A population genetics approach to species delimitation in the genus *Selliera* (Goodeniaceae).

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Kay Margaret Pilkington

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ABSTRACT

Selliera is a genus in the Goodeniaceae described as a small creeping herb. Currently there is only one internationally recognised species of *Selliera, Selliera radicans*. In New Zealand, three species have been described based on morphology and geographic location although there is disagreement about whether these actually constitute different species. *Selliera rotundifolia* is distinguished from *S. radicans* by rounder leaves and a preferred dune habitat compared to the estuary habitat of *S. radicans. Selliera microphylla* is distinguished from *S. radicans* by a smaller size and inland location. However, *S. microphylla* reverts to a size similar to *S. radicans* when grown in the same environment, but a single chromosome count for *S. microphylla* on the Central Volcanic Plateau is 2*n*=56. Both *S. rotundifolia* and *S. radicans* have chromosome counts of 2*n*=16. Species delimitation is important in biology, conservation, and evolutionary studies but remains a difficult task. I applied a population genetics approach combined with morphological analysis of leaves and existing karyotype data to determine the species boundaries within *Selliera.*

Microsatellite markers are ideal for use in population genetics due to the higher mutation rate, genotyping ease and their co-dominant nature. No microsatellite markers previously existed for use in *Selliera.* In this study, next generation sequencing was used to develop microsatellite markers for *Selliera.* From 8,101 independent sequence contigs, 107 microsatellite loci were detected and primer pairs designed for these. Forty-three of these primer pairs were chosen to be screened and nine of these were reliably amplifiable and polymorphic. These nine markers were genotyped over 618 samples from *Selliera* comprising the three described species.

Populations within all three described species showed high differentiation and *S. radicans* was variable for population structure. Leaf morphological analyses suggested there was a distinct difference between the three species. Microsatellite data revealed two genetic clusters in *S. microphylla* which clustered into the North Island and South Island populations. Two genetic clusters were also observed in *S. rotundifolia* which each clustered with different *S. radicans* populations suggesting

round leaves may have had multiple origins. Hybridization was observed at one sympatric site between *S. radicans* and *S. rotundifolia* and apparent reproductive isolation for *S. rotundifolia* was observed at another site.

These results suggest that the South Island *S. microphylla* population may be an inland variant of *S. radicans* which may continue to diverge if it remains isolated, while the North Island populations should retain the *S. microphylla* name due to the $2n$=56 chromosome count, geographic isolation and genetic distinction although this needs further review. There is evidence of reproductive isolation for *S. rotundifolia* at one of the sympatric sites suggesting this is a distinct species but it appears round leaves may have had multiple origins so may not be suitable to describe the species according to the lineage species concept. This study provides insights into the population structure within and between the described species and has identified interesting areas of future study.

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ABBREVIATIONS

%P	- percentage polymorphic loci
A	- number of alleles
AMOVA	- Analysis of molecular variance
ANOVA	- Analysis of variance
bp	- base pairs
cm	- centimeters
CTAB	- cetyltrimethylammonium bromide
d.f	- degrees of freedom
DNA	- deoxyribonucleic acid
dNTP	- deoxyribonucleotide triphosphate
F_{IS}	- factor in Sewell Wrights F statistic. F_{IS} is the inbreeding coefficient that is the proportion of the variance in the subpopulation within an individual.
FIJI	- FIJI is just imageJ (image analysis software)
F_{ST}	- factor in Sewell Wrights F statistic. F_{ST} is the proportion of the total genetic variance in the subpopulation contained in an individual.
G_{ST}	- coefficient of genetic differentiation. Defined as the expected heterozygosity for the total population minus the expected heterozygosity within the subpopulations divided by the expected heterozygosity for the total population.
Ho	- observed heterozygosity
He	- expected heterozygosity
H_T	- species-wide expected heterozygosity
ISSR	- inter simple sequence repeat
ITS	- internal transcribed spacer
n	– chromosome number in a haploid
N	- number of individuals
NA	- number of alleles
Ne	- number of effective alleles
NZGL	- New Zealand Genomic Limited

P value	- probability of obtaining a test statistic at least as extreme as the one observed assuming the null hypothesis is true
PCR	– polymerase chain reaction
RNA	- ribonucleic acid
Rxy	- correlation coefficient of Mantel test
SNP	- single nucleotide polymorphism
STE	- sucrose, tris, EDTA
Ta	- melting temperature
TE	- Tris-EDTA buffer

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Chapter 1

Introduction

1.1 The Genus Selliera

Selliera Cav. is a genus within the Goodeniaceae family. The Goodeniaceae are predominately found in the Southern Hemisphere and consists of 12 genera and at least 300 species (Allan 1961). They are easily distinguished by the indusium, a unique cup-shaped structure, located at the top of the style. In New Zealand only four indigenous species are present; three endemic species and one non-endemic (FloraCommittee. 2013). *Selliera* is described as a small creeping herb (Allan 1961) and occurs in Australia, Chile and New Zealand. There are three described species in New Zealand, *Selliera radicans* Cav., *S. rotundifolia* Heenan., and *S. microphylla* Colenso. In 1876, Cheeseman reported findings on fertilization in *S. radicans* suggesting that self-fertilization cannot take place as the pollen is shed and dispersed long before the stigma is mature. Therefore transfer of pollen is thought to be carried out by insects, primarily a species of Diptera although other insects have been observed visiting the flowers. However, although this suggests *Selliera* is an out-crosser, all species can also be clonally propagated easily from division of whole plants.

1.1.1 *Selliera* in New Zealand

Selliera radicans was first described in 1799. *Selliera radicans* is described with spathulate elongated leaves (Figure 1.1) but is noted to be highly polymorphic in form and size (Allan 1961) and occurs in Australia, Chile and New Zealand. It has a chromosome number of 2*n*=16 (Dawson et al. 2000) although a count of 2*n*=48 has been reported in Tasmania (Jackson 1958). It mainly occurs in coastal estuary habitats, although it can be found in dune habitats, and has also been recorded inland in the South Island and around Lake Taupo on the North Island (Allan 1961). Ogden (1974) performed experimental garden studies with spathulate–leaved *S. radicans* from an estuary habitat and a round-leaved dune form collected from the Manawatu coast. These two forms retained the leaf shape and dimensions when grown in identical conditions implying they are genetically distinct although

Ogden still regarded these forms as ecotypes rather than distinct species (Ogden 1974).

In 1997, Heenen described the round-leaved "ecotype" as a new species, *Selliera rotundifolia* as the distinguishing characteristics of the two forms were retained in identical environments. *S. rotundifolia* is distinguished from *S. radicans* by rotund leaves (Figure 1.1) with a distinct petiole from the leaf lamina (Heenan 1997). It has the same chromosome number as *S. radicans,* 2*n*=16 (Dawson et al. 2000), but is endemic only to the lower western North Island where it is mainly found in dune habitats (Heenan 1997). This species is rated in decline due to faster and taller growing weed species that are rapidly modifying the dune habitats (de Lange et al. 2009).

Figure 1.1 Examples of *Selliera* species in New Zeland. *Selliera radicans* (A) showing elongated spathulate leaves, *S. rotundifolia* (B) showing the rounder leaf with distinct petiole and leaf lamina and *S. microphylla* (C) distinct from *S. radicans* only in size.

The third *Selliera* species, *Selliera microphylla,* was described by Colenso in 1889. It is indistinguishable from *S. radicans* except for a smaller size and inland location, however, when cultivated it reverts to a larger size making it indistinguishable from *S. radicans.* This has lead to suggestions that it is in fact a mountain version of *S. radicans* which is known to be highly polymorphic with a large variation in habitat (De Lange 2014) even with the segregation of *S. rotundifolia* (Murray and de Lange 2013). Endemic to New Zealand, *S. microphylla* is mainly found on the Central Volcanic Plateau and adjacent mountains but has also been collected from the Eyre Mountains in the Southland Land district. A single chromosome count of 2*n*=56 for *S. microphylla* has been

reported for the Central North Island population in Matea Swamp but the South Island populations still retained a chromosome number of 2*n*=16 (Murray and de Lange 2013). As a result of these observations, the status of these three 'species' needs further investigation. To date, no genetic studies have been performed on any of these species. In this thesis a population genetics approach was employed to attempt to determine the species boundaries within *Selliera.*

1.2 Taxonomy

Taxonomy is the classification of living organisms. It involves grouping organisms based on relationships and can be at the species level or higher. This forms a taxonomic hierarchy where the higher groupings contain all the groups below them, for example, the phylum Magnoliophyta contains all angiosperms which are then grouped further into order, family, genus and species. Understanding the classification of organisms is important for all biological research providing continuity across disciplines. In conservation it is important to make sure effort and funding are best utilised. For these reasons species delimitation is an important part of taxonomy essential for many fields of study to aid in knowledge and conservation of threatened species.

Yet species delimitation remains one of the most difficult tasks in biology (Birky et al. 2010) particularly on younger islands where there is generally less time for speciation processes to complete (Whittaker et al. 2007). The properties of species, such as monophyly, reproductive isolation, distinct morphology and habitat are often used as criteria to delimit species but can cause disagreements between species concepts as these are biological properties (De Queiroz 2007). This disagreement between species concepts has been called the "species problem" (Hey 2001) with at least 24 different species concepts historically described (Mayden 1997).

1.2.1 Species concepts

To be able to delimit species we must have a species concept to describe what a species is. There are at least 24 species concepts in the literature (Hey 2001) which

try to define a species but these concepts often don't apply to all organisms, for example, the biological species concept (Dobzhansky 1935; Mayr 1942) uses reproductive incompatibility, a major factor in speciation, to distinguish species but plants can have high rates of hybridization within and between genera (Whitney et al. 2010) so many described species of plants are not reproductively isolated. Furthermore, allopatric species, which are geographically isolated, may not develop reproductive isolation over time even if they become morphologically and genetically differentiated from each other and may still be capable of reproduction if introduced together again leading to confusion over whether these are the same species or not under the biological species concept. Also ring species such as *Ensatina eschscholtzii* Gray. (Plethodontidae) can be difficult to delimit under this concept as all subspecies can interbreed with the adjacent subspecies except for the geographically terminal and most distantly related subspecies thereby making it difficult to determine where to draw the species boundaries (Wake 2006). The biological species concept encounters even more problems with fossils, where evidence of reproductive isolation can no longer be determined, or typically asexual organisms such as bacteria which do not undergo typical forms of sexual reproduction (Mayden 1997).

Another example, the phylogenetic species concept is often used in systematics and defines a species as the smallest group of organisms that share a common ancestor and can be distinguished from other such groups. This concept often requires monophyly but many species, especially plant species, are not monophyletic due to hybridization or polyploidy. Chrisp and Chandler (1996) tested monophyly in several studies which suggested that estimates of paraphyly range from 20 -50%. Bacon et al. (2012) tested monophyly in *Pritchardia* Seem & H Wendl. (Arecaceae) species with only 5 of 27 species showing monophyly although these five species also only had weak support in a maximum parsimony analysis. Ring species can be an example of paraphyletic species which could be considered monophyletic under this concept even if reproductive isolation occurred between some of the 'species' so the divergence of these potential 'species' would not be recognised by the phylogenetic concept (Crisp and Chandler 1996). This is important as these paraphyletic species could produce misleading results if

assumed monophyletic when they are not. These species concepts are based on single characters, particularly ecological and biological characters, which cause disagreements between different taxa and even between the different concepts.

De Queiroz (2007) suggested that these species concepts had an underlying unity and the incompatibility of them is based on biological properties such as character states, reproduction or ecological niche and habitat, and proposed an alternative concept now called the "general lineage concept" (Figure 1.2). This concept defines a species as a lineage that is evolving separately to other lineages. During the existence of a species lineage some or all of the properties of a species, that result in incompatibilities between previous species concepts, may be acquired by the evolving "species" which is considered evidence that a lineage is a distinguished species (De Queiroz 2007). At the symposium for the Society of Systematic Biologists there was general agreement by almost all present that species are lineages (Wiens 2007).

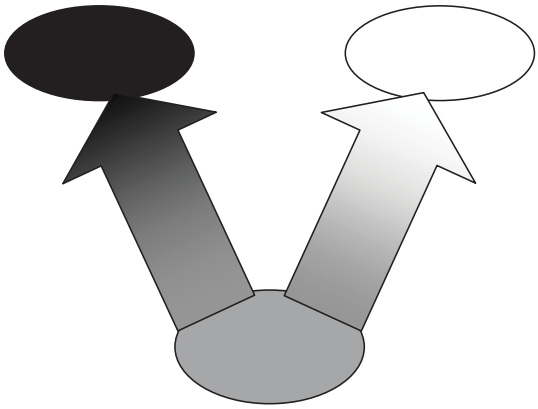

Figure 1.2 Representation of the general lineage species concept. The arrows indicate the process of speciation where species properties are acquired and so species delimitation is difficult during this time. The black and white circles represent the two new distinct species that can easily be differentiated. Figure modified from De Queiroz (2007).

The important part of the lineage concept is that it does not define a separate lineage based on only one or more biological properties but uses all these properties as evidence for the separation of lineages. However a disadvantage of the lineage concept is that lineages that haven't completed speciation or are currently diverging will be difficult to identify as they will not show clear boundaries from the ancestor

species (De Queiroz 2007). Also there is no way of knowing whether these lineages will complete speciation rather than return to one (the ancestor) species or become extinct (Crisp and Chandler 1996).

The species description used in this thesis will be the "general lineage concept" where the biological properties of species, reproductive isolation, morphology and genetic structure will be used to determine species boundaries. To aid determination of species boundaries it may be helpful to consider how species arise.

1.3 Speciation

1.3.1 Models of speciation

Speciation is the process whereby new species arise. As the forces that drive genetic differentiation, random genetic drift, mutation, migration and natural selection, act on populations, we could consider species to be groups of populations that contain distinguishing characteristics and remain distinct (Futuyma 2005). There are several models of speciation. Firstly allopatric speciation occurs when populations become geographically isolated and gene flow between them is disrupted resulting in the populations evolving into distinct species. The Dobhansky-Muller-Bateson (Orr 1996) model of speciation describes how reproductive isolation can evolve between two geographically isolated populations. In this model isolation between populations over time leads to an accumulation of incompatible mutations. These incompatible mutations, when fixed in at least two loci will prevent reproduction between populations. If the separated populations integrate again they are reproductively isolated, due to incompatibilities of the loci involved, leading to the formation of a new species (Bateson 1909; Dobzhansky 1934; Muller 1942). Reproductive isolation is not required to consider allopatric species as separate species under the general lineage concept as long as they have acquired some of the biological properties that constitute a species and remain distinct lineages. Instead the two geographically isolated populations may acquire all or just some of the properties that provide evidence of a new species (De Queiroz 2007). This can be seen in plants as hybridization occurs frequently

between described species (Whitney et al. 2010) and reproductive isolation is not required to form separate species under the general lineage concept.

Sympatric speciation occurs within a population where a genetic mutation or niche selection for some of the individuals provides a reproductive isolating mechanism allowing each new lineage to evolve leading to a potential new species if they remain reproductively or habitually isolated (Smith 1966). Disruptive selection is often proposed as a method of sympatric speciation (Futuyma 2005) where homozygous genotypes have a higher fitness than the heterozygote genotypes.

1.3.2 Speciation by polyploidy

Polyploidy has been observed as an important method of speciation especially in plants (Wendel 2000). For example, diploid parents form tetraploid offspring that produce sterile triploids when backcrossed with the diploid parents, but these can potentially produce fertile offspring with other tetraploids. This situation leads to isolation from the parents and may result in speciation as there will be reduced gene flow between the parental species and the polyploids. However, how these polyploid species establish is unknown because if crossing with parents produces sterile offspring, then the reproductive success would be lower than the parents. However, self-fertilization, higher fitness, a new environment from the diploid or vegetative reproduction could help a polyploid population establish (Rodriguez 1996). In polyploids chromosomal rearrangements are often observed (Chester et al. 2012; Lagercrantz 1998; Soltis and Soltis 1999) which also can result in reproductive incompatibility from the parental diploids. Also polyploidy, especially allopolyploids formed from two different species, may have the potential to adapt to environments that are unsuitable for the parents, due to the doubling of chromosome number and therefore the genetic material, allowing ecological and morphological divergence from the diploid parents. Speciation by polyploidy has been observed in treefrogs where the tetraploid species *Hyla versicolor* Le Conte. (Hylidae) has multiple origins from the diploid *H. chrysoscelis* Cope. with each tetraploid lineage more closely related to different diploid lineages (Ptacek et al. 1994). In plants polyploid speciation has been observed in *Asplenium cimmeriorum* Brownsey & de Lange. (Aspleniaceae) and *A. gracillimum* Colenso. Each of these

species contains at least two allopolyploids that are genetically distinguished even in sympatry indicating they can be considered distinct species (Perrie et al. 2010).

