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Testing techniques for tracing the provenance of green-lipped mussel spat washed up on Ninety Mile Beach

New Zealand Aquatic Environment and Biodiversity Report No. 164

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ISSN 1179-6480 (online)
ISBN 978-1-77665-134-4 (online)

December 2015

New Zealand Government

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EXECUTIVE SUMMARY

Dunphy, B.J.; Silva, C.; Gardner, J.P.A. (2015). Testing techniques for tracing the provenance of green-lipped mussel spat washed up on Ninety Mile Beach.
***New Zealand Aquatic Environment and Biodiversity Report No. 164.* 41 p.**

Aquaculture of the green-lipped mussel (*Perna canaliculus*, Gmelin, 1791) has recently become New Zealand's largest aquaculture export earner. However, the industry is reliant on wild caught larvae ("spat") that wash ashore attached to foliose seaweeds and hydroids at Ninety Mile Beach within the quota management area of GLM 9. Whilst a small industry is based on the collection and relaying of these spat to farming operations, the industry is vulnerable to reductions in supply arising from variable spat fall at this one location. Moreover, the location(s) of broodstock populations that supply these larvae are currently unknown, thus risks to the adult beds supplying spat could be better managed if the provenance of spat was known.

There are a number of methods available to determine the provenance of marine invertebrate larvae, with the two most popular being genetic and geochemical population markers. Previous work on genetic population markers of *P. canaliculus* has been able to discriminate North Island populations from those in the lower South Island, but has typically struggled to resolve the local population structure necessary for management. However, there is some promise in the recent development of microsatellite markers for this species which may offer the required spatial resolution to identify discrete populations within GLM 9. Alternatively, geochemical markers comprise site-specific signatures of chemical elements laid down in the shell. While this method has been shown to resolve *P. canaliculus* populations on the scale of tens of kilometres, this was only for populations within the Auckland region, and thus geochemical markers remain largely untested around Ninety Mile Beach.

Accordingly, our objective was to test the resolution of microsatellite and geochemical markers in discriminating adult populations along the west coast of the North Island of New Zealand. Sites selected were separated on scales of hundreds to tens of kilometres and fell within the boundaries of GLM 9 (Scott Point, Ahipara, Tanutanu Beach, Mitimiti, Whatipu) and GLM 8 (Oakura). At each site, 50 juvenile mussels were collected and the flesh dissected out for genetic analyses and the shell used to test the efficacy of geochemical markers.

We found that use of 10 microsatellite loci developed for *P. canaliculus* further confirmed a north-south split in northern and southern green-lipped mussel populations. Furthermore, success of assigning samples back to their site of collection varied between 30 and 81 %, with a mean success rate of 50%. In an expanded analysis including a further 14 sites, mussels from all northern sites collected clustered together (with the exception of Mitimiti), suggestive of a high degree of genetic differentiation of northern from southern populations, and providing evidence of genetic differentiation within the northern group.

Quantifying geochemical markers within the shells of mussels revealed that eight elemental ratios (Sr, Zn, Ba, Ni, Mn, Co, Cu, and Li:Ca) could be used to successfully assign mussels back to their site of collection with between 88 (Oakura) and 100 % (Whatipu) success. Most promisingly, mussels collected from two sites separated by 4 km of coast (i.e., Ahipara and Mokorau Stream) could be discriminated with 94% success indicating the potential of geochemical markers for discriminating *P .canaliculus* populations within GLM 9.

However, greater power could be achieved by a larger long-term study that encompasses more sites and makes use of the different pieces of information that both these methods provide. An assessment of temporal stability in provenance of spat at Ninety Mile Beach is needed given the highly dynamic nature of the environment and breeding biology of the species. Furthermore, the use of LA-ICP-MS methods that can sample different parts of an intact shell would also be of benefit, as temporal variation in elemental signatures over the lifetime of the mussel could be quantified.

1. INTRODUCTION

Aquaculture of the green-lipped mussel, *Perna canaliculus* (Gmelin, 1791), is New Zealand's most valuable aquaculture export producing over 100 000 t a year, and an export value of approximately $NZ 160 million per annum (Alfaro et al. 2011). The industry has undergone rapid growth over the last 30 years placing increasing demands on supplies of early juveniles or spat (Jeffs et al. 1999). The industry is almost wholly reliant on spat caught from the wild, with up to 80% coming from the collection of wild spat from one location, Ninety Mile Beach (Alfaro et al. 2010). More than 100 t of spat-bearing material is harvested from this site in northern New Zealand where juveniles are washed ashore attached to algae and hydroids (Alfaro et al. 2004). Given the planned expansion of this industry, the reliance on wild spat sources is viewed as a potential constraint to further production gains of farmed *P. canaliculus* given that the source broodstock populations that supply these spat are yet to be identified (Dunphy et al. 2011, Jeffs et al. 1999). However, while hatchery production methods for this species are well established, there still remains a strong interest in the use of wild harvested spat by industry due to comparative cost savings.

Identifying the provenance of marine larvae often relies upon some form of marker or tag, with genetic markers and geochemical tags being the most common (Levin 2006). In terms of genetic markers, geographical surveys of genetic variation can provide an indirect means of tracing movements of marine larvae and offer evidence of whether populations are closed (no genetic connectivity with other populations, such that recruitment to the population in question is self-recruitment only) through to situations where populations are completely open (100% of the recruits to the population come from other populations) (Hellberg et al. 2002). To this end microsatellites offer an excellent genetic marker because they consist of short, simple repeat sequences (e.g. CGTCGTCGT) and these short motif repeats occur throughout the genome of all eukaryotic organisms and are generally, but not always, non-coding (Guichoux et al. 2011). Because they are non-coding they tend to be under much reduced selection pressure which means that they can vary in length (the number of repeats of the motif), which in turn means that they are highly variable (MacAvoy et al. 2008). It is these properties of being highly variable (polymorphic) and selectively neutral that makes them ideal markers for genetic studies (Wei et al. 2013).

Geochemical tags rely upon elements being incorporated into the matrix of biogenic calcium carbonate in amounts related to the dissolved concentrations within local seawater (Vander Putten et al. 2000). As long as sufficient variation exists in elemental composition of seawater among locations then an elemental "signature" can be incorporated into the developing calcium carbonate structure (Thorrold et al. 2007). Typically this method is applied to the testing of natal origins of juvenile fish using otolith chemical composition (Elsdon et al. 2008). There is however, a growing body of work on marine invertebrates, particularly commercially valuable bivalves (Cathey et al. 2014).

Obtaining accurate, high resolution measurement of trace elements within calcified structures is achieved via inductively coupled plasma mass spectrometry (ICP-MS) and variants thereof e.g. LA-ICP-MS (Limbeck et al. 2015). Statistical algorithms are then employed to deduce the elemental signatures arising from site differences in the chemical composition of water, with a commonly used example being multivariate discriminant function analysis (DFA) (Thorrold et al. 2002). By comparing the elemental composition of shells from known locations, the accuracy of the DFA can be ascertained and the natal origin of "source unknown" larvae referenced back and identified (Becker et al. 2007, Carson 2010). Becker et al. (2007) used elemental analysis to reveal patterns of larval exchange in the mussel species *Mytilus californianus* and *M. galloprovincialis* from southern California. They found regional differences in dispersal for both species, with export of larvae to adjacent areas occurring for southern populations and self-recruitment for northern populations. Carson (2010) was able to demonstrate that the majority of Olympia oyster (*Ostrea lurida*) recruits in San Diego Bay were self-recruiting and that a lesser exchange of larval recruits into surrounding

estuaries occurred up to 75 km away. However, given the expense of performing ICP-MS analyses, identification of the most discriminatory shell elements is desirable to assist the efficacy of future studies (Gillanders et al. 2001, Levin 2006, Thorrold et al. 2002). Whilst Dunphy et al. (2011) were able to use LA-ICP-MS to discriminate sites differences in elemental signatures for *P. canaliculus* shells, their focus was predominantly on the Auckland region, and not Northland.

Finally, before genetic and/or geochemical studies can be undertaken an understanding of their resolving power must be obtained with the study species (Carson et al. 2013). Whilst identification of natal origins relies upon spatial variation in elemental/genetic signatures, the spatial resolution at which the DFA model (or other) can accurately distinguish sites must be proven using sites of varying distances from one another (Gillanders et al. 2001). For the green-lipped mussel, assessments of pelagic larval duration and coastal physical oceanography suggest that spat arriving at GLM 9 are derived from one or more source populations in the region from Hokianga Harbour south to Taranaki (Alfaro et al. 2010).

Therefore, we sought to determine the utility of microsatellite markers and geochemical tags to discriminate juvenile mussels collected from six sites along the west coast of the North Island of New Zealand. Our key aim was to determine the spatial resolution at which each method could discriminate populations and thus inform future attempts at identifying the provenance of mussel spat washing ashore at Ninety Mile Beach. The specific objective of this contract was that we should "utilise marker technology (natural or otherwise) to determine the smallest scale at which the source of GLM 9 spat from adult mussel beds can be determined".

Key activities were:

1. the collection of mussel samples from six sites along the west coast of the North Island
2. LA-ICP-MS analysis (note: modified to ICP-MS) of the six sites
3. genetic analysis using microsatellites of the six sites
4. determining the ability of the genetic and the shell geochemistry analyses as methods to discriminate among the six new sites
5. placing the results for the six new sites in a wider context (larger spatial scale) where possible.

2. METHODS

2.1 Collection and processing of juvenile mussels

In January of 2015 juvenile mussels between 14 and 20 mm in shell length were collected from intertidal reefs located at six sites within the North Island of New Zealand (see Table 2.1 for grid coordinates and shell lengths and Figure 2.1 for map of locations). Mussels of this size were expected to have settled within the last twelve months, particularly for northern sites where Hickman (1979) recorded an annual growth rate of 31 mm for juvenile intertidal mussels in Northland. Mussels collected were free of obvious epibiota and had an entire periostracum from umbo to shell edge. Once collected, mussels were stored in plastic zip lock bags and frozen at -20 °C within 1 hour of collection. Once back at the laboratory, mussels were thawed and photographed to record shell length via ImageJ software. Using plastic tweezers, mussels were opened and the flesh removed and placed in tubes containing 4 mL of 95% EtOH for genetic analysis. Valves were stored in plastic vials and refrozen for ICP-MS elemental analysis. Sex of mussels was unable to be determined as all mussels collected were sexually immature.

Table 2.1: Sampling location, sample size and mean shell length of juvenile green-lipped mussels (*P. canaliculus*) collected in January 2015 from northern New Zealand. Note sites are listed in order of increasing latitude and those in italics were only included in ICP-MS analyses. Name in parentheses indicates coding in genetic analyses.

Site	Grid coordinates	n analysed	Mean shell height (± S.D)
Scott Pt (SCO)	34°31’35”S, 172°43’29”E	50	16.3 (± 3.5)
Maunganui Bluff (MGB)	*34°41’13”S, 172°53’32”E*	*50*	*17.7 (± 3.3)*
Ahipara/Shipwreck Bay (SHI)	35°10’40”S, 173°07’56”E	50	16.4 (± 4.4)
Mokorau Stream (MOS)	*35°10’07S, 173°05’18”E*	*50*	*19.4 (± 4.7)*
Tanutanu Beach (TAN)	35°12’14”S, 173°04’17”E	50	20.1 (± 3.4)
Mitimiti Beach (MIT)	35°25’51”S, 173°16’12”E	50	19.0 (± 3.7)
Whatipu (WHA)	37°02’41”S, 174°30’35”E	50	14.5 (± 4.4)
Oakura Beach (OAK)	39°06’33”S, 173°57’17”E	50	17.0 (± 3.4)

