa, 2) test for a north to south barrier to gene flow and 3) compare population genetic structuring for these two species. Both species have a significant amount of genetic structure, with two distinct regional genetic assemblages of *R. plebeia* and two to four assemblages for *R. leporina*. While *Rhombosolea plebeia* had an isolation by distance pattern among the North Island localities and west coast of the South Island, the southeast part of the South Island was significantly differentiated in allele frequencies and in other analyses. *Rhombosolea leporina* populations were well connected along the west coast and between western and eastern coasts of the North Island, but had limited gene flow among other regions. Neither species demonstrated significant differentiation of allele frequencies along the west coast, therefore there was no support for a north to south split. *R. plebeia* generally had more connectivity among localities, while *R. leporina* showed greater differentiation. This study fills major gaps in our knowledge about the connectivity among sandy, soft bottom and estuarine habitats and challenges the assumption that populations of animals with long PLDs are generally open.

Introduction

Population genetic connectivity, defined here as the exchange of genes between and among populations, is determined by oceanography, ecology, life history traits, behaviour and stochastic events (Cowen et al. 2006, Levin 2006). The connectivity of marine populations is not only strongly affected by biology (Rocha et al. 2007, Scheltema 1986), larval behaviour (Kingsford et al. 2002), and currents (Galindo et al. 2006, White et al. 2010), but also the spatial arrangement of suitable habitats for recruitment (Parsons 1996). The full range and distribution of marine species are influenced by oceanographic factors, such as currents and temperature, although the scale and persistence of these effects are poorly known (Pringle 2007).

Most marine fish have a pelagic larval dispersal (PLD) stage (Moser 1996, Nybakken and Bertness 2005) whereby larvae and spores are transported through the water column for a period of hours to months. The transport, duration, behaviour, and recruitment of larvae are the important in determining marine population connectivity (Cowen and Sponagule 2009, Shanks 2013, Levin 2006). Earlier meta-analyses have shown a correlation between PLD and dispersal distance (Shanks et al. 2003, 2009, Ross 2009), however this positive relationship does not hold for organisms with very short larval durations (Ross 2009) and it overestimates “realized,” or successful, dispersal distance for organisms with longer PLDs (Shanks 2009). Weersing and Toonen (2009) found that the regression between larval duration and dispersal distance is largely anchored by direct developers, and patterns are inconsistent when different genetic markers are used. Riginos et al. (2011) noted that biogeographic patterns and egg type were more significant in predicting population structure in marine organisms than the PLD. These findings indicate that there is no simple relationship between PLD and dispersal potential, or indeed between PLD and realized dispersal.

The diminutive size of marine larvae makes them difficult to track, so indirect methods, such as genetics, are used to measure connectivity between populations. Using genetics to estimate population connectivity among settled recruits and adults is necessarily measuring “realized dispersal,” as only organisms that have survived to the point in their life stage where they were sampled, are included in the study. There is very high mortality during the PLD (Hixon et al 2002); therefore patterns after this

potential selection event are of great interest to biologists and fisheries managers. Exchange of larvae between populations varies in space and time, but is essential to connecting populations, replenishing fish stocks and providing genetic diversity. Current play a role in the transport of larvae (Galindo et al. 2006, White et al. 2010), but passive dispersal does not explain all of the spatial patterns observed.

Larval behaviour (Kingsford et al. 2006, Gerlach et al. 2007) and the spatial arrangement of suitable habitats for recruitment (Parsons 1996) may limit realized dispersal. Adult movements and habitat preference may also structure populations by promoting local retention of larvae through choice of spawning grounds (Frisk et al. 2013). While the adult population may move in a largely homogenous habitat, for example, sandy coastal areas, juveniles may have separate habitat requirements, such as estuarine areas. Many species are known to have specific nursery and spawning grounds and regular migration patterns, known as philopatry (Svendang et al. 2010, Cardinale et al. 2011). Philopatry can counteract the homogenizing effects of dispersal and drift with behavioural adaptations (Svendang et al. 2007).

The distributions and persistent spatial patterns across many species are defined as biogeographic patterns (Jones 1987). Biogeographic patterns provide testable hypotheses about possible barriers to dispersal that can guide researchers to investigate the physical and biological interactions with the environment (Spalding et al. 2007). Biogeographic areas may or may not correspond to genetic differentiation between populations, depending on how they are defined or what evolutionary phenomenon they are intended to explain (Briggs and Bowen 2012). Genetic markers can be used to identify spatially explicit genetic patterns by providing independent evidence about connectivity between habitats and organisms (Palumbi 1996).

New Zealand has very high levels of marine biodiversity and endemism (Gordon et al. 2010). The mainland (here referring to the North Island, South Island and Stewart Island) waters border on the southern-most edge of subtropical waters and span 13 degrees of latitude to the sub-polar regions. The oceanography consists of a complex current system and upwelling, overlain upon unique geological history (Wallis and Trewick 2009). The predominant current, the Tasman Current, moves

from west to east, and when it encounters the landmasses of the New Zealand, it splits into many smaller current systems and gyres.

There are several hypotheses about the location of biogeographic regions around New Zealand based on qualitative and quantitative assessments of its marine biota (Figure 1.1, Chapter 1). Shears et al. (2008) used presence/ absence data for 151 species of macroalgae and macroinvertebrates to evaluate six biogeographic classifications, and proposed 2 major biogeographic provinces north and south of Cook Strait and 11 regions where communities were significantly grouped. They noted a break around the Cook Strait, which marks a major change in species distributions and assemblages, indicating the possibility of a significant barrier to dispersal. In a review of 58 population genetic studies of 42 New Zealand species, Gardner et al. (2010) found an overall pattern whereby 20 studies noted genetic differentiation across the top of the south island, between 41° and 42° South (Figure 1.1). This area represents a major break in animal distributions, community structure (Apte and Gardner 2002, Ayers & Waters 2005, Shears et al. 2008), and changes in habitat classifications (Walls 2009). The barrier could be caused by the high energy D'Urville current passing through the Cook Strait or strong upwelling regions on the west and east coasts of the north part of the South Island (Wallis and Trewick 2009).

The review by Gardner et al. (2010) also highlighted a surprising paucity of studies focusing on estuaries and sandy soft bottom habitats. Out of 58 studies, only 9 studies were conducted in estuarine systems, and 2 focused on sandy, soft bottom habitats, while 36 studies took place in rocky reef and intertidal systems. Coastal and soft bottom habitats are rarely studied for population connectivity, perhaps under the assumptions that these habitats are continuous, homogenous and well connected. For some soft bottom dwellers, estuaries are key habitats in early life history stages of these animals. Estuaries are areas of the coast that many organisms rely on as nursery grounds due to their sheltered, productive and biodiverse nature (Elliot and Hemingway 2008). A nursery area is one that contributes a large proportion of recruits to the adult population, therefore is essential to conservation and management (Beck et al. 2001, Sunblad 2013).

Unfortunately, estuaries are also disproportionately impacted by anthropogenic activities, including industrial, municipal and agricultural pollution

(Little 2000), which can lead to loss of function as critical nursery habitat (Pape et al. 2007). In New Zealand, many species of migratory fish rely on estuaries (McDowall 1976), therefore the levels of connectivity among these habitats, and the contribution of estuaries to other ecosystems is a crucial to our understanding of marine biodiversity, ecological function and evolution.

Two commercially, recreationally and culturally important representatives of estuarine and sandy soft bottom habitats are *Rhombosolea plebeia* (sand flounder) and *R. leporina* (yellowbelly flounder). The flatfish fishery for the New Zealand market and exports was estimated to be worth 8 million dollars in 2008 (Forest and Bird 2012), and is a popular recreational fishery for spearfishing. The flounder, or pātiki, is offered at Māori celebrations as a symbol of hospitality and is incorporated into Māori art as a symbol of abundance. *Rhombosolea* remains have been found in middens as old as the fourteenth century in New Zealand (Anderson 1981). The flounder fishery was later vital to the livelihood of the European settlers, with debates about how best to manage the fishery documented as far back as the late 1800's (Anonymous 1891).

R. plebeia is widely distributed in shallow coastal waters throughout New Zealand to 100 m. The sister species, *R. leporina*, is less common and distributed to 50 m and both rely heavily on estuaries for early life history stages (Ayling and Cox 1982). The PLD of fishes of the genus *Rhombosolea* has only been studied in *R. tapirina*, and was measured from fertilization to metamorphosis as an average of 69 days (Crawford 1984), and from hatching to metamorphosis as 35 days (Jenkins 1987), which varies widely with temperature (Chambers and Leggett 1992). Juvenile *R. plebeia* (TL) have been observed recruiting to estuaries at 4.5 mm total length (Roper 1986). At two years of age, flounders move out of estuaries onto shallow areas of the continental shelf (Mundy 1968, Colman 1978). Adults meet in large spawning aggregations at the mouths of harbour and estuaries during the austral winter and spring months (September through December) (Mundy 1968, Ayling and Cox 1982). Females of the sister taxa, *R. tapirina*, can ovulate multiple times during a spawning season (Barnett and Pankhurst 1999), which means there can be several pulses of recruits within a season.

The dispersive stage for *Rhombosolea* may be the PLD, given that the movements of adults are thought to be restricted. An intensive tagging study of *Rhombosolea* in the Hauraki Gulf (Colman 1974) and around the Banks Peninsula (Colman 1978) confirmed this observation of regular periodic aggregations as well as limited movements of adult fish. The farthest any individual travelled was 400 km, which was rare as most fish stayed within a 100 to 200 km area over the 4 year study. This may indicate some philopatric behaviour and the potential for separate populations.

Colman (1976) performed morphometric analysis of dorsal and anal fin counts and found strong differentiation among 5 areas, although there was significant variation between years, though it has been widely reported that number of vertebrae, fin and dorsal rays are strongly affected by temperature (Hubbs 1922, Barlow 1961, Kinoshita et al. 2000), rather than genetic variation. These studies are the basis for the current flatfish quota management areas (QMA) which include 8 species of flatfish under one management plan (Ministry of Primary Industries, MPI 2013a).

The goal of this study was to use the microsatellite DNA markers developed in chapter 2 to: 1) describe the population genetic structure of *R. plebeia* and *R. leporina* within New Zealand, 2) test for potential barriers (biogeographic, contemporary oceanographic, previously unidentified) to dispersal, and 3) compare the spatial genetic patterns of *R. plebeia* and *R. leporina*. This study fills major gaps in our knowledge about the coastal connectivity among estuarine and soft bottom habitats, which is severely lacking within New Zealand (Gardner et al. 2010) and globally (Caveen et al. 2012). This study contributes to the management of these commercially and culturally important species.

Methods

Sample Collection

Sample collection took place during the spring and summer of 2010/2011 and 2011/2012. Collection locations (Table 3.1) were chosen to include at least one site within each of the 9 mainland biogeographic region according to definitions of

Shears (2008) and Walls (2009) (Figure 1.1). There have been reports of *Rhombosolea* in the Fiordlands, but it was not possible to obtain samples from this location. To test the north to south differentiation highlighted by Gardner et al. (2010), I attempted to collect both species on either side of the Cook Strait. *R. plebeia* were collected from eight of the biogeographic regions, and *R. leporina* were collected in seven regions.

Flounders were collected using a 4 m wide beach seine with 9 mm mesh at low tide or incoming tide, yielding predominantly juvenile samples. Juveniles were euthanased in compliance with VUW animal ethics approval permit numbers 2008R5 and 2011R15, and then the whole animal was preserved in 80 % ethanol for later processing. Total length, head length and a photograph were recorded for each individual to confirm species identification by morphometric analysis (Eldon and Smith 1986). Larger fish caught using the beach seine were photographed, a fin clip was taken and preserved in 80% ethanol, and the animal was returned live to the estuary. For each location, both species of flounder were sought, but often locations only had one species of flounder, with the exception of Karamea River Estuary (KM) and Westport (WS) and Avon Heathcoat in 2011 (AH).

Adult flounder tissue samples were also collected on inshore commercial vessels by New Zealand Ministry of Primary Industries (Fisheries observers). A fin clip of approximately 2 cm² was cut from the pectoral fin on the blind side and preserved in 80% EDTA DMSO (Seutin et al. 1991) to comply with airline transportation requirements. Total fish length, site location and latitude/longitude were recorded for spatial analysis. Remaining adult specimens were sampled from fresh catch directly from fishermen or at fish markets. Adult specimens were identified on the basis of morphological characters and photographed. The total length recorded and a fin clip was sampled, placed in a 2 ml tube and fixed in 80% ethanol.

Figure 3. 1 *Rhombosolea plebeia* and *R. leporina* collecting localities

Table 3.1. *Rhombosolea plebeia* and *R. leporina* collecting locality descriptions, sample sizes, dates and coordinates

<i>R. plebeia</i> collection localities					
Code	Locality Description	N	Date Collected	Latitude	Longitude
DB-10	Dicks Bay, Bay of Islands, Te Huruhi Bay, east side of Dicks Bay Manawaora I	38	15 Nov 2010	-35.2678	174.2181
DB-11	Bay of Islands, Te Huruhi Bay, east side of Dicks Bay Manawaora Road	51	06 Sep 2011	-35.2678	174.2181
PA	Paihia, Bay of Islands, South of Paihia, end of Tohitapu Road	24	14 Nov 2010	-35.2938	174.0989
HK	Hokianga Harbour, east side of town of Rawene along Clendon Esplanade	48	14 Nov 2010	-35.3966	173.5060
MK	Manukau Harbour, Blockhouse Bay, Flounder Bay boat ramp	23	12 Nov 2010	-36.9265	174.7067
HB	Hawke Bay, 5 km east of Clive	45	07 Jan 2011	-39.5236	177.0645
AR	NNW corner of Ahuriri Estuary	47	22 Nov 2010	-39.4807	176.8788
FX	Foxton Estuary, 340 m west of car park towards end of Hartley Street	48	21 Nov 2012	-40.4714	175.2325
PH	Te Onepoto Bay, Porirua Harbour protected estuary on NW end	42	05 Oct 2010	-41.1079	174.8548
LF	Wairapa, West side Lake Onoke at Beach Road crossing	30	10 Apr 2012	-41.3835	175.1066
TB	Tasman Bay, ~ 8km north of town of Nelson	48	01 Nov 2010	-41.0545	173.2652
MH	Mapua Harbour, end of Tahi road on west side	47	25 Feb 2011	-41.2608	173.0985
KM	Karamea River Mouth behind Karamea Lodge on Otumahana Lagoon	23	21 Feb 2011	-41.2643	172.1047
WS	Orowati River Estuary, End of Snodgrass Road, off State Hwy 67	20	21 Feb 2011	-41.7438	171.6344
AH -11	Avon Heathcote Estuary by evaporating ponds	49	12 Feb 2011	-43.5311	172.7236
AH-12	Avon Heathcote Estuary by Scott Park	49	25 Feb 2012	-43.5561	172.7106
LH	Lyttleton Harbour	50	18 Dec 2011	-43.5833	172.8333
LE	Lake Ellesmere	49	19 Dec 2011	-43.7920	172.4970
OT	Mouth of Otago Harbour	22	23 Feb 2012	-45.7780	170.7196
TE	Waipori River, Taieri Mouth, east side, just south of bridge	50	24 Feb 2012	-46.0495	170.1927
AB-11	North side of Awarua Bay, off Awarua Bay Road, Bluff Harbour	29	16 Feb 2011	-46.5668	168.4727
AB-12	North side of Awarua Bay, east of Tiwai Road Bridge, Bluff Harbour	26	22 Feb 2012	-46.5610	168.4265
<i>R. leporina</i> collection localities					
MG	Mangonui Harbour	50	10 Sep 2011	-34.9914	173.5468
HK	Hokianga Harbour, Rawene	26	07 Sep 2011	-35.3966	173.5060
HB	Hawke Bay, 5 km east of Clive	49	07 Jan 2011	-39.5850	176.9689
TB	Tasman Bay, ~ 8km north of town of Nelson	47	01 Nov 2010	-41.0545	173.2652
BL	Wairau Bar, at mouth of Tuamarina River, Blenheim	50	11 Feb 2011	-41.5044	174.0552
KM	Karamea River Mouth behind Karamea Lodge on Otumahana Lagoon	11	21 Feb 2011	-41.2643	172.1047
WS	Orowati River Estuary, end of Snodgrass Road, off State Hwy 67	27	21 Feb 2011	-41.7438	171.6344
AH	Avon Heathcote Estuary by evaporating ponds	46	12 Feb 2011	-43.5311	172.7236
LH	Lyttleton Harbour	48	18 Dec 2011	-43.5833	172.8333
HS	Hapuku Estuary, Okuru River, southwest of Haast	50	20 Feb 2011	-43.8992	168.9191

Genotyping

DNA was extracted from fin and muscle tissues using a phenol-chloroform protocol modified from Barker (2005), with the Genomic DNA Minikit by Geneaid, or PrepGEM DNA extraction kit by ZYGEM, and stored in Tris-EDTA buffer at -20°C. Primers were developed from genomic DNA sequence data (454 Roche Sequencer) for both species separately, with the exception of locus 5, which cross-amplified (refer to Chapter 2). Multiplex PCRs were performed with 3 to 4 pairs of primers. The multiplex PCR mix of 15 µL contained Tris-HCl pH 8.8, 0.2 mM dNTPs, 0.6 mg/µL BSA, 2-3 pmol each of forward primer, 2-3 pmol each of reverse primer, 0.75 mM MgCl₂, 0.5 U *Taq* polymerase (Fisher Biotech) and 100 ng genomic DNA.

Thermal cycling was conducted on an Applied Biosystems GeneAmp 2700 PCR System machine using a touchdown protocol at one of two different temperature regimes; 65°C-55°C touchdown and 55°C-45°C touchdown, adapted from Rahman et al. (2000). The 65°C-55°C touchdown program was as follows: 95°C 3 min, 20 cycles of 30 s each at 95°C (denaturation), 65°C (annealing) with stepwise lowering of annealing temperature by 0.5° each cycle, 72°C (elongation); followed by 15 cycles of 30 s each at 95°C, 55°C and 72°C; and a final extension of 72°C for 15 min. The 55°C-45°C touchdown program was as follows: 95°C 3 min, 20 cycles of 30 s each at 95°C (denaturation), 55°C (annealing) with stepwise lowering of annealing temperature by 0.5° each cycle, 72°C (elongation); followed by 15 cycles of 30 s each at 95°C, 45°C and 72°C; and a final extension of 72°C for 15 min.

PCR products were electrophoresed in a 1.5% agarose gel and a Hyperladder I (Bioline) molecular weight standard was used to check the quality of the amplification. The genotypes of the resultant PCR products were determined using an Applied Biosystems 3730 Genetic Analyzer (Massey University Genotyping Service). Allele calling was conducted using the software GeneMarker version 2.2.0 (SoftGenetics) and was repeated twice by the author.

Evaluation of Loci and Power Analysis

Microchecker software (Oosterhout et al. 2004) was used to analyze the loci for both species for null alleles, stutter and homozygote excess with a 95% confidence interval and 1,000 iterations with suspect data included. Allele discovery curves over the entire dataset were generated using the R software package PopGenKit (Rioux Paquette 2011).

The observed heterozygosity (H_o), expected heterozygosity (H_e), fixation index (F_{IS}) was calculated for each locus and population combination by the software package GenAlEx 6.4 (Peakall and Smouse 2006). The number of private alleles (PA) and allelic richness (Ar) were calculated by the software package HPRare (Kalinowski 2005). Allelic richness was calculated for each locus with rarefaction using 40 individuals for *R. plebeia* and 26 individuals for *R. leporina*. A two-tailed test of Hardy-Weinberg Equilibrium (P_{HWE}) was performed by the software package GenePop on the web (Raymond and Rousset 1995).

To test for loci under selection, which would violate the assumption of neutrality, outlier analyses were performed by the software package LOSITAN (Antao et al. 2008) by comparing a global F_{ST} value to heterozygosity for 50,000 simulations with a confidence interval (CI) of 0.95 and a False Discovery Correction (FDR) (Verhoeven et al. 2005) of 0.05 assuming the infinite alleles model and stepwise mutation model using the neutral mean F_{ST} model. Linkage disequilibrium was tested by the software package GENEPOP 4.2 on the web (Raymond and Rousset 1995), implementing the log likelihood ratio statistic with 1,000 dememorizations, 100 batches and 1,000 iterations per batch.

A power analysis was conducted by the software package POWSIM 4.1 (Ryman and Palm 2006) running N_e of 500, 1000, 5000 and 10,000 for 20 iterations to estimate probability of detecting differentiation of an F_{ST} at the 0.001, 0.0025, 0.005 and 0.01 levels. Simulations were run 20 times to detect alpha levels.

Population Genetic Structure

Samples of *R. plebeia* that had been collected over two years, as two sampling seasons were needed to obtain satisfactory spatial coverage for both species as well as provide temporal replicates. I pooled the samples from locations where two years data were collected, Dicks Bay (DB), Avon Heathcote (AH) and Awarua Bay (AB) (Table 3.1) for the spatial analysis as there was no differentiation between years (refer to Chapter 4).

Allele frequency differences between populations were measured using a number of metrics. F_{ST} (Wright 1935, 1965) is a measure of allele frequency divergence from an idealized Hardy Weinberg Equilibrium (HWE) proportions due to subpopulation structure. The original F statistics were developed based on a single biallelic locus. F_{ST} was adapted by Wier and Cockerham (1984) for many multiallelic loci, is unbiased to different sample sizes and uses an ANOVA approach to standardize within and among population variation. The Weir and Cockerham (1984) analogue to F_{ST} , formally known as theta (θ), and is implemented in the widely used GENEPOP (Raymond and Rousset 1995) and Fstat (Goudet 2001), but here reported as F_{ST} , as it is commonly refer to in the literature. Pairwise F_{ST} values between sampling locations were calculated by the software package Fstat 2.9.3.2 (Goudet 2001) not assuming Hardy-Weinberg Equilibrium (HWE) with 5,000 iterations and pairwise tests of allelic differentiation. R_{ST} is an F_{ST} analogue that assumes a stepwise mutation model for microsatellites, and pairwise R_{ST} values were generated by the software package Arlequin 3.5.1.2 (Excoffier and Lischer 2010). All p-values were corrected using a False Discovery Rate Correction (FDR) (Verhoeven et al. 2005).

Theoretically, F_{ST} ranges from 0 to 1, however, with highly variable loci, such as microsatellites, the maximum value of F_{ST} is limited by the amount of genetic variation, or heterozygosity (Waples 1998, Hedrick 1999, Jost 2008). Therefore F_{ST} analogues were developed, including F'_{ST} , which standardizes the value of F_{ST} based on a theoretical maximum value (Hedrick 2005, Meirmans and Hedrick 2011). There is no p-value associated this standardized FST measure because it is calculated from a

theoretical data set (Meirmans 2006). Here I report pairwise population values of F_{ST} for each analysis from the program GenoDive 2.0b23 (Meirmans and Tienderen 2004).

Jost (2008) argues that aside from maximum F_{ST} measures being reduced when diversity is high, this statistic cannot detect the difference between low allelic frequencies and when two populations have no alleles in common at all. He proposed a metric, D , that will take total variation and differentiation into account. Here I also present Jost's D for comparison, which has no p-value associated with it. Pairwise Jost's D values were calculated using the software package GenoDive 2.0b23 (Meirmans and Tienderen 2004).

Mantel tests were calculated by ISOLDE in GENEPOP (Raymond and Rousset 1995) to test for isolation by distance pattern using F_{ST} and shortest coastal distances as measured on Google Earth. The Analysis of Molecular Variation (AMOVA) was conducted using the software package GenAlEx 6.4 with 99 permutations. The sampling locations were grouped by northern group (DB to MH) and southern group (KM to AB), western group (HK to HS including FX, PH, TB, MH) versus eastern group (MH to AB including LF and BL) samples and sampling year (see Table 3.1, Figure 3.1), based on F_{ST} . PCA analyses were also performed in GenAlEx using codominant genetic distance (Smouse and Peakall 1999).

Assignment Tests and Clustering Analysis

Neighbor joining (NJ) dendrograms were generated using the software package Poptree 2 (Takezaki et al. 2009) and were based on F_{ST} values with 1000 bootstrap replicates. Bayesian cluster analyses were performed using Structure 2.3.4 (Pritchard et al. 2000), which minimizes HW disequilibrium to establish the number of “k” clusters. Analyses were performed with a parameter set of 100,000 burn-ins and 1×10^6 assuming the admixture model for k=1 through k=8 five times with and without locprior settings using population labels. Results were evaluated using Structure Harvester's DeltaK analysis (Earl and vanHoldt 2012). Cluster analyses were also performed using AWClust, which does not make any assumptions about HWE or linkage disequilibrium (LD) (Gao and Starmer 2008). The program was originally developed for SNP data, but

each allele within a locus can be coded as SNPs, with two for homozygote, one for heterozygote and zero for no allele in a genotype, following the approach of Gruber et al. (2013).

Assignment analysis was performed using the software package GeneClass2 (Piry et al. 2004), which is a Bayesian analysis that does not assume HWE, or that all potential populations have been sampled, and uses a Markov chain Monte Carlo (MCMC). GeneClass 2 was used to assign individuals to the most likely population of origin using the Rannala and Mountain Bayesian method and estimate numbers of recent migrants between sampling locations.

Results

A total of 872 *R. plebeia* adults and juveniles were genotyped at 12 loci from 19 locations. Subsequently 10 individuals were removed as they had missing data for six or more loci, leaving a total of 862 fish for the final analyses. A total of 412 *R. leporina* adults and juveniles were genotyped at 14 loci from 10 populations. Subsequently 8 individuals were removed as they had missing data at seven or more loci, leaving a total of 404 for the final analyses.