1.3.3 Speciation and Hybridization

Hybridization is among one of the most complicating factors in species delimitation and has an important role in the evolution of many lineages. In plants hybridization between species and some genera is common (Whitney et al. 2010). Whitney et al. (2010) found that hybrids occurred in 40% of the plant families examined, although these were in only about 16% of genera surveyed, indicating hybridization is common but not pervasive. However the estimation of hybridization frequency in this study likely underestimates the actual frequency because many species cannot be inspected throughout their range so many hybrids may not be documented and hybrids may not be suspected if the parental taxa are not obvious, for example the parental species have become extinct (Whitney et al. 2010).

Hybridization between species is a form of gene flow and can potentially dissolve the barriers between species, prevent divergence of populations into new species or cause extinction of less aggressive species, as has been happening with some island species (Rhymer and Simberloff 1996). Therefore for species to remain isolated while hybridization is occurring, there must be strong selection on each of the parent species to allow them to remain diverged (Pinho and Hey 2010). Alternatively post- and pre-zygotic mechanisms are strategies that prevent hybridization or establishment of hybrids and therefore gene flow between species. Post-zygotic isolation, such as hybrid sterility, prevents gene flow between species, which can allow them to remain distinct, but this also has the effect of wasted reproductive effort (Allendorf et al. 2001) which can be significant especially in endangered populations. In post-zygotic isolation, the hybrid zygote is formed but either doesn't develop or develops but is sterile or inviable; this is considered an isolating mechanism (Orr and Turelli 2001) as there is consequently no effective gene flow between populations leading to reproductive isolation and separate evolutionary lineages. Prezygotic mechanisms act before the zygote is formed to prevent hybridization and, therefore, gene flow between species (Zenkteler 1990). An example of a prezygotic mechanism is behavioural isolation, which can be seen

in pollinator preference in *Mimulus lewisii* Pursh. (Phrymaceae) and *M. cardinalis* Douglas ex Benth. *M. lewisii* flower traits are preferred by bees whereas *M. cardinalis* flower traits are preferred by hummingbirds preventing reproduction between the two plant species (Schemske and Bradshaw 1999).

Most initial hybrids display intermediate morphologies relative to the parental species. However hybridization can also result in speciation as hybrids may be able to survive in environments that are unsuitable for the parent species so gain new selective pressures or could out-compete the parents due to hybrid vigour where the hybrids gain increased fitness relative to the parental species. This can create difficulties in delimiting species as the hybrid species may still contain ancestral polymorphisms or if hybrids backcross with one of the parents, evidence of the other parental species may confuse the results (Reeves and Richards 2007). An example of hybrid speciation is in the genus *Helianthus* L. (Asteraceae) which has produced three hybrid species, *H. anomalus* S.F. Blake.(Rieseberg et al. 1991), *H. paradoxus* Heiser. (Rieseberg et al. 1990), and *H. deserticola* Heiser. (Gross et al. 2003), from the same progenitor species *H. annuus* L. and *H. petiolaris* Nutt. These hybrid species were analysed with molecular markers such as allozymes, chloroplast RFLPs, and microsatellites. *Helianthus paradoxus* showed combined alleles from the parental species with no unique alleles. It also had the rDNA repeat regions of both parents and the chloroplast of *H. annuus* providing good evidence that *H. paradoxus* was derived by hybridization (Rieseberg et al. 1990). *Helianthus anomalus* combines the allozymes and ribosomal DNA repeats of the parent species and individuals of the hybrid species have chloroplast haplotypes of either parent indicating *H. anomalus* arose by reciprocal hybridization (Rieseberg et al. 1991). *Helianthus deserticola* contains chloroplast haplotypes of both parental species and, is polyphyletic with *H. annuus* when microsatellite markers inherited from *H. annuus* are used, but monophyletic with *H. petiolaris* when markers derived from *H. petiolaris* are used (Gross et al. 2003). These results indicate a hybrid origin of *H. deserticola.* Gross et al. (2003) also showed that, in *H. deserticola,* fertility between populations is high despite the different chloroplast haplotypes and polyphyletic nature of this species, indicating *H. deserticola* fits the definition of a "good" species. In these studies, the hybrid lineages were considered

species when they are reproductively isolated from the parents and have a stable but fertile genotype.

1.4 Methods of species delimitation

Species delimitation is often carried out by a morphological or phylogenetic approach. Morphological species delimitation in plants is difficult because plants often have plastic phenotypes (Stearns 1989), meaning their phenotype can vary depending on the environment they are growing in. To differentiate between genetic and environmental effects, a common garden experiment must be performed which is often time consuming and difficult so is not a common practice. Morphological based species delimitation can also incorrectly delimit cryptic species. Cryptic species are defined as two or more species that are morphologically similar and so can be classified as the same species but are actually distinctly evolving lineages (Paris et al. 1989). An example of a cryptic species is *Ceratopteris thalictroides* Brongn. (Pteridaceae) which was discovered to consist of three distinct types based on allozyme analysis and chloroplast DNA analysis, but which cannot be morphologically distinguished due to the high morphological plasticity of the species. Crossing experiments determined that one of the discovered types (the south type) was completely cross-sterile with the other two types. The other two types, North and third type, were partially cross-sterile but maintained the genetic distinctiveness despite sporadic crossing suggesting the species actually contains cryptic species (Masuyama et al. 2002). Therefore morphological studies should be used in conjunction with genetic analyses (or reproductive tests) in species delimitation when possible.

Phylogenetic studies use phylogenetic trees to represent relationships between species and often rely on monophyly (Sites and Marshall 2004). In plants, speciation by hybridization and recurrent polyploidy are common (Whitney et al. 2010), meaning species are not always monophyletic. As speciation can occur at the population level, but populations are comprised of genetically diverse individuals, phylogenetic trees can also disagree between the gene tree and the species tree. Other causes of phylogenetic incongruence can be caused by horizontal transfer through hybridization (Doyle 1992), gene duplication (Page

1993) or extinction and lineage sorting. This disagreement between phylogenetic trees can make species delimitation difficult using a phylogenetics approach especially as usually only one or a few markers are included in the analysis which may yield different trees depending on the molecular markers used.

The ideal markers for phylogenetics should be single copy, be easy to align, all sites in a codon should be equally able to vary, have substitution rates high enough to provide enough informative sites but not too low that reversion mutations can occur, have approximately equal base composition, and have universal primers (Cruickshank 2002). Nuclear DNA sequence markers, such as ITS, and chloroplast markers, are commonly used in phylogenetics (Duminil and Michele 2009). Chloroplast markers are often highly conserved between species as most plants are homoplasmic for the organelle genomes so no recombination occurs (Ouborg et al. 1999) making chloroplast markers more suited for use at higher taxonomic levels (Palmer et al. 1988). ITS, a commonly used nuclear marker, evolves faster than the chloroplast genome (Gielly and Taberlet 1994) making it suitable for more closely related species. However ITS can have problems with lineage sorting (Cubas et al. 2006) and concerted evolution (Alvarez and Wendel 2003) confounding species relationships. ITS and chloroplast markers have universal primers available for PCR amplification so they can be easily used (Duminil and Michele 2009). However, the common problem with these markers is the low amount of variation between closely related species, even in ITS (Baldwin et al. 1995), leading to low species-level resolution. AFLPs or RAPDs are also used in phylogenetic analyses (Hodkinson et al. 2000) but they are dominant markers, which means heterozygotes cannot be differentiated from homozygotes, and they can have problems with homoplasy (Despres et al. 2003). Homoplastic characters are those shared by species but not present in a common ancestor, thereby confusing inferences about historical relationships (Duminil and Michele 2009).

Thus phylogenetic trees are potentially poor indicators of good species especially where there is gene flow (Reeves and Richards 2007), which may prevent characters from becoming fixed and causing collapsed nodes. However phylogenetic trees allow comparison between morphological and molecular data

which is useful for inferring species relationships. As the forces driving speciation, natural selection, mutation, migration, and random genetic drift, act on populations; studying the population structure may help in determining species boundaries. Therefore a population genetics approach may succeed in delimitating species where other methods struggle.

1.5 Population genetics

Population genetics is the study of the genetic structure of populations which is determined mainly by gene flow between populations and the maintenance of genetic diversity (Michalski and Durka 2007). These factors are influenced by natural forces such as mutation, migration, assortative mating, natural selection and population size (Whitlock and McCauley 1999). When these forces are not acting on a population, the genotype frequencies of any locus are simply dependent on the allele frequencies, which is termed Hardy-Weinberg equilibrium as it was described separately in 1908 by Hardy and Weinberg (Wigginton et al. 2005). Testing whether a population conforms to Hardy-Weinberg equilibrium is common in population genetic studies where deviations from Hardy-Weinberg equilibrium may indicate that any of the natural forces, such as natural selection, are acting on the population (Thompson et al. 2007). Alternatively a deviation can indicate problems with markers or genotyping (Wigginton et al. 2005). Population structure can also be evaluated by genetic distances (Kalinowski 2002), fixation indices such as F_{ST}, and other statistical tests such as ANOVA, which compares genetic variation between and within populations. This genetic structure is detected usually by analysis of neutral molecular markers such as microsatellites (Michalski and Durka 2007) or SNPs with many individuals sampled from each population. In species delimitation studies, these data can also be analysed by phylogenetic trees or networks to indicate species relationships.

1.5.1 Markers used in population genetics

A population genetic approach uses the genetic structure of populations or collections of individuals for species delimitation, and therefore, requires markers different from those used in phylogenetics. In population genetics, markers should be easily assayed by PCR, comparable, have many loci available, and be easily

screened (Sunnucks 2000). Protein molecular markers such as allozymes were the first molecular markers used for population genetics studies (Hubby and Lewontin 1966). Allozymes are based on enzyme variation in individuals but have mainly been replaced by DNA markers. However, Duminil et al. (2007) suggest proteomics can be a useful tool in studying the relationships between genotype and phenotype and to resolve issues on ecology and evolution of plant species or populations, but that the area still needs to be further developed. Single nucleotide polymorphisms (SNPs) are quicker and easier to analyse than microsatellites (Jones et al. 2007). They are commonly found throughout the genome, but are less variable than microsatellites and many other markers, so require more loci to be informative.

Microsatellite markers are being used increasingly in population genetics studies. The high amount of variability, smaller number of loci needed when compared to single nucleotide polymorphisms, and genotyping ease (Ashley and Dow 1994) mean they will most likely continue to be used especially with advances in their discovery, including next-generation sequencing which increases the efficiency of developing markers (Gardner et al. 2011). Microsatellite markers are abundant throughout the genome and di-nucleotide repeat microsatellite loci are often used as they are highly polymorphic and evolve faster than other repeats, making them useful for closely related taxa that may not contain a lot of variation, but can stutter more making genotyping difficult (Gardner et al. 2011). Stutter in microsatellites can be caused by slip-strand pairing leading to differently sized fragments (Miller and Yuan 1997). Gardner et al. (2011) recommends using tetranucleotide repeats as they have high polymorphism and low stutter but the allele ranges can be larger. Another advantage of microsatellites is that they are expected to be neutral, reducing the effect of selection pressures, and are co-dominant markers (Duminil and Michele 2009), so can differentiate between heterozygotes and homozygotes, which is important for detecting hybridization especially in population genetic analyses. Microsatellite markers are more species-specific than other markers where cross-amplification within or between a genus many not always be possible although this may require markers to be developed for each species or genus studied (Selkoe and Toonen 2006). Microsatellites can be homoplasious, but using

multiple loci can minimise the effect (Duminil and Michele 2009). These advantages mean microsatellite markers can provide information on species delimitation and on the genetic structure of populations (Duminil and Michele 2009), and therefore, are excellent markers for use in a population genetics approach to species delimitation.

A good example of the power of microsatellites for species delimitation in a group where other markers failed is the study of *Schoenoplectiella* Lye. (Cyperaceae) (Kim, et al. 2012). They used microsatellite markers to delimit species in this taxonomically difficult group and to determine the genetic relationships within the genus. They resolved the differing results between morphology and ribosomal sequence data for the hybrid *S. x trapezoidea* (Koidz.) J. Jung & H. K. Choi. using microsatellites due to their high level of variability. However the methods used were to score microsatellite bands as presence or absence as the ploidy level of some species was unknown. This means they lost the information of codominant markers but retained more reliable data as variation in ploidy level, when it is unknown, makes scoring microsatellites difficult (Kim et al. 2012). Microsatellite markers are becoming the favoured method to use in species delimitation and detection of natural hybrids due to their resolving power and reproducibility (Kim et al. 2012).

1.5.2 Analysis methods in population genetics

There are two types of analyses for species delimitation, non-tree based methods which calculate gene flow or genetic differences between populations or species by calculating genetic distances and number of migrants (Sites and Marshall 2004) and tree-based methods, which often use phylogenetic trees using the phylogentic species concept. Non-tree methods make assumptions about gene flow and certain properties of populations, which, if not met, can produce incorrect relationships between species (Sites and Marshall 2003). F statistics are commonly used in population genetics (Wright 1951). F_{IS} measures the ratio of observed to expected heterozygosity in a population where a positive value indicates a homozygote excess and a negative value indicates a heterozygote excess within the population. Homozygote excess may indicate inbreeding within the population but also can be

the result of null alleles or the loci being under selection. A heterozygote excess may indicate out-crossing between populations. F_{ST} measures the genetic differentiation between populations where a higher value indicates there is a greater differentiation between populations and a low value indicates less difference between populations. Tree methods often lean toward monophyly to describe a species (Sites and Marshall 2004). However, in recent divergences and especially, in plants, where hybridization is common, monophyly may not be upheld, which can cause anomalies such as polytomies in bifurcating trees or evidence of hybridization can be lost during the tree-making process (Reeves and Richards 2007). Also single gene trees may not be representative of phylogenetic relationships (DeSalle et al. 2005) especially where hybridization has occurred in the past as different gene trees may show different relationships.

Phylogenetic networks can resolve this problem because they can identify evolutionary events like hybridization and recombination (Cardona et al. 2009) so can provide a more accurate portrayal of species relationships. Bacon et al. (2012) used phylogenetic trees in their study of *Pritchardia* (Arecaceae); their results showed unresolved relationships for several species which could be due to the high rates of gene flow common between species in this genus. As phylogenetic trees assume that a lack of gene flow between species maintains monophyly (Sites and Marshall 2004), they are not useful for species without reproductive isolation where monophyly may not be observed. Phylogenetic networks use distance or other based methods to produce a network that can represent gene flow events.

STRUCTURE (Pritchard et al. 2000) provides another form of analysis common in population genetics. This program calculates the proportion of the genome derived from hypothetical ancestral populations calculated independently of the genome assignment of individuals thereby showing which individuals are genetically similar. The K value determines the number of ancestral populations which can be analysed to find the optimum K value by assessing the likelihood of a model fitting the data.

1.6 Focus of this research

Three species of *Selliera* have been previously described in New Zealand based on morphological characteristics, particularly leaf morphology and geographic location. Only one species of *Selliera* is broadly recognised, *Selliera radicans,* (Mabberley 2008) which is known be highly polymorphic and can be found in a variety of habitats (De Lange 2014) leading to disagreement about the status of some species. No genetic studies have previously been performed on this genus. A population genetics approach, using microsatellite markers, is employed here with aims to:

1. Develop reliable and polymorphic microsatellite markers to use for *Selliera.*
2. Determine the species boundaries within *Selliera.*
3. Examine the population structure within *Selliera* species.

