MOLECULAR PHYLOGENY OF THE GENUS *METROSIDEROS* AND POPULATION GENETICS OF SOME NEW ZEALAND SPECIES WITHIN THE GENUS

A thesis submitted in partial fulfilment of the requirements for the Degree

of Doctor of Philosophy in Biology

in the University of Canterbury

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University of Canterbury

2017

Acknowledgment

First of all, I would like to thank the almighty God for keeping me healthy and giving me the stamina to pursue and complete my PhD study in the field of genetics, which I have liked most since my school days. I would also like to thank my supervisors Assoc Professor Hazel Chapman from University of Canterbury and Dr Gary Houliston from Landcare Research at Lincoln for their wonderful and uninterrupted supervision and support in every step of my research progress. Without their consistent advice and supervision, the completion of my study would not have been realized. I would like to thank Dr Pieter Pelser from University of Canterbury for extending his unreserved help in gap coding of nucleotide sequence data that were used for the molecular phylogeny work, and also for the valuable discussions I had with him regarding the Bayesian and Maximum Parsimony tree building methods. My thanks also go to Landcare Research at Lincoln for providing me the opportunity to use the molecular lab and the reagents therein for all my PhD research, and also for granting me access to Allan Herbarium to take leaf samples of *Metrosideros* species for my molecular phylogeny work. I am grateful to Dr Dagmar Goeke, Caroline Mitchell and Katherine Trought for their technical support in the Molecular Lab at Landcare Research, which facilitated my lab work and also made my stay at Landcare Research enjoyable. I would like to thank the University of Canterbury (UC) for granting me the financial support (UC Doctoral Scholarship) to pursue my PhD. Last but not least, I would like to thank Drs Peter de Lange and Jeremy Rolfe from the Department of Conservation for providing leaf samples of *Metrosideros bartlettii* trees for DNA extraction for genetic diversity analysis.

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Chapter 1: General Introduction

This thesis attempts to answer three questions about the phylogeny and genetics of the New Zealand genus *Metrosideros* which contains iconic tree species of New Zealand. The first question (Chapter 2) is at the scale of the genus and attempts to resolve the phylogenetic relationships within the *Metrosideros* group. At the same time, based on the molecular phylogeny, inference is made on the dispersal route of the genus from New Zealand to the Pacific. In doing so, this chapter tests two hypotheses (1) the current classification of genus *Metrosideros* is supported by the genetic data and (2) the current distribution of genus *Metrosideros* in the Pacific is a result of long distance dispersal from New Zealand rather than due to ancient Gondwanan connection among the Pacific islands. Molecular phylogeny studies have shown that the biogeography of New Zealand species is shaped mainly by transoceanic dispersal rather than Gondwanan connection (Pole, 1994; McGlone *et al*., 2001; Winkworth *et al*., 2002; Perrie & Brownsey, 2005; 2007; Cantley *et al*., 2016). Hence, this chapter attempts to test if this is true for the New Zealand genus *Metrosideros*.

The second question (Chapter 3) is at the scale of the population with a focus on the critically endangered New Zealand endemic *Metrosideros bartlettii*. Population genetics are used to assess the genetic diversity and structure of this species with applications for conservation management. This chapter tests the hypothesis that *M. bartlettii* populations have a low genetic diversity relative to equivalent species because of the very small size of the current populations. In other words, the chapter attempts to test if the correlation between population size and genetic diversity is positive as reported in the literature (e.g. Cole, 2003; Leimu *et al*., 2006; Solórzano *et al*., 2016).

Chapter 4 addresses the third question, which is again at the population scale and investigates hybridisation between two New Zealand endemic species (*M. excelsa* and *M. robusta*) on Rangitoto Island. The extent, pattern and implications of hybridisation between these two species are discussed in detail. This chapter tests the hypothesis that hybridisation occurs between *M. excelsa* and *M. robusta* on Rangitoto Island as reported by several sources (Cooper, 1954; Allan, 1961; Julian, 1992; Wilcox, 2007; Dawson *et al*., 2010b).

The broad theme that connects the three chapters is the issue of conservation of our biological resources. All the three chapters can have direct implications to the conservation of species.

With regard to chapter 2, the role of phylogeny in conservation has been highlighted in earlier studies (e.g. Erwin, 1991; Vane-Wright *et al*., 1991; Faith, 1992; Crozier, 1997). In recent

years, the contribution of phylogeny and evolutionary biology to nature conservation has received even more emphasis (e.g. Mace & Purvis, 2008; Hendry *et al*., 2010; Rolland *et al*., 2012; Winter *et al*., 2013; Forest *et al*., 2015). One contribution of phylogeny to conservation is setting conservation priorities; tree topologies and genetic distance (branch lengths) from molecular phylogeny can help to identify which species are evolutionary distinct and therefore deserve more attention. Priority may be given to the conservation of such species (Faith, 1992; Crozier, 1997; Rodrigues *et al*., 2005; Forest *et al*., 2015). For instance, in the present study tree topologies show that *M. bartlettii* is distinct from its closest relatives, *M. excelsa* and *M. robusta*. Hence, apart from the genetic diversity assessment done in the present study, the phylogeny work would help to justify the conservation of this particular endangered species.

Evolutionary models used in molecular phylogeny can help to estimate and compare the rate of evolution across lineages, thereby helping to identify clades that have low evolutionary potential or greater chances of extinction than others. Management efforts can then be directed towards such vulnerable lineages (Morlon *et al*., 2010; 2011; Rolland *et al*., 2012). Molecular phylogeny also helps us to identify the most closely related relatives to species that are low in genetic variation and which we may want to increase diversity through interspecific hybridization. For instance, by looking at tree topologies in the current study, we can see that *M. robusta* and *M. excelsa* are the closest relatives of *M. bartlettii* so that they are the best candidates to be used to boost the genetic diversity of *M. bartlettii* through interspecific hybridization. This is very important because significant improvement of genetic diversity of this species is unlikely to be achieved via intraspecific hybridization due to the very small size of all the populations.

With regard to chapter 3, the contribution of genetic diversity studies to the conservation of species is basic; genetic diversity studies help to assess the genetic status of endangered species and inform management to take conservation actions. The assessment of genetic diversity and structure helps to prioritize populations and management actions for conservation of species (Toro & Caballero, 2005; Markert *et al*., 2010; Arponen, 2012; Ottewell *et al*., 2016).

With regard to chapter 4, hybridisation studies help to assess the level and pattern of hybridization among species. Because hybridisation may cause extinction of the numerically inferior parental species via genetic assimilation (Gomez *et al*., 2015; Todesco *et al*., 2016),

the estimation of the level and pattern of hybridization helps to design management actions such as removal of hybrids, and/or the collection of parental germplasm for future propagation. The detection of hybrids through molecular work also helps to avoid the collection of germplasm from the wrong individual trees. On the other hand, hybridisation can be an importance process for improving genetic diversity, evolution of adaptive traits and new species (Mallet, 2007; Thompson *et al*., 2010; Abbot *et al*., 2013). In such cases, hybridisation studies help to highlight the conservation value of hybrids. Generally, hybridisation studies help to predict the consequences of hybridization, important in order to assess which conservation action to take.

In summary, taken together, the three chapters help us to adequately know our biological assets, assess their current status and take remedial action before we lose them. This is especially crucial given that we are living in a rapidly changing world that can drastically affect our biological resources and their course of evolution.

1.1 Resolving phylogenetic relationships in the genus *Metrosideros*

The genus *Metrosideros* belongs to the family Myrtaceae, which is a large family of around 140 genera and more than 5,000 species (Govaerts *et al*., 2015). A substantial number of *Metrosideros* species occur in Australia and South America, while fewer species are found in Africa (Wilson, 1996). *Metrosideros* is widely distributed in the Pacific with representative species in the Philippines, New Guinea, New Caledonia, New Zealand and many of the Pacific Islands. It is, however, absent from Australia, Indonesia and Micronesia (Wilson, 1996). The genus contains 53 species (Dawson & Stemmerman, 1999), 12 of which are from New Zealand, including the iconic *M. excelsa* (pōhutukawa or New Zealand Christmas tree). *Metrosideros* species are often referred to as 'iron-hearted' trees because of the durability and hardness of their wood (Dawson & Stemmermann, 1999).

The current classification of *Metrosideros*, proposed by Dawson (1976) and based on morphological characteristics, recognizes three subgenera: *Metrosideros*, *Mearnsia* and *Carpolepis*. Since then, several authors have questioned the phylogenetic relationships among some of the species, subgenera and sections and suggested the relationships between several species in the genus should be revised (Briggs & Johnson, 1979; Wright *et al*., 2000a; James *et al*., 2004; Percy *et al*., 2008; Harbaugh *et al*., 2009).

One of the phylogenetic uncertainties involves the subgenus *Carpolepis*, which includes three species from New Caledonia. Even Dawson, who originally recognised it as subgenus (Dawson, 1976), changed his mind and later gave it a generic rank by segregating it from genus *Metrosideros* (Dawson, 1984). Dawson made this change because *Carpolepis* has yellow flowers and winged seeds, which are uncommon among other species of *Metrosideros*. However, Wilson (1996) disagreed with Dawson and placed *Carpolepis* back within the the genus *Metrosideros*. Later, Wright *et al*. (2000a) did molecular phylogeny based on nuclear ribosomal DNA regions and suggested that this group best fits as a subgenus of *Metrosideros* rather than as separate genus.

Another uncertainty involves the subgenus *Mearnsia*. According to Dawson's (1976) classification, which is still in use, this subgenus is further divided into two sections (*Mearnsia* and *Calyptropetala*), which are further divided into subsections (*Crassiviens* and *Trivalvis* within *Mearnsia*, and *Exsertis* and *Inclusis* within *Calyptropetala*). However, the lack of adequate morphological homogeneity among sections and subsections of this group led Briggs and Johnson (1979) to suggest the group be split into several smaller genera. Moreover, the molecular phylogeny study by Wright *et al*. (2000a) showed that subgenus *Mearnsia* is not monophyletic.

Further uncertainties have been raised over the species within subgenus *Metrosideros*, especially the *M. collina* group and the Hawaiian *Metrosideros* group. Smith (1973) recognised the *M. collina* group as a monophyletic group consisting of three varieties (*M. collina var. collina*, *M. collina var. villosa* and *M. collina var. fruticosa*). However, Wright *et al* (2000b) showed through molecular analysis that the *M. collina* group is not a monophyletic group; instead they form two distinct groups. One group consists of taxa from French Polynesia (Rurutu and Tahiti) and the Cook Islands (Rarotonga) and the other group consists of taxa from Samoa, Vanuatu and Fiji. Moreover, *M. collina var. collina* is found in both of these distinct groups, which raises the question of whether one or more new varieties or species might not yet be recognized in the current classification. The taxonomic uncertainty within the *M. collina* group is also reflected in the confused names and identities of cultivars grown in New Zealand (Dawson *et al*., 2010b).

The relationship of the subgenus *Metrosideros* in relation to the Hawaiian *Metrosideros* group remains unclear. In Hawaii there are five species of *Metrosideros*, one of which (*M. polymorpha*) has five varieties, all known for their significant morphological variations (Dawson & Stemmermann, 1999; Wright & Ranker, 2010). However, the discovery that

these species have little genetic variation despite large morphological differences (e.g. James *et al*., 2004; Percy *et al*., 2008; Harbaugh *et al*., 2009) suggests that current taxa should be collapsed into varieties of a single hypervariable species. In contrast, other studies have reported significant genetic differences among the varieties within the group (e.g., DeBoer & Stacy, 2013; Stacy *et al*., 2014).

These phylogenetic and taxonomic ambiguities show that the phylogenetic relationships within the *Metrosideros* group are still poorly understood. This thesis attempts to fill this knowledge gap through conducting a molecular phylogeny of the group using the nuclear ribosomal DNA (nrDNA) spacer regions (Internal Transcribed Spacers, or ITS, and External Transcribed Spacers, or ETS), and chloroplast regions (*matK* and *trnL intron*). All of these markers are all widely used in plant molecular phylogenetic studies (e.g, ITS and ETS in Li *et al*., 2007; Masuda *et al*., 2009; Logacheva *et al*., 2010; Caze *et al*., 2013; Ipek *et al*., 2014; Jiménez-Mejías et al., 2016; *matK* and *trnL intron* in Chen *et al.,* 2005; Scharaschklin & Doyle, 2005; Dong *et al*., 2012; Shinohara *et al*., 2013; Ipek *et al*., 2014; Saha *et al*., 2015; Tekpinar *et al*., 2016).

1.2 Population genetics of *M. bartlettii*

The second question this thesis attempts to address is the genetic status of the critically endangered New Zealand endemic, *M. bartlettii* (Townsend *et al*., 2008; de Lange, 2014). Currently, only three extremely small populations of this species remain in the far north of the North Island (Radar, Unuwhao, and Kohuronaki). The major factors that caused its drastic decline include browsing by introduced mammals (mainly possums), change in land use and fire (de Lange, 2003; Simpson, 2005). Possums appear to be the major threat to remaining populations.

Understanding the genetic diversity and structure of extremely rare species such as *M. bartlettii* can assist with their conservation (Toro & Caballero, 2005; Laikre, 2010; Ottewell *et al*., 2016). Such data allows the identification of populations and even individual trees with unique alleles and higher genetic diversity, so that they can be used in cultivation and restoration programs. It also helps to determine whether populations have already experienced high inbreeding, so that they can be avoided in revegetation programs. The genetic structure of populations across species also indicates the amount of past and current gene flow among populations. In places where gene flow is minimal or non- existent, it may

be possible to establish new populations to serve as bridges connecting fragmented populations.

Given the significance of population genetic studies for the conservation of endangered species, this thesis attempts to assess the genetic diversity and structure of *M. bartlettii* for conservation management. The study used two molecular markers, Amplified Fragment Length Polymorphism (AFLP) and microsatellites (SSR), both of which have been widely used in population genetic studies of rare and endangered plant species (e.g., Zawko *et al*., 2001; Armstrong & de Lange, 2005; Grueber & Jamieson, 2011; Bian *et al*., 2015; Wu *et al*., 2015; Szczecińska *et al*., 2016).

1.3 Genetic confirmation of hybridisation between *M. excelsa* and *M. robusta*

My third and last research question is the genetic confirmation of natural hybridisation between *M. excelsa* and *M. robusta* on Rangitoto Island, and its implications for the conservation of the parental species and their hybrids.

Natural hybridisation between species is common among plants (Genovart, 2009; Abbott *et al*., 2013), having occurred in approximately 70% of angiosperms (Masterson, 1994; Whitney *et al*., 2010a). Moreover, natural hybridisation is as common in New Zealand as it is elsewhere (Cockayne, 1923; Cockayne & Allan, 1934; Rattenbury, 1962; Morgan-Richards *et al*., 2009). For example, intergeneric hybridisation has been reported between *Helichrysum lanceolatum* and *Anaphalioides bellidioides* (Smissen *et al*., 2007), *Anaphalioides bellidioides* and *Ewartia sinclairii* (McKenzie *et al*., 2008) and *Disphyma australe* subsp. *australe* and *Carpobrotus edulis* subsp. *Edulis* (Heenan & Skyes, 2010). Examples of interspecific hybridisation include *Convolvulus verecundus* and *C. waitaha* (Smissen & Heenan, 2011), and within the *Plantago* group (Meudt, 2011; Wong & Murray, 2014). Such hybridisation can happen when two species have overlapping distribution (sympatry) and flowering time (Arnold, 1997), both of which are true for *M. excelsa* and *M. robusta* on Rangitoto Island (Dawson *et al*., 2010a).

In addition to 'natural' hybridisation, hybridisation can be facilitated by anthropogenic disturbance and/or interference such as the introduction of species to new locations and modification of habitats that may increase the proximity of previously isolated species (Gilman & Behm, 2011; Kahilainen *et al*, 2011; van Hengstum *et al*, 2012; Guo, 2014).

Human-induced hybridisation of plant and animal species can be found in several studies in New Zealand and Australia (e.g., Harris *et al*., 1992; Rhymer *et al*., 1994; Esa *et al*., 2000; Lamont *et al*., 2003; Murray *et al*., 2004; Morgan-Richards *et al*., 2004; de Lange *et al*., 2005).

Regardless of the cause of hybridisation, the consequences can be negative or positive. The major negative consequence of hybridisation is that it may lead to the extinction of the numerically inferior parental species through demographic swamping and genetic assimilation (Levin *et al*., 1996; Wolf *et al*., 2001; Gomez *et al*., 2015; Todesco *et al*., 2016). However, extinction of parental species may not occur if the rate of introgression is limited and if parental species have comparable abundances (Lepais *et al*., 2009), in which case hybridisation can help to boost the genetic diversity of the parental species (e.g., Nettel *et al*., 2008; Yu *et al*., 2014; Song *et al*., 2015). This may be important for the survival of rare parental species. Furthermore, growing evidence suggests hybridisation enhances the genetic diversity of species, the evolution of adaptive traits, and the formation of new species through hybrid speciation (e.g., Seehausen, 2004; Whitney *et al*., 2010b; Clay *et al*., 2012; Abbott *et al*., 2013; Hufford *et al*., 2013; Dittrich-Reed & Fitzpatrick, 2013; Vallejo-Marín & Hiscock, 2016; Pedersen *et al*., 2016; Goulet *et al*., 2017).

Because of the consequences of hybridisation, the detection and confirmation of hybrids in natural populations is important. Specifically, understanding the extent and pattern of hybridisation helps predict its most likely consequences, which may be crucial in designing effective management practices to conserve parental and/or hybrid types. To this end, this thesis provides the first genetic confirmation of natural hybridisation between *M. excelsa* and *M. robusta* on Rangitoto Island, a 600-year-old volcanic island that is home to more than 200 native plant species (Wilcox, 2007).

1.4 Summary of key findings

Resolving phylogenetic relationships in genus *Metrosideros*

This molecular phylogenetic study of the *Metrosideros* genus supports the current recognition of three subgenera: *Metrosideros*, *Mearnisa*, and *Carpolepis*. However, the study highlights some phylogenetic questions that still need to be addressed. Species within the subgenus *Metrosideros* were found to be monophyletic and more homogeneous than species within the

subgenus *Mearnsia*. Within the subgenus *Metrosideros*, the relationships among taxa in the *Metrosideros collina* group especially might require reconsideration.

This study corroborates Wright et al. (2000b)'s findings that *M. collina* varieties are polyphyletic, forming two completely distinct groups separated by *M. bartlettii* of New Zealand. One group consists of taxa from French Polynesia (Rurutu and Tahiti) and the Cook Islands (Rarotonga) while the second group consists of taxa from Samoa, Vanuatu and Fiji. It is surprising to find the same variety (*M. collina var. collina*) in both of these two geographic groups, while different varieties of *M. collina* that are from different regions fall into the same phylogenetic group. This suggests that perhaps the *M. collina var. collina* in one of the two clades was wrongly named. In other words, one or more new varieties or species may not yet be recognized in the current classification of this group.

With regard to the Hawaiian *Metrosideros* group, I found very few genetic differences within the group despite the taxa's large morphological differences. Based on this result alone, it is difficult to suggest the collapse of the group into one or a few hypervariable species. Hawaii is well-known for adaptive radiation and it is possible that the *Meterosideros* group might be the result of recent adaptive radiation of an ancestral species. The low genetic differences within the group can be explained by the limited power of the markers used to resolve the group, by the relatively recent colonisation of the Hawaiian Islands by *Metrosideros* (3.9 million years ago; Percy *et al*., 2008), or by the involvement of a few genes with a major effect on morphology.

Results showed that sections and subsections of the subgenus *Mearnsia* are not monophyletic; rather they form four separate clades that appear to have equivalent rank as subgenus *Metrosideros*. The group is very diverse with little genetic cohesion among the different sections and subsections, which suggests that the subgenus might need to be split into a few small subgenera, as was suggested earlier by Briggs and Johnson (1979). On the other hand, the genetic diversity may simply reflect poor resolution of the subgenus, which can be improved using more powerful markers. Further phylogenetic studies using coding and non-coding nuclear and plastid regions are needed to determine whether subgenus *Mearnsia* should be split into two or more subgenera.

Contrary to Wilson (1996), I found no association of the subgenus *Carpolepis* with subgenus *Metrosideros*. Species within the subgenus *Carpolepis* were monophyletic, and although Bayesian inference results showed the nesting of the subgenus with the New Caledonian

group (Sect. *Mearnsia* subsect. *Crassiviens*), this result had very low support. Moreover, the maximum parsimony test did not support this nesting; instead the results strongly supported a monophyletic clade equivalent in rank to the other two subgenera. In summary, my results strongly suggest that the current classification of this group as a separate subgenus is appropriate and does not need revision.

The phylogenetic analysis suggested that the dispersal route of genus *Metrosideros* was most likely from the most species-rich landmasses of New Zealand and New Caledonia into the other islands, often via 'stepping stone' islands. The presence of the oldest fossil records of *Metrosideros* in New Zealand, which are absent in other adjacent Gondwanana islands, suggests that the presence of the genus across the Pacific is most likely due to long distance dispersal events originating from New Zealand, rather than due to a Gondwanan connection between New Zealand and other Pacific Islands. This is a plausible explanation given the genus has several attributes for efficient long-distance wind dispersal (Wilson, 1996).

Population genetics of *M. bartlettii*

The population genetic study of *M. bartlettii* showed that this species has lower genetic diversity than both its most related taxon (*M. excelsa*) and the average reported values for long-lived, outcrossing, and wind-dispersed species. This low genetic diversity may be the result of a combination of factors, such as forest destruction, fire, land-use change and possum grazing.

Cultivated samples were expected to have a very low genetic diversity, because they were sourced from only a few trees in wild populations. However, they showed a similar level of genetic diversity to the wild populations, although they had only two private alleles compared with 11 in the wild populations. The genetic structure analyses showed that, despite the proximity of the populations and the species' extensive dispersal capacity, populations of *M. bartlettii* had significant genetic differentiation. This suggests limited gene flow between populations, which could be expected given the extremely small number of trees and the resulting small pollen and seed production that is inadequate to attract many pollinators. The conservation implications of the findings are discussed in detail later in this thesis.

Genetic confirmation of hybridisation between *M. excelsa* and *M. robusta*

The genetic testing of hybridisation between *M. excelsa* and *M. robusta* on Rangitoto Island confirmed the presence of high introgression between these species. Of the 95 individuals tested, the AFLP markers detected 26 and 31 hybrids using STRUCTURE and NEWHYBRID programs respectively, using an arbitrary probability threshold of $P \geq 0.9$. (This threshold means that an individual is identified as a hybrid if the proportion of its genome inherited from either parent is less than 90% in STRUCTURE or if its genotypic frequency for either parental type is less than 90% in NEWHYBRIDS.) The SSR markers similarly detected 27 and 28 hybrids using STRUCTURE and NEWHYBRID respectively.

With AFLP markers, the NEWHYBRIDS detected 12 *M. excelsa* backcrosses, 7 *M. robusta* backcrosses, 7 F1 hybrids, and 3 F2 hybrids, while the SSR markers detected 11 *M. excelsa* backcrosses, 5 *M. robusta* backcrosses, 7 F1 hybrids, and 4 F2 hybrids. The large number of backcrosses using both markers suggests high introgression between *M. excelsa* and *M. robusta* on Rangitoto Island. Furthermore, both AFLP and SSR markers suggested that F2 hybrids are lowest in the sample, which may be due to the lower viability of these hybrids. It is unlikely to be due to a limited resolving power of the markers, as a number of other advanced level hybrids (backcrosses) were detected. The presence of more *M. excelsa* backcrosses than *M. robusta* backcrosses suggests a bidirectional but biased introgression towards *M. excelsa*, which can be explained by the low abundance of *M. robusta* trees compared with the very large population of *M. excelsa* on Rangitoto Island.

1.5 Future research directions

To further explain the molecular phylogeny of the genus *Metrosideros*, future research should be conducted with multiple nuclear and plastid markers to investigate if the Hawaiian group has genetic divergence that is similar to their large morphological divergence. Moreover, the heritability of the diagnostic phenotypic characteristics should be tested to determine if their large morphological differences are the result of phenotypic plasticity in response to the environmental heterogeneity of the Hawaiian Islands. There are few studies that have addressed this issue within varieties of *M. polymorpha* (e.g Stacy *et al*., 2016; Tsujii *et al*., 2016). The use of multiple nuclear and plastid markers may also help to further resolve sections and subsections of the subgenus *Mearnsia*. It will also determine if the subgenus *Carpolepis* should be nested with the New Caledonian group (Sect. *Mearnsia* subsect.

Crassiviens) and therefore be recognised as a new section or subsection of the subgenus *Mearnisa* rather than as a separate subgenus.

The level of introgression between *M. excelsa* and *M. robusta* can be made more precise using a larger sample size, more powerful markers such as SNPs that have better genome coverage and polymorphism, and new technologies such as RAD sequencing technologies that can analyse large samples rapidly. The use of large samples, powerful markers and new sequencing technologies will also help to better understand how biased the pattern of hybridisation really is. This can help determine if symmetrical introgression is occurring and if so, how fast the parental populations are collapsing into a hybrid swarm. This information is crucial for management decisions, such as the storing of germplasm for future revegetation programs.

Controlled crossing experiments are also needed to investigate if there is a significant difference in fitness between *M. excelsa* and *M. robusta* backcrosses, which would help explain a biased introgression towards *M. excelsa*. Future research should also evaluate the conservation value of hybrids by subjecting them and parental species to stresses such as drought and fire. In this way, the dynamics and fate of whole populations can be predicted under the most likely disturbances that they are expected to face in future in order to make timely and appropriate management decisions.

1.6 Implications of myrtle rust on *M. bartlettii* conservation

The fungal disease myrtle rust was detected for the first time in New Zealand in early May 2017 (http://www.mpi.govt.nz/). The disease is caused by the pathogen *Austropuccinia psidii* (Beenken, 2017) and originated in Central and South America. It mainly affects species within the Myrtaceae family, and it has spread around the world including to Africa, Australia and New Caledonia (Glen *et al*. 2007; Roux *et al*., 2013; Silva *et al*., 2013). The fungus was detected for the first time in Australia in April 2010 (Carnegie *et al*., 2010; Carnegie and Cooper, 2011).

In New Zealand, the disease was first reported at a Northland nursery and has now been confirmed in Northland, Taranaki and Waikato. Kriticos *et al*. (2013) predicted the climatic suitability of New Zealand for the pathogen through climate modelling, which showed that most of the North Island and a small area of the northern South Island are climatically suitable. Myrtle rust thrives in climates associated with the wet tropics and subtropics, with

prevailing moist conditions and moderate temperatures. The arrival of this disease in New Zealand was also predicted by Clark (2011) and is unsurprising given that rust fungi are among the most globally mobile pathogens due to the abundance and wind dispersal of their spores (Brown and Hovmøller, 2002; Stokstad, 2007). Spores could easily be blown to New Zealand from nearby landmasses such as Australia and New Caledonia.

Myrtle rust is known to affect leaves, shoots, buds, flowers, fruits and new growth (Tommerup *et al*., 2003; Glen *et al*. 2007; Pegg *et al*., 2014). Potential hosts include plants in the Myrtaceae family (Teulon *et al*., 2015) such as the iconic native New Zealand species pōhutukawa, rātā, and mānuka. There is high probability that *M. bartlettii* will be affected because of its location in the North Island and because a closely related species, *Metrosideros polymorpha*, is already infected in Hawaii and Japan (Uchida *et al*., 2006; Silva *et al*., 2014; Kawanishi *et al*., 2009). Similarly, *M. collina* and *M. kermadecensis* are infected in Queensland, Australia (Pegg *et al*., 2014).

Swift management actions are therefore required to protect populations of *M. bartlettii* as well as other susceptible species. *M. bartlettii* is currently represented by very few trees within close proximity, and it is likely that if one individual is infected, the disease will disseminate quickly. It is therefore important that seeds are collected as soon as possible and repeatedly over the next several years to capture genetic diversity for future propagation. Rangitoto Island may also be a useful source of seed material for other *Metrosideros* species, as the populations contain both pure parental species and hybrids, which have high genetic diversity and novel genotypes that may confer disease resistance.

Investigation of 122 taxa representing 15 tribes of the Myrtaceae family in Australia showed that some taxa might have genetic resistance against Myrtle rust (Morin *et al*., 2012). Tobias *et al*. (2016) also reviewed resistance to Myrtle rust among Australia Myrtaceae. Inoculation experiments with 441 individuals of *Eucalyptus pellita* in Brazil also confirmed the presence of rust-resistant genotypes in this species (Santos *et al*., 2014). Similar investigations in New Zealand taxa should be conducted as soon as possible, in order to store and propagate genetic material from resistant individuals.

In addition, new populations of *M. bartlettii* could be established in cooler areas that are not suitable for the fungus. The locations would need to be assessed to ensure that ecological conditions are suitable for *M. bartlettii*, and care should be taken to avoid using seeds and seedlings that are infected with this disease. Nursery sites especially need monitoring, as they

are sources of plant material for *in situ* recovery and *ex situ* planting. In the future when it is possible to prevent the dissemination of the disease in current *M. bartlettii* populations, new populations should be established close to current populations to improve gene flow and connectivity amongst the fragmented populations. This will reduce the risks associated with small population size such as inbreeding, genetic drift and loss of genetic variation.

In general, it is also important to raise public awareness of the disease and to teach people how to handle affected plants. The introduction of plant material such as flowers from regions of high infestation should be banned or at least closely monitored, and germplasm should be collected and stored as quickly as possible from healthy plants of susceptible species. Wherever the disease is detected, it is important to contain the pathogen by applying fungicides. Although the application of fungicides can be expensive and ineffective, it appears to be the only quick solution until other effective control methods are developed. New Zealand could learn about the nature and application of effective fungicides from other countries that have already experienced rust disease outbreaks.

As a long-term strategy, research should be done to learn more about the potential disease impacts, including further investigation of the most climatically suitable regions for the pathogen and the range of New Zealand host plant species and their degree of resistance. Propagation techniques should be developed for susceptible species in order to manage the disease in the future.

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Chapter 2: Molecular phylogeny of the genus *Metrosideros*

Abstract

The genus *Metrosideros* belongs to the Myrtaceae family, which consists of 140 genera and more than 5,000 species. *Metrosideros* is a widely distributed genus with representatives in the Philippines, New Guinea, New Caledonia, New Zealand and many of the Pacific Islands. The relationships amongst taxa in the *Metrosideros* group are poorly undertood, with no current molecular phylogenetic data on the entire group. This study investigates the molecular phylogeny of the group using nuclear ribosomal DNA (nrDNA) spacer regions (Internal Transcribed Spacers, or ITS, and External Transcribed Spacers, or ETS) and chloroplast regions (*matK* and *trnL intron*). These data are used to evaluate how well the current taxonomy reflects the genetic data and to explore the possible routes by which the genus may have spread from New Zealand into the Pacific. Generally, the molecular phylogenetic study supports the current recognition of the three subgenera *Metrosideros*, *Mearnsia*, and *Carpolepis*. However, the study highlights some phylogenetic questions that need to be addressed or reconsidered. The phylogenetic analysis suggests that the dispersal route of *Metrosideros* was most likely from the most species-rich landmasses of New Zealand and New Caledonia into the other islands in the Pacific, which would have been aided by the group's efficient wind-dispersal mechanism.

2.1 Introduction

The genus *Metrosideros* is widely distributed within the Asia Pacific region, with species in the Philippines, New Guinea, New Caledonia, New Zealand and many of the Pacific Islands (Figure 2.1). One out-group, *M. angustifolia*, occurs in South Africa (Dawson, 1975). The genus is absent from Australia, Indonesia and Micronesia (Wilson, 1996). The most well-known *Meterosideros* species are the iconic native tree species of Hawaii (*M. polymorpha* / Ohia lehua) and New Zealand (*M. excels*a /pōhutukawa/ New Zealand Christmas tree).

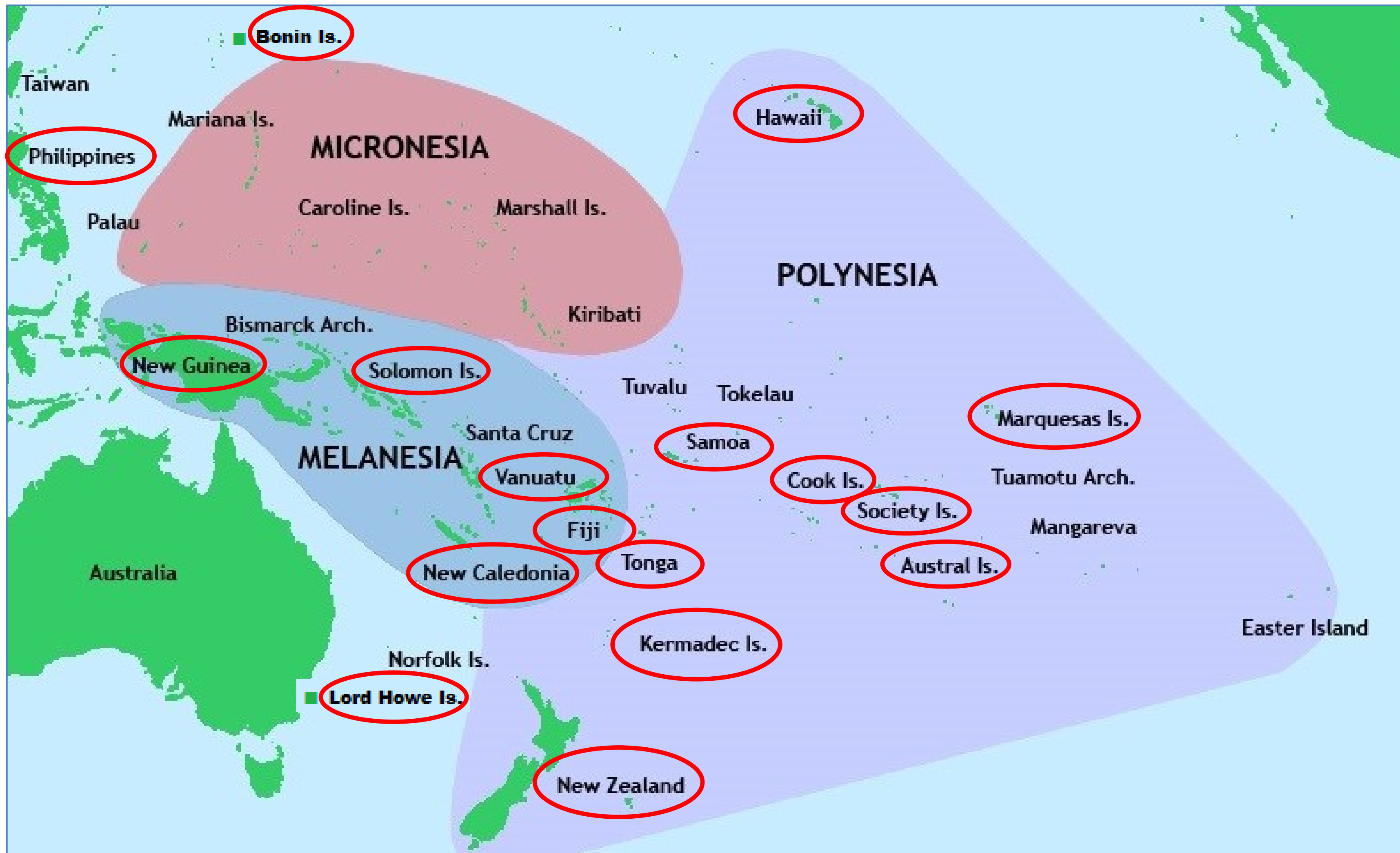

Figure 2.1 Distribution of the genus *Metrosideros*.

The genus *Metrosideros* contains 53 species distributed across the Pacific (Dawson and Stemmerman, 1999). New Zealand, which has 12 *Metrosideros* species (Table 2.1), is considered the origin of the genus based on fossil evidence. Long records of pollen fossil have been found in New Zealand as far back as the late Paleocene to early Eocene (Mildenhall, 1980; Lee *et al*., 2012), and leaf and fruit macrofossils have been found here from the early Miocene (Pole, 1993**;** Pole, 2008; Lee *et al*., 2012). Wright *et al*. (2000a) suggested New Caledonia was also a centre of diversity for the genus, but although it has the largest number of species (18) of *Metrosideros*, there is no fossil or other evidence to support this.

Tarran *et al*. (2016) reported the discovery of 40-30 million year old (between Eocene and Oligocene) fossil fruits and flowers in Little Rapid River, Tasmania, Australia, that shared several characteristics with species of *Metrosideros*. The authors argue that these fossils represent the oldest record of the genus. They argued that the pollen fossils discovered in New Zealand cannot be reliably regarded as the oldest record of *Metrosideros* as the pollens are similar to pollens of other Myrtaceae genera. Their argument would also imply that Australia, rather than New Zealand, could possibly be the landmass of origin of *Metrosideros*. The discovery of more fossils in the future may offer a clearer picture of the origin of the genus.

Table 2.1 *Metrosideros* species of New Zealand and New Caledonia

Metrosideros species of New Zealand	subgenus	*Metrosideros* species of New Caledonia	Subgenus
M. bartlettii	*Metrosideros*	*M. cherrieri*	*Metrosideros*
M. excelsa	*Metrosideros*	*M. engleriana*	*Metrosideros*
		M. humboldtiana	*Metrosideros*
M. kermadecensis	*Metrosideros*	*M. microphylla*	*Metrosideros*
M. robusta	*Metrosideros*	*M. nítida*	*Metrosideros*
		M. oreomyrtus	*Metrosideros*
M. umbellata	*Metrosideros*	*M. puncanta*	*Metrosideros*
M. albiflora	*Mearnsia*	*M. tetrasticha*	*Metrosideros*
		M. brevistylis	*Mearnsia*
M. carmínea	*Mearnsia*	*M. cacuminum*	*Mearnsia*
M. colenosi	*Mearnsia*	*M. dolichandra*	*Mearnsia*
		M. longipetiolata	*Mearnsia*
M. diffusa	*Mearnsia*	*M. operculata*	*Mearnsia*
M. fulgens	*Mearnsia*	*M. panensis*	*Mearnsia*
		M. patens	*Mearnsia*
M. parkinsonii	*Mearnsia*	*M. porphyrea*	*Mearnsia*
M. perforata	*Mearnsia*	*M. rotundifola*	*Mearnsia*
		M. whitakeri	*Mearnsia*

Figure 2.1 shows that many of the islands that contain *Metrosideros* are remote, indicating the importance of long-distance dispersal to the group's distribution (van Balgooy, 1971). Wilson (1996) pointed out that the adaptive features that favour the dispersal of this genus are: 1) prolific flowering, 2) pollination mainly by nectar-feeding birds, 3) dehiscent capsules that release large numbers of seeds, 4) light seeds that are easily wind-dispersed, and 5) the ability to colonize exposed sites, including recent lava flows.

Taxonomy of *Meterosideros*

Based on morphology, Dawson (1976) proposed three subgenera of *Metrosideros*: *Metrosideros* (25 species), *Mearnsia* (25 species) and *Carpolepis* (3 species). He recognised four sections within subgenus *Mearnsia* (*Mearnsia, Calyptropetala, Adnatae* and *Crystalla*), and further divided sections *Mearnsia* and *Calyptropetala* into subsections. However, Dawson (1984) later revised this assessment, promoting *Carpolepis* to genus. Later, Wright *et al*. (2000b) conducted a phylogenetic analysis based on nrDNA and concluded that *Carpolepis* should be reverted back to subgenus. The current arrangement of species in the three subgenera and a comparison of these subgenera are given in Table 2.2 and Table 2.3 respectively.

