**A population genetic analysis of the New Zealand
spotty (*Notolabrus celidotus*) using mitochondrial
DNA and microsatellite DNA markers**

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Abstract

Notolabrus celidotus (the New Zealand spotty) is a common rocky reef species that is endemic to New Zealand. This species is the most abundant demersal reef fish in New Zealand, and is distributed throughout the North and South Islands, and Stewart Island. *Notolabrus celidotus* consumes a wide variety of small invertebrates, and juveniles are reliant on coastal kelp forests as nursery habitats. Because *N. celidotus* is such a common species on New Zealand rocky reefs it is a good model species for population genetic studies.

The primary goal of this research was to investigate new genetic markers and add new sample locations to bolster previous genetic population data from *N. celidotus*. The thesis research utilised DNA sequences obtained from a 454 massively parallel DNA sequencer and reports six new microsatellite loci for *N. celidotus*. These loci are the first microsatellite DNA markers to be developed for this species. Additional mitochondrial DNA (mtDNA) control region sequences were obtained from new samples of *N. celidotus* and combined with previously reported mtDNA sequences. Increasing the sample size improved the genetic coverage of *N. celidotus* populations around coastal New Zealand. The mtDNA sequences were analysed to examine the population connectivity and demographic history of *N. celidotus*. The microsatellite DNA loci reported in this study were also used to examine the levels of genetic diversity and population structure in *N. celidotus*.

Results of the combined genetic analyses revealed extremely high levels of genetic diversity among the population sample of the mtDNA control region. Both the mitochondrial DNA and microsatellite DNA analyses showed a distinct lack of population genetic structuring, which suggests there is constant mixing of *N. celidotus* among sites. The results of this study have the potential to inform the expectations about the genetic structure of closely related wrasse species, such as *Notolabrus fucicola*, as well as other coastal species that have a similar life history, dispersal power, and New Zealand-wide distribution.

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Table of Contents

Chapter One

General Introduction	8
Connectivity and population structure	8
Population genetic marker types.....	10
Marine population connectivity	12
Study species: <i>Notolabrus celidotus</i> (New Zealand spotty)	16
The aims of the thesis research	17

Chapter Two

Development of novel microsatellite markers for <i>N. celidotus</i>	19
2.1 Introduction	19
2.2 Materials and methods	21
2.3 Results.....	24
2.4 Discussion	28

Chapter Three

Analysis of population structure and demographic history using mitochondrial DNA and microsatellite DNA markers	30
3.1 Introduction	30
3.2 Materials and methods	33
3.2.1 Sample collection	33
3.2.2 Mitochondrial DNA sequencing	35
3.2.3 Microsatellite DNA genotyping	35
3.2.4 Mitochondrial DNA sequence analysis	36
Genetic diversity	36
Population structure	37
Demographic history.....	38
3.2.5 Microsatellite DNA genotype analysis	39
Genetic diversity	39
Population structure	39
3.3 Results.....	41
3.3.1 Mitochondrial DNA sequencing	41
3.3.2 Microsatellite DNA genotyping	50
3.4 Discussion	58
Genetic diversity	58
Population structure	59
Demographic history.....	60

Chapter Four

General Discussion	62
Genetic diversity of <i>Notolabrus celidotus</i>	62
Population structure of <i>Notolabrus celidotus</i>	64
<i>Notolabrus celidotus</i> as a model species	65
Future directions	66

Appendix	69

References	71

List of Tables

Chapter Two

2.3.1	Microsatellite primer information and annealing temperatures	26
2.3.2	Comparisons between <i>N. celidotus</i> and other species	26

Chapter Three

3.2.1	Sample collection sites within provinces	34
3.3.1	Outline of the new samples collected for this study	41
3.3.2	Summary of population information (mitochondrial DNA)	42
3.3.3	Measures of population differentiation using G_{ST}	44
3.3.4	AMOVA results (mitochondrial DNA)	44
3.3.5	Results of Tajima's D and Fu's F_S tests	48
3.3.6	Summary of population information (microsatellite DNA)	50
3.3.7	Pairwise population comparisons using F_{ST}	53
3.3.8	Pairwise population comparisons using Jost's D_{est} and G'_{ST}	53
3.3.9	Population assignment, in provinces where $N < 20$	53
3.3.10	AMOVA results (microsatellite DNA)	55

List of Figures

Chapter One

1.1	Three general types of population structure	9
1.2	Map of New Zealand showing major ocean currents	15

Chapter Two

2.3.1	Lengths of all 454 sequencing reads	25
2.3.2	Frequency plot showing the number of repeat motifs	25

Chapter Three

3.2.1	New Zealand map showing provinces and sample sizes	33
3.3.1	Haplotype rarefaction plot	43
3.3.2	Multidimensional scaling plot of pairwise G_{ST} values	43
3.3.3	Isolation-by-distance plot	45
3.3.4	Minimum spanning network of all haplotypes	46
3.3.5	Private and shared haplotypes from each province	47
3.3.6	Mismatch distribution	49
3.3.7	Bayesian skyline plot	49
3.3.8	Allele rarefaction curve for Spot07	51
3.3.9	Allele frequency distribution for all loci	52
3.3.10	Factorial component analysis (FCA)	54
3.3.11	Bayesian Structure plots for all provinces	56
3.3.12	Bayesian Structure plots for Abel, Banks and Cook	56
3.3.13	Delta K output from Structure analysis (all provinces)	57
3.3.14	Delta K output from Structure analysis (Abel, Banks and Cook)	57

Appendix

Appendix 1	Allele rarefaction curves for all microsatellite loci	69
Appendix 2	Visual comparison of haplotype networks	70

Chapter One

General Introduction

Connectivity and population structure

Connectivity forms an important part of biological systems. It describes the connectedness of reproductive units (populations), which are the units of evolutionary change in a species. Connectivity links a variety of biological disciplines, including evolution, population genetics, dispersal and migration, as well as conservation (Kool et al., 2013). Historically, connectivity has been defined as the degree to which the landscape facilitates movement among resource patches (Taylor et al., 1993), but has since been revised to include the importance of larval settlement, post-larval survival and contribution to the gene pool in marine environments (Pineda et al., 2007). Population structure describes the degree to which populations are subdivided (Meirmans & Hedrick, 2011). Species that exhibit population structure have some degree of disconnection from one another, and can therefore be genetically or demographically disconnected (Ovenden, 2013).

Understanding the levels of population connectivity of a species can have many useful applications. It can inform many aspects of the marine environment as oceanic dispersal cannot often be observed directly, due to the logistical challenges in studying the large and complex three-dimensional structure of the ocean (Unsworth et al., 2008). Identifying levels of connectivity has particular applications in the fishing industry: recent work to identify biological units, and matching them to managed units has gained popularity, as an attempt to reduce the likelihood of stock depletion from overfishing (Branch et al., 2011). Connectivity can inform the management of invasive species when the potential dispersal abilities of a species can be mapped, and management actions taken (Handley et al., 2011). In designing marine protected areas, information about connectivity can be used to predict levels of local recruitment and the carrying capacity of a particular protected area (Moland et al., 2013).

Figure 1.1 Three general types of population structure, as adapted from Laikre *et al* (2005). Representations of homogenous populations (panmixia, left), continuous variation (isolation by distance, centre) and genetically distinct stocks (right) are shown.

Patterns of population structure can generally be classified in three ways (Laikre *et al.*, 2005). Firstly, populations can be genetically homogenous over space: this form of population structure contains large enough levels of gene flow that populations can be classified as a single panmictic unit. Secondly, the genetic composition of populations changes gradually over space: this is also known as isolation-by-distance. Thirdly, individuals can form distinct populations, where small levels of gene flow cause genetic divergence between populations (Figure 1.1). Phylogeographic splits between distinct populations can arise from ecological or historical separation, limiting gene flow from one population to another (Wallis & Trewick, 2009).

Population structure and connectivity in marine environments can be studied in both direct and indirect ways. Direct methods of study can include tagging or tracking individuals, as well as isotopic analysis. Tagging and tracking individuals can be useful in identifying breeding grounds or migratory routes of a species (Drenner *et al.*, 2012). Isotopic analysis, where the ratios of trace elements are analysed in an individual and matched to a location, such as a harbour or river, can reveal natal origins of individuals (Morrison, 2008). These direct methods of study tend to provide precise information about individual organisms, and do not necessarily reflect the behaviour of all individuals in that species. Additionally,

these analyses can be time-consuming and costly, and logistically difficult in the case of tracking studies in particular (Drenner et al., 2012). Indirect methods of studying population structure and connectivity, such as genetic analyses, can provide a larger scope for analysis of a larger number of individuals (Ovenden, 2013). Genetic studies are often used to assess connectivity due to the difficulties in measuring dispersal at large spatial scales (Lowe & Allendorf, 2010). Various genetic markers can be used to study population structure, and the ideal marker depends largely on the scope and aims of the project and the intended analyses (Schlotterer, 2004).

Population genetic marker types

The genetic marker used in a study can determine the resolution of population structure that can be detected. In the past, most population genetic studies have used neutral molecular markers, because the genetic patterns observed in these markers are expected to reflect the evolutionary processes of genetic and migration that act on the whole genome (Laikre et al., 2005). Loci influenced by selection can only provide information about the selective pressures acting on that particular part of the genome, and some of the patterns seen may only be applicable to the locus studied (Avice, 2004). However, studying markers under selection can reveal information about types of selective pressures and areas that promote local adaptation. In some cases, studying local adaptation can help to explain physiological differences within a species, by studying single nucleotide polymorphisms, and correlating them with environmental variation (Coop et al., 2010; Dionne et al., 2008).

It can be advantageous to use more than one type of molecular marker in genetic population analyses. Some studies that have used both mitochondrial DNA and nuclear DNA to estimate a population's effective size found discrepancies depending on the marker that was used. These differences were attributed to features of the species' life history and behaviour (Schlotterer, 2004). For example, in species with sex biased dispersal, where females are largely philopatric and males disperse, calculations of population size using mitochondrial DNA were

smaller than when using nuclear DNA (Peters et al., 2012). Using multiple marker types in a study can be useful in species that have sex-biased dispersal or are hermaphroditic, since the different features of various marker types can reveal more information about patterns of inheritance, reproduction and dispersal (Kolleck et al., 2013).

The most common molecular markers used in studies of population structure are mitochondrial DNA and microsatellite DNA (Meirmans & Hedrick, 2011). Mitochondrial DNA sequencing involves identifying haplotypes of a particular region of the mitochondrial genome. The non-coding control region (or D-loop) is popular, as it typically contains regions that are less constrained by negative mutations than protein-coding genes (Portnoy & Heist, 2012). Mitochondrial DNA is maternally inherited, and is advantageous in that haplotypes are unlikely to be identical unless the individuals are related (Hughes et al., 2013). Mitochondrial DNA has been widely used in phylogeographic studies as it can detect genetic differences within a species. Comparing mitochondrial haplotypes can also have the power to detect populations that had been historically separated but have now re-joined, due to the unbroken and unrecombined matrilineal lineages of mitochondrial DNA (Avice, 2004).