These aims will be addressed in the following two chapters which have been written as manuscripts for publication. Chapter 2 was written following the 'primer note' guidelines for *Applications in Plant Sciences.* Chapter 4 includes a conclusion and summarises the results from the previous chapters.

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Chapter 2

Microsatellite primers in *Selliera radicans* (Goodeniaceae)

2.1 Abstract

Premise of the study: Microsatellite markers were developed for species of *Selliera* (Goodeniaceae) to investigate population structure and species boundaries within the genus.

Methods and Results: Using 454 sequencing, 9 primer pairs were isolated from *S. radicans.* The primer pairs amplified di- and tri-nucleotide repeats with 1-5 alleles per locus. All 9 primer pairs amplified in the three species screened; *S. radicans, S. rotundifolia* and *S. microphylla.*

Conclusions: The new primers provide an important resource for future investigations in the genus *Selliera* for both population genetics and species boundary research.

2.2 Introduction

Selliera is a genus within the Goodeniaceae. In New Zealand three species of *Selliera* have been described. *Selliera radicans* is described as having spathulate elongated leaves but is also known to be highly polymorphic (Allan 1961), and has a chromosome count of 2*n*=16 (Dawson et al. 2000). *Selliera rotundifolia* is distinguished by rounded leaves and also has a chromosome count of 2*n*=16 (Dawson et al. 2000; Heenan 1997). It is endemic to the lower western North Island (Heenan 1997). Both *S. radicans* and *S. rotundifolia* are coastal species. *Selliera microphylla* is found inland on the central volcanic plateau on the North Island and in the Eyre mountains on the South Island (Colenso 1889). It differs from *S. radicans* only by smaller size and inland location and some believe it is simply a mountain form of *S. radicans*; however a population of *S. microphylla* from Matea swamp in the North Island has given a chromosome count of 2*n*=56 (Murray and de Lange 2013). As these species have been described based only on morphology or geography, and two are sympatric in part of their distribution, the status of these species is questionable.

Future study, using a population genetics approach, may aid species delimitation in *Selliera* and provide information on the population structure within groups. Microsatellite markers are effective tools in population genetics due to their co-dominance (Duminil and Michele 2009), abundance in the genome, ease of genotyping (Ashley and Dow 1994), and high variability compared to other markers meaning fewer loci are needed. High variability also means they are potentially useful in delimiting closely related species where traditional sequence-based markers have failed. High-throughput sequencing has made the development of microsatellite markers more efficient than previous methods (Gardner et al. 2011). Here, using 454 pyrosequencing, microsatellite markers were developed for *Selliera* species to be used to assess population structure and determine species boundaries in subsequent work.

2.3 Methods and results

Genomic DNA was extracted from silica dried leaf tissue using a modified cetyltrimethylammonium bromide (CTAB) method with an initial STE (sucrose, Tris, and EDTA) wash (Shepherd and McLay 2011) and an additional RNAse step. The DNA was dissolved in 100µL of TE buffer. DNA from *Selliera radicans* collected from a population at Moana roa beach was chosen for 454 sequencing. The sample had a concentration of 84.8ng/µL and a 260/280 of 1.99 quantified as measured on a Nanodrop 2000 (Thermo-Scientific) and was run on a 1% agarose gel to ensure RNA had been successfully removed. This genomic DNA was then sequenced by shotgun sequencing on the 454 GS-FLX (Roche) by the NZGL (New Zealand Genomic Limited).

The sequence data received was assembled into contigs in GENEIOUS (v5.6.7) (Drummond et al. 2010) to increase the reliability of microsatellite detection and preventing locus duplication. MSATCOMMANDER v0.8.2 (Faircloth 2008) was used to search the *Selliera* contigs for di-, tri-, and tetra-nucleotide repeat motifs with a minimum of seven uninterrupted repeats and with the requirement to design primers at least 50 bp from the repeat region using Primer3 (Rozen and Skaletsky 2000). Criteria for primer pairs selected for testing included size between 150-350bp, with no long repeats in the region surrounding the microsatellite (for example mononucleotide repeats) and primers optimally would have 60% GC content with a GC clamp at the 3' end.

The total number of sequences generated by the 454 run was 57,561 with an average sequence length of 407 bp. These sequences assembled into 8,101 contigs where the average sequence length was 672 base pairs. From these contigs MSATCOMMANDER (Faircloth 2008) detected 227 repeat motifs. Of the 227 repeat motifs found, 196 were dinucleotide repeats (86%), which had a high frequency of AT repeats (51%), 30 were trinucleotide repeats (13%) and one tetranucleotide repeat (0.4%). PRIMER3 designed primers for 107 of the 227 repeats detected. Of these 107, 90 primer pairs were designed for the dinucleotide repeats, 17 primer pairs were designed for the trinucleotide repeats and no primer pairs could be designed for the tetranucleotide repeat.

From the 107 microsatellite loci detected, 43 suitable primer pairs were chosen for initial testing. Selected primer pairs were screened initially on 15 individuals consisting of individuals from *S. radicans* and *S. rotundifolia* populations. PCR amplification was performed in a volume of 10μL with 1X buffer BD (Solis Biodyne, Tartu, Estonia), 50 μM of each of the dNTPs, 2.5 μM $MgCl_2$, 0.5U of Firepol polymerase (Solis Biodyne, Tartu, Estonia), 20 nM of forward primer, 450 nM of reverse primer and 450 nM M13 primer. Amplification by PCR was attained by: 95ºC for 3 minutes, 35 cycles of 95ºC for 30 seconds, 53º C for 30 seconds and 72ºC for 1 minute and a 72ºC hold step for 20 minutes. The ABI3730 DNA analyzer at the Massey Genome Service was used to genotype samples with alleles scored manually in GENEMAPPER (v4.0) using CASS size standard (Symonds and Lloyd 2004). The observed and expected heterozygosity and the number of alleles per locus were calculated using GenAlEx 6.5 (Peakall and Smouse 2012).

Loci that were consistently amplifiable, clean, and amplified in all three species were then chosen for further testing over six different populations. These populations consisted of 20 individuals from each of the populations collected for the three species. *S. radicans* populations consisted of Moana Roa, Napier, Tauranga, and Ohiwa. Himatangi and Central Volcanic Plateau are populations of *S. rotundifolia* and *S. microphylla,* respectively (Table 2.3). From the 43 primer pairs, 24 were suitable for screening over the larger sample size of 100 individuals which resulted in 9 markers that amplified consistently (Table 2.1). All nine of these were polymorphic. Observed heterozygosity ranged from 0.000 to 0.737 and expected heterozygosity ranged from 0.000 to 0.672 (Table 2.2).

Table 2.1. Characteristics of nine microsatellite primer pairs developed for use in *Selliera.*

Locus	Primer sequence		Repeat motif	Size range (bp)	*Ta*
SR17	F	AATGGAAAGCAACCAATCCC			57.31
SR17	R	TGTTCCAACGATTTGACCAAC	AT^12	252 - 258	58.05
SR24	F	GGCAAGTAGGAAATGTGGGC			59.94
SR24	R	TCTCCTGAACCAGCAACCG	AT^8	203 - 209	60.38
SR29	F	GAACGGCAGCAAACTACCC			59.86
SR29	R	AGCCTCCAAGAGACTTGACC	AG^7	397 -399	59.79
SR31	F	CAGCCGAGTGCCTACCTTC			60.9
SR31	R	CGCCCAACTATCAAGCCAC	CT^7	369 - 373	59.64
SR37	F	TCAAGCCTTTGGCAAGATAGTC			59.42
SR37	R	ACTCGTGGACGTAGGTTCTG	AG^10	296 - 302	59.87
SR45	F	CTGCGATAGCGTCGATTCC			59.3
SR45	R	GTGGTTGATCCATATTACCAGGC	GAT^9	271 - 283	59.95
SR6	F	GAGCTTGAGCTGGTTCCTC			55.5
SR6	R	CCAGTCTCAGTCACAAGCG	GA^8	208 – 214	55.6
SR11	F	GCTCTACTTGCACGGCTTC			56.3
SR11	R	GTGCTCACATGGGATCTGC	GT^8	182 - 194	56.2
SR13	F	AGGTCCTCACCCTCTTGAAC			56.3
SR13	R	CAGCATGTTTGAAGCTACGTG	TC^7	313 - 315	54.5

***Ta* =annealing temperature**

Table 2.2. Results of the primer screening in populations of *Selliera*.

	Moana Roa			Napier			Ohiwa			Tauranga			Himatangi			Central Volcanic Plateau		
Locus	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e
SR17	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	2	0.000	0.188	2	0.050	0.289	2	0.000	0.255
SR31	2	0.368	0.361	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	3	0.600	0.521	2	0.100	0.095
SR45	4	0.737	0.619	2	0.050	0.139	2	0.100	0.095	3	0.737	0.659	3	0.800	0.614	2	0.450	0.399
SR37	3	0.056	0.563	1	0.000	0.000	2	0.700	0.500	4	0.421	0.630	1	0.000	0.000	3	0.105	0.586
SR6	2	0.053	0.051	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	2	0.200	0.320	2	0.200	0.180
SR11	2	0.056	0.239	1	0.000	0.000	1	0.000	0.000	2	1.000	0.500	1	0.000	0.000	1	0.000	0.000
SR24	5	0.684	0.672	2	1.000	0.500	2	0.421	0.388	2	0.000	0.500	4	0.158	0.395	2	0.150	0.139
SR29	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
SR13	2	0.211	0.266	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000

A, number of alleles; H_O, observed (heterozygosity and H_e, expected heterozygosity.

2.4 Conclusion

Developing microsatellite markers for *Selliera* from next generation sequencing was successful with nine polymorphic markers developed suitable for population genetic analysis. All microsatellite markers successfully amplify in all described species: *S. radicans, S. microphylla* and *S. rotundifolia*. These nine markers will be used to determine population structure in *Selliera* and attempt to determine species boundaries using a population genetics approach.

Table 2.3 Voucher information for *Selliera* populations used in this study.

Population (Location)	Geographic co-ordinates	Sample size	Voucher
Moana Roa Beach	-40º16'48.43"S, 175º13'30.14"E	19	MPN 47847
Napier	-39º28'4.15"S, 176º52'30.14"E	20	MPN 47843
Ohiwa	-37º59'14.38"S, 177º9'37.47"E	20	MPN 47844
Tauranga	-37º43'27.84"S, 176º11'25.04"E	19	MPN 47840
Himatangi	-40º21'44.53"S, 175º13'57.32"E	20	MPN 47848
Central Volcanic Plateau	-38º54'34.95"S, 176º27'20.8" E	20	MPN 47841

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Chapter 3

Population genetics and species delimitation in the genus *Selliera* (Goodeniaceae)

3.1 Abstract

Species delimitation is an important but difficult task in biology. A good example of the difficulties inherent in species delimitation is in the genus *Selliera* as species have been described based on morphological features or geographic location. *Selliera* consists of three described species; *Selliera radicans, S. rotundifolia* and *S. microphylla. Selliera rotundifolia* is distinguished from *S. radicans* by rounder leaves whereas *S. microphylla* is distinguished from *S. radicans* by an overall smaller size and inland location. Here we applied a population genetics approach using nine microsatellite markers combined with analysis of leaf morphology and existing karyotype data to attempt to determine species boundaries within *Selliera.* Two of the species, *S. rotundifolia* and *S. microphylla,* both had high interpopulation divergence and two genetic lineages were identified within each of these species which clustered with different *S. radicans* populations. Morphology analysis of leaf shape suggested there are distinct morphologies between the three species. Hybridization was observed between *S. rotundifolia* and *S. radicans* at one of the sympatric sites but no gene flow was observed with *S. rotundifolia* at the other site suggesting reproductive isolation in *S. rotundifolia* may have evolved although there is evidence of possible introgression from *S. rotundifolia* to *S. radicans*. Round leaves which are used to distinguish *S. rotundifolia* may have originated multiple times and there is some evidence for reproductive isolation between *S. rotundifolia* and *S. radicans* suggesting that at least one lineage of *S. rotundifolia* is a distinct species. We suggest that the South Island *S. microphylla* lineage is a mountain variant of *S. radicans* while the North Island lineage is maintained as *S. microphylla* due to genetic distinction, geographic isolation and

polyploidy. This study provides important insights into species boundaries within *Selliera* and has revealed interesting possibilities for future study with regard to species boundaries especially in the areas of sympatry.

3.2 Introduction

Species are described as the fundamental unit of biology (Hull 1977). As such, the species is often the subject of study so it is important to be able to properly identify the organism of interest. Identifying species is also important in conservation to ensure that limited resources receive the proper focus. Historically, this has made species delimitation an essential task however it has also proven difficult. Many different species concepts have been described but none of these have been suitable for all organisms. The disagreement between species concepts has been termed the 'species problem' (Hey 2001).

De Queiroz (2007) suggested a unified species concept, the general lineage concept, where species are described as separately evolving metapopulations and the biological properties of a species that were previously used to define species concepts are evidence for separately evolving lineages. There is general agreement among biologists that species are lineages (Wiens 2007), reducing the conflict caused by different species concepts, but delineating the lineages still remains a difficult task. Some suggest that species should be treated as hypotheses that can be revised as evidence from natural populations becomes available (Sites and Crandall 1997; Templeton 2001); however, in delimiting species, evidence from several sources should be considered, for example molecular and morphological, with a sample size large enough to reduce the likelihood of sampling artefacts. Even with multiple lines of evidence, events like hybridization, introgression, polyploidy and incomplete lineage sorting can further increase the difficulty in delimiting species, especially on younger islands where the evolutionary processes may not have had time to complete (Birky et al. 2010) and ancestral signals may still be present in divergent taxa.

Hybridization is common in plant species. Whitney, et al. (2010) observed hybrids in at least 40% of the plant families they reviewed. Most initial hybrids have intermediate morphologies to the parents although sometimes effects like hybrid vigour can result in offspring that are larger and more successful than the parental species. For species to remain distinct there must be strong selection against hybrids, for example diversifying selection (Chapman et al. 2013), or reproductive

isolating measures such as infertile hybrids preventing backcrossing with the parental species. Hybridization is a form of gene flow which has many effects, including dissolving the boundaries between species and even speciation (James and Abbott 2005; Wolfe et al. 1998) making this an interesting issue, especially with regard to speciation and the development of reproductive isolation. However for species delimitation hybridization can result in conflicting phylogenetic signals making species boundaries difficult to determine.

A further complicating factor in species delimitation is interspecific variation. Individual variation within a species is important to allow the maintenance of genetic diversity preventing inbreeding depression or extinction. This means that not all individuals within a species will be identical although most will share similar distinguishing features. Populations of a species that are experiencing no or low gene flow may be exposed to different environmental conditions and so will acquire different allele frequencies and possibly morphologies, due to different selective pressures which is termed local adaptation (Williams 1966). However, isolated populations may also acquire different allele frequencies (and morphologies) via genetic drift. Local adaptation may result in the evolution of reproductive isolation, and therefore speciation, if populations are isolated over a long period of time with no gene flow (Lenormand 2012). In addition to genetic variation, plants often demonstrate phenotypic plasticity (Stearns 1989), resulting in variable morphology simply caused by environmental factors. A common garden experiment can often identify if the variation in morphology is due to genetic or environmental factors although these can be difficult and time consuming to conduct. When studying species boundaries we need to differentiate interspecific variation from intraspecific variation. As a population genetics approach involves determining interpopulation variation it can be a suitable approach to use in species delimitation, especially for closely related species.

Population genetic approaches would appear well-suited to species delimitation in closely related plant groups given the often larger sample sizes, the utilization of more markers with greater resolution, and more flexible analytical approaches than traditional systematics. Indeed such strategies have been successfully applied to

identify species where other methods have failed (Bacon et al. 2012; Edwards et al. 2009; Kim et al. 2012) and this approach is steadily growing in popularity. The ideal marker for population genetics should be easily assayed by PCR, comparable, have many loci available, and be easily screened (Sunnucks 2000). Microsatellite markers are commonly used in population genetics due to the ease of genotyping (Ashley and Dow 1994), their co-dominant nature (Duminil and Michele 2009), and the relatively faster evolution compared to other markers, thereby making them suitable for use in closely related species where hybridization may be expected.