Evaluation of Loci and Power Analysis

Microchecker identified loci 25, 65, 79, 25, 124 and 145 as possibly having null alleles due to an overall homozygote excess, but no scoring errors due to stutter or long allele drop out were detected. Locus 145 had the most missing data at 9.51% and locus 133 had the least missing data at 0.85%, with an average of 4.71% across loci. When examining whether populations were in HWE at each locus, loci 25, 57, 65, 79 and 145 were identified as being significantly out of HWE at more than half of the populations after FDR correction for multiple tests (Appendix 1). Most loci had reached the asymptote for number of alleles discovered over the samples for the entire study, with the exception of locus 11 (Appendix 3). Therefore loci 145 and 65 were excluded from

further analysis for a combination of possibly being under selection, missing data and being significantly out of HWE across many populations.

For *R. leporina*, no loci were identified as candidates for selection and no patterns of linkage disequilibrium were found across populations or loci. Microchecker indicated that locus 1 had an excess of homozygotes and null alleles, and locus 22 had a homozygote excess and null alleles, but no scoring errors due to stutter or long allele drop out were detected. Locus 22 had the most missing data at 9.06% and locus 20 had the least missing data, with an overall average of 2.82%. When examining whether populations were in HWE, loci 20, 22, 23 and 37 were identified as being significantly out of HWE at more than half of the populations after FDR correction for multiple tests (Appendix 2). Locus 5 had yet to reach the asymptote of number of alleles discovered by 400 individuals (Appendix 4). Locus 22 was excluded from further analysis due to a combination of factors.

For *R. plebeia*, outlier analysis performed in Lositan identified locus 65 as a candidate for strong positive selection, locus 57 as a candidate for weak positive selection and locus 131 was identified as being under weak balancing selection when samples were split into 22 localities (years separated). When all localities were combined, locus 145 was a candidate for strong positive selection. Results were similar under the stepwise mutation and infinite alleles model. No consistent patterns of linkage disequilibrium were found across localities or loci. Power analyses for the *R. plebeia* data set indicated that pairwise F_{ST} values less than 0.0025 would be difficult to detect regardless of the N_e , and the alpha, or probability of detecting a false positive, was 5%, and increased as N_e increased (Appendix 5). Power analyses of the *R. leporina* data set indicated that pairwise F_{ST} values less than 0.0025 would be difficult to detect, and alpha values ranged from 5% to 10% (Appendix 6).

Table 3.2. Descriptive population statistics for *R. plebeia* and *R. leporina*. Sample size (N), observed heterozygosity (H_o), expected heterozygosity (H_e), allelic richness (A_r), probability of being out of Hardy-Weinberg Equilibrium (P_{HWE}), fixation index (F_{IS}) and number of private alleles (PA). P-values for localities significantly out of HWE after FDR correction for multiple tests labeled in bold

<i>R. plebeia</i>							
Code	N	H_o	H_e	A_r	P_{HWE}	F_{IS}	PA
DB	89	0.405	0.475	4.27	0.000	0.170	1
PA	24	0.399	0.468	3.84	0.000	0.162	1
HK	48	0.422	0.464	4.13	0.000	0.143	1
MK	23	0.390	0.463	4.00	0.001	0.185	0
HB	45	0.365	0.433	4.01	0.000	0.154	0
AR	47	0.456	0.486	4.50	0.005	0.099	1
FX	47	0.424	0.467	4.38	0.000	0.044	0
PH	42	0.398	0.469	4.27	0.001	0.190	0
LF	30	0.431	0.488	4.53	0.004	0.134	0
TB	48	0.432	0.496	4.66	0.000	0.188	0
MH	47	0.417	0.473	4.54	0.000	0.152	0
KM	23	0.480	0.477	4.62	0.399	-0.028	1
WS	20	0.420	0.464	4.60	0.055	0.133	0
AH	97	0.463	0.503	4.49	0.000	0.070	0
LH	50	0.423	0.477	4.23	0.004	0.124	0
LE	48	0.454	0.457	4.42	0.392	0.013	1
OT	29	0.453	0.466	4.34	0.037	0.043	0
TE	50	0.441	0.479	4.82	0.000	0.164	1
AB	55	0.438	0.508	4.63	0.000	0.112	0
Totals / Averages	862	0.427	0.474	4.38	0.047	0.119	0.368
<i>R. leporina</i>							
MG	50	0.525	0.548	5.79	0.124	0.025	4
HK	26	0.525	0.567	5.79	0.025	0.072	0
HB	49	0.498	0.535	5.8	0.013	0.060	3
TB	47	0.535	0.548	5.72	0.005	0.017	0
BL	50	0.497	0.556	4.71	0.000	0.070	0
KM	11	0.485	0.534	n/a	0.001	0.107	1
WS	27	0.542	0.534	5.26	0.403	-0.012	0
AH	46	0.497	0.561	6.36	0.000	0.149	5
LH	48	0.500	0.554	6.22	0.000	0.066	1
HS	50	0.520	0.568	5.81	0.000	0.072	2
Totals / Averages	404	0.484	0.522	5.17	0.057	0.075	1.080

Population Genetic Structure

Overall, the locality-specific summary statistics for *R. plebeia* (Table 3.2) showed slightly lower levels of observed heterozygosity, average $H_o = 0.427$, than expected heterozygosity, average $H_e = 0.474$. However, 16 of 19 populations were significantly out of HWE after FDR correction for multiple tests. Allelic richness (A_r) averaged 4.38, F_{IS} ranged from -0.028 at KM to 0.190 at PH, with an average of 0.119, and seven private alleles were found at seven locations. Locality-specific data for *R. leporina* (Table 3.2) also showed overall slightly lower levels of observed heterozygosity, average $H_o = 0.484$, than expected heterozygosity, average $H_e = 0.522$, and eight out of ten locations were significantly out of HWE. Average allelic richness was 5.17, F_{IS} ranged from -0.012 at WS to 0.149 at AH, with an average of 0.075. The number of private alleles was 16 across six populations.

F_{ST} values for all pairwise comparisons of localities of *R. plebeia* over all loci (Table 3.3) ranged from 0 to 0.039. Of the 171 tests of allelic differentiation, 93 were significant after FDR corrections for multiple tests where the final and highest p-value of statistical significance was $p = 0.0275$. The LE and HK localities had the greatest number of significant comparisons. F'_{ST} values ranged from 0 to 0.066 between LE and HK (Table 3.3). R_{ST} (Table 3.4) values ranged from 0 to 0.110 (LE and PH, respectively), however, of the 171 tests, only 16 were significant after FDR corrections where the final and highest p-value of statistical significance was $p = 0.0041$. Pairwise comparisons of R_{ST} values had only a third as many significant comparisons, with Lake Ellesmere and Dicks Bay being the most differentiated. Jost's D values ranged from 0 to 0.0356 (Table 3.4). The sample comparisons that had the most significant differences when compared to other locations were LE, DB and HK. There was a weak isolation by distance signal in *R. plebeia* ($p < 0.001$, $R^2 = 0.1409$) (Figure 3.2) using F_{ST} and minimum coastal distances.

F_{ST} values for all populations of *R. leporina* over all loci (Table 3.5) ranged from 0 to 0.0456. Of the 45 tests, 36 were significant after FDR correction for multiple tests where the final and highest p-value of statistical significance was $p = 0.0422$. KM and

LH locations had the greatest number of significant pairwise population comparisons. F'_{ST} values (Table 3.5) ranged from 0 to 0.091, with LH and KM having the highest values. R_{ST} (Table 3.6) ranged from zero to 0.0820 (WS and LH), but only 14 values were significant after FDR correction for multiple tests where the final and highest p-value of statistical significance was $p = 0.0178$. Jost's D values ranged from 0 to 0.061 (Table 3.6). For *R. leporina*, the F_{ST} values for HB, TB and BL populations were highly differentiated at all nine comparisons with other populations, but R_{ST} was only significant for six comparisons for BL, four for TB and two for HB. There was no isolation by distance relationship for *R. leporina* ($p = 0.308$, $R^2 = 0.002$) (Figure 3.2).

AMOVA analysis for *R. plebeia* regions grouped by northern populations and southern populations showed 79.534% of the variation was explained within populations, 19.220% was explained among individual within populations, 0.630% was explained among populations within groups and 0.616% was explained among groups, and all of these percentages were significant at the 0.05 level (Table 3.7). Results for east coast and west coast populations were similar, and all of these percentages were significant at the 0.05 level (Table 3.8). *R. leporina* samples grouped with regions north and south showed 85.529% of variation was explained within populations, 12.744% among individuals within populations, 1.461% among populations and 0.266% among regions, with all percentages significant at the 0.05 level (Table 3.9). *R. leporina* samples grouped east and west showed similar results with 85.548% of variation was explained within populations, 12.747% among individuals within populations, 1.497% among populations and 0.209% among regions, with all percentages significant at the 0.05 level (Table 3.10). Temporal differences did not explain any of the variation in either data set at the 0.05 level (see Chapter 4 for more details).

Table 3.3. Pairwise F_{ST} values between *R. plebeia* sampling locations below the diagonal, significant values indicated in bold after False Discovery Rate (FDR) correction for multiple tests. F'_{ST} values above the diagonal

	DB	PA	HK	MK	HB	AR	FX	PH	LF	TB	MH	KM	WS	AH	LH	LE	OT	TE	AB
DB		-0.012	0.012	-0.003	0.004	0.002	0.024	0.014	0.003	0.007	0.013	0.010	-0.001	0.039	0.023	0.051	0.019	0.049	0.035
PA	-0.004		-0.008	-0.015	-0.016	-0.007	0.002	-0.015	-0.023	-0.016	-0.013	-0.010	0.012	0.005	-0.008	0.024	-0.012	0.015	0.000
HK	0.008	-0.003		-0.013	0.001	0.006	0.022	0.013	0.001	0.014	0.025	0.001	0.031	0.029	0.022	0.066	0.010	0.047	0.031
MK	0.001	-0.004	-0.006		-0.030	-0.019	-0.004	-0.014	-0.008	-0.010	0.008	-0.014	0.012	0.014	-0.003	0.046	0.001	0.036	0.019
HB	0.005	-0.002	0.004	-0.010		-0.005	0.003	0.002	-0.001	-0.004	0.006	-0.013	0.008	0.020	0.006	0.045	0.004	0.033	0.018
AR	0.003	-0.001	0.004	-0.007	0.001		0.011	-0.002	-0.004	-0.004	0.006	-0.003	0.015	0.017	0.012	0.037	0.018	0.034	0.018
FX	0.015	0.005	0.013	0.001	0.007	0.008		-0.008	-0.006	0.008	0.010	-0.010	0.033	0.016	0.001	0.033	0.014	0.037	0.016
PH	0.011	0.000	0.011	0.001	0.009	0.003	0.001		-0.016	0.000	0.000	-0.015	0.003	0.014	-0.005	0.024	0.002	0.026	0.010
LF	0.004	-0.008	0.002	0.000	0.007	0.001	0.000	-0.002		-0.001	0.000	-0.004	0.023	0.008	-0.006	0.025	0.000	0.024	0.009
TB	0.006	-0.005	0.009	-0.001	0.002	0.000	0.007	0.006	0.003		-0.016	-0.006	-0.006	-0.005	-0.005	0.010	-0.006	-0.002	-0.011
MH	0.010	-0.001	0.017	0.011	0.010	0.007	0.010	0.007	0.006	-0.005		0.004	-0.002	-0.001	-0.005	-0.007	-0.005	-0.005	-0.011
KM	0.009	-0.002	0.002	-0.004	0.001	0.001	-0.002	0.000	0.002	0.000	0.009		0.003	0.014	0.016	0.045	0.016	0.032	0.004
WS	0.002	0.008	0.017	0.008	0.013	0.010	0.021	0.010	0.016	0.000	0.005	0.004		0.031	0.029	0.044	0.021	0.030	0.021
AH	0.023	0.007	0.018	0.012	0.015	0.012	0.011	0.012	0.008	0.000	0.003	0.012	0.021		-0.004	0.004	-0.010	-0.003	-0.010
LH	0.015	0.000	0.014	0.003	0.009	0.009	0.003	0.002	0.001	0.000	0.001	0.013	0.019	0.001		0.000	-0.019	0.002	0.000
LE	0.031	0.019	0.039	0.031	0.034	0.024	0.022	0.021	0.019	0.009	0.001	0.031	0.030	0.006	0.004		-0.001	-0.001	0.007
OT	0.014	-0.001	0.008	0.006	0.011	0.013	0.012	0.010	0.006	0.001	0.004	0.015	0.016	-0.001	-0.006	0.006		-0.007	-0.003
TE	0.028	0.011	0.026	0.022	0.024	0.020	0.022	0.019	0.016	0.001	0.001	0.020	0.018	0.001	0.004	0.003	0.000		-0.006
AB	0.033	0.016	0.029	0.023	0.026	0.019	0.022	0.022	0.020	0.007	0.008	0.014	0.024	0.006	0.015	0.019	0.017	0.007	

Table 3.4. Pairwise R_{ST} values between *R. plebeia* sampling locations below the diagonal, significant values indicated in bold after False Discovery Rate (FDR) correction for multiple tests. *Jost's D* values above the diagonal

	DB	PA	HK	MK	HB	AR	FX	PH	LF	TB	MH	KM	WS	AH	LH	LE	OT	TE	AB
DB		-0.003	0.007	0.001	0.005	0.003	0.014	0.010	0.004	0.005	0.009	0.008	0.002	0.022	0.014	0.029	0.013	0.027	0.021
PA	0.001		-0.003	-0.004	-0.002	-0.001	0.004	0.000	-0.007	-0.004	-0.001	-0.002	0.007	0.007	0.000	0.016	-0.001	0.010	0.005
HK	0.007	0.032		-0.005	0.003	0.004	0.012	0.010	0.002	0.008	0.015	0.002	0.015	0.018	0.013	0.036	0.007	0.025	0.019
MK	0.002	0.028	-0.004		-0.009	-0.007	0.001	0.001	0.000	-0.001	0.010	-0.003	0.007	0.012	0.002	0.028	0.006	0.021	0.016
HB	-0.001	-0.005	0.009	0.010		0.001	0.006	0.008	0.006	0.002	0.009	0.001	0.011	0.014	0.008	0.028	0.009	0.021	0.014
AR	0.008	0.036	-0.006	-0.008	0.011		0.007	0.003	0.000	0.000	0.007	0.001	0.009	0.012	0.009	0.022	0.012	0.019	0.013
FX	0.000	0.022	-0.008	-0.005	0.000	-0.010		0.001	0.000	0.007	0.009	-0.002	0.019	0.011	0.003	0.020	0.011	0.021	0.011
PH	-0.008	-0.008	0.012	-0.001	0.007	0.008	0.001		-0.002	0.005	0.007	0.000	0.009	0.012	0.002	0.019	0.009	0.018	0.011
LF	-0.004	0.016	-0.012	-0.008	-0.005	-0.012	-0.013	-0.009		0.003	0.005	0.002	0.015	0.008	0.001	0.017	0.006	0.015	0.010
TB	0.003	0.026	-0.006	-0.001	0.009	-0.005	-0.009	0.007	-0.012		-0.004	0.001	0.001	0.000	0.000	0.009	0.001	0.001	-0.002
MH	0.010	0.027	0.010	0.022	0.029	0.010	0.002	0.016	-0.007	-0.003		0.008	0.005	0.003	0.001	0.001	0.004	0.001	-0.001
KM	0.006	0.029	0.008	-0.017	0.015	0.000	0.000	-0.008	-0.003	0.004	0.023		0.003	0.013	0.012	0.028	0.014	0.020	0.008
WS	-0.006	0.009	-0.008	-0.004	-0.003	-0.004	-0.015	-0.021	-0.018	-0.016	-0.015	-0.003		0.021	0.018	0.027	0.015	0.017	0.017
AH	0.050	0.097	0.015	0.026	0.061	0.012	0.017	0.051	0.014	0.015	0.020	0.035	0.021		0.001	0.005	-0.001	0.001	-0.003
LH	0.017	0.054	-0.004	-0.002	0.028	-0.008	-0.005	0.022	-0.010	-0.002	0.013	0.008	-0.002	0.002		0.003	-0.005	0.003	0.003
LE	0.057	0.110	0.025	0.043	0.081	0.021	0.025	0.072	0.019	0.019	0.021	0.050	0.024	-0.006	0.012		0.005	0.002	0.008
OT	0.004	0.038	-0.016	0.000	0.018	-0.011	-0.010	0.025	-0.019	-0.014	-0.003	0.007	-0.023	-0.005	-0.011	0.003		0.000	0.004
TE	0.031	0.060	0.012	0.009	0.046	0.007	0.010	0.007	0.007	0.003	-0.008	0.015	0.009	0.000	-0.003	-0.007	-0.014		0.000
AB	0.027	0.065	0.006	0.013	0.042	0.003	0.005	0.035	0.002	0.000	0.004	0.020	0.005	0.000	0.000	-0.002	-0.011	-0.006	

Table 3.5. Pairwise F_{ST} values between *Rhombosolea leporina* sampling locations below the diagonal, significant values indicated in bold after False Discovery Rate (FDR) correction for multiple tests. F'_{ST} values above the diagonal

	MG	HK	HB	TB	BL	KM	WS	AH	LH	HS
MG		0.021	0.010	0.025	0.040	0.037	0.014	0.044	0.040	0.024
HK	0.011		0.023	0.026	0.038	0.009	-0.009	0.022	0.033	-0.031
HB	0.006	0.012		0.002	0.014	0.053	0.015	0.014	0.020	0.021
TB	0.012	0.013	0.002		0.004	0.088	0.021	0.017	0.011	0.018
BL	0.019	0.018	0.008	0.003		0.079	0.038	0.020	0.020	0.033
KM	0.023	0.015	0.032	0.045	0.043		0.036	0.022	0.091	0.024
WS	0.007	-0.002	0.008	0.010	0.019	0.025		0.007	0.020	-0.011
AH	0.024	0.017	0.011	0.012	0.013	0.032	0.010		-0.011	0.024
LH	0.018	0.015	0.010	0.005	0.010	0.046	0.010	-0.001		0.027
HS	0.012	-0.011	0.011	0.009	0.016	0.020	-0.003	0.016	0.013	

Table 3.6. Pairwise R_{ST} values between *Rhombosolea leporina* sampling locations below the diagonal, significant values indicated in bold after False Discovery Rate (FDR) correction for multiple tests. *Jost's D* values above the diagonal

	MG	HK	HB	TB	BL	KM	WS	AH	LH	HS
MG		0.014	0.007	0.015	0.024	0.028	0.009	0.032	0.024	0.016
HK	0.004		0.016	0.017	0.025	0.021	-0.003	0.024	0.020	-0.015
HB	0.003	-0.008		0.002	0.010	0.040	0.010	0.014	0.012	0.014
TB	0.012	0.005	0.004		0.004	0.058	0.013	0.016	0.007	0.012
BL	0.028	0.016	0.026	0.027		0.057	0.024	0.018	0.013	0.022
KM	-0.005	0.009	0.007	0.029	-0.025		0.031	0.043	0.061	0.027
WS	0.042	0.011	0.017	0.026	0.065	0.063		0.013	0.012	-0.003
AH	0.022	0.007	0.016	0.025	-0.006	-0.019	0.042		-0.002	0.022
LH	0.051	0.029	0.050	0.042	0.002	0.006	0.082	0.003		0.017
HS	0.015	-0.007	0.006	0.014	0.014	-0.001	0.011	0.001	0.026	

Figure 3.2. Mantel test regressing transformed F_{ST} values with straight line coastal geographic distance for *R. plebeia* and *R. leporina*

Table 3.7. AMOVA for *R. plebeia* using F_{ST} with North and South as regions

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Variance	Estimated Variance	% Variation	Differentiation Indexes	Significance
Between Regions (N vs S)	1	18.6	18.564	0.016	0.616%	$F_{RT} = 0.006$	0.010
Among Populations	17	77.5	4.556	0.017	0.630%	$F_{SR} = 0.006$	0.010
Among Individuals	843	2606.8	3.092	0.504	19.220%	$F_{ST} = 0.012$	0.010
Within Populations	862	1797.0	2.085	2.085	79.534%	$F_{IS} = 0.195$	0.010
Total	1723	4499.8		2.621	100%	$F_{IT} = 0.205$	0.010

Table 3.8. AMOVA for *R. plebia* using F_{ST} with East and West as regions

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Variance	Estimated Variance	% Variation	Differentiation Indexes	Significance
Between Regions (E vs W)	1	7.0	7.043	0.002	0.085%	$F_{RT} = 0.001$	0.030
Among Populations	17	87.9	5.173	0.023	0.889%	$F_{SR} = 0.009$	0.010
Among Individuals	843	2607.8	3.093	0.504	19.292%	$F_{ST} = 0.010$	0.010
Within Populations	862	1797.0	2.085	2.085	79.734%	$F_{IS} = 0.195$	0.010
Total	1723	4499.8		2.615	100%	$F_{IT} = 0.203$	0.010

Table 3.9. AMOVA for *R. leporina* using F_{ST} with North and South as regions

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Variance	Estimated Variance	% Variation	Differentiation Indexes	Significance
Between Regions (N vs S)	1	14.2	14.242	0.011	0.266%	$F_{RT} = 0.003$	0.010
Among Populations	8	74.3	9.293	0.060	1.461%	$F_{SR} = 0.015$	0.010
Among Individuals	393	1795.5	4.569	0.524	12.744%	$F_{ST} = 0.017$	0.010
Within Populations	403	1418.5	3.520	3.520	85.529%	$F_{IS} = 0.130$	0.010
Total	805	3302.6		4.115	100%	$F_{IT} = 0.145$	0.010

Table 3.10. AMOVA for *R. leporina* using F_{ST} with East and West as regions

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Variance	Estimated Variance	% Variation	Differentiation Indexes	Significance
Between Regions (E vs W)	1	13.1	13.136	0.009	0.209%	$F_{RT} = 0.002$	0.010
Among Populations	8	75.4	9.431	0.062	1.497%	$F_{SR} = 0.015$	0.010
Among Individuals	393	1795.5	4.569	0.524	12.747%	$F_{ST} = 0.017$	0.010
Within Populations	403	1418.5	3.520	3.520	85.548%	$F_{IS} = 0.130$	0.010
Total	805	3302.6		4.114	100%	$F_{IT} = 0.145$	0.010

Figure 3.3. Principal component analysis for *R. plebeia* and *R. leporina* using pairwise genetic distances

The results of the PCA for *R. plebeia* showed that Axis 1 explained 50.08% of the variation in the data and Axis 2 explained 17.09% (Figure 3.3). The plot showed a tight grouping of the North Island populations on the right side of the axis in addition to WS and KM, and the remaining South Island populations were on the left. There was one particularly close grouping of HB and AH, which are 10 km apart. The PCA plot for *R. leporina* showed that Axis 1 explained 44.81% and Axis 2 explained 28.55% of the variation (Figure 3.3). East coast localities, except for TB on the right side of the axis. West coast localities, in addition to MG were on the left side of the axis.

Assignment Tests and Clustering Analysis

For *R. plebeia*, the NJ dendrograms showed most of the southern populations grouping together with the exception of WS and KM (Figure 3.4), but with low support (50-55% bootstrap values). Lake Ellesmere (LE) and TE grouped (63%), and DB and WS grouped together with 51% support. For *R. leporina*, NJ dendrograms showed 78% support for most of the east coast populations grouping together, with the exception of MG, but also included the TB population. There was 68% support for a grouping with HK and HS. The main structure of the tree did not change when KM was removed. There was very high bootstrap support (96%) for a grouping between AH and LH for *R. leporina*, which are approximately 8 km apart (Figure 3.4).

The cluster analysis in STRUCTURE for *R. plebeia* had the greatest DeltaK value of 2 clusters (Figure 3.5), and the bar plot showed a cline of proportions of clusters with a change in proportions from AH south (Figure 3.6). Cluster analysis for *R. leporina* showed the greatest DeltaK value for four clusters (Figure 3.5) and the bar plot showed three spatially separated clusters and a rarer fourth cluster labeled in yellow (Figure 3.6). The west coast populations grouped in the predominantly green labeled cluster, while the eastern populations contained a greater proportion of red cluster. A blue cluster included Tasman Bay and populations on the eastern part of the south island (Figure 3.6). The gap analysis from AWClust showed *R. plebeia* with 5 clusters by a narrow margin with widely overlapping error bars (Figure 3.7). The gap analysis for *R. leporina* showed a strong signal for two main clusters (Figure 3.7).

Figure 3.4. Neighbor Joining population tree based on F_{ST} values for *R. plebeia* and *R. leporina*

Figure 3.5. Delta K values from 2 to 7 (Evanno et al. 2005) for *R. plebeia* and *R. leporina*

Figure 3.6. Cluster analysis from Structure bar plot K = 2 for *R. plebeia* and k = 4 for *R. leporina*

R. plebeia

R. leporina

Figure 3.7. AWClust results for $k=1-8$ for *R. plebeia* and *R. leporina*. The graph on the left shows plots of the log of the expected (E) and observed (O) pooled within cluster sum of squares (W_k) plotted against the number of clusters. The graph on the right shows the gap statistics between the observed and expected (W_k). The largest gap indicates the most appropriate number of clusters for the data.

Assignment analysis for *R. plebeia* showed an average correct assignment of 35.69% for the localities (Table 3.11). The locality with the highest level of correct assignment was KM at 60.00% and the lowest was AH at 21.65%. The individuals from AH were most likely to assign to locations further south. The locality OT received 6.52% of the total number of misassigned individuals. Assignment analysis for *R. leporina* (Table 3.11) had an average of 66.81% correct assignment to the original localities, the highest at KM, with 81.82% and the lowest at HS at 38.00%. The majority of incorrectly assigned individuals were assigned to HK or WS, both at 6.19%.