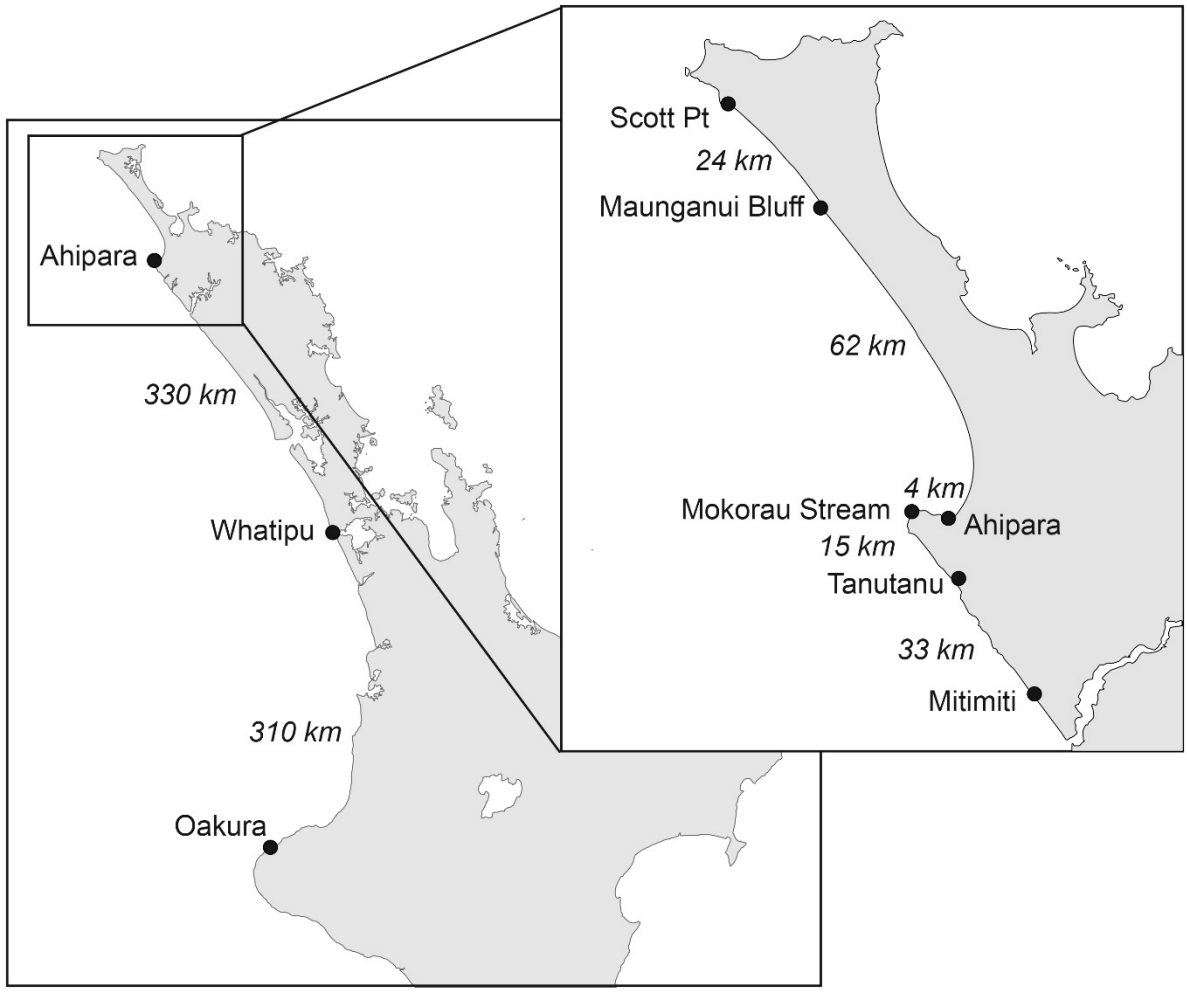

Figure 2.1: Location of study sites where green-lipped mussels (*P. canaliculus*) were collected along the west coast of the North Island of New Zealand in January 2015.

2.2 Microsatellite marker methods

DNA extraction and genotyping

Total DNA was extracted from mantle or gill tissue using Geneaid Genomic DNA Mini Kits (Tissue) following the manufacturer´s instructions. DNA concentrations and the A260/A280 ratios were quantified using a NanoDrop™ ND-1000 (Thermo Scientific). Following Wei et al. (2013), specimens were genotyped at 10 polymorphic microsatellite loci (*Pcan1-25*, *Pcan2-20*, *Pcan2-60*, *Pcan6-17*, *Pcan10-36*, *Pcan1-27*, *Pcan1-29*, *Pcan2-17*, *Pcan10-44* and *Pcan22-11*) developed by MacAvoy et al. (2008). Microsatellite loci were PCR-amplified in reactions (15 µL) containing 70 ng of DNA template, 0.5 units/µL *Taq* DNA polymerase, 67 mM Tris–HCl pH 8.8, 16 mM $(NH_4)SO_4$, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM of Forward and Reverse primers and ddH_2O to volume. PCR products were visualised with an automated sequencer (ABI PRISM 3730 DNA Sequencer, Applied Biosystems) with the GeneScan-500 (LIZ) internal size standard. The software GeneMarker V2.2.0 (SoftGenetics) was used to analyse electropherograms for allele scoring and the alleles were binned with manual checking using the AutoBin program (Guichoux et al. 2011).

Scoring of locus-specific alleles was conducted between the 6 new populations and the published 14 populations by comparing allele frequencies and then matching the identity of those alleles at highest frequencies in the two length-frequency distributions.

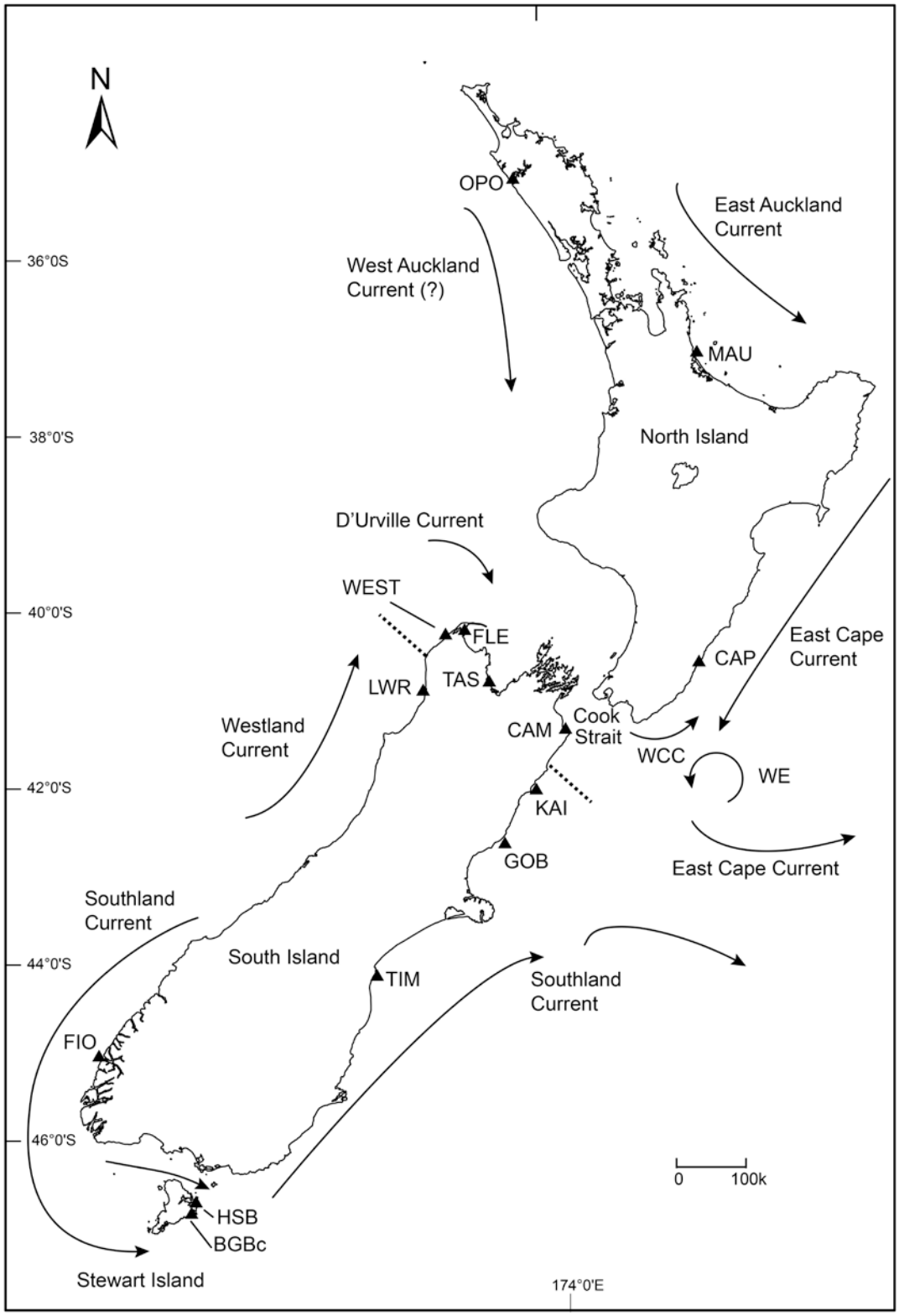

Figure 2.2: Collection sites of mussels from 14 populations used in tests to compare with the mussels from the six new populations collected for this study. Opononi (OPO); Maunganui (MAU); Castlepoint (CAP); Westhaven (WEST); Fletchers Beach (FLE); Tasman Bay (TAS); Cape Campbell (CAM); Little Wanganui River (LWR); Kaikoura (KAI); Gore Bay (GOB); Timaru (TIM); Fiordland (FIO); Horseshoe Bay (HSB); Big Glory Bay (BGB). Filled circle = northern group; filled triangle = southern group; dotted lines = areas of coastal upwelling; c = cultured mussels, WE = Wairarapa Eddy, WCC = Wairarapa Coastal Current.

Theoretical framework for testing

We employed two approaches to data testing. First, data from the 6 mussel populations collected in 2015 were analysed as a single data set to provide new information about these specific populations and about genetic diversity and potential connectivity among populations on the west coast of the North Island. Second, data from the 6 mussel populations collected in 2015 were combined with previously published data for 14 populations throughout New Zealand (Wei et al. 2013 – Fig. 2.2) to provide a larger geographic scale and context for the present study. Generally, analyses as described below were first carried out for the 6 populations and then repeated for the 20 populations.

Analysis of genetic diversity

Genotyping artefacts were assessed using the software Micro-Checker v.2.2.0.3 (Van Oosterhout et al. 2004). Analysis of departure from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were performed using the software GenePop on the web using the Markov chain method and Fisher´s exact test (Rousset 2008). False discovery rate (FDR) control (Verhoeven et al. 2005) was applied to p-values in all statistical analyses that included multiple comparisons. GenAlEx 6.5 (Peakall & Smouse 2012) was used to quantify the number of different alleles per site (Na), observed (H_O) and expected (H_E) heterozygosity and the fixation index (F_{IS}).

Population genetic analyses

Genetic differentiation among populations was assessed using different approaches. Pairwise F_{ST} values were calculated using the software GenePop on the web (Rousset 2008). This F_{ST} value, formerly known as theta (θ), was adapted by (Weir & Cockerham 1984) for use with multiallelic loci, unbiased to different sample sizes and uses an ANOVA approach to standardize variation within and among populations. An exact G-test (Goudet et al. 1996) was also calculated in GenePop (Markov-chain parameters: 10 000 dememorisation steps, 1000 batches and 10 000 iterations per batch) for each population pair using the G log likelihood ratio. All p-values were corrected using the False Discovery Rate (FDR) (Verhoeven et al. 2005) and comparisons were considered significant at $p < 0.05$. Modified pairwise statistics (F'_{ST}) were calculated using the software GenoDive 2.0b25 (Meirmans & Van Tienderen 2004). The F'_{ST} index is based on the AMOVA (analysis of molecular variance) framework (Excoffier et al. 1992). It is a standardised measure based on a maximum possible value given the observed amount of within-population diversity and therefore has no p-value associated with it.

To investigate the possibility of group structure within the dataset we used a variety of different approaches. A UPGMA rooted tree with bootstrap support values (10 000 replications) of population relatedness was constructed using the software package POPTREE2 (Takezaki et al. 2010). This analysis places the most dissimilar population as the root of the tree and then clusters subsequent populations according to their degree of similarity to reveal group structure. Principal coordinates analysis (PCoA) was employed in GenAlEx 6.5 (Peakall & Smouse 2012) to explore and visualise the relationships (distances) among populations.

GenAlEx 6.5 (Peakall & Smouse 2012) was used to conduct hierarchical Analysis of Molecular Variation (AMOVA) using 999 permutations to test for structure within the data set. The six populations were analysed separately and also grouped into three regions (MIT, WHA and remaining four populations), according to the PCoA results and the F_{ST} values.

The software package STRUCTURE was employed to examine multilocus similarity among individuals and to test for the existence of different numbers of possible groups. STRUCTURE is a parametric analysis and assumes both HWE and no linkage disequilibrium, both states which are generally not true, in particular for data from marine bivalve molluscs. Nonetheless, STRUCTURE is

a powerful and easy to interpret tool for the analysis of group structure within the data set. The software package AWclust (Gao & Starmer 2008) was employed to examine multilocus similarity among individuals and to test for the existence of different numbers of possible groups. AWclust is a nonparametric analysis and does not assume either HWE or linkage disequilibrium. Like STRUCTURE it is a powerful and easy to interpret tool for the analysis of group structure within the data set. For further details refer to (Wei et al. 2013).

Estimates of migration

Assignment tests were used to identify first-generation migrants as well as the proportion of individuals recruiting to a location. The Bayesian program Geneclass 2 (Piry et al. 2004) was used with the Markov chain Monte Carlo (MCMC) resembling the algorithm of Paetkau et al. (2004). Following Wei et al. (2013), to determine the most likely source of individuals in each sampled population, self-assignment tests were performed (Paetkau et al. 2004), using the leave-one-out procedure, in which the individual under consideration is removed during computations for their population of collection (Piry et al. 2004). The probability of assignment was based on 10 000 simulated individuals and an exclusion threshold of $P<0.05$. Individuals that were excluded from their population of collection were assigned to another sampled population when $P>0.1$. For detection of first-generation migrant individuals, we used the 'L_home' likelihood computation method to allow for the possibility that not all source populations were sampled, which is highly likely in this situation. Individuals identified as first-generation migrants were removed from the data set, and the remaining sampled individuals were used as reference populations for further assignment tests to identify the most likely source population for each first generation migrant. Where the results indicated more than one possible sampled source population ($P>0.1$), the individual was assigned to the population with the highest probability. Thus, the self-assignments are 'home' individuals (self recruits), whereas the first-generation migrants are 'away' individuals (a migrant from one known population to another). Other analytical approaches to the quantification of migration such as BAYESASS (Wilson & Rannala 2003) could not be employed in the analysis of the six new populations because F_{ST} values were all too small.