An excellent group to assess species delimitation with a population genetics approach is *Selliera. Selliera* is a genus in the Goodeniaceae which are identified by their distinctive pollen cup or indusium. Members of the genus are described as small creeping herbs which can form large mats and are mainly found in coastal areas. Currently there is only one generally acknowledged species of *Selliera*, *Selliera radicans* (Mabberley 2008; The Plant List 2013). *Selliera radicans* is described with spatulate elongated leaves (Figure 3.1) and is found in Australia, New Zealand and Chile; it can be found throughout the coast of New Zealand. Chromosome counts reveal a chromosome number of 2*n*=16 (Dawson et al. 2000) although a count of 2*n*=48 has been reported in Tasmania (Jackson 1958). *Selliera radicans* is commonly found in estuarine areas although can be found in other habitats, such as in dunes and on stream banks, and is noted to be highly polymorphic (Allan 1961).

In New Zealand three species of *Selliera* (*S. radicans, S.rotundifolia* and *S.microphylla*) have been described based on morphology or geographic location although no genetic studies have been performed previously. *Selliera rotundifolia* is distinguished from *S. radicans* by rounder leaves (Figure 3.1) (Heenan 1997) that retain their shape in a common garden experiment (Ogden 1974). It is endemic to the southwestern coast of the North Island and prefers a dune environment. There is some overlap between the ranges of *S. radicans* and *S. rotundifolia* which suggests the potential for hybridization to occur. Chromosome counts for *S. rotundifolia* also have a chromosome number of 2*n* = 16 (Heenan 1997).

Figure 3.1. Representatives of *Selliera radicans* (A), *S. rotundifolia* (B) and *S. microphylla* (C) with insets showing the typical leaf shape found in each of the species and scale bar at 10mm.

Selliera microphylla is described based on its overall smaller size and inland distribution on the Central Volcanic Plateau and Eyre Mountains (Colenso 1889) but is otherwise indistinguishable from *S. radicans* (Figure 3.1) and reverts to a similar size when grown in a common garden (De Lange 2012a). However a chromosome count for one population on the Central Volcanic Plateau has given 2*n*=56 (Murray and de Lange 2013) although it is noted as being doubtfully distinct (De Lange 2012a).

In this study we used a population genetics approach utilizing new microsatellite markers combined with morphology analysis of leaf shape and existing karyotype data to assess the species boundaries within the three species of *Selliera* in New Zealand. Using the general lineage concept which considers evidence from the biological properties of species such as reproductive isolation, morphological differences and genetic similarity we attempt to 1) Determine the species boundaries within *Selliera* and 2) Examine the population structure within all *Selliera* species.

3.3 Methods

3.3.1 Sample collection

A total of 618 individuals were collected from 35 populations (Table 3.1) representing all described species of *Selliera* from throughout New Zealand (Figure 3.2). All sampling was completed from November 2012 to March 2013. Several leaves were collected from each plant sampled and placed in silica gel to preserve the tissue until DNA extraction . At the time of collection three leaves from each plant sampled were chosen as representative of the plant and photographed using a digital camera next to a cm ruler for a scale for analysis of leaf morphology. Twenty individuals per population were collected except where the population size did not permit. Species designations were made at the time of collection based on previous species descriptions including leaf shape and geographic location.

3.3.2 DNA extraction and genotyping

DNA was extracted from dried tissue using a modified CTAB method (L. E. Doyle and Doyle 1987) and then frozen until needed. Each sample was screened at nine microsatellite loci described previously (Chapter 2). PCR amplification was performed in a volume of 10 μL with 1x buffer BD (Solis Biodyne, Tartu, Estonia), 50 μM of each DNTP, 2.5 μM $MgCl_2$, 0.5U of Firepol polymerase (Solis Biodyne, Tartu, Estonia), 20 nM of M13-tailed forward primer, 450 nM of reverse primer and 450 nM dye-labelled M13 primer. Amplification by PCR was attained by: 95ºC for 3 minutes, then 35 cycles of 95ºC for 30 seconds, 53º C for 30 seconds and 72ºC for 1 minute follwed by a 72ºC hold step for 20 minutes. One of three fluorescent dyes, FAM, NED, or VIC, was incorporated into each marker and three markers each containing a different dye were pooled for genotyping. The samples were genotyped on a ABI3730 DNA analyzer at the Massey Genome Service (Palmerston North, New Zealand) with alleles scored manually in GENEMAPPER (v4.0) using CASS size standard (Symonds and Lloyd 2004).

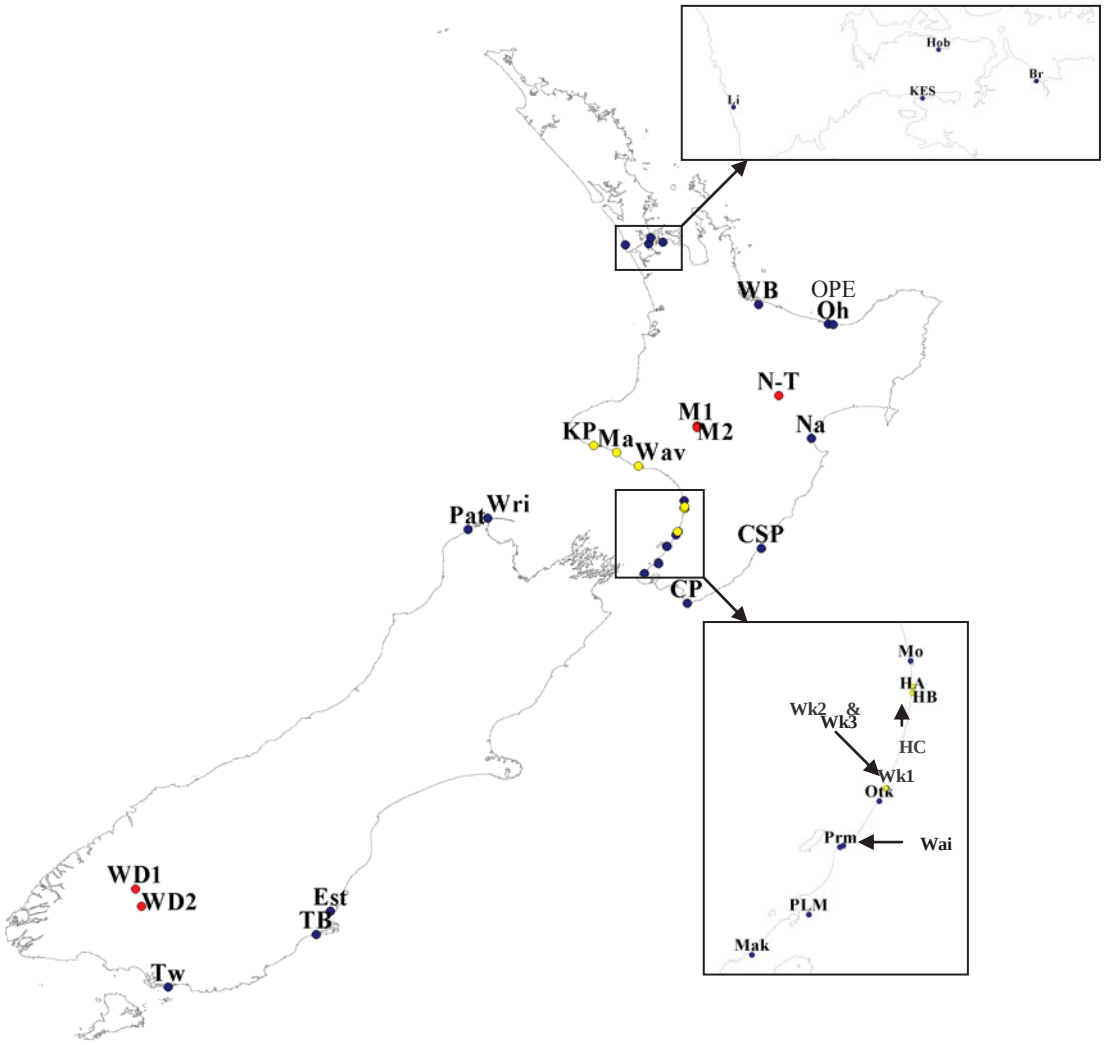

Figure 3.2 Distribution map of populations of *Selliera* collected for this study. Map was created in DIVA-GIS v7.5. *Selliera radicans* in blue, *S. rotundifolia* in yellow and *S. microphylla* in red. Insets show distribution of populations in closer detail for Auckland area and the Lower Western North Island.

Table 3.1: Population information and genetic diversity estimates for 35 populations in *Selliera.* Populations are ordered by designated species.

Population	Code	N	NA	Ne	%P	Ho	He	F_{IS}
S. rotundifolia								
Kaupokonui	KP	20	1.333	1.024	33.33	0.012	0.022	0.486
Manutahi	Ma	20	1.778	1.327	55.56	0.189	0.198	0.078
Himatangi	Ha	20	2.000	1.338	66.67	0.222	0.191	-0.140
Himatangi	HB	10	1.667	1.109	66.67	0.167	0.153	-0.038
Himatangi	HC	20	2.000	1.467	55.56	0.201	0.238	0.180
Waikawa	Wk2	20	1.667	1.347	55.56	0.208	0.195	-0.043
Waverly*	Wav	26	2.000	1.727	77.78	0.300	0.356	0.175
S. radicans								
Castlepoint	CSP	20	1.889	1.429	44.44	0.132	0.212	0.399
Waikawa	Wk1	20	2.222	1.634	66.67	0.250	0.289	0.164
Waikawa	Wk3	20	2.111	1.368	66.67	0.133	0.219	0.414
Tauranga	WB	20	1.889	1.652	55.56	0.240	0.275	0.156
Hobson Bay	HOB	20	1.111	1.006	11.11	0.006	0.005	0.000
Lion Rock (Piha)	Li	20	1.333	1.133	33.33	0.088	0.082	-0.038
Cape Palliser	CP	20	1.444	1.244	33.33	0.132	0.119	-0.014
Napier	Na	20	1.222	1.129	22.22	0.117	0.071	-0.629
Plimmerton	PLM	20	1.333	1.141	33.33	0.107	0.081	-0.280
Moana Roa	Mo	20	2.444	1.695	77.78	0.240	0.308	0.246
Paterau	Pat	15	1.556	1.357	33.33	0.141	0.172	0.214
Whaririki beach	Wri	10	1.889	1.510	44.44	0.128	0.209	0.432
Makara	MAK	20	2.000	1.437	44.44	0.141	0.204	0.332
Otaki	Otk	20	2.222	1.362	66.67	0.119	0.188	0.391
Waikanae	Wai	10	1.333	1.333	33.33	0.333	0.167	-1.000º
Auckland	KES	10	1.111	1.111	11.11	0.111	0.056	-1.000º
Auckland	Br	15	1.333	1.127	22.22	0.119	0.070	-0.684
Paraparaumu	Prm	20	2.111	1.489	55.56	0.144	0.227	0.385

Population	Code	N	NA	Ne	%P	Ho	He	F_{IS}
Dunedin	EST	20	2.111	1.560	55.56	0.146	0.248	0.433
Ohope	OPE	20	1.556	1.218	33.33	0.139	0.128	-0.063
Ohiwa	Oh	20	1.333	1.193	33.33	0.136	0.109	-0.217
Tunnel Beach	TB	20	1.778	1.351	44.44	0.068	0.178	0.633
Tiwai Point	Tw	20	1.889	1.517	55.56	0.113	0.262	0.588
S. microphylla								
West Dome	WD1	20	1.444	1.240	44.44	0.044	0.137	0.689
West Dome	WD2	20	1.778	1.384	55.56	0.045	0.208	0.795
Central Volcanic Plateau	NT	20	1.778	1.323	66.67	0.112	0.184	0.414
Central Volcanic Plateau	M1	1	1.111	1.111	22.22	0.222	0.111	NA
Central Volcanic Plateau	M2	1	1.111	1.111	22.22	0.222	0.111	NA

N, number of individuals; NA, number of alleles/locus Ne, number of effective alleles; (averaged for all markers) %P, percentage polymorphic loci; Ho, observed heterozygosity; He, expected heterozygosity; and F_{IS} (fixation index) population mean. * denotes the Waverly population that consisted of both *S. rotundifolia* and *S. radicans* plants. º indicates populations where all individuals were identical and either all homozygous or all heterozygous for a given marker. H_O and He are calculated including the homozygous markers but F_{IS} is calculated excluding the homozygous markers resulting in the discrepancy in F_{IS} value.

3.3.3 Data Analysis

Individuals that had missing data for four or more microsatellite markers were removed from the data set (six individuals: PLM9, PLM15, PLM18, Mo10, WB16, and Li10). Data were organised in GenAlEx v6.5 (Peakall and Smouse 2012) which was used to export data to formats suitable for MSA (Dieringer and Schlotterer 2003), FSTAT (Goudet 1995), and STRUCTURE (Pritchard et al.

2000). SPLITSTREE4 (Huson 1998) was used to create a population neighbornet from a distance matrix generated with MSA (Dieringer and Schlotterer 2003). In creating the distance matrix with MSA pairwise population distance and proportion of shared alleles were selected. For calculation of F_{ST}, AMOVA, ANOVA and the Mantel test, populations were grouped by the designated species (Table 3.3) with the Waverly (Wav) and Waikawa (Wk3) populations excluded as they were determined to potentially be hybrid populations. Genetic variation was assessed using FSTAT (Goudet 1995) which calculated F_{IS} and $F_{ST.}$ Both Weir and Cockerham's F_{ST} (theta) (Weir and Cockerham 1984) and Nei's G_{ST} (Nei 1975) were calculated. GenAlEx v6.5 (Peakall and Smouse 2012) was used to calculate observed and expected heterozygosity. Arlequin was used to perform an AMOVA to test for genetic differences among species. For the Mantel test pairwise F_{ST} was calculated and transformed into $F_{ST}(1\text{-}F_{ST})$ and tested against the log of geographic distance in GenAlEx v6.5. Genetic structure was also assessed by STRUCTURE (Pritchard et al. 2000). Parameters used for STRUCTURE analyses were: 10 replicate runs for each K (putative ancestral population) value from 1 – 8, assumed admixture, allele frequencies correlated, and 100,000 iterations of burn-in and 1,000,000 iterations of data collection. The K value with the bet fit to the data was determined following Evanno (2005). The K=6 run (K=6 was found to be the best fit (Figure 3.5)) with the highest probability score was used to generate a STRUCTURE plot.

3.3.4 Morphological analysis

Images of three leaves per plant were taken at the time of collection using a digital camera. A cm ruler was used as the scale for the images. These images were analysed in FIJI (Schindelin et al. 2012). The scale was calibrated by a known distance of 20mm and this distance and the units recorded. To reduce the issue of shadows caused by taking images in the field, the blue colour channel was used and the option "subtract background" applied. Thresholding was performed on the images and the leaves were selected with the wand tool and individually analyzed. Since *S. rotundifolia* is described by rounder leaves, the roundness of leaves was of interest to see if this measure was distinct from the more variable *S. radicans*. In leaves that were difficult to determine where the leaf blade ended and petiole began

the leaves were measured from the top of the leaf to the narrowest point. Leaf measurements excluded the petiole. Leaves that were damaged, were not lying flat or leaves that could not undergo thresholding were not analysed. If possible three leaves were measured per sample and the average roundness score was then used for the morphology analysis. An ANOVA was performed on the morphology data using existing species as groups (Table 3.3) with Waverly (Wav) and Waikawa (Wk) omitted as they are suspected hybrid populations. Frequency data from the morphology analyses were graphed to assess the shape of the data for further analysis. Microsoft Excel was used to run a one way ANOVA and to produce the frequency graphs for morphology analysis. Following the ANOVA, pairwise species comparisons were made by Tukey's HSD test using the Harmonic mean for each comparison. Morphological comparisons of flowers were not made due to the difficulties of measuring on site, small size of the flowers and absence of flowers in many populations at the time of collection.