The relationships among all the taxa within the *Metrosideros* group are poorly understood and no molecular phylogenetic study of the entire group had been undertaken until after I had finished this chapter. After this, but before the production of this thesis, Pillon *et al*. (2015) published their work on molecular phylogeny of the entire *Metrosideros* group. Other studies that included broad phylogenetic analyses within *Metrosideros* with a more restricted ultimate focus include Warren and Hawkins (2006), Lucas *et al*. (2007), and Papadopulos *et al*. (2011). The few other molecular phylogenetic analyses of *Meterosideros* that have been conducted (e.g., Wright *et al*., 2000a, 200b; Wright *et al*., 2001; Wright *et al*., 2003; Gardner *et al.,* 2004; Harbaugh *et al*., 2009b) have focused on species from a particular region (mostly New Zealand and Hawaii) or within a certain subgenus (mostly *Metrosideros*). As a result, the complete phylogenetic picture of the genus is lacking. My objective is therefore to analyse the phylogenetic relationships among all taxa within the *Metrosideros* genus and suggest the possible routes the genus might have taken in its dispersal across the Pacific region.

Table 2.2 Subgenera of *Metrosideros* and their member species (Dawson *et al*., 2010a).

Metrosideros	***Mearnsia***	***Carpolepis***
M. bartlettii J.W.Dawson - Bartlett's rātā (New Zealand)	*M. albiflora* (New Zealand)	*M. elegans* (New Caledonia)
M. boninensis (Bonin Islands)	*M. angustifolia* (South Africa)	*M. laurifolia* (New Caledonia)
M. cherrieri (New Caledonia)	*M. brevistylis* (New Caledonia)	*M. tardiflora* (New Caledonia)
M. collina (Vanuatu)	*M. cacuminum* (New Caledonia)	
M. engleriana (New Caledonia)	*M. carminea* W.R.B.Oliv. - (New Zealand)	
M. excelsa Gaertn. - Pōhutukawa (New Zealand)	*M. colensoi* (New Zealand)	
M. gregoryi (Samoa)	*M. cordata* (New Guinea)	
M. humboldtiana (New Caledonia)	*M. diffusa* (New Zealand)	
M. kermadecensis - (Kermadec Islands)	*M. dolichandra* (New Caledonia)	
M. macropus A.Gray - *Lehua mamo* (Hawai‘i)	*M. fulgens* Sol. ex Gaertn. - (New Zealand)	
M. microphylla (New Caledonia)	*M. halconensis* (Philippines)	
M. nervulosa - Mountain Rose (Lord Howe Island)	*M. longipetiolata* (New Caledonia)	
M. nitida (New Caledonia)	*M. operculata* (New Caledonia)	
M. ochrantha (Fiji)	*M. ovata* (New Guinea)	
M. oreomyrtus (New Caledonia)	*M. paniensis* (New Caledonia)	
M. polymorpha Gaudich. - *‘Ōhi‘a lehua* (Hawai‘i)	*M. parkinsonii* (New Zealand)	
M. punctata (New Caledonia)	*M. patens* (New Caledonia)	
M. robusta A.Cunn. - Northern rātā (New Zealand)	*M. perforata* (New Zealand)	
M. rugosa A.Gray - *Lehua papa* (Hawai‘i)	*M. porphyrea* (New Caledonia)	
M. salomonensis (Solomon Islands)	*M. ramiflora* (New Guinea)	
M. sclerocarpa (Lord Howe Island)	*M. rotundifolia* (New Caledonia)	
M. tetrasticha (New Caledonia)	*M. scandens* (New Guinea)	
M. tremuloides - *Lehua ‘āhihi* (Hawai‘i)	*M. whitakeri* (New Caledonia)	
M. umbellata Cav. - Southern rātā (New Zealand)	*M. whiteana* (New Guinea)	
M. waialealae (Hawai‘i)	*M.* n. sp. (unnamed) (Solomon Islands)	

Table 2.3 Comparison of subgenera of *Metrosideros* (adapted from Simpson, 2005).

	Metrosideros	***Mearnsia***	***Carpolepis***
Growth habit	Trees, shrubs, hemi-epiphytes	Trees, shrubs, vines, hemi-epiphytes	Trees (all hemi-epiphytes)
Branching	Sympodial	Monopodial	Monopodial
Bud scales	Present	Absent	Present
Inflorescence	Terminal (in clusters at tips, although each inflorescence is axillary)	Terminal, axillary, or ramiflorous (on older stems)	Below the leaves at the beginning of each season´s growth
Flowers	Sepals equal Petals free, persistent Ovary semi-superior Mostly red, sometimes yellow or white	Sepals equal Petals free, persistent; or fused together and caduceus (falling off) Ovary inferior to semi-superior Red, pink or white	Sepals unequal Petals free, persistent Ovary inferior to semi-superior Bright yellow
Fruit	Base of style and placentas separating by tissue extension between them Seeds released through capsule	Base of style and placentas not separating Seeds released through capsule, or capsule and hypanthium	Base of style and placentas separating by tissue extension Seeds released through capsule
Seeds	Not winged	Not winged	Winged

2.2 Molecular markers, phylogenetic tree building methods, and nucleotide substitution models

2.2.1 Molecular markers used in the study

Ribosomal DNA (rDNA; Figure 2.2) is popular in phylogenetic studies (Poczai and Hyvonen, 2010), because it has lots of repeats, which makes the amplification and sequencing of this region technically easy (Baldwin *et al*., 1995; Weider *et al*., 2005; Soltis *et al*., 2008). Moreover, this region is subject to concerted evolution (Zimmer *et al*., 1980; Moritz and Hillis, 1996), which homogenizes rDNA repeats within individual genomes so that phylogenetic relationships can be analysed based on interspecies sequence differences (Weider *et al*., 2005; Soltis *et al*., 2008). Concerted evolution is an evolutionary process of homogenizing nucleotide sequences of a multigene family found at various locations of a genome (Elder and Turner, 1995; Liao, 1999). The homogenization is accomplished through gene conversion, unequal crossing over, or large deletions (Elder & Turner, 1995; Liao, 1999; Muir *et al*., 2001; Ganley & Kobayashi, 2011). Gene conversion is a non-reciprocal

transfer of genetic material, where a DNA segment at a given locus is replaced by a homologous DNA segment from another locus (Chen *et al*., 2007). Which of these molecular processes is the main driver of gene homogenization is debated (Koch *et al*., 2003).

The rDNA consists of a large multigene family occurring in tandem at one or several locations across the plant genome (Hillis & Dixon, 1991). Each rDNA repeat unit is composed of coding and noncoding regions (Figure 2.2). The coding regions contain three highly conserved genes (5.8S, 18S, and 26S) that code for rRNAs that are structural components of the smaller and larger subunits of ribosomes. The fact that the sequences of these genes are so conserved among all eukaryotes reflects the functional similarity of this region across all organisms and the strong selection against mutations that affect the functions of the ribosomal subunits (Caetano-Anolle´s, 2002; Poczai and Hyvonen, 2010).

The noncoding regions of the rDNA, unlike the coding regions, are variable due to accumulation of mutations (Baldwin *et al*., 1995; Alvarez and Wendel, 2003). These regions are therefore widely used in phylogenetic studies of plants (Soltis *et al*., 2008; Poczai and Hyvonen, 2010). These regions consist of three useful spacer regions: the Internal Transcribed Spacers (ITS1 and ITS2), the External Transcribed Spacers (ETS) and the Intergenic Spacer (IGS). These spacers and their associated coding regions are found in hundreds to thousands of tandem repeats per chromosome, separated by the IGS (Prokopowich *et al*., 2003).

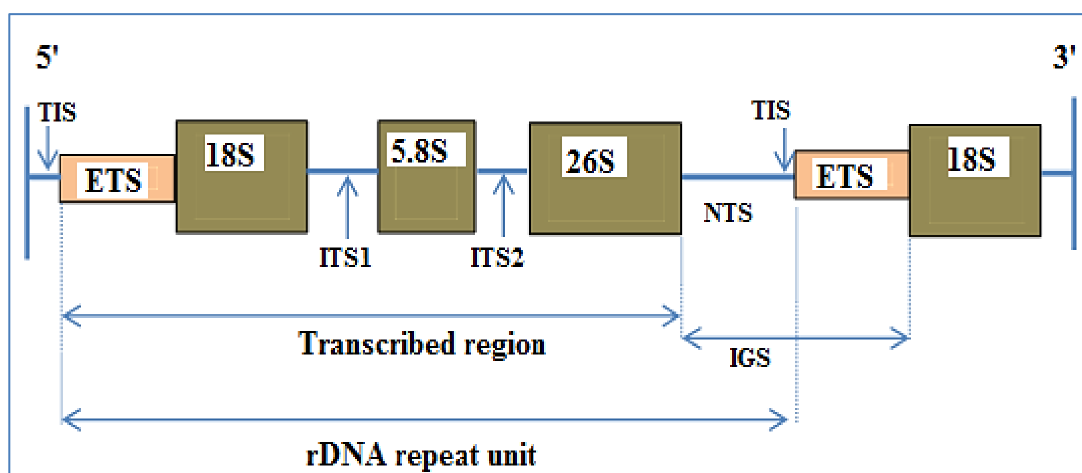

Figure 2.2 The structure of ribosomal DNA of higher plants. 18S, 5.8S, and 26S are highly conserved genes for ribosomal subunits, ETS is an external transcribed spacer, ITS1 and ITS2 are internal transcribed spacers, NTS is a non-transcribed spacer, IGS is an intergenic spacer separating repeat units, and TIS is a transcription initiation site.

Internal Transcribed Spacers (ITS)

The Internal Transcribed Spacer (ITS) region varies between 500 and 750 bp in angiosperms (Baldwin *et al*., 1995) and consists of three parts: *ITS1, ITS2*, and the highly conserved 5.8S rDNA exon. Many phylogenetic studies have used this region in their analysis (e.g., Markos and Baldwin, 1998; Wright *et al*., 2000a, 200b; Wright *et al*., 2003; Markey & de Lange, 2003; Mavrodiev *et al*., 2005; Li *et al*., 2007; de Lange *et al*., 2013; Caze *et al*., 2013; Ipek *et al*., 2014; Jiménez-Mejías *et al*., 2016). In fact, Alvarez and Wendel (2003) estimated that 66% of phylogeny papers that were published between 1997 and 2002 used ITS data at the generic level or below. Soltis *et al*. (2008) also mentioned that ITS sequences are among the nucleotide sequences most frequently submitted to Genebank.

The popularity of the ITS region for phylogenetic studies is largely because of the following advantages:

- **Biparental inheritance** – More variation can be captured compared to the maternally inherited chloroplast genomes (Baldwin *et al*., 1995; Alvarez & Wendel, 2003).
- **Universality** – Primers can amplify this region from most plant and fungal phyla and there is no need for prior sequence knowledge to design primers (Baldwin *et al*., 1995).
- **Simplicity** - Because there are hundreds to thousands of nuclear rDNA repeats in plant genomes, they can be easily isolated and amplified with little expertise. Both the high copy number and the small size of the target DNA fragment facilitate ITS amplification by PCR, even from ancient material such as herbarium specimens (Baldwin *et al*., 1995; Baldwin *et al*., 1995; Weider *et al*., 2005; Soltis *et al*., 2008). Moreover, the availability of highly conserved flanking ribosomal genes for primer annealing makes the PCR amplification of this spacer region easy (Baldwin, 1992; Linder *et al*., 2000).
- **Intragenomic uniformity** – ITS sequences, like other multigene families, can be subject to concerted evolution via unequal crossing over or gene conversion (Zimmer *et al*., 1980; Moritz & Hillis, 1996). In the presence of a complete homogenization, sequence variation within genomes can be eliminated, leaving only species-specific sequences for phylogenetic analysis (Baldwin *et al.*, 1995; Alvarez & Wendel, 2003).
- **Intergenomic variability** – The degree of ITS sequence variations obtained through nucleotide polymorphisms or common insertions/deletions are suitable for

phylogenetic inference at species, generic or even family levels (Baldwin *et al.*, 1995; Linder *et al*., 2000; Alvarez & Wendel, 2003).

Despite these advantages and its popularity, the use of the *ITS* region for phylogenetic studies also has drawbacks. Because ITS sequences are not coding regions, they lack protein coding exons, which serve as guides in the alignment of nucleotide sequences (Alvarez & Wendel, 2003). The accumulation of indels (insertions or deletions of bases in the DNA of the ITS region) may also complicate the sequence alignment procedure (Baldwin *et al.*, 1995). However, if the amplified sequences include a good portion of the conserved ribosomal genes, the placement of gaps and the alignment of sequences can be minimized. Moreover, the predominance of substitution mutation over indel mutations (Baldwin *et al.*, 1995) means that the effect of varying indel treatments (i.e., treating them as missing data, informative fifth character, binary data, or deleting them) may not have a significant effect on tree topologies.

The other drawback with ITS in phylogenetic studies is related to incomplete concerted evolution. The application of ITS and ETS in phylogeny assumes that concerted evolution is complete and hence that the tandem repeats of rDNA show little or no variation within genomes (Weider *et al*., 2005; Soltis *et al*., 2008). However, concerted evolution may be absent or not rapid enough to cause a complete homogenization of rDNA repeats, as observed in some plant groups (e.g., genus *Passiflora* in Lorenz-Lemke *et al.*, 2005; genus *Rosa* in Wissemann & Ritz*,* 2005 and Koehler-Santos *et al.*, 2006; genus *Mammillaria* in Harpke & Peterson, 2006; genus *Pyrus* in Zheng *et al*., 2008; and *Camellia sinensis* in Xu *et al*., 2015).

Incomplete concerted evolution is usually associated with polyploidy and hybridisation, as these evolutionary processes lead to the incorporation of different parental rDNA types into a single individual genome at a faster rate than the different rDNA types can be completely homogenised by concerted evolution (Won & Renner, 2005; Grimm & Denk, 2008; Xiao *et al*., 2010). The presence of different rDNA paralogs (rDNA copies with different genealogies) in single genomes has been reported for several polyploid and hybrid plant species (e.g., Wendel *et al.*, 1995; Zheng *et al*., 2008; Tang *et al*., 2015; Xu *et al*., 2017)

The occurrence of different rDNA paralogs in a single genome due to an incomplete homogenization of the rDNA types will result in a mixture of rDNA paralogs for phylogenetic analysis, which will lead to incongruence between a gene tree and a species tree (Doyle, 1992; Sanderson & Doyle, 1992) as depicted in Figure 2.3.

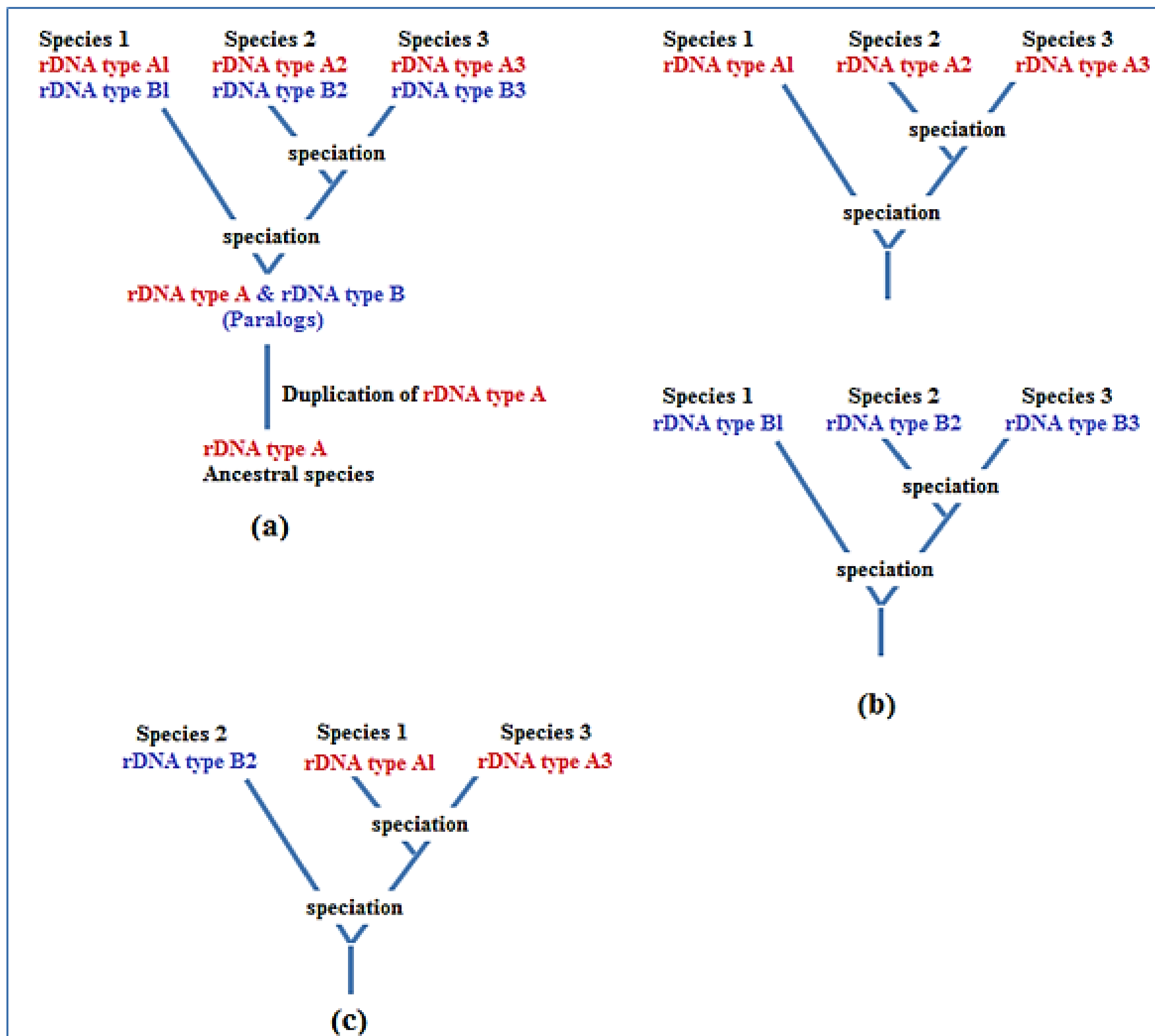

Figure 2.3 Incongruence between the species tree and gene tree due to paralogs of rDNA repeats. An ancestral rDNA type A undergoes gene duplication where one copy may evolve over time in to rDNA type B. (a) When the ancestral species evolves in to multiple species, its rDNA paralogs (rDNA type A and rDNA type B) may evolve into orthologous rDNA types in several species (rDNA type A1, rDNA type A2, and rDNA type A3 are orthologous; rDNA type B1, rDNA type B2, and rDNA type B3 are another orthologous group). (b) If orthologous rDNA types are sampled from all individuals, the species phylogeny can be recovered with the genetic data. (c) If a mixture of paralogous and orthologous rDNA types are chosen (rDNA type B2 from species 2 is paralogous to the two orthologous rDNA types – rDNA type A1 from species 1 and rDNA type A3 from species 3), then the species tree cannot be recovered from the genetic data (adapted from Doyle, 1992).

The figure shows that a gene duplication event can produce two genes that are paralogs of each other. If complete concerted evolution does not occur to homogenize these paralogs, one of them may diverge through mutation as a different gene lineage. The duplicated genes evolve into two sets of orthologous types through speciation (one set is rDNA type A1, rDNA type A2, and rDNA type A3; the other set is rDNA type B1, rDNA type B2, and rDNA type B3).

As Figure 2.3b shows, if orthologous rDNA types are sampled for phylogenetic analysis, the species phylogeny can be recovered using the genetic data. However, if a mixture of paralogous and orthologous rDNA types are used (Figure 2.3c), there will be incongruence between the species tree and the gene tree and the phylogeny will not be recovered. Figure 2.3c shows that species 1 and 3 are more related to each other than they are to species 2, while the correct relationship should be that species 2 and 3 are more closely related to each other than to species 1.

To solve the problem of paralogy, Alvarez and Wendel (2003) recommend the routine use of single-copy nuclear genes as an alternative to ITS for phylogenetic analysis, since such genes are not subject to concerted evolution and contain codon regions that help to minimize nucleotide sequence alignment uncertainties. Alternatively, pseudogenes and paralogs can be isolated with considerable investment in amplification and analysis (Poczai & Hyvonen, 2010).

In this study, nucleotide sequences of *Metrosideros* species were not difficult to align despite the presence of indels. Moreover, my phylogeny analysis is unlikely to be affected by paralogy and incomplete concerted evolution because polyploidy, which is associated with the presence of different rDNA types in individual genomes, has never been reported for any of the species in this genus. All *Metrosideros* species are diploid with chromosome number 2n = 22 (Dawson, 1987).

External Transcribed Spacers (ETS)

The External Transcribed Spacer (ETS) region separates the repetitive 18S-5.8S-26S ribosomal gene blocks from each other. In addition to having the useful characteristics of the ITS, the ETS is known for its faster evolution and for containing more informative sites than ITS (e.g., Baldwin & Markos, 1998; Linder *et al*., 2000; Markos & Baldwin, 2002; Plovinich & Panero, 2004; Hidalog *et al*., 2006; Masuda *et al*., 2009). As a result, the combined use of ETS and ITS data has been found to improve the branch support and phylogenetic resolutions of the trees constructed compared with ITS data alone (e.g., Baldwin & Markos, 1998; Wright *et al*., 2003; Li *et al*., 2005; Li *et al*., 2007; Jiménez-Mejías *et al*., 2016).

Despite its usefulness in phylogenetic studies, ETS are not used as frequently as ITS, because their more variable sequence length and composition means that amplification with universal primers is not always successful (Poczai & Hyvonen, 2010). Moreover, the ETS region lacks a primer annealing site at its 5' end due to the lack of a highly conserved flanking region

except the short transcription initiation sequence (Volkov *et al*., 1996; Baldwin & Markos, 1998). As a result, less than half of the entire ETS sequence is usually available for use (Baldwin & Markos, 1998; Linder *et al*., 2000).

Chloroplast markers maturase K (*matK*) and *trnL intron*

Chloroplast markers are routinely used in phylogenetic studies of plants (Dong *et al*., 2012; Patwardhan *et al*., 2014). Chloroplasts have suitable attributes such as uniparental (maternal) inheritance and genomes free of processes such as gene deletion and duplication, pseudogene formation and concerted evolution. Such processes are common in nuclear genomes and can potentially distort the phylogenetic history of plants (Palmer, 1985; Palmer *et al*., 1988). Moreover, chloroplasts are found in high copy number in plant cells which simplify the isolation, amplification, and sequencing of their genes (Palmer 1985; Soltis & Soltis, 1998).

Despite the fact that chloroplast genes have a low rate of sequence evolution, different genes within the genome evolve at different rates and non-coding regions evolve relatively faster than coding regions (Patwardhan *et al*., 2014). Therefore, the identification of chloroplast regions with an appropriate sequence length and nucleotide substitution rate is important for phylogenetic studies. This is especially crucial for plants that are recently evolved and therefore have little genetic differentiation since the use of less variable chloroplast regions for such plants would not be useful. Fortunately, the availability of more than 191 sequences of entire chloroplast genomes (Dong *et al*., 2012) makes the selection of appropriate chloroplast markers relatively easy.

Chloroplast markers that are currently used in phylogeny include *trnL intron, trnL – trnL F spacer, matK, rbcL, ndhF, rpl16,* and *atpB*. Of these markers, the *matK* (*maturase K*) gene (Figure 2.4), which is used in this study, has the fastest mutation rate (Dong *et al*., 2012; Shinohara *et al*., 2013; Ipek *et al*., 2014; Neubig *et al*., 2015). The gene is about 1500 bp in length and is embedded within the intron of another chloroplast gene *trnK* (which codes for the lysine tRNA). The *matK* gene codes for an enzyme called *maturase*, which is involved in splicing type II introns from RNA transcripts (Liang & Hili, 1996).

The *matK* gene has a good species discrimination power, its sequence recovery is high quality, and its sequence can be easily amplified and aligned (Dong *et al*., 2012; Ipek *et al*., 2014; Jiménez-Mejías *et al*., 2016). Because of these attributes, this marker has been used in several phylogenetic studies (e.g., Gao *et al*., 2008; Hilu *et al*., 2008; Kuo *et al*., 2011;

Shinohara *et al*., 2013; Jiménez-Mejías *et al*., 2016**;** Tekpinar *et al*., 2016). Its high discrimination power also means the *matK* gene is one of the few chloroplast regions that is used for plant barcoding (e.g., Hollingsworth, 2011; Dong *et al*., 2012; Bandara *et al*., 2013; Ipek *et al*., 2014; Tallei & Beivy, 2015). A DNA barcode is a short DNA sequence (usually less than 700bp) used to identify species based on a reference sequence (Dick & Kress, 2009). A candidate chloroplast region for barcoding needs to be powerful enough to discriminate between plants that have little genetic difference (Janzen, 2009; Dick & Kress, 2009); such as those that evolved recently.

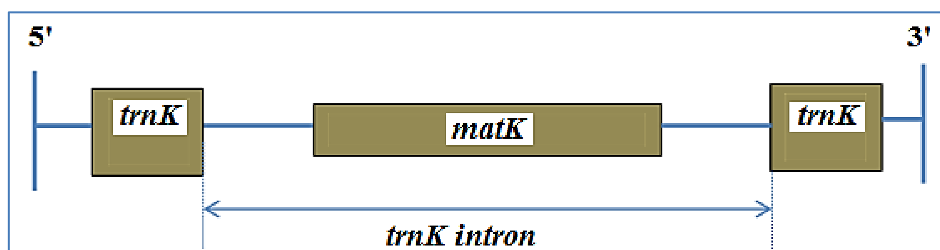

Figure 2.4 Location of *matK* gene within the *trnK* intron.

The other chloroplast region used in this study is the *trnL* (UAA) intron (Figure 2.5). This region has been widely used to study phylogenetic relationships among closely related species (e.g., McDade *et al.,* 2005; Chen *et al.,* 2005; Scharaschklin & Doyle, 2005; Dizkirici *et al*., 2013; Saha *et al*., 2015; Tekpinar *et al*., 2016). It is not among the most variable non-coding chloroplast regions (Shaw *et al*., 2005) but it has some unique merits. It has very good universal primers to easily amplify the region and resolve closely related genera (Taberlet *et al*., 1991; Gielly & Taberlet, 1996). Also, its evolution is well analysed and understood (Quandt & Stech, 2005) and it has a conserved secondary structure with alternation of conserved and variable regions. This alignment of different sequences might allow the development of versatile primers in the conserved regions to amplify the interspersed variable regions (Taberlet *et al*., 2006).

Figure 2.5 Location of the *trnL* intron and the universal primers *trnL*-c and *trnL*-d that were used to amplify it.

2.2.2 Phylogenetic tree-building methods

There are several tree-building methods that can be used to infer phylogenetic relationships. Most of these methods can be categorized as either distance-based or character-based (Rizzo & Rouchka, 2007; Staton, 2015).

2.2.2.1 Distance-based methods

Distance-based methods, also called algorithmic methods, transform nucleotide sequence data into a pair-wise distance data matrix to construct the phylogenetic tree (McCormack & Clewley, 2002). In other words, the distance matrix generated is a measure of the number of nucleotides that differ between two compared taxa (Egan & Crandall, 2006; Sleator, 2011). Although distance-based methods are computationally faster than character-based methods, they produce a single tree which does not provide an opportunity to compare other trees that might be more informative and accurate (Scott & Gras, 2012; Sardaraz *et al.*, 2012; Staton, 2015). Another major limitation of distance-based methods is the loss of information due to the transformation of sequence data into a distance matrix, which makes it impossible to identify the specific character changes that support a particular tree branch (Felsenstein, 1988; Soltis & Soltis, 2003). The most popular and routinely used distance-based methods are the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Neighbour Joining (Rizzo & Rouchka, 2007; Roy *et al.*, 2014).

a) UPGMA Method

The UPGMA method (Sokal & Michener, 1958) is a clustering algorithm which assumes a molecular clock by which all nucleotide sequences evolve at a constant rate over time across all lineages (Egan & Crandall, 2006; Roy *et al.*, 2014). Although the UPGMA method is fast and simple, which enables the analysis of large data sets, its major limitation is its molecular clock assumption, since in reality mutation rates vary among lineages and nucleotide

positions (Scott & Gras, 2012; Sardaraz *et al*., 2012). Hence, this method generates accurate tree topologies only when nucleotide sequences diverge based on the molecular clock analysis, and it is not used often today.

The UPGMA method constructs phylogenetic trees in a stepwise manner, beginning by grouping a pair of taxa that have the least divergent sequences. In the next step, the original distance matrix is collapsed by one row and column, as the first paired taxa are treated as one composite taxon, assuming a single position (column and row) in the new distance matrix. In the computation of the new distance matrix, the distance of other taxa from the originally grouped pair will be the average of each taxon's distance from each of the paired taxa. In the subsequent steps, this process is repeated until all taxa join the cluster and form a single UPGMA tree (Egan & Crandall, 2006; Rizzo & Rouchka, 2007; Staton, 2015).

b) Neighbour Joining Method

The Neighbour Joining Method (Saitou and Nei, 1987) is the most popular distance-based method (Egan & Crandall, 2006). Its advantage over the UPGMA method is that it does not rely on an unrealistic molecular clock; it rather adjusts for the variation in evolutionary rates among branches (Egan & Crandall, 2006; Rizzo & Rouchka, 2007). Compared to UPGMA, the Neighbour Joining Method is relatively rapid and yields a more accurate result. However, this method may generate different trees depending on the order in which sequences are entered (Sleator, 2011).

The construction of Neighbour Joining trees begins with a complete, unresolved star-like tree from which a fully resolved tree is produced through successive insertion of branches between the most similar terminals and the remaining terminals of the star tree. To identify which pair of terminals are most similar and should be joined, each pair is joined and the total branch length of the resultant tree is computed. The pair that gives the lowest total branch length is considered the closest neighbour and is then joined. Once the most similar terminals are identified and joined, a new branch is inserted between them and the remaining terminals of the star tree. The branch length is then recalculated. This process is repeated several times until only one terminus remains (Egan & Crandall, 2006; Rizzo & Rouchka, 2007). Figure 2.6 shows how this tree-building algorithm works.

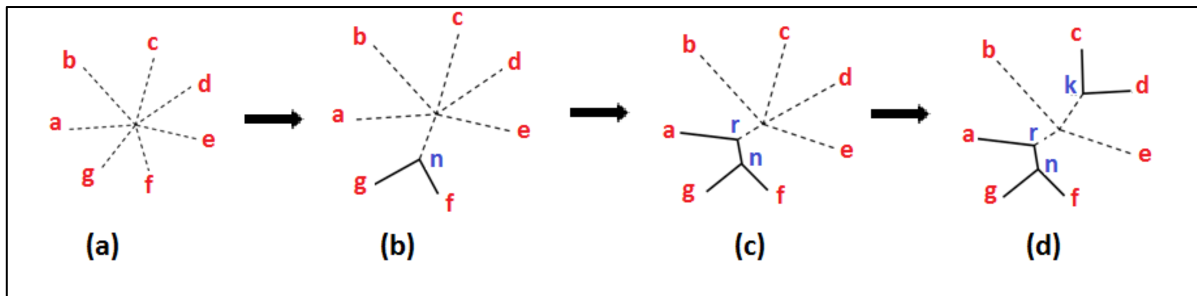

Figure 2.6 The construction of a Neighbor Joining tree by star decomposition. (a) The construction of the tree begins with an unresolved star-like tree. (b) Distance matrix is computed to identify the most similar pair of nodes (taxa f & g) which will be grouped and linked to the remainder of the tree through a new branch (node n). (c) Treating taxa f & g as one composite taxon represented by node u, a new pair-wise distance matrix involving nodes a, b, c, d, e, and n is generated from which a new pair of most similar taxa are identified (nodes a and n). This pair of taxa is linked to the remainder tree through a new branch (node r). (d) The process is repeated until the tree is resolved completely (adapted from Saitou and Nei, 1987).

2.2.2.2 Character-based methods

Unlike distance-based tree-building methods, character-based methods compare several tree topologies and select the best tree based on certain optimal criteria (i.e., minimum evolution in Maximum Parsimony, highest likelihood score in Maximum Likelihood, and highest posterior probability in Bayesian Inference; Egan & Crandall, 2006; Sleator, 2011). Unlike the distance-based methods, the character-based methods avoid loss of information because they use the original sequence data for phylogenetic inference and take into account the mutation events accumulated on the nucleotide sequences (Sardaraz *et al.*, 2012; Patwardhan *et al.*, 2014). The character-based methods use a more complex algorithm and generate trees that are more accurate than the distance-based methods (Scott & Gras, 2012; Patwardhan *et al.*, 2014). Some earlier studies (e.g., Huelsenbeck & Hillis, 1993; Huelsenbeck, 1995) showed that trees constructed with character-based methods are more frequently closer to the true phylogenetic tree than those constructed with distance-based tree-building methods.

The major limitation of these algorithms is that they are very time consuming; the possible number of tree topologies increases rapidly with the number of nucleotide sequences (Egan & Crandall, 2006; Sardaraz *et al.*, 2012; Staton, 2015). Although they use heuristic search methods to avoid the need for evaluating every tree topology, many different trees still need to be evaluated and hence they take more time to construct than the distance-based trees. The three commonly used character-based tree building methods are Maximum Parsimony, Maximum Likelihood and Bayesian Inference (Sleator, 2011; Roy *et al.*, 2014).

c) Maximum Parsimony

The Maximum Parsimony method (Sober, 1983) aims to find the best (most parsimonious) tree that has a topology explained by the minimum number of nucleotide substitutions among the sequences to be analysed. The main premises of this concept are: (1) mutations are such extremely rare events that the fewest nucleotide substitutions are more likely to explain phylogenetic relationships; and (2) the use of nucleotide substitution models to explain such unlikely events as mutations is probably incorrect. The method assumes the fewest evolutionary changes in order to reduce the occurrence of homoplasy, which is the sharing of identical nucleotides by two nucleotide sequences (taxa) due to independent multiple mutations rather than sharing a common ancestor (Egan & Crandall, 2006).

However, this method may yield inaccurate outcomes when used to analyse sequences with large divergence, since homoplasy is more pronounced in largely divergent sequences that involve several mutation events (Egan & Crandall, 2006). In such cases, two taxa that have largely divergent sequences can be wrongly grouped together as closely related taxa. This phenomenon is known as long-branch attraction (Huelsenbeck, 1995) which means long branches (taxa with divergent sequences) become artificially connected because the number of non-homologous similarities that the sequences have accumulated exceeds the number of homologous similarities they have retained with their true closest relatives (Figure 2.7). However, there are ways to minimize this limitation by using what is known as weighted parsimony, where different weights are given for different substitution types (e.g., more weight is given for transversions than transition mutations; Hillis *et al*., 1994).

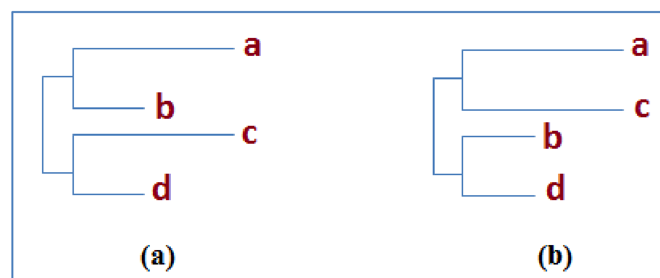

Figure 2.7 Long-branch attraction (a) The true topology. (b) Maximum Parsimony recovers the wrong topology due to the grouping of long-branched unrelated taxa a & c

The major strengths of Maximum Parsimony include its simplicity and its amenability to rigorous mathematical analysis (Yang & Rannala, 2012); it is robust and its computation is faster than the Maximum Likelihood method, although it is slower than distance-based

methods (Egan & Crandall, 2006; Sardaraz *et al*., 2012). It also provides diagnoses for each clade and branch lengths in terms of the number of changes on each branch, and it can identify informative or problematic characters (Sleator, 2011).

Apart from the long-branch attraction problem mentioned above, limitations of this method include: 1) its unrealistic assumption of consistency in evolutionary rates across nucleotide sites and among lineages (Foster, 2009; Sleator, 2011); 2) its lack of an explicit evolutionary model to account for differences in substitution rates among lineages and nucleotide sequences; 3) its use of only informative characters rather than the whole data set; and 4) there can be more than one equally parsimonious trees (i.e., with equal total branch length), which means it is impossible to identify which tree best reflects the phylogeny. The last point is an important issue especially when a single evolutionary hypothesis is required to explain relationships among taxa (Egan & Crandall, 2006).

d) Maximum Likelihood

The Maximum Likelihood method (Felsenstein, 1981) evaluates different tree topologies based on their probability of explaining the data under a nucleotide substitution evolutionary model. The best tree has the maximum likelihood score, or the highest probability of explaining the data (Brocchieri, 2001).

The major advantages of this method include: (1) it has strong statistical foundations and it allows the comparison of different topologies, models and parameters; 2) it makes use of all the sequence data, unlike Maximum Parsimony which only uses informative characters (Sleator, 2011; Yang & Rannala, 2012); (3) it uses an evolutionary model to make explicit assumptions about evolutionary processes and to account for differences in nucleotide substitution rates among lineages and nucleotide positions, thereby resolving phylogenetic relationships in a more realistic way (Egan & Crandall, 2006; Foster, 2009); and (4) it is robust to violations of model assumptions (Felsentein, 2003). Perhaps the major limitation of Maximum Likelihood that is often mentioned in literature is that it is so computationally intensive that it requires much more time than other methods (Brocchieri, 2001). However, this not an issue in current times where advanced computers and programs can run large data sets in minutes.

e) Bayesian Inference

Bayesian Inference (Huelsenbeck *et al*., 2001) is based on a likelihood function similar to Maximum Likelihood. However, whereas a Maximum Likelihood score explains the probability of obtaining the data given a hypothesis (i.e., a tree topology), the Bayesian Inference score represents the probability that the hypothesis (tree topology) is correct given the data (Egan & Crandall, 2006). Bayesian Inference differs from Maximum Likelihood in that model parameters (branch length, tree topology, etc.) are treated as random variables with statistical distributions rather than as parameters (Douady *et al*., 2003; Yang & Rannala, 2012).

In the Bayesian Inference method, a prior distribution is assigned to parameters before data analysis, and this distribution is then combined with the likelihood of the data to compute the posterior probability of each possible topology (Egan & Crandall, 2006). The inference relies on Bayes's Theorem, which states that the posterior probability is proportional to the prior probability times the likelihood of the data. In other words, the posterior information is the prior information plus the data information as depicted in the equation below:

$$\mathrm{P}(T,\theta|D) = \frac{\mathrm{P}(T,\theta)\mathrm{P}(D|T,\theta)}{\mathrm{P}(D)}$$

where $\mathrm{P}(T,\theta)$ is the prior probability of tree T with model parameter θ (including branch length, the ratio between transition and transversion rates, etc.), $\mathrm{P}(D|T,\theta)$ is the likelihood or probability of the data given tree T and model parameter θ, and $\mathrm{P}(T,\theta|D)$ is the posterior probability of tree T with model parameter θ given the data, D. The denominator $\mathrm{P}(D)$ is a normalizing constant to ensure that $\mathrm{P}(T,\theta|D)$ sums over the trees and integrates over the parameters to one

Because the calculation of posterior probabilities of trees involves a multi-dimensional computation over all possible parameter θ values, the Bayesian Inference relies on Markov Chain Monte Carlo (MCMC) algorithms to approximate the estimation of the posterior probabilities (Douady *et al*., 2003; Foster, 2009). The MCMC is a simulation algorithm, where trees are sampled in fixed intervals in the tree and parameter space thousands or millions of times. The posterior probability of a given tree topology is approximated by the proportion of times that topology is visited (sampled) by the algorithm (Tierney, 1994; Yang & Rannala, 1997). The best tree has the highest posterior probability.

The advantage of Bayesian Inference over Maximum Likelihood is its faster computational speed (Douady *et al*., 2003; Steane *et al.*, 2003), which means that large data sets can be analysed quickly. Because of its reliance on the MCMC algorithm, Bayesian Inference can analyse large data sets with models that are complex and rich in parameters (Mar *et al*., 2005; Foster, 2009). Another advantage of Bayesian Inference over Maximum Likelihood is that it directly informs the probability that a tree is accurate (Mar *et al*., 2005; Egan & Crandall, 2006). In addition, the MrBayes 3 program (Ronquist & Huelsenbeck, 2003), which is used to construct Bayesian trees, allows the combined analysis of data sets with different nucleotide substitution models. Finally, additional bootstrap analyses, which are hard to interpret, are not required because estimation of branch support accompanies tree estimation (Steane *et al.*, 2003).