Microsatellite DNA (also known as short tandem repeats) has gained popularity recently as a tool to study genetic structure (Selkoe & Toonen, 2011). Microsatellite repeats tend to be highly polymorphic, which offers greater resolution when studying population structure, and are distributed throughout the genome. If levels of polymorphism are adequately high, it may be possible to conduct kinship analyses by comparing the genotypes of different loci (Waldbieser & Bosworth, 2013). Microsatellites can be prone to homoplasy, where identical individuals are not necessarily identical due to descent, but due to converging mutations (Dickey et al., 2013). Additionally, it can be a costly process to screen for and develop microsatellite loci, since they are commonly species-specific; although recent work has focused on developing microsatellites that can be employed across a range of species in the same genus or family for this reason (Gardner et al., 2011). It can be unclear whether the number of repeats in a microsatellite locus

changes in a step-wise way (the step-wise mutational model), or whether they can jump to any size (the infinite allele model), which is a limitation in the use of microsatellite markers. This limitation can be countered by applying both models during data analysis.

Fine-scale population differentiation can be more easily detected using markers with greater resolution, such as microsatellite markers (Harrison et al., 2014). However, the choice of molecular marker largely depends on the aims of the project. To fully understand connectivity in a species, it can be useful to employ both direct and indirect studies: such as genetics coupled with otolith microchemistry. Genetic methods can provide insight to demographic connectivity when genetics is combined with data about migratory behaviour, and reproductive success (Lowe & Allendorf, 2010; Ovenden, 2013).

Marine population connectivity

In the past, it was generally held that populations of oceanic species were largely open, and therefore showed no population structure, due to the ability of ocean currents to disperse larvae or eggs from different populations over long distances, and the ability for the adults of some species to swim from one location to another (Kool et al., 2013). The opposite has been found in coral reefs: a study that involved chemical tagging of tropical clownfish and butterflyfish found that up to 60% of larvae self-recruited to the natal reef (Almany et al., 2007). Several factors have been suggested as important in determining whether a population will show population structure or not, including the pelagic larval duration and reproductive habits of a species, habitat patchiness and the oceanic currents around particular habitats (Pinsky et al., 2012). It is not yet clear which of these factors is more important, since it is impossible to study these factors in isolation, and the interactions between life history and environment can be difficult to untangle (Lowe & Allendorf, 2010).

Biological factors that can determine population structure are related to a species' life history. One of the most commonly suggested factors is the pelagic larval

duration (PLD), since an organism that spends a longer period of time in the plankton will have a greater chance of being dispersed further (Mora et al 2012; Trembl et al., 2012). The PLD is sometimes cited as the only factor that influences population structure, but there are several other factors of a species' life cycle and life history that also influence population structuring. Firstly, species that have actively swimming larvae (larvae that are able to swim against water movements, rather than passively swimming larvae which cannot) are more able to select a habitat, and therefore may show less connectivity with other populations (McManus & Woodson, 2012). It is also important to know about the spawning patterns of a species. Spawning aggregations, in which individuals from many different locations gather to spawn at an established site, can allow for gene flow between individuals from many geographically disparate populations. For example, shallow population structure was observed in the grouper *Hyporthodus acanthistius*, and this was linked to the species' spawning aggregations (Beldade et al., 2014). Additionally, philopatric species, in which individuals return to natal grounds for spawning, limits the degree of connectivity between populations, and is therefore expected to result in stronger genetic structuring (Kinitz et al., 2013).

Habitats that already have a well-established population of a particular species may limit genetic connectivity between habitats because there are limited opportunities for migrants to successfully integrate into the resident population (Waters et al., 2013). This is possible if a population has reached its carrying capacity in an area: even if an individual has dispersed to this habitat, it may not be able to recruit successfully due to the lack of available space, resources or available mates (Abesamis & Russ, 2005). The amount of physical space available for an organism to occupy limits successful recruitment: in the case of sessile or territorial species, all available physical spaces in an environment may be occupied, and very little or no successful recruitment will occur. The amount of food that is available to a population can also limit the degree of successful recruitment. Limited food supplies can cause intraspecific competition, and recruits are likely to be excluded from access to food resources (Bertness, 1989). For example, populations of California spiny lobster are genetically differentiated even though they have a long PLD and no geographic barriers to gene flow were

observed (Iacchei et al., 2013). It was thought that high levels of local recruitment restricted the number of larvae from other populations that were able to settle in each habitat, thereby reducing the level of observed genetic connectivity, even though current flows facilitated connectivity.

Non-biological factors can also determine levels of population connectivity. An important factor is the system of currents that operate around the species' range: strong offshore currents can sweep larvae away from natal grounds, and currents may restrict the range of locations that an individual from a particular population can be dispersed to (Galarza et al., 2009).

Connectivity in the marine environment has been studied extensively in New Zealand. New Zealand waters encompass sub-tropical, temperate and sub-Antarctic climates (Ross et al., 2009). Major current systems divide as they reach New Zealand, creating smaller surface currents, shown in Figure 1.2. These currents interact with local geography, as well as variable tides, winds and waves, making phylogeography difficult to predict (Ross et al., 2009). Deeper water currents around New Zealand can also carry larvae: whether a species has larvae that inhabit shallow or deep waters will influence the phylogeography of the species. Ocean current patterns in New Zealand have been hypothesised as the cause of phylogeographic breaks in species that have low dispersal abilities (Apte & Gardner 2002; Wallis & Trewick, 2009).

A review of genetic population structure studies in New Zealand found that of the 29 species analysed, eight taxa showed no genetic differentiation, 16 taxa showed north-south differentiation, and four taxa showed east-west differentiation (Ross et al., 2009). This result suggests that population structure is influenced strongly by the life history of a species: the pelagic larval duration, whether larvae are active or passive swimmers, and the presence or absence of mass spawning aggregations.

Figure 1.2 Map of New Zealand showing major ocean currents (adapted from Ross *et al.*, 2009). The grey band represents the sub-tropical convergence, a joining of sub-Antarctic and sub-tropical waters.

In a review by Ross *et al* (2009), phylogeographic breaks were identified in several species, where gene flow is restricted over a particular area. Various causes of these phylogeographic breaks have been suggested, including the strong tidal flows and turbulent mixing of Cook Strait, and regions of upwelling. Cook Strait is the most commonly cited cause of phylogeographic breaks (Waters & Roy, 2004). It has been suggested that regions of upwelling around the 42°S latitude line, which crosses the northern part of the South Island, act as a barrier to gene flow between North Island and South Island populations of certain species (Apte & Gardner, 2002). However, in the multiple studies of invertebrates and vertebrates,

no phylogeographic breaks have been identified that are common to all species (Ross et al., 2009).

Study species: Notolabrus celidotus (New Zealand spotty)

The New Zealand spotty, *Notolabrus celidotus*, is an endemic member of the wrasse family (Labridae). The Labridae family is the second largest marine fish family, and is one of the most diverse families in terms of morphology and ecology in the world, containing over 500 species in 70 genera (Cowman et al., 2009; Westneat & Alfaro, 2005). *Notolabrus celidotus* is distributed throughout New Zealand, including Stewart Island, but it has not been recorded in New Zealand sub-Antarctic Islands or the Chatham Islands. It is a sister species to the New Zealand banded wrasse (*Notolabrus fucicola*), another common reef species. Spotties can grow up to 25 cm in length, and are the most common demersal fish on coastal rocky reefs (Willis & Anderson, 2003). Spotties are not commercially fished in New Zealand, due to the small size and the difficulty of removing the flesh from the bones (Scott, 2010), but are often caught by children fishing from wharves (personal observations).

Notolabrus celidotus is a protogynous hermaphroditic species, a reproductive feature that is common to other wrasse species. All *N. celidotus* fish begin life as females, and the sex change is usually accompanied by a change in colour (Jones, 1980). Almost all male *N. celidotus* individuals occupy the 'terminal phase' of the life cycle: some fish change sex prematurely to become males, and are able to mimic females to gain access to territories and mating opportunities (Jones, 1980). The sex change occurs around 4–5 years of age. *Notolabrus celidotus* eggs hatch after 5–10 days (Welsford et al., 2004), and the larval duration of *N. celidotus* is estimated to be as long as five months (Scott, 2010). Juvenile fish are dependent on macroalgal forests on rocky reefs as a nursery habitat, particularly *Ecklonia radiata* algae (Choat & Ayling, 1987; Jones, 1984a). Higher levels of juvenile recruitment have been observed in kelp forests than rocky reefs or sediment flats, and the fish are negatively affected by decreases in kelp density (Anderson & Millar, 2004). There is also some association with seagrass beds as juvenile

habitats (Parsons et al., 2013). *Notolabrus celidotus* utilise a wider range of habitats as they grow, and adults are most closely associated with rocky reefs (Jones, 1984b). Density-dependent growth and mortality appear to restrict the input of juveniles to adult populations when recruitment is high (Jones, 1984a).

Notolabrus celidotus feed on a wide range of small invertebrates, including copepods, amphipods and small molluscs (Jones, 1980). Certain bivalves and crabs account for a large part of the diet (Russell, 1983). Juveniles tend to feed on amphipods in kelp forests, while adult *N. celidotus* use their forward-jutting teeth to rake food from rocky reef substrates. The diet of *N. celidotus* was found to differ between shallow turf flats and deeper reef flats (Jones, 1988). The first detailed analyses of a New Zealand rocky reef species, in terms of its population dynamics and demography, were conducted on *N. celidotus*, due to its ubiquity in reef communities (Jones, 1988).

Only one study to date has investigated the population genetics of *N. celidotus*. An analysis of the mitochondrial genome control region found no differentiation between populations around New Zealand (Scott, 2010), and the results suggested that *N. celidotus* populations were expanding. The suggested explanation for the expanding populations of *N. celidotus* was the long pelagic larval duration allowing for high levels of gene flow.

The aims of the thesis research

The general aim of this thesis study is to increase understanding of the population genetics of *Notolabrus celidotus*. The first section of this study, Chapter Two, describes the development of novel microsatellite markers for *N. celidotus*. Next-generation sequencing methods were used to identify 17 potential microsatellite loci, which were tested for polymorphisms and amplification success. This resulted in the successful development of six microsatellite loci.

Chapter Three expands the previous genetic work of Scott (2010) in analysing the genetic population structure of *N. celidotus*. Individuals were added to previously

reported sequence data for more thorough analyses of mitochondrial DNA, including individuals from regions that had not been previously sampled. This chapter also details the genetic analyses of the microsatellite loci developed in Chapter Two. This chapter aims to determine the levels of genetic diversity, as well as investigate the population structure and demographic history of *N. celidotus* populations based on various analyses of mitochondrial DNA and microsatellite loci. Chapter Four discusses the results and applications of this study.