3.4 Results:

Nine markers were screened in 612 *Selliera* individuals. Success rate of marker amplification ranged from 73 – 100% across individuals (Table 3.2).

Table 3.2: Characteristics of 9 microsatellite markers in *Selliera.*

Loci	A	Size range	Ho	Ht	F_{IS}	F_{ST}	% amplification
SR17	4	252 - 258	0.001	0.399	0.980	0.818	99.2
SR31	5	369 - 373	0.181	0.192	-0.345	0.298	99.2
SR45	8	271 - 283	0.356	0.654	-0.053	0.483	99.2
SR37	7	296 - 302	0.102	0.670	0.684	0.519	97.1
SR6	3	208 – 214	0.070	0.135	-0.170	0.556	99.1
SR11	4	182 - 194	0.087	0.246	-0.027	0.790	73.2
SR24	5	203 - 209	0.416	0.726	0.071	0.382	90
SR29	2	397 -399	0.013	0.089	0.739	0.444	98.7
SR13	2	313 - 315	0.122	0.318	0.027	0.604	99.2

Number of alleles (A), size range in bp, observed heterozygosity (Ho), species-wide expected heterozygosity (Ht), percentage amplification (%) and estimates of Wright's fixation index (F_{IS} and F_{ST}) for all micorsatellite markers.

3.4.1 Genetic variation

Average F_{IS} for all populations was 0.124 however F_{IS} per population varied from -0.629 to 0.795 (Table 3.1) (excludes populations Wai and KES; see Table 3.1) with only four populations, all *S. radicans*, showing a significant heterozygote excess with negative values. F_{ST} for all populations was 0.525 showing a high degree of differentiation between populations. All currently described species also had high F_{ST} values (Table 3.3) indicating populations within the species have a high degree of differentiation.

Table 3.3. F statistics of populations for species not including hybrid populations and for all populations. Populations are grouped into the currently described species and Nei's Gst and Weir and Cockerham's θ are reported for each of the species and for all populations combined.

Species	Populations	Nei's Gst	W-C θ
S. microphylla	M1,M2,NT,WD	0.477	0.563
S. rotundifolia	HA, HB, HC KP, Ma	0.441	0.497
S. radicans	CP, CSP, MAK, Otk, Wai, EST, KES, Na, Oh, Tw, WB, Wri, Mo, TB, PLM, HOB, Li, Pat, Prm, OPE, Br,	0.434	0.453
All	All populations	0.516	0.525

AMOVA suggests that 25.41 percent of the variation occurs between the currently described species while 37.49 percent occurs among populations within species and 37.10 percent of the variation occurs within populations (Table 3.4), indicating there is greater variation within species than between species. Mantel tests suggest there is isolation by distance for both *Selliera microphylla* and *Selliera rotundifolia,* although it is not significant in *Selliera microphylla* (Table 3.5) likely due to the

small sample size. The same test indicates that there is no correlation between genetic distance and geographic distance *Selliera radicans* (Figure 3.3).

Table 3.4. AMOVA results for *Selliera* species, including the degrees of freedom (d.f), sum of squares, variance components and percentage variation. $p < 0.01$.

Source of Variation	d. f.	Sum of Squares	Variance components	Percentage of variation
Among species	2	223.239	0.34914 Va	25.41
Among populations within species	28	535.993	0.51520 Vb	37.49
Within populations	1097	559.207	0.50976 Vc	37.10
Total	1127	1318.439	1.37410	

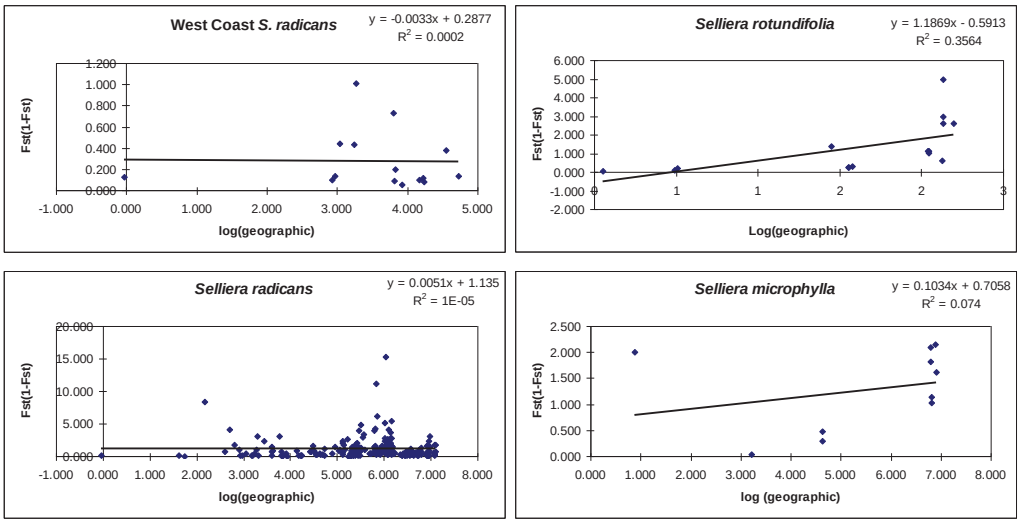

Figure 3.3 Mantel test results displayed in graphs of log(geographic distance) against $F_{ST}(1-F_{ST})$ for each of the species in *Selliera, S. microphylla, S. rotundifolia* and *S. radicans. S. rotundifolia* and *S. microphylla* both show a positive correlation between geographic distance and genetic distance. A mantel test for population of *S. radicans* along the described range of *S. rotundifolia* (West Coast *S. radicans*) was also performed but no correlation was observed in either analyses for *S. radicans*.

Table 3.5 Mantel test results. Rxy and *p* values for each of the species are reported.

Species	**Rxy**	***p* (rxy-rand >= rxy-data)**
S. microphylla	0.272	0.238
S. rotundifolia	0.597	0.002
S. radicans	0.004	0.490

Rxy = correlation coefficient of the Mantel test. P(rxy-rand >= rxy-data) = the probability of a positive autocorrelation (one tailed t test).

3.4.2 Morphological analysis

A one way ANOVA was used to test for a significant difference in leaf morphology among the three species as described in Table 3.3. Leaf morphology differed significantly between the three species, $F(2, 382) = 452.6793$, $p < 0.001$. *S. rotundifolia* and *S. radicans* both show a normal distribution and are mostly distinct with a small overlap whereas *S. microphylla* leaf morphology falls within the upper range of *S. radicans* (Figure 3.4). Waverly is a site where hybridization is suspected between *S. radicans* and *S. rotundifolia*; individuals at Waverly show a range in leaf morphology spanning from *S. radicans* and *S. rotundifolia* (Figure 3.4). Waikawa is another site of sympatry for *S. rotundifolia* and *S. radicans* where hybridization may occur but no hybrid morphologies were found with a clear distinction in morphology between the species (Figure 3.6).

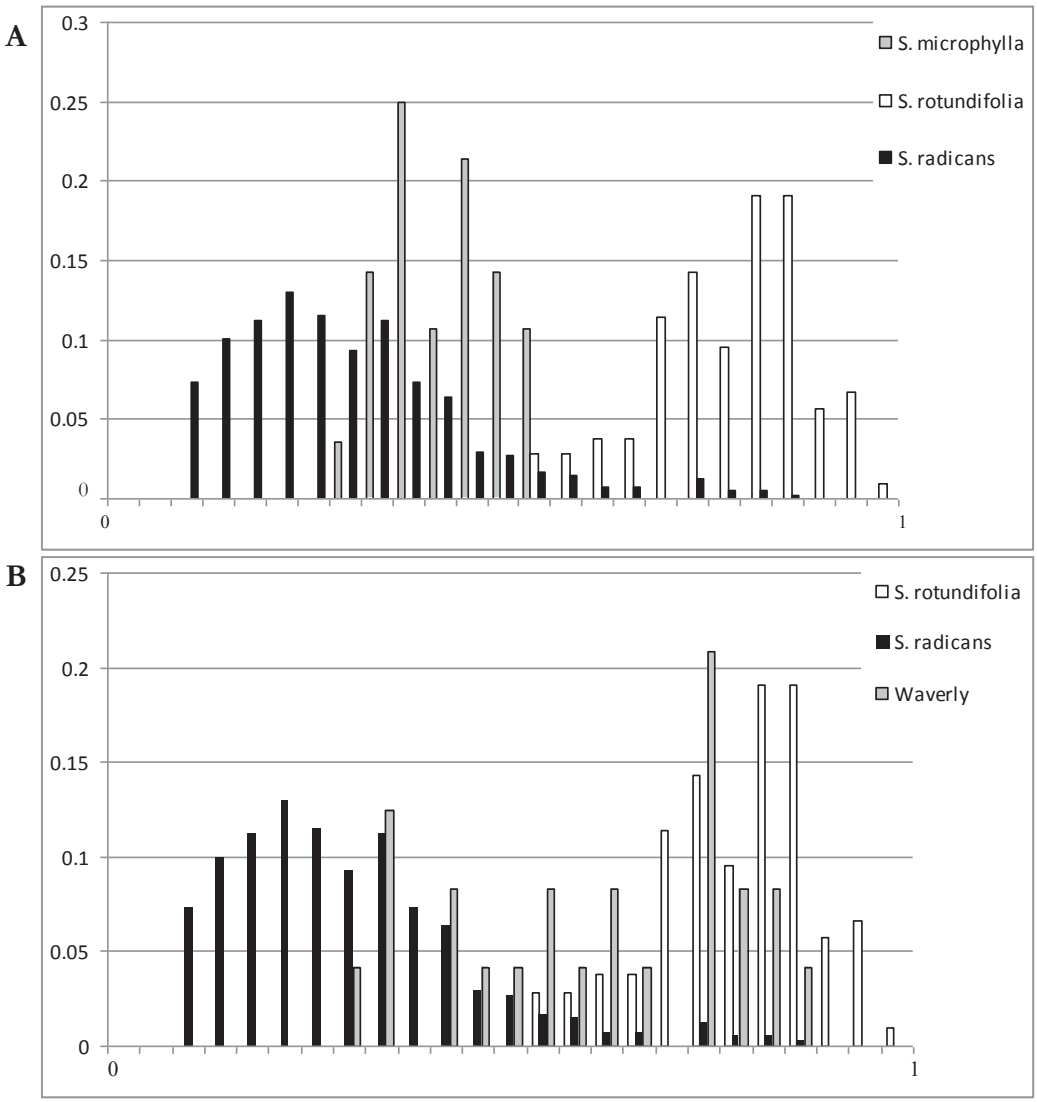

Figure 3.4 Histograms of the leaf morphology roundness index. Comparison of the three species (A) showing *S. radicans* in black, *S. rotundifolia* in white and *S. microphyllaa* in grey. A small overlap in leaf morphology is observed between *S. radicans* and *S. rotundifolia. S. microphylla* leaf morphology falls within the range of *S. radicans.* A comparison between *S. radicans* (black), *S. rotundifolia* (white) and individuals from Waverly (grey) where hybridization is suspected between these species is shown in (B) where Waverly can be seen to consist of a range of morphologies from *S. radicans* to *S. rotundifolia.*

3.4.3 STRUCTURE

In the STRUTURE analyses, K=6 was revealed to be the best fit of the data (Figure 3.5). *S. radicans* populations show four main clusters but many populations show admixture with all ancestral genotypes present within the described species (Figure 3.6). Many *S. radicans* clusters correlate with geographic locality, for example,

Ohope (OPE) and Ohiwa (Oh) cluster which are both North Island populations collected from the Bay of Plenty (Figure 3.2) But often the clusters in STRUCTURE for *S. radicans* do not correlate with geographic location where populations from the North Island may cluster with South Island populations and not populations geographically closer to them, for example Ohope (OPE) and Ohiwa (Oh) also cluster with the South Island populations Tunnel Beach (TB) and Dunedin (EST). In another example, from the North Island populations, Cape Palliser (CP) clusters with Napier (Na), and Plimmerton (PLM) but not Makara (MAK) or Castlepoint (CSP) which are geographically closer. Many of the *S. radicans* populations cluster with some populations described as *S. microphylla* or *S. rotundifolia* suggesting these species may have originated from those populations. The population WB from Tauranga revealed a clear genetic distinction between the first and second halves of the population suggesting a lack of gene flow between these individuals within this population.

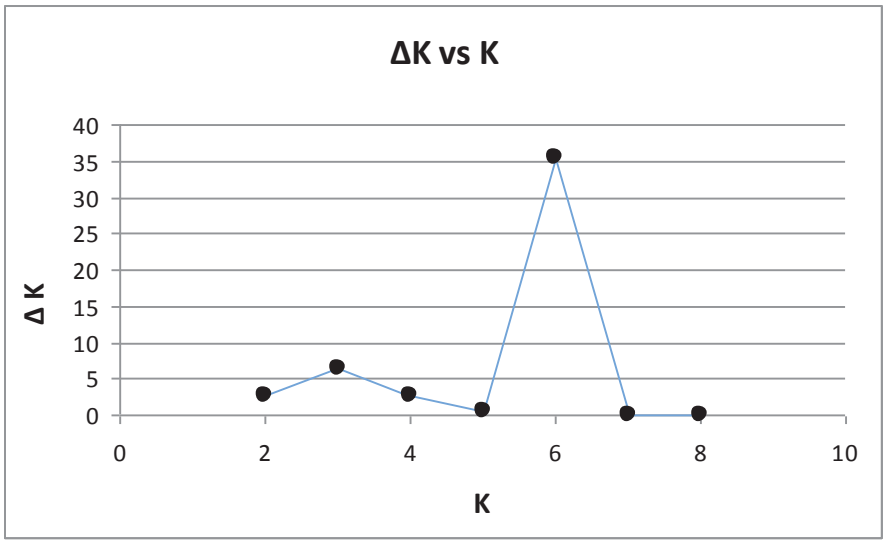

Figure 3.5 Plot of ΔK vs K for STRUCTURE results following Evanno (2005). K = 6 was the best fit to describe the data. Results are based on 10 replicates for each K value.

Populations that have been described as *Selliera microphylla* show two genetic clusters, one made up of the populations from the central volcanic plateau (NT/M1) which clusters with several *S. radicans* populations while the other forms the South Island populations of *S. microphylla* collected from West Dome (WD) near the Eyre Mountains. The West Dome populations only cluster with one other population, Tiwai Point (Tw), which is the most proximal *S. radicans* population collected. Populations from *Selliera rotundifolia* also show two genetic clusters (Figure 3.6). The three Himatangi (H) populations cluster together along with the Waikawa round leaved population (WK2) and *S. radicans* from Castlepoint (CSP). The second cluster consists of populations Kaupokonui (KP) and Manutahi (Ma) found north of Himatangi which also cluster with many other *S. radicans* populations.

The Waverly (Wav) population, which consists of sympatric *S. rotundifolia,* and *S. radicans* individuals, has genotypes representing both species. This is consistent with the range in leaf morphology, both of which support hybridization at this site. The Waikawa (Wk) population is another population where *S. radicans* and *S. rotundifolia* grow in sympatry. An additional *S. radicans* estuary population also was collected at this site. The *S. rotundifolia* individuals at Waikawa mostly cluster with other *S. rotundifolia* populations from Himatangi (H). *S. radicans* growing sympatrically, and at the estuary, consist of genotypes from *S. radicans* and a small percentage of *S. rotundifolia* genotype.