There is controversy as to which character-based method is preferable, but model-based likelihood methods are widely recognized as superior because of their stronger statistical foundation and ability to compare different trees, parameters and models (Sleator, 2011; Yang & Rannala, 2012). However, Maximum Parsimony is still commonly used alone or in combination with Maximum Likelihood or Bayesian Inference, as it usually offers reasonable outputs and is computationally efficient (Yang & Rannala, 2012).

Generally, every method of building phylogenetic trees has its advantages and disadvantages. The value of each method may depend on the type and amount of data, the nature of question we want to answer, and how quickly results need to be available. For instance, although problems of long-branch attraction are associated with the maximum parsimony method if largely divergent taxa are analysed, this method can well be effective for analysing taxa that are not largely divergent. On the other hand, the Neighbour Joining method may be preferable over character-based methods in order to have a quick topology of many taxa, which would require a tremendous amount of time to analyse with character-based methods.

2.2.3 Nucleotide substitution models

Some nucleotide substitution types are more frequent than others; for example, substitutions are generally more frequent between biochemically similar bases. The four types of transition (A to G, G to A, C to T, T to C) occur more frequently than the eight types of transversion (A to C, A to T, C to G, G to T, and the reverse; Wakely, 1996; Brinkman & Leipe, 2001). Such biases will affect the estimation of sequence divergence and tree topology if they are not

corrected for in the phylogenetic analysis. In addition, substitution rates may vary among lineages and nucleotide positions, which can also affect the outcomes of the phylogenetic tree construction (Swofford *et al*., 1996). To explain the phylogenetic relationships of taxa in a more realistic way, tree-building methods need to have mathematical models such as nucleotide substitution models to describe and account for biased base frequencies and nucleotide substitution rates.

Commonly used nucleotide substitution models incorporate parameters that describe biases in base frequencies and the type of base substitutions (i.e., transition vs. transversion; Verbruggen & Theriot, 2008). To account for substitution rate heterogeneity among nucleotide positions, the nucleotide substitution models assume a gamma distribution (*G*) of substitution rates across nucleotide sites (Yang *et al*., 1994; Swofford *et al.*, 1996). In addition, the nucleotide substitution models incorporate the proportion of invariable sites (*I*) to estimate the proportion of sites with zero probability of change, while the remaining sites are assumed to vary with equal probability (Steel *et al*., 2000). The combined approach (*G*+*I*), which makes the nucleotide substitution model more complex, assumes that a proportion of sites are invariant while the remaining sites vary according to a gamma distribution (Verbruggen & Theriot, 2008). The following five nucleotide substitution models are most commonly used in molecular phylogenetic studies.

Jukes Cantor (JC) model

The Jukes Cantor (JC) model is the simplest substitution model and assumes that all four bases have equal frequencies and that any base can change to any other base with equal probability (Jukes & Cantor, 1969).

Kimura 2 Parameter (K2P) model

The Kimura 2 Parameter model assumes that all four bases have equal frequencies, but unlike the JC model, it assumes that transversions and transitions have different substitution rates (Kimura, 1980).

Felsenstein (F81) model

The Felsenstein model assumes variable or unequal base frequencies within a sequence but equal frequency for each base across sequences. The model assumes that all substitutions are equally likely (Felsenstein, 1981).

Hasegawa, Kishino and Yano (HKY85) model

The HKY85 model can be considered a combination of the K3P and F81models. It assumes that transversions and transitions have different substitution rates and that the four bases have unequal frequency (Hasegawa *et al.*, 1985).

General Time Reversible (GTR) model

Tavaré (1986) first proposed the GTR model in a more general form. The model assumes unequal base frequencies and different substitution rates among all combinations of bases (AC, AG, AT, CG, CT, and GT). It uses six parameters to describe the relative substitution rates among these base combinations (Verbruggen & Theriot, 2008).

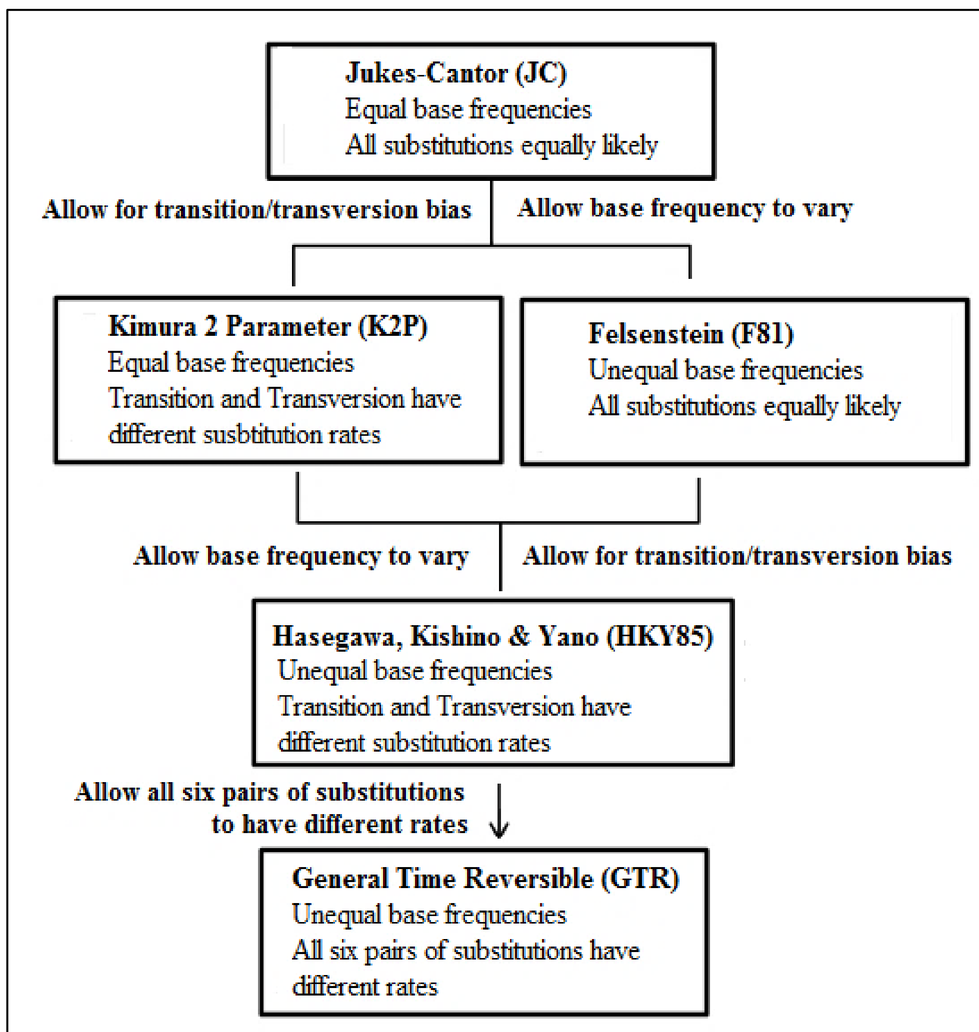

Figure 2.8 Overview of nucleotide substitution models.

2.3 Materials and Methods

2.3.1 Acquisition and alignment of nucleotide sequence data

This study is based on nuclear sequence data (*ITS* and *ETS* regions of rDNA) obtained for all species from the NCBI gene database and chloroplast data (*matK* and *trnL intron*) obtained from newly sequenced samples. The chloroplast phylogenetic analysis was done only for species available at Allan Herbarium at Landcare Research in Lincoln. Accordingly, leaf samples of 20 species were obtained from the herbarium. Chloroplast DNA was extracted from 20mg of leaf samples following the manufacturer's protocol for the NucleoSpin® Plant II DNA Extraction Kit (Macherey-Nagel, Duren, Germany).

The *matK* chloroplast region (≈ 800bp) was amplified using 2521F and 2519R *matK* primers (Gadek *et al*., 1996). Amplifications were performed with an initial denaturation period at 94°C for 2 minutes, then 35 cycles of denaturation at 94°C for 20 seconds, annealing at 45°C for 20 seconds, and extension at 72°C for 2 minutes with final extension at 72°C for 10 minutes. The *trnL* (UAA) *intron* chloroplast region (≈ 500bp) was amplified using *trnL-c* forward and *trnL-d* reverse primers (Taberlet *et al*., 1991). Amplifications were performed with an initial denaturation period at 94°C for 2 minutes, then 30 cycles of denaturation at 94°C for 20 seconds, annealing at 57°C for 10 seconds, and extension at 72°C for 40 seconds with final extension at 72°C for 5 minutes. All sequence data were obtained using an ABI PRISM 3100 Genetic analyser. The sequencing was conducted at Landcare Research in Auckland.

Table 2.4 Primers used to amplify the *matK* and *trnL* intron chloroplast regions.

	Primer	**Sequence 5' to 3'**	**Source**
matK	*2521F*	TTCACATTTAGATTATGTG	Gadek *et al*. (1996)
	2519R	TTTACGAGCCAAAGTTTTAA	
***trnL* intron**	c (code B49317)	CGAAATCGGTAGACGCTACG	Taberlet *et al*. (1991)
	d (code A49855)	GGGGATAGAGGGACTTGAAC	

Once the sequence data were obtained, both the nuclear (ITS and ETS) and chloroplast sequence data were aligned using the computer program ClustalW (Larkin *et al*., 2007) in Geneious 7.1 (Kearse *et.al*., 2012). As indel positions are not always perfectly aligned, the alignments were visually inspected and corrected manually. Sequence alignment is a critical step in phylogenetic analysis, as it greatly impacts the topology of the trees generated

(Patwardhan *et al*., 2014). Using Gapcode.py 2.1 (Richard Ree, Field Museum, Chicago, IL, USA; available from http://www.reelab.net), indels were coded as binary characters using the simple coding method of Simmons and Ochoterena (2000). A concatenated alignment of ITS & ETS and *matK* & *trnL intron* were produced after aligning each data set separately.

2.3.2 Data analysis

Once the nucleotide sequences were aligned, phylogenetic trees were constructed using the Maximum Parsimony (MP) and the Bayesian Inference (BI) tree builders. MP analyses were carried out in TNT 1.1 (Tree analysis using New Technology; Goloboff *et al*., 2008) using the New Tech Search option. Default settings were selected for Sectorial Searches (RSS, CSS and XSS), Ratchet, Tree Drifting and Tree Fusing. Ten initial random addition sequences were used and the search was terminated after minimum length trees were found five times. Bootstrap support (Felsenstein, 1985) was calculated with Poisson independent reweighting using 1,000 replicates.

The BI analyses were performed using the program MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Before the analysis, the best nucleotide substitution model for each data set was identified using the Akaike Information Criterion (AIC) in JModeltest 0.1.1 (Posada, 2008). The best evolutionary model for the ITS data set was GTR + I + г, whereas GTR + г was selected as the best model for ETS and the chloroplast data. The concatenated data sets were analysed as partitioned data using the selected models and also as unpartitioned data using the GTR + г model. Indel characters were included as 'restriction type' data in the BI analyses.

The BI analyses were performed using two independent and simultaneous runs. The Markov Chain Monte Carlo analyses (Geyer, 1991) were run for one million generations with four chains per analysis, sampling one tree every 1,000 generations using the specific nucleotide substitution model for each data set and default settings for other parameters. The burn-in period used for each run was 250,000 generations, using trees from the remaining MCMC generations in order to generate the consensus tree and its posterior probablities. The one million generation run gave an average standard deviation of split frequencies between the two independent runs below 0.01, which indicates a good convergence between the runs (Ronquist *et al.,* 2005).

2.4 Results

To construct the phylogenetic trees, I used *Cloezia floribunda* as an outgroup species. *Cloezia floribunda* is endemic to New Caledonia, and is classified within the *Metrosideros* alliance of the *Myrtaceae* (Briggs & Johnson, 1979). I constructed the trees using nucleotide sequences from five data sets: 1) ITS, 2) ITS - ETS combined, 3) *matK*, 4) *trnL intron*, and 5) *matK* - *trnL intron* combined. Table 2.5 presents a summary of these molecular markers.

Table 2.5 Characteristics of the molecular markers used in the study.

Characteristics	ITS	ETS	ITS-ETS Combined	*matK*	*trnL*	*matK-trnL* combined
Sequence length including gaps (bp)	633	446	1079	742	491	1233
GC content %	54.9	48.1	50.97	32.7	33.8	33.1
Mean sequence divergence	0.029	0.034	0.038	0.01	0.01	0.01
No. of variable characters (%)	171 (27%)	159(35.65%)	330(30.58%)	57 (7.68%)	33 (6.72%)	90 (7.29%)
No. of parsimony informative characters (%)	82 (12.95%)	77 (17.26%)	159(14.74%)	13 (1.75%)	7 (1.43%)	20 (1.62%)

2.4.1 Results of nuclear sequence data

The trees generated from both ITS and ITS - ETS combined data (Figures 2.9 & 2.10) showed the subgenus *Metrosideros* as one large monophyletic clade containing smaller monophyletic clades. This was strongly supported by bootstrap analysis [ITS: Posterior Probability (PP) 0.95; ITS-ETS combined: Bootstrap Support (BS) 95%, PP 1.00]. The ITS tree (Figure 2.9) showed that within this subgenus, clade A3 was a predominantly New Caledonian group with four embedded species from New Zealand (*M. umbellata*), the Solomon Islands (*M. salomonensis*), Bonin Islands (*M. boninensis*), and Fiji (*M. ocharantha*). Clade A1 contained a widely distributed group with representative samples from Lord Howe Island to the Polynesian Islands, including the remote Hawaiian Islands. Both phylogenetic trees (Figures 2.9 & 2.10) strongly supported the New Zealand species *M. bartlettii* as a sister taxon to this subgroup (ITS: BS 78%, PP 0.95; ITS - ETS combined: BS 59%, PP 0.99).

Another poorly resolved subgroup A2 (Figure 2.9) was also embedded within the subgenus *Metrosideros.* This group contained two taxa from Melanesia (Vanuatu and Fiji) and another

two from Polynesia (Samoa). The two big clades (A1 and A3) showed geographical grouping of species, where New Caledonian species fell into clade A3 and Hawaiian species fell into clade A1.

Phylogenetic trees constructed from the ITS - ETS combined data set (Figure 2.10) resulted in a much better resolved and supported subgenus *Metrosideros*. The monophyletic status of the whole subgenus was very strongly supported (BS 95%, PP 1.00). Within this subgenus were three strongly supported monophyletic clades: A1, A2 and A3. These clades were better resolved than in the ITS-based tree (Figure 2.9), with the two groups that exclusively comprise *M. collina* varieties (clades A2 and A3) split from the rest of subgenus *Metrosideros* with strong support (clade A2: BS 97%, PP 1.00; clade A3: BS 98%, PP 1.00).

The tree strongly supported an almost exclusively Hawaiian group (clade A1) as a separate clade (BS 73%, PP 0.99). The only non-Hawaiian member of this clade was *M. collina,* from the Marquesas Islands. The grouping of this *M. collina* variety with the Hawaiian group rather than with *M. collina* varieties of southeastern Polynesia (clade A2 and A3) was unexpected. Moreover, southeastern Polynesia is much closer than Hawaii is to Marquesas Island. Like the ITS-based tree, the ITS - ETS combined tree suggested the genetic distinctiveness of *M. bartlettii* from its close relatives, *M. robusta* and *M. excelsa*. *M. umbellata* from New Zealand appeared to be a sister taxon to other species in the subgenus *Metrosideros*, suggesting that it could be the earliest diverged taxon in this subgenus.

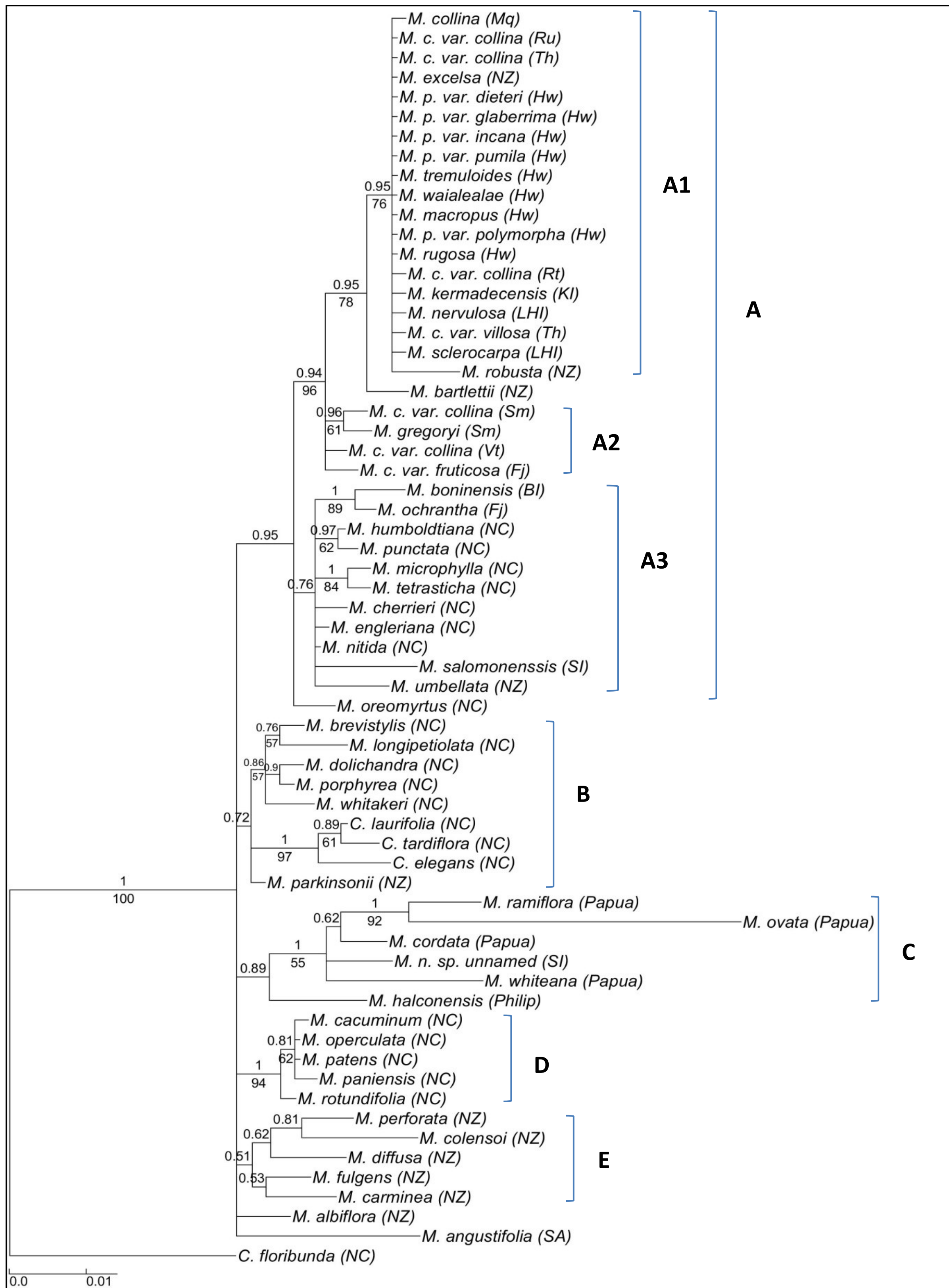

Figure 2.9 Bayesian phylogenetic tree obtained from ITS data set. Numbers above branches indicate posterior probabilities obtained from Bayesian analysis and numbers below branches indicate bootstrap values obtained from Maximum Parsimony analysis. BI - Bonin Is, Fj – Fiji, Hw – Hawaii, KI - Kermadec Is, LHI - Lord Howe Is, Mq – Marquesas Is, NC - New Caledonia, NZ - New Zealand, Papua – Papua (New Guinea), Philip – Philippines, Rt – Rarotonga, Ru – Rurutu, SA – South Africa, SI -Solomon Is, Sm – Samoa, Th – Tahiti, Vt – Vanuatu.

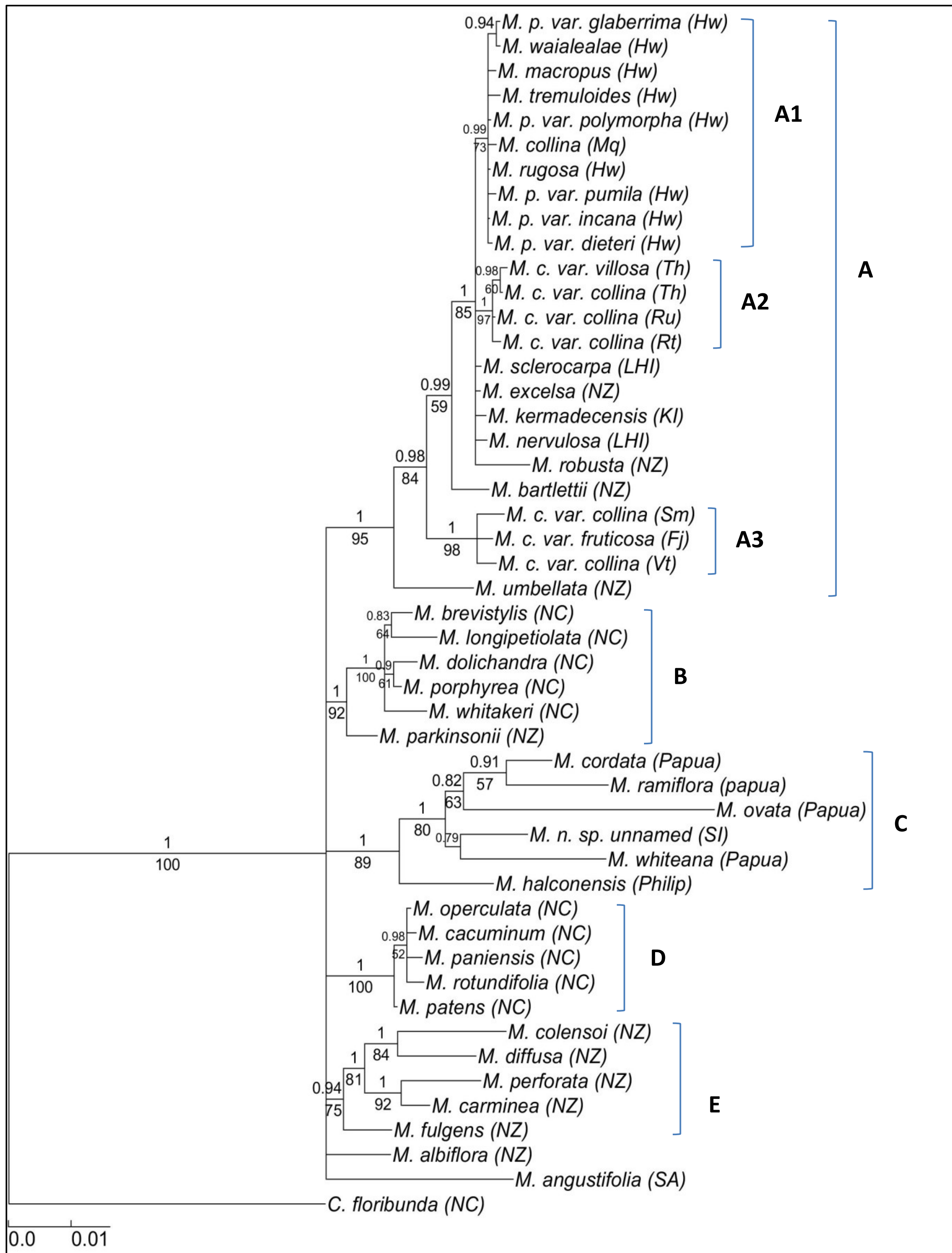

Figure 2.10 Bayesian phylogenetic tree obtained from ITS - ETS combined data set. Numbers above branches indicate posterior probabilities obtained from Bayesian analysis and numbers below branches indicate bootstrap values obtained from Maximum Parsimony analysis. Fj – Fiji, Hw – Hawaii, KI - Kermadec Is, LHI - Lord Howe Is, Mq – Marquesas Is, NC - New Caledonia, NZ - New Zealand, Papua – Papua (New Guinea), Philip – Philippines, Rt – Rarotonga, Ru – Rurutu, SA – South Africa, SI -Solomon Is, Sm – Samoa, Th – Tahiti, Vt - Vanuatu.

Both trees (Figures 2.9 & 2.10) showed that the subgenus *Mearnisa*, unlike subgenus *Metrosideros*, was not monophyletic. It was a very heterogeneous group with member species falling into several small monophyletic clades or subsections. To illustrate the heterogeneity of the three subgenera, Table 2.6 lists mean sequence divergence (number of base substitutions per site averaged over all sequence pairs) for the three subgenera. The mean sequence divergence of the subgenus *Mearnsia* (0.047) was three times that of the subgenus *Metrosideros* (0.016).

Table 2.6 Comparison of mean sequence divergence among subgenera of *Metrosideros*

Subgenus	**ITS mean sequence divergence**	**ETS mean sequence divergence**	**Mean of ITS and ETS mean sequence divergences**
Metrosideros	0.013	0.018	0.016
Mearnsia	0.035	0.058	0.047
Carpolepis	0.011	Data not available	0.011

Both phylogenetic trees (Figures 2.9 and 2.10) showed geographical differentiations within the subgenus *Mearnsia*, with New Zealand, New Guinea and New Caledonia species grouped according to their respective regions. These region-based groupings were more strongly supported in the ITS - ETS combined data set than in the ITS data, as is the case for the subgenus *Metrosideros*. The subgenus *Carpolepis* (see Figure 2.9) was a strongly supported monophyletic group (BS 96%, PP 1.00) with a weak association (PP 0.72) with the New Caledonian group (clade B). In the Maximum Parsimony tree (not shown), even a weak association did not exist; rather *Carpolepis* was placed separately with equivalent rank to subgenus *Metrosideros* and subsections of subgenus *Mearnsia*.

The ITS - ETS combined trees resulting from the Maximum Parsimony and Bayesian Inference analyses were identical both in topology and their degree of resolution. The ITS trees of Maximum Parsimony and Bayesian Inference also had very similar topologies except that clades A3 and E (Figure 2.9) did not exist in the Maximum Parsimony tree. Species of these two clades formed a polytomy in the Maximum Parsimony tree without forming monophyletic clades. This is not surprising, as these two clades had very weak support in the Bayesian tree (clade A3: PP 0.76; clade E: PP 0.51).

2.4.2 Results of chloroplast data

The chloroplast analysis suggested that the *trnL intron* chloroplast region is highly conserved among species, as its resolving power is much lower than the *matK* region, leaving many species unresolved and many others falling in one group (Figure 2.12). The Maximum Parsimony (MP) analysis gave an almost identical topology with poor resolution. On the other hand, the *matK tree* (Figure 2.11) revealed several monophyletic groups within the subgenus *Metrosideros* (clades A1, B and C) and subgenus *Mearnsia* (Clade A2), which were also supported by the nuclear data.

The tree based on the *matK-trnL intron* combined data set (Figure 2.13) resolved the group better than the *trnL intron* region but worse than the *matK* region. For instance, in the combined tree, *M. fulgen* split from *M. diffusa* to pair with *M. collina* of Rarotonga. Moreover, *M. umbellata, M. bartlettii* and *M. collina* of Fiji - which were revealed as a strongly supported group in the *matK* tree (clade C) - split in the combined tree. Perhaps the inclusion of the *trnL intron* data decreases the resolving power of the *matK* data in the combined data set.

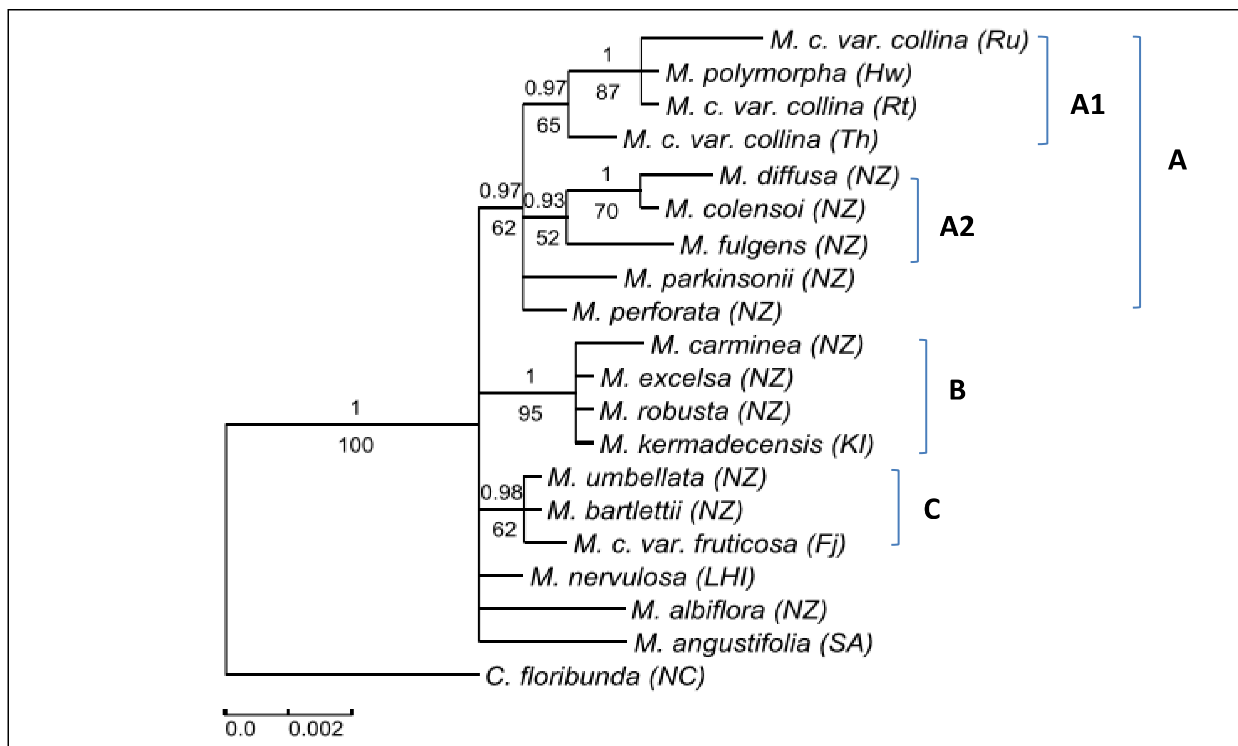

Figure 2.11 Bayesian phylogenetic tree obtained from *matK* data set. Numbers above branches indicate posterior probabilities obtained from Bayesian analysis and numbers below branches indicate bootstrap values obtained from Maximum Parsimony analysis. Fj – Fiji, Hw – Hawaii, KI - Kermadec Is, LHI - Lord Howe Is, NZ - New Zealand, Rt – Rarotonga, Ru – Rurutu, SA – South Africa, Th – Tahiti.

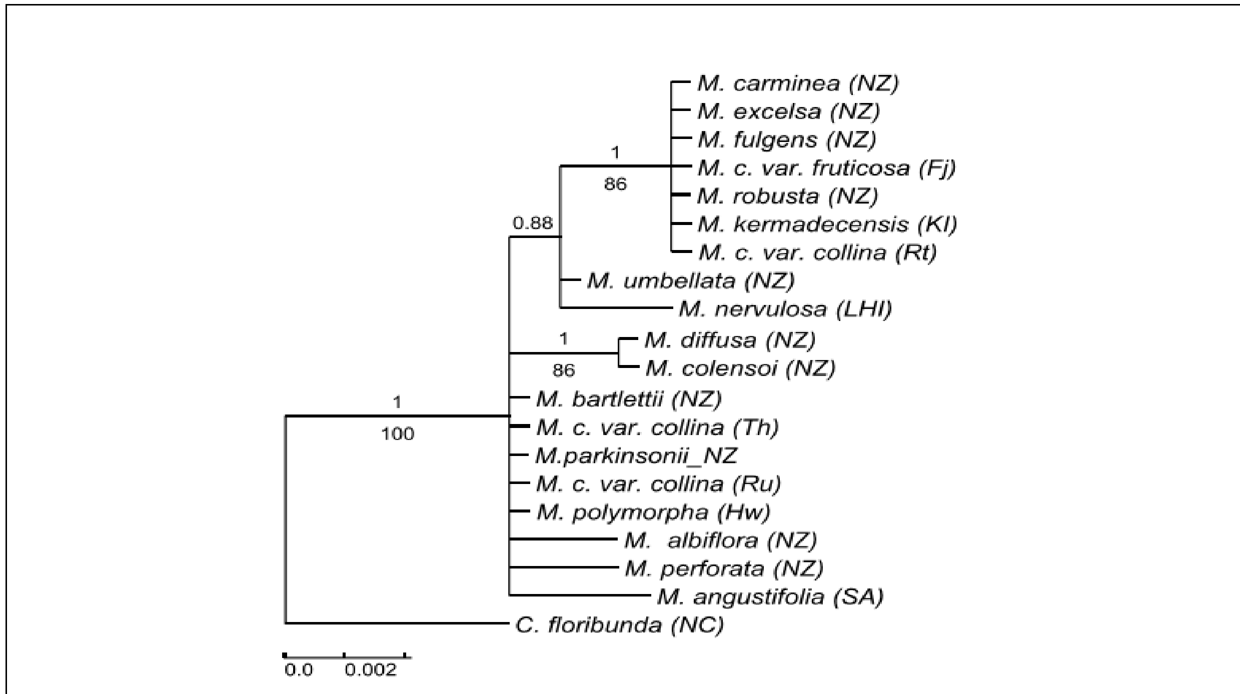

Figure 2.12 Bayesian phylogenetic tree obtained from *trnL intron* data set. Numbers above branches indicate posterior probabilities obtained from Bayesian analysis and numbers below branches indicate bootstrap values obtained from Maximum Parsimony analysis. Fj – Fiji, Hw – Hawaii, KI - Kermadec Is, LHI - Lord Howe Is, NZ - New Zealand, Rt – Rarotonga, Ru – Rurutu, SA – South Africa, Th – Tahiti.

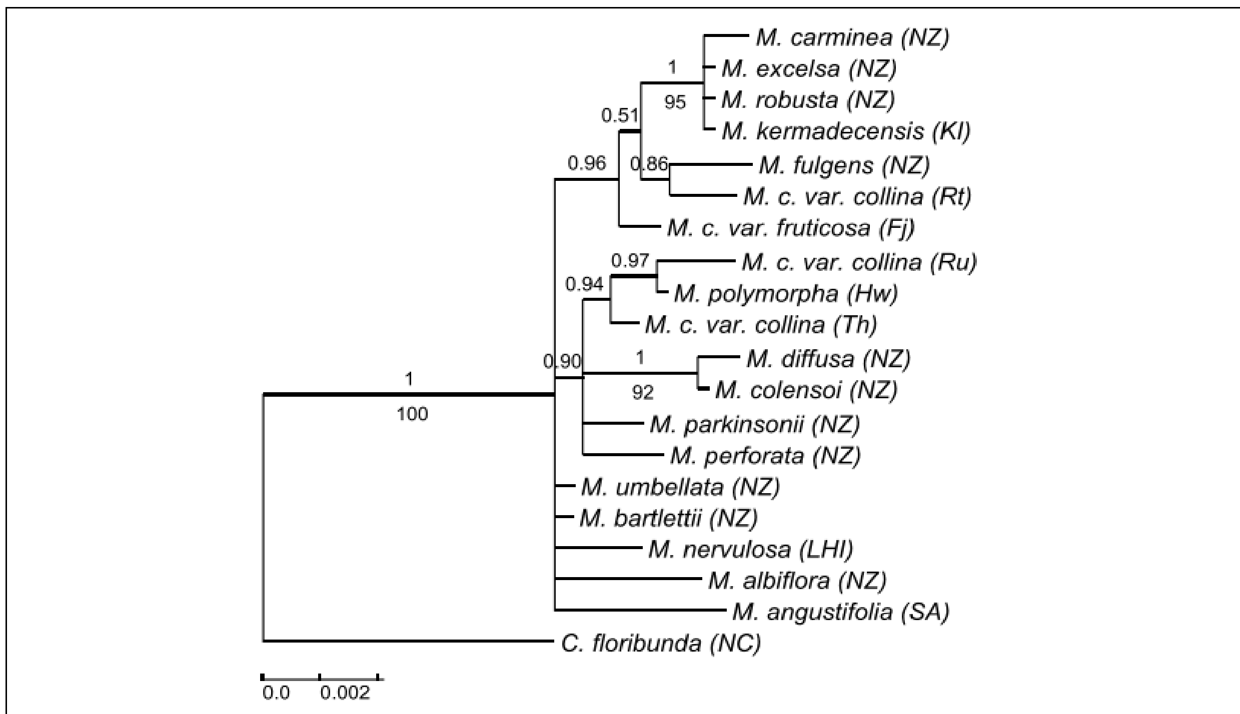

Figure 2.13 Bayesian phylogenetic tree obtained from *matK - trnL intron* combined data set. Numbers above branches indicate posterior probabilities obtained from Bayesian analysis and numbers below branches indicate bootstrap values obtained from Maximum Parsimony analysis. Fj – Fiji, Hw – Hawaii, KI - Kermadec Is, LHI - Lord Howe Is, NZ - New Zealand, Rt – Rarotonga, Ru – Rurutu, SA – South Africa, Th – Tahiti.

2.5 Discussion

This study is the first phylogenetic analysis of the genus *Metrosideros* using ETS and ITS sequences. Combining ETS and ITS data gave a better resolved and more strongly supported tree than the tree obtained with ITS data alone. Similar observations have been reported in other studies (e.g., Clevinger & Panero, 2000; Li *et al*., 2005; Li *et al*., 2007; Jiménez-Mejías *et al*., 2016). The enhanced phylogenetic resolution using the ETS region can be explained by the fact that this region evolves more rapidly and contains more informative sites than the ITS region (Bena *et al*., 1998; Vander, 2003).

Table 2.5 shows that the ETS data had a higher percentage of parsimony informative characters, more variable characters and higher mean sequence divergence than the ITS data. Other studies on molecular phylogeny of angiosperms using the ETS sequence have also showed that the ETS region has a faster evolutionary rate and a higher percentage of informative characters than the ITS sequence (e.g., Tronchet *et al*., 2005; Masuda *et al*., 2009; Poczai and Hyvönen, 2010; Logacheva *et al*., 2010). Hence, although the ETS sequence used has a shorter sequence length than the ITS sequence, it provides more informative variation for phylogenetic resolution, and so should be considered as a valuable marker for future phylogenetic studies of *Metrosideros*.

2.5.1 Phylogenetic analysis of the genus *Metrosideros*

2.5.1.1 Phylogeny of the subgenus *Metrosideros*

Trees from both the ITS and ETS data sets (Figures 2.9 & 2.10) suggest that the subgenus *Metrosideros* is monophyletic (Clade A in both trees); all members fall into one large strongly supported clade with strong bootstrap support. In contrast, the subgenus *Mearnsia* is polyphyletic; it comprises several smaller monophyletic clades. Subgenus *Metrosideros* comprises species from various islands (New Zealand, Hawaii, Lord Howe Island, Tahiti, Rarotonga, Rurutu, and Marquesas) with few nucleotide differences.

Table 2.6 shows that the genetic heterogeneity (mean sequence divergence) in subgenus *Mearnsia* (0.047) is three times that in subgenus *Metrosideros* (0.016), which suggests that subgenus *Metrosideros* is genetically more homogeneous and may have evolved later than subgenus *Mearnsia.*The fact that subgenus *Mearnsia* is closer to the root of the tree than subgenus *Metrosideros* also supports this conclusion. The resemblance of the oldest (early

Miocene) leaf and fruit fossil records to current *Mearnsia* species (Pole, 2008; Lee *et al*., 2012) also suggests the early evolution of the subgenus.