Chapter Two

Development of novel microsatellite markers for *N. celidotus*

2.1 Introduction

Since their discovery around 30 years ago, microsatellite markers have become a popular genetic marker for ecological and evolutionary studies (Guichoux et al., 2011). They have become popular for a number of reasons: firstly, they are highly polymorphic. Secondly, microsatellites are abundant and widely distributed throughout a genome, which means they give a general impression of the levels of variation across the genome, and are not biased towards particular regions of the genome (Avisé, 2004). However, there are often high costs associated with developing microsatellite markers, as they tend to be species-specific. There can also be some technical difficulties associated with designing and optimising primers (Miah et al., 2013). Recent work has focussed on markers that can be used across species and genera, in order to reduce the cost and time of developing new microsatellite markers (Barbara et al., 2007).

Many applications of microsatellite DNA genotyping have been reviewed in the literature (Chistiakov et al., 2006; Dudgeon et al., 2012). Particularly in regard to fish species, microsatellite DNA has been able to offer insights into population connectivity, relatedness indices of natural populations and population dynamics. These markers have been used in identifying genetic structure to inform fisheries management (Ensing et al., 2013), as well as aiding in discriminating cryptic species (Harrison et al., 2014). The polymorphic and codominant features of microsatellite markers offer information about patterns of reproduction and recruitment in species where this cannot often be studied directly. These include parentage analysis, identifying natal populations, and measuring levels of larval transport (Berry et al., 2012).

Next-generation sequencing is a technique that offers rapid and cost-effective processing of genomic DNA, compared to older microsatellite identification techniques. Previous methods involved highly labour-intensive cloning methods that could only target a few types of microsatellite marker: next-generation sequencing can detect all types of microsatellite, and is usually conducted by an external sequencing service (Kang et al., 2013). This technique consists of digesting a whole genome into small fragments or reads, which are then sequenced (Ellis et al., 2011). A previously popular sequencing method was Roche 454 Sequencing: this method tends to produce larger average size fragments than other methods, which increases the likelihood of a fragment containing a repeating section having adequate space on the flanking regions of the repeat to allow for primer design (Abdelkrim et al., 2009). However, this method is now obsolete, and other methods such as Illumina Sequencing have become popular due to the low costs and high sequence yields that can be obtained (Zavodna et al., 2014).

The aim of the research presented in this chapter was to develop novel microsatellite markers for *Notolabrus celidotus*, for use in genotyping analysis.

2.2 Materials and methods

This chapter describes the process of developing microsatellite loci for *Notolabrus celidotus*. Selection of suitable loci and optimisation of laboratory protocols were completed by Angela Fleming and Jack Du. Additional analyses of the original dataset were completed by the author.

DNA was extracted from one *N. celidotus* individual using a standard phenol-chloroform protocol (Sambrook et al., 1989). The purified DNA was sent to Otago University's High Throughput DNA Sequencing Unit, and run on the Roche 454 Genome Sequencer FLX system. The resulting library consisted of over 160,000 reads. These reads were analysed in Geneious 8.0.5 (Kearse et al., 2012) to determine appropriately sized reads containing repeating sections. The Geneious plug-in Phobos (http://www.rub.de/spezzoo/cm/cm_phobos.html) was used to identify the number and type of repeat sequences. Sequence reads containing repeating sections were detected with QDD (Meglecz et al., 2010), and primers were designed for the selected reads by Primer3 (Koressaar & Remm, 2007; Untergasser et al., 2012). Primer3 returned the data in an Excel file, consisting of around 22,000 repeats with primer pairs. The number of repeat motifs in each sequence read was calculated in Tandem Repeats Finder (Benson, 1999).

Potential microsatellite candidate loci were ranked based on a number of factors. These included the length and motif type of the repeating sequence, as well as features of the primers: the matching of forward and reverse primer melting temperatures and GC content. The position of the repeat sequence in the genome was also considered, to allow for pool-plexing design. Imperfectly repeating sequences (such as (AT)₇GC(AT)₁₀) can lead to some inconsistencies in identifying alleles, since multiple sequences can correspond to a single length fragment (Guichoux et al., 2011). Perfect repeats (eg. (AT)₂₀) were favoured for this reason, and perfect repeats mean the microsatellite locus follows the stepwise mutational model, which is useful in further analysis of genetic structure and relatedness. Smaller motif types (such as dinucleotide and trinucleotide repeats) are often preferred when designing multiple microsatellite loci for one species: it allows for

greater efficiency when designing multiplexing or poolplexing combinations (Guichoux et al., 2011). However, small motifs, particularly dinucleotide repeats, are prone to stutter bands, which are often attributed to DNA polymerase slippage during amplification. Stutter bands make allele peaks difficult to discern. Larger motif types, such as pentanucleotide and hexanucleotide repeats, allow for clearer interpretation of allele sizes, due to the gaps between possible allele sizes being greater (Chistiakov et al., 2006).

Seventeen potential microsatellite loci were selected for further analysis based on these factors, and the computed primer sequences were synthesised. The locus primer pairs were tested by PCR amplification of 20 individuals from six locations. Firstly, loci were amplified separately for PCR optimisation, then a M13 fluorescently-labelled primer labelled with VIC, FAM or PET dyes was added, and the samples sent for genotyping at Massey University Genome Service. This step was carried out to analyse loci separately for the range of allele sizes present, and to identify polymorphic loci.

PCRs consisted of 67 mM Tris-HCl pH 8.8, 16 mM $(\text{NH}_4)_2\text{SO}_4$, 2.0 mM MgCl_2 , 0.2 mM dNTPs, 0.1 mM forward primer, 0.3 mM reverse primer, 0.3 mM fluorescently-labelled M13 primer, 0.4 $\mu\text{g}/\mu\text{L}$ Bovine Serum Albumin (BSA), one unit of BIOTAQ™ DNA Polymerase (Bioline) and 1 μL of template DNA in 15 μL volumes. Thermocycling conditions were 95°C for 10 minutes, 30 cycles of 94°C for 30 seconds, 54°C for 30 seconds and 74°C for 30 seconds, followed by an additional 8 cycles of 94°C for 30 seconds, 53°C for 30 seconds and 74°C for 30 seconds. The final eight cycles allowed for the binding of the fluorescently labelled M13 primer, when it was used. This was followed by a final extension time of 10 minutes at 72°C. The amplified products were confirmed after electrophoresis in 3% agarose gel, staining in ethidium bromide and viewing under a UV light source.

Purified amplified products were then sent to Massey University Genome Service, and the allele sizes identified using an ABI3730 Genetic Analyzer. The GeneScan™ 500 LIZ™ Size Standard was used for fragment sizing, and allele peaks were viewed and analysed in Genemarker (<http://www.softgenetics.com>). Allele sizes

were exported into an Excel worksheet, where the alleles were placed manually into 'bins' set for each allele. All loci were checked for the presence of null alleles, large allele dropout and scoring errors in Microchecker 2.2.3 (van Oosterhout et al., 2004). Deviation from Hardy-Weinberg equilibrium and linkage disequilibrium were measured in Arlequin 3.5 (Excoffier & Lischer, 2010). After analysing the loci, pool-plexing combinations were identified by Multiplex Manager 1.2 (Holleley & Geerts, 2009).

2.3 Results

The sequence library generated from 454 sequencing contained 162,855 reads, ranging from 52 base pairs to 1200 base pairs in length. The reads had a mean length of 513.2 base pairs, with a standard deviation of 97.2 (Figure 2.3.1). Nucleotide frequencies for all reads were $\pi_A = 0.290$, $\pi_C = 0.206$, $\pi_G = 0.217$, $\pi_T = 0.285$. Approximately 150,000 reads were over 400 base pairs in length, which is a suggested minimum read length to allow for primer design on either side of the repeat sequence. Searching the sequence reads in the Phobos 3.3.12 revealed 67,465 dinucleotide repeats, 80,269 trinucleotide repeats, 47,335 tetranucleotide repeats and 36,699 pentanucleotide repeats. Approximately 22,000 repeats were selected as potential candidates for microsatellite loci by QDD (Meglecz et al., 2010) and the corresponding primer pairs were identified by Primer3 (Untergasser et al., 2012). 44% of these sequence reads contained one repeat motifs, 15% contained two separate repeat motifs and 9.6% contained three separate motifs. The frequency plot is shown in Figure 2.3.2. The largest number of repeats detected in one read was 34.

Seventeen potential loci were selected for primer synthesis. These loci consisted of six dinucleotide repeats, eight trinucleotide repeats and three tetranucleotide repeats, and were labelled Spot01 to Spot17. Eleven loci failed to amplify or were found to not be polymorphic. The six polymorphic loci remaining that amplified correctly consisted of one dinucleotide repeat, three trinucleotide repeats and two tetranucleotide repeats. Optimisation of PCR thermocycling conditions identified the following annealing temperatures: 60°C for Spot07, Spot11, Spot13, Spot 14 and Spot17, and 54°C for Spot05. The six selected loci did not show any signs of null alleles or deviation from Hardy-Weinberg equilibrium.

Figure 2.3.1 Sequence lengths for all 454 sequencing reads

Figure 2.3.2 Frequency plot showing the number of repeat motifs within a sequence read, of the potential microsatellite loci that were evaluated.

Table 2.3.1 *N. celidotus* microsatellite loci showing primer information and annealing temperatures.

Locus	Repeat unit	Size (bp)	5' Forward Primer 3'	5' Reverse Primer 3'	Annealing temperature (°C)	M13 primer
Spot05	AC	174-236	AGA CAT CAA GAG GCG AAG GA	TGT GAG GGC GTG AGT GTA AG	54	FAM
Spot07	CAA	110-161	GAT GAA CAT GCG GGG TAA CT	CCA CAC CTG CTC ATC CAC TA	60	PET
Spot11	AGAT	128-245	AAG GAG AAG AGG TGG AGA GGT T	CCG TCA TCA ATG GGA CTC TT	60	VIC
Spot13	ATGG	106-142	CCA ACC CTG GAT AAG CAG AA	AAT CTG CAC AGG ATT TTG CC	60	VIC
Spot14	TCT	119-150	GAG AAC GCT TAC AGC CAA CC	AAA GAA TGA GCA GTC ACA ACC A	60	PET
Spot17	ATA	127-200	TCC ACA AGG GAA ATC TAC TAC TTA CA	TGA TGC AAA GGA GGA CTT ATA GC	60	FAM

Table 2.3.2 Comparisons of microsatellite loci developed using next-generation sequencing methods for *Notolabrus celidotus* and other species.