2.3 Geochemical markers in shell

Although contracted to undertake analyses of six sites, due to a cost saving opportunity, ICP-MS analyses were able to be performed on a further two sites i.e. Maunganui Bluff and Mokorau Stream.

Shell Preparation for ICP-MS

In the laboratory *P. canaliculus* shells were thawed. The valves were individually placed into 5 ml plastic vials and sonicated for two minutes, the water was then drained. All plastic ware used to handle and store *P. canaliculus* after the sonication step was acid washed for 12 hours in 10% HNO_3 (Sigma-Aldrich trace element grade) and rinsed three times in de-ionised water.

The left valve of each individual was chemically cleaned to remove the periostracum and any remaining organic matter. The chemical treatment procedure was based on techniques used by Strasser et al. (2008) to prepare juvenile softshell clam (*Mya arenaria*) shell for digestion based ICP-MS. Each valve was soaked in a 1% H_2O_2 (Fisher Scientific trace analysis grade) solution buffered in 1 N NaOH (Sigma-Aldrich trace analysis grade) at 80 °C for 10 minutes. Shells were then rinsed by running under de-ionised water for 10 seconds and stored in clean acid washed 5 ml plastic vials, until digestion.

Digestion, ICP-MS and analysis

The digestion process was as follows: shells were weighed into Maxi-44 80 ml Teflon vessels and combined with 5 ml HNO_3 (65%) and 1 ml HCl (37%). They were digested using a Milestone ETHOS-UP microwave digestion system. The digests were diluted with 50 ml dH_2O and the final weights were obtained. The final solutions were clear and the shells were completely dissolved. These solutions were diluted 10× with matrix matched diluent and run using an Agilent 7700 ICP-MS

at the Mass Spectrometry Centre, Auckland Science Analytical Services, The University of Auckland, Auckland, New Zealand. Instrument settings were RF Power (W) = 1550, with a He gas flow rate of 4.3 L min^{-1}. The elemental suite monitored consisted of 12 elements and was based on those found to contribute to the classification success of *P. canaliculus* by Dunphy et al. (2011). The elemental suite consisted of: ^{43}Ca, ^{66}Zn, ^{55}Mn, ^{11}B, ^{88}Sr, ^{25}Mg, ^{138}Ba, ^{63}Cu, ^{7}Li, ^{60}Ni, ^{47}Ti, and ^{59}Co.

Raw elemental concentrations were converted to molar concentrations i.e. mmol mol^{-1}, and trace element:calcium ratios were calculated in order to standardise elemental concentrations. Correlation matrices were generated for each site using MANOVA, and due to differing covariance estimates among sites a quadratic discriminant function analysis (Q-DFA) was performed. Furthermore, due to non-normality, data were rank transformed (Bello 1993). For shells where the concentration of an element was below detection limits of the instrument, an imputation procedure was applied. This consisted of substituting the recorded 0 value with half of the minimum value of that element recorded in all shells from all sites combined (K. Ruggiero, Dept. Statistics, UoA, pers. comm. 2015) The Q-DFA was performed stepwise by firstly running the analysis on all elemental ratios and recording the overall classification success. Then the elemental ratio with the least discriminatory influence was removed and the Q-DFA ran again with the difference in classification success noted. This was repeated for all elemental ratios in order to identify the point at which no increase in the classification success was observed and thus what combination of elements could provide successful discrimination of sites. Mean canonical scores were calculated for shells from each site and a classification success table generated in order to gauge the success of the Q-DFA in assigning mussel shells back to their site of collection based on elemental ratios. Scores for the Receiver Operating Characteristic (ROC) curve were calculated in order to provide a measure of the sensitivity of the Q-DFA. All analyses were carried out in JMP 11.0 software (SAS institute, North Carolina, USA).

3. RESULTS

As noted earlier, the genetic (microsatellite variation) analyses focused on the six new populations from the west coast of the North Island of New Zealand. However, these new results have been placed in a wider context – that of the previously analysed 14 populations from throughout New Zealand. Results presented below focus on all 20 New Zealand populations, with the detailed results for the six new west coast North Island populations reported in Appendices. This is because the results for the six new populations are consistent with the results for all 20 populations. Nonetheless, reference to the six new populations is specifically made on occasions because the spatial scale differences of the two data sets (west coast of North Island only versus all of New Zealand) have a profound impact on our ability to identify with high levels of confidence where recruits come from. That is, it is much easier to identify a northern recruit in the southern populations than it is to identify a northern recruit within the northern populations.

3.1 Microsatellite marker results

In total, 288 of 300 individuals were genotyped for variation at 10 microsatellite loci. Due to homozygote excess at each locus Micro-Checker identified putative null alleles at *Pcan1-27, Pcan1-29, Pcan6-17, Pcan10-44* and *Pcan22-11*. No long allele dropout was detected. After false discovery rate (FDR) correction for multiple tests, *Pcan1-27* was identified as being significantly out of HWE at all populations and *Pcan10-44* at more than half of the populations. No evidence of significant linkage disequilibrium was detected between locus pairs. These findings are consistent with the results for 14 other *P. canaliculus* populations (Wei et al. 2013). Whilst mean number of different alleles per site (Na) for the six populations was slightly greater than for the 14 populations (10.633 versus 9.064, *t*-test, p = 0.004), all other indices (observed heterozygosity (H_O), expected heterozygosity (H_E) and the fixation index (F_{IS})) were not significantly different between the two groups. These results indicate that processing of the new mussels from the 6 populations and

interpretation of their genetic variability was not different from mussels from the 14 populations (Wei et al. 2013). Descriptive statistics for all 20 populations are presented in Table 3.1.

Table 3.1: Descriptive measures of genetic variation in *Perna canaliculus* for 10 loci and 20 populations. Number of samples (N), mean number of different alleles per site (Na), observed heterozygosity (H_O), expected heterozygosity (H_E) and the fixation index (F_{IS}).

Pop	N		Na	H_O	H_E	F_{IS}
SCO	50	Mean	11.00	0.64	0.73	0.13
		SE	2.37	0.05	0.05	0.04
SHI	50	Mean	10.80	0.64	0.72	0.10
		SE	2.24	0.05	0.05	0.07
TAN	50	Mean	10.40	0.63	0.71	0.10
		SE	1.89	0.05	0.05	0.07
MIT	47	Mean	10.90	0.67	0.75	0.09
		SE	2.11	0.06	0.04	0.08
WHA	41	Mean	10.40	0.64	0.72	0.10
		SE	2.02	0.06	0.05	0.05
OAK	50	Mean	10.30	0.62	0.70	0.11
		SE	1.80	0.06	0.06	0.06
CAP	30	Mean	11.100	0.67	0.75	0.10
		SE	2.18	0.04	0.05	0.03
MAU	29	Mean	10.300	0.64	0.70	0.09
		SE	1.84	0.05	0.05	0.03
OPO	27	Mean	9.400	0.64	0.72	0.09
		SE	1.51	0.03	0.04	0.05
TAS	20	Mean	9.400	0.61	0.75	0.18
		SE	1.83	0.04	0.05	0.04
CAM	25	Mean	9.30	0.63	0.71	0.09
		SE	1.78	0.05	0.05	0.05
FLE	14	Mean	7.80	0.60	0.72	0.17
		SE	1.41	0.06	0.05	0.05
WEST	13	Mean	6.90	0.64	0.72	0.11
		SE	1.01	0.05	0.04	0.07
KAI	16	Mean	8.00	0.66	0.71	0.04
		SE	1.60	0.04	0.06	0.07
LWR	26	Mean	9.90	0.70	0.73	0.03
		SE	1.97	0.06	0.05	0.05
TIM	22	Mean	8.10	0.63	0.66	0.02
		SE	1.66	0.07	0.09	0.07
FIO	19	Mean	8.40	0.63	0.68	0.05
		SE	1.85	0.05	0.07	0.06
GOB	28	Mean	10.00	0.61	0.71	0.10
		SE	2.07	0.03	0.06	0.06
BGB	20	Mean	8.60	0.68	0.69	0.00
		SE	1.63	0.07	0.07	0.06
HSB	22	Mean	9.70	0.60	0.73	0.19
		SE	1.64	0.06	0.05	0.05

Population genetic structure

Tests of significance of the pairwise F_{ST} values for all 20 populations of *Perna canaliculus* (Table 3.2) confirmed patterns reported by Wei et al. (2013). The six new northern NZ populations tend to fall into the northern group of populations which shows greatest difference from the southern group of populations. Of the 190 tests reported in Table 3.2, 151 are significant after correction for multiple testing (the FDR test). For the six new populations, all tests for differentiation when compared to southern populations were significant, and approximately half of the tests were significant when compared to northern populations. These results indicate that the six new northern populations are characterised by genetic variation that is different from many other northern populations (and all southern populations). Refer to Appendix I for results for the six new northern populations.

The UPGMA tree of population relatedness for all 20 New Zealand populations (Fig. 3.1) confirms the northern group status of the six new populations, but also provides evidence of the differentiation of the MIT population from the other five populations, all of which cluster together in a poorly supported separate group within the UPGMA tree. There is no clear relationship between geography and genetic relatedness within the northern grouping as revealed by the UPGMA tree. UPGMA analysis of the six new populations alone reveals clear evidence of structure (i.e., genetic differentiation) at this regional scale (Appendix II).

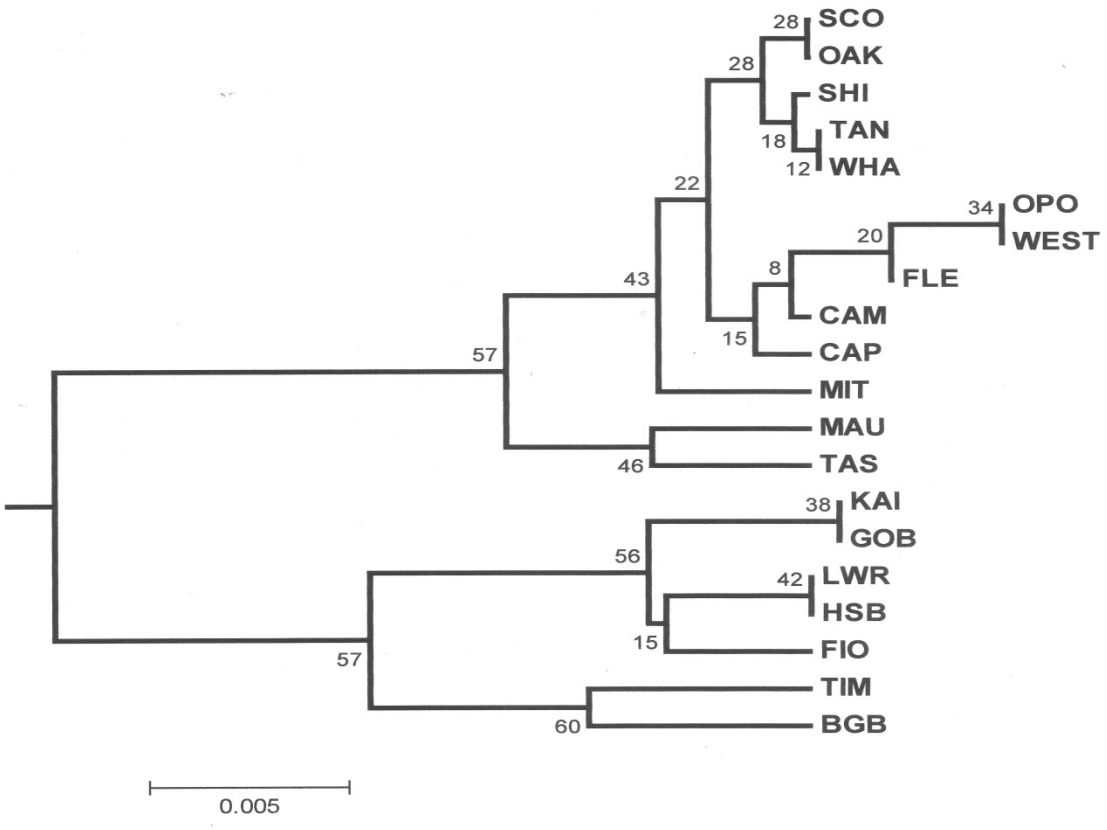

Figure 3.1: Relationships between 20 New Zealand mussel populations inferred from a UPGMA tree reconstructed on the basis of Nei's corrected F_{ST} values (percentage bootstrap support values are indicated at nodes).

Table 3.2: Pairwise F_{ST} values for *Perna canaliculus* collected from 20 locations using 10 loci are below the diagonal and F'_{ST} values are above the diagonal. Significant values after FDR testing are in bold.