The ITS tree (Figure 2.9) shows that species of subgenus *Metrosideros* are clustered in two monophyletic clades (A1 and A3) and one poorly resolved group (A2), which the combined data tree strongly supported as a monophyletic group mainly composed of *M. collina* (clade A3 in Figure 2.10). Members of the *M. collina* group are found from Vanuatu in the southwest to French Polynesia in the east. Smith (1973) recognized three varieties (*M. collina var. collina, M. collina var. villosa* and *M. collina var. furticosa*), which are still currently accepted. However, the combined data tree (Figure 2.10) indicates that *M. collina* is not a monophyletic group in Oceania. Rather, the group forms two distinct clades (A2 and A3) separated by an interposed basal New Zealand species (*M. bartlettii*). This suggests at least two independent dispersal events from New Zealand occurred: one from New Zealand to Samoa and Melanesia (Vanuatu and Fiji) giving rise to clade A3, and the second from New Zealand to Polynesia giving rise to clade A2.

Comparison of ITS sequences of *M. collina* in clades A2 and A3 show that clade A2 is more closely related to *M. excelsa* (their closest New Zealand relative) than clade A3, which suggests the dispersal from New Zealand to Polynesia must have occurred later than the dispersal from New Zealand to Melansia and Samoa. The position of the *M. collina* group in clade A3 (Figure 2.10) suggests that this group evolved from an extinct New Zealand ancestor that was transitional between *M. umbellata* and *M. bartlettii*.

The presence of one variety (*M. collina var. collina*) in these two distantly related clades is particularly interesting. It is unlikely that two identical varieties of *M. collina* would fall in two different clades (A2 and A3), while other varieties of this species from different regions fall into the same clade (e.g., *M. collina var. collina* and *M. collina var. villosa* belong to A2*; M. collina var. collina* and *M. collina var. furticosa* belong to A3). On the other hand, the strongly supported monophyletic clade A3 (Figure 2.10) appears to have an equal rank to all other species of the subgenus combined. It is therefore possible that the several *M. collina var. collina* varieties in clades A2 and A3 have been incorrectly given the same name. In other words, one or more new varieties or species may not yet be recognized in the current classification of this group. The taxonomic uncertainty of the *M. collina* group is also reflected in the confused names and identities of cultivars of this group grown in New Zealand (Dawson *et al*., 2010b).

The other monophyletic clade within subgenus *Metrosideros*, A3 (Figure 2.9), consists of all New Caledonian species and four other species from widely dispersed locations (*M. umbellata* of New Zealand, *M. ochrantha* of Fiji, *M. salomonensis* of Solomon Islands and *M. boninensis* of the Bonin Islands). The ITS sequences showed that the three species from Fiji, the Solomon Islands, and the Bonin Islands are all closer to all species of New Caledonia in clade A3 than they are to *M. umbellata* of New Zealand. Hence, the grouping of these three species with the New Caledonian species might suggest their evolution from an ancestral New Caledonian species. In other words, New Caledonia might be a landmass of origin for some species within subgenus *Metrosideros*, although New Zealand may still be the initial origin of diversification and dispersal for the subgenus (Wilson, 1996).

The third monophyletic clade within subgenus *Metrosideros*, A1 (Figure 2.9), consists of species from Lord Howe Island, Hawaii and various Polynesian islands. All these species are either identical to or have little sequence difference to their closest New Zealand relative (*M. excelsa*). This also supports the recent evolution of this group following dispersal of an ancestral New Zealand species. All the Hawaiian species are included in this group, and although they are known for their significant morphological variation, they have identical ITS sequences (Figure 2.9) and very little sequence difference in the combined data (Figure 2.10). A detailed analysis of the Hawaiian group is given below.

The Hawaiian *Metrosideros* group

The Hawaiian Islands are home to five endemic species of *Metrosideros* that are readily distinguished from one another by their leaf characteristics (Wanger *et al*., 1999). One of these species, *M. polymorpha*, is the most dominant and variable species with five varieties scattered over seven of Hawaii's ten climatic zones (Dawson & Stemmermann, 1999). The varieties are based on morphological variation including stature, flower colour, seed size, leaf size and leaf pubescence (Corn, 1972; Dawson & Stemmermann, 1999).

Despite the large morphological differences among species and varieties of Hawaiian *Metrosideros*, I found very few genetic differences among species and varieties, a pattern that has been reported for other Hawaiian plant and animal species. For instance, the 19 species and eight subspecies of Hawaiian *Bidens* exhibit little genetic differentiation for isozyme loci although there is substantial morphological and ecological differentiation (Helenurm &

Ganders, 1985). Similarly, Hawaiian *Drosophila* species show great variation in morphology and behaviour yet exhibit relatively little cytogenetic differentiation (Carson *et a1*., 1970).

Previous studies also found little genetic divergence among species and varieties of Hawaiian *Metrosideros* (e.g., Aradhya *et al*., 1991, 1993; Treseder & Vitousek, 2001; James *et al*., 2004; Percy *et al*., 2008; Harbaugh *et al*., 2009b). These findings led some authors (e.g., James *et al*., 2004; Harbaugh *et al*., 2009b) to question whether the taxa are different species or varieties. Based on microsatellite analysis, Harbaugh *et al*. (2009b) found no genetic structure among Hawaiian *Metrosideros* species by taxonomy or geography, which they interpreted as evidence that the taxa are varieties of a single hypervariable species (*M. polymorpha*) rather than independent species. On the other hand, cluster analysis of AFLP data by James *et al*. (2004) showed a high degree of overlapping of different varieties of *M. polymorpha* and found a continuum of morphologies, including morphotypes that were intermediate between the different varieties. These two observations led the authors to question the recognition of varieties as distinct taxa.

Aradhya *et al.* (1991) also showed through an isozyme diversity analysis that in spite of distinct morphological differences, *M. rugosa, M. tremuloides,* and *M. polymorpha* did not have the anticipated level of gene divergence. The authors suggested that the morphological variability among populations may represent recent and incipient speciation. Isozyme diversity analysis of *M. polymorpha* varieties also showed little genetic variation despite large morphological differences (Aradhya *et al*., 1993; Treseder & Vitousek, 2001).

In contrast, other studies have found significant gene divergence among the species and varieties of Hawaiian *Metrosideros*. For instance, a microsatellite analysis by DeBoer and Stacy (2013) showed significant genetic differentiation among the three varieties of *M. polymorpha* (*M. polymorpha* var. *incana*, *M. polymorpha* var. *glaberrima*, and *M. polymorpha* var. *polymorpha*). Another microsatellite-based analysis of 23 populations of *M. polymorpha* covering the full range of all varieties showed the genetic clustering of trees by variety rather than by location (Stacy *et al*., 2014). The F_{st} values (measure of population genetic differentiation) for pairs of populations in the same variety were lower than F_{st} values for pairs of populations in different varieties regardless of geographic distance between populations. Moreover, the genetic cohesion within each variety was stronger than the homogenization of different varieties by hybridisation in the overlapping zones. The authors concluded that Hawaiian varieties of *M. polymorpha* are distinct evolutionary units that should be recognized as they are currently classified.

The contrasting findings described above could be explained by the type of molecular markers used and the sampling intensity of the studies. For instance, Percy *et al*. (2008) used chloroplast markers, and the lack of haplotype diversity among *M. polymorpha* varieties could be due to hybridisation that can lead to homogenization of the sampled trees through chloroplast capture. Or simply, the taxon might have radiated faster than the cpDNA evolves. On the other hand, the small genetic variation detected among the three species (*M. rugosa, M. tremuloides* and *M. polymorpha*; Aradhya *et al.*, 1991) and among varieties of *M. polymorpha* (Aradhya *et al*., 1993; Treseder & Vitousek, 2001) could be explained by the fact that isozyme markers are not as informative as microsatellites.

Isozymes are codominant markers that represent different forms of an enzyme coded by alternative alleles at a given locus (Markert & Moller, 1959). Most previous investigations of genetic variation within plant species have been based on the analysis of isozymes, and several papers are repeatedly cited (Hamrick, 1983; Hamrick & Godt, 1990). However, although isozyme loci are relatively easy to screen and have value for estimating gene frequencies and heterozygosity, they represent a limited number of coding regions (Schaal *et al*., 1991; Schlötterer, 2004). Moreover, coding regions are conserved regions and hence reveal a much lower genetic variation than neutral markers that are not under selection pressure. The number of alleles per locus and the percentage of polymorphic loci are also lower for isozymes than for other codominant markers such as microsatellites (Butcher *et al*., 1998).

Another explanation for the lack of genetic variation among species and varieties could be the use of insufficient sample sizes. For instance, Percy *et al*. (2008) sampled 97 trees on five islands, and 80 of these were *M. polymorpha*. Each of the other four species (*M. macropus*, *M. rugosa, M. tremuloides* and *M. waialealae*) was represented by fewer than 6 trees. In contrast, Stacy *et al*. (2014) sampled 404 trees from 23 populations, ensuring that each of the five varieties of *M. polymorpha* was represented by trees from all geographical ranges.

In summary, the extremely low genetic divergence observed in the present study among Hawaiian *Metrosideros* species in contrast to their high morphological divergence may be explained by one or a combination of the following factors:

1- ITS and ETS molecular markers are not as powerful as microsatellite markers in revealing the genetic differences between species and varieties. Moreover, the ITS and ETS regions are neutral markers whose sequence divergence may not positively correlate

with the level of divergence in protein-coding genes that cause morphological differences. Although the strength of correlation between neutral and adaptive genetic variation is debatable, multiple studies and reviews have showed that the correlation is weak (e.g., Reed & Frankham, 2001; Holderegger *et al*., 2006; Whitlock, 2014).

2- Epigenetic processes such as DNA methylation and histone protein modifications can be stimulated by environmental conditions, thereby affecting phenotypes through a permanent change in gene expression without changing the genes themselves (Duncan *et al*., 2014). Such processes can cause phenotypic plasticity, which is the expression of different phenotypes in response to varying environmental cues (Pigliucci *et al*., 2006). Thus, the morphological differences among varieties of Hawaiian *Metrosideros* might be induced by environmental changes rather than genetic differences. Earlier studies (Corn & Hiesey, 1973; Stemmermann, 1983; Joel *et al*., 1994) showed that morphological differences among *M. polymorpha* varieties are strongly influenced by environmental conditions such as elevation, rainfall and temperature. Cordell *et al.* (1998) similarly showed through an analysis of physiological and morphological variation in *M. polymorpha* that several characteristics, including anatomical features, were mainly induced by the environment and not genes. However, this same study also showed that traits that are used to identify *M. polymorpha* varieties (leaf size, leaf margin, leaf pubescence, and lengths of petiole and internode) appeared to be controlled by genes. Moreover, the occurrence of different morphotypes growing side by side in the same environment (Percy *et al.*, 2008) makes phenotypic plasticity a less likely explanation. However, further studies such as those of Stacy *et al*. (2016) and Morrison & Stacy (2014) are required to fully understand the heritability of morphological traits in Hawaiian *Metrosideros* species.

3- The large morphological differences among the Hawaiian *Metrosideros* species and varieties may represent a recent adaptive radiation (speciation) that involved few coding genes experiencing strong selection in the environmentally heterogeneous islands of Hawaii. Environmental heterogeneity is important for the divergence of species and varieties (Gonzalez-Martinez *et al*., 2006; Savolainen *et al*., 2007). In such cases, the taxa might show big morphological differences for a few coding regions, while most portions of their genomes remain similar or identical. Moreover, distinctive features such as leaf morphology could be controlled by a few genes (Hilu, 1983; Gottlieb, 1984; Crawford *et al*., 1987), and so small genetic differences could result in large morphological differences. In addition, a recent speciation would mean small genetic differences are

expected among species. Aradhya *et al.* (1991) suggested that the small isozyme diversity among *M. rugosa, M. tremulodies,* and *M. polymorpha* might be the result of recent and incipient speciation.

When facing a new environment, plant populations can show rapid local adaptation even in the presence of gene flow (Petit & Hampe, 2006). The Hawaiian Islands are well-known for adaptive radiations of plants and animals (Knope *et al*., 2012). For example, the silversword alliance (Asteraceae) is a classic example of adaptive radiation, where a single common ancestor from North America has given rise to more than 30 species in a wide array of habitats (Baldwin & Sanderson, 1998; Friar *et al*., 2006; McGlaughlin & Friar, 2011). The genus *Bidens* (Asteraceae) similarly includes 19 Hawaiian species that resulted from an adaptive radiation of a single ancestral species likely from North America, Mexico or Central America (Knope *et al*., 2012; Kimball & Crawford, 2004). The genus *lobelia* (Campanulaceae) is a third well-known example, with 23 Hawaiian species resulting from adaptive radiation of an ancestral species from Polynesia, Africa or Southern Asia (Givnish *et al*., 2009).

Adaptive radiation has also been documented in several Hawaiian bird and insect species (e.g., *Drosophila* in Craddock & Kambysellis, 1997; honeycreepers in Fleischer *et al*., 1998; spiders in Gillespie, 2004), which may have led to co-adaptation and co-diversification with host Hawaiian *Metrosideros* species. On the other hand, the diversification of *Metrosideros* could likewise have stimulated the diversification of Hawaiian honeycreepers and other endemic insect groups that depend on this plant for food (Percy *et al*., 2008).

Generally, the Hawaiian *Meterosideros* group might be the result of recent adaptive radiation of an ancestral species. The small genetic differences within the group can be explained by one or a combination of the above listed factors.

2.5.1.2 Phylogeny of the subgenus *Mearnsia*

The current classification based on Dawson (1976) suggests that the subgenus *Mearnsia* should be further divided into sections and subsections (Table 2.7). Figures 2.9 and 2.10 show that *Mearnsia*, unlike subgenus *Metrosideros*, is a heterogeneous group that is not monophyletic. Even species within each of the two major sections of this subgenus (*Mearnsia* and *Calyptropetala*) do not form monophyletic groups. Wright *et al*. (2000b) also thought that *Mearnisa* may not be monophyletic.

However, the phylogenetic relationships within subgenus *Mearnsia* remain poorly resolved in the current study. For instance, the closest relatives of the South African taxon (*M. angustifolia*) and the New Zealand taxon (*M. albiflora)* are not identified in this study. Also, the phylogenetic realtionships among subsections of *Mearnsia* remain unresolved. The subsections appear to have equal rank to subgenus *Metrosideros*. It seems that the subgenus is so highly divergent that several coding and non-coding regions might be required to fully resolve it. Alternatively, it may be appropriate for the genus *Metrosideros* to be split into several smaller genera, as suggested by Briggs and Johnson (1979).

The genetic heterogeneity of *Mearnisa*, as shown by its mean sequence divergence (0.047; Table 2.6), seems to be reflected in the morphology of the group. The species in this subgenus have a wide range of growth forms, including small trees, shrubs, epiphytes, hemi-epiphytes and vines. The group's genetic heterogeneity, as well as their proximity to the outgroup species *C. floribunda* in both phylogenetic trees, may suggest that *Mearnsia* species evolved earlier than species in subgenus *Metrosideros.*

Within subgenus *Mearnsia*, the grouping of species in clade B (Figure 2.10) has strong support (BS 92%, PP 1.00), which agrees with Dawson's (1976) classification of these species under sect. *Mearnsia* subsect. *Crassiviens* (Table 2.7). This study also strongly supports that the New Zealand species (*M. parkinsonii*) in this subsection is sister to the New Caledonian species of this group. This might suggest that the New Caledonian group evolved from a New Zealand ancestral species, supporting Wilson's (1996) hypothesis that *Mearnsia*, like subgenus *Metrosideros*, originated in New Zealand. This hypothesis is also supported by the presence in New Zealand of old (early Miocene) leaf and fruit fossils that resemble *Mearnsia* (Pole, 2008; Lee *et al*., 2012), while such fossils are lacking in New Caledonia.

The grouping of species in clade D (Figure 2.10) also has strong support (BS 100%, PP 1.00) and agrees with Dawson's (1976) classification of these species under sect. *Calyptropetala* subsect. *inclusis* (Table 2.7).

Table 2.7 Classification of species within subgenus *Mearnsia* (adapted from Dawson, 1976).

Section *Mearnsia*		**Section *Calyptropetala***	
Distinguishing features: Petals persistent, seeds release partly through openings in the lower part of the hypanthium		**Distinguishing features:** petals are caduceus (not persistent), seeds release entirely through free distal part of capsule	
Subsect. *Crassiviens*	**Distinguishing feature:** hypanthium does not separate into three valves	**Subsect. *Exsertis***	**Distinguishing feature:** terminal flowers are ebracteolate, capsule exserted
	Member species: *M. brevistylis_NC* *M. longipetiolata_NC* *M. whitakeri_NC* *M. dolichandra_NC* *M. porphyrea_NC* *M. halconensis_Philip* *M. n. sp. (unnamed)(Solomon Is)* *M. whiteana_Papua* *M. ovata_Papua* *M. cordata_Papua* *M. ramiflora_Papua* *M. parkinsonii_NZ* *M. fulgens_NZ*		**Member species:** *M. perforata_NZ*
Subsect. *Trivalvis*	**Distinguishing feature:** hypanthium separates into three valves	**Subsect. *Inclusis***	**Distinguishing feature:** terminal flowers are bracteolate, capsule included
	Member species: *M. albiflora_NZ* *M. colenosi_NZ* *M. diffusa_NZ* *M. carminea_NZ*		**Member species:** *M. patens*_NC *M. cacuminum*_NC *M. operculata*_NC *M. panensis*_NC *M. rotundifola*_NC

The third strongly supported (BS 89%, PP 1.00) group in this subgenus is clade C (Figure 2.10), which contains species from Philippines (*M. halconenesis*), Solomon Islands (*M*. n. sp. unnamed), and four species endemic to New Guinea (*M. whiteana, M. ovata, M. cordata and M. ramiflora*). The grouping of these equatorial Pacific species reflects the taxonomic classification by Dawson (1976) of all six species in sect. *Mearnsia* subsect. *Crassivenis* (Table 2.7).

The grouping of the species in clade C with five species from New Caledonia (clade B) within sect. *Mearnsia* subsect. *Crassiviens* might suggest the evolution of species in the Philippines, Solomon Islands and New Guinea from an ancestral New Caledonian species.

However, this taxonomic grouping is not supported by the phylogenetic analysis, which strongly supports clades B and C as two distinct groups rather than as one monophyletic group. Future research should be done with multiple nuclear and plastid markers to determine if these two clades have enough genetic cohesion to remain as a monophyletic clade, which would strengthen the argument that the equatorial Pacific species evolved from a New Caledonian ancestral species. Such research would also help to resolve the phylogenetic relationships within subgenus *Mearnsia* that remain poorly resolved in the current analysis.

Clade E in Figure 2.10 consists entirely of New Zealand species: three from sect. *Mearnsia* subsect. *Trivalvis* (*M. colenosi, M. diffusa,* and *M. carmínea*), one from sect. *Mearnsia* subsect. *Crassiviens* (*M. fulgens*), and another from a sect. *Calyptropetala* (*M. perforata*). This grouping had moderate support in the combined data set (BS 75%, PP 0.94). The grouping of *M. colenosi, M. diffusa,* and *M. carmínea* supports the current classification (sect. *Mearnsia* subsect. *Trivalvis*). However, the absence of *M. albiflora* from this group and the association of *M. fulgens* (from a different subsection) and *M. perforata* (from a different section) with this group might necessitate the reconsideration of sect. *Mearnsia* subsect. *Trivalvis*. *M. albiflora* of New Zealand remains unresolved in all analyses, and *M. perforata* of New Zealand groups with *M. carminea* with strong support. The presence of *M. perforata* and *M. fulgens* in this clade also means their current classification might require reconsideration.

The closest relative of *M. angustifolia* is unknown in the present study, but both phylogenetic trees show that this South African taxon has a closer proximity to subgenus *Mearnsia* than to subgenus *Metrosideros*. This supports Dawson's (1975) classification of this species in *Mearnsia*, based on its branching habit, vegetative bud form, inflorescence position, and capsular structure.

2.5.1.3 Phylogeny of the subgenus *Carpolepis*

The subgenus *Carpolepis*, which is endemic to New Caledonia, was first recognized by Dawson (1976), who later revised his classification to give it generic rank because of its bright yellow flowers, sympodial growth, and winged seeds (Dawson, 1984). Wright *et al*. (2000b) suggested that *Carpolepis* should be reverted to its subgenus status.

The ITS phylogenetic tree (Figure 2.9, clade B) strongly supported *Carpolepis* as a monophyletic group (BS 97%, PP 1.00) with a weak association with the New Caledonian group (sect. *Mearnsia* subsect. *Crassiviens*). The Maximum Parsimony tree (not shown) also strongly supported that subgenus *Carpolepis* is a monophyletic group (BS 96%) but shows that it has absolutely no association with any group of subgenus *Metrosideros* or subgenus *Mearnsia.* Instead, *Carpolepis* appear to have an equal rank with subgenus *Metrosideros*. Although Wilson (1996) mentioned the association of *Carpolepis* with subgenus *Metrosideros*, this is not supported by the phylogenetic tree, which suggests that the current recognition of this group as a separate subgenus is appropriate. However, the association of subgenus *Carpolepis* with the New Caledonian group (sect. *Mearnsia* subsect. *Crassiviens*), even if it is weak, should be tested further using several molecular makers. If a stronger association is detected, the recognition of *Carpolepis* as a subsection of *Mearnsia* may be more appropriate than recognising it as a subgenus of *Metrosideros*.

2.5.1.4 Phylogenetic analysis of chloroplast data

Figure 2.12 shows that the *trnL intron* chloroplast region did not resolve the group well. It seems this region is highly conserved with little or no variation among *Metrosideros* species. The Maximum Parsimony analysis of this region also gave the same topology with poor resolution. Although the *trnL intron* is widely used in phylogeny because of its good universal primers, this region is not the most variable non-coding chloroplast region (Shaw *et al*., 2005). In contrast, the *matK* region gave a much better resolved tree (Figure 2.11), which is unsurprising given its high rates of transversion mutation and substitution compared to other chloroplast genes (Plamer *et al.,* 1988).

The tree based on the combined data set (Figure 2.13) resolved the group better than the *trnL intron* region but worse than the *matK* region. For instance, the combined data split *M. fulgen* from *M. diffusa* to pair with *M. collina* of Rarotonga, and split the three species (*M. umbellata*, *M. bartlettii* and *M. collina var. fruticosa*) that were grouped together in the *matK* tree (clade C, Figure 2.11). The inclusion of the *trnL intron* data must have decreased the resolving power of the *matK* data.

The *matK* region not only gave a better resolution but also corroborated with the nuclear data set. For instance, within subgenus *Metrosideros*, species in each of the monophyletic groups A1, B and C were also together within one big monophyletic group in the ITS and ITS-ETS

combined trees (clade A in Figure 2.9 & 2.10). This result is interesting because both the ITS and ITS-ETS combined trees placed the three species in clade C of the *matK* tree (*M.umbellata, M.bartlettii* and *M. collina var. fruticosa*) outside the monophyletic clade that contains the three species in clade B of the *matK* tree (*M. excelsa, M. robusta*, and *M. kermadecensis*). Another interesting result is that *M. collina* from Fiji (clade C) does not group with *M. collina* varieties from Rurutu, Rarotonga and Tahiti in the *matK* tree (clade A1). This supports the ITS-ETS combined tree which places *M. collina* varieties from Rurutu, Rarotonga and Tahiti in a separate group (clade A2) to *M. collina* from Fiji (clade A3).

Within subgenus *Mearnsia,* the *matK* data again support the nuclear data, grouping *M. diffusa, M. coleonsi* and *M. fulgens* together (clade A2). However, in the *matK* tree, *M. perforata* and *M. carminea* are not part of this group as they are in nuclear data. Both the *matK* tree and the ITS-ETS combined tree could not resolve the close relatives of *M. albiflora* and *M. angustifolia*. The *matK* data generally supports the results from the nuclear data, but *matK* also groups species from different subgenera. For example, in clade B of the *matK* tree, *M. carminea* belongs to subgenus *Mearnsia* while all other species in the clade belong to subgenus *Metrosideros*. Again, species from subgenus *Metrosideros* (clade A1) are grouped with species from subgenus *Mearnsia* (*M. parkinsonii* and *M. peforata* from clade A2) to form one bigger group (clade A).

The grouping of species from different subgenera may be the result of chloroplast capture through interspecific hybridisation among morphologically different species of *Metrosideros*, which leads to the sharing of chloroplast genomes. A large degree of chloroplast sharing due to hybridisation has been reported for several genera including *Pinus* (Matos & Schaal, 2000), *Quercus* (Belahbib *et al*., 2001) and *Eucalyptus* (Steane *et al*., 1998; McKinnon *et al*., 2001), which belongs to the same family (Myrtaceae) as *Metrosideros*.

Gardner *et al*. (2004) reported sharing of chloroplast haplotypes among five endemic species of subgenus *Metrosideros* in New Zealand (*M. excelsa, M. robusta, M. bartlettii, M. umbellata and M. kermadecensis*), and they suggested this sharing is due to frequent historical hybridisation and introgression that was initiated during Pleistocene glacial events. These events forced frost sensitive species such as *Metrosideros* to be confined to and coexist in a few ecologically suboptimal refuges in the North and South Islands. Simpson (2005) also suggested that natural hybridisation has probably been an important factor in the evolution of *Metrosideros*.

Although hybridisation can explain the grouping of *Metrosideros* species from different subgenera in the present study, it does not explain Gardner *et al*.'s (2004) results, since their study did not include species from subgenus *Mearnsia*. Dawson *et al.* (2010a) mentioned that no known hybrids are known to involve species from subgenus *Mearnsia*, whereas hybridisation is common among species within subgenus *Metrosideros*. Moreover, the herbarium species that belong to different subgenera in the *matK* tree were sourced from different locations, and therefore since they are not sympatric, hybridisation is unlikely.

However, although no evidence shows hybridisation between species in different subgenera in recent times, these species might have hybridized earlier in their lineage divergence when reproductive isolation mechanisms were not strong or fully evolved. Hence, the role of hybridisation in sharing haplotypes should not be completely ruled out. Hybridisation experiments between species in different subgenera could assess the possibility of gene flow and hybridisation among these species.

The sharing of haplotypes may also be caused by incomplete lineage sorting of the chloroplast genome rather than hybridisation. Incomplete lineage sorting is the persistence and retention of ancestral polymorphisms through speciation events (Avise, 1994). Some studies (e.g., Mason-Gamer *et al.,* 1995; Comes & Abbott, 2001; Jakob & Balttner, 2006) show that incomplete lineage sorting can contribute to phylogenetic incongruence, and as a result shared haplotypes can be found in species that do not share the same geographic location.

After two lineages inherit polymorphic genes from their polymorphic ancestral lineage, the two lineages may over time lose one or the alleles due to genetic drift or selection. Depending on which allele the lineages retain, two species in different lineages can end up with genomic segments that do not match their species tree. Figure 2.15 shows the scenario under which incomplete lineage sorting can lead to phylogenetic incongruence.

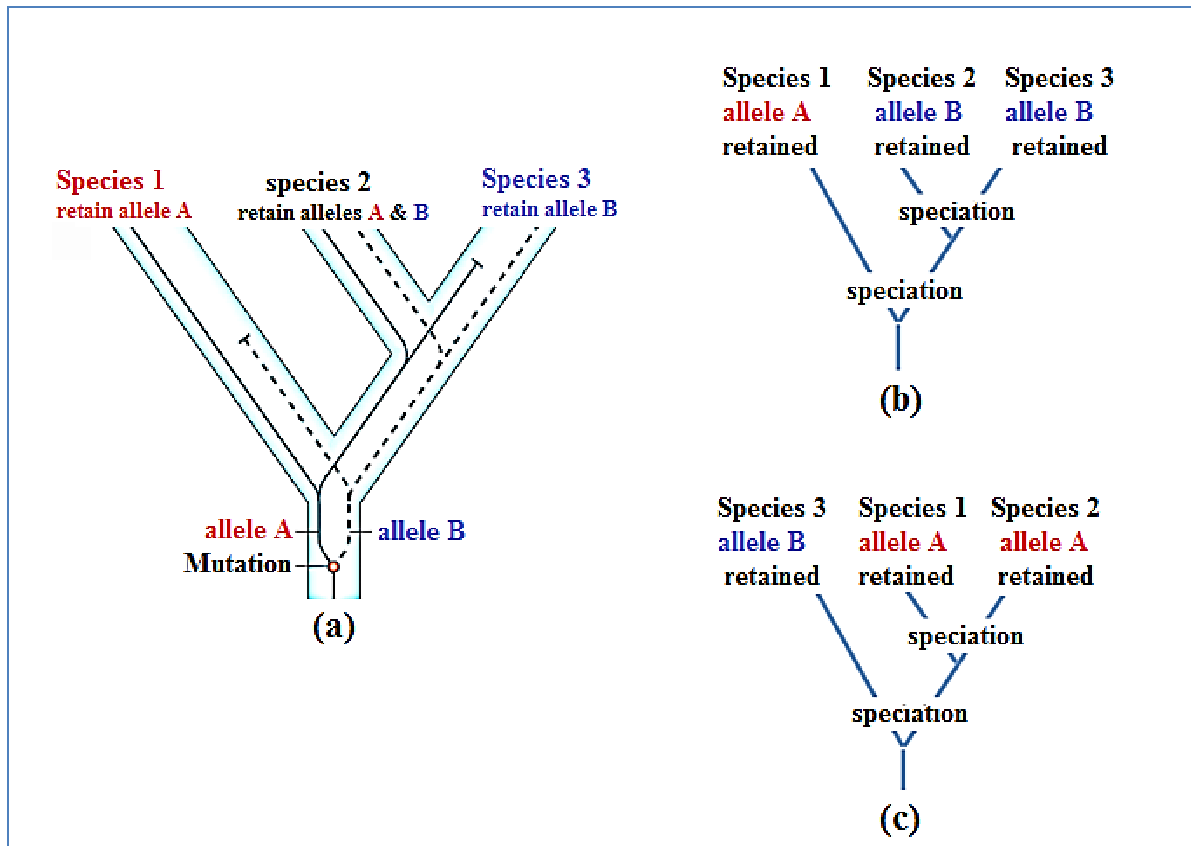

Figure 2.14 Incongruence between species tree and gene tree due to incomplete lineage sorting. (a) A species tree showing the evolution of a polymorphism (alleles A and B) into different species (species 1, 2, and 3). Over time, each lineage may experience different environments and lose either allele A or B due to genetic drift or selection. If species 2 retains allele B and loses allele A, then the gene tree will recover the species tree (b). If species 2 retains allele A and loses allele B, then incongruence between the species tree and gene tree will occur (c) (adapted from Rogers and Gibbs, 2014).

2.5.2 Dispersal routes

2.5.2.1 Dispersal routes for the subgenus *Metrosideros*

Fossil evidence suggests that New Zealand is the origin of the genus *Metrosideros* (Mildenhall, 1980; Pole, 1993; Pole, 2008) (but see Tarran *et al*., 2016 for claiming Australia as origin of *Metrosideros*). There are several possible dispersal routes the genus might have taken from New Zealand to the Pacific.

Both phylogenetic trees from ITS and ITS-ETS combined data show the high genetic similarity between *M. excelsa* of New Zealand and *M. kermadecensis* of the Kermadec Islands which are both in the same monophyletic group. Such grouping and high similarity suggest a recent dispersal event from New Zealand to these distant islands, as does the close grouping of New Zealand species (*M. robusta* and *M. excelsa*) with the species on Lord

Howe Island and Polynesian islands. The phylogenetic trees show that *M. excelsa* is the most likely species to have dispersed there, being the closest New Zealand relative to *Metrosideros* species on Lord Howe Island and Polynesian islands. Moreover, the abundance of *M. excelsa* on the coasts of northern New Zealand indicates that this species has the potential to colonise recent lava flows (Clarkson, 1990) and hence could have successfully colonized Pacific Islands with similar environmental conditions.

The extremely high similarity in nucleotide sequences between *M. excelsa* and Polynesian species also suggests the dispersal event took place recently. Based on the geologic ages of Rarotonga (1.1 to 2.3 my; Thompson *et al*., 1998) and Tahiti (0.62 to 1.2 my; Duncan & McDougall, 1976), Wright *et al*. (2000a) suggested a minimum mutation rate of one nucleotide substitution per 1-2 million years for ITS sequences. The ITS sequences of species in clade A1 (Figure 2.9) are either identical or differ by only one nucleotide from the ITS sequence of *M. excelsa*, which based on this rate would imply the dispersal of *Metrosideros* from New Zealand to Polynesian islands within the last couple of million years (i.e., as early as the Pleistocene).

Figure 2.10 (clade A1) shows strong support for all Hawaiian *Metrosideros* species and *M. collina* on the Marquesas Islands forming a monophyletic group. The combined data set shows one nucleotide ("T") that is shared only by Marquesan *M. collina* and all the Hawaiian taxa, while the corresponding positon in the rest of *Metrosideros* species is nucleotide "C". The nesting of this Marquesan species within the Hawaiian group rather than with *M. collina* from the geographically closer southeastern Polynesia (Rarotonga, Tahiti, Rurutu, and Samoa) is unexpected, especially since the Marquesas Islands are more than 3,000km from Hawaii with no intervening high islands between them. The Hawaiian Archipelago is one of the world's most isolated groups of islands, more than 2,600km from the nearest continent (Wieczorek *et al*., 2008; Harbaugh *et al*., 2009a).

The grouping of Marquesan *M. collina* with the Hawaiian *Metrosideros* group suggests that the dispersal of subgenus *Metrosideros* from New Zealand to Hawaii might have occurred through the Marquesas Islands as a stepping stone. The large distance between Hawaii and the Marquesas Islands makes multiple colonisations unlikely. A similar single dispersal event to Hawaii from a French Polynesian island was also reported for plant genus *Melicope* (Harbaugh *et al*., 2009a) based on ITS and chloroplast molecular phylogeny. The grouping of all Hawaiian species in one clade also could indicate the evolution of all Hawaiian species from a single dispersed seed from the Marquesas Islands. The weight of evidence also

suggests that a single colonization event by an ancestral species from the Marquesas Islands followed by an adaptive radiation on the Hawaiian Islands is the most likely scenario (Wright *et al*., 2000a; Wright *et al*., 2001; Percy *et al*., 2008).

M. collina may have reached the Marquesan Islands using other islands as stepping stones (Gilpin, 1980). *M. collina* occurs on all four eastern Polynesian islands (Rarotonga, Tahiti, Rurutu, and Samoa), which are located between New Zealand and the Marquesas Islands and might have served as a stepping stone for the dispersal of an ancestral species from New Zealand. Of the four Polynesian islands, Samoa is the least likely candidate for this, because *M. collina* here belongs to clade A3 (Figure 2.10), which is a genetically distinct group from the clade A2 (Figure 2.10) that contains *M. collina* from Rarotonga, Tahiti, and Rurutu. Both the ITS and combined trees (Figures 2.9 and 2.10) show that *M. collina* of the Marquesan Islands and *M. collina* of Rarotonga, Tahiti, and Rurutu belong to one monophyletic group that is genetically distinct from clade A2 with *M. collina* of Samoa. Rarotonga (1.1 to 2.3 million years old; Thompson *et al*., 1998) and Tahiti (0.62 to 1.2 million years old; Duncan & McDougall, 1976) are also unlikely to have been the stepping stone islands, as they are younger than the Marquesan Islands (six million years old; Bonneville *et al*., 2006). Hence, the most likely stepping island is Rurutu (part of the Austral archipelago) which is approximately 12 million years old (Percy *et al*., 2008).

The ITS sequence data show that *M. collina var. collina* from Vanuatu, *M. collina var. fruticosa* from Fiji and *M. collina var. collina* from Samoa are different from their closest New Zealand relative (*M. excelsa*) by six, seven, and eight nucleotide substitutions respectively. Based on Wright *et al*.'s (2000a) suggested minimum mutation rate of one nucleotide substitution per 1-2 million years for ITS sequences, the time gap between *M. excelsa* and the evolution of these three taxa would be 12, 14, and 16 million years, which suggests these dispersal events happened around the middle Miocene, long before the dispersal from New Zealand to the eastern Polynesian islands.

The ITS tree shows *M. umbellata* of New Zealand grouping with species of New Caledonia (clade A3), although this association has a weak support. However, interestingly, all subgenus *Metrosideros* species in New Caledonia and *M. umbellata* in New Zealand lack a six-base pair deletion (CACGCG in ITS sequence position 599 - 604) that is common in all other species from New Zealand (*M. robusta*, *M. excelsa* and *M. bartlettii*) and in most species distributed across the Pacific. This might suggest the dispersal of an ancestral New Zealand species to New Caledonia before a new lineage (with the six base pair deletion)

evolved within New Zealand and gave rise to most species in the Pacific with this deletion. This 6-bp deletion was also noted in Wright *et al*. (2000b). Because mutations are rare, it is more likely that an ancestral species had a single deletion event, rather than six independent events. The lack of fossil evidence from New Caledonia also strengthens the argument that New Zealand might be the origin of subgenus *Metrosideros*.

Despite all of this evidence that suggests New Zealand is the sole origin of genus *Metrosideros*, the grouping of *M. salomonensis* from the Solomon Islands, *M. ochranta* from Fiji and *M. boninensis* from the Bonin Islands with all the endemic species of New Caledonia (clade A3 in Figure 2.9) suggests that New Zealand may not be the origin of all species in subgenus *Metrosideros*; instead, the subgenus might have two origins (New Zealand and New Caledonia). This hypothesis might be strengthened if old fossils are discovered in New Caledonia. As I mentioned earlier, *M. salomonensis*, *M. ochranta* and *M. boninensis* are closer to all endemic species of New Caledonia than they are to *M. umbellata* of New Zealand within calde A3 which might suggest their origin from an ancestral New Caledonian species.

M. boninensis of the Bonin Islands and *M. ochrantha* of Fiji, despite being geographically separated by a great distance, are more closely related to each other than they are to any of the New Caledonian species. These two species share two nucleotides ("T" at position 120 and "C" at position 170) that are missing in all other species of *Metrosideros*. The strong support for the grouping of these two species (BS 89%, PP 1.00) might suggest a stepwise dispersal of an ancestral New Caledonian species to the relatively close Fiji and then to the more geographically distant Bonin Islands, as proposed by Wright *et al*. (2000a). These authors also suggest that extinct intervening islands or extant islands that no longer have subgenus *Metrosideros* species might have been involved in this long-distance dispersal route.

The ITS sequence data show that *M. ochranta* (Fiji), *M. boninensis* (Bonin Is.) and *M. salomonensis* (Solomon Is.) are different from their closest New Caledonian species (*M. nitidia*) by six, nine, and 12 nucleotide substitutions respectively. Given Wright *et al*.'s (2000a) minimum substitution rate of one nucleotide substitution per two million years, the dispersal of an ancestral species from New Caledonia to Fiji, the Bonin Islands and Solomon Islands probably occurred in the early to middle Miocene (24 to 12 million years ago).

In summary, the phylogenetic analyses suggest that the subgenus *Metrosideros* most likely originated in New Zealand and its subsequent dispersal led to the colonisation of New Caledonia and other Pacific islands as shown in Figure 2.15. The grouping of *M. umbellata* with all endemic species of New Caledonia and the fact that this species appears to be the sister taxon to other species of subgenus *Metrosideros* (see the ITS-ETS combined tree) suggests that *M. umbellata* could be the earliest diverged taxon in the subgenus *Metrosideros* that gave rise to the species of New Zealand and New Caledonia, which in turn dispersed and evolved to species of other Pacific Islands.

2.5.2.2 Dispersal routes for the subgenus *Mearnsia*

Interestingly, all New Caledonian species in clade B (Figure 2.10) have a strongly supported sister taxon from New Zealand (*M. parkinsonii*), which might suggest the dispersal of species within subgenus *Mearnsia* from New Zealand to New Caledonia. This is supported by the discovery of the oldest (early Miocene) fossil fruit in New Zealand, which shares similar characteristics with species of subgenus *Mearnsia* (Pole, 2008). Wilson (1996) also hypothesised New Zealand is the origin of both subgenera of *Metrosideros*.