Species	Family	Total number of sequence reads	Number of loci developed	Number of alleles per locus	Reference
<i>Notolabrus celidotus</i>	Labridae	162,855	6	6 to 15	-
<i>Megalabrama pellegrini</i>	Cyprinidae	257,497	26	2 to 5	Wang et al., 2012
<i>Raja pulchra</i>	Rajidae	453,549	14	2 to 10	Kang et al., 2012
<i>Schizothorax biddulphi</i>	Cyprinidae	3,476,226	29	2 to 6	Luo et al., 2012

The results of this study reflect the microsatellite development of many other fish species (Table 2.3.2). The loci developed for *Notolabrus celidotus* in this study were found to be more polymorphic than other loci that have been developed using next-generation sequencing methods. The cyprinid species *Megalobrama pellegrini* and *Schizothorax biddulphi* had 26 and 29 loci developed respectively (Luo et al., 2012; Wang et al., 2012). However, the loci developed for these cyprinids showed low levels of polymorphism in these studies. The suggested causes of the low genetic diversity seen in *M. pellegrini* are the changes that in the Yangtze River (the species' native habitat), that arose from the construction of dams and from over-fishing (Wang et al., 2012). Fewer microsatellite loci were developed for *Raja pulchra*, but they were more polymorphic than the cyprinid species (Kang et al., 2012).

2.4 Discussion

This study is the first to develop microsatellite loci for *Notolabrus celidotus*, with six microsatellite loci being successfully identified and developed. Over 162,000 Roche 454 sequencing reads revealed around 22,000 potential microsatellite loci, and 17 loci were selected for primer synthesis. The final selection of microsatellite loci showed adequate levels of polymorphism and no evidence of null alleles or deviation from Hardy Weinberg equilibrium.

In this study, loci were pool-plexed, which involved amplifying each locus separately then combining certain loci for analysis. A different method, known as multiplexing, where multiple loci are amplified in the same PCR reaction, is becoming increasingly popular (Guichoux et al., 2011). Developing suitable multiplexing combinations can be time-consuming and costly, but programs are available that can optimise primer design to allow for PCR condition matching, ultimately saving resources once they are developed (Jennings et al., 2011). Future work on *N. celidotus* could focus on developing multiplexes for genotyping: this may already be possible due to the relative consistency of annealing temperatures identified (Table 2.3.1).

In recent times, efforts have been made to develop microsatellite loci that are transferable between species (Kalia et al., 2011). The transferability of microsatellite markers can depend on features of the organism being studied: a review of transferable markers found that animal microsatellites generally have greater transferability than plant microsatellites (Barbara et al., 2007). Transferability of microsatellites is also more likely among closely related species (Kang et al., 2013). Success in developing transferable markers has been observed in some families of fish: Sparidae (Reid et al., 2012) and Cyprinidae (Gigliarelli et al., 2012). If the microsatellite markers developed in this study are found to be transferable, it is possible that genetic studies of closely related species, such as *Notolabrus fucicola* (New Zealand banded wrasse), could be undertaken using these loci, avoiding the costs and time required to develop novel microsatellites. There already appears to be evidence of marker transferability among different

genera of wrasses (Gardner et al., 2011). This suggests that the loci developed in this study may have the potential to be used in other wrasses.

Next generation sequencing techniques have enabled the rapid and cost-effective development of microsatellite markers in a range of organisms (Treangen & Salzberg, 2012). In fish, microsatellites are developed for a range of reasons: utilising microsatellite markers enables fine-scale population differentiation to be identified, and the resulting population genetic analyses can be applied to fisheries, conservation management and invasive species (Abdul-Muneer, 2014). The results of this study were compared with other fish species where microsatellites had been successfully developed using next-generation sequencing methods and found that although fewer loci were developed, they were found to contain higher levels of polymorphisms in initial analyses (Table 2.3.2). Loci that are highly polymorphic have the power to detect finer-scale population structuring than other markers (Guichoux et al., 2011). The levels of polymorphism found in this initial genotyping study suggest that the microsatellite loci that have been developed for *Notolabrus celidotus* will be able to detect any fine-scale population structure that is present in this species, when the markers are used in subsequent genetic population analyses.

Chapter Three

Analysis of population structure and demographic history using mitochondrial DNA and microsatellite DNA markers

3.1 Introduction

Levels of genetic connectivity between populations are influenced by a number of factors. In the marine environment, the pelagic larval duration (PLD) is a commonly attributed factor for explaining differences in genetic connectivity: species with a longer PLD tend to have a weaker population structure, due to the ability of the larvae or eggs to move greater distances before maturation and recruitment (Selkoe & Toonen, 2011). Non-biological factors that influence genetic connectivity relate to the physical conditions that an organism is exposed to during its dispersal phase. In marine systems, oceanic currents and upwelling patterns largely influence the dispersal of larvae, particularly those that cannot swim against currents: larval movement will be largely determined by currents and/or storm events, although in some cases larvae might only encounter suitable habitats for recruitment by chance (Huey et al., 2014). Additionally, the physical and chemical conditions encountered at sea can play a part in the connectivity of a species. If an organism can only survive in a narrow range of environmental conditions (eg. temperatures or salinity levels) it is more likely to show high levels of population structure because the risks of mortality or failed reproduction are high (Galarza et al., 2009).

Population genetic studies of fish have been used to understand how the marine environment appears to influence gene flow and levels of variation. The results for these types of studies have been used to inform fisheries and conservation management, and piece together how oceanographic processes structure the distribution of biota (Ovenden, 2013). The higher mutation rate, smaller effective population size and maternal inheritance of mitochondrial DNA (mtDNA) has made it a useful marker in studying gene flow (Okumus & Ciftci, 2003). Because

the mitochondrial genome is maternally inherited and does not undergo recombination, it can be used to study the demographic history of populations. For example, populations that were isolated thousands of years ago but have recently come back into contact, can still retain the historic patterns of isolation as distinct mtDNA lineages (Avice, 2004). Microsatellite DNA, a popular nuclear DNA marker used in population genetic studies, is bi-parentally inherited. These markers comprise short repeating sections of DNA, and are highly polymorphic, allowing for finer-scale detection of population differentiation compared to mtDNA markers (Guichoux et al., 2011). Microsatellite loci can be highly specific: often, microsatellite loci will need to be independently developed for each species, which adds considerable costs and time to the research (Morgan & Rogers, 2001).

New Zealand has a complex coastal environment that includes rocky shores, sandy shores, mudflats and mangrove forests (Ross et al., 2009). Genetic studies have been carried out on a range of New Zealand coastal marine organisms, but there has been inconsistency among the reported positions of phylogeographic breaks (summarised by Ross et al., 2009). New Zealand spotties (*Notolabrus celidotus*) are an endemic species of wrasse (Family: Labridae) and the most common demersal fish in New Zealand rocky reef ecosystems. They are one of the most abundant benthic carnivores, and consume a variety of small invertebrates, including copepods, amphipods and small molluscs (Jones, 1984a). The adults can grow up to 25 cm long and the juveniles are largely dependent on kelp forests for nursery grounds (Perez-Matus & Shima, 2010). The widespread distribution and common occurrence of this coastal, shallow water species make it an ideal model fish species for studying the population genetic structure of New Zealand coastal communities.

The first population genetic study of *N. celidotus* used mitochondrial DNA (mtDNA) control region sequencing and reported a lack of population differentiation between sampled locations around New Zealand (Scott, 2010). However, the scope of the analyses and genetic markers in that study was somewhat limited. The aim of the research presented in this chapter was to expand the sampling of the previous data set, conduct a more comprehensive set of analyses, and include

nuclear microsatellite DNA markers. The specific aims of the research presented in this chapter were to:

- 1) Increase coverage of New Zealand *N. celidotus* populations by adding the mtDNA control region sequences from additional individuals and locations, and conducting a more comprehensive phylogeographic analysis.
- 2) Analyse genotyping data from nuclear microsatellite DNA loci to determine the levels of variation and gene flow among *N. celidotus* sample locations.

The sampling and laboratory methods reported in this chapter were carried out with the assistance of other researchers.

3.2 Materials and methods

3.2.1 Sample collection

A total of 103 new individual *N. celidotus* samples were collected by Jack Du and Hayden Smith from nine sample sites across six biogeographic provinces in New Zealand, including the previously unsampled Banks province (Figure 3.2.1). Sample collection site information is shown in Table 3.2.1.

Figure 3.2.1 Map of New Zealand showing province boundaries (as proposed by Shears et al., 2008) and the number of samples collected from each province. Black dots represent sample collection sites.

Table 3.2.1 Sample collection sites within provinces

Province	Code	Location	Collection site	Latitude	Longitude
Abel	GOB	Golden Bay	Pohara	-40.824367	172.894765
Abel	MAP	Mapua	Mapua Wharf	-41.256247	173.102049
Abel	PAU	Pauatahahui	Mana Marina	-41.103006	174.868948
Abel	PAU	Pauatahahui	Pauahatani Harbour	-41.092942	174.892074
Abel	PIC	Picton	Picton wharf	-41.288068	174.007028
Banks	AKA	Akaroa	Akaroa Harbour	-43.808814	172.959054
Cook	KAIK	Kaikoura	South Bay	-42.426045	173.681584
Cook	WLG	Wellington	Karakia Bay	-41.304194	174.831712
Cook	WLG	Wellington	Seaview Pier	-41.252952	174.901411
Cook	WLG	Wellington	Evans Bay	-41.311188	174.799844
Northeastern	GBI	Great Barrier Island	Eastern coast	-36.240717	175.487249
Northeastern	GBI	Great Barrier Island	Western coast	-36.261738	175.436187
Northeastern	KAW	Kawau Island	Moorea Bay	-36.425873	174.849628
Northeastern	LEI	Leigh	Omaha Cove	-36.288551	174.811073
Northeastern	LNG	Long Bay	Long Bay Beach	-36.679538	174.750852
Northeastern	MOK	Mokohinau Islands	Fanal Island	-35.939331	175.150152
Northeastern	MOK	Mokohinau Islands	Lizard Isle	-35.90897	175.114168
Northeastern	RAN	Rangunu	Rangunu Bay	-34.853921	173.244501
Northeastern	TIRI	Tiritiri Matangi Island	Tiritiri Matangi jetty	-36.605963	174.888308
Northeastern	TOR	Torbay	Winstones Cove	-36.694633	174.761252
Northeastern	WHA	Whangarei	Whangarei Harbour	-35.775907	174.380902
Portland	NAP	Napier	Ahuriri Wharf	-39.477299	176.908526
Raglan	KAIP	Kaipara	Kaipara Harbour	-36.286125	174.225213
Raglan	KAWH	Kawhia	Kawhia Harbour	-38.08454	174.858709
Raglan	NPM	New Plymouth	Port Taranaki	-39.045195	174.063983
Stewart Island	HMB	Halfmoon Bay, Stewart Island	Oban jetty	-46.897152	168.131051
Stewart Island	HSB	Horseshoe Bay, Stewart Island	Horseshoe Bay pier	-46.87319	168.131687
Stewart Island	PWI	Port William, Stewart Island	Port William jetty	-46.836792	168.085655

3.2.2 Mitochondrial DNA sequencing

With the assistance of Jack Du, genomic DNA was extracted using a standard phenol-chloroform method (Sambrook et al., 1989). Species-specific mitochondrial DNA primers were used (Scott 2010). The forward primer (5'-AAT TAA GCT ACG CGA GCA GTT G-3') and reverse primer (5'-TYT AAC TCC CAC CCC TAA CTC C-3') were used to amplify a 790-base pair section of the mitochondrial control region between tRNA-Phe and tRNA-Pro. Polymerase chain reactions (PCR) consisted of 67 mM Tris-HCl pH 8.8, 16 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 800 µM dNTPs, 0.4 µM each forward and reverse primers, 0.6 µg/µL Bovine Serum Albumin (BSA), 0.6 units of BIOTAQ™ DNA Polymerase (Bioline) and 1 µL template DNA in 15 µL volumes.