For *R. plebeia*, OT, along with LH, were most likely to receive first generation migrants, at about 20% each (Table 3.12). PH was the least likely to receive first generation migrants at 0%. WS was the most likely to donate first generation migrants, with 15.00% emigration. For *R. leporina*, KM was the most likely to receive first generation migrants at 36.36% of the total population possibly being immigrants, but was the most likely to donate first generation migrants at 18.18% (Table 3.12). HK and WS localities were composed of about 15% immigrants, and HK was the second most likely to provide emigrants at 15.38% of the total sample size for that locality. There was no evidence for a source or sink relationship between any of the localities in either species

Table 3.11. Number of individuals assigned to collecting localities based on genotype for *R. plebeia* and *R. leporina*. Correct assignment is the percentage of individuals assigned to their original collection locality over the total number of individuals from that locality. Percent of total from other localities is the percentage of individuals assigned to a locality other than the original collection locality over the total number of fish.

Assignments to <i>R. plebeia</i> localities																				
Original collection locality	DB	PA	HK	MK	HB	AR	FX	PH	LF	TB	MH	KM	WS	AH	LH	LE	OT	TE	AW	Correct Assignment
	DB	27	15	4	4	7		3	5	4	3		3	4	2	2	2		2	30.34%
	PA		9			1	1		3	3				1		2	1	3		37.50%
	HK	6	3	23	3	2	2			1	1		1	1		2		3		47.92%
	MK		2	1	11	2	1		1				1				1	2	1	47.83%
	HB	2	6	3	5	15	1	2	3	1		1	3	1		1			1	33.33%
	AR	3	3	2	3	3	13	4	1		1	4	1	2		2	1	1	2	27.66%
	FX	2	4	3	1	3	3	15	6	1			1				5	2		31.91%
	PH	1	2	1	1	2	4	1	16	3	1	1	1	2		4		2		38.10%
	LF	3	2	2	2		1	1		9				4	1	2	1	1	1	30.00%
	TB	2	2	4	1	2	1	1	1		12	3	2	2	2		4	4	1	25.53%
	MH	1	1	1	2	6		3		3		14	1	4	1	2	4			30.43%
	KM		1	1		1		3	1				1	9	2	1		1	1	39.13%
	WS					1			1	1	1			12	1			1		60.00%
	AH	2	1	7	2	5	2	1	2	3	2	3	2	2	21	6	9	12	9	21.65%
	LH	2	1	2	2	3	1		3	4	1	1	3	1	1	13	4	5	1	26.00%
	LE	2	1		1	1		3	2			1			2	1	23	7	2	47.92%
	OT		1	1		1			1	1	2	1		1	1		5	12		41.38%
	TE	1	1	1	2	3	1	1			2	1			2	5	5	3	15	30.00%
	AW		4	2	1	3			1	4	3	1	1	1	2	4	1	7	2	31.48%
% of total from other localities	3.14%	5.82%	4.07%	3.49%	5.36%	2.10%	2.68%	3.61%	3.38%	1.98%	2.21%	2.33%	3.26%	1.86%	3.96%	5.12%	6.52%	2.33%	3.49%	Average correct assignment = 35.69%

Assignments to <i>R. leporina</i> localities												
Original collection locality		MG	HK	HB	TB	BL	KM	WS	AH	LH	HS	Correct Assignment
	MG	37		1	2	4	1	4	1			72.00%
	HK	1	16		1		1	3			4	61.54%
	HB	3	1	36	5			4				73.47%
	TB	2	1	4	27	4		2	2	3	2	57.45%
	BL	2	2	1	4	35		1	1	4		70.00%
	KM		1				9	1				81.82%
	WS		3	1				21		1	1	77.78%
	AH	1	3	3		2		2	30	3	2	65.22%
	LH	2	1		4			1	3	35	2	70.83%
	HS	1	13	2	5		2	7			20	38.00%
% of total from other localities		2.97%	6.19%	2.97%	5.20%	2.48%	0.99%	6.19%	1.73%	2.72%	2.72%	Average correct assignment = 66.81%

Table 3.12. Probability of first generation migrants to each population of *R. plebeia* and *R. leporina*. Individuals originate from the localities labeled vertically on the left side of the table and are assigned to the localities labeled horizontally across the top of the table. The number of immigrants to a new locality are tallied across the bottom of the table, along with the percent of the population that may be first generation immigrants (% of locality). Number of emigrants are tallied on the right side of the table with the percent of the locality that are emigrating. Averages in the bottom right corner of table.

		Immigrant receiving localities for <i>R. plebeia</i>																					% of
Original collection locality		DB	PA	HK	MK	HB	AR	FX	PH	LF	TB	MH	KM	WS	AH	LH	LE	OT	TE	AB	Emigrants	locality	
	DB		1							1		1		1		1					5	5.62%	
	PA					1															1	4.17%	
	HK										1					2		1	1		5	10.42%	
	MK			1														1			2	8.70%	
	HB				2							1									3	6.67%	
	AR		1												1				1	1	4	8.51%	
	FX		2	1														1			4	8.51%	
	PH							1					1				1				3	7.14%	
	LF	1											1		2						4	13.33%	
	TB			1										1					1		4	8.51%	
	MH																				0	0.00%	
	KM							1	1							1					3	13.04%	
	WS										1	1				1					3	15.00%	
	AH										1										1	1.03%	
	LH													1					1		2	4.00%	
	LE															1					1	2.08%	
	OT																1				1	3.45%	
	TE												1			1	1				3	6.00%	
	AB											1						1	2		4	7.41%	
Immigrants		1	4	3	2	1	2	1	0	3	2	5	2	4	4	6	2	6	2	2	Avg. No. emigrants = 3		
% of locality		1.12%	16.67%	6.25%	8.33%	2.22%	8.33%	2.13%	0.00%	10.00%	8.33%	10.64%	8.33%	20.00%	16.67%	12.00%	8.33%	20.69%	8.33%	3.64%	Avg. No. immigrants = 2.74		

Immigrant receiving localities for <i>R. leporina</i>													
Original collection locality		MG	HK	HB	TB	BL	KM	WS	AH	LH	HS	Emigrants	% of locality
	MG				1	1	1	1	1			5	10.00%
	HK						1				3	4	15.38%
	HB	1						2				3	6.12%
	TB		1			2			1		1	5	10.64%
	BL	1	1							4		6	12.00%
	KM		1			1						2	18.18%
	WS								1	1		2	7.41%
	AH		1					1		1		3	6.52%
	LH	1		1	3						1	6	12.50%
	HS				2		2					4	8.00%
Immigrants		3	4	1	6	4	4	4	3	6	5	Avg No. of emigrants = 4	
% of locality		6.00%	15.38%	2.04%	12.77%	8.00%	36.36%	14.81%	6.52%	12.50%	10.00%	Avg. No. of immigrants = 4	

Discussion

The aims of this chapter were to describe population genetic patterns of two species of endemic New Zealand flatfish and test for potential barriers to gene flow. Using biogeographic boundaries as a guide, I tested for differences in population connectivity between the northern and southern regions. Finally, I compared the genetic patterns of the two species.

Population structure

A low, but significant, level of population structure was found in both species of flatfish: *R. plebeia* showed a significant isolation by distance signal and a distinct southeastern population; and *R. leporina* showed a difference between eastern to western areas. There is gene flow among most locations for both species, with some level of structure.

Rhombosolea plebeia

There was a significant north to south split between *R. plebeia* locations north and south of AH on the east coast, which was consistently found in multiple analyses: F_{ST} , R_{ST} , AMOVA, PCA and STRUCTURE. Pairwise comparisons of allele frequencies showed more significant F_{ST} and R_{ST} values between the northern groups and southern groups than within either the northern or southern groups. The two localities KM and WS from the northwest part of the South Island consistently grouped with the North Island localities. The NJ tree had weak support for most of the localities from the south island together (TB, MH, AH, LH, LE, OT, TE and AW), but placed KM and WS with the North Island localities.

These results may indicate a north to south split below WS and above AH, however, the bar plot from the Bayesian cluster analysis in STRUCTURE showed a cline of northern and southern clusters to (Figure 3.6) which was mirrored in the mantel test (Figure 3.2). The second cluster analysis in AWClust does not assume HWE, which most of the descriptive statistics for localities did not meet (Table 3.2), and concerning the number discrete clusters due to the large confidence intervals. Assignment analyses

showed moderate levels of self-recruitment and detection of first generation migrants (Table 3.12) revealed the potential for exchange across most localities, north and south.

As far as the number of populations, the southern-most localities (AH, LH, LE, OT, TE and AW) appear to be one population. This group was highly differentiated from the other localities, but showed some exchange between them according to the STRUCTURE analysis and assignment tests. All of the other localities showed some differentiation among northern groups, but appear to be well connected through gene flow.

In Colman's meristic studies (1985, 1976) he found 5 distinct groups of fish based on significant differences in anal and dorsal ray counts. He stated that the southeastern group on the South Island were clearly differentiated from the west coast populations on the South Island and central populations. The populations on the west and east coasts of the north part of the North Island (Auckland to Cape Reinga) were also separated from one another. Colman concluded, based on his tagging studies (Colman 1974, 1978), that some populations may be their own distinct groups, such as Tasman Bay, Hawkes Bay and the Hauraki Gulf (Colman 1985). My results are somewhat consistent with Colman's findings, in that the southeast of the South Island is clearly a separate population, differentiated from all other localities. There appears to be a moderate amount of connectivity among North Island populations, central populations on the west coast of the South Island. This is driven by an isolation by distance signal of differentiation, which may be due to a stepping stone model of connectivity.

Colman (1978) tagged animals from the Banks Peninsula from three separate areas, the Avon Heathcote Estuary, Lyttleton Harbour and Akaroa Harbour, within 80 km of one another. Recaptured animals from Avon Heathcote and Lyttleton Harbour were collected in the greatest numbers in a bay north of the tagging area. Animals from Akaroa Harbour were caught in a bay south of the tagging area. Colman (1976) and Munday (1968) both discussed the possibility of there being two separate populations from the Banks Peninsula. In this current study, I did not have any samples from Akaroa Harbour, but this may be another separate population I did not sample. In Colman's meristic study of *R. plebeia* (1976) he found the greatest differences in fin ray counts

were between populations in Tasman Bay and Kapiti-Manawatu. The pairwise comparisons between Tasman Bay (TB and MH) and areas in the Kapiti-Manawatu region (FX and PH) showed that TB and MH were both significantly different from PH but not FX. This could indicate further fine scale differentiation, however only one analysis (the F_{ST} values) found this comparison significant, so it is inconclusive given all of the analyses available.

Rhombosolea leporina

R. leporina populations were split between east coast and west coast populations with some mixing across the top of the North Island according to allele frequency differences, F_{ST} , R_{ST} , PCA, dendrogram and STRUCTURE analysis. All pairwise population comparisons of F_{ST} were significant, with the exception of comparisons among west coast populations, and between AH and LH. The PCA results showed that all of the west coast populations grouped together on Axis 1, in addition to MG. In the dendrogram, the east coast populations, with the exception of MG, clustered together and had 78% bootstrap support. The cluster analysis in STRUCTURE showed four clusters, with the west coast locations as one cluster. The cluster analysis in AWClust showed two distinct populations (Figure 3.7) although I was not able to discern which populations were in which cluster. There was no evidence of a pattern of isolation by distance, most likely because of the structuring within subgroupings, rather than a cline.

There are most likely four populations of *R. leporina* around New Zealand given these data. While the STRUCTURE analysis showed four clusters, one of those clusters (labeled in yellow Figure 3.6) was mostly attributed to the KM location, which was composed of a small sample size ($n=11$). The effects of this small sample size may be skewing the other results. The AMOVA analysis (Tables 3.9 and 3.10) showed a significant east to west differentiation and north to south differentiation. This may be due to grouping the significantly differentiated AH and LH group with the southern group, which appears to be its own distinctive population. The other cluster analysis in AWClust showed only two groupings, and was most likely picking up the east to west differentiation.

These findings are consistent with Colman's observations of meristic and tagging studies (1974, 1976, 1978 and 1985). As for *R. plebeia*, the populations on the south east side of the South Island were completely differentiated from all other populations. TB was also differentiated from other west coast populations, but showed some connectivity between BL, another central population. HB was also very well differentiated from other populations, according to F_{ST} values and showed one migrant per generation joining the population, but 3 emigrants leaving the population.

Geographic patterns

Biogeographic patterns outlined by Shears et al. (2008) and Walls (2009) were used to direct the sampling design for this study, while I specifically tested the north to south split described by Shears et al. (2008) and Gardner et al. (2010). *R. plebeia* populations show significant differentiation between northern and southern locations, however, the location of this putative boundary was difficult to pinpoint given these data. Samples from Karamea River Estuary mouth (KM) and the Orowaiti Lagoon, Westport (WS) were significantly differentiated from some populations on the North Island according to F_{ST} values (Table 3.3), and all populations from the southeast side of the South Island, but not significantly different from TB or MH, which are just north of the biogeographic break proposed by Shears et al. (2008) and Gardner et al. (2010). PCA results and STRUCTURE analysis show KM and WS samples grouping with North Island populations (Figure 3.3, Figure 3.6) and there may be connectivity with locations further south, as with *R. leporina*, but there were no *R. plebeia* collected at the HS location. On the west coast, this does not support the proposed north to south provincial boundary by Shears et al. across the Cook Strait, or the differentiation found in the review by Gardner (et al. 2010) at Farewell Spit. On the east coast, the genetic break between northern and southern populations is much clearer, but whether it is correlated to Gardner's proposed break at Cape Campbell or Shear's proposed break south of the Hikurangi Trough at Kaikoura is unclear, as there no samples were collected on the north east end of the South Island for comparison.

The more striking pattern is the level of connectivity along the west coasts of both islands in *R. leporina*. STRUCTURE analysis (Figure 3.6) showed the predominance of a green labelled cluster along the west coast and around the top of the North Island. Samples do show differentiation between BL and AH, according to F_{ST} values (Table 3.5) and STRUCTURE analysis (Figure 3.6), but there is also the possibility of some exchange of migrants among the east and west coasts (Tables 3.11 and 3.12).

The prevailing southeasterly flowing Tasman Front splits over the top of the North Island into the West Auckland Current and the East Auckland current, however there is connectivity between HK and MG populations in *R. leporina*. The eastward flowing D'Urville Current, which cuts into Cook Strait around the NW tip of the South Island does not appear to be a migratory barrier to either of the species, as there appears to be gene flow across the Cook Strait.

The Banks Peninsula population appeared to be differentiated from all northern and western populations in both species, and features exchange between AH and LH. The geographic proximity of these populations may be facilitating exchange, which is supported by tagging experiments by Colman (1978). In this area, the Southland Current flows north along the Banks Peninsula and breaks into eddies around the Avon Heathcote estuary, which may retain larvae (Mundy 1968, Kilner 1974). This area was strongly differentiated in *R. leporina*, with no exchange with other populations. This could be due to the fact that it is the one area in the South Island that consistently shows the presence of *R. leporina* (www.NABIS.gov.nz), or may be due to the lack of samples from further south to compare with. Another possibility is the historical geography of the area. In the Pliocene (~5MYA), sea levels were at their highest in New Zealand, leaving the Banks Peninsula as an isolated island (Wallis and Trewick 2009), possibly segregating these localities from others, then reconnecting later in time.

Lake Ellesmere is a brackish lagoon, separated from the ocean by the Kaitorete Spit, periodically flooding and connecting with the open sea. This population is strongly differentiated from even neighbouring AH and LH in *R. plebeia*, although there is no differentiation between OT and TE to the south. The PCA shows Lake Ellesmere isolated from other populations. This is probably due to the geology of the area, but warrants

further investigation, as this could possibly reveal local adaptation for lower salinity due to freshwater inflows into the lake, especially during the winter months.

There is evidence of population differentiation between locations in the north and south and also from east to west in species that live on soft bottom, sandy and estuarine areas in New Zealand. Tuatua, *Paphies subtriangulata*, shows two main groupings, a northern grouping from Napier north and a central grouping which spanned the Cook Strait (Smith et al. 1989). Seagrass, *Zostera muelleri*, shows small amounts of gene flow between adjacent estuaries, but strong differentiation between the North and South Islands, and east and west coasts (Jones et al. 2008). A study of *Gracilaria chilensis* also found a population grouping in the north of the North Island, which did not include Napier, and an overall country group that was distinct from most of the north of the North Island populations (Intasuwan et al. 1993). The amphipod species *Chaetocorophium lucasi* shows strong differentiation among populations, especially the east and west coast populations of the north island, while *Paracorophium excavatum* showed differentiation between the north and south locations south of Nelson (Schnabel et al. 2000), which the authors attributed to a lack of pelagic larval stage and specific habitat requirements.

Comparison of *R. plebeia* and *R. leporina*

Rhombosolea plebeia and *R. leporina* have a very similar life histories and ecology (Ayling and Cox 1982), but have different patterns of population genetic structuring. *R. plebeia* shows more genetic connectivity among localities, and is geographically more widely distributed around New Zealand. *Rhombosolea leporina* had more significant differences between localities, less connectivity and higher levels of self-recruitment, and is mainly concentrated around the North Island and Banks Peninsula (www.NABIS.gov.nz). *R. leporina* is only distributed to 50 m, as opposed to *R. plebeia* which is distributed to 100m depth, and more commonly found further up rivers (Ayling and Cox 1982). *R. leporina* may be slower growing and later to mature (Colman 1970, Mutoro 2001). The Banks Peninsula populations (AH, LE, and LH) were significantly differentiated from other localities in both species.

Both *R. plebeia* and *R. leporina* have a relatively low amount of genetic diversity, according to these microsatellite markers, compared to other marine species with the number of alleles (N_a) averaging 10.3 and 9.64, and the mean heterozygosity (H_o) of 0.430 and 0.501 respectively. Freshwater fish show an overall average N_a of 7.5 and H_o of 0.46, anadromous fish have mean values of $N_a = 11.3$ and $H_o = 0.68$, while marine fish show $N_a = 20.6$ and $H_o = 0.79$ (DeWoody and Avise 2000). Higher levels of genetic diversity will increase the theoretical maximum of F_{ST} due to fewer alleles (Hedrick 1999, Waples 1998). Low genetic diversity has been shown in species as a direct consequence of high fishing pressure (Horau et al. 2005, Hauser et al. 2002), but without historical samples of *R. plebeia* and *R. leporina* to make a comparison, this is not possible to determine whether there have been any recent reductions in diversity.

Both *Rhombosolea* species exhibit genetic connectivity on a scale of 15 to 100 km, but differentiation on larger scales. These findings may be a reflection of restricted adult movements identified in the tagging studies by Colman (1978) showing local retention of adults on the scale of 100 to 200 km. These findings are supported by a study of the sister species *Rhombosolea tapirina*, the Tasmanian flounder which, based on an assessment of allozyme variation, found little differentiation between samples taken 10 and 30 km apart, but strong differentiation at the scale of 200 to 500 km and across the Tasman sea (van den Eden 2000). The authors attributed these genetic differences to the spatial arrangement of suitable habitats, which would indicate a stepping stone model of connectivity.

Despite their relatively long PLD and potential to disperse great distances, *Rhombosolea plebeia* and *R. leporina* show population structure around the New Zealand mainland. Shanks (2009) noted that genetic evidence tended to overestimate dispersal distance for organisms with PLDs of over 30 days. He suggested that this may be due to a rare long distance dispersal event evening out the genetic differences between populations. This could be the result of environmental factors either limiting dispersal of juveniles and adults and/or behavioral adaptations among adults and juveniles.

A comparison of population structure of species of the Family Tripterygiidae, a diverse endemic species flock in New Zealand, showed that shallow water species had lower genetic diversity and stronger population structure than deep water species (Hickey et al. 2009). This may reflect the effect of gaps in suitable habitat that restrict dispersal between estuaries habitats, as opposed to other habitat types that may be more connected. This same study suggested that cockabully, *Grahamina nigripenne*, shows an isolation by distance pattern and a north to south grouping, which is similar to my findings for *R. plebeia*. Interestingly, some Haast (HS) cockabully haplotypes grouped with the northern populations, which was also a pattern observed in *R. leporina*.

Many species of pleuronectids are known to have some site fidelity between breeding and foraging sites (Gibson 1998). Spawning behaviour may play a role in the local retention of larvae by aggregating at the mouths of harbours and estuaries to promote the transport of eggs into the nursery grounds (Frisk et al. 2013). Post flexion juvenile *R. plebeia* have been seen recruiting into estuaries (Roper 1986), which may be facilitated by the juveniles changing their position in the water column to migrate up to catch an incoming tide into the estuary and then deeper to be retained (Epifanio and Garvine 2001). These patterns may be reinforced over generations as parents return to these same estuaries to spawn, showing habitat preference or philopatry (Bailey 1997). Adult movements may play a larger than expected role in population structure (Frisk 2013). For example, the winter flounder (*Pseudopleuronectes americanus*) have resident and migratory populations within the same bays (Stoner et al. 2001, Crivello et al. 2004). Movements between nursery grounds and adult habitats could clarify some of these relationships (Gillanders et al. 2003)

This work highlights the importance of studying each species independently, even if they are closely related. Habitat preference or distribution can widely change overall patterns of genetic connectivity. These results also dispute the assumption that all sandy, soft bottom habitats are well connected, and that animals with longer PLDs have largely open populations. To examine what might be causing the boundary between northern and southern populations of *R. plebeia*, or eastern and western populations of *R. leporina*, biophysical models can be used (refer to Chapter 4). A more detailed and

explicit sampling design could be coupled with environmental models to investigate these spatial disjunctions.

Conclusions

Patterns of connectivity and differentiation among the two species show different patterns. *R. plebeia* has a distinct isolation by distance pattern for much of the country, but differentiation from the southeast part of the country and high levels of connectivity among these localities from AH south to AB. *R. leporina* shows no isolation by distance pattern, strong differentiation between west and east coast populations and up to 4 separate groupings or stocks. These genetic differences in *R. leporina* do not correspond with the biogeographic boundaries proposed by Shears et al. (2008) or Gardner et al. (2010), nor by the prevailing currents.

Chapter 4. Stability in spatial and temporal genetic structure of the sand flounder (*Rhombosolea plebeia*)

Abstract

Marine animals experience high mortality and oceanographically variable conditions during their pelagic larval phase, therefore spatial genetic patterns can change over time. The Sweepstakes Recruitment Hypothesis (SRS) posits that due to this variability, only a small portion of the successfully breeding adult population is represented in next year class. This could cause changes in spatial patterns between year classes and bias any spatial genetic patterns. In this chapter, I examine the temporal aspects of my data set to: 1) test for temporal stability of spatial patterns; 2) compare levels of relatedness among juvenile samples compared to adult samples; and 3) compare levels of diversity among juveniles and adults. There was no significant differentiation in allele frequencies between years, between juveniles and adults or between cohorts of juveniles. No evidence of relatives among juvenile samples collected in estuaries was found compared to adults collected from the open coast, therefore there was no evidence of family structure in estuaries. There was no evidence of a reduction in diversity among juvenile samples compared to adults, therefore no support for the SRS. This study is an important quality control check on the spatial results and explores an often overlooked dimension of population genetic studies.

Introduction

For highly fecund marine species, productivity, abundance and distribution can be variable on multiple temporal and spatial scales (Jennings et al. 2001). Changing oceanographic conditions, fluctuating levels of transport of eggs and larvae, and variable recruitment conditions across seasons, years and decades can result in complex genetic patterns after settlement (Johnson and Black 1982, Palumbi 1994). This temporal variability may have profound effects on the spatial structure of genetic diversity in marine populations (Waples 1998) and adds uncertainty to fisheries management (Larson and Julian 1999, Hauser and Carvalho 2008).

Population genetic structure is typically the result of a balance between the forces of genetic drift and gene flow (Slatkin 1987), although this only applies when using neutral markers. Spatial genetic patterns that we expect to see as a result of genetic drift and gene flow range from discrete populations, isolation by distance, to panmixia (Laikre et al. 2005). Even with some level of population structure and barriers to dispersal, a low level of gene flow through infrequent long distance dispersal is theoretically sufficient to reduce a weak signal of population structure (Wright 1931, Mills and Allendorf 1997).

Genetic differentiation can change dramatically across multiple temporal scales. Within a season there can be different patterns of larval transport, differential selection, and therefore different amounts of gene flow between spawning events, such as for the juvenile bicour damselfish (Hepburn et al. 2009), juvenile blue rockfish (Burford and Larson 2007), European eel (Pujolar et al. 2006) and juvenile orange-spotted grouper (Pumitinsee 2009). Differentiation in genetic structure between years has been documented in the Japanese eel (Han et al. 2010), icefish (Papetti et al. 2009) and red drum (Chapman et al. 2002). There may be genetic differentiation within the same

geographic area, river catchment or estuary or within a single season due to early and late spawners, as seen in salmonids (Quinn et al. 2000, Fillatre et al. 2003) or resident versus migratory populations, as seen in the winter flounder (Sagarese and Frisk 2011).

Temporal variability in gene flow can create many other complex spatial population genetic patterns on multiple scales. One possible outcome of this variability in space and time is a “genetic mosaic” of allele frequencies (Johnson and Black 1984), whereby individuals may have been transported together in a water mass and recruited to the same area, creating a pocket of heterogeneity in an overall homogenous mixture (Pujolar et al. 2006). This could include groups of relatives from a single spawning event being transported together (Planes et al. 2002, Planes and Lefant 2002).