On the other hand, the strongly supported grouping of *M. halconenesis* of the Philippines with species from the Solomon Islands and New Guinea (clade C in Figure 2.10) might suggest a dispersal route from either New Caledonia or New Zealand to the Solomon Islands and New Guinea, and then on to the more distant Philippines. Based on geographical proximity to New Guinea and the Solomon Islands, the source of the ancestral species is more likely New Caledonia than New Zealand. Generally, subgenus *Mearnsia* has more restricted dispersal compared with the widespread subgenus *Metrosideros*, as shown in Figure 2.15.

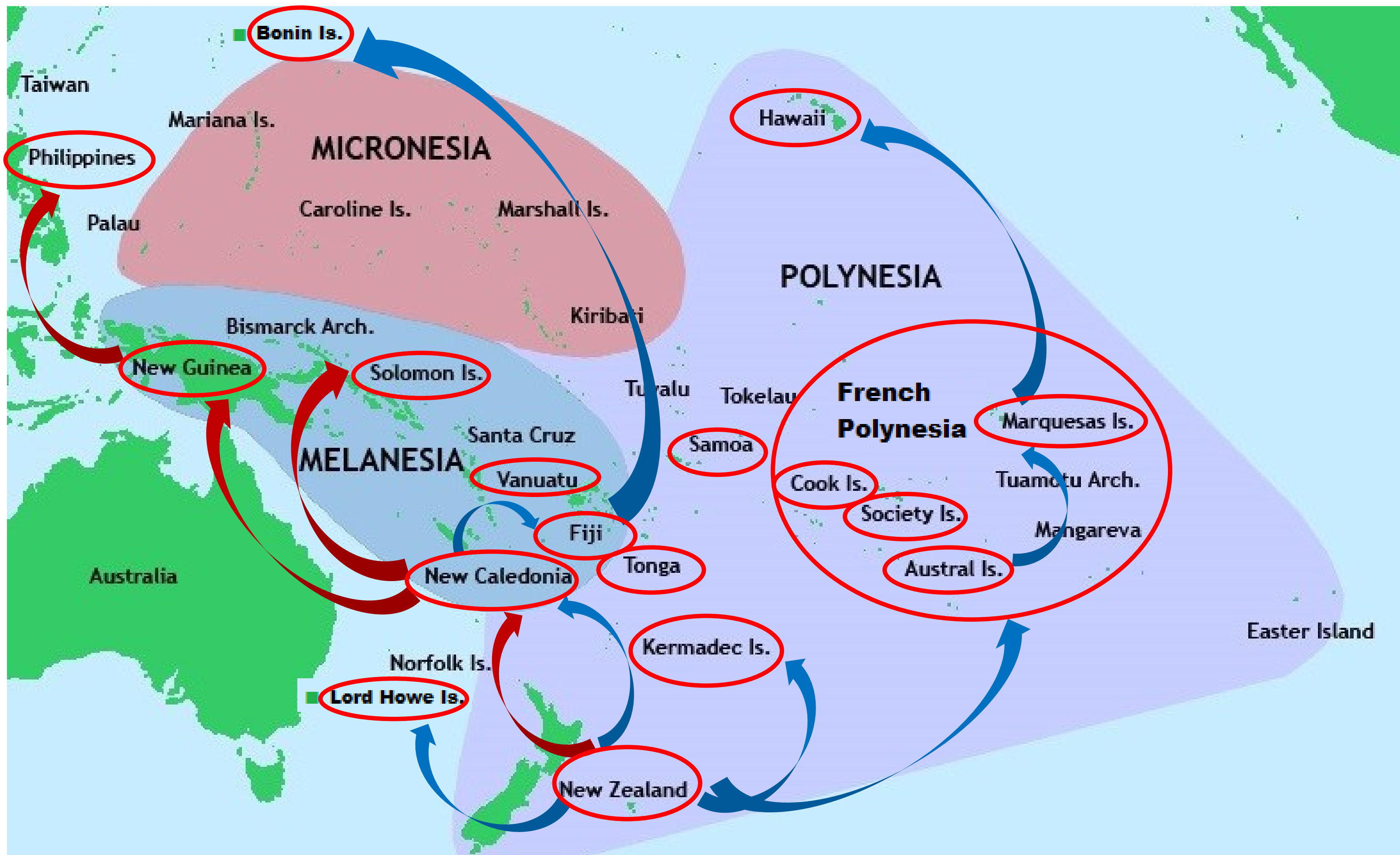

Figure 2.15 Dispersal route of subgenus *Mearnsia* (red arrows) and subgenus *Metrosideros* (blue arrows) inferred from the the present study.

2.5.2.3 Dispersal conclusions

Long-distance dispersal is a fundamental process that shapes the biogeography and evolution of fauna and flora of oceanic islands (Cowie & Holland, 2006). Molecular phylogeny studies have shown that the biogeography of New Zealand's flora has also been shaped by long-distance dispersal (e.g., Winkworth *et al*., 2002; Zhang & Renner 2003; Perrie & Brownsey, 2005; Perrie & Brownsey, 2007; Cantley *et al*., 2016). Because New Zealand was once part of the Gondwana supercontinent before it drifted away 80 million years ago, there have been uncertainties as to whether similar taxa occur in New Zealand and other Southern Hemisphere landmasses because of long-distance dispersal or simply because of Gondwanan connection and inheritance (McGlone *et al*., 2001; Winkworth *et al*., 2002). However, fossil evidence and phylogenetic studies have shown that transoceanic dispersal rather than Gondwanic inheritance is the major factor shaping the biogeography of species in New Zealand and elsewhere across the Pacific (Pole, 1994; McGlone *et al*., 2001; Winkworth *et al*., 2002; Perrie & Brownsey, 2007).

In genus *Metrosideros*, the presence of old fossil records in New Zealand while they are absent in other adjacent Gondwanana islands (but see Tarran *et al*., 2016 for Australian *Metrosideros* fossils) suggests that the presence of the genus across the Pacific is most likely due to long-distance dispersal events originating from New Zealand. Moreover, the young ages of oceanic islands (i.e., much younger than Gondwanaland) and the small genetic differences among species of subgenus *Metrosideros* suggest the recent arrival of these species on most of the oceanic islands, which could be explained by relatively recent dispersal events rather than by very old Gondwannan inheritance. This is very plausible given *Metrosideros* species are known for their efficient wind-mediated dispersal facilitated by light-weight seeds that are produced and released in abundance from dehiscent capsules (Wilson, 1996).

The previous discussions on the dispersal routes of the two subgenera indicate that they are most likely to have originated and diverged from New Zealand. While the initial radiation of species in subgenus *Metrosideros* probably happened in New Zealand, the radiation of species in subgenus *Mearnsia* might have happened both in New Zealand (seven species) and New Caledonia, which has the highest number of species (10) in subgenus *Mearnsia*.

The dispersal routes depicted in Figure 2.15 show that single dispersal events to remote islands are unlikely in most cases and therefore stepping-stones were probably important.

Other studies have also implied the importance of stepping-stone islands. For instance, the dispersal of alpine plants occurred from the Northern Hemisphere to New Zealand through New Guinea and Australia (Raven, 1973). Similarly, plants dispersed from New Zealand to South America through Antarctic islands (Swenson & Bremer, 1997; Renner *et al.*, 2000), and the genus *Astelia* originated from Australia, radiated in New Zealand and colonized western and eastern Pacific islands through stepping-stone islands (Birch & Keeley, 2013).The extremely species-rich genus *Cyrtandra* dispersed from Southeast Asia to various Pacific Islands via a northwest to southeast stepping-stone migration route (Cronk *et al.*, 2005).

The dispersal route shown in Figure 2.15 illustrates an important difference between the patterns of dispersal for the two *Metrosideros* subgenera from New Zealand. *Metrosideros* species show a widespread and mainly eastward transoceanic dispersal pattern, while *Mearnsia* species had a more restricted westward transoceanic dispersal from New Zealand.

The eastward transoceanic dispersal of subgenus *Metrosideros* could be attributed to the long-term dominance of west-wind drift, which has dictated the distribution of many plant species in Southern Hemisphere (Raven, 1973; Close *et al.*, 1978; Winkworth *et al.*, 2002). The west-wind drift is a weather phenomenon that causes the progressive movement of weather systems eastward at high southern altitudes (Winkworth *et al.*, 2002). The strongest west-wind drift is believed to have happened during the Pleistocene period (Stewart & Neall, 1984). Interestingly, the small genetic differences observed between species on several remote Polynesian islands and their closest New Zealand relative (*M. excelsa*) suggests the dispersal of the subgenus from New Zealand might have taken place as late as the Pleistocene.

Molecular phylogenetic studies have shown a similar eastward transoceanic dispersal pattern for several other endemic plant species in New Zealand. Examples include: the dispersal of genus *Astelia* from Australia to New Zealand and other Pacific Islands (Birch & Keeley, 2013); the dispersal of genus *Aciphylla* (Radford *et al.*, 2001) and *Asplenium hookerianum* (Shepherd *et al.*, 2009) from New Zealand to the Chatham Islands; the dispersal of genus *Sophora* sect. *Edwardsia* from New Zealand to South America and the Pacific Islands (Hurr *et al.*, 1999); the dispersal of *Coprosma oliveri* and *C. pyrifolia* from New Zealand to the Juan Fernandez Islands (Cantley *et al.*, 2016); and the dispersal of genus *Myosotis* (Winkworth *et al.*, 2002), genus *Coriaria* (Yokoyama *et al.*, 2000) and New Zealand's largest genus *Hebe* (Wagstaff & Garnock-Jones, 2000) from New Zealand to South America.

Unlike species in subgenus *Metrosideros*, species in subgenus *Mearnsia* showed a westward dispersal from New Zealand. Tree topologies show that *Mearnsia* species are closer to the roots of the trees than species of subgenus *Metrosideros*, suggesting that they evolved earlier. The discovery of an early Miocene fossil with similar characteristics to *Mearnsia* species (Pole, 2008) also indicates this subgenus was present in New Zealand well before the Pleistocene period. Hence, it is likely that *Mearnsia* species dispersed before the effect of west-wind drifts intensified during the Pleistocene period (Winkworth *et al*., 2002). Moreover, due to tectonic processes along the oceanic ridges of northern New Zealand, both climatic and landscape conditions were suitable for dispersal of species from New Zealand to New Caledonia during the late Oligocene through early Pliocene (McGlone *et al*., 2001). Hence, it is possible that *Mearnsia* species dispersed from New Zealand to New Caledonia during this period and later to other islands where the subgenus is currently represented (Solomon Islands, New Guinea and the Philippines).

Wilson (1996) attributed the widespread distribution of subgenus *Metrosideros* and the limited range of subgenus *Mearnsia* to the differing nature of their seed capsules. Most species in subgenus *Metrosideros* have exerted capsules projecting from the hypanthium and therefore seeds are released entirely through the free part of the capsule, making their release and dispersal very easy. On the other hand, *Mearnsia* species have fruits with enclosed capsules, and therefore seeds have to be released through small slits or irregular openings in the fruiting hypanthium. This makes the release and long-distance dispersal of seeds to remote Polynesian islands less likely.

This explanation is supported by Dawson (1970)'s floral descriptions that suggest all subgenus *Mearnsia* species in New Guinea, the Solomon Islands, the Philippines, and two species in New Caledonia (*M. porphyrea* and *M. dolichandra*) have fruits with included capsules that release seeds often through longitudinal slits or irregular openings in the fused capsule-hypanthum tissues. However, this explanation has limitations, since species in subgenus *Carpolepis* have exerted capsules but are restricted only in New Caledonia (Dawson, 1972a). Moreover, *M. perforata* and *M. operculata* belong to subgenus *Mearnsia* but have exerted capsules (Dawson 1972b) and are restricted to New Zealand and New Caledonia, respectively. Hence, the distribution of the two subgenera of *Metrosideros* is better explained by the effect of west-wind drift than by the nature of seed-releasing capsules.

The absence of *Metrosideros* species from Australia is surprising given its close proximity to New Zealand. However, from the Miocene onwards, Australia became more arid while New

Zealand's climate continued to cool until around the early Pleistocene (McGlone *et al*., 2001). Hence, the absence of *Metrosideros* in Australia could be due to unfavourable environmental conditions that might have caused the extinction of ancestral species from New Zealand. This is a plausible explanation since long-distance dispersal of most vascular plants to and from New Zealand occurred between the late Miocene and early Pleistocene (McGlone *et al*., 2001), the period that coincides with the development of sharp climatic differences between Australia and New Zealand. Moreover, the recent report of fruit and flower fossils of *Metrosideros* in Australia (Tarran *et al*., 2016) suggests that the absence of *Metrosideros* in Australia must have been due to extinction of the genus rather than the absence of dispersal. As mentioned, Tarran *et al*. (2016) claim the Australian fossil represents the oldest record of *Metrosideros*, which suggests Australia rather than New Zealand could be the origin of the genus. This debate will likely continue as new fossil evidence emerges in the future.

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Chapter 3: Genetic diversity and structure of *Metrosideros bartlettii* as revealed by AFLP and SSR markers

Abstract

Metrosideros bartlettii (Bartlett´s rata) is an IUCN Critically Endangered New Zealand endemic. The species is now confined to only three small populations in the far north of New Zealand. The genetic diversity and genetic structure of the populations were analysed using AFLP and SSR markers. Both markers revealed that the genetic diversity of *M. bartlettii* was low (AFLP: H_e = 0.17, SSR: H_e = 0.48) compared with both its closest relative (*M. excelsa*) and the average reported value for long-lived, outcrossing, and wind-dispersed species. This low genetic diversity may be the result of genetic drift and a demographic decline due to a combination of factors such as past Quaternary glaciation events, forest destruction, fire, land-use change and possum browsing (currently the major threat to the species). Cultivated samples from various locations showed a similar level of genetic diversity but with a very low number of private alleles (two) compared with the wild populations (11). The genetic structure analyses showed that, despite the proximity of the populations to each other and the extensive dispersal capacity of this species, populations had significant genetic differentiation (AFLP: F_{st} = 0.22, SSR: F_{st} = 0.20). This suggests limited gene flow, possibly due to the low population density, which is unlikely to produce adequate seeds to attract pollinating birds and insects. The implications of these results for conservation of the species are discussed in detail.

3.1 Introduction

3.1.1 Description of *Metrosideros bartlettii*

Metrosideros bartlettii (Figure 3.1) is an extremely rare and IUCN critically endangered tree species endemic to New Zealand (de Lange *et al*., 1999; de Lange, 2014). It is commonly called Bartlett's rata after John Bartlett, who discovered it in 1975 in Radar Bush, 9.5km southeast of Cape Reinga (Dawson, 1985).

A

B

Figure 3.1 *Metrosideros bartlettii* (A) adult tree and (B) new epiphytic growth. (Photos by Jeremy Rolfe, Department of Conservation)

The species was discovered as a small, isolated population of 30 individuals at the northern tip of the North Island (Bergin & Hosking, 2006). Trees of *M. bartlettii* grow up to 20m by 6m wide and have a papery bark. They usually develop as perching plants in the canopy of the host tree. Roots are formed and eventually reach to the ground, coalescing into a single trunk, which in due course replaces the host (Dawson, 1985; Bergin & Hosking, 2006).

M. bartlettiii belongs to the subgenus *Metrosideros*, which also contains some of the best known and tallest growing species in New Zealand such as *M. excelsa* (paöhutukawa or New Zealand Christmas tree), *M. robusta* (northern rätä) and *M. umbellata* (southern rätä).

Chromosome Number

Like other species in the *Metrosideros* genus, *M. bartlettii* is diploid with chromosome number 2n =22 (Dawson, 1987).

Distribution

M. bartlettii now naturally occurs only in three forest remnants near Spirits Bay, Te Paki: Radar Bush, Kohuronaki and Unuwhao Bush (de Lange, 2003). These fragments are mostly on private land and isolated from other forest fragments (de Lange *et al*., 1999).

Habitat and Ecology

Metrosideros bartlettii is an emergent or canopy species of northern coastal and lowland broad-leaved forest. It is associated with stream sides or wetlands, which probably reflects land clearance patterns rather than a genuine preference for such habitats (de Lange, 2014). Trees generally prefer dense forest and begin life as epiphytes on other host tree species such as *Cyathea* fern species. Sometimes trees are also found growing on harsh surfaces such as rocks and cliffs (Dawson, 1985; de Lange, 2014).

Trees have a spongy bark which has a pale grey to white colour. Young leaves are glossy and yellow green which turn into dark green leaves with hairy margins when they mature. Unlike most species in genus *Metrosideros* which have red flowers, *M. bartlettii* has white flowers that bloom between October and November (Dawson, 1985; de Lange, 2003).

Cultivation

M. bartlettii is occasionally cultivated from fresh seeds. Seedlings can also be raised from hardwood cuttings, although rooting is very slow. Although cultivated plants can tolerate a variety of conditions including mild frost and snow, they perform best in open, sunny conditions and well-drained soils (de Lange, 2003). Unfortunately, most cultivated specimens come from a single tree (*pers comm* Dr Gary Houliston), which was confirmed by their identical genetic identity in the present cluster analysis.

3.1.2 Background and significance of the study

While the IUCN classifies *M. bartlettii* as a Critically Endangered Species (de Lange, 2014), the New Zealand Threat Classification System categorises it as 'Threatened - Nationally Critical' based on Townsend *et al.* (2008) criterion A (1) that the total number of mature individuals is < 250.

Historically, *M. bartlettii* may have gone through a bottleneck during the last glaciation (McGlone *et al*., 2001), but the resulting small populations have been further reduced by factors such as introduced browsing animals, land-use change and fire. Possum browsing has been responsible for the death of many trees on private land (de Lange, 2003; Simpson, 2005). Trees have also been removed to clear land for new buildings, to improve views, or during general landscaping alterations. Moreover, potential host tree species for *M. bartlettii* have also declined in abundance (www.projectcrimson.org.nz).

Because *M. bartlettii* is a multipurpose tree that can be used for timber, medicine, honey, shelter and aesthetic values (Simpson, 2005), its conservation will not only prevent the species from going extinct but also boost the populations to a level where it can be harvested for timber, medicine, and other products. Like other *Meterosideros* species, *M. bartlettii* have mutual associations with other plants, animals, birds and insects (Simpson, 2005), and its conservation will therefore benefit the species with which it interacts. It is therefore extremely important to investigate the population genetics of *M. bartlettii* to assess its current genetic status and make informed management decisions for its conservation.

Conservation of genetic diversity is a fundamental concern in conservation and evolutionary biology, as genetic variation is the raw material for evolution in response to a changing environment (Booy *et al*., 2000; Reed & Frankham, 2003; Laikre, 2010). Estimation of

genetic diversity is especially important for endangered species to assess their genetic status, take conservation measures, and ensure their survival (Toro & Caballero, 2005). Quantifying the level and structure of genetic variation across populations can help in prioritizing sites and management choices that will capture and maintain that variation (Petit *et. al.,* 1998; Neel & Ellstrand, 2003; Arponen, 2012; Ottewell *et al*., 2016) and to determine the minimum number of populations that need to be conserved to avoid unnecessary costs and conflicts with competing land uses.

If most of the genetic diversity is contained within populations, then few populations need to be protected. On the other hand, if the genetic diversity is mainly contained between populations, then a larger sample of populations from different ecological conditions should be protected to capture a good representation of the total gene pool. Knowledge of genetic structure is also important to determine whether two populations should be treated as one or two different management units (Moritz, 1994; Crandall *et al*., 2000), as well as to predict the likelihood of outbreeding depression when individuals in genetically different populations are mixed to boost genetic diversity (Frankham *et al*., 2011).

Genetic diversity studies also help to explain the relative importance of different evolutionary processes (inbreeding, gene flow, genetic drift and selection) that structure the genetic diversity of an endangered species, so that future risks can be assessed and effective conservation strategies can be designed (Ceska *et. al*., 1997). For instance, if there is high inbreeding depression and low gene flow, re-vegetation programmes can be designed to avoid sourcing material from small populations, since a significant portion of the seeds collected might be produced through self-pollination or cross-pollination between closely related individuals (Young *et al*., 2001).

Currently, no data exist on the level and structure of genetic diversity of *M. bartlettii*. Therefore, the objective of this study is to analyse the genetic diversity and structure of this species using Amplified Fragment Length Polymorphism (AFLP) and microsatellite (SSR) markers. These markers are popular and have been successfully used in population genetic studies of New Zealand species and other *Metrosideros* species (Drummond *et al*., 2000; Zawko *et al*., 2001; Armstrong & de Lange, 2005; Broadhurst *et al*., 2008; Kaneko *et al*., 2008; Grueber & Jamieson, 2011; Bian *et al*., 2015; Stevens *et al*., 2015).

3.1.3 Molecular markers used in the study

3.1.3.1 Amplified Fragment Length Polymorphism (AFLP)

The AFLP technique was developed by Vos *et al*. (1995) and involves the digestion of a genomic DNA with a pair of restriction enzymes (e.g., *EcoRI* and *MseI*) and the ligation of two double-stranded DNA adapters to the sticky ends generated by the digestion of the DNA sample. A first amplification (pre-amplification) step is performed using primers that have a single selective nucleotide and a sequence that is complementary to the adapter sequences. Because the pre-amplification step generates an unmanageable amount of amplified product, a second and final amplification step (selective amplification) is performed using primers that have two or three selective nucleotides. In the final amplification, the *EcoRI* primer is labelled with a fluorescent dye for later visualization of bands using gel image analysing software. An outline of the major steps involved in the generation of AFLP markers is shown in Figure 3.2.

The AFLP technique is capable of generating hundreds of highly reproducible markers throughout genomes of individuals for a rapid genetic diversity assessment (Vos *et al*., 1995; Mueller & Wolfenbarger, 1999). The technique combines the features of the Restriction Fragment Length Polymorphism (RFLP) technique (Botstein 1980; Helentjaris *et al*., 1986) and PCR (Mullis & Faloona, 1987) through the digestion of genomic DNA with restriction enzymes and the amplification of digested fragments using PCR (Semagn *et al*., 2006).

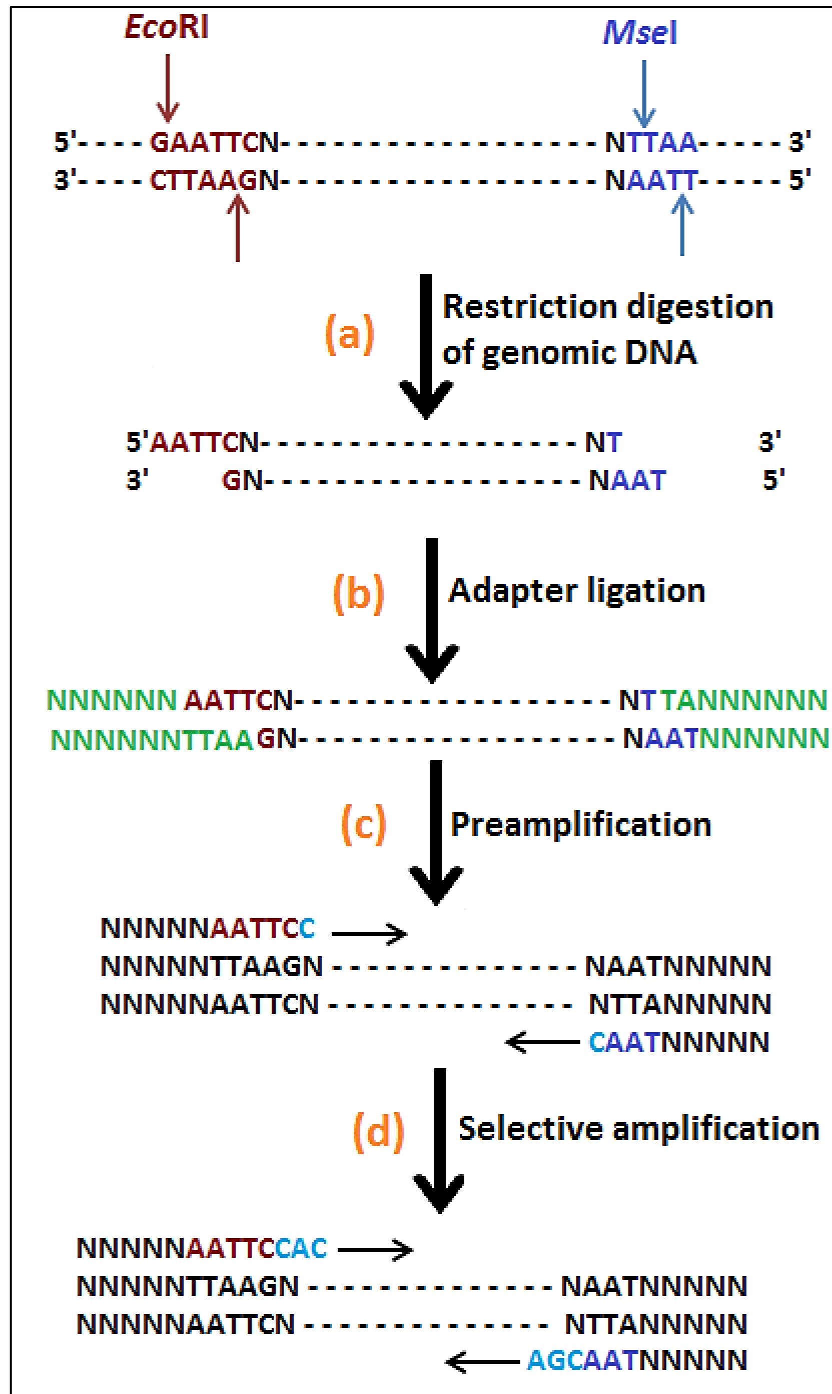


Figure 3.2 Generation of AFLP markers: (a) Genomic DNA is digested with two restriction enzymes, *Eco*RI and *Mse*I. (b) *Eco*RI and *Mse*I-specific adaptors are ligated to the ends of digested DNA fragments using ligase enzyme. (c) Preamplification is conducted on adapter-ligated fragments with primers having one selective nucleotide. (d) After pre-amplification of numerous fragments, a final selective amplification with primers having two or three selective nucleotides is performed to reduce the number of pre-amplified fragments to a manageable number.

The major limitation of ALFP markers is that they are dominant markers, which means they cannot distinguish heterozygote dominant genotypes from homozygous dominant genotypes.

They therefore can underestimate the actual level of genetic variability in populations. However, they have numerous advantages that can offset this limitation, such as they allow analysis of a large number of loci with genome-wide distribution; they have a high level of polymorphism and reproducibility and they do not require prior knowledge of the sequence of primer annealing sites in the genome (Powell *et al*., 1996). These attributes are particularly important in genetic studies of rare and endangered plant species, as genetic data can be generated quickly for swift conservation measures and a high level of polymorphism can be captured from a few available samples (Mba & Tohme, 2005). The AFLP technique demands a very good quality DNA, because secondary metabolites such as phenol in a poor quality DNA sample can affect the digestion of the DNA with restriction enzymes and ultimately lead to genotyping errors and a lack of reproducibility (Bonin *et al*., 2004; Semagn *et al*., 2006).

3.1.3.2 Simple Sequence Repeats (SSR)

Microsatellites, also known as simple sequence repeats (SSR), are one to six base tandem repeats of DNA that are mainly generated through slipped-strand mispairing, a major mechanism for evolution of DNA sequences (Levinson & Gutman, 1987; Schlotterer & Tautz, 1992). The genotypic differences among individuals arise from the difference in the number of the microsatellite repeat units. SSR markers are found in both coding and noncoding regions of DNA, with higher density in the latter (Toth *et al*., 2000; Li *et al*., 2002). Among the various types of SSR markers, di-, tri-, and tetra-nucleotides are the most frequently used repeat units in molecular genetic studies (Selkoe & Toonen, 2006).

SSR markers are well-suited to population genetic studies because these markers are co-dominant (i.e., both alleles of a gene can be differentiated), they have wide genomic distribution, and they are hyper-variable even among closely related individuals (Powell *et al*., 1996; Semagn *et al*., 2006; Stepien *et al*., 2007). They are also highly reproducible, require a low quantity of DNA (as little as 1ng), do not require high quality DNA, and can easily be automated for high throughput genotyping and multiplexing (Kumar *et al*., 2009; Kalia *et al*., 2011; Zalapa *et al*., 2012).

SSR markers have a high level of transferability across related species, and are useful markers not only for the assessment of genetic diversity but also for parental analysis, linkage mapping and evolutionary studies (Knapik *et al*., 1998; Varshney *et al*., 2005; Cavagnaro *et al*., 2010; Moe & Weiblen, 2011). The SSR markers remain the marker of choice for many

researchers due to their highly informative nature, the availability of tens of thousands of these markers in a single genome, and the relative ease of scoring them (Brown & Litt, 1992; Zane *et al*., 2002).

Traditionally, SSR markers are expensive and time-consuming to develop from genomic libraries, which involve the enrichment of recombinant DNA for a few targeted repeat motifs followed by the screening and sequencing of SSR-containing clones (e.g., Karagyozov *et al*., 1993; Armour *et al*., 1994; Hamilton *et al*., 1999). This approach is also extremely inefficient, especially for species with low SSR frequencies (Zane *et al*., 2002).

Recently, a new sequencing technology called Next Generation Sequencing (NGS) has become popular for its high throughout and quick generation of data, allowing the much faster and cost-effective development of SSR markers from a larger genomic portion of plants (Ekblom & Galindo, 2011; Zalapa *et al*., 2012). This method avoids the construction of SSR-enriched DNA libraries and allows a rapid generation of large volumes of sequence data that can be screened for the presence of SSR motifs with the aid of bioinformatics tools (Abdelkrim *et al*., 2009; Senan *et al*., 2014). Because the method allows multiplexing in sequencing, it can significantly reduce the cost associated with preparation and sequencing of several samples (Jennings *et al*., 2011). The NGS technology has already been used to develop cost-effective SSR markers in several plant species (e.g., Csencsics *et al*., 2010; Michalczyk *et al*., 2011; Kale *et al*., 2012; Fatemi *et al*., 2013; Motalebipour *et al*., 2016).

3.2 Materials and Methods

3.2.1 Plant material

A total of 49 leaf samples were collected for DNA analysis from all available trees in the three extant populations in the far north of New Zealand (Radar, Unuwhao, and Kohuronaki; Figure 3.3) and from cultivated samples from various sites. The descriptions of sample trees used in the study are given in Table 3.1.

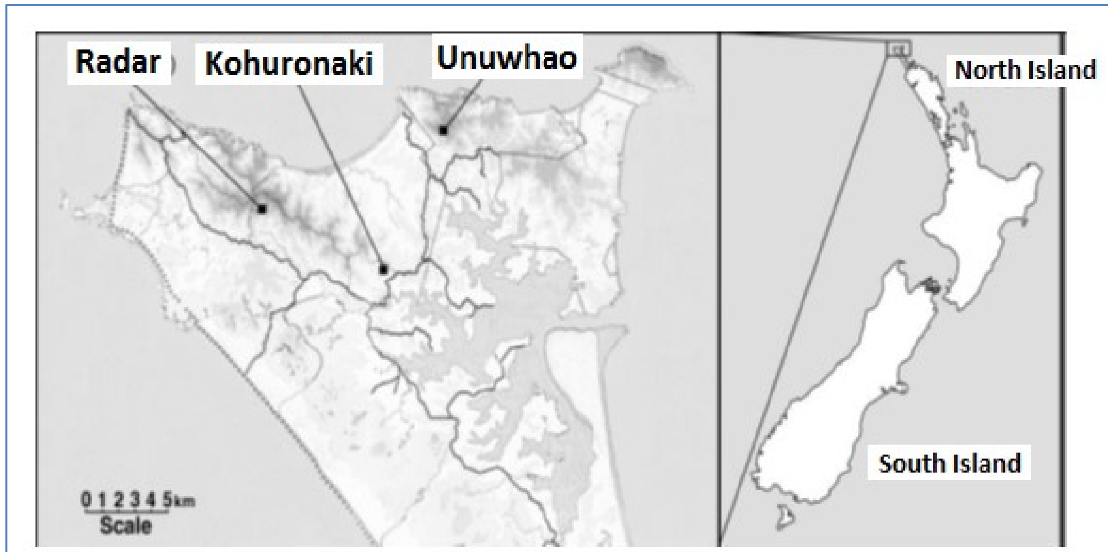


Figure 3.3 Locations of all extant populations of *Metrosideros bartlettii*.

Table 3.1 Description of sample trees used in the study.

	Tree ID.	Population	Remark		Tree ID.	Origin based on STRUCTURE analysis	Remark
Trees from Wild populations	1	Unuwhao	Maire Stream	**Trees from cultivation**	22	Radar	ABG 19910062 - Auckland Botanic Garden
	2	Unuwhao	bottom of cliff (previously known)		23	Radar	PγL 12824 - Auckland zoo
	3	Unuwhao	small tree near tree 4		24	Radar	JRR 15005 - Pinehaven, Upper Hutt
	4	Unuwhao	Big tree, Maire Stream		25	Radar	JRR 15006 - 6 Godley St, Lower Hutt
	5	Unuwhao	Maire Stream		26	Radar	JRR 15007- entrance to Percy Reserve
	6	Radar	Large tree near tree 7		27	Radar	JRR 15008 - Percy Reserve, near toilet
	7	Radar	Big tree		28	Radar	JRR 15009 - Percy Reserve, behind Weir
	8	Kohuronaki	Head of Broughton´s Gully		29	Radar	Pγ2 12714 - Ex-cult-Ferngles
	9	Kohuronaki	Big tree in Broughton´s Gully		30	Radar	Ex-Cultivated, origin said to be from Radar
	10	Kohuronaki	Sapling on Cyathea tree		31	Radar	Nursery origin - Vibrant Earth in Nelson
	11	Kohuronaki	Sapling on Cyathea tree		32	Kohuronaki	Pγ2 12690, Ex-cult, 14 Jesmond Terrace, Mt Albert
	12	Kohuronaki	Seedling on Cyathea tree		33	Kohuronaki	Pγ2 12710 - Ex-cult - ABG, Oratia Plant Nursery
	13	Kohuronaki	on Cyathea tree		34	Kohuronaki	Pγ2 12711 - Ex cult - Pokekohe, Langholm, origin said to be from Unuwhao
	14	Kohuronaki			35	Kohuronaki	Pγ2 12712 - Ex-cult-Great Bashel Island, Shoal Bay, origin said to be from Unuwhao
	15	Kohuronaki	seedling on Cyathea tree		36	Kohuronaki	PγL 12823 - Glendowie Park, Auckland
	16	Kohuronaki			37	Kohuronaki	PγL 12833 - Small tree, Thomas Building
	17	Kohuronaki	Big tree		38	Kohuronaki	JRR 15010 - Percy, Stanhope access road
	18	Kohuronaki			39	Kohuronaki	JRR 15011 - Percy, Stanhope access road
	19	Kohuronaki			40	Kohuronaki	JRR 15012 - Percy, Stanhope access road
	20	Kohuronaki	on rewarewa tree		41	Kohuronaki	Lower Hutt, Myrtle St, Gibbes Watson Conservatory
	21	Kohuronaki	wilting and dying tree next to tree 14		42	Kohuronaki	Ex-Cultivated Broughton Gully
					43	Kohuronaki	Ex-Cultivated Broughton Gully
					44	Kohuronaki	in cultivation, Koromiko Rd, Aro Valley, Wellington
					45	Kohuronaki	in cultivation, Te Papa Museum, Wellington
					46	Kohuronaki	Airstrip planting in Great Mercury
					47	Kohuronaki	Airstrip planting in Great Mercury
					48	Kohuronaki	Airstrip planting in Great Mercury
					49	Kohuronaki	Airstrip planting in Great Mercury

3.2.2 DNA Extraction and AFLP reaction

Genomic DNA was extracted from 20mg of young fresh leaves of *M. bartlettii* using the NucleoSpin® Plant II DNA Extraction Kit according to the manufacturer's protocol. The AFLP reactions were performed as described by Vos *et al*. (1995). The digestion and ligation steps were combined in one reaction tube with a mixture of total volume of 10µl. M*seI* and E*co*RI restriction enzymes were used to digest 20-50 ng of Genomic DNA and the fragmented DNA were ligated to M*seI* and E*co*RI adaptors using T4 Ligase enzyme in the presence of 10XT4 DNA ligase buffer. The mixture was incubated for 3 hours at 37°C followed by a heat inactivation step for 20 minutes at 65°C to stop the restriction endonuclease reaction.

The digested and ligated product was diluted ten-fold for use as template DNA for the next PCR pre-amplification step. This was performed in a total volume of 25µl using M*seI* and E*co*RI primers that matched the adapter sequences and carried one selective nucleotide at their 3' ends. The PCR conditions were 72°C for 2 min, 25 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 2 min, with a final extension at 60°C for 10 min. The success of the pre-amplification was checked using a 50 to 600bp smear of fragments in gel electrophoresis.

The pre-amplified products were again diluted ten-fold and used as template DNA for the final selective amplification step. The final step was performed in duplicates for ten test samples drawn proportionally from the three populations. The reactions were performed in a total volume of 20µl using 12 different combinations of E*co*RI and M*se*I primers, with two or three selective nucleotides at their 3' ends. The E*co*RI primers were labelled with JOE and NED fluorescent dyes for later visualization of bands.

The PCR conditions were an initial denaturation of 94°C for 2 min, 12 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 2 min, then 23 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 2 min with a final extension at 72°C for 10 min. To obtain the AFLP profiles, the selective PCR products were sent to Landcare Research, Auckland where samples were mixed with formamide and Genescan ROX 500 size standard and run on an ABI3100 sequencer (Applied Biosystems). Based on results from the duplicated test samples, four primer combinations (JOE E*co*RI-AGG + M*se*I-CT; JOE E*co*RI-AGG + M*se*I-CG; JOE E*co*RI-AGG + M*se*I-CTG; and NED E*co*RI-ACC+ M*se*I-CG) that showed high polymorphism and reproducibility were selected from the 12 primer combinations for the analysis of the rest of the samples (Table 3.2).

3.2.3 SSR Reaction

For the SSR analysis, the ten samples that were used to screen the AFLP primers were also used to screen and test the transferability of 15 pairs of SSR primers that were developed by Crawford *et al.* (2008) for *M. polymorpha.* Seven of these markers were also successfully used by Harbaugh *et al.* (2009) across five different species of *Metrosideros* to study the genetic structure of the *Metrosideros* complex in the Hawaiian Islands. In the present study, ten of these 15 pairs of polymorphic primers were found to be polymorphic and reproducible. They were used to amplify microsatellite loci, which are characterised in Table 3.2.

Table 3.2 AFLP and SSR primers used in this study (n = 49 individuals).

AFLP			SSR			
Primer combination	**No. of bands**	***P* (%)**	**Locus**	**Primer sequence (5' to 3')**	***K***	**Size (bp)**
JOE E*co*RI-AGG + M*se*I-CT	35	15 (42.87%)	MePo501	M13F: TCTTTCGCCGGATTACTT R: AGTGCCTTATTCATGCTATGT	7	119-155
			MePo502	M13F: ATGAGGAGGATTACGTATTA R: CGTATTTACTCCCAATTATCA	3	221-255
JOE E*co*RI-AGG + M*se*I-CG	34	23 (67.67%)	MePo503	M13F: CTCACATCGCTTGTCTA R: CCAAATTAAGAACGATACAT	4	189-207
			MePo505	M13F: TAATGTTGGTTGTGTTAT R: CATTTGGACTAGCAAGTTAC	3	263-269
			MePo506	M13F: ATCCCCTCACGATTATAG R: ATGGGCTGACGAATATA	2	158-164
JOE E*co*RI-AGG + M*se*I-CTG	25	11 (44.01%)	MePo507	M13F: TTTTCTTGGCAYGACTCT R: CGTCTACAAATCCAAGTAAA	5	172-244
			MePo509	M13F:GTTCGCGGTTGATTACTA R: GAATCACAAGCCATTAG	4	197-214
			MePo512	M13F:ACGAATTGCTTTATTGATATAC R: TAAATTGGGCCACATAGA	3	132-189
NED E*co*RI-ACC+ M*se*I-CG	40	23 (57.52%)	MePo513	M13F: CAGAACTGGTATCCTGATATA R: AATGAGTTGGGATTTAGAG	4	178-220
			MePo514	M13F: GCTTTCCTGCAACAGAGTAG R: GTGCATCAACCGCTACAT	5	184-208
Total	**134**	**72 (53.73%)**			**40**	

P = Proportion of polymorphic loci at 5% level; *K* = Total number of alleles

The PCR amplification of the microsatellite loci involved two amplification steps. In the first step, each locus was amplified using a M13-tagged forward primer (M13F sequence: 5'TGTAAAACGACGGCCAGT3') and a reverse primer, both specific to each locus. The first amplification step used 50ηg of DNA. In the second step, a 1µl of the PCR product obtained from the first amplification step was amplified using the reverse primer and a universal 6FAM labelled M13-tagged forward primer, which was complementary to the M13-tagged forward primers that were used in the first amplification step.