Code	SCO	SHI	TAN	MIT	OPO	WHA	MAU	OAK	CAP	TAS	CAM	FLE	WEST	KAI	LWR	TIM	FIO	GOB	BGB	HSB
SCO		0.000	0.000	0.000	0.003	0.000	0.047	0.000	0.006	0.023	0.000	0.000	0.000	0.065	0.101	0.241	0.124	0.094	0.208	0.099
SHI	**0.001**		0.000	0.020	0.000	0.000	0.061	0.000	0.027	0.067	0.009	0.000	0.000	0.078	0.129	0.284	0.167	0.12	0.226	0.114
TAN	0.000	**0.000**		0.009	0.000	0.000	0.040	0.000	0.018	0.052	0.000	0.000	0.000	0.066	0.11	0.247	0.137	0.096	0.192	0.095
MIT	0.001	**0.009**	0.007		0.006	0.000	0.068	0.02	0.027	0.033	0.000	0.000	0.000	0.03	0.075	0.187	0.117	0.080	0.130	0.056
OPO	**0.003**	**0.003**	**0.000**	**0.004**		0.000	0.042	0.000	0.000	0.013	0.009	0.000	0.000	0.063	0.120	0.231	0.132	0.070	0.182	0.094
WHA	0.000	0.000	0.000	0.004	**0.004**		0.017	0.000	0.006	0.000	0.000	0.000	0.000	0.000	0.051	0.163	0.073	0.036	0.120	0.016
MAU	**0.015**	**0.020**	**0.015**	**0.021**	**0.011**	**0.015**		0.044	0.075	0.019	0.058	0.059	0.019	0.085	0.132	0.26	0.123	0.098	0.241	0.131
OAK	0.000	0.002	0.000	**0.011**	**0.000**	0.005	**0.017**		0.011	0.048	0.003	0.000	0.000	0.071	0.148	0.261	0.165	0.102	0.243	0.135
CAP	**0.004**	**0.010**	**0.008**	**0.009**	0.000	**0.010**	**0.020**	**0.007**		0.004	0.015	0.022	0.000	0.056	0.106	0.190	0.127	0.064	0.157	0.087
TAS	**0.010**	**0.022**	**0.019**	**0.012**	0.003	**0.014**	0.005	**0.019**	0.001		0.033	0.015	0.000	0.018	0.088	0.18	0.091	0.038	0.147	0.058
CAM	**0.002**	**0.006**	**0.004**	**0.003**	0.003	**0.003**	**0.016**	**0.006**	0.004	0.009		0.004	0.000	0.058	0.101	0.236	0.155	0.101	0.216	0.083
FLE	**0.004**	**0.000**	**0.001**	**0.003**	0.000	0.000	0.016	**0.008**	0.005	0.004	0.001		0.000	0.000	0.067	0.169	0.115	0.020	0.128	0.003
WEST	**0.000**	**0.000**	**0.000**	**0.004**	0.000	0.000	0.005	**0.001**	0.000	0.000	0.000	0.000		0.001	0.072	0.187	0.108	0.029	0.138	0.030
KAI	**0.022**	**0.027**	**0.024**	**0.012**	**0.016**	**0.012**	**0.023**	**0.028**	**0.014**	0.005	**0.016**	0.000	0.000		0.027	0.061	0.051	0.000	0.064	0.005
LWR	**0.030**	**0.038**	**0.034**	**0.022**	**0.031**	**0.024**	**0.036**	**0.047**	**0.026**	**0.022**	**0.027**	**0.017**	**0.018**	**0.007**		0.12	0.000	0.022	0.104	0.000
TIM	**0.075**	**0.090**	**0.081**	**0.057**	**0.068**	**0.064**	**0.079**	**0.089**	**0.053**	**0.050**	**0.071**	**0.049**	**0.055**	**0.018**	**0.035**		0.058	0.046	0.030	0.056
FIO	**0.039**	**0.053**	**0.046**	**0.036**	**0.037**	**0.036**	**0.036**	**0.056**	**0.034**	**0.024**	**0.045**	**0.031**	**0.029**	**0.014**	0.000	**0.018**		0.000	0.088	0.032
GOB	**0.029**	**0.037**	**0.031**	**0.024**	**0.019**	**0.021**	**0.027**	**0.034**	**0.017**	**0.010**	**0.028**	0.005	0.008	0.000	**0.006**	**0.014**	0.000		0.052	0.016
BGB	**0.061**	**0.069**	**0.061**	**0.039**	**0.050**	**0.049**	**0.069**	**0.079**	**0.041**	**0.038**	**0.061**	**0.035**	**0.037**	**0.017**	**0.028**	**0.010**	**0.026**	**0.015**		0.035
HSB	**0.029**	**0.034**	**0.030**	**0.017**	**0.024**	**0.017**	**0.035**	**0.043**	**0.021**	**0.014**	**0.022**	0.001	0.007	**0.012**	0.000	**0.016**	0.009	0.004	**0.009**	

The PCoA plot of the 20 New Zealand populations revealed a group of southern populations (to the left of the plot) and a group of northern populations (including the six new northern populations) to the right of the plot (Fig. 3.2). The status of the WHA population as an outlier from the main northern group is particularly apparent. Refer to Appendix III for results for the six new northern populations.

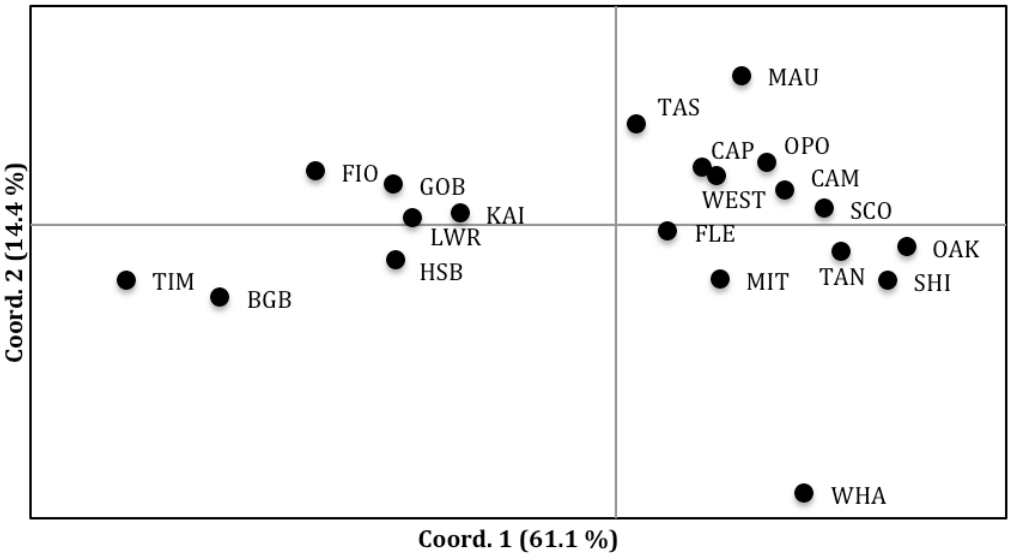

Figure 3.2: Principal coordinate analysis (PCoA) for 20 populations of *Perna canaliculus* using pairwise genetic distance based on variation at 10 microsatellite loci.

Inclusion of the six new populations within an AMOVA testing for difference among the northern and southern groupings of 20 New Zealand populations confirmed the status of the six new populations as members of the northern group (Table 3.3). Refer to Appendix IV for results for the six new northern populations.

Table 3.3: Analysis of molecular variance (AMOVA) for 20 populations from two regions (North: SCO, SHI, TAN, MIT, OPO, WHA, MAU, OAK, CAP, TAS, CAM, FLE, WEST; South: KAI, LWR, TIM, FIO, GOB, BGB, HSB) using 10 loci.

Source of variation	Degrees of Freedom	Sum of Squares	Estimated Variance	% Variation	Differentiation Indices	Significance (p value)
Among Regions	1	60.0	0.117	3.0%	0.030	0.001
Among Populations	18	128.3	0.047	1.2%	0.013	0.001
Among Individuals	579	2 501.1	0.616	15.9%	0.042	0.001
Within Populations	599	1 849.0	3.087	79.8%	0.166	0.001
Total	1197	4 538.4	3.867	100.0%	0.202	0.001

Note: Among regions – northern versus southern groupings; Among populations – among all 20 populations; Among individuals – among all mussels regardless of population or region.

As background and for context the STRUCTURE analysis plot from the data of Wei et al. (2013) for 14 New Zealand populations is shown in Figure 3.3. Although not part of the analyses of that paper, these results confirm the very pronounced split between northern (mostly red, on the left of the plot) and southern (mostly green, on the right of the plot) populations, consistent with the other grouping analysis (AWclust) used by Wei et al. (2013).

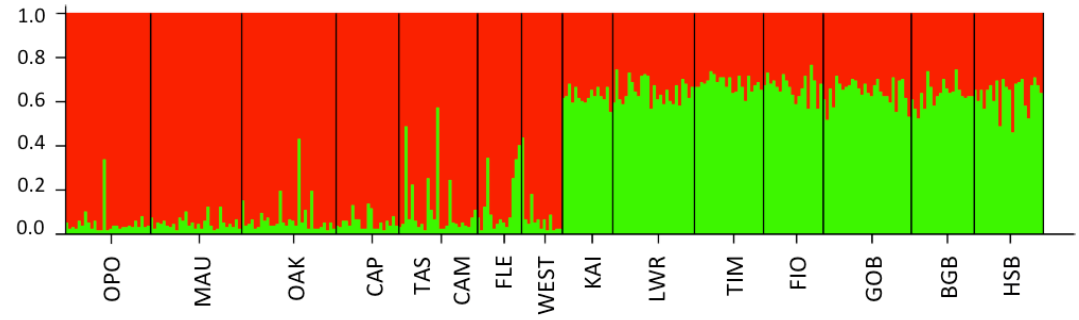

Figure 3.3: Bayesian cluster analysis (Structure) results for *Perna canaliculus* using 10 loci from dataset by Wei et al. (2013) (K=2). Populations are arranged from north to south (left to right).

Not surprisingly, analysis of all 20 New Zealand mussel populations using STRUCTURE confirmed the northern group membership of the six new populations (i.e., mostly green) (Fig. 3.4). However, the six new populations have greater northern affinity than those northern populations previously sampled, including Opononi (OPO – just inside Hokianga Harbour) which is geographically very close to the SCO, SHI, TAN and MIT populations. In Figure 3.4 the change in proportion from green (northern) to red (southern) group membership is apparent and is important in the context of trying to identify the source of the spat in GLM 9.

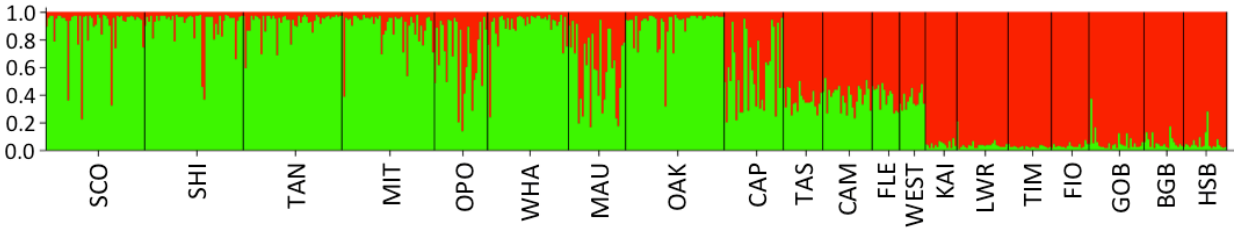

Figure 3.4: Bayesian cluster analysis (Structure) results for *Perna canaliculus* using 10 loci from 20 locations, individuals collected in 2015 and dataset by Wei et al. (2013) (K=2). Populations are arranged from north to south (left to right).

AWclust results for population-specific group membership confirm the STRUCTURE results. AWclust identified two main groups (northern and southern as previously defined), but do not add any new insights. Refer to Appendix VI for results for the six new northern populations.

Estimates of migration

Assignment probabilities (mean ± SD) for mussels from all 20 populations were 60% ± 19.6, for the seven northern populations were 54.1% ± 19.8, for the seven southern populations were 74.6% ± 12.4 and for the 6 new northern populations were 49.8% ± 18.5 (Table 3.4). Within the new northern group the WHA population stood out as being very different, with an assignment estimate of 81%. These results confirm at large spatial scales that there are genetic differences amongst the populations, sufficient to allow for moderately high levels (in the range of about 50 to 70%) of assignment success. Refer to Appendix VII for results for the six new northern populations.

Estimates of first generation migrants amongst all 20 New Zealand populations (Table 3.5) revealed that only 27 first generation migrants were detected, and these were mostly between pairs of populations that were, on average, closer together than further apart (as might be expected). In total, 14 migrants were exchanged between northern populations, 3 moved from south to north, 4 moved from north to south, and 6 migrants were exchanged between southern populations (Table 3.5). For the 6 new northern populations it was predicted that 6 immigrants were derived from the northern group of populations and only 1 immigrant was derived from the southern group of populations. Interestingly, the recipient population of the southern immigrant was OAK, on the Taranaki coast, the

most southerly of the 6 new populations. Refer to Appendix VII for results for the six new northern populations.

Table 3.4: Percentage of individuals of *Perna canaliculus* collected from each sampling location, assigned to each sampled potential source location. "Total other locations" is the percentage of individuals recruited from locations other than the collection site. Results include data for 14 New Zealand populations taken from from Wei et al. (2013) and the six new populations. Bolded and italicised text on the diagonal indicates percentage of migrants from any one population back to that same population (i.e. self recruits).