Thermocycling conditions consisted of an initial denaturation period of 94°C for 30 seconds, followed by 35 cycles of 94°C for 30 seconds, 52°C for 30 seconds and 74°C for one minute. This was followed by a final extension time of 10 minutes at 74°C. Amplification was confirmed by electrophoresis of samples through a 1% agarose gel, staining with ethidium bromide and viewing under a UV light source. PCR products were purified with ExoSAP-it and sequenced using an ABI 3730 Genetic Analyzer (Massey University Genome Services).

3.2.3 Microsatellite DNA genotyping

With the assistance of Angela Fleming, DNA was extracted from samples using the same standard phenol-chloroform method described in Section 3.2.2. The PCR primers used for each locus are described in Table 2.3.1. All loci were amplified separately, in PCR reactions consisting of 67 mM Tris-HCl pH 8.8, 16 mM (NH₄)₂SO₄, 2.0 mM MgCl₂, 0.2 mM dNTPs, 0.1 mM forward primer, 0.3 mM reverse primer, 0.3 mM fluorescently-labelled M13 primer, 0.4 µg/µL Bovine Serum Albumin (BSA), one unit of BIOTAQ™ DNA Polymerase (Bioline) and 1 µL of template DNA in 15 µL volumes.

Thermocycling conditions were 95°C for 10 minutes, 27 cycles of 94°C for 30 seconds, annealing temperature (Table 2.3.1) for 30 seconds and 74°C for 30 seconds, followed by an additional 8 cycles of 94°C for 30 seconds, 53°C for 30 seconds and 74°C for 30 seconds. The final eight cycles allowed for the binding of the fluorescently labelled M13 primer. This was followed by a final extension at 72°C for 10 minutes. The amplified products were viewed after electrophoresis in 3% agarose gel, staining in ethidium bromide and viewing under a UV light source.

The PCR products were pool-plexed into two combinations of loci, and the pooled amplicons were genotyped using an ABI3730 Genetic Analyzer (Massey University Genome Service). The GeneScan™ 500 LIZ™ Size Standard was used for fragment sizing, and allele peaks were viewed and analysed in Genemarker (<http://www.softgenetics.com/GeneMarker.html>). Allele sizes were exported into an Excel worksheet, where the alleles were placed manually into 'bins' set for each allele.

3.2.4 Mitochondrial DNA sequence analysis

Genetic diversity

Mitochondrial DNA control region sequences were aligned using the ClustalW algorithm implemented in MEGA6 (Tamura et al., 2013). Summary statistics for the levels of variation at each sample location were determined using DnaSP 5.10.1 (Librado & Rozas., 2009): the number of polymorphic sites, the number of total and private haplotypes, the haplotypic diversity and average nucleotide diversity, and the average number of nucleotide differences among samples from each sample site. Haplotype rarefaction curves were calculated using Analytic Rarefaction 1.3 (<http://strata.uga.edu/software>).

Population structure

The level of genetic differentiation between provinces was determined using pairwise calculations of G_{ST} (Nei, 1973). This estimate of differentiation determines the difference between sample locations based on the presence and absence (and the frequency) of haplotypes, rather than nucleotide-level differences between haplotypes. The analysis was conducted in Arlequin 3.5 (Excoffier & Lischer, 2010) and levels of significance were assessed using 110 permutations. The values of G_{ST} were also displayed in a multi-dimensional scaling plot, implemented in the Excel plug-in XLSTAT (<http://www.xlstat.com/en/download.html>).

Genetic diversity was measured among and within populations with Analyses of Molecular Variance (AMOVA) implemented in Arlequin 3.5. All population groupings were tested with 10,000 permutations. Three population groupings were tested: 1) Two regions: North Island and South/Stewart Island samples, 2) Two regions: Northern and Southern sample sites divided at the 42° latitude line, 3) Two regions: Eastern and Western sample sites. These population subdivisions were based on putative phylogeographic breaks in New Zealand's coastal environment (Ross et al., 2009).

An isolation-by-distance (IBD) analysis was undertaken by comparing the pairwise G_{ST} with the shortest marine distance (measured in km) between these sample sites. Previous analyses had only compared provinces; the analysis conducted in this study compared individual sample sites within those provinces. This approach was chosen because it was important to have a precise geographic location for the comparisons, instead of analysing the averaged distances between provinces. Samples from Stewart Island and the Auckland regions were each analysed as single sample sites, because of the close proximity of the sample locations within each area. Some sample sites were excluded from the IBD analysis because of the low number of samples available: locations with fewer than 10 samples (including New Plymouth, Pauahatani and Kawhia) were not included in the IBD analysis to minimise the potential for bias, and because these sample sites were not in close proximity to any other sample locations. The pairwise comparisons were based on

the G_{ST} estimates of genetic difference and the shortest marine geographic distances (measured in km) estimated using Google Earth 7.1. The significance of the correlation between genetic and geographic distances was determined using a Mantel test with 10,000 replicates that was implemented on the Isolation by Distance Web Service (Mantel, 1967; Jensen et al., 2005).

A Minimum Spanning Network was constructed using PopART (<http://popart.otago.ac.nz>). Haplotype distribution was also displayed in a haplotype frequency distribution plot, showing the proportion of private haplotypes in each geographic province, as well as the proportion of haplotypes that are shared with other provinces.

Demographic history

Tests for a mutation-drift balance were conducted, which can be used to determine whether population size has been stable or there has been a significant bottleneck or expansion event. Tajima's D , Fu's F_S statistics and a mismatch distribution were calculated using DnaSP 5.10.1.

Effective population size was calculated using the formula $\theta = 2N_e\mu$, where θ is the level of diversity present, and μ is the mutation rate per site per generation. From a conservatively estimated 5% change per million years in the wrasse family (Ludt et al., 2012) and a generation time of two years for *N. celidotus* (Scott, 2010), μ was estimated at 1.2×10^{-5} . The θ values used in the calculations were calculated in DnaSP, and were based on the nucleotide diversity, number of segregating sites and total number of mutations.

BEAST v1.8 was used to generate Bayesian skyline plots that showed a time-stamped population history (Drummond et al., 2012). The chain length was 50 million iterations with a burn-in time of five million iterations. The mutational model that best fits that data set was determined using jModelTest (Darriba et al., 2012; Guindon & Gascuel, 2003). Based on previous studies of Ludt *et al* (2012), the substitution rate was conservatively set to 5% per million years. The resulting

skyline plot and the post-posterior Bayesian information were viewed using Tracer (<http://beast.bio.ed.ac.uk/Tracer>).

3.2.5 Microsatellite DNA genotype analysis

Genetic diversity

The presence of null alleles and scoring errors was assessed using Microchecker 2.2.3 (van Oosterhout et al., 2004). Deviations from Hardy-Weinberg Equilibrium in all populations and loci were tested in GenoDive 2.0b27 (Meirmans & Van Tienderen, 2004) with 10^4 permutations. Pairwise tests of linkage disequilibrium were carried out in Arlequin 3.5 (Excoffier & Lischer, 2010).

Estimates of the level of genetic diversity, including allelic richness, observed and expected levels of heterozygosity, numbers of private alleles and allele frequencies, were determined using GenoDive. Rarefaction curves for each locus were obtained using Analytic Rarefaction 1.3 (<http://strata.uga.edu/software>) and indicated whether a sample size was adequately large to capture the genetic variation present.

Population structure

Population differentiation was measured using several calculations: firstly Weir and Cockham's (1984) F_{ST} (θ) test; G'_{ST} : a comparison of heterozygosity within and between population, with a correction for bias that may occur from sampling a limited number of populations (Nei, 1987), and Jost's D , a measure of population differentiation that is not dependent on the amount of within-population diversity (Jost, 2008).

A factorial components analysis (FCA) was also calculated in GenoDive, to examine relationships based on pairwise F_{ST} . A population assignment analysis was implemented in GenoDive. This analysis determines which population an

individual is most likely to come from, given the allele frequencies of that population (Paetkau et al., 1995). Individuals can only be assigned to populations where $N > 5$, so individuals from the Northeastern, Portland, Raglan and Stewart Island provinces were excluded from this analysis.

Three analyses of molecular variance (AMOVA) were carried out in Arlequin 3.5, to measure genetic diversity within and between groups of populations. All groupings were tested with 10,000 permutations. These population groupings were: 1) Two regions: North Island and South Island/Stewart Island samples, 2) Two regions: Northern and Southern sample sites divided at the 42° latitude line, 3) Two regions: Eastern and Western sample sites. The population groupings were based on previously suggested phylogeographic breaks around the New Zealand coastline (Ross et al., 2009).

Bayesian Structure analysis was carried out in STRUCTURE 2.3.4 (Pritchard et al., 2000). This analysis assigns individuals to population clusters (K) based on the similarity of that individual to the allele frequencies characteristic of the cluster. The simulations were carried out 10 times for each value of K (the number of clusters), which ranged from K=1 to K=7. Each Markov Chain Monte Carlo simulation ran for 10^5 iterations, with a burn-in period of 10^4 iterations. Structure Harvester (Earl & vonHoldt, 2012) was used to identify the most probable value of K.

3.3 Results

3.3.1 Mitochondrial DNA sequencing

DNA sequences were obtained from 103 individuals from nine sample sites in six provinces: when combined with previously reported sequences the total data set comprised 293 individuals sampled from seven provinces.

Table 3.3.1 Outline of the new samples collected for this study, and how many were collected from each province.

Province	Number previously reported	Number added	Total number of samples
Northeastern	37	2	39
Raglan	7	3	10
Portland	13	2	15
Cook	19	52	71
Abel	72	15	87
Banks	0	29	29
Stewart Island	42	0	42
Total	190	103	293

The section of the mitochondrial control region sequenced was 791 base pairs in length, and had 268 variable sites. Nucleotide frequencies were: $\pi_T = 0.293$, $\pi_C = 0.174$, $\pi_A = 0.274$, $\pi_G = 0.260$. Overall nucleotide diversity was 0.01341. There were 247 haplotypes present in the sampled sequences, of which 224 were private (restricted to a single province). The average haplotypic diversity was 0.998. The haplotype rarefaction plot (Figure 3.3.1) does not show flattening of curves, which indicates that the sample sizes are not large enough to capture all the haplotypic variation present in *N. celidotus* populations.