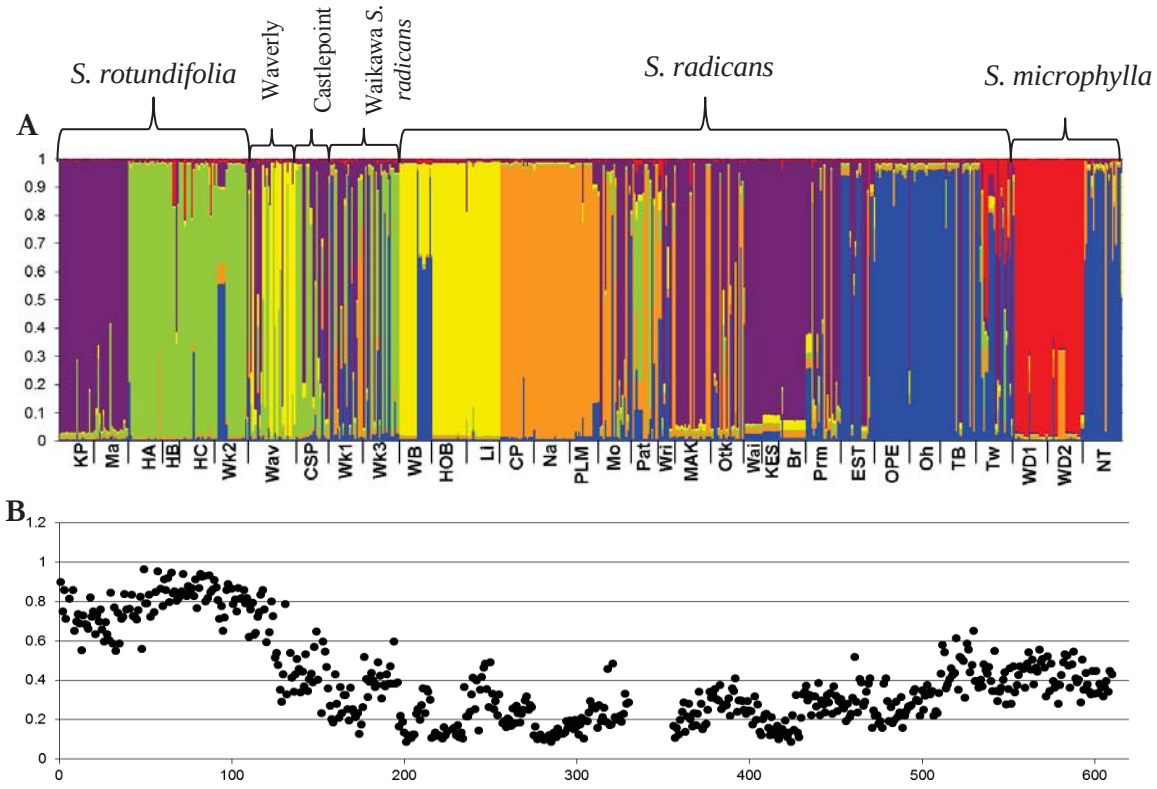

Figure 3.6 STRUCTURE cluster assignment and morphology score for individuals in *Selliera.* A, K=6 STRUCTURE cluster plot for *Selliera* individuals grouped by current species descriptions. Two genetic clusters can be observed in both *S. rotundifolia* and *S. microphylla. S. radicans* consists of four main clusters although all genotypes are present in this species. B, morphology score for individuals corresponding to STRUCTURE. Morphology data for two populations was not collected, Pat and Wri. M1 and M2 individuals (not labelled) are the last two individuals next to the NT population.

3.4.4 NEIGHBORNET

Patterns similar to those identified in the STRUCTURE analyses were observed in the population-level Neighbor-Net (Figure 3.7). *S. microphylla* populations show the same distinct groupings. The West Dome (WD) populations cluster together and are most closely related to Tiwai Point (Tw). The Central Volcanic Plateau *S. microphylla* populations (NT) are placed next to the West Dome populations meaning the *S. microphylla* populations appear to cluster together although the Central Volcanic Plateau population also clusters with other *S. radicans* populations. This is further considered in the discussion.

S. rotundifolia populations also are positioned near each other but show two main clusters. The first is a cluster with the Himitangi populations (H), round leaved Waikawa (Wk2), Castlepoint (CSP) and some *S. radicans* populations (Wk1, Wk3 and Mo) which is the same pattern observed in STRUCTURE. The second main cluster consists of Kaupokonui (KP) and Manutahi (Ma) which is also observed in the STRUCTURE results. KP/Ma is also closely related to several other *S. radicans* populations.

S. radicans populations do not form a single cluster in the NEIGHBORNET with many populations of *S. radicans* more closely related to the other described species in *Selliera.* However, many of the clusters observed in STRUCTURE are also present in the NEIGHBORNET. The sympatric site of *S. radicans* and *S. rotundifolia*, Waverly (Wav), is not clustered with any other populations possibly due to hybridization at this site. Waikawa individuals, from the other sympatric population, are closely related to the Himatangi cluster although the Waikawa round leaved population (Wk2) is distinct from the *S. radicans* populations at Waikawa (Wk1 and Wk3) which are more closely related to an *S. radicans* population (Mo).

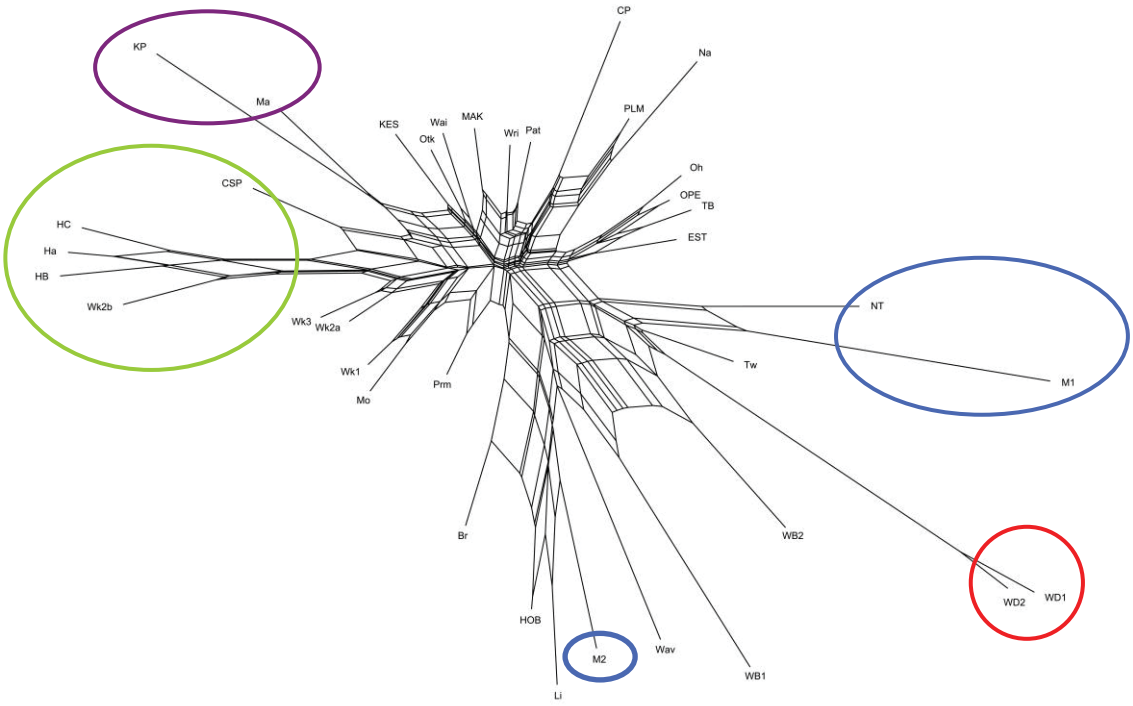

Figure 3.7 Neighbornet based on pairwise genetic distances between all populations of all *Selliera* species. The neighbornet was generated in SPLITSTREE4. *S. rotundifolia* population lineages observed in STRUCTURE are indicated by the green and purple circles and *S. microphylla* population lineages observed in STRCUTRE are identified by the red and blue circles. Populations Wk2 and WB were each split into two groups, Wka, Wkb, Wb1 and Wb2 respectively, due to the distinct genotypes observed in STRCUTURE in each of these populations.

3.5 Discussion:

Species delimitation is an important but difficult task in biology (Birky et al. 2010). While most agree that species can be defined as lineages that are separately evolving from other such lineages (Wiens 2007), identifying these lineages can be problematic. Hybridization, polyploidy, introgression and incomplete lineage sorting are examples of biological processes that increase the difficulty of species delimitation particularly in plants where these processes can occur frequently (Wendel 2000; Whitney et al. 2010) and instances where speciation processes have not had time to complete such as younger islands (Whittaker et al. 2007). Furthermore, both local adaptation and random genetic drift (Williams 1966) can result in divergent populations within a species, so among the difficulties is distinguishing between intraspecific and interspecific variation. Natural selection, geographic isolation and gene flow, which influence the genetic structure of populations, are often described as the driving forces of speciation (Harrison 2012). As these forces act at the population level a population genetic approach, which examines the genetic structure of populations, may aid in species delimitation especially in closely related species where speciation processes may not be complete.

An example of a genus where species delimitation has been difficult is *Selliera,* a genus in the Goodeniaceae. There have not been many population genetic studies performed within the Goodeniaceae. One study on *Scaevola hainanensis* Hance. (Goodeniaceae) revealed low levels of genetic differentiation (GST = 0.172) between the study sites indicating gene flow between sites was likely high (Ho et al., 2005). Only 6 of 233 ISSR bands were polymorphic indicating low genetic diversity overall however *S. hainanensis* is an endangered species and was restricted to a small site of less than 1ha. Another study on *Scaevola plumieri* Vahl. (Goodeniaceae) used ISSR markers to investigate population structure. *S. plumieri* is a primary dune coloniser from southern Africa, described as a small shrub which can be propagated by seed, sprawling stems and stem cuttings, similar to propagation in *Selliera*. They revealed high differentiation between populations and concluded individual isolated dunes usually contained individual plants or clones but sexual reproduction occurred between populations and dunes (Barker, Harman,

Ripley, & Bond, 2002). Using a population genetics approach we attempted to determine the species boundaries within *Selliera*. Nine microsatellite markers were analysed in 35 populations consisting of samples from each of the species to attempt to determine species boundaries and population structure within *Selliera.*

3.5.1 Variation a*mong Selliera*

The genus-wide F_{ST} was 0.525 (Table 3.3) suggesting high differentiation between populations which may not be surprising if there are distinct species within the genus. AMOVA results suggest there is greater variation between populations within a species than between species (Table 3.4) suggesting low diversity among the described species consistent with the F_{ST} estimation that showed high distinction between populations for all species.

The ANOVA results comparing leaf morphology showed a significant difference between the leaf shape among species ($F(2, 382) = 452.6793$, $p < 0.001$). Frequency graphs show a normal distribution for both *S. radicans* and *S. rotundifolia* with a small overlap in leaf shape between these species while *S. microphylla* is within the range of *S. radicans* (Figure 3.4). This result supports a morphological distinction between *S. radicans* and *S. rotundifolia* although as *S. rotundifolia* is primarily described based on rounder leaves this result was expected. Post-hoc tests identified significant differences among all three species. These results provide evidence for the existence of the three species in *Selliera* which is considered in greater detail in the following sections.

3.5.2 Species in *Selliera*

Selliera radicans:

The F_{ST} value for all populations considered to be *S. radicans* was 0.453 which is lower than the values for both of the other species (Table 3.3). This suggests populations in the *S. radicans* grouping have greater genetic similarity than the populations of *S. rotundifolia* and *S. microphylla* although there is still considerable differentiation between populations in *S. radicans*. The *S. radicans* F_{ST} value is slightly lower than the value for all *Selliera* populations (0.525) which is expected as the inclusion of the different species should decrease genetic similarity between populations although it still remains high suggesting there is still divergence

between some populations within *S. radicans*. Species with large geographic ranges, such as *S. radicans*, are expected to maintain genetic diversity so they would have higher F_{ST} values than species in more restricted ranges (Hamrick & Godt, 1996).

The Mantel test was performed on each of the species separately (Figure 3.4) and suggests no isolation by distance for *S. radicans* (Table 3.5). Theoretical research has suggested that founder effects will likely lead to greater differentiation between populations (Slatkin 1977; Wade and McCauley 1988) where differences between populations can be a result of colonization history rather than migration or selection. This situation has been observed in several field and laboratory trials (Kolbe et al. 2012; Moya et al. 1995; Travisano et al. 1995) and has been suggested to be the cause of population differentiation in an island bird species *Anthus berthelotii* (Motacillidae) where genetic structure within the species was not consistent with isolation by distance or adaptation (Spurgin et al. 2014). The lack of isolation by distance in *S. radicans* may be caused by colonization history where population differentiation is based on the ancestral coloniser resulting in geographically close populations highly differentiated due to different colonization events. This founder effect and restricted gene flow between populations could result in speciation through adaptation to different habitats although founder effects do not necessarily result in speciation (Moya et al. 1995). A lack of gene flow between close populations or gene flow over large distances may also increase genetic distance and explain the lack of isolation by distance.

Gene flow over large distances could be explained by pollinator movement which, in *Selliera,* is thought to be a species of Diptera (Cheeseman 1876). *Sitodiplosis mosellana* Gehin. (Diptera: Cecidomyiidae) has been observed to travel distances up to 195 Km, suggesting long distance pollen dispersal may be possible although it is not known if this is a mechanism for pollen dispersal in *Selliera.* Gene flow over a large distance could also be due to sea currents as *S. radicans* habitat is coastal and seeds could be dispersed by the sea allowing long distance dispersal. *Selliera* seeds have no significant mortality after treatment in a

saline solution (Partridge and Wilson, 1987) indicating survival in salt water may be possible. Seed dispersal by sea currents was shown to allow gene flow between populations of *Cakile maritima* Scop. (Brassicaceae), which had no evidence of isolation by distance, and showed that seed buoyancy was important in allowing dispersal and survival of seeds (Gandour, et al. 2008) which has not been assessed in *Selliera*. There is a greater amount of admixture between the *S. radicans* populations than in the other described species (Figure 3.6). STRUCTURE showed a value of K=6 to best describe the data (Figure 3.5) with all six ancestral genotypes present in *S. radicans* populations which indicates that the other species may have evolved from *S. radicans.* This result might also suggest that divergence of the other species is relatively recent.

There are four main genetic clusters within *S. radicans* populations. One of these clusters consists of two Auckland populations, HOB and Li, and half the population at Tauranga (WB). The Tauranga population revealed an interesting pattern with a clear distinction between the first half of samples from the population and the second half. This can be explained by the distribution of individuals within this site which consists of two separate areas. The first area of samples was taken from a muddy estuarine area around a small drain, while the second half of samples were taken from a grassy bank next to the estuary. The spacing between these two areas is ~10 meters. The distinct genotypes found within this population suggest there is a lack of gene flow between these sites within this population despite the small geographic distance. This could be due to selection pressures in the different environments at this site selecting for certain genotypes that confer an advantage to the plants in that environment, for example, flood tolerance or soil composition differences between the muddy estuary and the grassy bank. However it is possible gene flow is resticted at this site through other means, such as a lack of pollinators, as it is next to an inhabited area in the city which may deter insects.

A cluster of three *S. radicans* populations, Cape Palliser (CP), Napier (Na) and Plimmerton (PLM) show almost no evidence of gene flow with other populations. These populations are not geographically close, with CP found at the lower eastern

North Island, Na on the East Coast and PLM on the West Coast of the North Island, but there is evidence of some gene flow to other populations from these three. Why these three geographically distant populations cluster together is not clear, although it may be a result of colonization history as suggested in other systems (Kolbe et al. 2012; Spurgin et al. 2014). The lack of genotypes from nearby populations suggests lack of gene flow although Na and PLM have significant negative F_{IS} values, -0.629 and -0.280 repectively, (Table 3.1) indicating heterozygote excess within the populations. Another obvious cluster of *S. radicans* populations is Ohiwa (Oh) and Ohope (OPE) which also show no evidence of gene flow from other populations. Oh also has a significant negative F_{IS} value of -0.629 suggesting a heterozygote excess in this population, but as these populations are geographically close it is possible gene flow occurs between them. Negative F_{IS} values can also be attributed to gene flow, selection for heterozygotes, increased genetic drift in small populations or marker error.