Location	Assigned location																				
	SCO	SHI	TAN	MIT	OPO	WHA	MAU	OAK	CAP	TAS	CAM	FLE	WEST	KAI	LWR	TIM	FIO	GOB	BGB	HSB	Total other locations
SCO	***30***	2	2	2		52		12													70
SHI	6	***44***	4	2		42		2													56
TAN			***34***	4		48		14													66
MIT	2			***55***		36		7													45
OPO			4		***30***	40		7		11							4			4	70
WHA	10	2	2			***81***		5													19
MAU	4		4			32	***56***	4													44
OAK	6		2			37		***55***													45
CAP			3			20			***74***	3											26
TAS						15			5	***80***											20
CAM						40				12	***36***				4				4	4	64
FLE						21				7		***65***						7			35
WEST				8		22			8	8		8	***38***							8	62
KAI				6		13			6					***69***			6				31
LWR				4		4									***92***						8
TIM				4		23										***59***				14	41
FIO	5					5		5		5					5		***75***				25
GOB	3		3			25			4	4								***61***			39
BGB				5		10	5												***80***		20
HSB						9			5											***86***	14

Populations are arranged in north to south direction in the left hand column. Northern populations from Wei et al. (2013) are OPO, MAU, CAP, TAS, CAM, FLE, WEST; Southern populations from Wei et al. (2013) are KAI, LWR, TIM, FIO, GOB, BGB, HSB; new populations from the west coast of the North Island are SCO, SHI, TAN, MIT, WHA, OAK.

Table 3.5: Number of first generation (F0) migrants detected for *Perna canaliculus* at each sampled location. Results include data for 14 New Zealand populations taken from Wei et al. (2013) and the six new populations.

Receiving location	Source of F0 migrants																				
	SCO	SHI	TAN	MIT	OPO	WHA	MAU	OAK	CAP	TAS	CAM	FLE	WEST	KAI	LWR	TIM	FIO	GOB	BGB	HSB	Total F0 migrants
SCO		1		1																	2
SHI																					0
TAN																					0
MIT	1					1															2
OPO							1			1							1				3
WHA	1																				1
MAU						1				1											2
OAK	1																			1	2
CAP					1																1
TAS									1												1
CAM								1													1
FLE														1							1
WEST							1														1
KAI																1		1			2
LWR																		1			1
TIM																	1				1
FIO												1				1					2
GOB	1													1							2
BGB							1														1
HSB									1												1

Populations are arranged in north to south direction in the left hand column. Northern populations from Wei et al. (2013) are OPO, MAU, CAP, TAS, CAM, FLE, WEST; Southern populations from Wei et al. (2013) are KAI, LWR, TIM, FIO, GOB, BGB, HSB; new populations from the west coast of the North Island are SCO, SHI, TAN, MIT, WHA, OAK.

3.2 Geochemical marker results

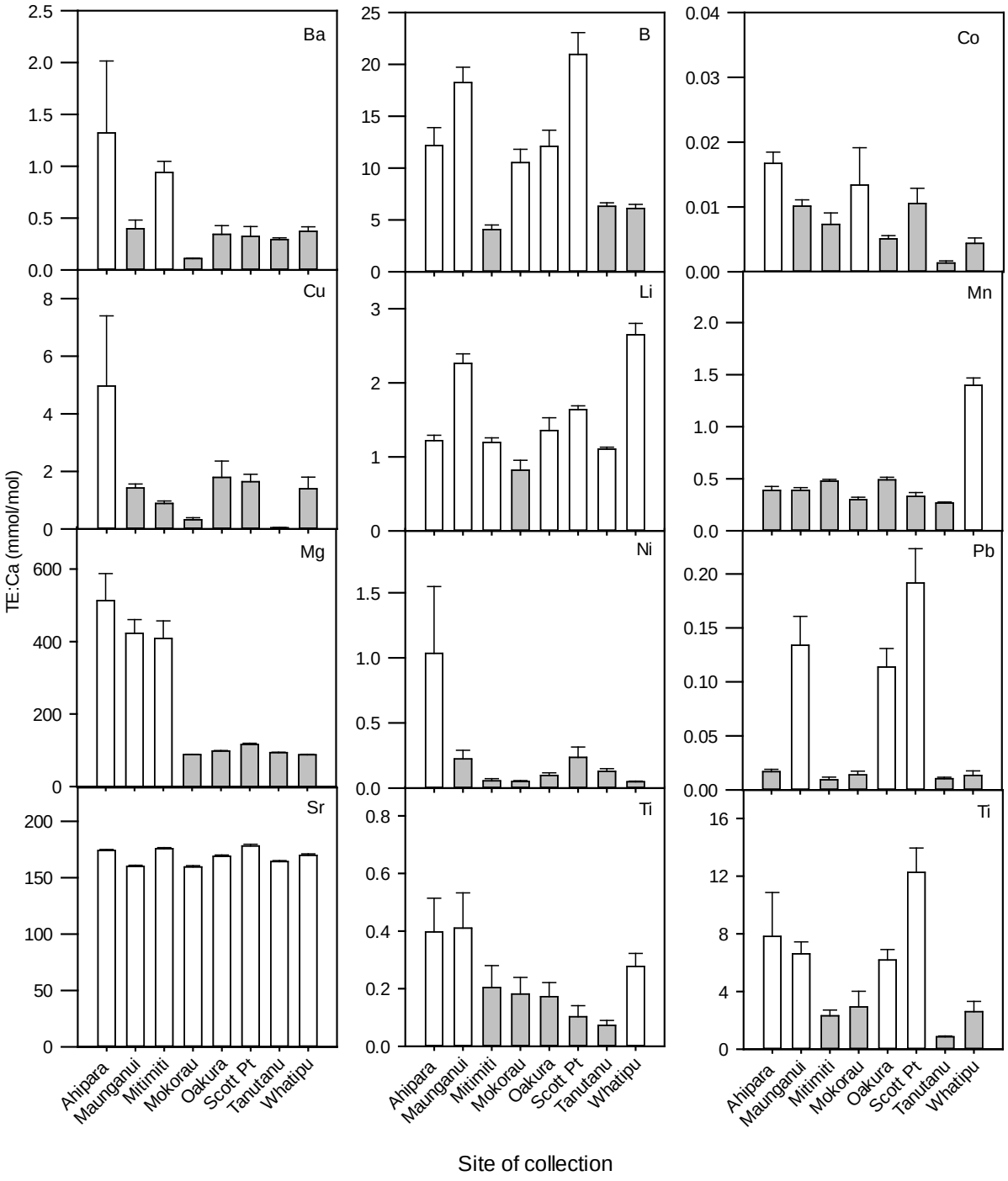

Figure 3.5: Elemental ratios (mean ± S.E.M.) quantified via ICP-MS in shells of juvenile green-lipped mussels (*P. canaliculus*) collected from the west coast of the North Island, New Zealand in January 2015.

Levels of elements varied among sites (Figure 3.5) with Sr and Mg being the most abundant, and Co and Pb the least abundant of the 12 elements quantified in *P. canaliculus* shells.

Spatial variation in elemental ratios of eight sites

Running the Q-DFA with the addition of the two extra sites of Maunganui Bluff and Mokorau Stream revealed that eight elemental ratios (Sr:Ca, Zn:Ca, Ba:Ca, Ni:Ca, Mn:Ca, Co:Ca, Cu:Ca, and Li:Ca) dictated the majority of the classification success of the Q-DFA (Table 3.6). Furthermore, these results are supported by the overall percent classified figures of the Q-DFA model whereby 95.2% of shells were classified to their site of collection when using the above eight ratios. The addition of further elemental ratios i.e., Ti, Ba, and Mg:Ca, provided marginal to no improvement in classification success (Table 3.6).

Table 3.6: Results of stepwise variable selection of discriminant function analysis model for comparing elemental ratios of juvenile shells of green-lipped mussel, *Perna canaliculus*, for mussel shells collected from Scott Pt, Maunganui Bluff, Ahipara, Mokorau Stream, Tanutanu Beach, Mitimiti, Whatipu and Oakura in January 2015. Shaded rows denote the elemental ratios used in subsequent quadratic discriminant function analyses (Q-DFA).

Elemental ratio (*X*:Ca)	Total % classified
Sr	42.7
Zn	63.1
Ba	79.4
Ni	84.4
Mn	91.2
Co	92.0
Cu	93.5
Li	95.2
Ti	95.5
Ba	97.5
Mg	97.7

Position of sites within canonical score 1 and 2 is indicated in Figure 3.6 which shows an excellent degree of separation among sites, and no overlap of 95% confidence intervals.

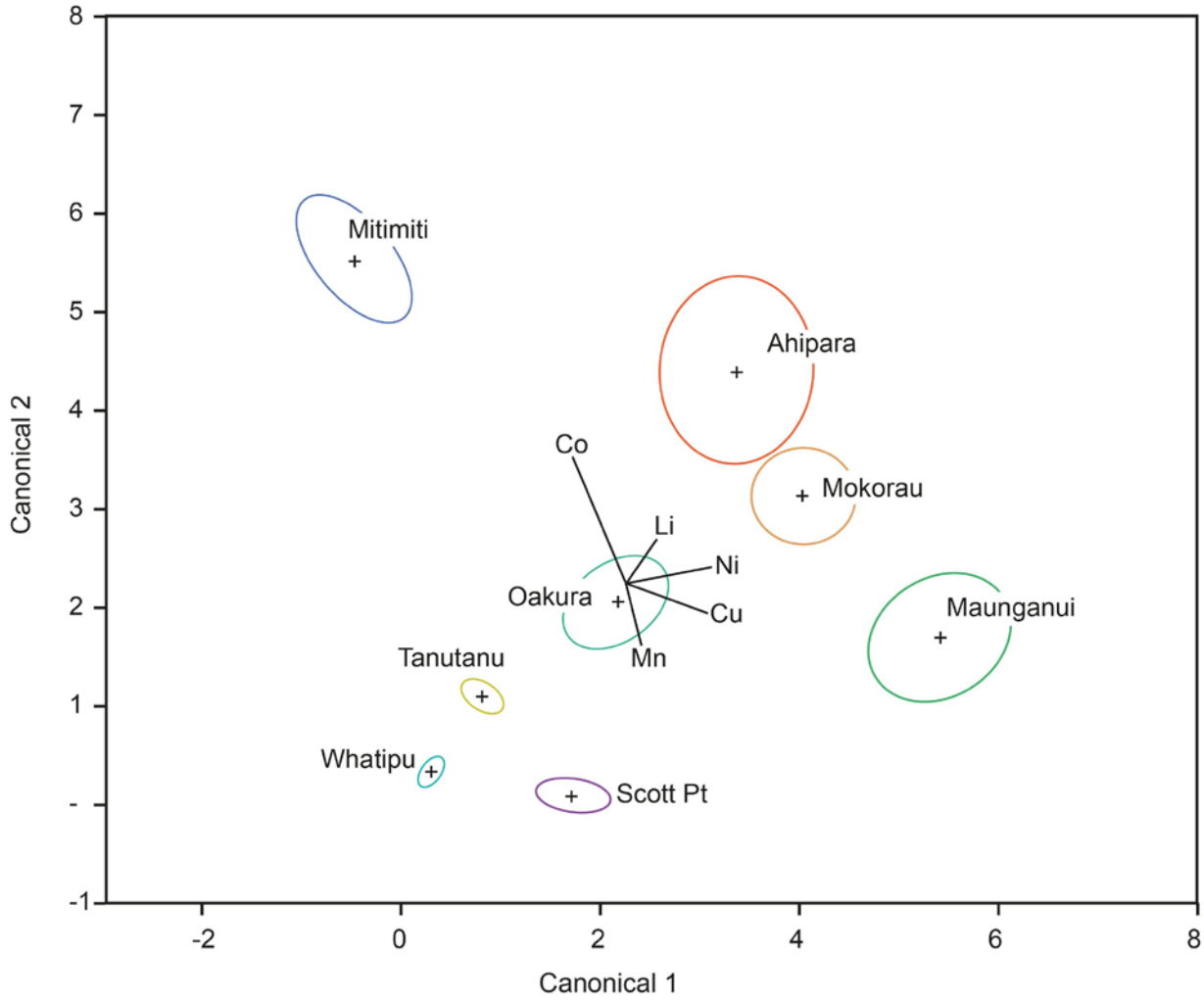

Figure 3.6: Average and 95% confidence intervals (ellipse) of canonical scores from quadratic discriminant function analysis of Sr, Zn, Ba, Ni, Mn, Co, Cu, and Li:Ca ratios in early juvenile shells of *P. canaliculus* collected from eight sites within the North Island of New Zealand in January 2015. Bivariate vector plots of Ba, Zn and Sr:Ca ratios occupy the centre and are too small to be seen.