The PCR conditions for the first amplification step were 94°C for 5 min, 30 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 45min, with a final extension at 72°C for 30 min. The PCR conditions for the second amplification step were identical except the annealing temperature was 51°C instead of 54°C. The PCR mixture in both amplification steps was identical with the following composition: 1.5µl of 10X PCR buffer (with 20mM $MgCl_2$), 1.5µl of 10mM dNTPs (2.5mM each), 1µl of 10µM forward and reverse primers, and 0.2µl of Taq DNA polymerase (5U/µl). The total volume of the PCR mix was 15µl. For measuring the size of the alleles, the selective PCR products were diluted five to ten times based on the intensity of bands on the gel image and sent to Landcare Research, Auckland where samples were mixed with formamide and Genescan ROX 500 size standard and run on an ABI3100 sequencer (Applied Biosystems).

3.3 Data analysis

3.3.1 Genetic diversity

Raw AFLP data were scored using GeneMarker V 2.6.4. AFLP bands were scored automatically as present (1) or absent (0). The automatic scoring was also inspected visually to make sure that the software had scored correctly. The binary data matrix was analysed using AFLP-SURV 1.0 (Vekemans, 2002). For each population, allelic frequencies were computed using a Bayesian method with non-uniform prior distribution of allele frequencies (Zhivotovsky, 1999), assuming no deviation from Hardy Weinberg Equilibrium.

This method computes the frequency of the null allele at each locus from two numbers: the sample size and the number of individuals in the sample that lack the AFLP fragment, using a Bayesian method that assumes a non-uniform distribution of allelic frequencies. It estimates the distribution of allele frequencies based on the variation over loci of the frequencies of AFLP fragments in the sample. When the data concerns several populations, the distribution

of allele frequencies is estimated separately for each population. Once the frequency of the null allele is computed, the frequency of the marker allele is computed as one minus the frequency of the null allele.

The Bayesian method of calculating allelic frequencies is computationally intensive and therefore requires more time. However, it provides the most accurate results (Zhivotovsky, 1999) and has been shown to efficiently reduce the bias of the square-root method (Krauss, 2000), where the frequency of the null allele at each locus is computed as the square root of the proportion of individuals in the sample that lacks the AFLP fragment (i.e., the square root of one minus the frequency of the AFLP fragment in the sample).

Once allelic frequencies are computed with Bayesian methods, the AFLP-SURV 1.0 program uses these allelic frequencies to estimate genetic diversity and population genetic structure based on the approach of Lynch & Milligan (1994). This approach uses the average expected heterozygosity of the marker loci (Nei's gene diversity) as a measure of genetic diversity. To estimate the genetic diversity, the proportion of polymorphic loci (*P%*) at the 5% level, the expected heterozygosity (Nei´s genetic diversity; H_e) and the total gene diversity (H_t) were computed.

The proportion of polymorphic loci is the number of polymorphic loci divided by the total number of loci (polymorphic and monomorphic). This study defined a gene as polymorphic if the frequency of one of its alleles was less than or equal to 0.95. The unbiased expected heterozygosity (UH_e) was calculated based on the following equation (Nei, 1978; Bonin *et al.*, 2007):

$$H_e = \frac{\sum_{i=1}^{L}(1 - p_i^2 - q_i^2)}{L}$$

$$UH_e = \frac{2N}{2N - 1}\frac{\sum_{i=1}^{L}(1 - p_i^2 - q_i^2)}{L}$$

Where, i is the locus number, p_i is the frequency of plus-allele for locus i, q_i is frequency of the null-allele for locus i, N is the number of individuals and L is the number of loci.

For the microsatellite data, the raw codominant SSR data were analysed and SSR bands were scored for each locus across all individuals using GeneMarker V 2.6.4. To estimate the genetic diversity within populations, the number of alleles per locus (N_a), number of effective alleles per locus (N_e), observed heterozygosity (H_o) and expected heterozygosity (H_e) were calculated using GenAlex 6.5 (Peakall & Smouse, 2012).

No. of effective alleles = $N_e = \frac{1}{\sum p_i^2}$

Where p_i is the frequency of the ith allele for the population and the sum of p_i^2 is the sum of the squared population allele frequencies.

Expected Heterozygosity = $H_e = 1 - \sum p_i^2$

The mean H_e is the average H_e across the populations.

Observed Heterozygosity = H_o = No. of Heterozygotes / Total number of individuals (N)

The mean H_o is the average H_o across the populations.

The level of inbreeding in each population was also computed using the inbreeding coefficient (F_{is}) according to Weir and Cockerham (1984). The inbreeding coefficient is a measure of excess homozygosity beyond the expectations from Hardy-Weinberg Equilibrium. It was computed as follows:

Inbreeding coefficient = $F_{is} = \frac{meanH_e - meanH_o}{meanH_e}$

3.3.2 Genetic structure

To investigate genetic structure, Wright's (1951) F_{st} was computed with 10,000 permutations using AFLP-SURV 1.0 (for AFLP data) and GenAlex 6.5 (for SSR data) as follows.

$F_{st} = \frac{H_t - meanH_e}{H_e}$, where $H_t = 1 - \sum tp_i^2$ and $H_e = 1 - \sum p_i^2$

Where H_t is the total expected heterozygosity, tp_i is the frequency of the ith allele for the total and sum tp_i^2 is the sum of the squared total allele frequencies, H_e is the expected heterozygosity within population, p_i is the frequency of the ith allele for the

population and the sum of p_i^2 is the sum of the squared population allele frequencies, and the mean H_e is the average H_e across the populations.

Wright's (1951) F_{st} is the most commonly used method to assess the structure of populations. It is a measure of genetic distance assuming population divergence is caused by genetic drift. In this method, groups of individuals must be predefined as populations and then the genotype information of individuals in populations is used to compute variances in the frequencies of alleles to determine the structure of the populations.

The F_{st} value also gives an indication of the extent of gene flow, which can be defined as the movement of genes between populations (Slatkin, 1985). The higher the F_{st} value, the higher the genetic differentiation between populations, and the lower the level of gene flow. F_{st} values in the range of 0 to 0.05 are considered low, 0.05 to 0.15 moderate, 0.15 to 0.25 large, and 0.25 to 1.0 very large (Hartl & Clark, 1997). Values close to zero indicate little differentiation among populations (i.e., most genetic diversity is within populations), while values close to one indicate high differentiation (i.e., most diversity is among populations). The gene flow among populations (Nm) was estimated from F_{st} value using the following equation (Rousset, 1997):

$$Nm = \frac{1 - F_{st}}{4F_{st}}$$

Gene flow and migration are often used interchangeably, but the latter does not necessarily imply the successful establishment of immigrant genotypes and their subsequent involvement in reproduction of the new population (Woodruff, 2001). While gene flow facilitates homogenization of populations, the lack of gene flow leads to differentiation of populations.

Analysis of molecular variance (AMOVA) was also performed to calculate the proportion of genetic variation within and between populations. The AMOVA approach as described by Excoffier *et al* (1992) generates squared Euclidean distances between pairs of individuals and then partitions this variation within populations and among populations. It can also be used to partition variation at higher levels of structure in nested analyses (e.g., by geographic region or species).

The genetic structure of *M. bartlettii* was also analysed using cluster analysis. To group individual samples into clusters, both distance-based and model-based methods were used. The distance-based methods used were the principal coordinate analysis (PCO) and the Unweighted Pair Group Method with Arithmetic averaging (UPGMA), both of which were

performed using the multivariate statistical package MVSP 3.22 (Kovach, 2007). The distance-based clustering methods used a pairwise genetic distance matrix between every pair of individuals to display the clustering in a more convenient graphical representation (Pritchard *et al*., 2000). Unlike the Wright's (1951) $F_{st,}$ the distance-based methods do not require the definition of populations for inference of the genetic structure (Evanno *et al*., 2005).

UPGMA is the most common approach used in cluster analysis. In this method, the distance at which a cluster is formed corresponds to the average of all pair-wise distances between populations that are joined together in that particular step. Thus, pair-wise differences of populations that had already been joined in earlier steps are not included (Kindt *et al*, 2009). In PCO, a pairwise distance matrix is subjected to an analysis that expresses observed differences in positions along a small number of principal axes of variation. The positions can then be visually compared in two- or three-dimensional diagrams, providing an overview of the spread of variation within stands (Kindt *et al*, 2009).

Although the distance-based methods are easy to apply and are visually appealing, they are associated with some disadvantages. For example, the resulting clusters may heavily depend on the distance measure and graphical representation chosen, and it is difficult to assess confidence in the meaningfulness of the clusters obtained. Other problems include a lack of fine statistical inference and difficulty in incorporating additional data such as the geographic sampling locations of samples (Pritchard *et al*., 2000).

Model-based methods do not have these problems, so to complement the distance-based methods, the model-based Bayesian clustering method was implemented using the software STRUCTURE v2.3.4, developed by Pritchard *et al.* (2000) and freely available at http://pritchardlab.stanford.edu/home.html. The program assigns individual samples probabilistically to genetic populations or clusters (K), where K is set by the user. The model demarcates clusters of individuals on the basis of allele frequency at multiple loci using a Bayesian model-based clustering algorithm. This algorithm estimates admixture proportions (Q) for each individual, which is the proportion of its genome originating from each inferred population. The individuals´ proportion of membership for each cluster was calculated without consideration of sampling localities (i.e., no *a priori* population information was used).

The analysis can be performed using either of two models: admixture and non-admixture (Pritchard *et al*., 2000). The non-admixture model is a simple model that assumes each individual originated only in one of *K* genetic populations, which each has its own characteristic set of allele frequencies. The admixture model is an expansion of this model to allow for admixed individuals (i.e., those originating from more than one *K* genetic population) by introducing a vector *Q* to denote the admixture proportions for each individual. For our purposes, the analyses were performed under the admixture model assuming independent allele frequencies.

In the structure analysis, the number of clusters, *K*, needs to be specified *a priori* by the user and the best *K* value was determined using the following two approaches:

1. **Based on an estimate of posterior log likelihood (probability) of data for each *K*, *LnP(D)* = *L(K)***: Falush *et al.* (2007) suggested the lowest value for *K* be adopted for which the *LnP(D)* values have begun to plateau, and for which results appear biologically meaningful. When *K* is approaching the best value, the *L(K)* usually shows the largest increase and then plateaus (or keeps increasing slightly) and finally shows high variance between runs for larger *K* values. For each value of *K*, the analysis is repeated several times (usually 10-20) to explore the consistency (variance) of *LnP(D)* values. Another important decision for obtaining meaningful results in STRUCTURE is the number of steps in the burn-in period and the number of subsequent Markov Chain Monte Carlo (MCMC) repetitions to perform (Kindt *et al*, 2009). The authors of STRUCTURE suggest using 10,000 - 100,000 steps in both phases. Obviously, longer times are required for a larger number of steps. The number of steps required can be decided by repeating the analysis with various numbers of steps and checking when *LnP(D)* values and end results become stable (Kindt *et al*, 2009).
2. **Based on an ad hoc quantity (ΔK)**: Evanno *et al* (2005) tested the ability of the Bayesian algorithm in the STRUCTURE program to detect the best *K* value when the pattern of dispersal among populations is not homogenous. They found that the *LnP(D)* did not give the correct estimation of *K*, whereas an ad hoc statistic ΔK (the rate of change in the log probability of data between successive *K* values) accurately detected the best *K* values. The ΔK shows a clear peak at the true value of *K* (i.e., the best K is the one corresponding to the maximum value of ΔK).

In this study, to quantify the variation of the likelihood of data for each *K* varying from one to ten, ten runs were performed using a 100,000-steps burn-in period followed by 500,000

MCMC repetitions. These values were selected because the burn-in was long enough to stabilize log (alpha) and *Ln* likelihood, and the MCMC chain was long enough to obtain consistent end results. No *a priori* population information was used.

The best value of *K* was determined based on the estimated posterior log probability of the data, *L(K),* and the rate of change in probability (ΔK) between successive *K* values, following Evanno *et al*. (2005). The visualization of the structure output and implementation of Evanno's method were performed using the Structure Harvester program (Earl & vonHoldt, 2012), which is available at http://taylor0.biology.ucla.edu/structureHarvester/.

3.4 Results

3.4.1 Genetic diversity

Of the 12 AFLP primer combinations tested, four (JOE E*co*RI-AGG + M*se*I-CT, JOE E*co*RI-AGG + M*se*I-CG, JOE E*co*RI-AGG + M*se*I-CTG, and NED E*co*RI-ACC+ M*se*I-CG) were found to be polymorphic and highly reproducible and therefore were used in the analysis. At the species level, the four primer combinations gave a total of 134 markers over the entire data set, of which 72 (53.7%) were polymorphic at the 5% level (Table 3.2). The SSR primers gave a total of 40 alleles over ten loci with an average of four alleles per locus.

In natural populations, the proportion of polymorphic loci (*P%* at 5% level) for AFLP markers at the population level ranged from 14.2% for Kohuronaki to 89.6% for Unuwhao with an average of 60.23% (Table 3.3). The mean expected heterozygosity (H_e) within a population for these markers ranged from 0.09 for Kohuronaki to 0.23 for Unuwhao with an average of 0.17 (Table 3.3). As expected, the SSR markers gave a higher estimate of genetic diversity (H_e = 0.48) than the AFLP markers. The inbreeding coefficient (F_{is}) was 0.04, suggesting an absence of excess homozygosity. All genetic diversity parameters for both AFLP and SSR showed that the Unuwhao population had the highest genetic diversity despite containing only five trees.

The estimation of genetic diversity after dividing the samples into two 'populations' (i.e., the three wild populations versus all samples from cultivation sites) showed that both populations had a very similar genetic diversity for both markers (wild population: AFLP H_e = 0.17, *P%* = 36.6; SSR H_e = 0.50; Cultivated samples: AFLP H_e = 0.18, *P%* = 33.6; SSR H_e = 0.51;

Table 3.4). However, the wild population had a far greater number of private alleles (11) than the cultivated samples (2). Private alleles are found only in a particular population.

Table 3.3 Genetic diversity of wild populations based on AFLP and microsatellite markers.

Population	*N*	**AFLP**			**SSR**						
		P%	***He***	***Total F_{st}***	***N_a***	***N_e***	***N_{pa}***	***H_o***	***H_e***	***F_{is}***	***Total F_{st}***
Radar	2	76.9	0.20	0.22*	1.90	1.79	1	0.50	0.52	0.03	0.20*
Unuwhao	5	89.6	0.23		2.90	2.25	10	0.54	0.59	0.08	
Kohuronaki	14	14.2	0.09		2.20	1.64	5	0.43	0.35	-0.23	
Mean		**60.23**	**0.17**		**2.33**	**1.89**	**5.33**	**0.49**	**0.48**	**-0.04**	
Mean reported values (Nybom, 2004) for:											
Long lived perennials			0.25					0.63	0.68		
Outcrossing species			0.27					0.63	0.65		
wind dispersed specie			0.27					0.54	0.61		
Metrosideros excelsa, widespread (Broadhurst *et al*, 2008)		70.4	0.20								
Metrosideros boninensis, endangered & extremely rare (Kaneko *et al.,* 2008)		12.9	0.024		2.752			0.336	0.334		
Rhododendron protistum var. Giganteum, extremely endangered (Wu *et al*., 2015)		57.61	0.22								

N = Population size; *P%* = proportion of polymorphic loci; H_j = expected heterozygosity (Nei's genetic diversity, analogous to H_e); N_a = Number of alleles per locus; N_e = Effective number of alleles per locus; N_{pa} = Number of private alleles; H_O = Observed heterozygosity; H_e = expected heterozygosity (Nei's genetic diversity); F_{is} = Inbreeding coefficient; F_{st} = Wright´s Fixation index interpreted as genetic differentiation among populations.

* Significant deviations from zero.

Table 3.4 Comparison of genetic diversity of samples obtained from the three wild populations and from various cultivation sites.

Population	N	AFLP		SSR				
		$P\%$	He	N_a	N_e	N_{pa}	H_o	H_e
Wild populations	21	36.6	0.17	3.80	2.13	11	0.47	0.50
Cultivation	28	33.6	0.18	2.90	2.17	2	0.55	0.51

3.4.2 Genetic structure

The analysis of population genetic structure based on AFLP (Table 3.5) and the AMOVA analysis based on SSR markers (Table 3.6) showed that a greater portion of the total genetic diversity was contained within populations (77% for AFLP and 74% for SSR), with a smaller portion between populations (23% for AFLP and 26% for SSR). Although between-population variation accounted for a smaller portion of the total variation, it nevertheless represents a significant differentiation among populations as revealed by the F_{st} values of both markers (0.22 for AFLP, 0.20 for SSR; Table 3.3). The level of gene flow (*Nm*) based on F_{st} was calculated as 0.89 for AFLP and 1.00 for SSR, which are both similar to the minimum value (Nm=1) required to prevent population differentiation via genetic drift (Slatkin, 1987), suggesting a very restricted number of migrants between populations.

Table 3.5 Population genetic structure based on AFLP markers

	H_t	H_w	H_b	% variation
	0.2262	0.1739	0.0523	Within-populations = 76.88
SE		0.04	0.00	Among-populations = 23.12

H_t = total genetic diversity; H_w = mean within population genetic diversity (analogous to Nei´s H_s); H_b = average between population genetic diversity (analogous to Nei's D_{st}).

Table 3.6 Analysis of molecular variance (AMOVA) based on SSR markers

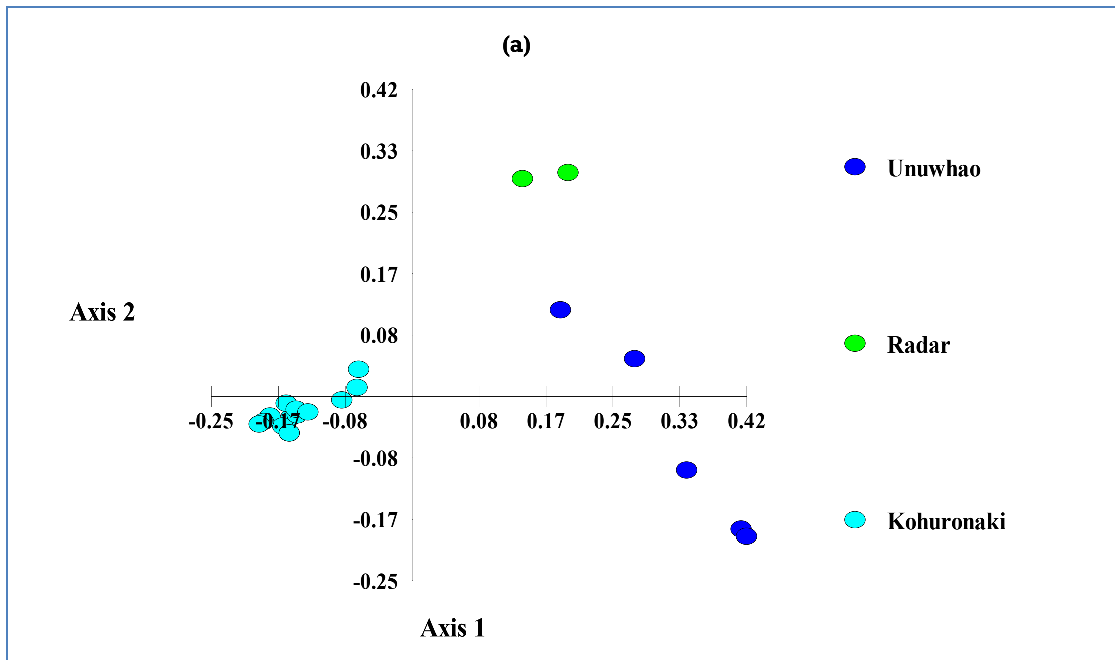
Source of variation	degree of freedom	Sum of squares	Mean square	Estimated variance	% variation
Among Pops	2	20.62	10.31	0.83	26%
Within Pops	21	49.00	2.33	2.33	74%
Total	23	69.62	12.64	3.16	100%

The SSR marker-based pair-wise F_{st} between populations (Table 3.7) showed a significant genetic differentiation for two pairs of populations (Unuwhao-Kohuronaki: $F_{st} = 0.19$, $P < 0.001$; Radar-Kohuronaki: $F_{st} = 0.25$, $P < 0.05$). The table shows a lack of statistical significance ($P > 0.05$) for the Unuwhao-Radar pairwise F_{st} value (0.12) which is likely due to the very small population sizes of these two populations. Based on Wright (1978) and Hartl and Clark (1997), these F_{st} values indicated a moderate to large genetic differentiation among the populations. Pair-wise F_{st} values for AFLP markers could not be computed due to the small sample size of the Radar population. The AFLP-Surv program requires a minimum of three individuals per population to compute pair-wise F_{st} values.

Table 3.7 Pair-wise F_{st} estimates of genetic divergence between populations obtained with SSR markers. F_{st} values below the diagonal. Probability, P (rand >= data) based on 999 permutations is shown above diagonal.

	Unuwhao	Radar	Kohuronaki
Unuwhao	0.000	0.431	0.000
Radar	0.121	0.000	0.013
Kohuronaki	0.186	0.247	0.000

The PCO analyses based on AFLP (Figure 3.4a) and SSR (Figure 3.4b) gave very similar results, with the Kohuronaki population forming a distinct group clearly separated from the other two populations, which were grouped together. The first and second axes explained 39.46 % and 12.32% of the variation respectively for the AFLP data and 34.60% and 11.20% of the variation respectively for the SSR data.



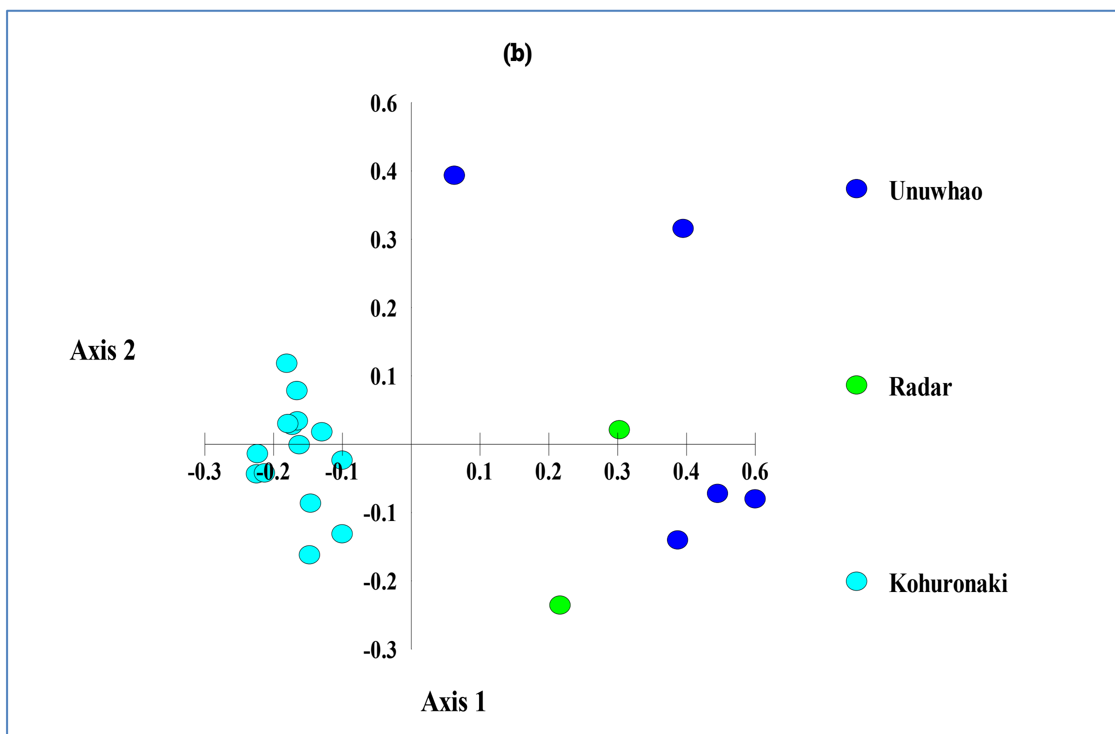
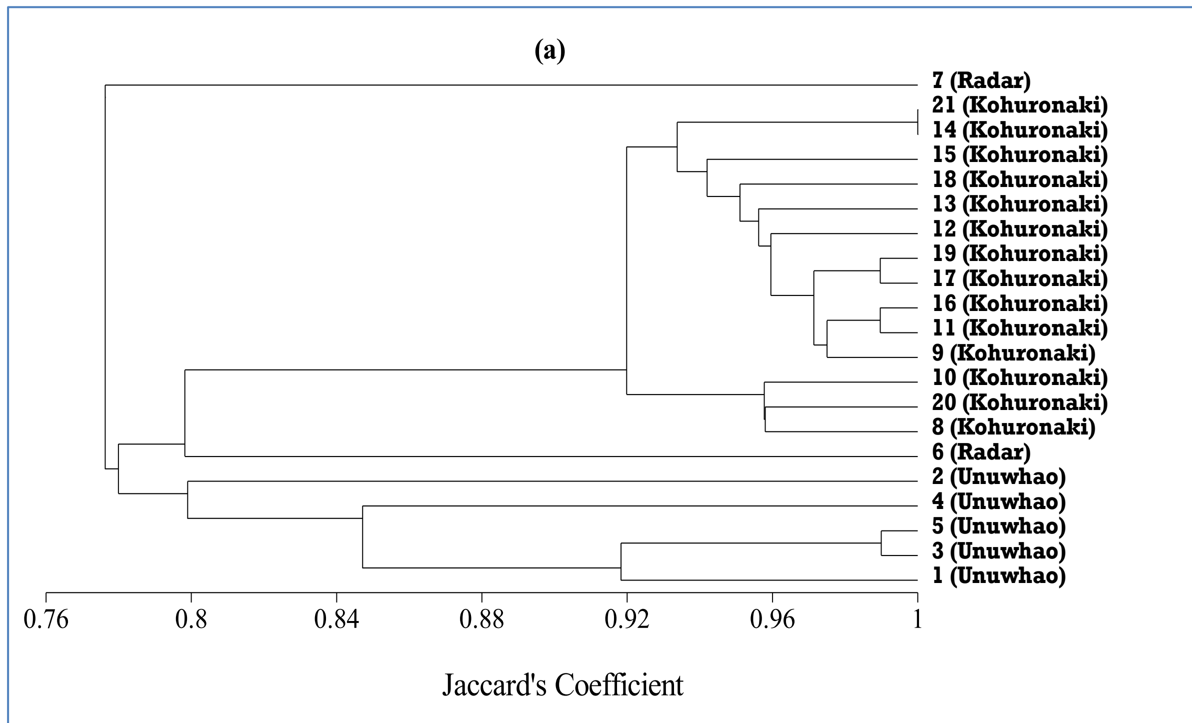


Figure 3.4 Principal coordinate analysis of 21 individuals of wild populations *Metrosideros bartlettii* based on (a) AFLP markers and (b) SSR markers. The first and second axes explained 39.46 % and 12.32% of the variation respectively for the AFLP data and 34.60% and 11.20% of the variation respectively for the SSR data.

The UPGMA trees based on Jaccard's similarity coefficient were similar for both markers (Figure 3.5a & 3.5b). The Kohuronaki population again formed a very distinct group clearly separated from the other two populations. The UPGMA tree based on SSR data showed the

association of the Radar with Unuwhao populations, although this association was not evident in the AFLP-based UPGMA tree.



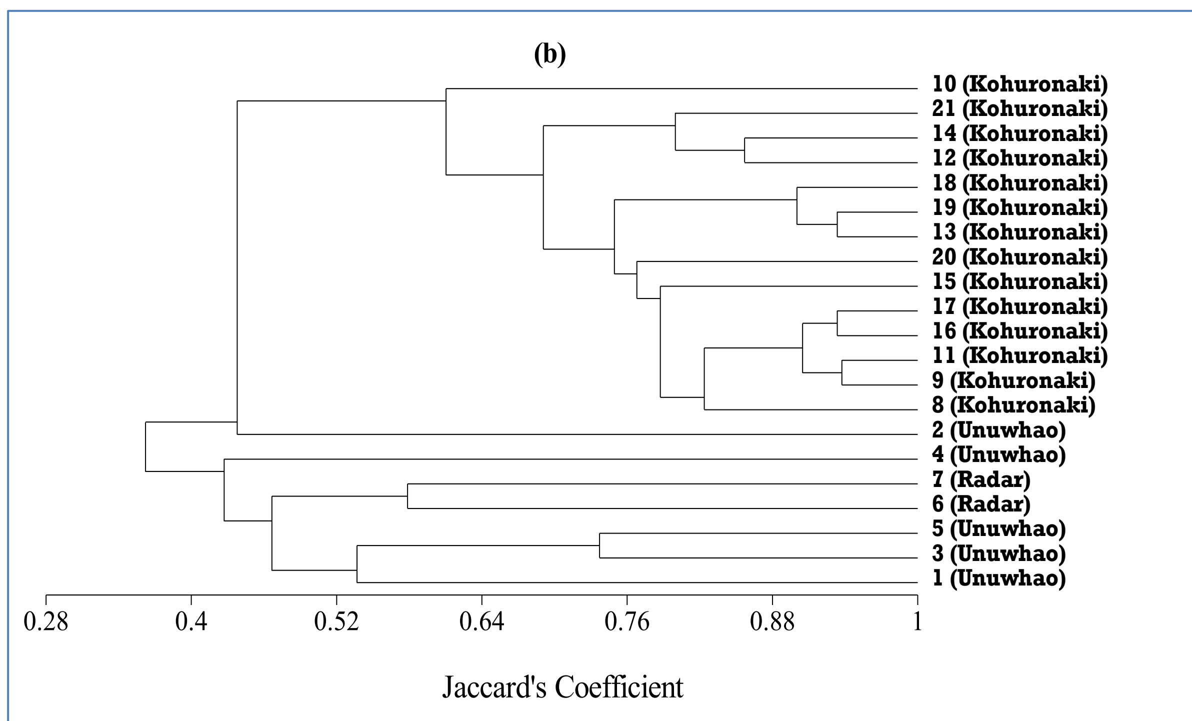


Figure 3.5 The UPGMA dendrogram showing the genetic association of 21 *M. bartlettii* individuals based on (a) AFLP markers and(b) SSR markers. Tree numbers are as indicated in Table 3.1.

In the Bayesian model-based STRUCTURE analysis, the *ΔK* test (Evanno *et al*., 2005) showed that the highest *ΔK* value was found for $K = 2$ both with AFLP (Figure 3.6a) and SSR (Figure 3.6b). This suggests two genetic populations, with the Kohuronaki population as one distinct cluster and the other two populations forming another cluster with few admixture individuals. This supported results from the PCO and UPGMA analysis, where the Kohuronaki population formed a clearly distinct group and the other two populations were grouped together.

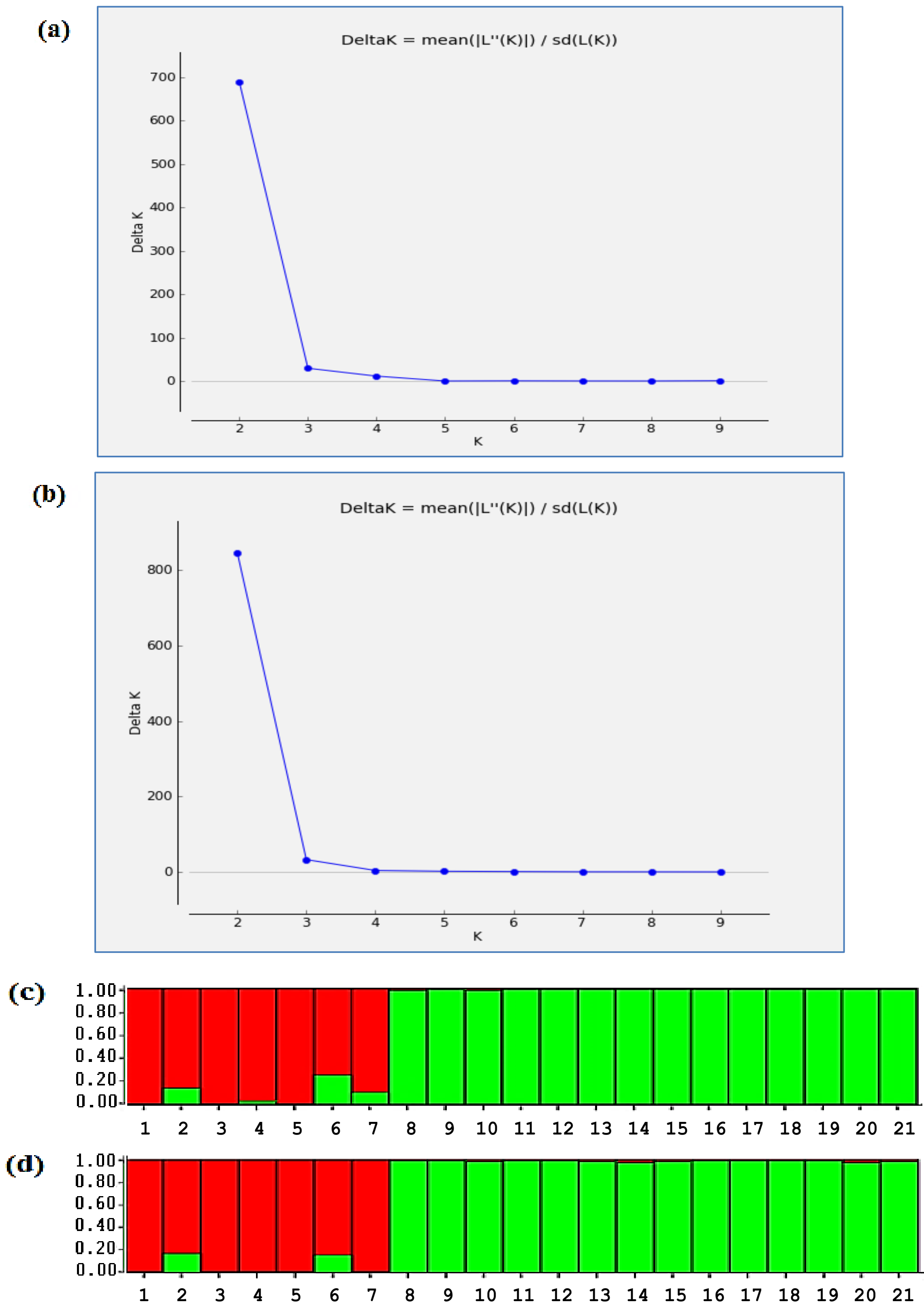


Figure 3.6 Genetic structures of *M. bartlettii* inferred from Bayesian clustering using AFLP and SSR markers. The most likely number of clusters, K = 2, based on the ΔK estimation using (a) AFLP and (b) SSR data. The assignment of individuals into two genetic populations is shown for (c) AFLP and (d) SSR data. Each individual is represented by a vertical bar, coloured according to the assigned group(s). Numbers on the x-axis refer to individual tree ID as indicated in Table 3.1. Trees 1-5 are from Unuwhao; 6-7 are from Radar; and 8-21 are from Kohuronaki. Numbers on y-axis refer to the probability of membership in the inferred clusters.

3.5 Discussion

3.5.1 Genetic diversity

My aim in this study was to determine the genetic diversity and structure of *M. bartlettii* populations within New Zealand, and from these data, to assess its long-term viability. Based on the results of the AFLP and SSR analysis, the expected heterozygosity (H_e) of *M. bartlettii* was markedly lower than for comparable species elsewhere. Based on AFLP data, the mean expected heterozygosity for *M. bartlettii* was 0.17, which is low compared with mean genetic diversities reported for other long-lived perennials (0.25), outcrossing species (0.27), and wind-dispersed species (0.27), as compiled by Nybom (2004) from 307 studies. The microsatellite analysis also gave an expected heterozygosity (H_e = 0.48) lower than the average reported by Nybom (2004) for long-lived perennials (0.68), outcrossing (0.65), and wind-dispersed species (0.61).

In another comparison between *M. bartlettii* and an endangered large tree species in China, *Rhododendron protistum var. giganteum* (Wu *et.al,* 2015), again *M. bartlettii* was markedly lower in genetic diversity (Table 3.3). *M. bartlettii* also has lower AFLP diversity than its closest widespread New Zealand endemic relative, *M. excelsa* (P = 70.4%, H_e = 0.20 - Broadhurst *et al*., 2008). However, it has a greater genetic diversity compared with another endangered and extremely rare *Metrosideros* species of the Bonin Islands of Japan (*M. boninensis*; AFLP: P = 12.90%, H_e = 0.024; SSR: H_e = 0.334 - Kaneko *et al.,* 2008).

Genetic diversity as measured with AFLP vs SSR markers

The two different genetic markers used in this study produced different genetic diversity statistics. The genetic diversity of *M. bartlettii* obtained with SSR markers was three times the estimate obtained with AFLP markers. This finding agrees with several other studies that have used and compared dominant (AFLP/RAPD) and codominant (SSR) markers (e.g., Mariette *et al*., 2001; Maguire *et al*., 2002; Kaneko *et al*, 2008; Tang *et al*., 2008; Li *et al*., 2011). This may be a result of the different ways that AFLP and SSR markers reveal polymorphisms; while SSR markers detect multiple alleles at a given locus, the AFLP markers detect multiple loci distributed throughout a given genome (Powell *et al*., 1996; Hamblin *et al*., 2007).

The higher level of genetic diversity associated with SSR markers can be explained by the fact that SSR markers are codominant and show higher levels of mutation rates among individuals (Levinson & Gutman, 1987; Hedrick, 1999; Schlotterer, 2000; Woodhead *et al*., 2005). The replication slippage that generates the SSR polymorphism is thought to occur more frequently than nucleotide mutations and insertion/deletion events, which generate the AFLP polymorphisms (Powell *et al*., 1996).

Other explanations for the higher level of genetic diversity with SSR markers could be that while the AFLP analysis involves both monomorphic and polymorphic loci, the SSR analysis involves only polymorphic loci. In addition, dominant markers such as AFLP can underestimate the actual level of genetic diversity because they do not discriminate homozygous dominant genotypes from heterozygous dominant genotypes.

Explanations for the current levels of genetic diversity in *M. bartlettii*

The genetic diversity of a species can be shaped by several factors including geographic range, mating system, gene flow among populations and natural selection (Hamrick & Godt, 1996b; Nybom & Bartish, 2000; Gitzendanner & Soltis, 2000; Duminil *et al*., 2007). Of these factors, the species range appears to most greatly affect the within population diversity (Gibson *et al*., 2008; Huang *et al*., 2009). Generally, geographically limited species are expected to show lower genetic diversity than widespread species due to their small population size (Frankham, 1996; Cole, 2003; Leimu *et al*., 2006; Solórzano *et al*., 2016).