Selliera radicans is known to be highly polymorphic in leaf shape but *S. rotundifolia* is described primarily based on leaf shape; specifically, a rounder leaf shape with the apex and base obtuse (Heenan 1997). Morphological analysis on leaf roundness reflects this polymorphic leaf shape in *S. radicans* (Figure 3.6) and shows a small overlap between the leaf morphology of *S. rotundifolia* and *S. radicans* (Figure 3.4), but reveals a distinction between the two species. The likely hybrid population, Waverly (Wav), does contain a range in morphology between the 'typical' *S. radicans* and *S. rotundifolia* forms, which is consistent with the range of genotypes (Figure 3.6) suggesting hybridization occurs at this site. This is in stark contrast with the Waikawa (WK) site where these two species also occur. There are essentially two distinct classes of leaf morphology (*S. radicans*-like, Wk1 and 3, and *S. rotundifolia*-like, Wk2) at Waikawa (Figure 3.6), although there is evidence of introgression from *S. rotundifolia* into *S. radicans* suggesting hybridization has occurred in the past. These sites are discussed further in the section on hybridization.

Selliera rotundifolia:

Populations described as *Selliera rotundifolia* form two distinct clusters in both the Neighbornet and STRUCTURE results (Figure 3.6 and 3.7), suggesting that the species may not be monophyletic. These clusters each also cluster with different *S. radicans* populations. The F_{ST} value for *S. rotundifolia* is 0.497 indicating populations are distinct from each other which supports the distinct genetic clusters of *S. rotundifolia* seen in the different analyses. This suggests there may have been multiple origins, at least two, of round leaves appearing in *S. rotundifolia* which, under the lineage species concept, may not accept these different lineages as one species. Alternatively, these two lineages may have resulted from isolation of populations from a single origin of *S. rotundifolia* leading to divergence due to lack of gene flow between populations, but this is unlikely as each lineage shows a closer relationship with different *S. radicans* populations indicating round leaves has arisen multiple times. The Mantel test suggests a positive relationship in *S. rotundifolia* (Figure 3.3) which may be a result of the two clusters within this species (Figure 3.6) which are geographically distinct from each other.

Currently, *S. rotundifolia* has been described as endemic to the lower western North Island (Heenan 1997) which is where all round-leaved populations occur. However, a population on the East Coast of the lower North Island, Castlepoint (CSP), also clusters with the Himatangi/Waikawa grouping of *S. rotundifolia* in both the Neighbornet and STRUCTURE results (Figures 3.6 and 3.7). The results of the morphological analysis show that Castlepoint individuals do not have as strongly rounded leaves as those of other *S. rotundifolia* populations, but they are at the top end of the measurements for *S. radicans* samples. STRUCTURE reveals genotypes from both *S. rotundifolia* and *S. radicans* in the Castlepoint samples, suggesting this may be a population where hybridization has occurred between species although this will need to be investigated in more detail. The KP/Ma *S. rotundifolia* grouping clusters with many *S. radicans* species in STRUCTURE (Figure 3.6) and is more closely related to some of the same *S. radicans* populations than the other *S. rotundifolia* populations in the Neighbor-net (Figure 3.7), indicating a distinct origin for rounder leaves in this group.

The shape of leaves in *Selliera* may not be a suitable method to describe species, as round leaves appears to have multiple origins. Matching the current geographic distribution of *S. rotundifolia*, round-leaved plants were located in the lower western North Island and so far no other locations have produced similarly shaped round leaves. While it is documented that the round leaf morphology is genetically determined (Ogden 1974), it is unknown whether the genetic variation for roundness only occurs in the southwest region of the North Island.

There are only two sampled sites where *S. rotundifolia* and *S. radicans* are clearly sympatric, Waverly and Waikawa. At Waverly, the morphology indicates there is a range of hybrid morphologies from round-leaved plants to more elongated, spatulate leaves. This can also be observed in the STRUCTURE results where there is a range in the percentage of genotype from each species (Figure 3.6), indicating there is gene flow between these species. The range of morphology suggests leaf shape is controlled by multiple genes. The *S. radicans* genotype at Waverly clusters with the more northern populations of *S. radicans* (HOB/Li/WB) and both *S. rotundifolia* genotypes are present, which may suggest hybridization between these distinct lineages is possible, possibly supporting the two *S. rotundifolia* lineages considered as a single species.

At Waikawa, morphology does not show a gradient between the distinct morphologies of *S. rotundifolia* and *S. radicans* plants that are completely interspersed (Figure 3.6). The round-leaved *S. rotundifolia* plants of Waikawa (WK2) mostly cluster with the Himatangi *S. rotundifolia* grouping and appear to be reproductively isolated from the sympatric *S. radicans* as there is no evidence of gene flow despite complete sympatry. The sympatric *S. radicans* population, despite having typical *S. radicans* leaf morphology, shows possible gene flow from *S. rotundifolia*. STRUCTURE results reveal that this population contains genotypes mostly from *S. radicans* but also contains some *S. rotundifolia* genotypes. The low degree of similarity to the sympatric *S. rotundifolia* and the *S. radicans*-like morphology indicate there may be introgression occurring from *S. rotundifolia* into *S. radicans* although this needs further study. Further, there is evidence of a low level of gene flow with the estuary population of *S. radicans* at the Waikawa site

(Wk1), which is not sympatric with the other Waikawa populations, suggesting possible hybridization with *S. rotundifolia* or the sympatric *S. radicans.*

As the Waikawa round-leaved population (Wk2) clusters with the Himatangi *S. rotundifolia* grouping (HA/HB/HC), which is fairly distinct from most *S. radicans* populations (Figure 3.7), it may be more distant from *S. radicans* and so may have evolved reproductive barriers with *S. radicans* at Waikawa. Reproductive isolation would indicate a species under the general lineage concept as they would form distinct lineages if gene flow is resticted, however more research on this would be needed especially crossing experiments to see if populations are indeed reproductively isolated. There are other possibilities for why hybridization occurs differently at the Waverley and Waikawa sites such as pollinator preference where pollinators may prefer flowers that resemble the parent so can be a barrier to reduce gene flow; however in *Selliera* the flowers are indistinguishable among the described species, differing only slightly in size (Heenan 1997) so pollinator preference is unlikely to play a significant role.

The rounder leaves of *S. rotundifolia* may not confer any significant advantage to the plant in the dune habitat, but may be pleiotropic with traits that are under selection or linked to genes that do give an advantage to the plants, for example genes that aid water stress as water may not be as easily available in a dune environment compared to an estuary environment. *Linanthus parryae* (Polemoniaceae) is an example of a species with an obvious morphological polymorphism linked to a more cryptic variable trait under strong selection. *L. parryae* has two flower colour morphs, blue and white, which differ in frequency in different populations. Initially this was thought to be the effect of random genetic drift (Wright 1943), but later studies showed that natural selection appeared to be the driving force in maintaining the flower colour polymorphism as the soil types differed between regions. The authors suggested a pleiotropic effect where differential cation uptake by the different flower morphs was the mechanism of selection (Schemske and Bierzychudek 2007).

Selliera microphylla:

As currently described, *S. microphylla* is found on the Central Volcanic Plateau in the North Island and the Eyre mountains in the South Island. F_{IS} for all populations was positive (Table 3.1) indicating significant homozygote excess. Significant homozygote excess is likely a result of inbreeding, reducing the amount of heterozygotes in the population (Wright 1951), especially as *Selliera* can form large mats and spread clonally. However, small population sizes or null alleles can also result in a high level of homozygosity. Inbreeding is particularly an issue in geographically isolated populations where gene flow with other populations is not possible, such as in *S. microphylla.* Consequently, this high homozygosity in *S. microphylla* is expected as gene flow between the North and South Island populations or with other *Selliera* species is unlikely and the geographically isolated nature of these populations from other *Selliera* populations may promote inbreeding. Unsurprisingly, F_{ST} for *S. microphylla* populations was the highest of the three species at 0.563 suggesting *S. microphylla* populations are strongly genetically differentiated from each other. A positive relationship was observed in the Mantel test for *S. microphylla* (Figure 3.3) but was not significant (Table 3.5) likely due to the smaller sample size.

This lack of gene flow and distinct population structure within *S. microphylla* was seen in the STRUCTURE results and Neighbour-net which revealed two clusters for *S. microphylla* one including populations from the North Island and one for the South Island populations (Figures 3.6 and 3.7). The North Island *S. microphylla* populations cluster with Oh/OPE and a few individuals also cluster with the CP/Na/PLM cluster. Tiwai Point (Tw) is the only *S. radicans* population that contains any genotypes from the South Island *S. microphylla*; Tw is the closest known *S. radicans* population to the Eyre Mountain *S. microphylla* populations. In the Neighbor-net the *S. microphylla* populations form a cluster, but each is also more closely related to *S. radicans* populations than each other; the STRUCTURE results support this, showing the North Island and South Island *S. microphylla* samples to be unique. The apparent clustering of all *S. microphylla* in the Neighbor-Net is likely due to a long branch attraction (Bryant and Moulton 2004) anomaly more than biological reality. The high differentiation between populations

of *S. microphylla* and closer relationship to *S. radicans* populations that are geographically proximal indicate separate colonisations of *S. radicans* into the mountain habitat rather than a single species of mountain *Selliera* that has spread throughout the islands. As these populations are isolated from the other coastal populations of *S. radicans* they may continue to diverge and could potentially form new species, but there is no evidence to cluster the North and South Island *S. microphylla* as a single species. The shared smaller size difference may be due to the environmental conditions of the mountains, given that plants are usually smaller at higher altitudes (Billings and Mooney 1968) and *S. microphylla* plants revert to a similar morphology to *S. radicans* when grown in the same habitat (De Lange 2012a and K Pilkington personal observe.) suggesting habitat differences cause the shared morphology in the currently described *S. microphylla*.

As the South Island populations have the same chromosome number as *S. radicans*, 2*n*=16 (Murray and de Lange 2013), they likely are an inland population of *S. radicans* although further experiments on reproductive isolation may be helpful to understand if these populations can still reproduce with other *S. radicans* populations, particularly as they appear highly genetically distinct from most populations in *Selliera*. These populations have low heterozygosity (Table 3.1) and with geographic isolation from other populations could suffer from inbreeding depression affecting their future. North Island populations of *S. microphylla* have shown a single chromosome count of 2*n*=56 (Murray and de Lange 2013), much higher than the 2*n*=16 of *S. radicans* indicating these plants have undergone polyploidy. In Tasmania there has been a report of 2*n*=48 (Jackson 1958) for *S. radicans*, which was found in the same location as the diploid, suggesting that polyploidy has occurred in this species in other sites, although in New Zealand only *Selliera microphylla* in the North Island has revealed a higher chromosome number so far.

In *Arabidopsis* Heynh. the frequency of diploid male gamete production increased in periods of cold stress (De Storme et al. 2012) and cold shock is known to result in polyploidy, not only in plants (Wang et al. 2010), but also in fish (Donaldson et al. 2008) and shrimp (Wood et al. 2011). The higher altitudes which harbour colder

temperatures may facilitate the production of unreduced gametes and therefore result in polyploidy which may be a factor of the increase in chromosome number in *Selliera microphylla*. Analysis of Artic plants showed that at least 40% of these plants were polyploid and this frequency increased further northwards (Brochmann et al. 2004) where the temperature is colder and where plants likely experience cold shock. It is also possible that polyploidy increases the survival chances of plants at higher altitudes and is a result of adaptation to the environment. Induced tetraploids of *Dendranthema nankingense* (DC.) Des Moul. (Asteraceae) showed increased tolerance to cold, drought and salinity stress compared to the diploids (Liu et al. 2011) indicating these polyploids had a greater potential to survive in more extreme environments.

As the mountain habitat is vastly different to the coastal habitat of *Selliera* the potential to adapt to the differing conditions would give an advantage to plants growing there and increase the capacity for divergence into a distinctly evolving lineage. Due to the large difference in chromosome number, it is unlikely that the Volcanic Plateau *Selliera* plants could reproduce with other *S. radicans* populations, although this still needs to be investigated further with crossing experiments. However, the strong genetic divergence, increased chromosome number, and geographic isolation of this population support the North Island *S. microphylla* be maintained as a distinct species.

Species delimitation in *Selliera* remains difficult, however this study has revealed evidence for each of the described species, although with modified boundaries. We suggest that the *S. microphylla* name is maintained for the North Island volcanic plateau populations, but the South Island Eyre Mountain populations be named a variant of *S. radicans*. While the South Island populations are genetically distinct and are unlikely to experience gene flow with any other *Selliera* populations, there is currently no evidence for reproductive isolation. There is evidence that *S. rotundifolia* is reproductively isolated from *S. radicans* at one site of sympatry suggesting that this is a distinct species. However, two lineages were identified within the currently described *S. rotundifolia* suggesting not all round-leaved plants may be considered a single species under the general lineage concept but possible

evidence of gene flow between these lineages was observed at Waverly but needs further investigation. It appears as though there may be multiple origins of *S. rotundifolia* and there is some variation in the completeness of the speciation process among sites and potentially among origins.

3.5.3 Hybridization and introgression in *Selliera*

Hybridization between different species is not uncommon and has the potential to dissolve the barriers between species. Whitney et al (2010) found hybridization in 40% of the plant families they studied, although these were in only about 16% of genera surveyed, indicating hybridization is common but not pervasive. However, the estimation of hybridization frequency in this study may underestimate the actual number, because many species cannot be inspected throughout their range so many hybrids may not be documented and hybrids may not be suspected if the parental species are not obvious, for example the parental species have become extinct (Whitney et al. 2010). If hybrid offspring are infertile, this results in a barrier to gene flow as wasted reproductive effort, which may be a significant loss for endangered species. Alternatively, hybrids may be able to survive in environments that are less suited to the parents so inhabit a different niche (Kunte et al. 2011; Mao and Wang 2011) or are selected against (Vamosi and Schluter 1999) allowing the parental species to remain distinct especially if the parents are more successful in the original habitat. Hybridization accompanying polyploidy may also allow expansion into new niches or habitats relative to the parents (Ramsey and Schemske 1998) although most initial hybrids are morphological intermediates of the parental species. Hybrids can also result in extinction of one or both of the parental species if they out-compete the parents (Hegde et al. 2006). In New Zealand hybridization has played an important role in the evolution of many endemic species, both plant and animal. Additionally many distinct species remain able to hybridise, resulting in low levels of gene flow, but still maintain their distinctive lineages (Morgan-Richards et al. 2009).

Within *Selliera* there are two locations where both *S. rotundifolia* and *S. radicans* are currently found growing sympatrically, Waverly and Waikawa. At these sites there is the possibility for hybridization between these two species. At Waverly the

morphology of leaf roundness shows a range from round leaves, like that of *S. rotundifolia,* to elongated leaves, similar to those of *S. radicans,* with various intermediate forms indicating hybridization is occurring and producing intermediate leaf shapes. The range in leaf morphology is mirrored by the genotypes in the STRUCTURE results (Figure 3.6) indicating there is gene flow between *S. rotundifolia* and *S. radicans.* Hybridization between these two species, at Waverly, indicates they have not yet evolved reproductive isolation but does not necessarily mean they are not distinct species. Hybrid infertility or forces that maintain the genetic lineage of the parental species despite hybridization can allow a species to maintain its lineage and therefore remain distinct despite hybridization. In New Zealand at least 19 hybridization events between endemic species have been observed where the parental species maintain themselves as distinct species (Morgan-Richards et al. 2009). Further testing of hybrid individuals at Waverly may indicate whether hybrids are able to backcross with one or both parents and provide further insights into reproductive barriers between *Selliera* species.

For the two species to remain distinct at Waverly, the hybrids would need to be infertile to prevent backcrossing with the parental species. Alternatively, selection pressures on the hybrids that prevent them from inhabiting the preferred habitat of the parental species may allow the parental species to remain distinct. If the hybrids can backcross with the parents, introgression may allow them to expand into the parental habitat. Further, if the hybrids are more successful than the parents, then they may out-compete them and cause extinction of the parental species at Waverly or dissolve the boundaries between the parental species by forming a hybrid swarm.