Whilst the ability of the subsequent Q-DFA to correctly assign mussels to their actual site of collection varied (Table 3.7) the classification success of the model was still considerably high. All mussels collected from Whatipu were correctly assigned back to site of collection (100% classification success), whereas mussels from the remaining sites (Scott Pt, Maunganui Bluff, Ahipara, Mokorau Stream, Tanutanu, Mitimiti and Oakura) were correctly classified back to their point of collection with between 94 and 98% success. In general misclassified shells were assigned to sites immediately adjacent (Table 3.7), the exception being Oakura with shells assigned to Scott Pt, Ahipara and Mokorau, respectively. Finally, sensitivity of the model was high with receiver operating characteristic (ROC) curves for the DFA model ranging between 0.997 and 1.000 (Table 3.7).

Table 3.7: Classification success of the quadratic discriminant function analysis model comparing the elemental ratios of juvenile shells of the green-lipped mussel (*P. canaliculus*) collected from sites encompassing differing spatial scales along the west coast of the North Island of New Zealand January, 2015. Sites listed from most northern to southern. The % Classification success column indicates how many shells collected from a site were correctly assigned back to that site by the Q-DFA.

	Scott Pt	Maunganui	Ahipara	Mokorau	Tanutanu	Mitimiti	Whatipu	Oakura	n	% Classification success	ROC curve
Scott Pt	**48**	0	0	0	0	0	0	1	49	98	0.999
Maunganui	1	**48**	0	1	0	0	0	0	50	96	0.999
Ahipara	0	1	**47**	0	0	1	0	0	49	96	0.997
Mokorau	0	1	1	**48**	0	0	0	0	50	96	0.998
Tanutanu	0	0	0	0	**48**	0	0	2	50	96	1.000
Mitimiti	0	1	2	0	0	**46**	0	1	50	92	0.999
Whatipu	0	0	0	0	0	0	**50**	0	50	100	1.000
Oakura	3	0	1	2	0	0	0	**44**	50	88	0.995

4. DISCUSSION

We have assessed the ability of microsatellite and geochemical marker systems to discriminate juvenile green-lipped mussels collected from six sites along the west coast of the North Island of New Zealand. Where possible, this work has been placed in a larger spatial scale to provide more context and to highlight the relative accuracies of assignment methods as a function of spatial scale.

4.1 Microsatellite markers

As expected, the levels of genetic diversity reported for the six new northern populations using the existing 10 microsatellite markers for *Perna canaliculus* (MacAvoy et al. 2008) are comparable to previously published findings, and their membership of the northern group of populations, as previously defined, is confirmed (Gardner & Wei 2015, Wei et al. 2013). What is particularly interesting and of direct relevance here is the level of genetic differentiation that exists among the six new northern populations, both as a group and also within the context of the 20 New Zealand populations for which we now have data.

For genetic data, the ability to detect the source of spat arriving at GLM 9 depends on levels of genetic variation existing between source populations (or between the source population and all other (non-source) populations) and our ability to employ a genetic marker type that has sufficient power to detect the similarity between spat in GLM 9 and mussels from the source population(s). At the moment it is not clear if the source population is one or many, or if there is, for example, temporal variation in the supply of spat to GLM 9 that reflects different source populations (and this one-off study was never designed to address this question).

Based on assessments of pelagic larval duration and coastal physical oceanography it seems likely that spat arriving at GLM 9 are derived from one or more source populations in the region from Hokianga Harbour south to Taranaki (Alfaro et al. 2010). This is a reasonably large area, spanning several hundreds of kilometres of coastline, much of which is rugged, at times not easily accessible, and is often not particularly well characterised for its biota. Based on earlier work (Gardner & Wei 2015, Wei et al. 2013) we know that this whole region falls into the northern group of *P. canaliculus* populations which tends to be reasonably homogeneous and without much evidence of genetic structure. Wei et al. (2013) showed that the Opononi population (which is located within Hokianga Harbour) was most similar to the Westhaven (on the South Island west coast of Farewell Spit) and Fletcher's Beach (on the South Island, just inside Golden Bay) in their analysis which had no North Island west coast samples south of Opononi. These results suggest that populations within the northern group on the west coast of New Zealand are exchanging genes (larvae), but in the absence of samples between Opononi (about 35.5047 °S) and Westhaven (about 40.5811 °S) or Fletcher's Beach (about 40.6784 °S) a source population for spat at GLM 9 could not be identified.

All of the analyses reported here for molecular variation highlight an informative level of genetic differentiation that exists within or among the six new northern populations, and also among all populations on the west coast of New Zealand. The most northern population of SCO exhibits greatest similarity to the most southern (Taranaki) population of OAK and to the population at TAN. The populations at WHA (west coast of Auckland), MIT and SHI (far north) all show varying degrees of genetic differentiation from the other three new northern populations, sufficient to allow reasonably high (in the range of 30 to 81%, mean of 50%) levels of success with the assignment tests.

The use of microsatellite markers to identify the source population(s) for spat arriving at GLM 9 will ultimately depend on the use of a variety of different assignment tests. The underlying principle is simple – spat at GLM 9 will show high levels of affinity with the source population(s), meaning that the spat cannot easily (with a high probability of success) be differentiated from mussels (the presumptive parental stock) from the source population(s). Populations that do not contribute spat to GLM 9 are expected to be differentiated from the GLM 9 spat and can therefore be assigned with a

high degree of accuracy to their appropriate locations. The tests of genetic variation carried out on the six new northern mussel populations strongly suggest that population-specific levels of multilocus genotypic variation among mussels from locations on the west coast of New Zealand, at least as far south as Westhaven, are sufficient to permit successful assignment testing of the origin of spat at GLM 9 at rates in the range of 50 to 70%.

Assignment testing, by its very nature, is only as informative as the populations that are sampled. If, for example, the source population of mussels for spat at GLM 9 is a large bed off the Taranaki coast that is not sampled in subsequent testing, then the power of the testing is, of course, greatly reduced. Nonetheless, based on what is presently known, the results reported here strongly suggest that multi-allelic microsatellite markers have sufficient population-specific differences as multilocus genotypes to permit identification of the source population(s) for spat at GLM 9 if a more comprehensive study involving the collection of mussels from many more locations is carried out. Two further important points need consideration.

4.2 Geochemical markers

Although not contracted to do so, we were able to incorporate an extra two sites (Maunganui Bluff and Mokorau Stream) in our analyses. These had the benefit of further reducing the spatial distance between sites, and for Maunganui Bluff, to incorporate a significant coastal feature along an otherwise vast sandy stretch of coastline. Eight elemental ratios (Sr, Zn, Ba, Ni, Mn, Co, Cu and Li:Ca) were able to provide an excellent level of discrimination, assigning mussels back to their site of origin with 88–100% success. This finding is valuable for future studies looking to use this method to discriminate mussel populations along this coast by reducing the number of elemental ratios needed to be analysed, thus optimising analytical procedures and reducing costs. It is nonetheless, a higher number than Becker et al. (2005) required to distinguish populations of *Mytilus californianus* and *M. galloprovincialis* along the California coast. In their study, populations of these two species could be discriminated using the three ratios of Ca to Pb, Ba and Mn. However, it is similar to the number needed by Cathey et al. (2014) to discriminate populations of the Northern quahog (*Mercenaria mercenaria*) using ICP-MS. Furthermore, incorporating these two extra sites allowed us to discriminate sites ranging over a range of scales, that is, between hundreds of kilometres (i.e. Whatipu and Oakura), down to tens of kilometres (Scott Pt, Maunganui Bluff, Tanutanu, Mitimiti).

Reliable discrimination of mussels from sites encompassing similar distances have been recorded for a number of marine taxa, including the Olympia oyster, *Ostrea lurida* (Carson 2010) and the Chilean abalone, *Concholepas concholepas* (Zacherl et al. 2003). With regard to mussels, Sorte et al. (2013) were able to discriminate blue mussel (*M. edulis*) populations at approximately 50 km distance using geochemical tags whereas Becker et al. (2005) could distinguish populations of *M. californianus* and *M. galloprovincialis* 20 km apart. Finally, Dunphy et al. (2011) were able to discriminate green-lipped mussel populations at a distance down to 12 km at the more southerly locations within the Auckland region. Perhaps of greater importance is that in this study we were able to discriminate green-lipped mussels collected from sites separated by as little as 4 km (i.e., Ahipara and Mokorau Stream) with up to 96% classification success. Such a result appears unprecedented in terms of spatial discrimination for a marine invertebrate population occupying the open coast, with 10–20 km tending to be the normal limit (Cathey et al. 2014).

Comparing the elemental ratios that provided the majority of classification success in this study with data in Dunphy et al. (2011) reveals Ca:Zn, Mn, Sr, Ba and Cu ratios to be integral in discriminating west coast populations in Northland, despite the four year gap between the respective studies. This information is valuable as it limits the number of elements needing to be quantified in future geochemical tag studies on this species along this coast.

Populations occupying estuaries or those near streams or rivers are often easily discriminated using geochemical tags (Becker et al. 2005, Gillanders et al. 2001) because localised terrigenous input of elements with freshwater is important in controlling the availability of at least some elements (Burton

1976). Oakura, Whatipu and Mokorau Stream are located near consistent sources of freshwater and accordingly were readily distinguished from other sites. The remaining sites were also able to be discriminated from one another with equal if not better success. Reasons for this could lie in the fact that the west coast of the North Island of New Zealand is an area of active terrigenous sedimentation due to the active plate boundary running through New Zealand (Gillespie & Nelson 1996). This results in considerable uplift with erosion through a varied mix of volcanic and sedimentary rock catchments (Churchman et al. 1988, Nelson et al. 1988). In Northland the geology comprises a complex mix of allochthononous igneous rock, Miocene sedimentary rock and mixed accumulated sediments from the Pleistocene and Holocene periods (Payne 2008). Accordingly, it may well be that the elemental composition of surrounding coastal waters is complex due to being fed by riverine inputs draining a complex geological landscape.

Biological and environmental controls on the incorporation of elements into bivalve shell are equally important. For example, the uptake of magnesium and strontium are known to be controlled by physiological processes (i.e. diffusion across cell membranes) as opposed to abiotic controls on crystal formation (Gillikin et al. 2005, Lorens & Bender 1980). Incorporation of manganese can be affected by microbial action which form manganese oxides and thus remove manganese ions from surrounding waters, which may lead to increased misclassification rates (Klinkhammer & McManus 2001). For those elements whose incorporation into calcite/aragonite structures is influenced by temperature e.g. Ba, spatial variation in element concentrations may not be important as long as consistent differences in environmental temperature are present to create differential uptake among sites of interest (Strasser et al. 2008).

Whilst the exact mechanisms of elemental incorporation into bivalve shell may be more of academic interest the spatio-temporal stability of geochemical tags is vital if they are to be informative. Some species, such as the gastropod *Kelletia kelletii*, demonstrate significant temporal variability in geochemical tags among years (Zacherl 2005), whereas other invertebrates show stability in geochemical tags between seasons i.e. 6–8 months e.g. *Mercenaria mercenaria* (Cathey et al. 2014). Whilst geochemical tags were shown to be consistent in green-lipped mussels by Dunphy et al. (2011) this was for one site and over a 6 month window. Thus, it would be prudent to devote more effort to characterising the temporal stability (or lack thereof) of geochemical tags in shells of this species at a greater range of sites for longer time periods.

Limitations of ICP-MS

Lastly, one limitation of the ICP-MS approach is the destruction of the whole shell, thus the geochemical tags of early D-stage larval structures are unable to be compared to later post settlement shell structures. One method to overcome this in future work is the use of laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) which can target and analyse small (approximately 20 µm) portions of shell. Traditionally, this has meant comparing Prodissoconch shell with late-settler stages. Such methods are reliant upon the generation of reference signatures whereby hatchery reared larvae are housed within specially designed rearing tubes and deployed at select locations along the coast (i.e., Becker et al. 2007, Gomes et al. 2014). Here they are allowed to grow for a short period and briefly incorporate the local elemental "signature" of the surrounding waters. Newly settled larvae at the site of interest (i.e. Ninety Mile Beach) are then analysed and via a DFA matched back to their site of origin using the reference signatures generated above. Due consideration to appropriate experimental design, on both spatial and temporal scales, would be required; as would the logistics of working on a very turbulent and challenging coastline. But with the right resourcing is not impossible.

5. MANAGEMENT IMPLICATIONS

Firstly, it seems unlikely based on what we know of mussel connectivity and recruitment dynamics that the spat at GLM 9 are derived from a single source population. It seems far more likely that the spat are a mix of recruits from several source populations. This presents a challenge to the identification of the source populations, given that the genetic signal of the spat at GLM 9 will exhibit

a mixed genetic signal, reflecting the extent of contribution from the different source populations. Interpreting this mixed signal is difficult, but not impossible, and is akin to the use of mixed source models employed to test the origin of stable isotopes in diet analysis (e.g.,Phillips 2008, Phillips & Gregg 2003, Phillips et al. 2005). It is also important to bear in mind that the spat arriving at GLM 9 at different times of the year may originate from different source populations, with the result that identifying a single source population is impossible and the identification of multiple source populations at different times of the year is practically very difficult.

Sufficient genetic diversity exists within the six new northern populations when assessed with ten highly polymorphic microsatellite loci to indicate that mussels from these populations can be reasonably accurately assigned to population of origin. The similarity of the OAK (Taranaki) population to at least two of the most northern populations is, at the very least, intriguing and suggestive of genetic connectivity amongst them. The genetic differentiation of populations such as MIT and WHA is informative insomuch as it demonstrates a reduced level of genetic connectivity between this pair of populations and the other four populations used in this study. There is clearly enough genetic variation amongst these six populations to support the suggestion that a wide scale sampling programme, replicated in time and space, can answer the question about the provenance of *P. canaliculus* spat at GLM 9.