Several genetic diversity studies of small populations have also confirmed this hypothesis (e.g., Godt *et al*., 2005; Gibson *et al*., 2008; López-Pujol *et al*., 2013; Ilves *et al*., 2013; Szczecińska *et al*., 2016). A meta-analysis of 52 plant species by Honnay and Jacquemyn (2007) showed that smaller populations consistently contained significantly less variation than larger populations. Ellstrand and Elam (1993) compared the amount of genetic variation in 11 sets of geographically restricted species and their widespread congeners and found that geographically restricted species generally contained less genetic variation than their widespread congeners. A review of the relationship between genetic variation and population size by Frankham (1996) also showed a positive correlation between the two factors.

While several similar studies have implied the association of smaller population size or rarity with a reduced level of genetic diversity, this may not always be the case as low genetic variation is not a universal feature of rare species (Gitzendanner & Soltis, 2000; Brodie,

2007; Bouzat, 2010). For instance, despite having extremely small wild populations, the Madagascar endemic species, *Voanioala gerardii*, has unexpectedly high genetic diversity (Shapcott *et al*., 2012). Similar observations have been reported in other studies (e.g., Nickrent & Wiens, 1989; Henderson *et al*., 2006; Shapcott *et al*., 2007; Torres-Florez *et al*., 2014).

Where there is no correlation between genetic variation and population size, historical factors may be more important than population size in explaining the magnitude of the genetic diversity (Ellstrand & Elam 1993). In the case of *M. bartlettii*, the association between small population size and reduced genetic diversity appears to hold, but additionally, historical influences may have contributed to its low genetic diversity.

M. bartlettii is confined to a very small geographical range with a very small number of trees and only three populations left in the wild. The primary reason for the reduction in size of *M. bartlettii* populations may have been the shrinking of its geographical ranges into isolated refuges during Pleistocene glaciation events. Extreme low temperatures might have restricted *M. bartlettii*, which is frost-sensitive, to the Cape Reinga area, where the few surviving populations exist today.

This process has been reported for other New Zealand plant species (McGlone *et al*., 2001) and for species in other regions of the world (e.g., Dubreuil *et al*., 2008; Beck *et al*., 2008; Beatty & Provan, 2011). When such population declines are accompanied by a reduction in genetic diversity, it is often called a genetic bottleneck (Barrett & Kohn, 1991), and such events have been used to explain the general tendency for New Zealand tree species to have relatively low genetic diversity compared with similar plants in other countries (Hawkins & Sweet, 1989; Billington, 1991; Haase, 1993).

Similar bottlenecks may be caused in the future by climate change. In New Zealand, the impacts of climate change have already been observed and are predicted to increase further in the future (Schiel *et al*., 2004; Morrisey *et al*., 2010; Lundquist *et al*., 2011; Wethey *et al*., 2011). The impacts include mortality, change in species abundance and distribution, rise in sea levels reducing coastal habitats, fire risk, erosion and sedimentation (Lundquist *et al*., 2011).

Browsing by introduced possums appears to be the major reason for the recent decline of populations of *M. bartlettii* (de Lange, 2003). The buds, flowers, fruits, young shoots and seedlings are very palatable to possums, which may have a devastating effect on the

reproduction and re-vegetation of *M. bartlettii*. Grazing of flowers and shoots has been reported to have caused the decline of other rare and endangered species such as *Anemone patens* (Uotila, 1996) and *Pulsatilla patens* (Szczecinska *et al*., 2016).

Possums were introduced to New Zealand from Australia in 1858 for a fur trade (Cowan, 2005; Jones *et al*., 2012). Since their introduction, they have become widespread (c.30 million possums according to Warburton *et al*., 2009) and have become one of the major threats to New Zealand´s biodiversity (Norton, 2009; Innes *et al*., 2010; Byrom *et al*., 2016). They have caused the decline of several New Zealand plant species (Burns *et al*., 2011; Szczecinska *et al*., 2016), including southern rata (*M. umbellata*), northern rata (*M. robusta*; Atkinson *et al*., 1995), and kohekohe (*Dysoxylum spectabile*), which has been browsed to near extinction (Nugent *et al*., 2002). Possums have also caused similar impacts on global biota (Mack *et al.*, 2000).

Despite current control efforts and improved knowledge of their abundance and impact, possums and other mammal pests are still affecting 81% of the country´s indigenous forest (MPI, 2015). One of the difficulties with controlling possums is that they can obtain their food from various sources, including indigenous birds and non-forested areas such as pasture and farmland (Dodd *et al*., 2006, 2011). Hence, even if they are kept outside forested areas, they can continue to survive and reproduce.

Although possums are the major threat to populations of *M. bartlettii*, the species might have experienced its biggest historical decline due to changes in land use following the settlement of New Zealand by Polynesian people 750 years ago and by Europeans in the 19th and 20th century (MPI, 2015). The early Maori burned forests to clear land for agriculture, to favour the growth of bracken fern (*Pteridium aquilinum*), to facilitate cross-country travel and to make moa hunting easier (Bussell, 1988; McGlone, 1989). Europeans further destroyed forests for timber, expansion of agriculture, settlement and social infrastructures (Kirch, 1982; Ewers, 2004). Approximately half of New Zealand's original forest was destroyed in 1840s and 1850s following widespread settlement by Europeans (McGlone, 1989).

Over the past 800 years, humans have caused the loss of many endemic species in New Zealand (MfE, 2007), including seven plant species (de Lange *et al*., 2013). Prior to human settlement, the forest cover of New Zealand was about 90% (Gillman, 2008). Recent satellite-based estimates show that the current forest cover of the country is about 38% (MPI, 2015).

Forest and habitat fragmentation are threats to global biological diversity, affecting species survival and ecosystem functions (Wu *et al*., 2003; Board, 2005; Pimm & Brooks, 2013). Habitat fragmentation has affected the pattern and composition of plant communities, caused the loss of epiphytes, the decline of species richness, and facilitated the establishment of invasive species in New Zealand (Walker *et al*., 2006; MPI, 2015) and elsewhere (Kupfer *et al*., 2006; Fischer & Lindenmayer, 2007; Arroyo-Rodriguez *et al*., 2007; Allnutt *et al*., 2008; Gibson *et al*., 2011).

Apart from directly affecting the abundance and distribution of plant species, habitat fragmentation can have also an indirect effect on the fitness of populations by reducing gene flow between populations and thereby enhancing mating within populations leading to inbreeding depression (Wright *et al*., 2008; Charlesworth & Willis, 2009). Hence, forest fragmentation can have both short-term demographic and long-term genetic effects that may drive populations to extinction (Alharbi & Petrovskii, 2016). Mathematical models also show that extinction is more likely to happen in small and fragmented populations (e.g., Richter-Dyn & Goel, 1972; Lande, 1987; Dennis 1989; Alharbi & Petrovskii, 2016).

Fire is also a threat to *M. bartlettii* (de Lange, 2003). While natural fire was a significant factor in the destruction of New Zealand´s forest long before Polynesian settlement (McGlone, 1989), the intentional destruction of forests with fire for various purposes has been a major cause of the transformation of New Zealand´s landscape following Polynesian settlement (Kirch, 1982).

Generally, the decline of *M. bartlettii* can be attributed to a combination of factors including possum grazing, habitat and forest destruction, fire, and more. These factors might have contributed to the very small bottlenecked populations of *M. bartelettii* that exist today. Population bottlenecks strongly influence the maintenance of rare alleles (Nei *et al*, 1975), which make a significant contribution to the genetic diversity of endemic species (Ellstrand & Elam, 1993). As populations decline in number, they will inevitably face the two main genetic consequences of small population size: genetic drift and inbreeding (Bouzat, 2010).

Genetic drift is defined as random fluctuations in the frequency of alleles between generations, which occurs because gametes transmitted from one generation to the next carry only a sample of alleles that were present in the parental generation (Ellstrand & Elam, 1993). Many genetic variations are maintained by the balance between mutation (adding

variation), selection (adding or removing variation) and genetic drift (removing variation; Rodriguez-Ramilo *et al*., 2004).

Because of the random nature of genetic drift, adaptive alleles can be lost and deleterious alleles can become fixed in populations just by chance (Lande, 1994; Hamrick & Godt, 1996a; Solórzano *et al*., 2016). This random loss and fixation of alleles leads to the decline of genetic variation (genetic erosion), which is evident in *M. bartelettii*. While random changes in allelic frequencies due to genetic drift are generally small in larger populations, such changes can be large and unpredictable in smaller populations (Barrett & Kohn, 1991). Hence, the loss of genetic diversity is more accelerated in smaller populations than in larger populations (Frankham, 1996; Gitzendanner & Soltis, 2000; Leimu *et al*., 2006).

The other genetic consequence of small population size is inbreeding, defined as mating between individuals of a given population that are more genetically similar than individuals drawn at random from the population (Hedrick & Kalinowski, 2000). A meta-analysis of 116 studies and 107 plant species by Angeloni *et al*. (2011) shows that inbreeding is common among plant species. Inbreeding decreases genetic diversity by increasing the relative proportion of homozygotes above levels expected under random mating, since the probability of mating between two genetically similar individuals is higher in smaller populations (Schaal & Leverich, 1996; Young *et al*., 1996).

Inbreeding can also cause inbreeding depression, which is a decline in the fitness (reproduction, survival, growth rate, or seed set) of individuals (Wright *et al*., 2008; Charlesworth & Willis, 2009). Inbreeding depression has been implicated in significant fitness decline in New Zealand plants (e.g., Garnock-Jones & Molloy, 1982; Delph & Lloyd 1996) and bird species (e.g., Jamieson *et al*., 2007; Grueber *et al*., 2010; Kennedy *et al*., 2014).

Two hypotheses may explain how inbreeding causes a decline in fitness. The 'over dominance' hypothesis suggests that heterozygotes have better fitness than homozygotes, and therefore a reduction in fitness is due to an increase in the relative proportion of homozygous loci caused by inbreeding (East, 1908). The 'partial dominance' hypothesis, which is more strongly supported, instead suggests that a decline in fitness is caused by an increase in the frequency of deleterious alleles due to inbreeding (Charlesworth & Charlesworth, 1999; Roff, 2002).

In the absence of factors that increase genetic variation (mutations and gene flow), the relationship between population size and loss of genetic diversity due to genetic drift and inbreeding can be described by the following equation (Wright, 1969):

$$H_t/H_0 = \left(1 - \frac{1}{2N_e}\right)^t = 1 - F$$

Where, H_t is heterozygosity (gene diversity) at generation t, H_o = initial heterozygosity, N_e = the genetically effective population size and F = the inbreeding coefficient (proportion of loss of genetic diversity).

This equation implies an exponential decline of genetic diversity with generations and that the rate of decline is greater in smaller populations than larger populations. This explains why the decline of genetic diversity due to inbreeding and genetic drift is more pronounced in smaller populations than larger populations.

It is important to note that the effective population size (N_e) determines the rate of loss of genetic diversity, and therefore it is more important to conservation genetics than the actual census population size (N) (Woodruff, 2001; Charlesworth, 2009). The effective population size (N_e) is the number of individuals in an ideal population (a population where there is random mating, discrete or non-overlapping generation, equal sex ratio, constant population size and equal probability for all individuals to contribute offspring to the next generation) that would have the same genetic response to random processes as the census population of size N (Wright, 1931).

The effective population size is often smaller than the census size due to biased sex ratios, overlapping generations, variation in progeny production, and fluctuations in population sizes (Crow & Denniston, 1988; Frankham, 1995; Rieman & Allendorf, 2001; Charlesworth, 2009). The average value for N_e/N ratio in unmanaged populations has been reported to be approximately 0.1 (Frankham, 1995).

The inbreeding coefficient (F_{is}) for *M. bartlettii* is very low (-0.04), which suggests that inbreeding is not a cause of decline in genetic diversity for this species. This makes sense because of the species' self-incompatibility and outcrossing mating system, which reduces selfing or mating between genetically similar individuals, probably to avoid the deleterious effects of inbreeding (Duminil *et al*., 2009). If this is the case, then genetic drift most likely is the remaining contributor to low genetic diversity.

The level of inbreeding and inbreeding depression can be influenced by several species characteristics such as longevity (perennial vs. annual), life form and mating system (Lande & Schemske, 1985; Duminil *et al*., 2009). Duminil *et al*. (2009) reported a strong association among longevity, mating system, and level of inbreeding among plant species. They showed that most long-lived plant species (perennials) are outcrossing, while most short-lived plant species (annuals) are selfing. They also showed that long-lived outcrossing species have lower inbreeding coefficients than short-lived species. This could be because short-lived plants generally have a short stature, which may reduce gene flow and facilitate mating between closely-spaced and genetically related individuals. Alternatively, long-lived plants might be more sensitive to inbreeding depression than short-lived plants, so that individuals carrying deleterious alleles are eliminated through natural selection, thereby lowering the inbreeding coefficient in perennial plants.

Scofield and Schultz (2006) suggested that the deleterious effects of inbreeding depend on the number of deleterious mutations that occur through errors in DNA replications. Because perennial woody plants undergo more mitotic cell divisions per cycle than short-lived plants, the number of deleterious mutations is expected to be higher in perennials, and therefore perennial plant species are more sensitive to inbreeding that combines these deleterious alleles and affects the fitness of individuals.

The life form and breeding system of *M. bartlettii* might therefore be the reason for its low level of inbreeding and for its relatively robust genetic diversity, given the current number of trees and populations. Long-lived outcrossing species can still maintain a higher genetic diversity than expected despite extreme demographic decline (Young *et al*, 1996; Hamrick & Godt, 1996b; Nybom & Bartish, 2000).

Genetic variation within populations

Of the three populations, the Unuwhao population showed the highest genetic diversity (AFLP H_e = 0.23; SSR H_e = 0.59). This population also had a considerably higher number of private alleles (10) than Radar (one private allele) and Kohuronaki (five private alleles). Moreover, all the private alleles in the Unuwhao population were in high frequencies ($\geq$ 0.1), while only two of the five private alleles of the Kohuronaki population had frequencies $\geq$ 0.1 and none from the Radar population. Hence, the Unuwhao population will have a greater

contribution to the conservation of *M. bartelettii* than the other two populations due to its highest number of unique alleles.

The presence of ten private alleles (Unuwhao) and five private alleles (Kohuronaki) in such small populations and the low level of gene flow among the three populations strongly suggest that the reduction of genetic diversity of *M. bartlettii* has been caused by genetic drift and geographic isolation of populations. In summary, it is likely that the current genetic diversity of *M. bartletti* reflects both historical bottlenecks from past climatic changes, deforestation, and land-use change as well as current threats such as fire and browsing by introduced possums.

3.5.2 Genetic structure

The genetic structure of a species refers to the distribution of the genetic variation within and among its populations. It is the result of interaction among factors such as selection, genetic drift, gene flow and mating system (Loveless & Hamrick, 1984; Duminil *et al*., 2007; Sork, 2016). Natural selection leads to the transmission of alleles with adaptive value from generation to generation. On the other hand, maladaptive alleles are selected against and eliminated from populations. When different selection pressures operate in two different populations, alleles that are selected for in one population may be selected against in another population and therefore the allelic frequencies of the two populations become increasingly different over time, leading to divergence (Hamilton, 2009)

Genetic drift not only decreases genetic diversity, but also increases the genetic divergence among populations (Loveless & Hamrick, 1984; Ewens, 2004). This is because when two populations of a species are isolated and become smaller in size, each population will experience independent genetic drift leading to the fixation and elimination of different alleles in different populations. In this way, the random process of genetic drift leads to changes in the frequency of alleles between populations and later the populations become genetically divergent (Hamilton, 2009).

Gene flow is the active or passive movement of individuals, gametes, or seeds from one population to another and involves not only the dispersal of immigrant genotypes but also their successful establishment in the new population (Slatkin 1985; Woodruff, 2001). While genetic drift and selection facilitate the divergence of populations, gene flow tends to

homogenize populations (Slatkin, 1985; Young et al. 1996; Ouborg *et al*., 2006; Lowe *et al*., 2005).

The mating system of plants is another factor that dictates the spatial distribution of genetic variation within and among populations (Loveless & Hamrick, 1984; Nybom & Bartish, 2000; Duminil *et al*., 2007). Outcrossing species have a larger portion of the total genetic diversity within populations, whereas selfing plants have a larger portion of genetic diversity between populations. This is because outcrossing reduces the effect of inbreeding (i.e., increases diversity within populations) and increases gene flow (i.e., reduces genetic differentiation or variation among populations). On the other hand, selfing increases homozygosity within populations and reduces gene flow among populations (Loveless & Hamrick, 1984).

The genetic structure analyses of *M. bartlettii* based on both AFLP and SSR markers shows that a larger portion of the total genetic diversity of *M. bartlettii* is contained within populations than among populations, which is typical of a long-lived, outcrossing species (Gitzendanner & Soltis, 2000; Honnay & Jacquemyn, 2007). Results from the total F_{st} analyses of both markers showed a high level of population genetic differentiation according to the categorization of F_{st} values by Wright (1978) and Hartl and Clark (1997). The population pair-wise F_{st} estimates also show a moderate (between Unuwhao and Radar) to large (between Unuwhao and Kohuronaki, and between Radar and Kohuronaki) genetic differentiation. Although the P value for the estimate of Unuwhao-Radar genetic differentiation ($P = 0.431$) does not suggest a significant genetic differentiation between these two populations, the F_{st} value (0.12) is quite high for conspecific trees, and therefore the lack of statistical significance of this F_{st} value could be due to the very low population sizes of these populations.

An estimation of sound F_{st} requires a quality estimate of allele frequencies which in turn requires a good sample size (Selkoe and Toonen, 2006). However, this should not be a concern as census populations rather than samples were used in the present study. The UPGMA (SSR) and PCO cluster analyses (AFLP and SSR) also revealed a somehow moderate level of genetic differentiation between Unuwhao and Radar populations while the Kohuronaki population appear as a distinct genetic population which is also supported by the structure analysis.

The level of gene flow (*Nm*) based on F_{st} values was 0.89 for AFLP and 1.00 for SSR, both of which are similar to the minimum value (*Nm* = 1) required to prevent genetic differentiation between populations via genetic drift (Slatkin, 1987). However, this minimum gene flow threshold may not be enough to homogenize small populations (Couvet, 2002; Wang, 2004). The F_{st} value, therefore, suggests that there has been a minimal gene flow between populations of *M. bartlettii*, resulting in an overall high genetic differentiation among populations.

Given that the populations are no more than eight km apart and given *M.bartlettii* is an outcrossing species whose seeds can be easily wind dispersed farther than eight km, the relatively high genetic differentiation among the populations was unexpected. It is likely that some gene flow may still be occurring among populations, but insufficient to homogenize populations. This may be due to the extremely low number of trees in the wild, which means that not enough individuals are available to provide successful seed dispersal.

The small number of trees in each population also means that bird pollinators may not frequently visit the populations, because as they do not offer a guaranteed food source. Most pollinators are generalists and would prefer to visit other plant species when their preferred species occurs in low numbers (Ashman *et al*., 2004; Gascoigne *et al*., 2009). de Lange (2003) suggested that one threat to *M. bartletttii* is lack of sufficient viable seed set due to a lack of nectar-feeding pollinators. Little is known about the biology and ecology of *M. bartlettii*, but studies of closely related *Metrosideros* species (Carpenter, 1976) suggest that *M. bartlettii* is probably pollinated by nectar-feeding native birds.

In addition, when the density of a plant species is low, generalist pollinators may pick up pollen from surrounding species and block the plant's stigma with the wrong pollen. This has been reported for other plant species such as *Cassia biflora* (Silander, 1978) and *Clarkia concinna* (Groom, 1998).

The density of individuals within a population can affect the viability and reproductive success of the population through what is known as the Allee effect (Knight *et al*., 2005; Berec *et al*., 2007; Kramer *et al*., 2009). The concept was introduced by W.C. Allee (Allee, 1931) and refers to the positive correlation between the size and density of populations and their fitness. Animal-pollinated plant species may suffer from low seed set due to pollen limitation if the size or density of their population is too small or too isolated to attract their biotic pollinators (Jennersten, 1988; Lande, 1988; Groom, 1998). Therefore, the very low

density *M. bartlettii* populations may have failed to attract bird pollinators that would have enabled the production of sufficient seeds to enhance gene flow and increase population size through seed dispersal. The positive correlation between population size of a plant species and the abundance and frequency of pollinator visitation has been documented in several other studies (e.g., Groom, 1998; Brys *et al*., 2004; Spigler & Chang, 2008; Duffy *et al*., 2013).

In New Zealand since human colonization around 1280 AD, deforestation and the introduction of predatory mammals have caused the extinction of 41% of the country´s bird species (Holdaway *et al*., 2001). This has in turn led to the disruption of ecological interactions, including pollination (Kelly *et al*., 2010). For instance, *Sophora microphylla* has experienced high pollen limitation (58% decline in seed production) due to low visitation by endemic bird pollinators (Van Etten *et al*., 2015). The pollen limitation also resulted in a high selfing rate (61% of offspring were selfed) and high inbreeding depression (selfed individuals were 86% less fit).

Schmidt-Adam *et al*. (2000) similarly implicated the decline of native bird pollinators in increased selfing in *M. excelsa*. Natural populations of *M. excelsa* consist of a mosaic of self-compatible and self-incompatible individuals (Schmidt-Adam *et al*., 1999), which makes a shift to self-fertilization possible. In the absence of sufficient pollination, plants may be forced to shift their mating system towards self-pollination (Lennartsson, 2002; Herlihy & Eckert, 2002; Quesada *et al*., 2003; Ashman *et al*., 2004; Delmas *et al*., 2015), resulting in enhanced selfing and inbreeding depression, and ultimately a decrease in fitness and genetic diversity. Hence, the low abundance of pollinators affects not only the quantity of seeds but also the quality of seeds and offspring. In addition, when populations fail to attract their main pollinators, they tend to rely on less preferred or ineffective pollinators, which might also lead to a decline in fecundity (Duffy *et al*., 2013).

The abundance and behaviour of pollinators can have a huge effect on gene flow and will determine the ability of species to counteract the effect of genetic drift (Aguilar *et al*., 2008). Bergquist (1989) mentioned that the tui (*Prosthemadera novaeseelandiae*), one bird species that pollinates *Metrosideros*, forages across distances of 15km, which is farther than the distance separating *M. bartlettii* populations. Hence, the low gene flow among populations is not because the populations are beyond the reach of the pollinators but rather the low density of the populations probably fails to attract such birds.

3.5.3 Implications for *M. bartlettii* conservation

The conservation and management of many endangered species has been successfully guided and improved by genetic data (Ellstrand & Elam, 1993; Scribner *et al*., 2016; Gardiner *et al*., 2017). If genetic factors are ignored in the management of threatened species, inappropriate management actions and resource allocations are more likely to result (Frankham, 2003). For example, populations with low genetic diversity may be wrongly used for reintroduction. Very different evolutionary units may be mixed, resulting in reduced fitness. Gene flow levels cannot be known and correctly managed, and problems may arise related to the loss of diversity of self-incompatibility alleles (Frankham, 2005). Hence, it is hoped that this population genetics study of *M. bartlettii* will contribute to the conservation of this species by providing genetic data for *M. bartlettii* that will help its conservation.

The low genetic diversity and the few trees and populations left in the wild suggest that *M. bartlettii* does require urgent conservation interventions. Without such interventions, the limited genetic diversity of *M. bartlettii* will likely further decline and eventually lead to the extinction of this species. Smaller and isolated populations are more susceptible to extinction due to environmental, demographic and genetic stochasticity (Shaffer, 1981; Ellstrand & Elam, 1993; Lande, 1993; Frankham, 1996; Groom, 1998; Matthies *et al*., 2004; Wootton & Pfister, 2013).

Demographic stochasticity arises from random factors that affect the birth and death rates of populations (Lande, 1993). For instance; a random variation in sex ratios and reproductive potentials in females can decrease the size of populations and lead them to extinction. The effects of such events are more pronounced in smaller populations, whereas they tend to average out across time and space in larger populations (Lande, 1988; Lande, 1993; Jeppsson & Forslund, 2012).

Environmental stochasticity arises from such external factors as rainfall, temperature, availability of food, competitors, predators and diseases (Rai, 2003). Although environmental stochasticity can affect the viability of both large and small populations, its effect is greater in smaller populations that are isolated from each other (Lande, 1963; Hedrick & Miller, 1992).

Genetic stochasticity refers to random fluctuations in gene frequencies due to genetic drift and inbreeding (Rai, 2003). I have already discussed why the effects of genetic drift and inbreeding are greater in small populations. These genetic factors can increase local extinction through the fixation and expression of deleterious mutations (Lynch *et al*., 1995;

Bijlsma *et al*., 2000; Frankham, 2005). Some biologists argue that demographic and environmental stochasticities are more important than genetic factors in causing extinction (e.g., Simberloff, 1988; Caro Laurenson, 1994; Wootton & Pfister, 2013). However, although the role of genetic stochasticity in species extinction is debated, there is ample compelling evidence for the role of genetic factors in species extinction (Caro & Laurenson, 1994; Spielman *et al*., 2004; Frankham, 2005; Vilas *et al*., 2006; O´Grady *et al*., 2006).

A clear example of the role of genetics in species extinction is *Hymenoxys acaulis var. glabra*, a self-incompatible species from, USA (DeMauro, 1993). A lack of diversity in self-incompatible alleles in this species effectively led to the extinction of the one remaining population, where all plants were a single mating type. Another example of direct evidence of extinction due to loss of genetic variation is the herb *Clarkia pulchella*, which Newman and Pilson (1997) planted in an experimental population, founded with a low level of genetic diversity. They showed this population exhibited 75% extinction rates over three generations in the wild, while populations with a higher level of genetic diversity had only a 21% extinction rate. It is therefore important to realize that *M. bartlettii* may be facing an extinction risk from the genetic consequences of small population size, unless efforts are made to boost the genetic diversity and abundance of this species.

3.5.4 Conservation management recommendations

3.5.4.1 Protection of existing populations

As a short-term strategy for conservation of *M. bartlettii*, the first priority should be to protect all existing populations and individuals from further decline. To this effect, fences should be constructed to prevent grazing animals. In New Zealand, fencing to exclude grazing animals is a commonly used management tool (Burns *et al*., 2011; 2012) and it has helped in the recovery of fragmented populations of other species such as kahikatea (*Dacrycarpus dacrydioides*; Smale, 2004; Smale *et al*., 2005) and tawa (*Beilschmiedia tawa*; Innes 2009; Dodd *et al*., 2011). These studies also showed that where possums were not controlled, regeneration of other palatable canopy species such as mangeao (*Litsea calicaris*) was supressed in favour of less palatable sub-canopy tree species. Exclusion of stock by fencing has also been reported to improve the conditions of Australian native species (e.g., Pettit *et al*., 1995; Spooner & Briggs, 2008)

However, fencing may not be effective against possums and may also be expensive (Burns *et al.*, 2012). Hence, other control methods such as banding trees with aluminium and poisoning or trapping possums could be used (Gormley *et al.*, 2012; Byrom *et al.*, 2016). However, poisoning and trapping cannot be long-term solutions since surviving possums can quickly breed and boost the declining population. Moreover, although the application of aerial poisoning has helped to reduce the density of possums and their impact on New Zealand's vegetation (e.g., Nugent *et al.*, 2010; Byrom *et al.*, 2016) and on native birds and invertebrates (Powlesland *et al.*, 2003; Byrom *et al.*, 2016), such application may not be effective as it may cause damage to the environment and other native species. Moreover, private landowners may not be open to aerial poisoning as a control option and in fact that may partly explain why the populations are currently in such poor state.

Perhaps, the most effective strategy is to use a biological control method to decrease possum numbers. Currently, Landcare Research is developing an immunocontraceptive vaccine to reduce possum fertility. The vaccine is hoped either to prevent female possums from producing eggs or to affect fertilization of eggs if they are produced (http://www.landcareresearch.co.nz/publications/factsheets/possums).

For species that have most genetic diversity within rather than between populations, theoretically only a few populations need to be preserved to maintain the majority of genetic variation (Neel & Ellstrand, 2003). However, in this case only three populations with a few individuals remain and so all populations and individuals need to be preserved. The large trees in each of the populations deserve special attention, as these individuals are reservoirs of genetic variability acquired over a long time. This variability can be helpful to maintain the reproductive fitness and evolutionary potential of a species in the face of unpredictable and changing environmental conditions (Mosseler *et al.*, 2003; Cruse-Sanders *et al.*, 2005).

3.5.4.2 Increasing current populations

Once populations have been protected, the next priority and longer-term conservation strategy should be to increase the population sizes and restore the fragmented habitat of *M. bartlettii*. Soule (1980) suggested that an effective population size (N_e) of 50 is needed to avoid extinction and ensure short-term survival of populations. To maintain adequate genetic variation for long-term survival and evolutionary potential, Franklin (1980) suggested an effective population size (N_e) of 500. The 50/500 rule has been used to calculate the

minimum viable population size (MVP) that should be maintained to avoid extinction and ensure short-term and long-term survival of species.

Shaffer (1981) gave this tentative and arbitrary definition for MVP:

> *A minimum viable population for any given species in any given habitat is the smallest isolated population having a 99% chance of remaining extant for 1000 years despite the foreseeable effects of demographic, environmental, genetic stochasticity, and natural catastrophes.*

Generally, a MVP can be defined as the minimum population size at which a population is likely to persist over some defined period of time with a given probability of extinction (Jamieson & Allendorf, 2012). Below this size, populations will decline and fall into an extinction vortex, as described by Gilpin and Soule (1986).

Frankham (1995) suggested that the average value for the N_e /N ratio in unmanaged populations is approximately 0.1. Based on this ratio, the MVP that is required to ensure short-term and long-term survival of populations or species are 500 (50 x 10 = 500) and 5,000 (500 x 10 = 5,000) individuals, respectively. Some authors have proposed a much larger effective population size is needed to ensure the long-term survival and evolutionary potential of populations. For instance, Lande (1988) and Franklin and Frankham (1998) suggested N_e = 5,000 while Frankham *et al*. (2014) recently recommended the doubling of the 50/500 rule (i.e., N_e = 100 to avoid extinction in the short-term and N_e = 1000 to maintain genetic diversity and evolutionary potential for long-term survival.

The 50/500 rule and MVP are generally accepted by most researchers, despite debates over their application in species conservation (proponents include Rai, 2003; Traill *et al*., 2007; Traill *et al*., 2010; Brook *et al*., 2011; Jamieson and Allendorf, 2012; and Frankham *et al*., 2014, while opponents include Caughley, 1994; Henriksen, 1997; Flather *et al*., 2011a; 2011b; and Beissinger *et al*., 2011). The opponents of applying the 50/500 rule and MVP to species conservation argue that there should not be a single magic target number for the MVP size of all populations or species, because there is much variability in life history and environmental conditions that may affect the dynamics of populations and species.

The fact that different authors have reported different MVP sizes for various species supports this argument (e.g., Thomas, 1990; Reed & Bryant, 2000; Reed *et al*., 2003; Brook *et al*., 2006). Species-specific MVPs are often estimated using population viability analysis (PVA) models. PVA is a simulation method that incorporates several demographic parameters (e.g.,

population size, birth rate, growth rate), environmental factors, and genetic parameters (e.g., genetic drift, inbreeding) to analyse their combined effect and predict the probability that a given population may become extinct in the coming 50, 100, or 1000 years (Henriksen, 1997; Beissinger & Westphal, 1998; Akcakaya & Sjogren-Gulve, 2000; Brook *et al*., 2006). PVA models can also estimate the MVP size that is required to reduce the extinction risk of a given population to an acceptable level (Keedwell, 2004).

In his review of 12 years of research on genetics of small populations in New Zealand species, Jamieson (2015) criticized the use of a threshold N_e of 500 and its equivalent MVP of 5,000 individuals. In this review and several other co-authored papers (Jamieson & Allendorf, 2012; 2013), these threshold values are criticised for several reasons. First, this threshold value suggests that recovery programs that do not have a goal of reaching 5,000 individuals should be denied funding and resources. Second, the N_e/N ratio (0.1; Frankham, 1995), which is used to estimate MVP from N_e, is highly variable across species and even within species based on demography and life history traits. Third, the doubling of the N_e size of 500 by Frankham (2014) was not justified by any convincing new empirical evidence.

Hence, Jamieson (2015) suggested a two-stage approach to manage small populations rather than using the minimum threshold approach. The two-stage approach asserts that priority should be given first to securing a species from extinction by halting any further decline (e.g., fencing populations to exclude grazing animals, preventing overharvesting of trees, etc.). The second stage then includes the recovery of the secured species by implementing management actions that create suitable conditions for the growth and reproduction of populations (e.g., improving habitat, establishing new populations, etc.). The author emphasized that an MVP of 5,000 individuals should be understood as a long-term goal to maintain a genetically healthy population rather than as a magic threshold point to predict extinction or a criteria for allocating funding and resources.

Despite these criticisms, there are also strong arguments in support of using these threshold values. First, for generation of an accurate species-specific MVP estimate, PVA models require sufficient and extensive data (Reed *et al*., 2003; Keedwell, 2004; Frankham *et al*., 2014), which is not usually available for rare and endangered species. Moreover, many less developed nations may have financial and resource constraints that preclude intensive studies and the collection of sufficient data for each and every threatened species. Hence, in the absence of sufficient data and resources, a generalized MVP is important as the alternative will usually be unscientific and politically influenced (Belovsky *et al*., 2004; Brook *et al*.,

2011). Moreover, current extinction rates are so rapid that managers do not have time to wait for the collection of extensive, high quality data to make decisions (Lee & Jetz, 2008).

Second, using a single MVP can be justified because most species are likely to exhibit many similar characteristics and responses to small population size (Traill *et al*., 2010; Frankham *et al*., 2014). For instance, the correlation among genetic diversity, fitness and population size is positive in most species (Frankham, 2012), most species are negatively affected by inbreeding (Crnokrak and Roff, 1999), and most species have a comparable rate of mutation for quantitative traits (Houle *et al*., 1996).

Third, several meta-analysis studies found MVP sizes close to $N_e = 500$ (or census size of $N =$ 5,000; e.g., Thomas, 1990 - MVP = 5,500; Reed *et al*., 2003 - MVP = 5,816; Trail *et al*., 2007 - MVP = 4,169). This shows that MVPs based on the 50/500 rule could be good estimates for some species. However, this argument is not as convincing since several other studies reported very different MVP estimates for other species, as mentioned above.

Generally, it is complex and difficult to determine a specific point (MVP size) beyond which extinction is more or less likely to occur. However, a simplified approach is needed to make quick conservation decisions and to serve as a basic guideline especially in the absence of sufficient time, data or resources to generate specific MVP estimates for every threatened species that requires urgent management action. Thus, the simplicity and quick application of the 50/500 rule and MVP are used as a default by many managers (Woodruff, 2001; Traill *et al*., 2007).

However, care needs to be taken in interpreting the threshold values. Jamieson and Allendorf (2012) argue that the threshold values should be viewed as guiding principles to emphasize when genetic factors are likely to have an important role in the viability of populations, and as long-term goals for the maintenance of genetically healthy populations. They argue that they should not be used as absolute predictors of extinction or as thresholds to make decisions for conservation triage (a management action where populations with little chance of survival are considered hopeless and denied management intervention to direct conservation resources to populations and species that have a better chance of survival or are of high human interest; Traill *et al*., 2010).

This is important because some species may have a higher probability of long-term persistence despite having a population size well below the threshold MVP of 5,000 individuals. For instance, a PVA analysis of the threatened bog turtle in the United States

showed that as few as 15 individuals had greater than 90% probability of viability for more than 100 years. The authors concluded that the general MVP threshold may be too high for many long-lived organisms, and that very small populations can have great conservation significance for such species (Shoemaker *et al*., 2013). Importantly, small populations that are considered hopeless based on the general MVP rule of thumb might also contribute to the survival of other interacting species and to the functioning of their ecosystems.

The threshold value of N_e = 500 also helps to convey that thousands rather than hundreds of individuals are required to minimize the risk of extinction (Traill *et al*., 2010). Generally, a MVP estimate is useful as a simple and easily understandable guideline to communicate the science of conservation to policy makers (Tear *et al*., 2005). Hence, despite opposition from some biologists, the use of a generalized MVP across species is still receives significant attention from the scientific community for conservation decisions (Traill *et al*., 2010; Bradshaw *et al*., 2011; Brook *et al*., 2011).

In this study, the current population size of *M. bartlettii* is much lower than the minimum MVP based on the 50/500 rule. This suggests that there is a high probability of extinction for this species due to genetic, demographic and environmental stochasticities unless urgent conservation measures are put in place to boost its population size and increase its genetic diversity.

Ottewell *et al*. (2016) developed a simple decision-making tree for genetic management of populations based on the magnitude of three parameters: genetic diversity, inbreeding, and population genetic differentiation. The value of each parameter is designated as high or low by comparing it to the corresponding values for a genetically healthy reference population (i.e., a population with similar life history traits and other characteristics). *M. bartlettii* has low genetic diversity, a low inbreeding coefficient, and high population genetic differentiation. According to Ottewell *et al*. (2016)'s decision-making tree, this combination fits with his 7th management strategy, which recommends: (a) that the species' genetic diversity should be recovered *in situ* by increasing its population size, and (b) that in the absence of information about risks of outbreeding depression, individuals should be exchanged between populations to introduce new genetic diversity.

The *in situ* conservation approach is considered the most effective method to conserve endangered species (Shen *et al*, 2009). Population size in the wild can be enhanced by replenishing existing natural populations using seeds and transplants. To assist with this, *ex*

situ conservation will also be useful, as a source of stored germplasm for propagation. As an *ex situ* conservation strategy, germplasm can be maintained in seed banks and botanic gardens. Because this population is extremely small, the seed bank for *M. bartlettii* should consist of seeds collected from all trees and underground soil over several years. To avoid a shortage of seeds, small quantities of seeds should be collected frequently rather than collecting large quantities of seeds less frequently (Menges *et al*., 2004). At the same time, the current natural population is so small that it is unlikely adequate seeds can be collected even if frequent collections are taken. Moreover, *Metrosideros* species generally have low seed fertility (Dawson, 1968). Hence, plant propagation should also be considered to generate adequate plant material for revegetation programmes.

Botanic gardens not only serve as a source of germplasm for *in situ* conservation but also help to ensure the survival of the species in case unanticipated catastrophic events drive the natural populations to extinction. Botanic gardens also offer opportunities to hand-pollinate plants and use the resulting seeds to grow more plants. This approach has been used successfully for several species (e.g., Li *et al*., 2002; Namoff *et al*., 2010; Wang *et al*., 2015). *M. bartlettii* can grow well in average garden conditions (Hobbs, 1992) and has considerable potential as a street tree (Dawson *et al*., 2010), which presents an opportunity to grow it widely. The possibility of growing *M. bartlettii* trees on agricultural lands should also be investigated, as the species has both conservation and production values (e.g., windbreaks, erosion control, honey production, etc.).

As part of the *in situ* conservation programme, the genetic diversity of the current populations can be enhanced by using plant material sourced from different populations. However, this approach has already been attempted by the Department of Conservation and has failed because of disagreement over land ownership by iwi family members (*pers comm*, Dr Gary Houliston). One approach to convince private landowners to cooperate with DOC on such conservation efforts might be to assist them in managing their natural resources for apiculture or tourism, which can generate income. This would also require additional assistance, such as the improvement of local infrastructure to facilitate access for tourists and the creation of a market for selling honey. Incentives such as fencing permits, subsidised land rates, and financial support for the protection of species could also persuade landowner to assist with conservation efforts. While providing incentives, penalties and regulatory instruments such as controls on deforestation and habitat degradation should also be implemented. Controls on

the clearance of vegetation have been widely implemented throughout New Zealand (Davis & Cocklin, 2001).

Education can also be used as a tool to help land owners understand the consequences of misuse of natural resources and the wide benefits of conserving species, such as enriching soil nutrients, improving hydrological cycles, tourism, and property resale values. Other studies have shown that environmental education can motivate people to get involved in conservation practices (Brewer, 2006; Jordan *et al*., 2011; Chen *et al*., 2015).