The “sweepstakes reproductive success” (SRS) hypothesis proposed by Hedgecock (1994, 2011) has been used to explain the influence of temporal variability on recruitment patterns. This hypothesis states that high mortality during the pelagic larval duration (PLD) results in comparatively few larvae surviving to recruitment. As a result, only a small subset of the adult population successfully reproduces and contributes to the next generation. Even though groups of recruits may come from spatially or temporally separated spawning events, the resulting spatial pattern in the adult population over time would result in mixing from many sources and possibly panmixia (Han et al. 2010, Burford and Larson 2007, Chapman et al. 2002). The SRS predicts two results of this model: (a) reduced genetic diversity among the juveniles compared to the adults and (b) smaller effective population size (N_e) compared to the census size (N).

The effects of SRS may be problematic when using predominantly juvenile samples to determine population structure in the adult population due to non-random sampling (Waples 1998). New recruits collected from the same site may be close relatives that were transported together in a single water mass. Juveniles may have reduced genetic diversity among juveniles compared to adults, or a reduction in genetic heterogeneity in a single year class. This would make a sample of juveniles a poor genetic representation of the adult population and give a false signal of population structure (Allendorf and Phelps 1981).

Rhombosolea plebeia is a commercially and customarily important species in New Zealand (NZ), with a PLD of 70 days (Crawford 1984, Jenkins 1987). Variability in recruitment success of flounders is relative to the duration of the pelagic larval duration (PLD) (van der Veer et al. 2000). *R. plebeia*, the NZ sand flounder, spawns in the southern hemisphere during the Austral spring and summer months of September to December (Mundy 1968, Ayling and Cox 1982). Females of the sister taxa, *R. tapirina*, can ovulate multiple times during a spawning season (Barnett and Pankhurst 1999), which means there can be multiple pulses of recruits within a season. Given the long spawning season, it is likely that *R. plebeia* is also capable of ovulating multiple times. *R. plebeia* live for 5 to 8 years, therefore complete population turn-over can occur within 10 years. Colman (1985) also points out that the size limits for capturing flounder are so small, slower growing females may only spawn once before subjected to fishing pressure. Therefore the so the persistence of population structure may be short lived.

The overall aim of this chapter was to use polymorphic microsatellite loci to genotype *R. plebeia* individuals from 22 populations across the New Zealand mainland to examine genetic variation in the sand flounder. The majority of the individuals that were collected (in 17 populations) were below the known size for reproductive maturity and they were sampled in estuaries, which are known to be nursery area for flounders. Therefore, the majority of the samples included in this study were juveniles. The sampling design could be affected by spatial structuring due to non-random sampling or stochastic events that might reduce diversity during the PLD (Ruzzante 1996) such as SRS. Temporal sample replicates have been used to test whether patterns of geographic differentiation are consistent through time and not an artefact of juvenile sampling (Waples 1998). Samples were taken from three populations in the same or near the same locations between 2010 and 2012 to examine whether there are differences in population structure between years and recruitment pulses.

The goal of this chapter is to test for temporal stability of the spatial patterns observed in the chapter 3, and for any non-random effect of sampling juveniles of *Rhombosolea plebeia*. Specifically, to test for temporal stability (1) microsatellite allele frequencies were compared across age classes to determine whether there was inter-annual variation (a) across years; (b) between juveniles and adults collected in adjacent

localities; and (c) between pulses of recruits. The effects of genotyping a possibly non-random sample of juveniles (2) were investigated by comparing levels of relatedness within juveniles compared to adults and with-in cohorts. To test an aspect of the SRS hypothesis (3) levels of genetic diversity between adults and juveniles samples were compared. This study explores an important and often over-looked dimension of marine population genetic studies.

Methods

Sampling

Sample collection and genotyping is described in detail in Chapter 2. No flounder were dissected or sexed directly, so sexual maturity was estimated by size. All flounder < 145 mm total length (TL) collected by beach seine were considered juveniles and all flounder > 250 mm TL collected from commercial fisheries were considered adults (Mundy 1968, Colman 1972). There were no individuals capture between 145 mm and 250 mm.

There were four main collecting expeditions during which the majority of specimens were collected during the spring and summers of 2010 /2011 and 2011 /2012. The first one covered the North Island during November 2010 and the second covered the South Island in February 2011. In September 2011, a second trip to the North Island yielded the second *R. plebeia* sample for Dicks Bay (DB). In February 2012, a second year sample for Avon Heathcote (AH) and Awarua Bay (AB) were collected (Table 4.1). Because the spawning season ranges from September to January, populations were designated a sample season based on which summer they were collected (Table 4.1). Therefore, three sites were sampled at the same location two years in a row, Dicks Bay (DB-10, DB -11), Avon Heathcote (AH-11, AH-12) and Awarua Bay (AB-11, AB-12).

Figure 4.1. Map of collecting localities of *R. plebeia* included in temporal study

Four locations had both adult and juvenile populations within a putative biogeographic region (<80 km apart) sampled respectively, Hawke Bay (HB) and Ahuriri Estuary (AR), Tasman Bay (TB) and Mapua Harbour (MH), Lyttelton Harbour (LH) and Avon Heathcote (AH), and Otago Harbour (OT) and Taieri Mouth (TE) (Table 4.1, Figure 4.1)

Table 4.1. Sample collection sites for *R. plebeia*, life stage, date and location collected

Location	Code	N	Life Stage	Date Collected	Latitude	Longitude
Dicks Bay 2010	DB-10	38	Juvenile	15 Nov 2010	-35.2678	174.2181
Dicks Bay 2011	DB-11	51	Juvenile	06 Sep 2011	-35.2678	174.2181
Hawks Bay	HB	45	Adult	07 Jan 2011	-39.5236	177.0645
Ahuriri Estuary	AR	47	Juvenile	22 Nov 2010	-39.4807	176.8788
Tasman Bay	TB	48	Adult	01 Nov 2010	-41.0545	173.2652
Mapua Harbour	MH	47	Juvenile	25 Feb 2011	-41.2608	173.0985
Avon Heathcote 2011	AH-11	49	Juvenile	12 Feb 2011	-43.5311	172.7236
Avon Heathcote 2012	AH-12	49	Juvenile	25 Feb 2012	-43.5561	172.7106
Lyttleton Harbour	LH	50	Adult	18 Dec 2011	-43.5833	172.8333
Otago Harbour	OT	22	Adult	23 Feb 2012	-45.7780	170.7196
Waipori River, Taieri Mouth	TE	50	Juvenile	24 Feb 2012	-46.0495	170.1927
Awarua Bay, Bluff 2011	AB-11	29	Juvenile	16 Feb 2011	-46.5668	168.4727
Awarua Bay, Bluff 2012	AB-12	26	Juvenile	22 Feb 2012	-46.5610	168.4265

Cohort definition

To define cohorts of recruiting juveniles, total length of juvenile fish samples were measured from DB-10, DB-11, AH-11, AH-12, AB-11 and AB-12. I assumed that cohort lengths were normally distributed to be able to discern pulses of recruitment. Length-frequency histograms were generated in 5 mm increments and normal distributions within the histograms were identified as proxies for cohorts of similarly aged individuals. The length cohorts were identified using the Bhattacharya's analysis as implemented in FiSAT II 1.2.2 (Gayanilo et al. 2005), where lengths are transformed by natural logarithms and plotted in a line to choose standard deviations around the mean (Bhattacharya 1967) (Figure 4.2), representing the normal distribution and standard deviation.

Population Statistics

The observed heterozygosity (H_o), expected heterozygosity (H_e), and fixation index (F_{IS}) were calculated by GenAlEx 6.4 (Peakall and Smouse 2006) across loci for each locality. Allelic richness (Ar) was calculated with rarefaction using 40 individuals in HPRare and Genotype Viewer (Kalinowski 2005). A two-tailed test of Hardy-Weinberg Equilibrium (P_{HWE}) was performed by GenePop on the web (Raymond and Rousset 1995). All p-values were corrected using the false discovery rate method (FDR) (Verhoeven et al. 2005). The number of private alleles (PA) across all populations was calculated using Genotype Viewer (Kalinowski 2005). Once cohorts were designated for the Dicks Bay, Avon Heathcote and Awarua Bay populations these same population statistics were calculated for each cohort with allelic richness (Ar) based on a minimum of 7 individuals and private alleles (PA) compared within the same location as calculated in HPRare and Genotype Viewer (Kalinowski 2005).

Population Differentiation

To test whether there were significant differences between years, between adults and juveniles and between recruitment pulses, pairwise population F_{ST} values were calculated by Fstat (Goudet 1995) not assuming Hardy-Weinberg equilibrium with 5,000 iterations and pairwise tests of allelic differentiation. All p-values were corrected using the false discovery rate method (FDR). AMOVA were run by GenAlEx 6.4 with 99 permutations with populations grouped by sampling season and life stage as labeled in Table 4.1.

Relatedness

To test whether juvenile samples had a greater number of relatives than the adult populations, mean population relatedness (r) (Queller and Goodnight 1989) across all populations, was calculated by GenAlEx (Smouse and Peakall 2006) with 999 bootstrap permutations to test for significance. The measure r is the genetic similarity between

two individuals relative to that between random individuals in some reference population (Pamilo 1990). To test whether there might be relatives within cohorts of recruits, estimates of mean relatedness of fish from cohorts (Table 4.4) were calculated using the same method.

Diversity

Measures of genetic diversity were compared between pairs of adults and juvenile samples that were collected within 80 km, (HB and AR, TB and MH, LH and AH, OT and TE). Observed heterozygosity (H_o) and allelic richness (A_r) across loci were compared. The number of private alleles (PA) were calculated solely between the adult/juvenile pairing.

Results

Cohort definition

Length-frequency analysis of *R. plebeia* samples between years (Figure 4.2) revealed that all individuals captured in these locations were between 5 mm and 95 mm TL and had similar length ranges between years. To test for differentiation between cohorts, normal distributions were identified within the length-frequency histograms as a proxy for pulses of recruits. Samples with more than one pulse identified were separated out based on length and tested as separate cohorts. There were two cohorts each within Dicks Bay 2011, Avon Heathcote 2011 and Awarua Bay 2011 (Figure 4.2). The cohorts were designated as follows: DB-10-A and DB-11-B, DB-11-C, AH-11-A, AH-11-B, AH-12-C, AB-11-A, AB-11-B and AB-12-C (Table 4.2).

Figure 4.2. Length-frequency histograms of total length of juvenile samples across years at three locations with normal distribution outlined using Bhattacharya's method

Table 4.2. Cohort designation based on length-frequency histograms for *R. plebeia*. The minimum total length (Min Length) and maximum total length (Max Length) in millimeters of each cohort, the mean size (mm) and standard deviation (SD) and number of individuals in a designated cohort are shown.

Population	Code	Min Length	Max Length	Mean	SD	n
Dicks Bay 2010	DB-10-A	26.99	68.61	50	8.81	35
Dicks Bay 2011	DB-11-B	21.27	43.4	32.5	5.23	20
Dicks Bay 2011	DB-11-C	51.53	67.85	60.55	5.32	24
Avon Heathcote 2011	AH-11-A	19.58	34.38	28.25	7.94	15
Avon Heathcote 2011	AH-11-B	43.49	68.39	55.22	5.52	26
Avon Heathcote 2012	AH-12-C	13.15	31.92	21.63	5.83	48
Awarua Bay 2011	AB-11-A	20.33	40.88	29.85	6.51	35
Awarua Bay 2011	AB-11-B	43.94	63.65	53.09	5.47	12
Awarua Bay 2012	AB-12-C	21.24	47.27	35.85	5.57	22

Population Summary Statistics

Overall, the summary statistics for *R. plebeia* populations (Table 4.3) showed lower levels of observed heterozygosity compared to expected heterozygosity for all samples. Allelic richness (A_r) ranged from 3.84 within DB-11 to 4.82 within TE. F_{IS} ranged from 0.099 within AR to 2.49, and four private alleles were found in 13 populations. Among cohorts (Table 4.4), observed heterozygosity was lower than expected heterozygosity, except in the cohort AH-11-A. Allelic richness ranged from 2.99 to 3.66, with an average of 3.36. F_{IS} ranged from -0.076 (AH-11-A) to 0.439 (AB-11-B).

Table 4.3 Description statistics for sampling locations of *R. plebeia*. Sample size (N), observed heterozygosity (H_o), expected heterozygosity (H_e), allelic richness (A_r), probability of being out of Hardy-Weinberg Equilibrium (P_{HWE}), fixation index (F_{IS}) and number of private alleles (PA). P-values for localities significantly out of HWE after FDR correction for multiple tests labeled in bold

Code	N	H_o	H_e	A_r	P_{HWE}	F_{IS}	PA
DB-10	38	0.413	0.472	4.28	0.000	0.156	1
DB-11	51	0.407	0.461	3.84	0.098	0.185	0
HB	45	0.365	0.433	4.01	0.000	0.154	0
AR	47	0.456	0.486	4.50	0.005	0.099	1
TB	48	0.432	0.496	4.66	0.000	0.188	0
MH	47	0.417	0.473	4.54	0.000	0.152	0
AH-11	47	0.491	0.511	4.58	0.116	0.037	0
AH-12	50	0.434	0.489	4.40	0.000	0.096	0
LH	50	0.423	0.477	4.23	0.004	0.124	0
OT	29	0.453	0.466	4.34	0.037	0.043	0
TE	50	0.441	0.479	4.82	0.000	0.164	1
AB-11	29	0.368	0.526	4.69	0.000	0.249	0
AB-12	26	0.435	0.494	4.60	0.002	0.103	1

Table 4.4. Population statistics among cohorts of *R. plebeia*. Sample size (N), observed heterozygosity (H_o), expected heterozygosity (H_e), allelic richness (A_r), probability of being out of Hardy-Weinberg Equilibrium (P_{HWE}), fixation index (F_{IS}) and number of private alleles (PA) within each cohort of each population. P-values for localities significantly out of HWE after FDR correction for multiple tests labelled in bold

Code	N	H_o	H_e	A_r	P_{HWE}	F_{IS}	PA
DB-10-A	35	0.403	0.455	3.09	0.019	0.135	6
DB-11-B	20	0.38	0.437	2.99	0.062	0.118	1
DB-11-C	24	0.409	0.472	3.37	0.012	0.146	5
AH-11-A	15	0.509	0.472	3.17	0.594	-0.076	2
AH-11-B	26	0.466	0.517	3.52	0.124	0.089	4
AH-12-C	48	0.431	0.492	3.34	0.000	0.104	3
AB-11-A	35	0.401	0.525	3.56	0.000	0.196	5
AB-11-B	12	0.273	0.515	3.66	0.000	0.439	1
AB-12-C	22	0.442	0.503	3.54	0.018	0.096	3
Total/ Avg.	237	0.413	0.488	3.36	0.092	0.139	3.333

Population Differentiation

There were no significant differences between years (Table 4.5) or cohorts of recruits (Tables 4.6, 4.7 and 4.8). There were however significant differences between North Island and South Island populations (Tables 4.5 and 4.9), revealing a spatial, but not a temporal pattern of genetic differentiation. There were no significant differences between adults and juveniles within the same region. The AMOVA showed that no significant variation was explained by sample year (Table 4.10) or between juveniles and adults (Table 4.11).

Table 4.5. Pairwise tests for population differentiation between years are outlined, F_{ST} values below and p-values above diagonal. Bold values indicate significant values after FDR correction for multiple testing

	DB 2010	DB 2011	AH 2011	AH 2012	AB 2011	AB 2012
DB 2010		0.3600	0.0033	0.0033	0.0033	0.0233
DB 2011	0.0060		0.0033	0.0033	0.0033	0.0033
AH 2011	0.0222	0.0248		0.9500	0.5900	0.8167
AH 2012	0.0232	0.0241	-0.0005		0.5667	0.7200
AB 2011	0.0351	0.0263	-0.0020	-0.0026		0.4800
AB 2012	0.0098	0.0132	-0.0046	-0.0067	-0.0059	

Table 4.6. Tests for population differentiation between cohorts from Dicks Bay. F_{ST} values are below and p-values above diagonal

	DB-10-A	DB-11-B	DB-11-C
DB-10-A		0.7333	0.2833
DB-11-B	0.0000		0.4833
DB-11-C	0.0135	-0.0018	

Table 4.7. Tests for population differentiation between cohorts from Avon Heathcote. F_{ST} values below and p-values above diagonal.

	AH-11-A	AH-11-B	AH-12-C
AH-11-A		0.0333	0.4333
AH-11-B	-0.0012		0.6833
AH-12-C	-0.0022	0.0002	

Table 4.8. Tests for population differentiation between cohorts from Awarua Bay. F_{ST} values below and p-values above diagonal.

	AB-11-A	AB-11-B	AB-12-C
AB-11-A		1.0000	0.6500
AB-11-B	-0.0250		1.0000
AB-12-C	-0.0038	0.0012	

Table 4.9. Pairwise tests for population differentiation between adult and juvenile samples within the same region are outlined. F_{ST} values below and p-values above diagonal. Bold values indicate significant values after correction for multiple tests.

	HB	AR	TB	MH	LH	AH	OT	TE
HB		0.34643	0.20357	0.05536	0.00714	0.00357	0.00179	0.00179
AR	0.0011		0.47143	0.15893	0.01071	0.0125	0.00357	0.00179
TB	0.0023	-0.0001		0.81071	0.1375	0.28214	0.28214	0.23036
MH	0.01	0.0069	-0.0046		0.11429	0.56964	0.09464	0.26429
LH	0.0087	0.0089	0	0.001		0.46786	0.54643	0.12679
AH	0.0166	0.0108	-0.0012	-0.002	0.0016		0.74107	0.61607
OT	0.0112	0.0129	0.0008	0.0039	-0.0057	0.0008		0.60179
TE	0.0236	0.0197	0.0014	0.0006	0.0035	-0.0005	-0.0002	

Table 4.10. AMOVA for *R. plebeia* with years as regions. Degrees of freedom (DF), sum of squares (SS), mean, estimated variance (Est. Var.), percent variance (% Variance), Differentiation Indices and p-value in bold for significant values.

Source of Variation	DF	SS	Mean	Est. Var.	% Var.	Differentiation Indices	p-value
Among Regions (Sampling Season 1 versus 2)	1	11.75	11.745	0.003	0%	$\Phi_{RT} = 0.000$	0.220
Among Populations	20	203.49	10.174	0.103	2%	$\Phi_{PR} = 0.016$	0.010
Within Populations	862	5190.35	6.179	6.179	98%	$\Phi_{PT} = 0.017$	0.010
Total	883	5405.58	9.366	6.285	100%		

Table 4.11. AMOVA for *R. plebeia* with life stage as regions. Degrees of freedom (DF), sum of squares (SS), mean, estimated variance (Est. Var.), percent variance (% Variance), Differentiation Indices and p-value in bold for significant values.

Source of Variation	DF	SS	Mean	Est. Var	%	Differentiation Indices	p-value
Among Regions (Juveniles versus Adults)	1	13.344	13.344	0.002	0%	$\Phi_{RT} = 0.000$	0.350
Among Populations	20	240.142	12.007	0.144	2%	$\Phi_{PR} = 0.022$	0.010
Within Populations	862	5421.341	6.319	6.319	98%	$\Phi_{PT} = 0.023$	0.010
Total	883	5674.827		6.464	100%		

Figure 4.3. Mean within population pairwise relatedness (r) values for all localities. Juvenile localities on the left and adults on the right. The Mean is the mean relatedness values within a localitie. Upper and Lower are the confidence intervals for a randomized sample.

Table 4.12. Population mean relatedness of juveniles versus adults. Number of pairwise comparisons (N), mean Queller and Goodnight relatedness (Mean *r*) values within the data, (Upper 95%) and (Lower 95%) confidence intervals for 999 permutations of random relatedness values and p-value that the mean of the data is greater than random. Bold p-values indicate significance after FDR correction for multiple testing.

Population	Life Stage	N	Mean <i>r</i>	Upper 95%	Lower 95%	p-value
DB 2010	Juv	703	0.024	0.075	-0.068	0.209
DB 2011	Juv	1275	0.043	0.060	-0.065	0.079
PA	Juv	276	0.034	0.091	-0.094	0.208
HK	Juv	1128	0.055	0.061	-0.067	0.038
MK	Juv	253	0.032	0.097	-0.091	0.243
AR	Juv	1081	0.000	0.063	-0.064	0.437
FX	Juv	1081	0.041	0.061	-0.063	0.089
PH	Juv	861	0.020	0.063	-0.071	0.230
LF	Juv	435	-0.015	0.089	-0.081	0.589
MH	Juv	1081	0.024	0.063	-0.066	0.219
KM	Juv	253	0.007	0.098	-0.101	0.394
WS	Juv	190	0.036	0.105	-0.097	0.232
AH 2011	Juv	1081	-0.054	0.066	-0.064	0.939
AH 2012	Juv	1225	-0.029	0.058	-0.064	0.761
TE	Juv	1225	0.017	0.062	-0.066	0.241
AB 2010	Juv	1081	-0.096	0.059	-0.067	1.000
AB 2011	Juv	325	-0.042	0.091	-0.090	0.808
Mean of the means for juveniles			0.006			
HB	Adult	990	0.113	0.076	-0.066	0.004
TB	Adult	1128	-0.027	0.064	-0.066	0.780
LH	Adult	1225	0.012	0.064	-0.063	0.319
LE	Adult	1128	0.089	0.065	-0.066	0.004
OT	Adult	406	0.025	0.084	-0.083	0.268
Mean of the means for adults			0.042			

Figure 4.4. Mean within-cohort pairwise relatedness (r). The Mean is the mean relatedness values within a localitie. Upper and Lower are the confidence intervals for a randomized sample.

Table 4.13. Within cohorts population mean relatedness values. Number of pairwise comparisons (N), mean Queller and Goodnight relatedness (Mean r) values within the data, (Upper 95%) and (Lower 95%) confidence intervals for 999 permutations of random relatedness values and p-value that the mean of the data is greater than random. Bold p-values indicate significance after FDR correction for multiple testing.

Population	n	Mean	Upper 95%	Lower 95%	p-value
DB-10-A	595	0.101	0.062	-0.085	0.010
DB-11-B	190	0.122	0.120	-0.098	0.030
DB-11-C	276	0.059	0.090	-0.103	0.090
AH-11-A	105	0.042	0.101	-0.112	0.160
AH-11-B	325	-0.040	0.079	-0.083	0.740
AH-12-A	1128	-0.002	0.046	-0.063	0.470
AB-11-A	595	-0.071	0.063	-0.075	0.970
AB-11-B	66	-0.125	0.099	-0.148	0.950
AB-12-C	231	-0.034	0.088	-0.089	0.700
Mean		-0.0342			

Relatedness

Most populations had mean pairwise relatedness values around 0 ranging from -0.1 to 0.1 (Figure 4.3) and the means were within the upper and lower 95% confidence intervals of randomly generated values. However, three adult populations were significantly different from random: with AB2010 had a lower than expected average relatedness and HB and LE had a higher expected average relatedness than random. The overall average relatedness was 0.0140, while among juvenile populations it was 0.006 and among cohorts it was 0.042 (Table 4.12). The only populations that were significantly different from random after correction for multiple testing were the adult populations of Hawke Bay ($p=0.004$) and Lake Ellesmere ($p=0.004$).

Most cohorts had mean pairwise relatedness values around 0 (Figure 4.4), with an overall average of -0.0342. Dicks Bay 2010 had an average relatedness value that was significant after correction for multiple testing ($p=0.010$) (Table 4.13), although the population was in HWE and the F_{IS} value was very similar to other cohorts (Table 4.4).

Diversity

There was little difference in the genetic diversity values of juveniles compared to adults within the same region, based on observed heterozygosity (H_o) and allelic richness (A_r) measures (Table 4.14). Overall, juveniles had higher A_r values than adults, with the exception of Tasman Bay and Mapua Harbour. Juveniles generally had more private alleles than the adult population within the same region, with the exception of Mapua Harbour and Tasman Bay ($PA = 11$ in both cases).

Table 4.14. Comparisons of diversity between adults and juveniles for *R. plebeia*. Sample size (*N*), observed heterozygosity (*H_o*), expected heterozygosity (*H_e*), allelic richness (*Ar*), fixation index (*F_{IS}*) and number of private alleles (*PA*).

Location	n	Life Stage	<i>H_o</i>	<i>H_e</i>	<i>Ar</i>	<i>F_{IS}</i>	<i>PA</i>
HB	45	Adult	0.365	0.433	4.01	0.154	4
AR	47	Juvenile	0.456	0.486	4.5	0.099	11
TB	48	Adult	0.432	0.496	4.66	0.188	11
MH	47	Juvenile	0.417	0.473	4.54	0.152	11
LH	50	Adult	0.423	0.477	4.23	0.124	4
AH	98	Juvenile	0.463	0.503	4.49	0.07	6
OT	22	Adult	0.453	0.466	4.34	0.043	3
TE	50	Juvenile	0.441	0.479	4.82	0.164	16

Discussion

Temporal variability in spawning and recruitment success may strongly affect spatial population structure (Waples 1998). The aim of this chapter was to determine if there was any temporal variation in genetic structure of the NZ sand flounder that may influence the spatial patterns described in Chapter 3. The results suggest that there was no differentiation 1) between years, 2) between adults and juveniles in the same area or 3) between cohorts. Some significant levels of relatedness were found, but not in populations that would affect any of the spatial conclusions made in Chapter 3. There was no obvious reduction in diversity among juveniles compared to adults within the same regions, which is a finding that does not support the SRS hypothesis (Hedgecock 2011).