Table 3.3.2 Summary of population information. N: number of samples; S: number of segregating sites; H: number of haplotypes; H_P: number of private haplotypes; H_D: haplotype diversity; π : nucleotide diversity.

Site	N	S	H	H _P	H _D	π
Raglan	10	46	10	9	1	0.015
Northeastern	39	73	37	32	0.996	0.0108
Portland	15	45	15	12	1	0.011
Cook	71	155	65	48	0.997	0.0175
Abel	87	114	82	73	0.998	0.0117
Banks	29	181	29	20	1	0.0245
Stewart Island	42	66	40	30	0.997	0.0095
Total	293	284	247	224	0.998	0.01341

Pairwise G_{ST} comparisons showed only one significant difference at the 5% level between all populations compared. This was the comparison between the Northeastern province and the Stewart Island province ($p = 0.036$). However, this value was not significant after completing a Bonferroni correction (Rice, 1989). No other populations showed significantly different values of population differentiation. The multi-dimensional scaling plot showed strong clustering of samples, indicating that there is little genetic difference between them (Figure 3.3.2).

An analysis of molecular variance showed that over 99% of the observed variability was contained within a population in all scenarios that were tested. This suggests a lack of genetic differentiation between populations, contrary to suggested positions of phylogeographic breaks that have been observed in other species (Ross et al., 2009).

Figure 3.3.1 Haplotype rarefaction plot for *N. celidotus* individuals from various provinces.

Figure 3.3.2 Multidimensional scaling plot of pairwise G_{ST} values. Each dot represents a sample province.

Table 3.3.3 Measures of population differentiation using G_{ST} . G_{ST} values for each pairwise comparison are located above the diagonal, and corresponding p-values are below the diagonal.

	Abel	Banks	Cook	Northeastern	Portland	Raglan	Stewart Island
Abel	-	-0.00051	0.00042	0.0015	-0.00006	0.00073	0.001
Banks	0.685	-	-0.00218	0.00027	-0.0023	0	0.00035
Cook	0.279	0.919	-	0.0007	-0.00061	0.00131	-0.00065
Northeastern	0.081	0.477	0.342	-	0.0004	0.00218	0.00258
Portland	0.532	0.991	0.631	0.541	-	0	-0.00357
Raglan	0.342	0.991	0.243	0.568	0.991	-	-0.00113
Stewart Island	0.135	0.505	0.703	0.036	0.909	0.667	-

Table 3.3.4 AMOVA results from 1) North Island compared to South Island/Stewart Island samples; 2) Samples north of 42° latitude compared to samples south of 42° latitude; 3) Samples taken from the Eastern coasts compared to the Western coasts of New Zealand. D.f.: degrees of freedom; Var: variance component; %Var: variance component as a percentage of the total variation.

Regions	Among groups					Among populations within groups					Within populations				
	df	Var	%Var	ΦCT	p-value	df	Var	%Var	ΦCT	p-value	df	Var	%Var	ΦCT	p-value
NI/SI	1	-0.00014	-0.03	-0.00028	0.65554	21	0.00044	0.09	0.00087	0.1797	270	0.49866	99.94	0.00059	0.19525
N42/S42	1	0.00032	0.06	0.00065	0.21594	21	0.0002	0.04	0.00041	0.32535	270	0.49866	99.89	0.00105	0.21109
E/W	1	0.00026	0.05	0.00053	0.22604	21	0.00024	0.05	0.00048	0.28515	270	0.49866	99.9	0.00101	0.20475

The isolation-by-distance analysis, presented in Figure 3.3.3, revealed no significant correlation between pairwise genetic differences and the shortest marine distance between the sample locations ($p = 0.7495$). The Minimum Spanning Network calculated from the DNA sequences is complex, and shows the high number of haplotypes observed, and the low numbers of shared haplotypes (Figure 3.3.4). The haplotype distribution plot (Figure 3.3.5) shows high numbers of private haplotypes in each province; with a small number of haplotypes being shared among 2–4 other provinces.

Figure 3.3.3 Isolation-by-distance graph comparing nearest geographic distance (kilometres) with genetic distance ($G_{ST} / 1 - G_{ST}$)

Figure 3.3.4 Minimum spanning network of all *N. celidotus* haplotypes. The size of the circle indicates the number of individuals that share the haplotype, and the colour of the circle represents the province the sample originated from.

Figure 3.3.5 Private and shared haplotypes from each province.

The results of Tajima's D and Fu's F_S tests show significantly negative values when all provinces are analysed together. However, when each province is analysed separately, the Raglan province does not show a significantly negative value (Table 3.3.5). Negative values of these tests can indicate recent population expansion, whereas statistics equalling 0 can suggest a lack of recent population expansion or decline (Tajima, 1989).

Table 3.3.5 Results of Tajima's D and Fu's F_S tests. Values in bold are significant at the 5% level.

	Tajima's D	p-value	Fu's F_S test	p-value
Abel	-1.98628	0.003	-24.60782	<0.001
Banks	-2.25259	0.002	-14.85227	<0.001
Cook	-1.99484	0.008	-24.27088	<0.001
Northeastern	-1.85606	0.011	-24.83507	<0.001
Portland	-1.57986	0.049	-8.25995	0.001
Raglan	-1.3375	0.107	-3.02344	0.051
Stewart Island	-1.80344	0.014	-24.98553	<0.001
All populations	-1.83008	0.02771	-17.83356	0.00743

The mismatch distribution analysis compared the expected and observed frequencies of pairwise differences (Figure 3.3.6). The raggedness statistic for the observed frequency plot is $r = 0.0045$. A raggedness statistic of less than 0.03 is considered to be a smooth curve (Jobling et al., 2004). Smooth peaks in the observed frequency plot, as well as a unimodal distribution, can indicate a recent expansion in population size (Lopes et al., 2007). The Bayesian skyline plot showed a long period of stable population size followed by a slight decline, then a sharp population increase 150,000 years ago that appears to have been consistent since then (Figure 3.3.7).

Estimations of the effective population size of each province ranged from 800 to 3000 individuals, with a total size of around 8500 individuals. These calculated estimates appear to be too low when compared to the Bayesian skyline plot. It is possible that this is due to the presence of overlapping generations in *N. celidotus*: where more than one breeding generation is present in a mating system at a time.

Figure 3.3.6 Mismatch distribution showing expected and observed frequencies of various pairwise differences.

Figure 3.3.7 Bayesian skyline plot showing estimated population size and time (in years) since the present. Blue lines represent 95% confidence intervals.

3.3.2 Microsatellite DNA genotyping

Six microsatellite loci were used to genotype 101 individuals from seven New Zealand provinces. Tests for homozygote excess showed some evidence for null alleles at the Spot13 locus, but no evidence was found for large allele dropout or scoring errors at the 95% confidence interval at any loci. The summary statistics for each province are presented in Table 3.3.6. All loci were found to be highly polymorphic, with numbers of alleles ranging from 17 (Spot05 and Spot13) to 23 (Spot07). Expected heterozygosity ranged from 0.871 to 0.95, and the observed heterozygosity ranged from 0.567 to 0.917. The allele rarefaction plot for the most polymorphic locus, Spot07, is shown in Figure 3.3.8. The additional rarefaction plots are displayed in Appendix 1. Allele frequency plots are shown in Figure 3.3.9.

Individuals from the Raglan, Portland and Stewart Island provinces appear to deviate from Hardy-Weinberg equilibrium, however this is likely due to the low numbers of individuals sampled from these provinces. There is some evidence that the Spot11 locus also has some deviation from equilibrium, suggesting that this locus may not be neutral. There was no evidence of linkage disequilibrium observed between pairs of loci.

Table 3.3.6 Summary statistics of the microsatellite data collected. N: number of samples; H_0 : observed heterozygosity; H_E : expected heterozygosity; A_{avg} : average number of alleles; A_p : number of private alleles.

Province	N	H_0	H_E	A_{avg}	A_p
Abel	36	0.873	0.904	11.33	10
Banks	25	0.769	0.892	12.67	15
Cook	31	0.857	0.871	12.83	11
Northeastern	3	0.567	0.95	3.2	0
Portland	3	0.917	0.938	4	1
Raglan	2	0.625	0.875	3	0
Stewart Island	1	---	---	---	0
Total/Average	101	0.758	0.891	19.5	27

The allele rarefaction curves do not appear to reach a plateau for any of the microsatellite loci studied, which suggests that the sample sizes used in this study were too small to capture all the variation present in the population.

Figure 3.3.8 Allele rarefaction curve for Spot07

Pairwise F_{ST} comparisons between provinces showed no significant differences between the provinces studied (Table 3.3.7). Jost's D and Nei's D values reflected this result, showing no significant differences between provinces (Table 3.3.8). The population assignment analysis showed that individuals from the Cook province were most commonly assigned correctly (61% of individuals). 42% of individuals from the Abel province, and 36% of individuals from the Banks province were correctly assigned (Table 3.3.9).

Figure 3.3.9 Allele frequency distribution of six microsatellite loci, combined over all sample locations.

The analyses of molecular variance (AMOVA) showed that the majority of variation could be attributed to variability within populations. This was the case in all the putative phylogeographic break scenarios that were tested, suggesting that none of the tested scenarios adequately explain the variability patterns that were seen (Table 3.3.10). Results of the Factorial Component Analysis (FCA) showed a lack of

population clustering (Figure 3.3.11). The first five axes cumulatively explained 26% of the variation present.

The Bayesian structure analysis showed the most likely number of groups was four (Figure 3.3.11). However, it was suspected that the small sample sizes of the Northeastern, Portland, Raglan and Stewart Island provinces could be biasing the data, so in a subsequent analysis these individuals were removed from the analysis. The second analysis showed the highest Delta K value (and therefore most likely number of groups) was $k=2$ (Figure 3.3.14).

Table 3.3.7 Pairwise population comparisons using F_{ST} . Values of F_{ST} are above the diagonal, and corresponding p-values are below the diagonal.

	Abel	Banks	Cook	Northeastern	Portland	Raglan	Stewart Island
Abel		-0.002	0.004	0	-0.008	-0.004	0
Banks	0.578		-0.001	0.001	-0.006	0.019	0
Cook	0.221	0.57		0.026	0.014	0.054	0
Northeastern	0.435	0.45	0.131		0.001	-0.085	0
Portland	0.558	0.526	0.256	0.488		0.014	0
Raglan	0.518	0.273	0.064	1	0.618		0
Stewart Island	1	1	1	1	1	1	

Table 3.3.8 Jost's D_{est} above the diagonal, G'_{ST} below the diagonal. Asterisks indicate sample sizes too small to be analysed without bias.