For the ICP-MS work we have shown this method to have an excellent discriminatory capacity and to be able to discriminate populations separated by as little as 5 km along an open coast. In this (and previous work) the elemental ratios of Sr, Mn, Cu, Ba, and Zn:Ca appear to consistently discriminate mussel populations along the west coast of northern New Zealand and can form the basis of future investigation on geochemical tags for this species along this coast. Furthermore, it is vital that the spatio-temporal stability of these geochemical tags along this coastline be confirmed. Finally, given the differing pieces of information they can provide, a combined genetic and geochemical approach may be warranted. (Tanner et al. 2014) used this approach for delineating stocks of Mediterranean hake (*Merluccius merluccius*) and found that microsatellite methods provided broad scale geospatial information of gene flow on evolutionary timescales, whereas geochemical methods could provide fine scale geospatial information on ecological relevant timescales.

For any long term temporal study to be undertaken cost will be the deciding factor. Both the genetic and of geochemical methods applied here were approximately equal in terms of respective costs. However, if newer microsatellites are needed to be developed then this would result in an increase of approximately 50% of what was budgeted in this study (i.e. NZ$ 50 000).

6. ACKNOWLEDGMENTS

We wish to acknowledge Stuart Morrow (UoA) for ICP-MS work. Louis Ranjard and Craig Norrie assisted with sample collection and processing. Hiku o Te Ika Fisheries Forum, Maunganui Bluff Trustees, and Te Runanga o Te Aupouri provided site access and logistical support. This work was made possible by the generous funding provided by the Ministry of Primary Industries, Contract GLM2013/01.

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8. APPENDICES

This section contains the results of analyses of genetic data for the six new west coast North Island populations. Multi-allelic scoring between the present and the published study of 14 New Zealand populations (Wei et al. 2013) is consistent.

Additionally a subset of geochemical analyses is provided that includes the six sites originally contracted to be analysed in contract GLM2013/01

Appendix I – Table of pairwise F_{ST} values for the six new northern populations

Table A.1: Pairwise F_{ST} values for the six new northern populations of *Perna canaliculus* using 10 loci are below the diagonal and F'_{ST} values are above the diagonal. Significant values after FDR testing are in bold (significance testing for F'_{ST} values is not possible).

Code	SCO	SHI	TAN	MIT	WHA	OAK
SCO		0.000	0.000	0.000	0.000	0.000
SHI	**0.001**		0.000	0.020	0.000	0.000
TAN	0.000	**0.000**		0.009	0.000	0.000
MIT	0.001	**0.009**	**0.007**		0.000	0.020
WHA	0.000	**0.000**	0.000	**0.004**		0.000
OAK	0.000	**0.002**	0.000	**0.011**	0.005	

Population pairwise estimates of F_{ST} and F'_{ST} were all very low (Table A.1). This is to be expected given the geographic proximity of the sample collection sites. Nonetheless, eight of 15 tests for pairwise differences of F_{ST} values were significant, and this included all 5 tests involving the SHI population (Shipwreck Bay) and 4 of the 5 tests for the MIT (Mitimiti) population.

Appendix II – UPGMA tree for the six new northern populations

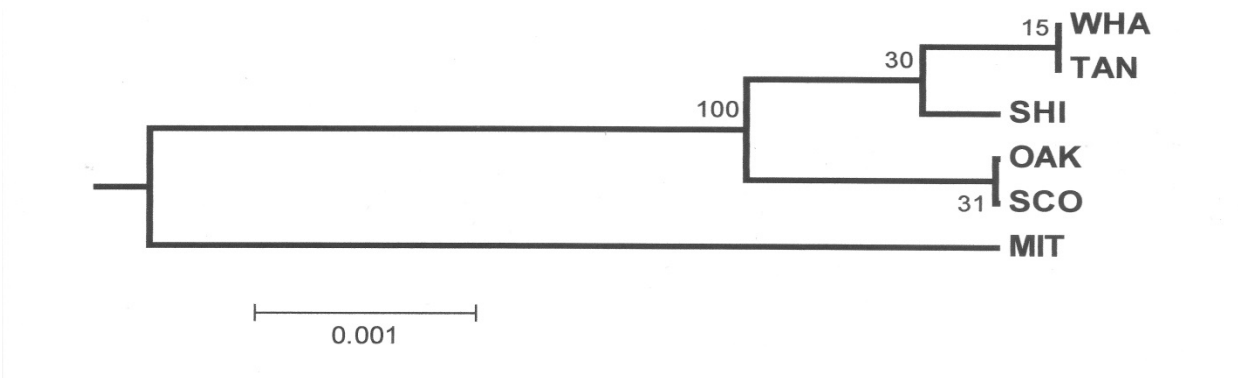


Figure A.2: Relationships among six northern mussel populations inferred from a UPGMA tree reconstructed on the basis of Nei's corrected F_{ST} values (percentage bootstrap support values are indicated at nodes).

The UPGMA tree of population relatedness indicates that the MIT population is the most distantly related of the six new northern populations (Fig. A.2). The split between the OAK and SCO versus the SHI, TAN and WHA populations is very well supported (100% bootstrap support), providing evidence of genetic differentiation among the six new mussel populations.

Appendix III - Principal coordinate analysis (PCoA) for the six new northern populations

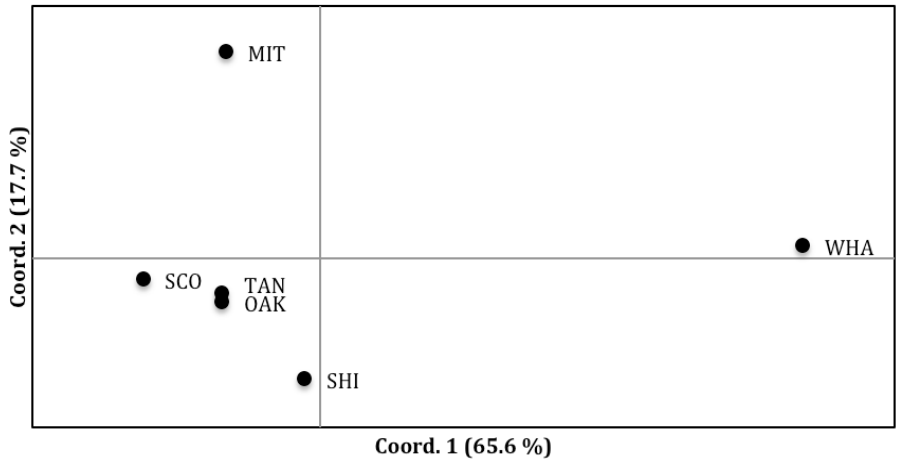


Figure A.3: Principal coordinate analysis (PCoA) for six populations of *Perna canaliculus* using pairwise genetic distance based on variation at 10 microsatellite loci.

Principal coordinate analysis (PCoA) for the six new northern populations (Fig. A.3) generally supports the findings of the other analyses. The WHA, MIT and SHI populations are respectively, the most dissimilar from the main grouping of the SCO, TAN and OAK populations. There is no obvious association between geography and the results of the PCoA plot.

Appendix IV - Analysis of molecular variance (AMOVA) for the six new northern populations

Table A.4: Analysis of molecular variance (AMOVA) for six populations from 3 regions (MIT, WHA and remaining populations) using 10 loci.

Source of variation	Degrees of Freedom	Sum of Squares	Estimated Variance	% Variation	Differentiation Indexes	Significance
Among Regions	2	27.7	0.058	1.5%	0.015	0.001
Among Populations	3	18.6	0.017	0.4%	0.004	0.006
Among Individuals	282	1272.7	0.773	20.3%	0.020	0.001
Within Populations	288	854.5	2.967	77.8%	0.207	0.001
Total	575	2173.6	3.815	100%	0.222	0.001

Analysis of molecular variance (AMOVA) for the six new northern populations by region (Table A.4) revealed significant differences among regions ($p < 0.001$) and among populations ($p < 0.001$). Although in both cases the percent of the variation explained was small (1.5% and 0.4%, respectively) these results provide evidence of genetic differentiation among mussels from the six populations along the west coast of the North Island.

Appendix V - STRUCTURE analysis of the six new northern populations

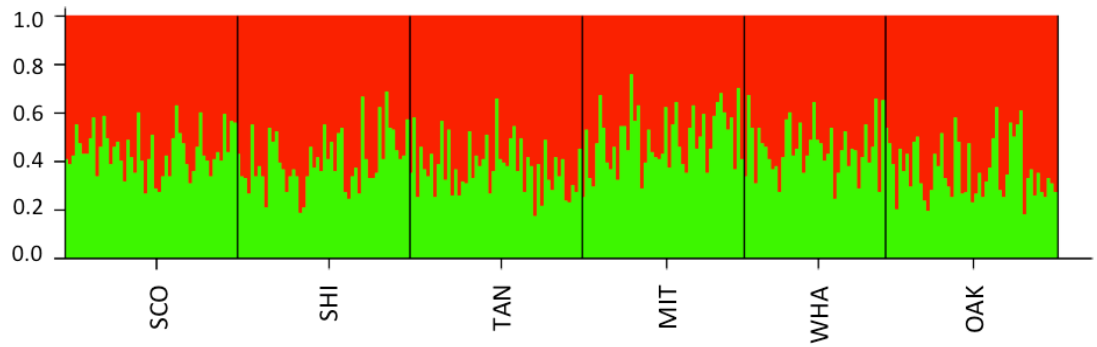
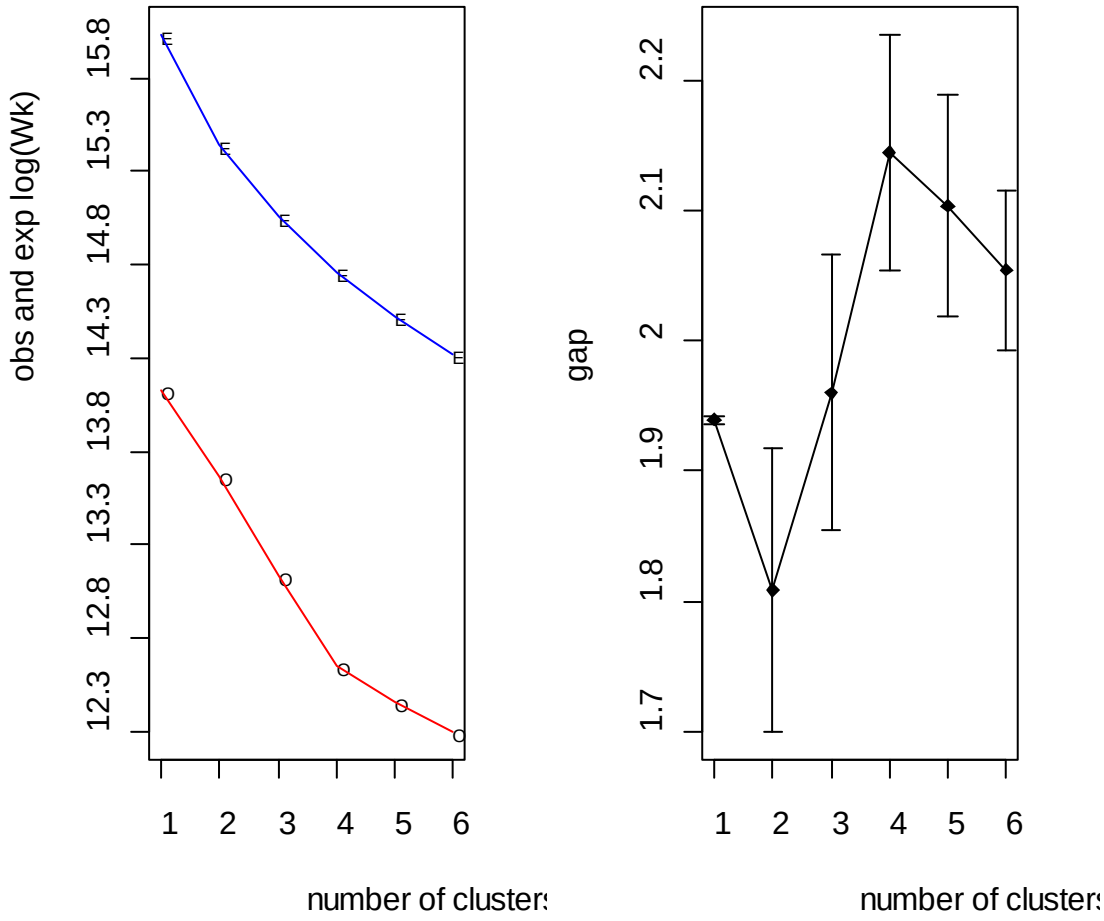


Figure A.5: Bayesian cluster analysis (STRUCTURE) results for *Perna canaliculus* using 10 loci for individuals collected in 2015 from six locations (K=2). Populations are arranged from north to south (left to right).