Temporal stability in allele frequencies

Pairwise comparisons of *F_{ST}* and allele frequencies between years, between juveniles and adults from the same region and between cohorts within a region showed no significant differences between temporal samples. In contrast, there was significant spatial differentiation between northern population and southern populations, which was consistent with the results from Chapter 3. AMOVA showed no significant proportion of the variation could be explained by sampling seasons or life stage. These

results contrast with other studies that have shown different genetic patterns among juveniles and adults (Burford and Larson 2007, Han et al. 2010, Chapman et al. 2002). A study of the coral reef fish *Amphiprion polymnus* showed significant temporal stability of spatial patterns over three years with varying levels of connectivity with other populations (Saenz-Agueldo et al. 2012). There was no differentiation among kelp rockfish (*Sebastes atrovirens*) between cohorts, juveniles and adults and no reduction in diversity between juveniles compared to adults (Gilbert-Horvath et al. 2006).

To define a true cohort of individuals born at the same time that have been transported together can be difficult as there may be mixing of several spawning events in the water column during dispersal (Hedgecock & Pudovkin 2011). In this study a 'cohort' is based on length-frequency data and assumes that the juvenile sample is the result of a single spawning event, which may not be the case (Ruzzante et al. 1996). Whether different size fish prefer different parts of the estuary, or where they move in relation to the tide was not controlled for during collection. Although standard methods were used at each collecting location, sampling was not spatially random. All collecting locations were chosen for convenience to road access and seining was done within a 500 m area or less. Due to practical constraints of carrying buckets of water through sticky mud, beach seining was performed within 50 m of the high tide mark, as further out was difficult to access. Therefore, the depth that the estuary was sampled was somewhat controlled, despite being sampled at different areas or tide levels.

Relatedness

To test whether samples of juveniles had significant numbers of relatives compared to adult populations (Allendorf and Phelps 1981, Waples 1998), the level of relatedness was measured within all juvenile, adult and cohort samples. Overall, juveniles did not have significant levels of relatedness compared to other samples, nor were levels of relatedness among juvenile samples greater than among adults. The results show that the average values of relatedness among juveniles ($r = 0.006$) and cohorts ($r = 0.034$) are lower than the average values of relatedness among adult populations ($r = 0.042$). While the AB-2011 had the largest FIS of 0.249, the r values

were lower than most other populations. Combined with the non-significant F_{ST} values between juveniles and adults there is little evidence to support juveniles having any family structure within estuaries and the juvenile samples are representative of the adult population.

There is high variability inherent in relatedness measurements (Lynch and Ritland 1999, Blouin 2003), and depending on levels of heterozygosity, 40 or more loci may be required to achieve accurate estimates of relatedness 90% of the time (Blouin et al. 1996). Significant values were only found among the adult populations, but this is most likely due to the confounding pattern of allele homoplasy. In a pedigree analysis, the exact value of r would be significant, but here I am using this measure to test whether there are more relatives within the juveniles than adults, which would bias the amount of genetic differentiation due to non-random sampling.

We might expect to find more relatives in groups of smaller or younger fish that may be transported together in the same water masse early during the pelagic larval duration, then lost due to the mixing of cohorts, which was the case in juvenile *Diplodus sargus* (Planes and Lenfant 2002). The juvenile flounder in this study were all post-settlement, and even the smallest juveniles could have had some control of their position in the water column (Chambers and Leggett 1992). Possibly at this stage in their development, they were quite mobile and had already moved away from their family groups to mix with other cohorts. Hoarau (et al. 2005) found that F_{IS} decreased with larval size in the plaice.

Sweepstakes recruitment

The level of genetic diversity among adult and juvenile samples in the same area was compared to determine whether there was reduced diversity among juveniles, possibly as a result of SRS (Hedgecock and Pudovkin 2011). Observed heterozygosity, allelic richness and number of private alleles among adult populations was less than or equal to that found in juvenile populations, which was not consistent with the expectation of SRS. If we compare the average allelic richness among cohorts (3.36) to the adult populations with rarefaction of 7 individuals (3.18) to make them comparable,

diversity is still not diminished among juveniles. Therefore, I have found no evidence of sweepstakes recruitment success, which is consistent with other studies that have defined cohorts to test the SRS in the European eel (Palm et al. 2009) and the Arctic surfclam (Cassista and Hart 2007).

The long PLD could allow significant mixing of cohorts, and of larvae from different locations. Variability in recruitment success is related to the duration of the pelagic stage in flatfish (van der Veer et al. 2000), 2 months provides ample time for variable conditions and stochastic events, which could reduce diversity. Alternatively, this could be due to the protracted spawning season with multiple spawning events confounding a much more temporally detailed signal. At one allozyme locus in *Rhombosolea plebeia*, Smith (1987) found homozygote excess, which he attributed to assortative mating between early and late spawners. May and Jenkins (1992) also found ‘winter’ and ‘spring’ cohorts of *Rhombosolea tapirina* through otolith microstructure. Although I only had three within-season cohorts to compare, I did not see any differences between cohorts of ‘early’ and ‘late’ spawners, therefore there is no evidence of different groups of adults spawning at different times in the estuaries sampled.

There are many alternate explanations for why juveniles and adults show similar levels of diversity despite the potential for high mortality during the PLD or at recruitment (van der Veer et al. 2000). First, only post-recruitment fish were sampled, which may have already experienced a selection event or high mortality (Cushing 1990). At this point, the recruits in the estuaries represent the adult genetic diversity. There is the possibility that adults suffer greater mortality compared to the juveniles, where there was at one point plenty of diversity among adults, but through fishing or other means, it has been reduced (Hoarau et al. 2005, Hauser et al. 2002). This may be evident if subadults (sexually mature, but before spawning) were sampled, however in this study, a large portion of lengths (hence presumptive ages) are missing between the maximum juvenile size of 95 mm and the fishery-legal adult size of 250 mm. It may be possible that the amount of genetic diversity is low in recruits, higher in subadults and reduced in legal size fish due to fishery pressure.

Another limitation to testing the SRS is the lack of a census size to compare an effective population size, which is a second testable hypothesis of the SRS. Since *R. plebeia* is managed within NZ's Quota Management System with seven other flatfish species, abundance information is a combination of many species (Ministry of Primary Industries, MPI 2013a). Therefore, a comparison of N_e versus census size was not possible.

In the absence of preserved material for genetic analysis, this study only covers two years of sampling and more temporal replicates are needed to support the conclusion of temporal stability. Waples and Yakota (2007) suggested that population samples should be taken across three to five generations for comparison for organisms with overlapping generations, but this has never been accomplished in marine population genetics (Hedgecock and Pudovkin 2011) to date.

This study also indicates that sampling predominantly juveniles does not necessarily bias population genetic studies, depending on the portion of the lifecycle of interest. In some cases, it is difficult to collect adult samples in large enough numbers for a population study, and juvenile samples are easier to obtain. If researchers must use juvenile samples, it's recommended to collect over multiple seasons (Waples 1998). Older and larger individuals may be more representative of the overall population as they move away from sibling groups (Planes and Lenfant 2002). Temporal replicates also provides an additional 'check point' in time to verify any structure observed. This may be especially important in high gene-flow species, where little to no spatial structure is detected.

The full temporal scale that these finding apply to is difficult to tease out. Given that there is actual spatial population structure among *R. plebeia*, this signal of structure probably evolved over a long time period. The evolution rate of microsatellites has been stated as 10^{-6} to 10^{-2} per generation (Schlotterer 2000), which would equate to one mutation per 10, 000 to 1,000 generations. For argument's sake, in terms of *R. plebeia* generation time of 5 years, this would be between 50,000 and 5,000 years per mutation. One of the microsatellites used had 22 alleles, which at this rate would take anywhere from 1.1 million to 110,000 years to evolve. Buonaccorsi (et al. 2002) used

computer models and microsatellite data to confirm that the genetic patterns they observed in copper rockfish (*Sebastes caurinus*) most likely originated 1700 generations ago in the Pleistocene. These calculations do not take into account that the alleles may be much older than the species, possibly evolving before the speciation event. Therefore a start date for the initial evolution of the allele is impossible to determine. Other lines of evidence are needed to confirm the time scale of this genetic data, such as a molecular clock or coalescent methods.

The PLD and recruitment are times of very high mortality and uncertainty for larval and juvenile fish. For species like *R. plebeia*, which may only spawn once or twice in their lifetime, variability in recruitment may have profound effects on year class strength, and hence overall abundance. In some cases, each part of the life history may be radically different, and each warrant study in detail with temporal replication. Long term investigation into the abundance of *R. plebeia* is necessary to determine whether there is demographic stability, in addition to the genetic stability observed here. Most studies are limited by funding and the time allotted, which makes historical samples and long term studies all the more valuable and informative.

Conclusions

The findings of this study indicate that there is temporal stability in the genetic patterns observed in *R. plebeia*. Although the sampling was only across a limited number of generations it indicates that the levels of genetic diversity are consistent across the two sampling seasons, and across the multiple age class cohorts. Given these data, there was no detectable differences in allele frequency, levels of relatedness or diversity between juveniles and adults, therefore juvenile samples are representative of the adult population.

Chapter 5. Estuary habitat, temperature and currents affect population genetic structure in *Rhombosolea plebeia* and *R. leporina* in New Zealand

Abstract

Barriers to connectivity in marine populations can be caused by a number of biological, oceanographic and physical factors. To explore the causes of these barrier to genetic connectivity among populations of *R. plebeia* and *R. leporina*, I tested environmental models to see which ones best explained 3 different measures of allelic differentiation, F_{ST} , F'_{ST} and Jost's D . Two environmental data sets were available, one for the marine exclusive economic zone of New Zealand, the other focusing on estuarine habitat types. Only the EEZ data set was used with *R. leporina*, and the results showed that a combination of latitude, sediment, mean orbital velocity and sea surface temperature best explained the measures of genetic differentiation observed. The best fitting model for *R. plebeia* was a combination of the EEZ and estuarine models, showing a combined influence of latitude, longitude, sea surface temperature, mean orbital velocity, the width of the estuary mouth and sediment type. These results suggest avenues of further study using candidate genes, such as rhodopsin or heat shock proteins to examine evidence of local adaptation.

Introduction

Seascape genetics is a rapidly growing multidisciplinary approach where population geneticists can increase the explanatory power of genetic data by combining it with oceanographic, chemical, biophysical or environmental models (Selkoe et al. 2008, Liggins et al. 2013). Population genetics research has historically relied on neutral genetic variation to give an overall signal of drift and gene flow acting on the entire genome (Slatkin 1987), because coding genes under selection evolve at different rates and along different trajectories in time and space. However, the actual environmental conditions that influence these processes are of major interest and provide testable hypotheses for investigating the effect of the environment on the evolution and maintenance of population structure.

Large-scale abiotic factors affect multi-species community structure (Menge and Olson 1990), biogeography (Horn et al. 2006) and population structure (Kritzer and Sale 2010). Biogeographic boundaries between neighbouring provinces can be maintained by any number of physical or abiotic environmental factors creating changes in allele frequencies at relatively small spatial scales. Examples include: the biogeographic break at Point Conception, the Southern California Bight (USA) is a major break due to the cold southward flowing California Current separating from the coastline and continuing southwest, while the warm Davidson Current flows north (Hickey 1979, Lynn and Simpson 1987); at Cape Agulhas in South Africa, where the cold Atlantic Ocean in the west meets the warmer waters of the Indian Ocean in the east (Turpie et al. 2000, Harrison 2002); and the Wallace Line separates Southeast Asian and Australian flora and fauna - a break which can be traced to the last glacial maximum (Barber et al. 2000).

The interactions between organisms and their physical environment play an important role in shaping population structure. During the pelagic larval duration (PLD), oceanographic and environmental factors strongly influence the dispersal and survival of pelagic larvae (Galindo et al. 2006, White et al. 2010). These factors can determine the successful recruitment of larvae to nursery habitat in estuarine species

(Beck et al. 2001). For example, sea surface temperature explains variation in genetic connectivity and population genetic structure of the greenshell mussel (*Perna canaliculus*) in New Zealand (Wei et al. 2013). In addition, variation in turbidity and photic environment has been shown to play an important role in the structure of intra-specific populations of the sand goby (*Pomatoschistus minutus*) in the North Atlantic (Laramuseau et al. 2009). For the sand goby, this variation was evident in neutral microsatellites markers, as well in selection on the spectral tuning sites in the rhodopsin gene.

In Chapters 2 and 3, I explored the population genetics of two species of endemic New Zealand flounder using neutral microsatellite markers. The analyses suggested there was a break in genetic connectivity of *R. plebeia* populations in New Zealand around the north part of the South Island on the east coast. A similar pattern has been documented in a wide variety of other New Zealand species (Apte and Gardner 2002, Shears et al. 2008, Gardner et al. 2010 et al.). The F_{ST} comparison of the northern and southern areas suggested that environmental or other forces are causing a discontinuity. For *R. leporina*, there is a higher level of population differentiation than in *R. plebeia*, but a very different spatial pattern. The most striking difference was found between the east and west coasts, with strong connectivity along most of the length of the west coast and separate populations along the east coast.

Despite the contrasting genetic patterns among populations of *R. plebeia* and *R. leporina*, there appear to be few ecological differences between the species. Both inhabit estuaries, harbours and shallow bays throughout the New Zealand mainland, but *R. plebeia* inhabits coastal areas up to 100 m in depth, whereas *R. leporina* are generally limited to 50 m depth (Aylng and Cox 1982). Juvenile *R. plebeia* and *R. leporina* are found in many of the same locations and at similar abundances (Webb 1968, Morrison 2002), but *R. leporina* has fewer known juvenile habitats than *R. plebeia* (NABIS). Webb (1973) found that stomach contents of juvenile *Rhombosolea* were very similar in the Avon-Heathcote Estuary. Livingston (1987) found that even though adult *Rhombosolea* have very similar morphology and feeding structures, in Wellington Harbour, *R. plebeia* fed primarily on ophiuriids and *R. leporina* fed primarily on crustaceans, with some overlap in other food sources. Environmental

factors related to feeding behaviour may be an important ecological distinction between these two species.

The mainland of New Zealand spans 13 degrees of latitude and 8.5 degrees of longitude in the south eastern hemisphere. The oceanography of the region consists of a complex current system and upwelling, characterized by a predominantly westward flowing Tasman Current, with the separation of the North and South islands by the D'Urville Current that flows through the Cook Strait (Wallis and Trewick 2009). Strong upwelling regimes may cause biogeographic discontinuities between northern groups and southern groups (Apte and Gardner 2002, Waters and Roy 2004). This heterogeneity of habitats effects species distributions and may also effect population dynamics.

There have been several environmental classification schemes designed to characterize the diverse abiotic and biotic geography of New Zealand's marine habitats (Shears et al. 2008, Walls 2009, Snelder et al. 2005, Hume 2007). The Marine Environmental Classification (MEC) system (Snelder et al. 2005) was developed for the entire exclusive economic zone (EEZ). The Estuarine Environmental Classification (EEC) system was first published by Hume et al. in 2007 and was published as a hierarchical classification system based on the morphology of lagoons, embayments and estuaries. Using these two well-established environmental data sets, the aim of this study was to identify some of the physical oceanographic characteristics that correlated with the genetic structure of *Rhombosolea plebeia* and *R. leporina* among the estuaries and bays of New Zealand. The analysis involved an evaluation of the environmental models used to determine which environmental factors, or combinations of factors best explain the genetic connectivity patterns seen in these two species. This is the first study to explore the relationship between the physical seascape and the genetics of a New Zealand marine fish species.

Methods

Adult and juvenile *R. plebeia* and *R. leporina* were collected at 10 and 19 locations respectively, as described in Chapter 3. The habitat at each sampling location was heterogeneous, ranging from thick sticky mud, to shell hash, sparse sea grass, sandy and silty substrate to mangrove areas. As the tide ebbed or flowed during sampling, habitat composition changed slightly as well, generally to sandier or muddier substrate the further away from the high tide line.

Genetic Data

Collection, PCR protocols, genotyping and data analysis are described in detail in Chapter 2 and 3 of this thesis. Pairwise population F_{ST} , F'_{ST} and Jost's D values were averaged across sampling locations to produce a single descriptive value for each sampling location for the GLM models following Wei et al. (2013) (Table 5.1). BEST analysis carried out in PRIMER (ver 6) with PERMANOVA (Clark and Gorley 2005), where individual allele frequencies for each locus were compiled for every individual per Wei et al. (2013).

Geospatial Data

The geospatial statistics of latitude and longitude were recorded in Google Maps via retrospective georeferencing using a modified point-radius method (Wieczorek et al. 2003). Coordinates were treated as point data, and no error radius was included for the purposes of collating data from GIS layers. Coastal distances were measured as the shortest over-water distance between two locations.

Environmental Data

Eleven coastal environmental variables at the collecting coordinates were extracted from the New Zealand Marine Environment Classification (MEC) data set (Snelder et al. 2005). GIS raster and vector shape files were mapped against collecting localities in ArcMap 10. If data layers did not overlap with collecting localities, these point data were adjusted up to 500 m to capture the closest edge of relevant data layers (Table 5.2). Eleven estuarine environmental variables were collated from the New Zealand Estuarine Environment Classification (EEC) data set (Hume et al. 2007) using the corresponding estuary name (Table 5.2). For two locations, Hawke Bay (HB) and Tasman Bay (TB), there were no estuarine data available because these locations are along coastal habitats and not near estuaries. For this reason, HB and TB had to be eliminated from the model whenever the *R. plebeia* models were run with estuarine variables. This also meant that I was unable to perform any modeling using the EEC data set for *R. leporina* because there would be large gaps in the spatial coverage of the models without the HB and TB collecting locations.

Table 5.1. Independent environmental variables from the Marine Environment Classification (MEC) and Estuarine Environment Classification (EEC)

Data set source	Name	Abbreviation	Units	Description
NZ MEC (Snelder et al. 2005)	Sediment	Sed	n/a	Sediment type as a categorical variable
	Summertime sea surface temperature anomaly	SSTAnom	°C	Spatial anomalies with scales between 20 and 450 km in late February when SST is typically highest
	Mean orbital velocity	MOrbVel	m/s	Orbital velocity at the bed for the mean significant wave height calculated from a 20 year wave hindcast
	Annual mean solar radiation	RadMean	W m ⁻²	Mean extra atmospheric solar radiation modified by mean annual cloud cover
	Annual amplitude of sea surface temperature	SSTAnnAmp	°C	Smoothed annual amplitude of SST
	Spatial gradient annual mean sea surface temperature	SSTGrad	°C km ⁻¹	Smoothed magnitude of the spatial gradient of annual mean SST
	Wintertime sea surface temperature	STTWint	°C	Mean of daily data from early September when SST is typically lowest
NZ EEC (Hume et al. 2007)	Spring tide range	SprTidRng	m	The tidal range for a mean spring tide
	Spring tidal prism	SprTidPrsm	m ²	The volume of water entering an estuary on the flood or incoming tide.
	Width of mouth	Wmouth	m	Width of the seaward boundary at the inlet constriction or where shoreline diverges up or down the coast
	Mean depth	D	m	The total estuary volume at spring high water divided by the estuary area at high water spring tide.
	Estuary area at low tide	ArLTid	m ²	The total water area at spring low tide.
	Estuary area at high tide	ArHTid	m ²	The total water area at spring high tide.
	Mean annual river discharge	AnnRivDis	cumecs	Mean annual river flow into the estuary.

Data Analysis

Correlation analyses and Principal Component Analyses were performed using the software package STATISTICA v10 (Stat Soft Inc. 2011) to test for spatial autocorrelation among variables. PCA plots for *R. leporina* MEC, *R. plebeia* MEC, EEC, EEC and MEC combined plots were examined and variables that were spatially autocorellated at the 0.05 level were removed from further analysis. The remaining variables, as defined in Table 5.1, were included in the final analysis. For the MEC and EEC combined model, the most significant variables that were not spatially autocorellated from each model were combined.

The generalized linear model (GLM) is a statistical way of regressing many independent variables (environmental variables) in multidimensional space to explain a single dependent variable, here mean F_{ST} , F'_{ST} , and *Jost's D* or the differences in allele frequency proportions attributed to population differentiation (Zar 1994). The GLZ's were performed in STATISTICA 10 (Stat Soft Inc 2011) using multiple combinations of independent variables to find the best fit model based on the lowest Akaike information criterion (AIC) value (e.g., Wei et al. 2013).

Once the best-fit GLZ model was obtained, the variables from this model were tested in PRIMER v6 (Clark and Gorley 2005) using the BEST non-parametric multivariate analysis (Clark 1993). The BEST protocol as implemented in PERMANOVA in PRIMER ver 6 is a nonparametric test that uses population-specific raw allele frequencies regressed against the environmental variables to produce a Spearman's rank correlation coefficient (r_s) to determine the best combination of environmental parameters (Clarke 1993). Spearman's r_s values are reported for each model. Multiple combinations of variables were also tested, but the highest r_s values tended to be the same set of variables as the GLZ analyses described above. The BEST routine was chosen as a non-parametric way of comparing two matrices, one with population specific allele frequencies and the other with normalized environmental variables and Euclidian distances that compares all of the possible combinations in each model (Balkenhol et al. 2009).

Results

Genetic Data

The largest mean F_{ST} , F'_{ST} and *Jost's D* values for *R. plebeia* was in the Lake Ellesmere (LE) site at 0.019, 0.026 and 0.017, respectively. The largest mean F_{ST} , F'_{ST} and *Jost's D* values for *R. leporina* were for the Karamea River Estuary site at 0.031, 0.049 and 0.041, respectively (Table 5.1).

Modelling Results

R. leporina

In comparing environmental variables from the MEC to F_{ST} in *R. leporina*, the best fit GLZ model included Lat + Sed + MOrbVel+ SSTGrad with a p-value of 0.001 (Table 5.3). The best fit models were significant at the $p < 0.05$ level, with Lat, Sed and MOrbVel each contributing significantly to explaining differences in F_{ST} across populations at the $p < 0.05$ level. Models using F'_{ST} and *Jost's D* were very similar, with the exception of SSTGrad contributing significantly to the models using F'_{ST} . The top 10 BEST analyses all showed an equivalent low Spearman's Rho (r_s) value of 0.117, which included a combination of Lat, Long, Sed, MOrbVel, SSTAnnAmp and SSTGrad. Only MOrbVel appeared in all top ten models (Table 5.4).

Table 5.2. Population summary statistics for a) *R. plebeia* and b) *R. leporina*. Sample size (*N*), observed heterozygosity (*Ho*), expected heterozygosity (*He*), allelic richness (*Ar*), significant deviations from Hardy-Weinberg Equilibrium (*P_{HWE}*) in bold, fixation index (*F_{IS}*) and number of private alleles (*PA*), average (*F_{ST}*), average (*F'_{ST}*) and average (*Jost's D*) across populations.