	Abel	Banks	Cook	Northeastern	Portland	Raglan	Stewart Island
Abel		-0.018	0.024	-0.042	-0.059	0.099	0.274
Banks	-0.002		-0.012	-0.033	0.050	0.179	0.119
Cook	0.003	-0.002		0.114	0.132	0.394	0.369
Northeastern	-0.005	-0.004	0.016		0.094	-1.131	1.25
Portland	*	*	*	*		0	1
Raglan	*	*	*	-0.063	*		-1.333
Stewart Island	0.045	0.018	0.074	0.042	0.167	-0.036	

Table 3.3.9 Results of population assignment, in provinces where $N < 20$. Bold values indicate individuals correctly assigned to their original province.

	Inferred population		
Original population	Abel	Banks	Cook
Abel	15	9	12
Banks	5	9	11
Cook	6	6	19

Figure 3.3.10 Factorial component analysis (FCA) showing axes 1 and 2

Table 3.3.10 AMOVA results from 1) North Island compared to South Island/Stewart Island samples; 2) Samples north of 42° latitude compared to samples south of 42° latitude; 3) Samples taken from the Eastern coasts compared to the Western coasts of New Zealand. D.f: degrees of freedom; Var: variance component; %Var: variance component as a percentage of the total variation.

Regions	Among groups					Among populations within groups					Within populations				
	d.f	Var	%Var	Φ_{CT}	p-value	d.f	Var	%Var	Φ_{SC}	p-value	d.f	Var	%Var	Φ_{ST}	p-value
NI/SI	1	0.00436	1.74	0.01744	0.85327	5	-0.01344	-5.38	-0.05472	1	195	0.25897	103.63	0.03633	1
N42/S42	1	0.00591	2.35	0.02354	0.47149	5	-0.01391	-5.54	-0.05677	1	195	0.25897	103.19	-0.0319	1
E/W	1	0.00749	2.99	0.02987	0.0998	5	-0.01567	-6.25	-0.06441	1	195	0.25897	103.26	0.03262	1

Figure 3.3.11 Results for Bayesian Structure plots for a) $k=2$; b) $k=3$. Each vertical line represents an individual, and the plots are ordered by province: 1: Abel; 2: Banks; 3: Cook; 4: Northeastern; 5: Portland; 6: Raglan.

Figure 3.3.12 Results for Bayesian Structure analysis (where provinces with $N < 20$ are excluded) for a) $k=2$; b) $k=3$. Each vertical line represents an individual, and the plots are ordered by province: 1: Abel; 2: Banks; 3: Cook

Figure 3.3.13 Delta K output from Structure analysis with all provinces included. The highest value of Delta K indicates the most probable number of groupings in the dataset.

Figure 3.3.14 Delta K output from Structure analysis with only Abel, Banks and Cook provinces included.

3.4 Discussion

The results of this study showed that there are extremely high levels of diversity in *N. celidotus* populations, and a lack of any apparent genetic differentiation among the different provinces of New Zealand.

Genetic diversity

Extremely high levels of haplotypic diversity were observed in the study of the mtDNA control region, with an average haplotypic diversity of $h = 0.998$, which is close to the maximum possible value of $h = 1.000$. The majority of the haplotypes observed were private (90.6% across all provinces). The high diversity may have led to the haplotypic rarefaction plot not showing any flattening, which indicates that the sample sizes present were not sufficient to capture all of the variation present in the population.

The results of the microsatellite DNA genotyping also showed high diversity. Rarefaction plots for all loci show more signs of flattening than the mitochondrial DNA haplotype rarefaction plot, suggesting that the microsatellite DNA analysis has captured more of the variation present than the mitochondrial DNA sequence analysis. The populations with smaller sample sizes show less flattening in the rarefaction plots, since less genetic diversity can be detected when smaller samples are taken (Leberg, 2002). Additionally, no individuals were sampled from the West Coast of the South Island, or the Otago region. It is likely that obtaining additional genotypes from these regions will show additional genotypes in *N. celidotus* populations. Increasing sample sizes of individuals from all populations is necessary, if the variation in microsatellite DNA is to be fully discovered.

Studies of the control region of the mitochondrial genome tend to show high levels of diversity when compared to other parts of the genome. For example, a study that sequenced both the control region and the cytochrome oxidase I gene (C01), another gene commonly used in population structure analyses, found an average haplotypic diversity of $h = 0.984$ for the control region, and $h = 0.639$ for the C01

gene, when analysing sequences from the same individuals (Ludt et al., 2012). The control region is a non-coding region of mtDNA, whereas the CO1 gene is a protein-coding gene. It is suspected that since the control region is non-coding, it allows mutations to accumulate that may otherwise be deleterious in a coding gene, such as stop codons (Moritz, 1994). High haplotypic diversity in the control region has been observed in other wrasse species (eg. Chen et al., 2004, D'Arcy et al., 2013). Comparing the haplotype networks generated from these studies with the network constructed in this study reveals similar patterns: there are a very large number of haplotypes, and many of them are only found in one location (Appendix 2).

Population structure

The analyses of both the mitochondrial DNA sequences and the microsatellite DNA genotypes show a distinct lack of genetic population differentiation in *N. celidotus*. Analyses of molecular variance of both markers showed over 95% of the variation present in a population was due to the differences within a population, rather than differences between populations. Additionally, no significant correlation was found when mtDNA genetic differentiation was compared to the geographic distances between sample locations ($p = 0.7495$). Isolation-by-distance analyses have been suggested as being more effective methods to measure population differentiation in species with high dispersal potential (such as many marine fishes), instead of more traditional measures such as pairwise F_{ST} comparisons (Palumbi, 2003). Isolation-by-distance methods can improve the sensitivity of F_{ST} where only slight genetic differentiation is found (Palumbi, 2003). No isolation-by-distance pattern was observed in *N. celidotus*, but some parts of the New Zealand coastline still remain unsampled. Future sampling efforts to cover these gaps will provide a more comprehensive sample and give additional confidence to the findings of this study. A complete sample might enable the detection of some weak population structure, or rule out the presence of any population differentiation or isolation-by-distance patterns.

Knowing the specific details of the species' life cycle is important information to consider when interpreting patterns of genetic population structure. Correlations

have been established between the pelagic larval duration (PLD) of a particular species and the observed population structure: species that have a long PLD tend to show less population structure, since there is greater opportunity for ocean currents to disperse larvae and mix with other populations (Ross et al., 2009). *Notolabrus celidotus* is thought to have a PLD that can last up to five months (Scott, 2010), and this study revealed a lack of population structure. Conversely, another wrasse species *Larabicus quadrilineatus* showed significant differentiation between populations in the Red Sea (Froukh & Kochzius, 2007). The PLD of *L. quadrilineatus* is unknown, but the population structuring in this species was attributed to low larval dispersal capabilities and the demographic history of the species.

Demographic history

The significantly negative Tajima's D ($p = 0.02771$) and Fu's F_s ($p = 0.00743$) statistics indicate that *N. celidotus* populations have undergone a recent, large population expansion. This demographic history can also be observed in the mismatched distribution: the single smooth peak seen suggests a recently expanded population. The Bayesian skyline plot generated from the mitochondrial sequence data suggests that this population expansion began around 150,000 years ago, and has continued at the same rate up to the present day. This time estimate assumed that the mutation rate used was appropriate for *N. celidotus* and did not vary significantly through time.

Notolabrus celidotus individuals live for approximately seven years in the wild, and reach maturity at 3–4 years of age (Jones, 1984a). Since adults can spawn each season, it is likely that there are overlapping generations in *N. celidotus*: where more than one breeding generation is present at one time (Turner et al., 2002). Simulations have shown that the presence of overlapping generations in a species can promote the maintenance of genetic diversity in an environment with a fluctuating optimum, where the relative fitness of a particular phenotype changes over time (Ellner & Hairston, 1994). To model the effective population size of a species with overlapping generations, more information about reproductive

behaviour is required. The number of reproductive opportunities will influence the number of offspring each individual can produce, with those with more opportunities having the potential to produce more offspring. Ultimately, the success of each reproductive event, in terms of the proportion of offspring that successfully recruit, will determine the effective population size (Hedgecock & Pudovkin, 2011).

Smaller effective populations sizes can result when only a small proportion of reproductive attempts are successful. The effective size and the census size of various populations can be estimated: visual counts of habitats can estimate the census size, while genetic studies can estimate the effective population size (Palstra & Fraser, 2012). Additionally, studying the genetic differences between juveniles and adults can be used to determine if there is a large difference between the census and effective population sizes: if juveniles are generally found to be less genetically diverse than adults, this can indicate an effective population size much smaller than the census size. This variable reproductive success has been observed in a number of marine species that exhibit Type III survivorship (where the greatest mortality is experienced in early life), and it has been attributed to environmental conditions that limit the success of reproductive events, such as strong currents or weakly swimming larvae (Hedgecock & Pudovkin, 2011). It is possible that there are discrepancies between the census and effective population sizes in *N. celidotus*, since it is a highly fecund species with many spawning opportunities. Future research could examine the genetic diversity of adult and juvenile populations, and determine the reproductive success of the species.

Chapter Four

General Discussion

Genetic diversity of Notolabrus celidotus

This thesis research analysed the genetic population structure of *N. celidotus* by sampling new locations and reporting new genetic markers. Chapter two described the development of six novel microsatellite loci for analysis of *N. celidotus*. In chapter three, extremely high levels of haplotypic diversity were reported based on an analysis of the mitochondrial DNA control region ($h = 0.998$: the maximum value that can be obtained is $h = 1.000$). No significant level of population structure was detected from either the mitochondrial DNA sequencing or the microsatellite DNA genotyping analyses. Analyses of the demographic history suggested that *N. celidotus* populations began to expand approximately 150,000 years ago.

Several studies of wrasses have reported high levels of genetic diversity in the mtDNA control region. This has been directly contrasted with the diversity found in other parts of the mitochondrial genome, such as the CO1 (cytochrome oxidase I) gene (Ludt et al., 2012). It has been suggested that the higher levels of diversity is because to the control region is non-coding, which means more mutations tend to persist because they are not removed by purifying selection (Messmer et al., 2012).

It has been hypothesised that in general, shallow-water species have lower levels of genetic diversity than deep-water species. The suggested reason is that the population size of shallow-water species is more sensitive to fluctuations in sea level arising from glacial cycles (Fauvelot et al., 2003). In species that inhabit coral reefs, species that inhabit the shallower lagoons were found to be less diverse than species that occupy the outer slopes of the reef, which tend to be more stable. This pattern seems to be restricted to coral reefs: the nature of these environments means they are highly susceptible to sea level fluctuations, and therefore repeated bottleneck events. Other habitats, such as rocky reefs and terrestrial lakes, do not

seem to share this pattern of increasing genetic diversity with depth. *Notolabrus celidotus* are shallow-water fish (Jones, 1984b), yet showed extremely high levels of genetic diversity in this study. Similar results were shown in African cichlid lake fish, where more genetic diversity was detected in shallow-water species, compared to deeper-water species (Nevado et al., 2013). A possible explanation for the higher levels of diversity is the larger population sizes of shallow water species arising from higher primary productivity (Leffler et al., 2012).