STRUCTURE analysis of the six new northern populations revealed very little specific differentiation among the populations (Fig. A.5). The best fit was K=2 groups of mussels (the red and the green), but population-specific membership of these two groups was approximately equal (i.e. the amount of red and green in the plot for each population was about the same).

Appendix VI – AWclust results for the six new northern New Zealand populations



Gap plot (to the right) shows that the most likely number of separate groupings is K = 2.

WHA	OAK	SHI	SCO	TAN	MIT
WHA210 1	OAK251 1	SHI400 1	SCO451 1	TAN550 1	MIT600 1
WHA211 2	OAK252 1	SHI401 1	SCO452 1	TAN551 2	MIT601 2
WHA212 1	OAK253 1	SHI402 1	SCO453 1	TAN552 2	MIT602 1
WHA213 1	OAK254 1	SHI403 1	SCO454 1	TAN553 2	MIT603 1
WHA214 1	OAK255 2	SHI404 2	SCO455 1	TAN554 1	MIT604 1
WHA215 1	OAK256 1	SHI405 1	SCO456 1	TAN555 1	MIT605 2
WHA216 1	OAK257 2	SHI406 1	SCO457 1	TAN556 1	MIT606 1
WHA217 1	OAK258 1	SHI407 1	SCO458 1	TAN557 1	MIT607 1
WHA218 1	OAK259 1	SHI408 1	SCO459 1	TAN558 1	MIT608 2
WHA219 2	OAK260 1	SHI409 1	SCO460 1	TAN559 2	MIT609 2
WHA220 1	OAK261 1	SHI410 1	SCO461 1	TAN560 1	MIT610 1
WHA221 1	OAK262 1	SHI411 1	SCO462 1	TAN561 1	MIT611 1
WHA222 1	OAK263 1	SHI412 1	SCO463 1	TAN562 1	MIT612 1
WHA223 2	OAK264 1	SHI413 1	SCO464 1	TAN563 1	MIT613 1
WHA224 2	OAK265 1	SHI414 1	SCO465 1	TAN564 1	MIT614 2
WHA225 1	OAK266 1	SHI415 1	SCO466 1	TAN565 1	MIT615 1
WHA226 2	OAK267 2	SHI416 1	SCO467 2	TAN566 1	MIT616 1
WHA227 1	OAK268 2	SHI417 1	SCO468 2	TAN567 2	MIT617 1
WHA228 2	OAK269 1	SHI418 1	SCO469 2	TAN568 2	MIT618 1
WHA229 1	OAK270 1	SHI419 1	SCO470 1	TAN569 2	MIT619 1
WHA230 1	OAK271 2	SHI420 1	SCO471 1	TAN570 1	MIT620 2
WHA231 1	OAK272 2	SHI421 1	SCO472 1	TAN571 1	MIT621 1
WHA232 1	OAK273 1	SHI422 1	SCO473 1	TAN572 1	MIT622 2
WHA233 1	OAK274 1	SHI423 1	SCO474 1	TAN573 1	MIT623 1
WHA234 1	OAK275 1	SHI424 1	SCO475 2	TAN574 2	MIT624 2
WHA235 1	OAK276 1	SHI425 1	SCO476 2	TAN575 2	MIT625 1
WHA236 1	OAK277 1	SHI426 1	SCO477 1	TAN576 1	MIT626 1
WHA237 1	OAK278 1	SHI427 1	SCO478 1	TAN577 2	MIT627 1
WHA238 1	OAK279 1	SHI428 1	SCO479 2	TAN578 1	MIT628 1
WHA239 1	OAK280 1	SHI429 2	SCO480 1	TAN579 2	MIT629 1

Table of group membership for all mussels from the six new northern New Zealand populations. Columns = populations, codes as in Figure 1. WHA210 1 – indicates that individual mussel called WHA210 belongs to group number 1.

Appendix VII - Estimates of migration

Table A.7: Percentage of individuals of *Perna canaliculus* collected from each sampling location, assigned to each sampled potential source location. "Total other locations" is the percentage of individuals recruited from locations other than the collection site. Bolded and italicised text indicates the percentage of individuals assigned to their source population.

Location	Assigned location							Total other locations
	SCO	SHI	TAN	MIT	WHA	OAK	Unassigned	
SCO	***29.8***	2.1	4.3	2.1	51.1	10.6	0	70.2
SHI	4.0	***46.0***	8.0	0	36.0	6.0	0	54.0
TAN	0	2.0	***32.6***	4.1	53.1	8.2	0	67.4
MIT	2.3	2.3	0	***59.1***	34.0	2.3	0	40.9
WHA	10.5	2.6	2.6	0	***79.0***	5.3	0	21.0
OAK	6.1	4.1	2.0	0	34.7	***53.1***	0	46.9

Assignment analysis (Table A.7) revealed that mussels from a location could be accurately assigned back to that location with a range of percentage probabilities, from a low of about 30% (SCO) to a high of about 80% (WHA). The mean ± SD assignment probability for all six populations was 49.9% ± 18.2. For these assignment tests to work with any degree of accuracy some level of genetic differentiation is required between pairs of populations (i.e., assignment accuracy will tend towards zero when all populations are genetically identical, and will tend towards 100% when all populations are genetically completely differentiated). Thus, the assignment estimates presented here (Table A.7) support the suggestion that six populations from the west coast of the North Island are genetically differentiated. Mean ± SD assignment probability for the four most northern populations that were only 124 km apart was 41.9% ± 13.5, indicating that even at a small spatial scale, genetic differences exist amongst the populations.

Table A.7.1: Number of first generation (F0) migrants detected among six populations of *Perna canaliculus* at each sampled location (n=50 from each location).

Receiving location	Source of F0 migrants						Total F0 migrants
	SCO	SHI	TAN	MIT	WHA	OAK	
SCO		1		1	1		3
SHI					1		1
TAN				1			1
MIT	2					1	3
WHA	1	2					3
OAK	1						1

Estimates of the number of migrants between pairs of populations from the six new northern populations are low (Table A.7.1). In total, only 12 migrants are predicted among the six populations. Because the numbers of migrants from source population to receiving location are so low, it is not possible to identify individual populations that are particularly important as sources or receiving locations. The general interpretation here is that low levels of gene flow (connectivity) exist among the six populations.

Appendix VIII

Spatial variation in elemental ratios of six sites (as specified in contract)

Running a stepwise variable selection procedure revealed that five elemental ratios (B:Ca, Ba:Ca, Co:Ca, Mn:Ca, and Zn:Ca) dictated the majority of the classification success of the Q-DFA (Table A.8). Furthermore, these results are supported by the overall percent classified figures of the Q-DFA model in which 94.3% of shells were classified to their site of collection when using the above five ratios. There was marginal to no improvement in classification success by the addition of further elemental ratios i.e., Ni, Ti, Sr, Li, Mg and Cu:Ca (Table A.8).

Table A.8: Results of stepwise variable selection of discriminant function analysis model for comparing elemental ratios of juvenile shells of green-lipped mussel, *Perna canaliculus*, for mussel shells collected from Scott Pt, Ahipara, Tanutanu Beach, Mitimiti, Whatipu and Oakura in January 2015. Shaded rows denote the elemental ratios used in subsequent quadratic discriminant function analyses (Q-DFA).

Elemental ratio (*X*:Ca)	Total % classified
B	61.4
Ba	76.2
Co	85.9
Mn	94.3
Zn	94.3
Ni	95.0
Ti	96.3
Sr	97.0
Li	97.7
Mg	98.0
Cu	98.0

Position of the six sites within Canonical score 1 and 2 is indicated in Figure A.8 which shows an excellent degree of separation among sites, and no overlap of 95% confidence interval ellipses.

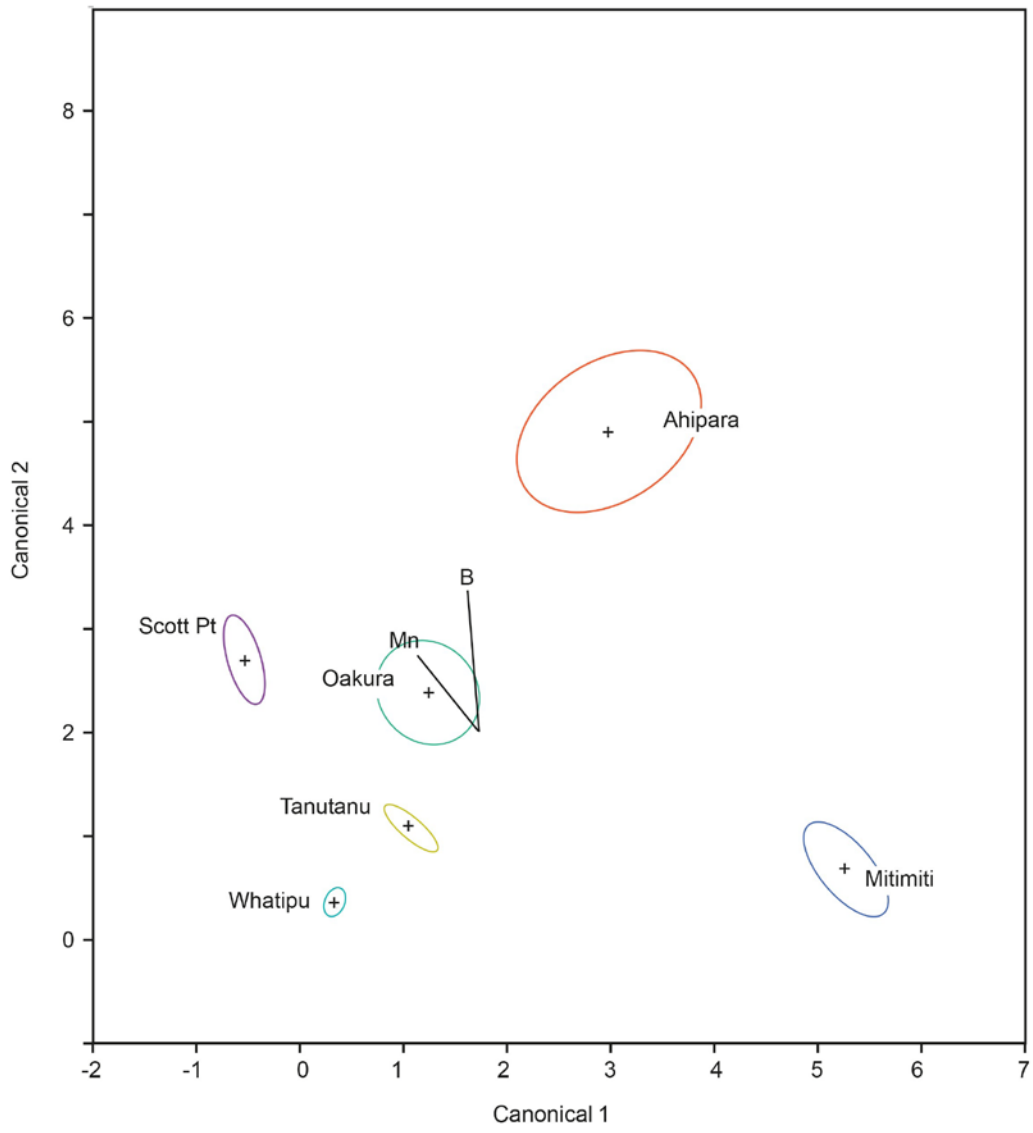


Figure A.8: Average (cross symbol) and 95% confidence intervals (ellipse) of canonical scores from quadratic discriminant function analysis of B, Mn, Mg, Ni, Ba, and Zn:Ca ratios in early juvenile shells of *P. canaliculus* collected from six sites within the North Island of New Zealand in January 2015. Bivariate vector plots of Ba, Co and Zn:Ca ratios occupy the centre and are too small to be seen.

The ability of the subsequent Q-DFA to correctly assign mussels to their actual site of collection varied (Table A.8.1), with mussels from Scott Pt, Ahipara, Tanutanu, Mitimiti and Whatipu able to be assigned back to their site of collection with an excellent level of discrimination e.g., between 100 – 92 % classification success. Conversely, the Q-DFA performed less well in assigning shells collected from Oakura to their correct site of collection (88% classification success) with a small number of shells assigned to the most northern sites of Scott Pt, Ahipara and Mitimiti. Nonetheless, receiver operating characteristic (ROC) curves were high for the DFA model (range 0.9843 – 0.9995) indicating that the sensitivity of the Q-DFA was high.

Table A.8.1: Classification success of the quadratic discriminant function analysis model comparing the elemental ratios of juvenile shells of the green-lipped mussel (*P. canaliculus*) collected from sites encompassing differing spatial scales along the west coast of the North Island of New Zealand. Sites listed from most northern to southern.

	Predicted collection site								
Collection site	**Scott Pt**	**Ahipara**	**Tanutanu**	**Mitimiti**	**Whatipu**	**Oakura**	**n**	**% Correct**	**ROC Curve**
Scott Pt	**47**	1	0	0	1	0	49	96	0.9970
Ahipara	0	**47**	0	1	0	1	49	96	0.9921
Tanutanu	0	0	**48**	0	0	2	50	96	0.9995
Mitimiti	0	2	0	**46**	0	2	50	92	0.9981
Whatipu	0	0	0	0	**50**	0	50	100	0.9992
Oakura	2	4	0	1	0	**43**	50	86	0.9843