5.2a. <i>R. plebeia</i>										
Code	<i>N</i>	<i>Ho</i>	<i>He</i>	<i>Ar</i>	<i>P_{HWE}</i>	<i>F_{IS}</i>	<i>PA</i>	<i>F_{ST}</i>	<i>F'_{ST}</i>	<i>Jost's D</i>
DB	89	0.405	0.475	4.27	0.000	0.17	2	0.012	0.017	0.011
PA	24	0.407	0.461	3.84	0.098	0.185	0	0.003	0.003	0.003
HK	48	0.422	0.464	4.13	0.000	0.143	1	0.012	0.018	0.011
MK	23	0.39	0.463	4.00	0.001	0.185	0	0.007	0.008	0.006
HB	45	0.365	0.433	4.01	0.000	0.154	0	0.010	0.008	0.008
AR	47	0.456	0.486	4.50	0.005	0.099	1	0.007	0.010	0.007
FX	48	0.424	0.467	4.38	0.000	0.044	0	0.010	0.013	0.009
PH	42	0.398	0.469	4.27	0.001	0.19	0	0.008	0.006	0.007
LF	30	0.431	0.488	4.53	0.004	0.134	0	0.006	0.005	0.005
TB	48	0.432	0.496	4.66	0.000	0.188	0	0.003	0.002	0.002
MH	47	0.417	0.473	4.54	0.000	0.152	0	0.006	0.004	0.005
KM	23	0.48	0.477	4.62	0.399	-0.028	1	0.007	0.008	0.007
WS	20	0.42	0.464	4.60	0.055	0.133	0	0.013	0.018	0.012
AH	98	0.463	0.503	4.49	0.000	0.07	0	0.009	0.012	0.009
LH	50	0.423	0.477	4.23	0.004	0.124	0	0.006	0.006	0.005
LE	49	0.454	0.457	4.42	0.392	0.013	1	0.019	0.026	0.017
OT	22	0.453	0.466	4.34	0.037	0.043	0	0.008	0.006	0.006
TE	50	0.441	0.479	4.82	0.000	0.164	1	0.013	0.020	0.012
AB	55	0.438	0.508	4.63	0.000	0.112	1	0.018	0.010	0.009
	858	0.427	0.474	4.38	0.052	0.12	0.421	0.009	0.011	0.008
5.2b. <i>R. leporina</i>										
Code	<i>N</i>	<i>Ho</i>	<i>He</i>	<i>Ar</i>	<i>P_{HWE}</i>	<i>F_{IS}</i>	<i>PA</i>	<i>F_{ST}</i>	<i>F'_{ST}</i>	<i>Jost's D</i>
MG	50	0.525	0.548	5.79	0.124	0.025	4	0.015	0.028	0.019
HK	26	0.525	0.567	5.79	0.025	0.072	0	0.011	0.019	0.015
HB	49	0.498	0.535	5.8	0.013	0.060	3	0.011	0.019	0.014
TB	47	0.535	0.548	5.72	0.005	0.017	0	0.012	0.024	0.016
BL	50	0.497	0.556	4.71	0.000	0.070	0	0.017	0.032	0.022
KM	11	0.485	0.534	n/a	0.001	0.107	1	0.031	0.049	0.041
WS	27	0.542	0.534	5.26	0.403	-0.012	0	0.010	0.017	0.012
AH	46	0.497	0.561	6.36	0.000	0.149	5	0.015	0.019	0.020
LH	48	0.500	0.554	6.22	0.000	0.066	1	0.014	0.029	0.018
HS	50	0.520	0.568	5.81	0.000	0.072	2	0.011	0.019	0.014
	408	2.403	0.339	2.90	0.057	0.063	1.600	0.015	0.025	0.019

Table 5.3. Ten best fitting models from GLZ analyses using MEC data for *R. leporina*. X's indicate which variables were included in each model. Bold figures indicate p-values significant at 0.05 level

MEC <i>R. leporina</i> GLZ								
F_{ST}								
Rank	AIC	p-value	Lat	Long	Sed	MOrbVel	SSTAnn Amp	SSTGrad
1	-83.599	0.001	x		x	x		x
2	-83.090	0.001	x		x	x		
3	-82.736	0.001	x	x	x	x		
4	-82.645	0.001	x		x	x	X	
5	-82.635	0.001	x	x	x	x		x
6	-81.713	0.002	x		x	x	X	x
7	-81.130	0.002	x	x	x	x	X	x
8	-80.718	0.002	x	x	x	x	X	x
9	-78.155	0.008	x			x		x
10	-76.946	0.014	x			x	X	x
p-value			0.000	0.315	0.001	0.000	0.777	0.197
MEC <i>R. leporina</i> GLZ cont.								
F'_{ST}								
1	-106.269	0.000	x		x	x		x
2	-104.777	0.000	x	x	x	x		x
3	-104.387	0.000	x		x	x	X	x
4	-102.789	0.000	x	x	x	x	X	x
5	-82.320	0.000	x	x		x	X	x
6	-80.977	0.000	x			x		x
7	-80.544	0.000	x			x	X	x
8	-79.518	0.000	x	x		x		x
9	-77.833	0.000	x		x	x	X	
10	-76.539	0.000	x	x	x	x	X	
p-value			0.000	0.525	0.000	0.000	0.912	0.000
Jost's D								
1	-77.058	0.001	x		x	x		x
2	-76.518	0.002	x		x	x		
3	-76.455	0.002	x		x	x	X	
4	-76.335	0.002	x	x	x	x		
5	-76.214	0.002	x	x	x	x		x
6	-75.332	0.002	x		x	x	X	x
7	-74.909	0.003	x	x	x	x	X	
8	-74.223	0.003	x	x	x	x	X	x
9	-72.732	0.008	x			x		x
10	-71.578	0.013	x			x	X	x
p-value			0.000	0.347	0.002	0.000	0.922	0.243

Table 5.4. Ten best fitting models from the BEST analysis for *R. leporina* with MEC data. Rank of the test, Spearman's Rho (r_s), X's indicate which variables were included in each model

MEC <i>R. leporina</i> Best							
Rank	r_s	Lat	Long	Sed	MOrbVel	SSTAnn Amp	SSTGrad
1	0.117				x		
2	0.117	x			x		
3	0.117		x		x		
4	0.117			x	x		
5	0.117				x	x	
6	0.117				x		X
7	0.117	x	x		x		
8	0.117	x		x	x		
9	0.117	x			x	x	
10	0.117	x			x		X

R. plebeia

The top MEC GLZ model for *R. plebeia* included Long, Sed, MOrbVel, SSTGrad, SSTAnom, RadMean and SSTWint with a p-value < 0.001. The top ten models were all significant at the $p < 0.05$ level, but Sed, MOrbVel, SSTGrad and SSTAnom had significant p-values with F_{ST} , F'_{ST} , and Jost's D at the $p < 0.05$ level (Table 4.5). The top two BEST models had Spearman's Rho (r_s) values of 0.437 and included Lat, MOrbVel, RadMean and SSTWint. Among the top ten BEST models, Long and SSTWint were included in every model (Table 5.6).

Table 5.5. Ten best fitting models from GLZ analyses using MEC data for *R. plebeia*. X's indicate which variables were included in each model. Bold figures indicate p-values significant at 0.05 level

MEC <i>R. plebeia</i> GLZ											
F_{ST}											
Rank	AIC	p-value	Lat	Long	Sed	SST		SST Grad	SST Anom	Rad Mean	SST Wint
						Morb Vel	Ann Amp				
1	-168.812	0.000		x	x	x		x	x	x	x
2	-168.628	0.000	x	x		x		x	x	x	x
3	-167.611	0.000	x		x	x		x	x		x
4	-167.141	0.000	x	x	x	x		x	x	x	x
5	-167.121	0.000		x	x	x	x	x	x	x	x
6	-166.682	0.000	x	x	x	x	x	x	x		x
7	-165.974	0.000			x	x		x	x		x
8	-165.917	0.000	x		x	x		x	x	x	x
9	-165.698	0.000	x		x	x	x	x	x		x
10	-165.690	0.000	x	x	x	x		x			x
p-value			0.706	0.063	0.006	0.001	0.724	0.007	0.018	0.435	0.001
MEC <i>R. plebeia</i> GLZ											
F'_{ST}											
1	-151.609	0.000	x	x	x	x	x	x	x		x
2	-150.648	0.000		x	x	x	x	x	x	x	x
3	-149.973	0.000	x	x	x	x		x	x		x
4	-149.823	0.000	x	x	x	x	x	x	x	x	x
5	-149.749	0.000	x		x	x	x	x	x		x
6	-148.855	0.000	x	x	x	x		x	x	x	x
7	-148.505	0.000	x		x	x	x	x	x	x	x
8	-148.499	0.000	x		x	x		x	x	x	x
9	-147.785	0.001		x	x	x		x	x	x	x
10	-147.742	0.001		x	x	x		x	x		x
p-value			0.275	0.074	0.017	0.000	0.081	0.000	0.008	0.650	0.000
Jost's D											
1	-173.050	0.000	x	x	x	x		x	x		x
2	-172.270	0.000		x	x	x	x	x	x	x	x
3	-172.208	0.000		x	x	x		x	x	x	x
4	-171.932	0.000	x		x	x		x	x		x
5	-171.921	0.000	x	x	x	x	x	x	x		x
6	-171.891	0.000	x		x	x	x	x	x		x
7	-171.415	0.001			x	x		x	x		x
8	-171.076	0.001	x		x	x		x	x	x	x
9	-171.057	0.000	x	x	x	x		x	x	x	x
10	-170.738	0.001		x	x	x		x	x		x
p-value			0.565	0.099	0.005	0.000	0.217	0.000	0.010	0.413	0.000

Table 5.6. Ten best fitting models from the BEST analyses for *R. plebeia* with MEC data. Rank of the test, Spearman's Rho (r_s), X's indicate which variables were included in each model

MEC <i>R. plebeia</i> BEST										
Rank	r_s	Lat	Long	Sed	Morb Vel	SST Ann Amp	SST Grad	SST Anom	Rad Mean	SST Wint
1	0.437		x		x					x
2	0.437		x		x				x	x
3	0.435		x				x			x
4	0.435		x				x		x	x
5	0.434		x		x		x			x
6	0.434		x		x		x		x	x
7	0.434	x	x		x					x
8	0.434	x	x		x				x	x
9	0.430		x							x
10	0.430		x						x	x

For *R. plebeia* the best fitted GLZ EEC model for F_{ST} , F'_{ST} , and *Jost's D* had an p-value < 0.001 and included Lat, Long, WMouth and ArLTid (Table 5.7). Among the top 10 GLZ models, Lat, Long and WMouth were all significant at the $p < 0.05$ level. For the BEST models, the Spearman's r_s value was 0.416 for the top two models and included Lat, Long and SprTidRng. No variables were present in all top ten BEST models, but Lat featured in 8 of 10 models (Table 5.8).

When the MEC and EEC variables were combined and tested in a GLZ to F_{ST} values in *R. plebeia*, the top model had a p-value of < 0.001, and included Lat, Long, SSTAnom, MORbVel, SSTWint, Wmouth, and Sed (Table 5.9). For all descriptive genetic statistics, the 10 best fitted GLZ models were significant at the $p < 0.001$, with Lat, Long, MORbVel, and Sed significant at the $p < 0.05$ level. However, different environmental variables explained the variation for F_{ST} , F'_{ST} and *Jost's D*. The model with F_{ST} also reported Wmouth as a significant variable and models using F'_{ST} reported all of the variables as significant except for SSTAnom. The models using *Jost's D* were more similar to the models using F_{ST} , but Wmouth was not a significant factor. The two top BEST models had a Spearman's r_s value of 0.461 and included Long, SSTAnom, SSTGrad and SSTWint (Table 5.10).

Table 5.7. Ten best fitting models from GLZ analyses using EEC data for *R. plebeia*. X's indicate which variables were included in each model. Bold figures indicate p-values significant at 0.05 level

EEC <i>R. plebeia</i> GLZ											
F _{ST}											
Rank	AIC	p-value	Lat	Long	Spr Tid Rng	Spr Tid Prsm	W mouth	D	ArL Tid	ArH Tid	Ann Riv Dis
1	-151.379	0.000	x	x			x		x		
2	-150.877	0.000	x	x	x		x		x		
3	-150.551	0.000	x	x			x		x	x	
4	-150.549	0.000	x	x		x	x		x		
5	-150.458	0.000	x	x		x	x			x	
6	-149.659	0.000	x	x	x	x	x			x	
7	-149.629	0.000	x	x	x	x	x		x		
8	-149.587	0.000	x	x			x		x		x
9	-149.576	0.000	x	x	x		x		x	x	
10	-149.458	0.000	x	x			x	x	x		
p-value			0.004	0.000	0.357	0.840	0.007	0.960	0.875	0.936	0.689
F' _{ST}											
1	-130.967	0.002	x	x			x		x		
2	-130.780	0.002	x	x		x	x			x	
3	-130.777	0.002	x	x		x	x		x		
4	-130.698	0.002	x	x			x		x	x	
5	-129.918	0.003	x	x	x		x		x		
6	-129.766	0.003	x	x		x	x	x	x		
7	-129.732	0.003	x	x			x	x	x	x	
8	-129.666	0.003	x	x		x	x	x		x	
9	-129.298	0.004	x	x	x	x	x			x	
10	-129.241	0.004	x	x	x	x	x		x		
p-value			0.012	0.011	0.643	0.743	0.010	0.478	0.903	0.916	0.775
Jost's D											
1	-157.784	0.000	x	x		x	x			x	
2	-157.650	0.000	x	x		x	x		x		
3	-157.433	0.000	x	x			x		x	x	
4	-156.964	0.000	x	x		x	x	x		x	
5	-156.867	0.000	x	x		x	x	x	x		
6	-156.850	0.000	x	x			x		x		
7	-156.591	0.000	x	x			x	x	x	x	
8	-156.032	0.000	x	x	x	x	x		x		
9	-155.850	0.000	x	x	x	x	x		x		
10	-155.830	0.000	x	x		x	x			x	x
p-value			0.003	0.002	0.875	0.581	0.001	0.308	0.970	0.793	0.754

Table 5.8. Ten best fitting models from the BEST analyses for *R. plebeia* with EEC data. Rank of the test, Spearman's Rho (r_s), X's indicate which variables were included in each model

EEC <i>R. plebeia</i> BEST										
Rank	r_s	Lat	Long	Spr TidRng	Spr TidPrsm	W mouth	D	ArLTid	ArHTid	Ann RivDis
1	0.416	x	x	x						
2	0.416	x	x							
3	0.373	x		x						
4	0.358	x								
5	0.345	x	x	x			x			
6	0.339	x	x				x			
7	0.301		x							
8	0.285		x	x						
9	0.279	x		x			x			
10	0.267	x					x			

Table 5.9. Ten best fitting models from GLZ analyses using MEC and EEC data for *R. plebeia*. X's indicate which variables were included in each model. Bold figures indicate p-values significant at 0.05 level

MEC and EEC <i>R. plebeia</i> GLZ											
F_{ST}											
Rank	AIC	p-value	Lat	Long	SST Anom	SST Grad	Morb Vel	SST Wint	W mouth	ArLTid	Sed
1	-159.094	0.000	x	x	x		x	x	x		x
2	-158.513	0.000	x	x	x		x	x	x		x
3	-157.547	0.000	x	x		x	x	x	x		x
4	-157.498	0.000	x	x	x		x	x	x	x	x
5	-157.408	0.000	x	x			x	x	x		x
6	-156.735	0.000	x	x	x			x			x
7	-156.516	0.000	x	x		x	x	x	x	x	x
8	-156.453	0.000		x		x	x	x	x	x	
9	-156.291	0.000		x		x	x		x	x	
10	-155.930	0.000	x	x		x	x		x	x	x
p-value			0.006	0.001	0.195	0.538	0.031	0.059	0.000	0.563	0.001
F'_{ST}											
1	-139.622	0.000	x	x		x	x	x	x	x	x
2	-139.279	0.000	x	x	x	x	x	x	x	x	x
3	-138.149	0.000	x	x		x	x	x		x	x
4	-136.758	0.000	x	x	x		x	x	x	x	x
5	-136.172	0.000	x	x	x	x	x	x		x	x
6	-135.956	0.000	x	x			x		x	x	
7	-135.342	0.000	x	x		x	x	x	x		x
8	-134.797	0.000	x	x			x	x		x	x
9	-134.794	0.000	x	x		x	x		x	x	
10	-134.627	0.000	x	x			x	x	x	x	
p-value			0.000	0.000	0.119	0.031	0.001	0.001	0.007	0.008	0.000
Jost's D											
1	-164.012	0.000	x	x	x		x	x		x	x
2	-163.583	0.000	x	x			x		x	x	
3	-163.442	0.000	x	x		x	x	x		x	x
4	-162.705	0.000	x	x	x		x	x	x	x	x
5	-162.613	0.000	x	x	x	x	x	x		x	x
6	-161.967	0.000	x	x		x	x		x	x	
7	-161.745	0.000	x	x			x	x		x	x
8	-161.720	0.000	x	x			x	x	x	x	
9	-161.720	0.000	x	x	x		x		x	x	
10	-161.475	0.000	x	x		x	x	x	x	x	x
p-value			0.003	0.000	0.145	0.484	0.015	0.052	0.433	0.000	0.006

Table 5.10. Ten best fitting models from the BEST analyses for *R. plebeia* with MEC and EEC data. Rank of the test, Spearman's Rho (r_s), X's indicate which variables were included in each model

Mec & EEC <i>R. plebeia</i> BEST										
Rank	r_s	Lat	Long	SST Anom	SST Grad	Morb Vel	SST Wint	W mouth	ArLTid	Sed
1	0.461		x	x			X			
2	0.461		x	x	x		X			
3	0.456		x				X			
4	0.456		x		x		X			
5	0.434	x	x	x			X			
6	0.434	x	x	x	x		X			
7	0.434	x	x				X			
8	0.434	x	x		x		X			
9	0.416	x	x							
10	0.416	x	x		x					

Discussion

In Chapter 3, I discovered significant population structure in two species of endemic New Zealand flounder, *R. leporina* and *R. plebeia*. Although the species are in the same genus and have very similar life histories and ecologies, the spatial genetic patterns I found were dissimilar in some aspects, which indicates that more than just passive dispersal of larvae is connecting populations. Adult habitat choice and previously unobserved niche partitioning may be shaping the population structure of these animals. Here I use geospatial modeling to explore the physical factors that may be correlated with the patterns genetic differentiation.

The results showed that there is a complex combination of environmental variables that correlate with the population genetic structure in *R. leporina* and *R. plebeia*. The environmental variables latitude, sediment and mean orbital velocity correlated significantly with differences in population mean F_{ST} values in *R. leporina* according to the GLZ models. Latitude may be a proxy variable for any number of environmental or oceanographic variables that change with latitude, including

temperature, which is also present in the top model. Sea surface temperature gradient did not make a significant contribution to the models based on F_{ST} , but did using F'_{ST} . None of the BEST models were highly significant, producing a low Spearman's r_s value of 0.117, but mean orbital velocity stood out as an important factor in the top 10 models, which was consistent with the GLZ analysis (Table 5.2).

Mean orbital velocity was also a significant factor in all of the *R. plebeia* models using the MEC information. It may be intuitive that the populations with the highest F_{ST} values are most isolated by fast moving currents, however these data represent the current speeds at the collection location rather than current speeds between populations. Measures at a point in space versus measures between two points in space are an important distinction in seascape genetics (Liggins et al. 2013). Some landscape analyses take into account the “resistance distance” between values (McRae 2006, Spear et al. 2010), which not only measure geographic distance, but also include a level of difficulty of traveling between two points in space. This resistance distance could be greater when crossing a mountain range, for example. This resistance may be more relevant to marine species with oceanographic features that limit dispersal between populations, going against currents or regions of upwelling. Unfortunately, such data was unavailable, so I can only infer the effects of the environmental variables at the collection locality, rather than any effects between collection localities.

Sea surface temperature also explained much of the genetic variation in *R. plebeia* samples in the models using the MEC data. Sea surface temperature gradient, sea surface temperature anomaly and sea surface temperature winter all had significant contributions to the GLZ model, while sea surface temperature winter stood out in all top ten BEST models. In the northern latitudes, the sea surface temperatures are generally warmer and in the southern sub-polar latitudes the sea surface temperatures are generally colder, especially on the southeast coast of the South Island where the subarctic front encroaches in the winter. This may explain the north to south genetic break we see in both species along the southeast coast of the South Island. In a species which had a similar genetic break between northern and southern New Zealand, Wei et al. (2013) also found sea surface temperature explained the greatest amount of genetic variation in populations of greenshell mussel (*Perna canaliculus*) in New Zealand.

Their models used many of the same variables from the MEC data set to predict differences in F_{ST} and Phi_{ST} which they attributed to the possibility of there being warm water adapted populations north of $\sim 42^\circ$ South and colder water adapted animals in the south.

For *R. plebeia*, in the GLZ models using the EEC estuary data, the width of the mouth of the estuary (Wmouth) was a significant factor explaining population genetic differentiation among sites. A regression of Wmouth and F_{ST} alone shows a weak negative relationship. Estuarine features, like size and shape, contribute to their value as nursery grounds for fish and invertebrates (Boehlert and Munday 1988, Beck et al. 2001). This may be due to the ability of larvae to recruit to these estuary sites due to a larger area for recruitment, or simply a higher probability of recruiting to estuaries with larger openings than smaller ones facilitating greater connectivity. The width of the mouth of the estuary may be an important characteristic of the habitat where spawning aggregations of many species take place, such as in the blue crab (*Callinectes sapidus*) including *Rhombosolea* (Ayling and Cox 1982). The width of the mouth of the estuary may also related to the amount of water exchanging between the estuary and open coast during the tidal flux, which has been shown to facilitate recruitment to estuaries (Boehlert and Mundy 1988). However, in these models, the two variables related to flow (spring tidal range and spring tidal prism) were not significant factors in explaining genetic variation indicating the tidal flow was not an explanatory factor.

Sediment (Sed) is a categorical variable with very low resolution, based on 1:6,000,000 scale charts, and was not included in the final MEC classification for the EEZ, however, it may be important in defining habitat types. Abundance and distribution of Bering Sea pleuronectid species is related to sediment type, most likely due to prey availability and feeding specialization (McConnaughey and Smith 2000). In NZ, Livingston (1987) found that *R. plebeia* and *R. leporina* fed on benthic epifauna and infauna, and tended to be generalist compared to other pleuronectids in Wellington Harbour. Therefore, *Rhombosolea* species may have loose associations with sediment type due to feeding plasticity, however these models show sediment does play a part in explaining population genetic variation. The turbidity of the water will be affected by sediment type and orbital velocity, which affects feeding behaviour in greenback

flounder (*Rhombosolea tapirina*) (Shaw et al. 2006). Habitat type, slope and depth influence the habitat use of the southern flounder (*Paralichthys lethostigma*) (Furey et al. 2013).

While weighing the importance of the factors individually is useful, it is important to note that each of the best fitting models was a combination of factors interacting with one another. There were no significant tests where one single factor stood out as explaining all of the genetic variation. The most significant GLZ model for *R. plebeia* used both the MEC and EEC variables (Table 5.5) and was a combination of most of the variables in the model: latitude, longitude, sea surface temperature anomaly, mean orbital velocity, sea surface temperature winter, sediment and width of estuary mouth. Of these variables, four were significant across the top ten tests. These results highlight the complexity of abiotic interactions on genetic variation.

There are limitations to using the MEC data set to study coastal populations. It was developed to classify the very diverse environments across 4,300,000 km² of the New Zealand exclusive economic zone (EEZ), rather than subtle differences among coastal sites. The scale of the MEC GIS layers are very low resolution and the data is more appropriate for the EEZ than estuaries. While the EEC is much more appropriate for the species and the aims of this thesis research, estuaries are an inherently variable environment which can change dramatically within a day, season or year. It is difficult to capture the dynamics of the system, but provides a useful guideline for overall characterization of the habitat (Hume et al. 2007). Ideally, long-term, fine scale environmental monitoring could be coupled with highly detailed demographic and genetic data to be able to tease out the effects of environment and genes.

This exploratory study has highlighted avenues for further inquiry into environmental factors correlated with genetic differentiation in *Rhombosolea plebeia* and *R. leporina*. Several candidate genes related to the environment can be identified for further study of selection and adaptation across spatial scales. A study of directional selection in heat shock proteins may elucidate some of the patterns related to sea surface temperature and how flatfish may adapt to climate change. A study of the rhodopsin gene in *Rhombosolea* would be enlightening as to adaptations in to spectral

tuning sites given different turbidities or sediment types, similar to the Larmuseau et al. study (2009). These factors may also apply to other estuarine dependent species. Besides genetic studies, any number of ecological studies, such as sediment type preference among *Rhombosolea* could tell us more about the ecological differences, and therefore distributional differences between the species in the genus. Studies which couple oceanography, currents and biophysical modelling have been at the forefront of the growing body of seascape genetic studies (Galindo et al. 2006, Selkoe et al. 2006), and this kind of insight would be invaluable to understanding connectivity of coastal organisms and the impact of orbital velocity on genetic patterns in flatfish.

With better and less expensive genomics techniques, the study of population genetics will be able to more easily identify genes under selective pressure and their functional role (Wenne et al. 2007). As we understand more about the structure and function of the genome, geneticists and conservationists may be able to identify and study the selective forces shaping local adaptation. We will have a more complete picture of the evolutionary processes to be described. Seascapes genetics may be the bridge to identifying the most significant environmental pressures driving the evolution of populations and species.

Chapter 6. General Discussion

Overview

The overarching goals of my thesis were to investigate levels of gene flow among two Pleuronectid species around the mainland of New Zealand. Using highly sensitive microsatellite DNA markers I developed specifically for these species, I was able to explore questions pertaining to the connectivity among sandy, soft-bottom and estuarine habitats. I challenged the assumption that animals with long PLDs have mostly open and connected populations, tested hypothesized biogeographic breaks, explored aspects of the SRS hypothesis, examined temporal variability and tested environmental models. This was the first study of New Zealand pleuronectids using a multidisciplinary approach with microsatellite DNA markers, GIS, and an array of bioinformatics software to study coastal connectivity on multiple spatial and temporal scales.

This study fills in major gaps in our knowledge about connectivity among shallow water coastal species. These habitats lie at the interface between human development and the marine environment, and play a significant role in economics, recreation and

culture. However, genetics research based on these habitats have been overlooked by researchers in New Zealand (Gardner et al. 2010) and globally (Caveen et al. 2012), possibly under the assumption that they are well-connected for mobile species.

To investigate these questions, I developed 15 and 12 polymorphic microsatellite markers from 454 data for each species, respectively. Using those markers, I genotyped 868 *R. plebeia* and 404 *R. leporina* from collection localities from biogeographic regions proposed by Shears et al. (2008) as a guideline for where barriers might occur. Using a number of bioinformatics techniques, I explored the allele frequencies and population structure of the species from many different angles. To ensure the spatial patterns observed were consistent across time, I tested for differentiation among year classes, relatedness among samples and for reduced diversity among juveniles. Finally, using two publicly available environmental data sets, I ran models to see which environmental variables best explained the differentiation observed. The results are a holistic approach to studying movement of genetic information in an open system.

Both species of *Rhombosolea* had significant population structure, but different spatial patterns of differentiation. Despite these species having high fecundity and mortality, there was little temporal variability among age classes. These spatial patterns are persistent across time and represent the coordination of multiple physical factors. Pelagic larval duration probably plays a major role in the connectivity of populations, but is not the only factor. The environmental models suggest that currents, sediment and sea surface temperature explain some genetic differentiation between localities.

While *Rhombosolea plebeia* had an isolation by distance pattern among the North Island localities and west coast of the South Island, the southeast part of the South Island was a distinct population. *Rhombosolea leporina* populations were well connected along the west coast and over the top of the North Island. The rest of the eastern populations from the North Island were significantly different from the west coast, and the southeast part of the South Island were similarly differentiated from the rest of the country.

Revisiting assumptions

At the outset of this study, it seemed likely that the PLD was the main factor connecting *Rhombosolea* populations. To control for factors related to passive dispersal and abundance, I used two widely distributed and ecologically similar species. Both had relatively restricted adult movements (Colman 1978) and I assumed a PLD of about 10 weeks for both species based on a study of *R. tapirina* (Crawford 1984). The results showed that the two species have different spatial patterns of genetic differentiation, even though subject to the same, or nearly the same oceanographic conditions given their sampling localities.