Species with high levels of genetic diversity tend to be more adaptable to changes to their environment, because there are potentially more advantageous alleles in large populations. Less diverse populations have a lower chance of advantageous alleles being present, so may not be able to respond to environmental challenges (Nevo, 2001). A review of proxies for population fitness found that genetic diversity was positively correlated with the fitness of a species (Reed & Frankham, 2003). Populations that have higher levels of heterozygosity are less likely to be vulnerable to inbreeding depression (Kardos et al., 2014). Based on these studies, the present research results suggest that *Notolabrus celidotus* is likely to be resilient to environmental changes due to its high genetic diversity and large population size. Understanding the potential resilience of populations is especially important if it is a commercially targeted species, but it is also useful to know the resilience of important ecosystem species (Folke et al., 2004).

Notolabrus celidotus forms an important part of rocky reef ecosystems. They provide a food source for larger, commercially valuable reef fish such as snapper and blue cod (Usmar, 2012), but also play a role in the health of the ecosystem. A trophic cascade in New Zealand rocky reef ecosystems has been identified between kelp, invertebrate grazers and predatory fish (Newcombe & Taylor, 2010). Various epifaunal invertebrates feed on the kelp, which is the basis of many rocky reef habitats. Invertebrates can damage the kelp while feeding on it, and this reduces primary productivity. *Notolabrus celidotus* consumes these epifaunal invertebrates, and is able to suppress the populations enough to promote kelp health. Kelp forests support a wide variety of organisms, and form habitats for many commercially important fish species in New Zealand (Cole et al., 1990).

While the life cycle and feeding habits of *N. celidotus* have been long known (Jones, 1984a), the broader role that this species plays in ecosystems is only now beginning to be understood: and it appears they are an important part of rocky reef ecosystems.

Population structure of Notolabrus celidotus

Life-history traits and environmental conditions both play an important part in determining connectivity and population structure (D'Arcy et al., 2013). Genetic methods can uncover large and small-scale population structure (Horne et al., 2013). Analysing a species' population structure can inform the management of fished stocks and marine reserves, and can identify cryptic species and levels of local recruitment (von der Heyden et al., 2014). Many factors can influence the degree of genetic structuring of fish populations, including their life history and habitat, historical events, and the marker chosen to study them (Selkoe et al., 2008).

Notolabrus celidotus are monandric hermaphrodites, which means there are one male and many female fish in each population (Allsop & West, 2004; Jones, 1980). Males defend territories and the group of female fish that occupy the area (Jones, 1980). The reproductive system of a species is thought to have an influence on the degree of population structuring that is observed, due to the biased sex ratios that are often observed in hermaphroditic species (Planes & Lenfant, 2002). However, a study has found no difference between levels of differentiation in gonochoristic (species that do not undergo a sex change) and hermaphroditic fish species, once the type of marker analysed and the dispersal ability were taken into account (Chopelet et al., 2009). It is suggested that while mating systems may be a factor in determining the genetic structure of a species, other factors such as dispersal ability are more significant (Barbosa et al., 2013). Although *N. celidotus* is a hermaphroditic species, it seems unlikely that this feature of its life history is related to the lack of population structure that has been observed.

Genetic studies cannot always show small-scale population structure, due to the resolution limits of the molecular marker that is used. Other methods can detect finer-scale population structure (such as detecting self-recruitment to a particular reef), including otolith microchemistry or chemical tagging of larvae (Haney et al., 2007). Otolith microchemistry techniques involve analysing various elemental ratios of a fish's otolith, and comparing those to physical locations, or other individuals to identify the original habitat of that individual. These techniques have been used to identify nursery grounds of important commercially fished species (Morrison, 2008), which often cannot be identified by genetic studies alone. Otolith microchemistry analyses have been used alongside molecular markers in studies of commercially exploited fish stocks (Collins et al., 2013; Miller et al., 2005). It can be particularly useful in the management of commercially fished migratory species, since it can be used to identify natal habitats and migratory paths that are not detectable through genetic studies (Feyrer et al., 2007). Otolith microchemistry has also been used in species that are not commercially fished, to identify natal habitats and juvenile recruitment patterns (Feutry et al., 2012). Otolith microchemistry methods have not yet been used in any studies of *N. celidotus* populations, but the technique could offer better scope to detect population differentiation, self-recruitment or migration patterns.

Notolabrus celidotus as a model species

Model species can provide insight into the features of other closely-related species that are more difficult to study directly (Biermann et al., 2003). The ubiquitous distribution of *N. celidotus* makes it a potential model species for studying population structure in New Zealand: the expected level of connectivity can be inferred in fish species that have a similar pelagic larval duration, and occupy similar habitats to *N. celidotus*. *Notolabrus fucicola* is a sister species to *N. celidotus* and another abundant rocky reef fish, which occupies similar habitats and has a similar life history: there have even been observed occurrences of hybrid *N. celidotus* x *N. fucicola* individuals in the wild (Ayling, 1980). While the pelagic larval duration of *N. fucicola* is unknown, it is likely to be similar to *N. celidotus*,

due to the close phylogenetic relationships of the two species (Russell, 1983). If the PLD is similar to that of *N. celidotus*, then it is likely that there will be high levels of population connectivity in *N. fucicola*. More broadly, the results of this study could be used to inform the expected levels of connectivity of species that have a PLD of at least 5-6 months, and are distributed throughout New Zealand.

Notolabrus celidotus has further potential to be a model species since it is not (and has never been) fished commercially. Certain fishing methods cause size-related selective pressure on fish species, when minimum size limits and the mesh size of nets only allow certain sized fishes to be captured. These methods can cause changes to the sex ratios, growth patterns and maturation time of a species, and have been observed in heavily fished species such as Atlantic cod and various salmon species (Hutchings & Fraser, 2008). Fisheries-induced evolution affects different species in different ways, but there is a tendency for species to mature earlier, and at a smaller size; the individuals that reproduce before reaching minimum size limits have a selective advantage (Kendall & Quinn, 2013). Since *N. celidotus* has never been commercially fished, population size fluctuations, sex ratios and growth-at-age can be studied in a species that has not been subject to fisheries-induced evolution. The changes in populations that occur can form a baseline that commercially targeted species can be compared with, in terms of population fluctuations and responses to environmental changes (Beninger & Boldina, 2014). Tracking the population dynamics of *N. celidotus* through time can give indications as to how reef fish populations respond to environmental changes. The changes in populations may mirror those that may occur in commercially targeted species, and these changes can be implemented into stock management plans to prepare for environmental changes.

Future directions

In addition to *N. celidotus* and *N. fucicola*, New Zealand is home to many other species of wrasse (Russell, 1983). Wrasses commonly occupy similar habitats and often share life history characteristics (such as mating systems and spawning behaviour), which means the results from this study could apply to other wrasses

(Westneat & Alfaro, 2005). Species that have more life history characteristics in common with *N. celidotus* are more likely to show similar patterns of population connectivity and genetic diversity. The other New Zealand wrasse species are not as well studied as *N. celidotus* (Russell, 1983), so the results of this study can give information about the general trends that could be observed in other species. Additionally, the same microsatellite loci developed for *N. celidotus* in this study could be used to study other New Zealand wrasses, depending on the transferability of these loci (Li et al., 2009). If the loci were found to be easily transferable between species, it would mean that the development costs associated with developing new microsatellite loci would be drastically reduced.

There are potential applications for *N. celidotus* in finfish aquaculture. Aquaculture is one of New Zealand's fastest growing primary industries, with the potential for a billion-dollar industry by 2025 (Alfaro et al., 2014). Aquaculturing finfish can pose risks of disease due to the high densities of cultured fish, and the potential of disease transmission to and from wild populations (Forrest et al., 2007). Kingfish (*Seriola lalandi lalandi*) has been identified as a potentially valuable aquaculture species, due to its high-value flesh and rapid growth (Symonds et al., 2014). However, kingfish are affected by several species of copepod ectoparasites, which can cause emaciation, anaemia and restriction of blood flow to the gills (Sharp et al., 2003). Infections can result in sickness or weight loss, which reduce the sale value of the fish. Recent research has examined the use of wrasses as cleaner fish in Norwegian salmon aquaculture, and wrasses have been used to successfully remove ectoparasites from aquacultured species (Skiftesvik et al., 2014). *Notolabrus celidotus* are generalist predators of invertebrates, so it is possible that these fish can act as cleaner fish in the same way. Another advantage of utilising *N. celidotus* as a cleaner fish is the reduced need for pesticides to reduce the parasite loads of kingfish (Sharp et al., 2004). Since kingfish aquaculture would most likely take place in marine cages or nets (Symonds et al., 2014), any pesticides will necessarily also be released into the ocean: and this is unlikely to be welcomed by the public (Burridge et al., 2014). If experiments find *N. celidotus* can successfully act as a cleaner fish, stock assessments will need to be implemented to ensure sustainable harvesting. Having a strong understanding of the genetic structure of

N. celidotus will aid greatly in the management of the species: since there is high genetic connectivity between locations, small-scale stock boundaries may not need to be established to protect populations from over-fishing.

Genetic markers that are selectively neutral are often chosen for studies of population connectivity, due to the ability of these markers to show the relationships and connectivity between populations (Schlotterer, 2004). Neutral markers are advantageous in studies of population structure since they tend to be highly variable, and therefore can identify fine-scale differences between populations (Avice, 2004). However, analysing selectively variable molecular markers can identify potential sources of selective pressure, and may help to explain phenotypic differences between populations (Landguth & Balkenhol, 2012). Different growth and maturity patterns have been observed between populations of *N. celidotus* in Auckland and Wellington: individuals in Wellington populations tend to mature later, and at a smaller size (Jones, 1980). So far, these differences have been attributed to the colder ocean environment in Wellington, and variation in adult density between these locations. It seems unlikely that genetic factors would play a part in these physiological differences, since no population structuring was detected: the differences are likely to be solely due to density-dependent and environmental differences between the sample locations.

Appendix

Appendix 1 Allele rarefaction curves for all microsatellite loci.

Appendix 2 Visual comparison of the haplotype network obtained for *N. celidotus* and those of different wrasse species. In all cases, each circle represents one haplotype, the colour of the circle represents the geographic origin, and the size of the circle indicates how many individuals share that haplotype. Networks adapted from Chen *et al* (2004) (B); Ludt *et al* (2012) (C – E) and D’Arcy *et al* (2013) (F).

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