At Waikawa there also are two distinct morphologies which are completely interspersed, round leaves and more elongated leaves that fall in the range of *S. radicans* but no intermediate morphology, so it appears that hybridization is not occurring between these species. However, genetic analyses indicate that hybridization may have occurred in the past as the *S. radicans* like plants show evidence of some gene flow from the *S. rotundifolia* plants. Genetic analyses for *S. rotundifolia* show no evidence of hybridization with *S. radicans* suggesting that at

this site gene flow from *S. radicans* to *S. rotundifolia* is no longer possible and *S. rotundifolia* may have evolved a reproductive isolating mechanism. This suggests that reproductive isolation may be preventing *S. rotundifolia* from crossing with *S. radicans* which would be evidence that they are distinct lineages and so distinct species. The estuary population at Waikawa (Wk1), which is not sympatric with the other Waikawa populations, shows some evidence of gene flow with *S. rotundifolia* but contains typical *S. radicans* morphology and could be the parental *S. radicans* population which may be able to cross with the dune *S. radicans* hybrids. It is possible that there is gene flow from *S. rotundifolia* to *S. radicans* directly but we would expect a hybrid morphology unless the elongated leaf morphology is dominant which is not seen in the Waverly hybrids. An alternative explanation for the presence of the *S. rotundifolia* genotype in the *S. radicans* populations at Waikawa is that *S rotundifolia* may have evolved from *S. radicans* at this site. The *S. radicans* populations may have retained some of the genotype that *S. rotundifolia* arose from, although it is also possible hybridization occurred between these species early in speciation.

Introgression has been suggested to create genetic variation and even aid adaptation or possibly adaptive radiation (Arnold and Martin 2009). At Waikawa, hybridization between *S. rotundifolia* and *S. radicans*, and subsequent backcrossing with *S. radicans* may have allowed the *S. radicans* hybrids to thrive in the dune flats rather than an estuary habitat which is their preferred habitat. If *S. radicans* can now out-compete *S. rotundifolia* this could pose a problem for the latter which is already rated in decline due to taller and faster growing weed species (De Lange 2012b). An example of adaptive introgression is in *Iris* L. between a flood tolerant species, *Iris fulva* Ker Gawl. and the dry adapted *Iris brevicaulis* Raf. The ability to survive flooding for up to four months was shown to have a genetic basis with genotypes of *I. fulva* surviving at significantly higher frequencies than *I. brevicaulis* genotypes. Hybridization and subsequent backcrosses to *I. brevicaulis* showed greater survival when they contained introgressed alleles from *I. fulva* involved in flood adaptation indicating that introgression can facilitate spread to new environments (Martin et al. 2006). Introgression has been observed in many New Zealand species such as, *Metrosideros* Banks ex Gaertn. (Gardner et al. 2004),

Coprosma J.R.Forst. & G.Forst. and alpine cicadas (*Maoricicada)* (Buckley et al. 2006) leading to questions whether this is a characteristic common to the New Zealand flora (Morgan-Richards et al. 2009).

Different hybridization rates among different sympatric populations have been observed previously in many different species, for example *Ipomosis* (Aldridge and Campbell 2009) and *Lomatia* R.BR. (McIntosh et al. 2014). The difference in hybridization rates is often attributed to ecological differences at the sites which can result in different selective pressures on hybrids (Aldridge and Campbell 2009). Alternatively, it is possible that there is a genotypic difference that prevents hybridization at some sites but not others, particularly as *S. rotundifolia* may have multiple origins so the different lineages could possess different genotypes some of which are more incompatible with *S. radicans* than others. These hybridization events between two *Selliera* species indicate that reproductive isolation occurs at one site providing strong evidence that the HB/HC/Wk cluster are a distinct species from *S. radicans*. Because *S. rotundifolia* has two genetic clusters that are more closely related to different *S. radicans* populations than to each other suggests that there are two lineages within *S. rotundifolia* which may explain the hybridization at Waverly but apparent reproductive isolation at Waikawa.

3.5.4 Future directions

These results provide insights into the population structure and species boundaries within *Selliera* but also raise further questions for investigation. It is not known if *S. microphylla* is capable of hybridizing with the other species so reproductive isolation tests and further chromosome analyses may be useful. It would be of interest to determine if the Central Volcanic Plateau population that has a chromosome count of $2n=56$ can hybridize with the other species, which is unlikely but would provide evidence that this is a distinct species. It also would be of interest to confirm the chromosome count and frequency of polyploids in other populations on the Central Volcanic Plateau. Studying the two sympatric sites of *S. radicans* and *S. rotundifolia* and the different lineages within *S. rotundifolia* in greater depth would be helpful in determining the status of these populations and the species. As hybridization appears to be restricted at Waikawa but not at

Waverly, comparing the differences between these sites including geographic and genetic factors that may be influencing the different rates of hybridization may help to identify the isolating mechanisms that prevent hybridization between the species and provide insights into the evolution of *S. rotundifolia* as well as determining species boundaries. An in depth study of the different preferred habitats for these species to investigate whether round leaves confers any advantage to *S. rotundifolia* in a dune environment compared to *S. radicans* preferred estuary habitat may reveal why the round-leaved form evolved and aid determination of species boundaries, although one might imagine that the round leaf form is either pleiotropic or genetically linked to another trait that is under selective pressure. As the Castlepoint population may be another *S. rotundifolia* population or a hybrid population where *S. rotundifolia* may have gone extinct, further investigation of this site may be an interesting study. As polyploidy has been documented in *Selliera radicans* in Tasmania (Jackson 1958) it would be interesting to make chromosome counts for populations of *Selliera radicans* from around New Zealand to assess if polyploidy is more extensive in New Zealand populations.

3.6 Conclusion

Species delimitation is an important task in biology but biological events such as hybridization, introgression and polyploidy can increase the difficulty in delimiting species. Population genetic approaches have been successful in delimiting closely related species that were previously difficult to determine (Bacon et al. 2012) and so is an ideal approach for use in *Selliera* which consists of closely related species described based on morphology or geographic location leading to disagreements about the status of some species.

We investigated the status of species in *Selliera* with microsatellite markers using a population genetics approach. Our results suggest there is evidence for the existence of each of the species previously described although this may need to be reviewed in the future. *S. rotundifolia* shows high differentiation between populations and distinct genetic lineages indicating round leaves may have arisen, twice in the history of *Selliera* in New Zealand and may not be suitable to identify a distinct species. There is evidence of hybridization between *S. rotundifolia* and *S. radicans* which differs between the two sympatric sites. At Waikawa *S.*

rotundifolia appears reproductively isolated from *S. radicans* although this needs to be confirmed through crossing studies but may suggest distinctly evolving lineages of *Selliera.* Introgression back to *S. radicans* at Waikawa may have allowed *S. radicans* to adapt to the dune environment that is preferred by *S. rotundifolia* which could pose a problem for *S. rotundifolia* which is already rated in decline due to faster and taller growing weed species. We also identified a further possible hybrid site at Castlepoint although this needs further investigation. *Selliera microphylla* also showed a high differentiation between populations. Two genetic lineages are each more closely related to different *S. radicans* populations suggesting that the South Island population may be an inland variant of *S. radicans* although if the population remains isolated it would be expected to continue to diverge into a new species.

3.7 References

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Chapter 4

Conclusion

4.1 Introduction

This study attempted to investigate the status of species in *Selliera,* (Goodeniaceae), which is controversial due to descriptions from only morphology and geographic distributions. Species delimitation is important in biology to provide consistancy, ensure the correct species is studied, conservation funding is spent where needed and may provide insights into evolution and the species process. However species delimitation remains one of the more difficult tasks in biology. The idea that species are lineages has been widely accepted by biologists (Wiens 2007) although this still leaves difficulty in defining a lineage, especially if the species are still in the speciation process, for example on younger islands (Birky et al. 2010). Factors such as hybridization, introgression, polyploidy, and incomplete lineage sorting can increase this difficulty in species delimitation. As these factors often act at the population level a population genetics approach, which studies the genetic structure of populations, may be informative in determining species boundaries. Population genetic approaches have been previously successful in delimiting species complexes where other methods have been unsuccessful (Bacon et al. 2012; Edwards et al. 2009; Kim et al. 2012) so may be an informative approach for *Selliera* where species are closely related and have the potential to hybridize.

Three species of *Selliera* have been previously described in New Zealand. *Selliera radicans* which is also present in Australia and Chile, *S. rotundifolia* (Heenan 1997), endemic to the lower Western North Island, and *S. microphylla* (Colenso 1889). *Selliera radicans* and *S. rotundifolia* are coastal species with *S. radicans* preferring an estuary habitat, although it can be found in a variety of habitats (De Lange 2014), and *S. rotundifolia* preferring a dune habitat (Heenan 1997). *Selliera*

microphylla is the only found inland mainly on the Central Volcanic Plateau (Colenso 1889) but has also been reported in West Dome in the South Island.

These species were described mainly based on geographic location and morphology. However in plants, morphology can be very plastic, where the environment can affect the phenotype seen, as plants cannot move once germinated, unlike animals, so need to be able to cope with minor environmental changes throughout their lifespan. *Selliera rotundifolia* has been described based on a preferred dune habitat and rounder leaves (Heenan 1997) than *S. radicans*, which is described with spathulate, elongated leaves although is noted to be highly polymorphic (Allan 1961). These rounder leaves were maintained in a common garden experiment suggesting that environmental conditions did not influence the rounder leaf shape (Ogden 1974). *Selliera microphylla* is noted to be smaller in size than *S. radicans* and grows inland although it has been noted to revert to similar morphology to *S. radicans* in similar environments (De Lange 2012a; K Pilkington, personal observ.).We utilised a population genetics approach combined with an analysis of morphology with aims to:

1. Develop reliable and polymorphic microsatellite markers to use for *Selliera.*
2. Determine the species boundaries within *Selliera.*
3. Examine the population structure within *Selliera* species.

Microsatellite markers were chosen for this study due to the ease of genotyping (Ashley and Dow 1994), co-dominance which allows detection of heterozygotes (Duminil and Michele 2009), and relative faster evolution (Li et al. 2002) making them informative for closely related species.

4.2 Findings

Nine microsatellite markers were developed for use in *Selliera* to attempt to determine species boundaries. The main findings were summarised within the previous chapters: Microsatellite primers in *Selliera radicans* (Goodeniaceae) and Species delimitation and population genetics in *Selliera* (Goodeniaceae). This section will use these findings to address the aims of the study:

1. Develop reliable and polymorphic microsatellite markers to use for *Selliera.*

454 next generation sequencing was utilized in sequencing *Selliera* DNA which was sorted into contigs and microsatellite loci detected by MSATCOMMANDER. Forty three detected loci were tested on samples of each of the described species in *Selliera* for reliable and polymorphic loci that could be used a markers for the second part of the study. Nine microsatellite markers were developed that amplified consistently and were polymorphic. These markers were used to screen all 618 samples of *Selliera* collected; only five of these samples failed to amplify for four or more markers and were removed from the analysis. Most markers amplified more than 90% of the samples with only one marker amplifying just 73% of the samples indicating these markers are reliable to use in a population genetics approach to delimiting species in *Selliera.* All markers were polymorphic with allele numbers ranging from 2 to 8 which may be low polymorphism but may be typical of the species.

2. Determine the species boundaries within *Selliera* and examine population structure within *Selliera* species.

Our results do suggest that the three species previously described in *Selliera* exist with some variation in species boundaries. There was greater variation within species than between them with only 25.4% of the variation attributed to between species, consistent with species in *Selliera* being closely related, and that the majority of populations within species were differentiated from others. Analyses showed that both *S. microphylla* and *S. rotundifolia* had high interpopulation differentiation and two genetic lineages were identified within each of the species indicating these species are not distinctly evolving lineages. Morphological analyses revealed distinct differences between the species with *S. rotundifolia* leaf morphology only slightly overlapping with *S. radicans* leaf shape and *S. microphylla* falling within the range of *S. radicans. Selliera radicans* showed greater admixture than the other species indicating more gene flow occurring between populations. *S. radicans* and *S. rotundifolia* are sympatric at two of the sampled sites which may result in

hybridization. We observed hybridization at one site, Waverly, with morphological and genetic intermediates and apparent reproductive isolation with possible past introgression, at another, Waikawa. We also identified another site, Castlepoint, where hybridization may have occurred although no *S. rotundifolia* plants were identified in this population.

The presence of reproductive isolation, distinct morphology and genetic distinction is strong evidence for a separate species, *Selliera rotundifolia,* and is also supported with previous analyses suggesting that rounder leaves is genetically determined (Ogden 1974). However the presence of two lineages in *S. rotundifolia* indicates rounder leaves may have evolved twice from *S. radicans* and therefore round leaves may not be a good indicator of a single species. This may explain why hybridization occurs at Waverly and not Waikawa with the different lineages of round leaves at different stages of evolution and have not yet evolved reproductive isolation so speciation is not complete for *S. rotundifolia* at all sites. With time and isolation all round leaved populations may evolve reproductive isolation and continue to diverge separately unless gene flow occurs between the two lineages which is possibly seen at Waverly and may indicate one species. Analyses suggest introgression back to *S. radicans* may have occurred in the past at Waikawa which may allow *S. radicans* to adapt to the dune environment of *S. rotundifolia* and could pose problems for *S. rotundifolia* as it is already threatened by faster and taller growing weed species (De Lange 2012b). Alternatively Waikawa may be the origin for the Himatangi/Waikawa lineage of *S. rotundifolia.*

Analyses suggest that the North and South Island *S. microphylla* are not consistent with one species and the South Island populations may in fact be an inland *S. radicans* population but, due to the isolation of this population, may diverge into a new species over time as analyses show this population is distinct from all others except one other South Island population (Tw) which may be due to shared ancestry. The North Island Central Volcanic Plateau population also clusters with other *S. radicans* populations but has a single chromosome count of $2n$=56 (Murray and de Lange 2013) which is much

higher than the 2*n*=16 of *S. radicans* and *S. rotundifolia.* We would expect a chromosome count this different to prevent reproduction with the other species indicating the Central Volcanic Plateau populations should retain the *S. microphylla* species name. Although reproductive isolation has not been confirmed yet in these populations and further cytological studies are required to examine the frequency of 2*n*=56 individuals.

These results will aid in the understanding of the species within *Selliera* and help to determine the species boundaries. The identification of two lineages within *S. rotundifolia* may be useful in future if conservation of this species is needed. However further research is needed to investigate the species boundaries. It may be helpful to investigate whether hybridization is possible between the Central Volcanic Plateau *S. microphylla* and the other species and confirm the higher chromosome count in other individuals and populations from the Central Volcanic Plateau. Investigation and comparison of the sympatric sites of *Selliera radicans* and *Selliera rotundifolia* and the frequency of hybridization within these sites may provide insights into the development of reproductive isolation, identify environmental or genetic factors that are involved and more clearly identify if introgression has occurred at Waikawa. Further investigation of the Castlepoint population may indicate whether hybridization has occurred or if this is a population of *S. rotundifolia* despite the *S. radicans* morphology.

4.3 Limitations

For this study there are a number of limitations to be considered. Morphological images were taken in the field using a digital camera due to time constraints which resulted in shadow from the sun on some images, and some leaves that would not lay flat which resulted in some leaves unable to be measured, which were excluded from the analysis but a large sample size was still obtained. *Selliera radicans* leaves do not always have a clear petiole from the leaf blade unlike *Selliera rotundifolia* so was measured from where the smallest part of the leaf could be found which may affect the results. The number of plants in a population was not estimated as it is difficult to tell separate plants apart due to the creeping nature of *Selliera*

although this was addressed by not sampling within one meter of other sampled plants but it is not known if individual *Selliera* plants can grow that large so it may still be possible sampling from the same plant occurred.

For marker development it was difficult to get the required quality of DNA from *Selliera* tissue which resulted in a lot of low quality reads returned although this was solved by forming contigs from the sequences. Sampling within *S. rotundifolia* and *S. microphylla* was smaller than for *S. radicans* and may not consist of a large enough sample size for some species however sampling was conducted on all populations that could be found at the time of collection for these species and this smaller size may be typical of the species. Finally, although microsatllite markers are assumed to be nearly neutral, we do not know if any markers are linked to selected phenotypes but using multiple markers should negate this effect. Despite the limitations within this study it provides an important initial assessment of population structure and species boundaries within *Selliera* where issues raised can be addressed in future with greater sampling of *S. microphylla* and *S. rotundifolia* and increased emphasis on sympatric populations. Future studies perhaps with more specific markers to study hybridization and introgression will further help in determining species boundaries.

4.4 References

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