The assumption that *R. leporina* and *R. plebeia* have a similar PLD as *R. tapirina* is based on studies that have found that the PLD of species is similar within a genus. This was found to be the case in 17 genera of Pleuronectids from the Northwest Pacific (Minami and Tanaka 1992) and 100 species of Pomacentrids (Wellington and Victor 1989). It is not probable that the PLDs of *R. plebeia* and *R. leporina* are radically different, but the actual laboratory measurement or aging experiment should be undertaken to understand this fundamental life history trait of these two economically important species. Robertson and Raj (1971) were able to rear *R. plebeia* eggs and larvae in the laboratory until 18 days after fertilization, but unable to keep them alive until metamorphosis. At the time of writing this, there are no measurements of the PLD of *R. plebeia* or *R. leporina* available.

I also assumed that the distribution of the two species was the same. Published information about the two species states that they are common around the entire mainland (Ayling and Cox 1982, www.NABIS.gov.nz) and are frequently collected together (Morrison et al. 2002, Kendrick and Francis 2002, Francis et al. 2005). My collecting methods did not collect the two species in the same estuaries except for within three estuaries on the South Island: Karamaea, Westport and Avon Heathcote. In the Morrison et al. (2002), and Francis et al. (2005) studies, they used 9 and 11 m nets deployed from a boat, which would yield a more representative sample of the entire biota of an estuary. In a study of presence and abundance of fish in the Avon Heathcote

estuary over time, Webb (1972) found that *R. leporina* was less common than *R. plebeia*, and were present in different parts of the estuary at different times, therefore movement patterns may be different in other locations as well. My collecting method of towing a 4 m seine from shore indicates that *R. plebeia* and *R. leporina* may cluster together by species on a smaller spatial scale.

Comparisons and contrasts

Assuming a similar PLD and distribution, why does *R. plebeia* show an isolation by distance signal along the west coast and *R. leporina* show strong connectivity? According to allele frequency results and the STRUCTURE analysis, *R. leporina* showed more differentiation and possibly four clusters, while *R. plebeia* showed greater connectivity and only two clusters. If *R. leporina* had a more restricted distribution, there should be less connectivity along the west coast, which is not the case. This is one indication that more than just passive dispersal during the PLD connecting populations and directing structure.

There was a strong barrier to gene flow between samples on the southeast side of the South Island and the rest of the country in both species. This break occurred somewhere somewhere south of Blenheim and north of the Banks Peninsula. This break did not seem to correspond with the north to south break outlined by Gardner et al. (2010) across the top of the North Island or by Shears (2008) across the Cook Strait because it only occurred on the east coast. In a phylogeographic study of the Asteroid *Patiriella regularis*, Waters and Roy (2004) were similarly unable to resolve where the barrier between northern and southern groups occurred at either of these locations. There is change in habitat type identified by Walls (2009) near the same location identified by Gardner, but it may also be a regional boundary identified by Shears, but this is unclear without a more detailed sampling protocol.

How many populations?

To answer the question of how many populations of *Rhombosolea plebeia* and *R. leporina* we must first define a population. This study primarily examined levels of genetic connectivity among populations, but not necessarily demographic connectivity, with the exception of the assignment tests in Chapter 3. Theoretically one migrant per generation (Spieth 1974) is thought to be enough to maintain genetic connectivity, but current conservation genetic management theory suggests that exchange is too low. Mills and Allendorf (1996) suggested 10 migrants per generation may be needed to maintain a healthy amount of diversity and exchange among populations. However, even this level of exchange is not enough to maintain demographic connectivity, which is the actual number of individuals moving between populations. This demographic connectivity is what fisheries and marine protected areas (MPAs) are generally concerned with.

Moritz (1994) defined management units (MUs) as “populations with significant divergence of allele frequencies at nuclear or mitochondrial loci, regardless of phylogenetic distance.” He made the phylogenetic distinction to differentiate between evolutionarily significant units (ESUs), which may be more applicable to the long term preservation of diversity within species, while MUs are concerned with short-term contemporary management goals. Palsboll et al. (2007) suggested that less than 10% migration of the effective population size should be the threshold for separate management units.

Rhombosolea populations meet Moritz’s criteria as MU’s as there is significant allele divergence of allele frequencies among populations, as evidenced by multiple analyses. According to the migration rate threshold, the results from the analysis of first generation migration (Chapter 3, Table 3.12) show a low range of migration and emigration rates for both species. *R. plebeia* shows an average of 7.03% of the localities emigrating and an average of 9.05% of localities contain composed of first generation migrants. For *R. leporina*, an average of 10.68% of each locality emigrating to another location and an average of 12.44% of the localities composed of first generation

immigrants. Under this definition, most localities along could be considered their own MU's even without significant differences in allele frequencies.

The results of this study suggest that there are two main populations of *R. plebeia*. Multiple analyses show differentiation between populations from the southeast of the country from the Banks Peninsula south. The rest of the country's localities appear to be connected along a cline. The story of *Rhombosolea leporina* is somewhat more complicated. While *R. leporina* has more significant differences in allele frequencies than *R. plebeia*, the AWClust analysis clearly shows 2 populations. This cluster analysis does not assume HWE, which many of the localities do not meet. It is clear the west coast of the North Island and South Island are well connected. The STRUCTURE analysis showed a fourth clade, composed mostly of individuals in the KM locality. This locality had the smallest sample size of 11 individuals and may be driving the signal for a fourth population. Overall, there are most likely 3 populations of *R. leporina*, a western population, north eastern population and south eastern population. More samples are needed, particularly in the KM locality to confirm this.

More populations and finer scale differentiation may exist, but the power to detect further differentiation may be limited. In our power analyses, given the number of loci and population sizes, F_{ST} values below 0.005 would be difficult to detect with certainty (Supplementary Figures 3 and 4). However, values as low as 0.001 were detected as significant, indicating that there may be more significant differentiation that we do not have the power to detect. There could be more highly localized stocks that we did not sample or were unable to detect with the sample size or methods used.

In the tagging work done by Colman (1978) he tagged animals from the Banks Peninsula from three separate areas, the Avon Heathcote Estuary, Lyttleton Harbour and Akaroa Harbour, within 80 km of one another. Recaptured animals from Avon Heathcote and Lyttleton Harbour were collected in the greatest numbers in a bay north of the tagging area, but animals from Akaroa Harbour were caught in a bay south of the tagging area. Colman (1976) and Munday (1968) both discussed the possibility of there being two separate populations from the Banks Peninsula. In my study, I did not have any samples from Akaroa Harbour, but this could be a separate, undetected population.

In Colman's meristic study of *R. plebeia* (1976) he found the greatest differences in fin ray counts were between populations in Tasman Bay and Kapiti-Manawatu. The pairwise comparisons between Tasman Bay (TB and MH) and areas in the Kapiti-Manawatu region (FX and PH) showed that TB and MH were both significantly different from PH but not FX (Table 3.3). This could indicate further fine scale differentiation, however only one analysis (the F_{ST} values) found this comparison significant, so it is inconclusive given all of the analyses available.

Further Exploring Habitat Connections

Several theories were discussed in Chapter 3 concerning the cause of the metapopulation structure, including upwelling, which could advect larvae off shore and provide an oceanographic barrier to pelagic dispersal, lack of shallow water habitat due to the Hikurangi Trough and the isolation of the Banks Peninsula during the last sea level maximum in the Pliocene. Further study is needed to test each of these theories, and experiments can be designed to address each in turn.

Current models may be able to identify whether areas of upwelling just at Kaikoura were responsible for limiting pelagic larval dispersal. Passive sensor drifter studies may be oversimplified in that they do not take into account larval behaviour, but the clincher would be whether *Rhombosolea larvae* were collected significantly offshore in an upwelling plume. *Rhombosolea leporina* are found in abundance close to shore off the Kaikoura Peninsula, but there was no clear pattern of abundance with distance away from shore (Hickford and Schiel 2003). Barring the possibility that these larvae may be able to return to shore and recruit successfully, this may be proof that upwelling is limiting dispersal through selection. If pelagic larvae were able to travel across this upwelling region, we might be able to see an isotopic signature of the water characteristics accreted in the otoliths of juvenile fish (Campana and Thorrold 2001). A recent study of *Prochilodus mariae* in a river system of Venezuela, populations showed an isotopic signature of multiple spawning grounds, but no population structure evidenced from a microsatellite analysis (Collins et al. 2013).

To test whether adult animals are able to cross the Hikurangi Trench, otolith microchemistry could be used. Tagging studies may be more feasible, however, as a direct observation of whether animals tagged north or south of the Trench are later collected on the other side. *Rhombosolea* would be an excellent representative of other bottom dwelling organisms as they are important to fisheries and Colman had such success with his tagging experiments with returns that continued two years after the initial tagging. A token reward of t-shirts were offered in this study (Colman 1978).

During the Pleistocene, the Banks Peninsula was a relict population for shallow water species during the last glacial minima. This could be tested by using a molecular clock that may give us an idea of the age of the metapopulation disjunction that separated Banks Peninsula populations from other localities. The contemporary population structure shows *R. plebeia* localities further south, including Otago Harbour, Taeiri Mouth and Awarua Bay are connected with the Banks Peninsula through gene flow. This strong signature of isolation and recolonization should be apparent in multiple species. A phylogeographic study using mitochondrial DNA would best facilitate investigating this pattern.

Results from models in Chapter 5 using the EEC data give insight into what the differences between genetic patterns between the species. The GLZ models were very similar between species. The best GLZ models for *R. leporina* showed latitude, sediment, mean orbital velocity and sea surface temperature gradient were significantly correlated with genetic differentiation. The best GLZ model for *R. plebeia* showed the same variables as significant, with the exception of latitude, and including sea surface temperature winter. The results of the BEST analysis, however were different. For *R. leporina*, mean orbital velocity was by far the most significant variable in the best 10 models. For *R. plebeia*, longitude and sea surface temperature winter were the most significant variables.

To explore the effect of these environmental factors on genetic variation, candidate genes could be utilized. As discussed in Chapter 5, orbital velocity may have a strong effect on turbidity by suspending sea floor sediments (Allen et al. 1980). The types and amount of sediment suspended in the water changes the optical properties

(Shi et al. 2013), which could have a strong effect on hunting abilities of flounder (Shaw et al. 2006). This may lead to local adaptations in the genes related to sight and spectral tuning. Larmuseau et al. (2009) demonstrated local adaptation of the rhodopsin gene in relation to different photic environments of the North Atlantic sand goby (*Pomatoschistus minutus*). In the Varela thesis (2013), a phylogenetic relationship between the depth species occupy and rhodopsin sequence was found. In this study a partial sequence of the rhodopsin gene was successfully sequenced in *R. plebeia* and *R. leporina* and found to have variation between the species.

Sea surface temperature was also a significant variable in the *R. plebeia* models that explained much of the genetic differentiation among localities between the north and south. If populations were warm and cold water adapted, we may see variation in genes related to heat shock proteins (HSPs). Local adaptation to thermal tolerance has been documented in the expression of HSPs in the European flounder *Platyichthys flesus* (Hemmer-Hansen et al. 2007). Similar variations in adaptation were found in killifish, *Fundulus heteroclitus*, alone and in combination with other environmental stressors (Schulte 2007). This may be particularly important in the face of climate change and the ecological effects it may have in a variable habitats, like estuaries. A recent study showed that the timing of the spawning of sole (*Solea solea*) in the North Sea has been early as a result of warming ocean temperatures (Fincham et al. 2013).

Management Implications

Although this research is not intended to be an investigation of fisheries stocks, the results have implications for the management of these species. The identification of a population is strongly tied to the identification of an evolutionarily significant management unit or a stock, so population genetics has inherently relevant to management goals. One of the main findings of this thesis is that the spatial patterns of connectivity among closely related species can be very different. The evolutionary forces that have separated two currently sympatric species may to this day delineate different habitat preferences, distributions, and connectivity. The current management of flounder acknowledges that stocks are fairly localized and the intent of the current quota

management areas is to provide flexibility in management, rather than to be spatially explicit as far as separate stocks are concerned (FLA doc 2013).

In the current management scenario, 8 species are being managed as a single fishery: yellowbelly flounder (*Rhombosolea leporina*), sand flounder (*R. plebeia*), black flounder (*R. retiaria*), lemon sole (*Pelotretis flavilatus*), New Zealand sole (*Peltorhamphus novaezeelandiae*), brill (*Colistium guntheri*), and turbot (*C. nudipinnis*). Significant differences in the biology of these species may confound an umbrella approach to multi-species managements. For example, while *Rhombosolea* are short lived species, living from 5-8 years, *Colistium guntheri* and *C. nudipinnis* live 21 and 16 years, respectively (Stevens et al. 2005). As noted by Colman (1970) *R. leporina* are more slow growing and larger at maturity than *R. plebeia*. Moturo (2001) showed that only 15% of *R. leporina* females are sexually mature by the time they are of the legal size limit of 25 cm size (Ministry of Primary Industries, MPI 2013a). These animals are only likely to spawn once during their lifetime before being captured by the fishery (Colman 1985), which may be causing the overall low genetic diversity observed in Chapter 3 and 4.

Even though *Rhombosolea* numbers are currently abundant, this study identifies genetic reasons why the metapopulations may be at risk. There are three main factors that put *Rhombosolea* populations at high risk of being severely impacted by an environmental disaster or over fishing. First, these species apparently have very low genetic diversity compared to other marine or anadromous fish. As discussed, there are several reasons why this may be the case, however reduced genetic diversity could have severe consequences in a species ability to recover quickly. A bottleneck on an already limited population would greatly reduce the amount of diversity available in the relic population. Secondly, if the fish have strong phylopatry and low levels of migration, the damaged area would not be re-colonized quickly. Third, given that these fish are so short lived and may only spawn once or twice in their lifetime, an entire generation could be wiped out in a single year. After two years, there may be no more evidence of this relic population.

Marine protected area (MPA) research shows there is a positive influence on the size and abundance of fish within MPAs (Lester et al. 2009). Spatially explicit reserves can be used as fishery management tools to increase fishery stocks, improve economic returns (Gaines et al 2010, Rassweiler et al. 2012) and protect nursery areas (Francis 2013). However, there are not many studies on MPAs in temperate regions or soft bottom habitats compared to tropical regions and rock reef habitats (Lester et al. 2009, Caveen et al 2012), which may indicate a lack of sandy and softbottom MPAs available. In New Zealand, there is only one MPA on the south east side of the South Island, the Pōhatu or Flea Bay Reserve on the Banks Peninsula (Department of Conservation, DOC 2013). Given the limited gene flow among *Rhombosolea* populations from this area with other parts of the country, a single reserve provides little protection.

Ongoing Monitoring

The basic population structure of a species is a baseline for understanding processes and connectivity, but on-going monitoring of a population is necessary, especially if the population is constantly being influenced by human forces. Simply obtaining the baseline information is time consuming and expensive, so there must be a way to constantly monitor the population's health that is un-invasive, cheap and effective. The benefit of using flounder as a model system is that there are ample tissue samples available from fisheries and most of the fishermen this author has communicated with are very interested in the research. Māori are similarly invested and active in monitoring the health of the environment (Harmsworth and Tipa 2006), so the need and the support for monitoring is available.

There has been an interest to use *Rhombosolea leporina* as a biological indicator species to monitor the health of New Zealand estuaries (Hack et al. 2006). Since *R. leporina* is a benthic, non-migratory resident of many New Zealand estuaries, the animals are exposed to contaminated sediments. A number of biological tests were developed to measure liver detoxification of contaminants, metabolites, stress proteins, and reproductive functions to measure the biological responses to known contaminants, like polychlorinated biphenyls (PCBs) and heavy metals. *Rhombosolea plebeia* is

currently being considered as an indicator species by the Department of Conservation for long term ecological monitoring of human impacts on the marine environment (Monks et al. 2013). Biomarker monitoring and population genetic studies are currently implemented using the dab (*Limanda limanda*) in UK waters (Tysklind et al. 2013).

My genetics study can inform these monitoring efforts, and in combination with long term modelling provide excellent research opportunities into the genetic effects of environmental change. Without species specific catch information, genetic monitoring (Schwartz et al. 2007) may be a good way to assess the diversity, and health of the population. Genetics monitoring could be used to track the effective population size, whether populations are mixing or migrating with sea surface temperature changes, whether diversity is being reduced due to fishing or human-induced size selection. With the growing field of genomics research becoming less expensive and more accessible, cDNA libraries can be assembled in tandem with the environmental stress response studies to study natural and selection, local adaptation, the health and diversity of populations, responses to environmental contaminants, reproduction and a whole suite of gene expression studies. These could be extrapolated to other estuarine fish and play an important role in the conservation of dynamic marine environments.

More research is needed to fully eliminate all competing hypotheses as to what is separating populations of *Rhombosolea*. Although this present research was extensive in spatial coverage, it was not exhaustive. To truly define where barriers to gene flow occur, more localities need to be sampled, possibly every estuary and lagoon in New Zealand over multiple years. Then physical data such as salinity, sea surface temperature, turbidity, and currents could be collected along with the specimens. Ideally this information could be coupled with physical models in a long term study.

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Appendices

Supplementary Table 1. Population statistics by locus for *Rhombosolea plebeia*. Number of alleles (N_a), allelic richness (Ar), observed heterozygosity (H_o), expected heterozygosity (H_e), probability of Hardy-Weinberg Equilibrium (P_{HWE}), inbreeding coefficient (F_{IS}).

	Locus 5								Locus 11							
	N	N_a	PA	Ar	H_o	H_e	F_{IS}	P_{HWE}	N	N_a	PA	Ar	H_o	H_e	F_{IS}	P_{HWE}
DB 2010	36	4	0	3.314	0.500	0.567	0.132	0.178	33	7	1	4.669	0.545	0.642	0.165	0.007
PA	24	4	0	3.383	0.542	0.544	0.026	0.989	24	4	0	3.5	0.583	0.662	0.14	0.585
DB 2011	51	4	0	3.723	0.667	0.623	-0.06	0.070	51	9	3	5.274	0.588	0.710	0.181	0.001
HK	48	4	0	3.461	0.479	0.518	0.086	0.740	48	9	0	5.35	0.708	0.712	0.015	0.000
MK	23	3	0	2.522	0.565	0.481	-0.153	0.729	23	4	0	3.519	0.391	0.634	0.402	0.017
HB	45	4	0	2.985	0.511	0.534	0.054	0.063	37	5	0	4.236	0.324	0.699	0.546	0.000
AR	47	6	0	3.465	0.553	0.553	0.011	0.986	46	6	0	4.61	0.630	0.685	0.09	0.000
FX	46	4	0	3.417	0.565	0.485	-0.155	0.576	47	7	0	4.641	0.532	0.615	0.146	0.479
PH	40	4	0	3.141	0.450	0.515	0.139	0.296	38	6	0	4.126	0.605	0.621	0.038	0.600
LF	30	4	0	3.523	0.567	0.522	-0.068	0.958	27	6	0	4.331	0.407	0.635	0.375	0.303
TB	48	5	0	3.604	0.479	0.570	0.169	0.252	48	9	0	4.689	0.688	0.709	0.041	1.000
MH	45	5	0	3.979	0.556	0.587	0.065	0.982	43	8	0	5.374	0.628	0.723	0.144	0.008
KM	23	4	0	3.299	0.609	0.532	-0.122	0.960	23	8	0	5.863	0.696	0.725	0.063	0.181
WS	20	4	0	3.789	0.450	0.556	0.216	0.169	20	8	1	6.38	0.650	0.768	0.178	0.383
AH 2011	49	5	0	3.795	0.592	0.540	-0.086	0.707	48	9	0	5.836	0.688	0.760	0.106	0.538
AH 2012	49	5	0	3.579	0.531	0.563	0.067	0.794	44	6	0	4.473	0.545	0.712	0.244	0.000
LH	49	5	0	3.581	0.469	0.505	0.08	0.849	43	6	0	4.498	0.651	0.682	0.057	0.896
LE	47	5	0	3.626	0.617	0.533	-0.148	0.004	45	7	0	5.256	0.644	0.713	0.108	0.970
OT	29	5	0	4.125	0.414	0.466	0.128	0.000	26	6	0	5.028	0.885	0.732	-0.189	0.038
TE	49	6	0	4.182	0.612	0.561	-0.081	0.988	49	10	0	5.665	0.633	0.734	0.148	0.720
AB 2011	45	4	0	2.877	0.289	0.542	0.476	0.026	42	8	2	5.806	0.667	0.787	0.164	0.000
AB 2012	26	5	0	3.847	0.538	0.590	0.107	0.721	26	9	0	6.621	0.731	0.766	0.065	0.412
Total/Average	869	4.50	0	3.510	0.525	0.540	0.040	0.547	869	4.50	0	3.510	0.525	0.540	0.040	0.547

	Locus 25								Locus 57							
	<i>N</i>	<i>Na</i>	<i>PA</i>	<i>Ar</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P_{HWE}</i>	<i>N</i>	<i>Na</i>	<i>PA</i>	<i>Ar</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P_{HWE}</i>
DB 2010	36	3	0	2.283	0.056	0.200	0.729	0.000	36	8	0	5.293	0.444	0.463	0.054	0.754
PA	21	3	0	2.751	0.048	0.217	0.789	0.000	24	5	0	4.445	0.583	0.472	-0.215	0.944
DB 2011	49	3	0	2.113	0.082	0.117	0.309	0.010	51	8	0	5.117	0.392	0.446	0.13	0.019
HK	47	2	0	1.939	0.064	0.173	0.638	0.000	48	7	0	4.203	0.313	0.362	0.147	0.878
MK	21	2	0	1.996	0.000	0.245	1	0.000	23	6	0	4.496	0.391	0.344	-0.115	1.000
HB	42	3	0	1.927	0.048	0.092	0.489	0.000	45	6	0	3.492	0.311	0.296	-0.04	1.000
AR	45	4	0	2.783	0.067	0.204	0.68	0.000	47	8	0	4.657	0.489	0.456	-0.063	0.995
FX	41	2	0	1.502	0.049	0.048	-0.013	0.873	48	10	0	5.215	0.479	0.490	0.032	0.228
PH	36	2	0	1.71	0.028	0.080	0.66	0.000	38	10	0	5.723	0.500	0.526	0.062	0.999
LF	29	3	0	2.304	0.034	0.160	0.791	0.000	30	7	0	4.753	0.533	0.464	-0.133	1.000
TB	42	3	0	2.315	0.024	0.156	0.85	0.000	47	9	0	5.746	0.553	0.615	0.111	0.683
MH	42	2	0	1.641	0.024	0.069	0.661	0.000	43	10	0	5.551	0.535	0.655	0.194	0.816
KM	21	2	0	1.571	0.048	0.046	0	0.911	23	5	1	3.801	0.348	0.338	-0.006	0.956
WS	20	2	0	1.6	0.050	0.049	0	0.909	20	7	0	6.159	0.600	0.583	-0.004	0.121
AH 2011	44	2	0	1.273	0.023	0.022	0	0.939	49	8	0	4.875	0.571	0.606	0.067	0.876
AH 2012	38	2	0	1.316	0.026	0.026	0	0.934	48	8	0	6.09	0.646	0.719	0.112	0.000
LH	49	2	0	1.963	0.061	0.199	0.698	0.000	49	6	0	4.477	0.571	0.581	0.027	0.176
LE	49	1	0	1	0.000	0.000	NA	Mono	48	8	1	5.044	0.729	0.654	-0.104	0.960
OT	29	3	0	2.074	0.103	0.099	-0.024	0.993	28	6	0	4.686	0.536	0.570	0.077	0.139
TE	46	3	0	1.912	0.000	0.084	1	0.000	49	9	1	5.763	0.735	0.685	-0.062	0.000
AB 2011	34	2	0	1.353	0.029	0.029	0	0.931	42	9	0	6.483	0.500	0.736	0.332	0.000
AB 2012	24	2	0	1.5	0.042	0.041	0	0.917	26	7	0	5.29	0.615	0.645	0.065	0.839
Total/Average	805	2.4	0	1.856	0.041	0.107	0.441	0.353	869	4.5	0	3.510	0.525	0.540	0.040	0.547

	Locus 65								Locus 79							
	<i>N</i>	<i>Na</i>	<i>PA</i>	<i>Ar</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P_{HWE}</i>	<i>N</i>	<i>Na</i>	<i>PA</i>	<i>Ar</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P_{HWE}</i>
DB 2010	36	6	0	5.178	0.611	0.718	0.163	0.242	37	4	0	3.283	0.324	0.449	0.29	0.025
PA	24	7	0	5.828	0.708	0.765	0.095	0.509	23	3	0	2.996	0.435	0.492	0.139	0.862
DB 2011	50	7	0	5.768	0.700	0.768	0.098	0.196	51	3	0	2.955	0.235	0.424	0.453	0.000
HK	48	6	0	4.876	0.500	0.662	0.254	0.060	47	3	0	2.939	0.340	0.541	0.38	0.001
MK	23	6	0	5.041	0.522	0.745	0.32	0.636	22	3	0	2.997	0.318	0.507	0.393	0.040
HB	38	10	0	6.948	0.737	0.781	0.07	0.000	45	3	0	2.946	0.222	0.471	0.536	0.000
AR	46	10	0	7.062	0.848	0.794	-0.057	0.750	44	3	0	2.966	0.318	0.434	0.277	0.008
FX	47	10	0	6.466	0.723	0.779	0.082	0.001	48	3	0	2.997	0.438	0.573	0.246	0.013
PH	41	7	0	5.706	0.805	0.763	-0.043	0.931	39	4	0	3.288	0.385	0.525	0.279	0.247
LF	12	7	0	7	0.417	0.781	0.5	0.106	30	3	0	2.96	0.267	0.460	0.434	0.007
TB	47	7	0	5.54	0.723	0.756	0.053	0.666	46	3	0	2.976	0.239	0.458	0.486	0.000
MH	45	7	0	5.217	0.756	0.727	-0.028	0.830	46	4	0	3.223	0.196	0.448	0.571	0.000
KM	21	9	0	7.504	0.714	0.825	0.158	0.113	23	3	0	3	0.391	0.586	0.352	0.091
WS	16	7	0	6.442	0.438	0.789	0.471	0.053	20	3	0	2.844	0.200	0.335	0.424	0.057
AH 2011	41	7	0	5.268	0.585	0.692	0.167	0.013	49	3	0	2.992	0.347	0.548	0.376	0.000
AH 2012	44	6	0	4.667	0.523	0.612	0.157	0.490	45	3	0	2.924	0.356	0.446	0.214	0.334
LH	41	5	0	4.098	0.268	0.643	0.591	0.000	49	3	0	2.969	0.306	0.470	0.357	0.003
LE	40	6	0	4.771	0.300	0.680	0.568	0.000	48	4	0	3.315	0.354	0.369	0.051	0.804
OT	18	7	0	6.228	0.667	0.796	0.19	0.819	29	3	0	2.981	0.345	0.474	0.288	0.079
TE	49	9	0	6.061	0.571	0.790	0.286	0.482	50	4	0	3.177	0.320	0.431	0.268	0.005
AB 2011	34	6	0	5.728	0.471	0.788	0.415	0.005	41	3	0	2.993	0.268	0.594	0.557	0.000
AB 2012	25	7	0	6.176	0.600	0.798	0.267	0.364	26	3	0	2.994	0.154	0.493	0.698	0.000
Total/Average	786	7.2	0	5.799	0.599	0.748	0.217	0.330	858	3.2	0	3.033	0.307	0.479	0.367	0.117

	Locus 108								Locus 124							
	<i>N</i>	<i>Na</i>	<i>PA</i>	<i>Ar</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P_{HWE}</i>	<i>N</i>	<i>Na</i>	<i>PA</i>	<i>Ar</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P_{HWE}</i>
DB 2010	37	3	0	2.324	0.568	0.487	-0.152	0.641	35	9	0	6.684	0.714	0.790	0.11	0.088
PA	24	3	0	2.5	0.250	0.385	0.368	0.244	24	8	0	6.138	0.625	0.749	0.186	0.956
DB 2011	51	3	0	2.235	0.412	0.469	0.132	0.694	51	11	0	7.367	0.627	0.789	0.214	0.311
HK	48	3	0	2.25	0.354	0.419	0.165	0.309	48	12	2	7.301	0.750	0.791	0.062	0.000
MK	23	3	0	2.522	0.348	0.396	0.144	0.832	23	9	0	8.022	0.826	0.830	0.027	0.470
HB	37	3	0	2.324	0.324	0.374	0.147	0.758	45	10	0	7.046	0.822	0.819	0.008	0.956
AR	47	3	0	2.255	0.404	0.424	0.058	0.465	47	12	1	8.062	0.851	0.822	-0.025	0.983
FX	48	4	0	2.83	0.375	0.382	0.028	0.001	45	10	0	7.645	0.533	0.844	0.378	0.000
PH	40	3	0	2.768	0.300	0.445	0.337	0.004	40	9	0	6.834	0.700	0.822	0.161	0.160
LF	28	3	0	2.678	0.429	0.442	0.048	0.901	29	10	0	7.257	0.552	0.778	0.307	0.000
TB	48	3	0	2.25	0.458	0.380	-0.196	0.492	46	12	0	8.611	0.826	0.857	0.047	0.528
MH	46	4	0	2.862	0.435	0.437	0.016	0.140	44	11	0	7.048	0.773	0.807	0.054	0.912
KM	22	3	0	2.545	0.500	0.408	-0.203	0.179	23	11	0	8.945	0.739	0.848	0.15	0.697
WS	20	2	0	2	0.500	0.480	-0.016	0.852	20	9	0	7.986	0.800	0.824	0.054	0.885
AH 2011	48	3	0	2.681	0.313	0.331	0.065	0.664	49	12	0	8.171	0.837	0.836	0.009	0.939
AH 2012	44	4	0	2.543	0.386	0.348	-0.1	0.984	48	10	0	7.189	0.833	0.817	-0.009	0.840
LH	47	4	0	2.947	0.340	0.353	0.046	0.461	50	10	0	7.781	0.600	0.830	0.287	0.334
LE	46	4	0	2.955	0.261	0.331	0.222	0.322	46	10	0	7.425	0.783	0.812	0.047	0.706
OT	27	4	0	2.888	0.296	0.360	0.195	0.899	27	7	0	5.722	0.815	0.778	-0.028	0.820
TE	49	2	0	1.998	0.245	0.325	0.256	0.085	50	12	0	8.347	0.660	0.818	0.203	0.133
AB 2011	36	3	0	2.707	0.278	0.361	0.243	0.198	42	10	0	7.78	0.619	0.822	0.258	0.110
AB 2012	25	3	0	2.48	0.320	0.374	0.163	0.786	26	8	0	6.869	0.692	0.808	0.163	0.051
Total/Average	841	3.2	0	2.525	0.368	0.396	0.089	0.496	858	10	0.1	7.465	0.726	0.813	0.121	0.495

	Locus 131								Locus 133							
	<i>N</i>	<i>Na</i>	<i>PA</i>	<i>Ar</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P_{HWE}</i>	<i>N</i>	<i>Na</i>	<i>PA</i>	<i>Ar</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P_{HWE}</i>
DB 2010	37	5	0	3.739	0.378	0.491	0.243	0.712	38	3	0	2.509	0.263	0.254	-0.024	0.344
PA	24	4	0	3.495	0.625	0.589	-0.041	0.897	24	4	0	2.998	0.292	0.345	0.174	0.454
DB 2011	50	4	0	3.409	0.520	0.588	0.126	0.640	51	3	0	2.766	0.294	0.290	-0.003	0.130
HK	48	4	0	3.244	0.729	0.600	-0.206	0.059	48	4	0	3.135	0.354	0.367	0.045	0.840
MK	22	4	0	3.598	0.409	0.499	0.203	0.056	23	3	0	2.978	0.391	0.419	0.088	0.786
HB	41	5	0	3.804	0.488	0.471	-0.024	0.013	45	4	0	3.334	0.378	0.374	0	0.701
AR	47	4	0	3.386	0.574	0.580	0.021	0.003	46	4	0	3.438	0.348	0.409	0.16	0.430
FX	48	5	0	3.82	0.500	0.552	0.105	0.008	48	4	0	3.203	0.417	0.434	0.05	0.991
PH	41	5	0	3.889	0.488	0.593	0.19	0.204	41	3	0	2.93	0.366	0.382	0.055	0.540
LF	30	6	0	4.902	0.667	0.682	0.039	0.101	30	4	0	3.599	0.533	0.453	-0.161	0.884
TB	48	5	0	4.182	0.479	0.582	0.187	0.273	48	4	0	3.349	0.396	0.397	0.013	0.745
MH	46	6	0	4.012	0.543	0.562	0.043	0.003	47	4	0	3.269	0.362	0.331	-0.082	0.965
KM	22	5	0	4.424	0.727	0.626	-0.139	0.538	23	4	0	3.756	0.565	0.502	-0.104	0.745
WS	20	4	0	3.692	0.500	0.510	0.045	0.872	20	4	0	3.2	0.400	0.396	0.016	0.995
AH 2011	48	5	0	4.087	0.604	0.578	-0.035	0.540	49	4	0	3.726	0.510	0.505	0.001	0.983
AH 2012	47	6	0	4.542	0.468	0.634	0.272	0.000	45	5	0	3.791	0.378	0.448	0.168	0.424
LH	49	4	0	3.069	0.510	0.508	0.007	0.854	50	4	0	3.389	0.380	0.345	-0.093	0.944
LE	45	5	0	3.539	0.533	0.555	0.05	0.984	48	3	0	2.737	0.292	0.290	0.004	0.779
OT	25	5	0	4.404	0.520	0.558	0.088	0.231	29	4	0	3.406	0.241	0.304	0.222	0.349
TE	50	5	0	4.042	0.660	0.584	-0.12	0.786	50	4	0	3.521	0.400	0.411	0.038	0.003
AB 2011	47	6	0	4.953	0.511	0.657	0.233	0.047	45	5	0	3.959	0.356	0.580	0.396	0.000
AB 2012	26	3	0	2.996	0.577	0.558	-0.014	0.846	26	4	0	3.666	0.346	0.362	0.064	0.063
Total/Average	861	4.8	0	3.874	0.546	0.571	0.058	0.394	874	3.9	0	3.303	0.376	0.391	0.047	0.595

	Locus 136								Locus 145							
	<i>N</i>	<i>Na</i>	<i>PA</i>	<i>Ar</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P_{HWE}</i>	<i>N</i>	<i>Na</i>	<i>PA</i>	<i>Ar</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P_{HWE}</i>
DB 2010	36	4	0	3.041	0.333	0.382	0.141	0.938	32	13	0	9.245	0.656	0.850	0.243	0.197
PA	24	2	0	1.945	0.083	0.153	0.471	0.026	23	14	0	10.728	0.565	0.873	0.372	0.108
DB 2011	51	3	0	2.498	0.176	0.228	0.236	0.115	50	14	0	9.235	0.660	0.874	0.254	0.383
HK	48	3	0	2.126	0.125	0.154	0.2	0.460	46	19	0	11.992	0.652	0.908	0.292	0.039
MK	23	4	0	3.254	0.261	0.271	0.06	0.589	22	12	0	10.119	0.727	0.870	0.186	0.229
HB	41	5	0	2.994	0.220	0.203	-0.067	1.000	38	15	0	9.258	0.711	0.813	0.139	0.000
AR	46	5	0	3.311	0.326	0.289	-0.119	0.998	37	17	0	10.753	0.486	0.863	0.448	0.000
FX	46	6	0	3.713	0.326	0.292	-0.107	1.000	48	18	1	10.952	0.625	0.887	0.305	0.000
PH	37	4	0	2.791	0.162	0.177	0.098	0.081	40	21	1	12.837	0.875	0.911	0.052	0.991
LF	28	4	0	2.843	0.321	0.279	-0.133	0.985	30	15	0	10.691	0.600	0.859	0.317	0.000
TB	45	4	0	2.679	0.178	0.238	0.264	0.340	42	16	0	8.916	0.667	0.839	0.217	0.001
MH	43	3	0	2.016	0.116	0.111	-0.04	0.983	42	16	0	10.027	0.667	0.880	0.254	0.339
KM	23	3	0	2.42	0.174	0.162	-0.054	0.976	23	10	0	9.047	0.609	0.871	0.322	0.008
WS	20	3	0	2.446	0.050	0.141	0.661	0.000	19	13	0	10.811	0.632	0.878	0.305	0.053
AH 2011	49	3	0	2.91	0.367	0.373	0.024	0.544	36	17	0	10.364	0.583	0.855	0.331	0.004
AH 2012	44	3	0	2.667	0.205	0.226	0.108	0.265	36	14	0	9.143	0.556	0.863	0.369	0.001
LH	50	5	0	3.112	0.340	0.297	-0.134	0.996	47	15	0	8.832	0.532	0.825	0.364	0.000
LE	42	5	0	3.509	0.310	0.313	0.025	0.036	41	14	0	8.897	0.561	0.791	0.302	0.000
OT	29	3	0	2.873	0.379	0.324	-0.154	0.662	26	13	0	8.181	0.615	0.706	0.148	0.024
TE	48	4	0	2.569	0.146	0.157	0.082	0.093	49	15	0	10.139	0.633	0.845	0.261	0.000
AB 2011	36	4	0	2.478	0.167	0.156	-0.053	1.000	42	18	0	10.816	0.643	0.892	0.29	0.000
AB 2012	24	5	0	3.894	0.333	0.298	-0.099	1.000	24	12	0	9.622	0.542	0.847	0.379	0.005
Total/Average	833	3.9	0	2.822	0.232	0.238	0.064	0.595	793	15	0.1	10.028	0.627	0.855	0.280	0.108

Supplementary Table 2. Population statistics by locus for *Rhombosolea leporina*. Number of alleles (Na), allelic richness (Ar), observed heterozygosity (H_O), expected heterozygosity (H_E), probability of Hardy-Weinberg Equilibrium, (P_{HWE}), inbreeding coefficient (F_{IS}).

	Locus 1								Locus 5							
	N	Na	PA	Ar	H_O	H_E	F_{IS}	P_{HWE}	N	Na	PA	Ar	H_O	H_E	F_{IS}	P_{HWE}
MG	50	3	0	2.488	0.320	0.381	0.169	0.272	50	2	0	1.411	0.060	0.058	-0.021	0.827
HK	24	3	0	2.560	0.250	0.517	0.532	0.011	26	2	0	1.525	0.077	0.074	-0.020	0.838
HB	45	3	0	2.791	0.467	0.458	-0.009	0.369	49	2	0	1.418	0.061	0.059	-0.021	0.825
TB	46	4	0	3.026	0.435	0.421	-0.022	0.000	47	2	0	1.829	0.149	0.173	0.150	0.338
BL	49	3	0	2.847	0.367	0.458	0.208	0.312	48	2	0	1.781	0.167	0.153	-0.080	0.529
KM	10	2	0	2.000	0.300	0.495	0.438	0.213	11	1	0	1.000	0.000	0.000	NA	0.000
WS	27	3	0	2.288	0.259	0.374	0.323	0.310	27	2	0	1.296	0.037	0.036	0	0.922
AH	30	3	0	2.852	0.400	0.491	0.202	0.221	46	3	1	2.042	0.196	0.213	0.092	0.899
LH	46	4	0	2.604	0.348	0.416	0.175	0.872	47	2	0	1.789	0.170	0.156	-0.082	0.524
HS	44	3	0	2.331	0.227	0.464	0.518	0.002	50	3	1	1.926	0.180	0.165	-0.078	0.921
Total/Average	371	3.1	0	2.579	0.337	0.447	0.253	0.258	401	2.1	0.2	1.602	0.110	0.109	-0.007	0.662

	Locus 10								Locus 13							
	N	Na	PA	Ar	H_O	H_E	F_{IS}	P_{HWE}	N	Na	PA	Ar	H_O	H_E	F_{IS}	P_{HWE}
MG	49	10	0	5.764	0.755	0.763	0.020	0.006	46	6	0	4.577	0.739	0.740	0.012	0.908
HK	26	11	0	6.942	0.731	0.774	0.075	0.878	26	5	0	3.791	0.615	0.598	-0.009	0.511
HB	47	11	1	6.971	0.745	0.783	0.059	0.072	49	9	1	4.765	0.592	0.677	0.135	0.944
TB	47	10	0	6.472	0.745	0.781	0.057	0.497	47	6	0	3.775	0.596	0.615	0.043	0.186
BL	50	9	0	6.568	0.860	0.832	-0.024	0.881	50	7	0	4.313	0.680	0.659	-0.022	1.000
KM	11	7	0	6.310	0.636	0.760	0.209	0.034	11	5	0	4.649	0.545	0.690	0.255	0.456
WS	27	11	0	7.122	0.815	0.819	0.024	0.057	27	4	0	3.610	0.741	0.619	-0.178	0.283
AH	45	10	0	6.941	0.756	0.834	0.105	0.633	44	7	1	3.940	0.659	0.646	-0.009	0.968
LH	47	11	0	6.949	0.809	0.832	0.039	0.989	47	6	0	3.719	0.596	0.615	0.042	0.855
HS	50	12	0	7.220	0.780	0.816	0.054	0.889	50	7	0	3.850	0.640	0.598	-0.061	0.000
Total/Average	399	10	0.1	6.726	0.763	0.799	0.062	0.494	397	6.2	0.2	4.099	0.640	0.646	0.021	0.611

Locus 15									Locus 19							
	<i>N</i>	<i>Na</i>	<i>PA</i>	<i>Ar</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P_{HWE}</i>	<i>N</i>	<i>Na</i>	<i>PA</i>	<i>Ar</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P_{HWE}</i>
MG	50	2	0	1.717	0.140	0.130	-0.065	0.595	50	7	1	5.200	0.760	0.753	0.001	0.834
HK	26	2	0	1.938	0.269	0.233	-0.136	0.428	25	4	0	3.866	0.720	0.719	0.019	0.160
HB	49	1	0	1.000	0.000	0.000	NA	0.000	49	6	0	3.948	0.551	0.641	0.150	0.001
TB	46	2	0	1.440	0.065	0.063	-0.023	0.819	47	6	0	4.212	0.553	0.628	0.129	0.885
BL	48	2	0	1.167	0.021	0.021	0.000	0.942	50	6	0	3.552	0.540	0.607	0.120	0.000
KM	11	2	0	1.935	0.182	0.165	-0.053	0.740	8	4	0	4.000	0.750	0.602	-0.183	0.896
WS	27	3	0	1.956	0.148	0.139	-0.045	0.982	27	5	0	4.056	0.630	0.708	0.129	0.545
AH	46	3	0	1.944	0.109	0.143	0.250	0.000	46	7	1	4.385	0.522	0.673	0.235	0.587
LH	47	2	0	1.685	0.128	0.120	-0.057	0.640	47	6	0	4.756	0.596	0.692	0.150	0.794
HS	50	2	0	1.869	0.220	0.196	-0.114	0.382	47	5	0	3.906	0.660	0.712	0.084	0.362
Total/Average	400	2.1	0	1.665	0.128	0.121	-0.027	0.553	396	5.6	0.2	4.188	0.628	0.673	0.083	0.506

Locus 20									Locus 21							
	<i>N</i>	<i>Na</i>	<i>PA</i>	<i>Ar</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P_{HWE}</i>	<i>N</i>	<i>Na</i>	<i>PA</i>	<i>Ar</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P_{HWE}</i>
MG	50	12	2	7.315	0.900	0.847	-0.053	0.503	50	1	0	1.000	0.000	0.000	NA	0.000
HK	26	9	0	6.808	0.654	0.836	0.236	0.029	25	1	0	1.000	0.000	0.000	NA	0.000
HB	49	10	0	6.863	0.918	0.843	-0.079	0.496	49	3	0	1.581	0.082	0.079	-0.024	0.993
TB	47	9	0	6.892	0.894	0.846	-0.046	0.647	47	4	0	1.796	0.106	0.103	-0.027	1.000
BL	50	10	0	7.235	0.660	0.857	0.239	0.003	48	2	0	1.676	0.125	0.117	-0.056	0.644
KM	11	7	0	6.167	0.636	0.793	0.243	0.010	8	1	0	1.000	0.000	0.000	NA	0.000
WS	27	9	0	6.824	1.000	0.842	-0.169	0.046	27	2	0	1.660	0.111	0.105	-0.040	0.760
AH	46	9	0	6.285	0.739	0.808	0.097	0.374	45	4	0	1.804	0.067	0.107	0.385	0.004
LH	47	10	0	6.594	0.702	0.796	0.128	0.002	47	4	0	1.916	0.128	0.122	-0.036	1.000
HS	49	10	0	6.329	0.653	0.824	0.218	0.000	48	2	0	1.307	0.042	0.041	-0.011	0.883
Total/Average	402	9.5	0.2	6.731	0.776	0.829	0.081	0.211	394	2.4	0	1.4739	0.066	0.0673	0.02729	0.5283

Locus 22									Locus 23							
	<i>N</i>	<i>Na</i>	<i>PA</i>	<i>Ar</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P_{HWE}</i>	<i>N</i>	<i>Na</i>	<i>PA</i>	<i>Ar</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P_{HWE}</i>
MG	44	5	0	3.099	0.318	0.432	0.273	0.000	50	9	0	6.553	0.780	0.836	0.077	0.000
HK	24	5	0	2.882	0.208	0.293	0.309	0.269	26	11	0	7.296	0.808	0.851	0.071	0.007
HB	46	6	1	3.635	0.196	0.545	0.647	0.000	48	9	0	6.090	0.792	0.799	0.020	0.000
TB	45	8	0	4.012	0.244	0.472	0.491	0.000	47	9	0	6.376	0.809	0.836	0.044	0.006
BL	44	7	0	3.321	0.182	0.323	0.446	0.000	50	14	0	7.526	0.620	0.865	0.293	0.000
KM	11	4	0	3.182	0.273	0.248	-0.053	1.000	11	9	1	7.972	0.727	0.843	0.184	0.120
WS	25	8	0	4.537	0.400	0.467	0.164	0.016	27	8	0	6.266	0.778	0.803	0.050	0.631
AH	30	5	0	2.868	0.167	0.270	0.397	0.038	44	17	0	8.500	0.705	0.857	0.189	0.000
LH	44	9	1	4.068	0.273	0.410	0.345	0.000	47	17	0	8.301	0.574	0.873	0.351	0.056
HS	47	4	0	2.099	0.106	0.160	0.343	0.000	50	12	1	6.914	0.780	0.848	0.090	0.000
Total/Average	360	6.1	0.2	3.370	0.237	0.362	0.336	0.132	400	12	0.2	7.179	0.737	0.841	0.137	0.082

Locus 29									Locus 37							
	<i>N</i>	<i>Na</i>	<i>PA</i>	<i>Ar</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P_{HWE}</i>	<i>N</i>	<i>Na</i>	<i>PA</i>	<i>Ar</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P_{HWE}</i>
MG	50	13	1	7.133	0.840	0.834	0.003	0.935	49	7	0	5.359	0.510	0.773	0.349	0.000
HK	25	9	0	5.729	0.880	0.774	-0.116	0.810	26	7	0	4.971	0.731	0.717	0.001	0.991
HB	49	11	0	6.214	0.735	0.804	0.096	0.452	48	8	0	5.314	0.521	0.753	0.318	0.000
TB	47	12	0	6.554	0.851	0.820	-0.027	0.004	47	8	0	5.522	0.574	0.784	0.277	0.056
BL	48	9	0	6.469	0.771	0.808	0.057	0.333	48	8	0	5.570	0.604	0.777	0.233	0.005
KM	8	7	0	7.000	0.750	0.789	0.116	0.765	11	8	0	7.100	0.818	0.826	0.058	0.024
WS	26	7	0	5.881	0.731	0.811	0.118	0.252	27	7	0	5.650	0.704	0.750	0.081	0.047
AH	40	8	0	5.350	0.875	0.781	-0.108	0.548	46	8	1	5.301	0.522	0.721	0.287	0.000
LH	47	14	1	6.000	0.766	0.798	0.051	0.358	47	8	0	5.439	0.574	0.739	0.233	0.002
HS	48	11	0	6.036	0.833	0.792	-0.042	0.995	49	8	0	5.104	0.633	0.718	0.129	0.000
Total/Average	388	10	0.2	6.236	0.803	0.801	0.015	0.545	398	7.7	0.1	5.533	0.619	0.756	0.197	0.113

Locus 40									Locus 47							
MG	<i>N</i>	<i>Na</i>	<i>PA</i>	<i>Ar</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P_{HWE}</i>	<i>N</i>	<i>Na</i>	<i>PA</i>	<i>Ar</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P_{HWE}</i>
HK	50	5	0	3.435	0.760	0.659	-0.143	0.678	49	2	0	1.847	0.204	0.183	-0.103	0.426
HB	26	5	0	3.822	0.462	0.686	0.344	0.000	26	4	0	2.987	0.269	0.357	0.265	0.000
TB	48	5	0	3.812	0.646	0.678	0.058	0.568	46	3	0	2.070	0.152	0.180	0.164	0.476
BL	47	4	0	3.529	0.681	0.692	0.027	0.785	45	3	0	2.151	0.200	0.184	-0.073	0.907
KM	50	4	0	3.394	0.600	0.666	0.109	0.577	49	4	0	2.404	0.224	0.223	0.004	0.887
WS	9	4	0	3.987	0.556	0.673	0.231	0.059	11	3	0	2.662	0.091	0.244	0.655	0.012
AH	27	4	0	3.288	0.704	0.660	-0.047	0.940	26	2	0	1.525	0.077	0.074	-0.020	0.838
LH	44	7	0	4.026	0.523	0.616	0.163	0.000	32	4	0	2.275	0.125	0.175	0.301	0.034
HS	47	4	0	3.497	0.638	0.617	-0.023	0.033	45	4	0	2.309	0.200	0.221	0.106	0.000
	50	5	0	3.698	0.540	0.689	0.226	0.000	50	4	0	2.716	0.260	0.325	0.210	0.000
Total/Average	398	4.7	0	3.649	0.611	0.664	0.095	0.364	379	3.3	0	2.295	0.180	0.217	0.151	0.358

Locus 49								
MG	<i>N</i>	<i>Na</i>	<i>PA</i>	<i>Ar</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	<i>P_{HWE}</i>
HK	50	10	0	5.743	0.580	0.720	0.204	0.002
HB	25	8	0	6.341	0.880	0.799	-0.081	0.576
TB	48	9	1	5.267	0.708	0.738	0.050	0.718
BL	47	9	0	5.386	0.830	0.731	-0.124	0.056
KM	50	8	0	5.275	0.720	0.742	0.040	0.669
WS	10	6	0	5.365	0.800	0.600	-0.286	0.996
AH	27	7	0	5.303	0.852	0.739	-0.135	0.820
LH	45	9	0	6.134	0.756	0.794	0.059	0.187
HS	47	8	0	5.535	0.766	0.753	-0.006	0.926
	47	8	0	5.842	0.830	0.766	-0.072	0.805
Total/Average	396	8.2	0.1	5.619	0.772	0.738	-0.035	0.575

Supplementary Figure 1. Allele discovery curves for *R. plebeia*

Supplementary Figure 1 continued

Supplementary Figure 2 Allele discovery curves for *R. leporina*

Supplementary Figure 2 continued

Supplementary Figure 2 continued

Supplementary Figure 3. Power analysis for *R. plebeia* microsatellite data

Supplementary Figure 4 Power analysis for *R. leporina* microsatellite data