In addition, maximum effort should be exerted to resolve land ownership disagreements to allow the introduction of new genetic material into the existing populations, which is crucial to this endangered species. Genetic rescue is the intentional introduction of new genetic material into genetically poor populations (Tallmon *et al*., 2004), which boosts genetic diversity and minimizes inbreeding in the beneficiary populations (Bouzat *et al*., 2009; Hedrick & Fredrickson, 2010). When implementing genetic rescue, the number of plants introduced to the existing populations should be kept low, to avoid a complete change in the current allele frequency and the elimination of private alleles (Frankham, 2015). Moreover, the fitness and genetic diversity of populations can be improved significantly by introducing a small number of individuals (Edmands, 2007).

A recent meta-analysis on the genetic rescue of small plant and animal populations showed that in 93% of the 156 total cases (of which 44 are plant taxa), the intentional introduction of new alleles into populations with poor genetic diversity consistently resulted in improved fitness of beneficiary populations (Frankham, 2015). A pollination experiment by Bossuyt (2007) similarly showed that the introduction of pollen of a perennial herb species (*Parnassia palustris*) from one population to another small population has significantly enhanced the seed set of the beneficiary population, enabling it to produce seeds that are larger, more numerous, and more viable. Genetic rescue has also been used to save several endangered plant species (e.g., Newman and Tallmon 2001; Willi *et al*., 2007; Bossuyt, 2007).

Another conservation strategy for endangered species is to establish new populations from material sourced from all existing populations, to capture as much of the genetic diversity as possible. When choosing a location for a new population of *M. bartlettii*, care should be taken to pick locations that are not climatically suitable for the fungal disease Myrtle rust (see Chapter One, section 1.6). This is an important consideration; because *M. bartlettii* is expected to be susceptible to the disease given other related species (e.g., *Metrosideros*

polymorpha, *M. collina* and *M. kermadecensis*) have already been infected (Uchida *et al*., 2006; Kawanishi *et al*., 2009; Pegg *et al*., 2014; Silva *et al*., 2014). Care should also be taken to avoid the use of infected seeds and seedlings for planting.

To increase the genetic diversity of *M. bartlettii*, the seeds and transplants that are used to establish new populations should be sourced from different populations. Controlled cross-pollinations can also be performed to produce seeds and transplants with higher genetic diversity. For cultivation programs, seeds sourced from different populations should be mixed to facilitate gene flow and enhance the genetic diversity of cultivated plants, which ultimately would contribute to the enhancement of genetic diversity in the wild populations as a whole. Cultivated plants could also be used to boost the species' genetic diversity; because despite having fewer private alleles than the wild populations, this study shows that their genetic diversity is equivalent to that of wild populations.

Because of the significant genetic differentiation revealed among the populations of *M. bartlettii*, the establishment of new populations by growing plants sourced from different populations side by side might risk outbreeding depression, which is a reduction in fitness due to hybridisation between individuals from genetically differentiated populations (Byrne *et al*., 2011; Weeks *et al*., 2011). Compared to inbreeding depression, outbreeding depression is so rarely studied that we do not know enough to predict which species and crossing scenarios will cause it (Edmands, 2007; Kramer & Havens, 2009; Ottewell *et al*., 2016). However, Frankham *et al*. (2011) used empirical data to estimate the probability of outbreeding depression due to adaptive differences among recently fragmented populations and concluded that current concerns about the risks of outbreeding depression are exaggerated. They studied the probability of outbreeding depression as a function of four variables: effective population size, genetic diversity, intensity of selection and number of generations the populations have been in isolation without gene flow. They showed that estimated probabilities of outbreeding depression resulting from the cross of two populations were elevated only when the populations were distinct species with fixed chromosomal differences (distinct karyotypes), and when they had exchanged no genes in the last 500 years or occupied different environments.

Although there is no information on how long the populations of *M. bartlettii* have been separated, it is unlikely that the populations have not exchanged genes in the last 500 years, since the populations are less than ten km apart. Moreover, the populations are found in similar environmental conditions and hence are expected to have similar adaptability. It is

therefore unlikely that the mating of individuals sourced from different populations would lead to the the breakdown of locally adapted genotypes and outbreeding depression.

Current climate changes may also mean that the seed transfer zone should be dynamic rather than rigid, as populations that have adapted to their local conditions in the past may no longer be optimally adapted to present conditions (Kramer & Havens, 2009). Empirical evidence shows that climate change has shifted the range of species, thereby increasing the proximity of populations and species and facilitating hybridisation (e.g., Garroway *et al*., 2010; Muhlfeld *et al*., 2014; Chunco, 2014; Canestrelli *et al*., 2017).

3.5.4.3 Improving habitat

Another long-term conservation strategy should be improving the general habitat in which *M. bartlettii* is found. Prevention of further forest clearance and possum control are not enough to protect the species. An active restoration of its habitat is necessary for the current population to persist into the future. Conserving habitat and ecological processes is the best strategy to conserve species, because species are directly impacted by the interactions they have with biotic and abiotic components of their community (Kramer & Havens, 2009).

Like other *Metrosideros* species, *M. bartlettii* has mutual associations with other plants and animals (Simpson, 2005). For instance, birds depend on it for food, and *M. bartlettii* likewise depends on birds for pollination and on other plants for its germination as an epiphyte. The improvement of its habitat will increase pollinating bird populations, which in turn wil improve the reproductive success of this species. Pollinating birds would likely benefit from growing other plant species that are food sources, but care should be taken to avoid exotic plant species that may compete with *M. barteletii* and affect its flowering and fruit set. Further research needs to be conducted to investigate if insects also pollinate *M. barteletii* and if so, to increase their populations as well. The management of host trees on which *M. bartlettii* is an epiphyte could also help the survival of this species. Host species include puriri (*Vitex lucens*), taraire (*Beilschimedia tarairi*), rewarewa (*Knightia excelsa*), and tree ferns (*Cyathea* spp.) (de Lange, 2003).

In general, the successful conservation of *M. bartlettii* requires an action plan that integrates both *in situ* and *ex situ* management, including the protection of existing populations through fencing and the control of threats such as possums, the reintroduction and translocation of trees into previous habitat or new suitable habitat, and the restoration of the current habitat.

New research is needed to develop methods for enhancing the viability and germination capacity of seeds and seedling establishment for successful propagation and planting, and to devise ways to monitor the success and contribution of propagated and transplanted individuals.

DOC could promote the protection of this species by providing the necessary financial and technical support for regional and local authorities, who have local networks and an understanding of the socioeconomic factors that influence whether private land owners protect the trees growing on their lands. Environmental education should also be used to create awareness among landowners and the general public of the economic, cultural and aesthetic values of this spevies. Revegetation and planting sessions are a unique opportunity for the community to participate and to raise awareness of conservation goals and outcomes (Husk *et al*., 2013).

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Chapter 4: Molecular detection of hybridisation between *Metrosideros excelsa* and *M. robusta* on Rangitoto Island

Abstract

Natural hybridisation among plants is common in New Zealand and elsewhere and can have positive consquences (e.g., enhancing genetic diversity, evolution of adaptive traits, and the formation of new species) or negative consequences (e.g., demographic swamping and genetic assimilation). Evaluating the extent and pattern of hybridisation is important to predict the most likely consequences and to design effective management actions. This study is the first genetic analysis of natural hybridisation between two closely related New Zealand endemic species, *Metrosideros excelsa* and *M. robusta*, on Rangitoto Island using AFLP and SSR markers, and two Bayesian clustering approaches implemented in the programs STRUCTURE and NEWHYBRIDS. The two markers gave comparable results, and the analysis confirmed the presence of high level of introgression between these species. Of 95 individuals tested, AFLP markers detected 26 (STRUCTURE) and 31 hybrids (NEWHYBRIDS) while the SSR markers detected 27 (STRUCTURE) and 28 hybrids (NEWHYBRIDS) using an arbitrary probability threshold of $P \geq 0.9$ to differentiate hybrids from parental classes. The NEWHYBRIDS program detected 12 *M. excelsa* backcrosses, 7 *M. robusta* backcrosses, 7 F1 hybrids, and 3 F2 hybrids, while the SSR markers detected 11 *M. excelsa* backcrosses, 5 *M. robusta* backcrosses, 7 F1 hybrids, and 4 F2 hybrids, suggesting a bidirectional but biased introgression towards *M. excelsa*. The conservation implications of the findings are discussed in detail.

4.1 Introduction

4.1.1 A history of plant hybridisation

Hybridisation has been defined as mating between taxa that have evolved separately over a long time (Barton & Hewitt, 1985; Genovart, 2009). Although the term is usually used to refer to interbreeding among two individuals of different species, it has also been used to refer to mating between individuals of two genetically distinct populations or subspecies of the same species (Rhymer & Simberloff, 1996). Since species boundaries are a continuum rather than distinct (Mallet *et al.*, 2007; Martin and Orgogozo, 2013), the presence of gene

flow and incomplete reproductive isolation results in the continuum of species and populations.

The importance of hybridisation to man dates back to the Neolithic era with the beginning of the domestication of plants and animals, but its mechanism was not understood. The first systematic study of plant hybridisation was performed in 1761 by Joseph Gottlieb Kölreuter, who crossed two herbaceous plant species, *Nicotiana paniculata* and *N. rustica*. By observing the F1 phenotype, he concluded that first-generation hybrids had intermediate morphology between each parent, and that hybrid formation is likely to occur when closely related species in different geographical locations come into contact through man-induced habitat disturbance (Kölreuter, 1761).

Perhaps the most important single contribution in the field of plant hybridisation was made in 1865 by the Austrian monk Gregor Mendel, whose work laid the foundation for modern genetics and the development of the chromosomal theory of inheritance (Sutton, 1903; Bridges, 1916). Mendel's work helped modern scientists understand how traits segregate to produce new plant varieties through hybridisation (López-Caamal and Tovar-Sánchez, 2014).

In the twentieth century, several discoveries advanced our understanding of the mechanisms and consequences of hybridisation. For example, Stebbins (1959) realized the role of hybridisation in evolution and speciation and Rieseberg (1997) demonstrated the mechanisms of hybrid speciation. Levin *et al*. (1996) recognised that hybridisation could lead to the extinction of rare plant species. Today, genome-wide sequencing (Allendorf *et al*., 2010; Hohenlohe *et al*., 2011; Amish *et al*., 2012; Lamer *et al*., 2014) and statistical models (Boecklen & Howard, 1997; Pritchard *et al*., 2000; Anderson & Thompson, 2002; Durand *et al*., 2011) are frequently used to detect complex hybridisation events and to explain the nature and consequences of hybridisation.

4.1.2 Causes and prevalence of plant hybridisation

Natural hybridisation is now understood as a common process among plants and animals (Abbott *et al*., 2013; López-Caamal & Tovar-Sánchez, 2014). It occurs in approximately 10% of animal species (Mallet, 2005), 40% of plant families (Whitney *et al*., 2010), and 70% of angiosperms (Masterson, 1994). The ability of plants to establish themselves through asexual reproduction might explain why natural hybridisation is more common in plants than it is in animals (Seehausen, 2004). Hybrid individuals that are sterile due to a mismatch of parental

chromosomes can maintain themselves through asexual reproduction and can become fertile if somatic chromosome doubling happens due to errors in mitotic cell division (Seehausen, 2004).

The wide occurrence of allopolyploidy in plants suggests that many plant species have a direct hybrid origin or are descended from hybrid species (Soltis & Soltis, 1995). A review of hybridisation in New Zealand by Morgan-Richards *et al*. (2009) showed that hybridisation is a common and important evolutionary process in New Zealand, as it is elsewhere. Hybridisation has been confirmed in at least 19 pairs of New Zealand native plants, insects, fish and bird species. The fact that many plant and animal species hybridize suggests that hybridisation is not a rare occurrence and that it may be an important process in shaping the evolution of many plant and animal species.

Hybridisation between species can happen naturally, or because of anthropogenic involvement such as the modification or disturbance of habitats or the introduction of species from one region to another (Gilman & Behm, 2011; van Hengstum *et al*., 2012; Lopez-Caamal & Tovar-Sanchez, 2014). Empirical evidence suggests that the introduction of species into another location can facilitate hybridisation by bringing together two closely related taxa that were formerly separated by geographical barriers (e.g., Novak & Mack, 2001; Richardson & Pysek, 2006; Ellstrand, 2009).

Anderson and Stebbins (1954) developed the "disturbance hypothesis", which proposes that habitat disturbance by humans can create new habitats that are suitable for the establishment and persistence of hybrid individuals. This hypothesis is supported by empirical evidence (e.g., Esa *et al*., 2000; Lamont *et al*., 2003; Hoban *et al*., 2012; van Hengstum *et al*., 2012). In Australia, habitat disturbance in eastern Victoria has resulted in the hybridisation of *Kunzea peduncularis* with *K. phylicoides* (de Lange *et al*., 2005). Anthropogenic disturbance has also promoted hybridisation between previously isolated *Banksia* species north of Perth, Western Australia (Lamont *et al*., 2003).

Habitat destruction has also been shown to promote hybridisation of New Zealand species. For instance, habitat destruction and the logging of kauri (*Agathis australis*) on Great Barrier Island following European settlement is thought to have resulted in a major hybrid swarm among *Kunzea ericoides var. linearis, K. aff. ericoides* and *K. sinclairill* (Harris *et al*., 1992; de Lange & Norton, 2004). Similarly, anthropogenic habitat disturbance has been implicated in the establishment of hybrids between *Pratia angulata* and *P. perpusilla* on Rotorua Golf

Course (Murray *et al*., 2004). The modification of waterways in Otago also led to the connection of two previously separated headwaters, causing the introgression of two galaxiid fish species (Esa *et al*., 2000).

A high incidence of hybrids at sites with lots of anthropogenic disturbance has been reported by several other authors (Culley & Hardiman, 2009; Thompson *et al*., 2010b; Hoban *et al*., 2012). Possible explanations for this association include: (1) open anthropogenic sites reduce competition for light and water, which facilitates the recruitment of hybrids; (2) habitat modification can facilitate the connectivity of hybridizing species that were previously unconnected; and (3) anthropogenic sites might serve as sources of species introduction.

Hybridisation can also be facilitated by natural and anthropogenic climate change (Garroway *et al*., 2010; Ortego *et al*., 2014; Gomez *et al*., 2015). Climate change can influence hybridisation by pushing the range of one species into another species' range or by changing the physiology, phenology and biotic interactions of species (Chown & Gaston, 2008). Profound climate changes have been reported to facilitate hybridisation between two Chinese species, *Buddleja crispa* and *B. officinalis*, by changing their floral composition, ranges and abundance (Liao *et al*., 2015). A huge amount of empirical evidence has demonstrated the role of climate change in hybridisation (e.g., Gerard *et al*., 2006; Garroway *et al*., 2010; Crispo *et al*., 2011; Muhlfeld *et al*., 2014; Chunco, 2014; Canestrelli *et al*., 2017).

4.1.3 Consequences of plant hybridisation

An interspecific hybridisation can have both useful and detremental effects for the species involved. Generally, hybridisation can have the following three consequences (Genovart, 2009): (i) formation of a stable hybrid zone causing no extinction of parental species (ii) extinction of one or both of the parental species involved in hybridisation; and (iii) formation of a new species (hybrid speciation).

4.1.3.1 Formation of a stable hybrid zone

Hybrid zones are regions that are formed when two populations with weak or incomplete reproductive barriers come in to contact and interbreed to produce hybrid individuals (Barton & Hewitt, 1985). Although the width of hybrid zones can vary, these zones generally form in narrow ecotones where two species meet and interbreed (Swenson & Howard, 2005). The rate of hybridisation and the degree of admixture in hybrid individuals not only depends on

the presence of a geographical overlap but also on other factors such as the suitability of the habitat for the hybridizing taxa and their hybrids (Anderson, 1948; Cullingham *et al*., 2012). In other words, while geographical contact creates the opportunity for hybridisation, environmental factors determine the success and persistence of hybrids and introgression.

There are four models that have been developed to explain how hybrid zones can be maintained in a stable manner over long periods. Prior to these models, hybrid zones were considered ephemeral (Buerkle *et al*., 2003).

Bounded hybrid superiority model

The bounded hybrid superiority model, which was developed by Moore (1977), proposes that a stable hybrid zone can establish in intermediate habitats because selection will favour hybrids that are intermediate to both species and therefore fitter in the hybrid zone. This model predicts a strong correlation between habitats and genotypes.

Dynamic equilibrium model (tension zone model)

Developed by Barton and Hewitt (1985), this model, unlike the hybrid superiority model, assumes hybrids have inferior fitness compared to parental species, because of lack of a complete gene complex of either parent, which they assume is necessary for adaptation to either side of the hybrid zone. According to this model, selection acts against hybrids independent of the environmental conditions and therefore it does not predict a correlation between habitats and genotypes. In this model, a stable hybrid zone can be established at the boundaries or between the distinct parental habitats through a balance or dynamic equilibrium between migration of parental individuals into hybrid zones and the selection against hybrid individuals that were produced.

Mosaic hybrid zone model

The mosaic hybrid zone model was developed based on observations of Harrison (1986) and Howard (1986) that hybrid zones may sometimes be maintained in mosaic habitats that include patches where the hybrids are more fit than parental species. In this model, selection can act either against or for hybrids, depending on the environment and the patchiness of the habitat (Howard *et al*., 1993). This model predicts a strong correlation between habitats and genotypes.

Evolutionary novelty model

The evolutionary novelty model is the most recent model, developed by Arnold (1997). It hypothesises that selection favours hybrids with certain genotypes under certain environmental conditions. Hence, the stability of hybrid zones is maintained by both intrinsic and extrinsic factors. In other words, the fitness of hybrids is predicted to vary depending on the genotype of the hybrid and the prevailing environmental conditions in its habitat. Hybrid individuals are predicted by Arnold's model to perform especially well in novel habitats but are not necessarily predicted to perform worse than parental individuals in the parental habitats.

Each of these four models has support from empirical studies (Bert & Arnold 1995; Buggs, 2007; Campbell and Waser, 2007; Carling & Zuckerberg, 2011; Cullingham *et al*., 2012; Ortego *et al*., 2014), and it is debatable which one is best supported by the majority of naturally occurring hybrid zones. Moreover, some naturally occurring hybrid zones fit into multiple models rather than only one (Bert & Arnold, 1995).

4.1.3.2 Extinction of parental species

The second consequence of hybridisation as discussed above is the extinction of parental species (Rhymer and Simberloff, 1996; Wolf *et al*., 2001). Although hybridisation is not usually mentioned as the major cause of species extinction, some biologists argue that the role of hybridisation in species extinction is greater than is generally considered (e.g. Rhymer and Simberloff, 1996; Levin and Francisco-Ortega, 1996; Wolf *et al*., 2001; Gomez *et al*., 2015). The authors argue that hybridisation can erode the gene pool of the parental species and ultimately drive them to extinction.

Interspecific hybridisation may cause extinction of species in two main ways (Levin *et al*., 1996; Todesco *et al*., 2016), i) – demographic swamping and ii) – genetic assimilation.

Demographic swamping is a decline in the population growth rate of the numerically inferior species when the resulting hybrids tend to have lower fitness compared to the parental species, which may be caused by outbreeding depression (Wolf *et al*., 2001). The decline in the population growth rate of the rare species is associated with the wasteful generation of maladaptive hybrid individuals (Rieseberg and Gerber, 1995; Gompert and Buerkle, 2016). Consequently, the parental species will exhibit a decline in seed set (Ellstrand and Elam, 1993). There are few empirical evidences that attribute local extinction of species to

demographic swamping (e.g. Prentis et al., 2007; Balao *et al*., 2015). Demographic swamping results in extinction of lineages or populations. Genetic swamping on the other hand results in the extinction of parental genotypes, but not necessarily parental alleles or traits which can be maintained in the hybrid populations (Todesco *et al*., 2016).

The other way hybridisation causes extinction of hybridizing species is genetic assimilation where the hybrids are so fertile and vigorous that they can displace one or both parental species through introgressive hybridisation (Riesberg *et al*., 1989). Introgressive hybridisation is the interbreeding of first generation hybrids back to their parents (Anderson, 1953). The mating of two plants of different species does not necessarily mean that introgression will follow next. While in some plant groups hybrids are not capable of reproducing, in other plant groups they are capable of crossing back to parental species. Genetic assimilation is more pronounced on island species because of smaller population size, lack of strong reproductive barriers and invasion of islands by a closely related exotic species (Riesberg *et al*., 1989; Rieseberg and Gerber, 1995).

If there is a limited rate of introgression and if the parental species have similar abundance, then the effect of introgression would be enhancing the genetic diversity within each of the parental species. On the other hand, if there is a significant difference in abundance between the parental species, the introgression may lead to the genetic assimilation of the less abundant parental species by the more abundant species (Levin and Francisco-Ortega, 1996; Rhymer and Simberloff, 1996; Fitzpatrick *et al*., 2010).

Some studies (Ellstrand and Elam 1993; Levin *et al*., 1996; Huxel, 1999; Wolf *et al*., 2001) predicted that interspecific hybridisation can lead to extinction of species, especially when the native species is rare and lacks a competitive advantage over the invading species. However, if the population size of the invading species is lower than the native species and if the hybrids resulting from them are selected against, then most of the newly introduced genome can be purged by the native species (Wolf *et al*., 2001).

A review on hybridisation in New Zealand (Morgan-Richards *et al*., 2009) showed that there are a number of cases of hybridisation involving endangered species with controversial evolutionary and conservation implications. For instance, mitochondrial DNA evidence shows that the introduction of mallard ducks (*Anas platyrhynchos*) in to New Zealand resulted in the rapid decline of populations of the indigenous grey duck (*A. superciliosa superciliosa*) (Rhymer *et al*., 1994; Miskelly *et al*., 2008). The most recent information

(Williams, 2013) shows that grey duck is so extensively hybridized with mallards that only few pure grey ducks may currently exist in New Zealand.

The Ethiopian Wolf (*Canis simensis*) is another example to demonstrate the effect of hybridisation on the genetic assimilation of an endangered species. The Ethiopian Wolf is an iconic endemic tourist attracting wildlife whose genepool is rapidly eroding due to hybridisation with local dogs (Gotelli *et al*., 1994) contributing to the loss of this precious endemic animal as well as the loss of revenue that could have been generated from tourism. Similarly, the hybridisation of European Wolf (*Canis lupus*) with domestic dogs has become a major conservation issue (Randi, 2011; Godinho *et al*., 2015). To pick an example from plant groups, several rare sunflower (*Helianthus*) species of the United States are reported to have been threatened due to hybridisation of these species with weedy species (*H. annuus*) (Rogers *et al*., 1982).

The role of hybridisation in extinction of rare native species can also be manifested through the evolution or further enhancement of invasiveness of alien species via hybridisation. The transportation of species far from their natural distribution has turned many species into invasive species due to higher competitive potentials for the resources in the regions they are introduced into (Mooney and Cleland, 2001). Hybridisation may further reinforce the competitive potential of these invasive species through recombination of new parental alleles that may confer this advantage (Schierenbeck and Ellstrand, 2009; Hovick *et al*., 2012). For instance, Whitney *et al*. (2006) reported that the introgression of genes from *Helianthus debilis* in to the weedy sunflower *H. annuus* through hybridisation rendered the latter species more resistant to herbivory.

Another very recent study on the role of hybridisation and precipitation on an invasive weedy *Raphanus* species showed that hybridisation enabled this hybrid weedy species to plastically respond to a range of drought and excess moisture conditions (Teitel *et al*., 2016). The success of some plant invaders has been attributed to their potential to use energy more efficiently than native species (e.g. Wu *et al*., 2013) and exhibit phenotypic plasticity in order to cope up with varied environmental conditions (Richards *et al*., 2006).

In New Zealand, *Hieracium* species, which are among the most aggressive weeds in the country, appear to have evolved invasiveness through hybridisation after their accidental introduction from Europe, most likely from the United Kingdom (Trewick *et al*., 2004). *Hieracium pilosella* has become an aggressive weed in South Island since its accidental

introduction in to New Zealand 100 years ago (Duncan *et al*., 1997). Once introduced, this alien species has hybridized with another introduced and most abundant related taxon (*H. praealtum*) to form pentaploid hybrids which are one of the most aggressive weeds in New Zealand (Trewick *et al*., 2004). Hybridisation gave these weedy species features such as the production of more number of seeds than parental species and possession of longer and more number of stolons than parental species that allow their successful colonization (Gadella, 1987). The role of hybridisation in the evolution of the highly invasive *Fallopia* taxa (*F. japonica*, *F. sachalinensis* and their interspecific hybrid *F.* × *bohemica*) in Europe and North America has also been implicated in Bailey *et al*. (2007). These taxa are also found in New Zealand and Australia (Bzdega *et al*., 2016).

Hybridisation also confers other advantages to hybrids such as altering the quantity and quality of their secondary metabolites in such away that it renders the hybrids less palatable to herbivores thereby also altering the ecosystem through changing the interaction between plants and their herbivores (Orians, 2000). For instance, Driebe and Whitham (2000) reported that hybrids of *Populus angustifolia* showed a slower leaf litter decomposition rate than their parental species due to the presence of high levels of tannin (a secondary metabolic product) which will affect the amount of nutrient contained in the soil thereby affecting the whole ecosystem.

In general, the detrimental effect of hybridisation depends on the habitat preference of hybrids and their parental species, population size of hybrids and parental species, migration rate among hybrids and parental species, the strength of reproductive isolation mechanisms, the vigour, fertility and competitive potential of hybrids (Ellstrand and Elam, 1993; Rhymer and Simberloff, 1996; Levin et al., 1996).

With regard to migration rate, results from Huxel (1999)'s simulation model showed that a rapid extinction (in less than 33 generations) of native species can happen under high rates of immigration from a non-native species even in the absence of hybridisation. A native allelic frequency of less than 0.01 was assumed to be extinction. Assuming the presence of hybridisation and production of hybrids which are fertile but less vigour than parental species, the model showed that extinction of the native species can happen under low migration rates from the non-native species. Assuming hybrids are infertile, the model showed that hybridisation had small effect on extinction of the native species provided that the production of hybrids had little impact on the population size. With regard to habitat preference, the occurrence of extinction through hybridisation is less likely if the invading

species, the native species and hybrids have a divergent requirement for habitat which is the case in many stable hybrid zones (Freeman *et al*., 1999; Kentner and Mesler, 2000).

To summarise, although there might be few cases where hybridisation is assumed to have a role in species extinction, hybridisation is not usually mentioned as a major cause of species extinction. Support for hybridisation-associated extinction risks mainly come from few old reviews (e.g. Levin *et al*. 1996; Rhymer and Simberloff, 1996) which, compared to recent reviews, are based on few case studies and theoretical predictions. Current reviews (e.g. Baskett and Gomulkiewicz, 2011; Lopez-Pujol *et al*., 2012; Todesco *et al*., 2016; Hamilton and Miller, 2016;) showed that hybridisation has much more positive outcomes for rare species such as the enhancement of the already endangered genetic diversity, evolution of adaptive traits and creation of new species which is discussed in the next section. Moreover, there are recent empirical evidences that show hybridisation between a rare species and a common relative species did not lead to extinction of the rare species but rather to the enhancement of genetic diversity and adaptive potential of the rare species (e.g. Fitzpatrick *et al*., 2010; Becker *et al*., 2013; Song *et al*., 2015).

In the future, as more advanced studies and analyses of hybridisation are done, the positive outcomes of hybridisation might outweigh its negative impacts. For instance, recent studies showed that risks of outbreeding depression due to intra-specific hybribization are not as universal as they seem to be (Frankham *et al*., 2011; Frankham, 2015). Some studies (e.g. Willi *et al*., 2007; Aitken and Whitlock, 2013; Whitley *et al*., 2015) showed that the benefits of gene flow between species outweigh by far the risks of outbreeding depression

In recent times many studies are emerging showing the role of hybrids and hybridisation in adaptation and evolution of parental species (e.g. Seehausen, 2004; Mallet 2007; Arnold *et al*., 2012; Lopez-Pujol *et al*., 2012). Therefore, as knowledge and evidence on the evolutionary and adaptive role of hybridisation continue to grow the positive roles of hybridisation will get the proper attention it deserves in the future. More importantly, advanced studies on hybridisation might give us a much better insight into the likelihood and relative importance of various outcomes of hybridisation.

4.1.3.3 Hybrid speciation

The assumption that most or all hybrids are less fit than their parents has led many biologists to assume that natural hybridisation has no effect on the evolution of species (Arnold *et al*., 1999). However, several authors have illustrated that hybrids are not always unfit and that hybridisation can therefore be an important process in the adaptation and evolution of species (e.g., Bell & Travis 2005; Mallet 2007; Abbot *et al*., 2013). Although it is generally assumed that hybridisation homogenizes two genetically distinct species, thereby working against speciation, this view has been challenged and hybridisation is now accepted to play an important role in the formation of new species (Mallet 2007; Soltis & Soltis 2009; Yakimowski & Rieseberg, 2014). New species occur through hybridisation when hybrids become reproductively isolated from parental species and become genetically stabilized (Buerkle *et al*., 2003).

There are two mechanisms by which a new and stable species can be created through hybridisation: homoploidy and allopolyploidy (Rieseberg & Willis, 2007). In allopolyploidy, new stable and fertile species are created by doubling somatic chromosomes of diploid hybrid individuals to form tetraploids that cannot produce a viable triploid offspring from mating with diploid parental types (Mallet, 2007). This mechanism has been experimentally proven in many plant species (Rieseberg, 1997) and is considered a prominent speciation mechanism in ferns and flowering plants (Soltis *et al*., 1993; Rasmsey & Schemske, 1998). Allopolyploidy has been reported in the Hawaiian Silversword alliance (Asteraceae) (Barrier *et al*., 1999).

The other mechanism, homoploid hybrid speciation, does not require duplication of hybrid chromosomes (Gross & Rieseberg, 2005; Abbott *et al*., 2010). Although homoploid hybrids have the same number of chromosomes as their parent species and can successfully backcross with parental species, homoploid speciation can occur when hybrid individuals acquire new genetic sterility barriers resulting from chromosomal rearrangements. These sterility barriers cause the reproductive isolation of hybrids from their parental species (Abbott *et al*., 2010). Homoploid speciation has been reported for *Helianthus* species (Gross *et al*., 2003). and several other plant species (e.g., Clay *et al*., 2012; Rentsch & Leebens-Mack, 2012; Balao *et al*., 2015; Pedersen *et al*., 2016).

Homoploid hybrid speciation can also happen if new transgressive traits are expressed in homoploid hybrids and allow them to colonise novel areas that are not occupied by or

suitable for parental species (López-Caamal & Tovar-Sánchez, 2014). This might ultimately lead to an ecological divergence between hybrids and parental species, paving the way for the formation of hybrid species without duplication of hybrid chromosomes.

Homoploid speciation is less frequent than polypoid speciation for two reasons (Rieseberg & Willis, 2007). First, the fitness of most early generation homoploid species can be so low that natural selection will eliminate them. Second, whereas somatic chromosome doubling helps newly formed polyploids maintain their integrity, there is no such mechanism for homoploids to avoid mating with parental types and to keep their integrity. Moreover, the rarity of homopolid speciation can be attributed to the failure to distinguish homoploid hybrids from parental types, since they show no change in chromosome number, unlike polyploids (Rieseberg & Willis, 2007).

In New Zealand, there have been numerous reports of new species and hybrid lineages created through hybridisation. For instance, *Anaphalioides hookeri* is a hybrid species created through allopolyploidy following hybridisation between *A. bellidioides* and *A. trinervis* (Breitwieser et al., 1999). *Podocarpus totara var. waihoensis* is a stable hybrid created from hybridisation between *P. acutifolius* and *P. totara* in south Westland (Wardle, 1972). Hybrid speciation has also been reported in New Zealand mountain buttercup (*Ranunculus nivicola*), which is an allopolyploid species created from hybridisation between *R. verticillatus* and *R. insignis* (Carter, 2006).

A recent study on the butterfly genus *Coenonympha* (Capblancq *et al.*, 2015) showed that the Darwin´s heath (*C. darwiniana*) is a hybrid species originating from hybridisation between the pearly heath (*C. arcania*) and the alpine heath (*C. gardetta*). Species resulting from hybridisation have also been reported in other animal groups such as birds (Brelsford *et al.*, 2011), fish (Nolte *et al.*, 2005), and mammals (Amaral *et al.*, 2014).

Although great progress has been made in understanding the mechanisms behind hybrid speciation, recent DNA sequencing technologies and the availability of large data sets from molecular markers such as SNPs will further investigations of the specific evolutionary processes and scenarios involved in the formation of new species through hybridisation. Such technologies have already been used to investigate hybrid speciation in butterflies (Capblancq *et al.*, 2015; Nice *et al.*, 2013) and plants (Sun *et al.*, 2014).

4.1.4 Hybrid detection methods

Interspecific hybridisation can be detected using morphological and/or molecular markers. Morphological markers may easily detect first-generation hybrids (F1 hybrids) when they have traits that are intermediate between the two hybridizing parents (Rieseberg & Ellstrand, 1993). However, this is not usually the case, and therefore morphology-based hybrid detection may not be reliable. In addition, the morphology-based hybrid method has other important limitations. First, morphological traits are highly dependent on environmental conditions. As a result, it is difficult to know whether a given phenotype is caused by hybridisation or environmental conditions (Lopez-Caamal & Tovar-Sanchez, 2014). Second, the morphological approach cannot identify plant species when the specimen is too old or badly damaged (Cennamo & Cafasso, 2002). Third, compared to molecular markers, there are fewer heritable morphological traits, which make a thorough analysis of complex hybrids difficult (Rieseberg & Ellstrand, 1993). Fourth, unlike molecular markers, morphological traits are often correlated, and therefore the number of independent characters available for diagnosis is limited (López-Caamal & Tovar-Sánchez, 2014).

Finally, the correct identification of hybrids beyond the first generation becomes extremely difficult using the morphological approach, because the hybrids increasingly tend to resemble one of the parental species (Rieseberg & Ellstrand, 1993; Rhymer & Simberloff, 1996). In fact, even F1 hybrids may not show intermediate phenotypes. Rieseberg and Ellstrand (1993) investigated 46 papers that studied the morphology of hybrids and found that 45% of the morphological traits seen in F1 hybrids were not intermediate between parental traits but rather resembled one of the parental species. Furthermore, 10% of the traits were transgressive traits (i.e., their values fell beyond the range of the parent species). The expression of transgressive traits is caused by a complementary action produced when parental alleles that have opposing effects within each parental species are combined in hybrids (Bell & Travis 2005; Stelkens & Seehausen 2009).

Because of the limitations of morphology-based hybrid detection, today the detection of hybrids generally relies on genetic data in addition to morphological data. In the twentieth century, one of the frequently used methods to detect hybrids was counting chromosome numbers, because hybrids were assumed to undergo duplication of chromosomes (allopolyploidy) right after hybridisation (e.g., Soltis *et al*., 2009; Gill *et al*., 2009; De Storme & Mason, 2014; Renny-Byfield & Wendel, 2014). However, although the chromosome count gives a better hybrid detection mechanism than the morphological approach, this method can

also underestimate the level of hybridisation, since homoploid hybrid plants have the same number of chromosomes as their parent species (Abbott *et al*., 2010). Moreover, counting the chromosomes of both parental and hybrid plants can be expensive and time-consuming (Lopez-Caamal & Tovar-Sanchez, 2014). Therefore, neither the morphological approach nor the chromosome count is a reliable hybrid detection method in the absence of additional genetic data.

The most reliable hybrid detection method is DNA fingerprinting using molecular markers that can be analysed using various statistical models and programs (e.g., Pritchard *et al*., 2000; Anderson & Thompson 2002; Buerkle, 2005; Durand *et al*., 2011). Molecular markers have the following advantages over the morphology-based detection mechanism (Lopez-Caamal & Tovar-Sanchez, 2014). The first advantage is that there are many molecular markers distributed throughout plant genomes, which detects hybrids more accurately, especially for complex hybrids which cannot be detected with few markers. Second, most molecular markers are unlinked; so many independent or uncorrelated markers are available for hybrid detection. Third, most molecular markers are found in noncoding regions of the plant genome and are therefore selectively neutral, which means the actual extent of introgression can be investigated. Finally, using powerful Bayesian-based software such as NEWHYBRIDS (Anderson & Thompson, 2002), molecular makers can be used to detect different classes of hybrids (e.g., F1, F2, backcross), which is not possible with morphological-based hybrid detection systems.

Chloroplast markers are one class of molecular markers that has been successfully used to analyse the pattern of hybridisation in plants (e.g., Zhou *et al*., 2008; Yu *et al*., 2014). Maternally inherited chloroplast DNA can determine the direction of hybridisation (i.e., whether the male parent always comes from one species and the female parent from the other species). However, the correct identification of all hybrids and the degree of introgression can only be revealed using biparentally inherited molecular makers (Rhymer & Simberloff, 1996).

4.1.5 Significance of hybrid detection

The identification of pure and hybrid individuals is useful to document introgression and gene flow among species (Anderson & Thompson, 2002). Moreover, interspecific hybridisation may have significant effects on the ecology and genetics of the species involved

(López-Caamal & Tovar-Sánchez, 2014). Hence, it is very important to accurately identify hybrid individuals to understand the magnitude of introgression and its effect on the species involved and ecosystem functioning.

The detection of hybrids is also important to check the genetic identity of a population in order to better understand its phylogeny and to identify the best germplasm for restoration or breeding programs (Ehrenberg *et al*., 1955). In the absence of such information, the wrong population or species could be used for restoration or breeding. For instance, in New Zealand, confusion over the morphological similarity between New Zealand endemic *Asplenium bulbiferum* and *Asplenium* x *lucrosum* (a hybrid of *A. bulbiferum* and Norfolk Island endemic *A. dimorphum*) led to the inappropriate use of the hybrid *Asplenium* x *lucrosum* for restoration purposes (Perrie *et al*., 2005). The detection of hybridisation is also important when it is implicated in the extinction of rare species (Rhymer & Simberloff 1996; Levin & Francisco-Ortega 1996; Wolf *et al*., 2001).

For all of these reasons, hybridisation deserves attention in the conservation and management of species, especially those that are rare or endangered. The first step in assessing the impacts of hybridisation should be to confirm whether or not interspecific hybridisation is occurring and, if so, to then estimate the frequency of parental and hybrid classes. Based on morphological observations, it is suspected that hybridisation occurs between *M. excelsa* and *M. robusta* (Cooper 1954; Julian 1992; Wilcox 2007; Dawson *et al*., 2010), but this has not been confirmed by genetic studies. This study aims to fill this knowledge gap.

4.2 Materials and Methods

4.2.1 Description of study species

M. excelsa and M. robusta are closely related *Metrosideros* species that are well-known in New Zealand. They are known for their beautiful summer flowers, which are important sources of honey and nectar for native birds (Bergin & Hosking, 2006**).** Although these two species do not naturally coexist, they do grow side by side on Rangitoto Island and are suspected to hybridise with each other (Cooper, 1954). Hybridization is known to occur among *Metrosideros* species in New Zealand (Gardner *et al*., 2004; Simpson, 2005; Dawson *et al*., 2010a).

M. excelsa

M. excelsa (pohutukawa) is a tree species that grows up to 25m and has multiple trunks of up to 2m diameter starting from the base. Branches are widespread and sometimes hover over the ground, and bark is firm and hard to detach. The bright masses of inflorescences open during summer from November to January (Allan 1961; de Lange, 2004a). *M. excelsa* is endemic to the rocky coastlines of northern New Zealand (Poole & Adams, 1990). In the west, its distribution ranges from North Cape to northern Taranaki and in the east it reaches Mahia Peninsula. The species has now been planted throughout New Zealand, and so its natural distribution in the south is difficult to demarcate (de Lange, 2004a). Although the species is still common throughout most of its range, it is on the verge of local extinction in areas where possums have unlimited access (de Lange, 2004a).

M. excelsa can grow in harsh conditions such as disturbed and exposed coastal areas, because of adaptations such as leathery leaves to capture moisture in exposed conditions, roots that can grow deep into rocky surfaces, and a trunk that can store food to endure drought conditions (Haines & Wilcox, 2007). Although *M. excelsa* has very low seed fertility, the abundant production of light-weight seeds enables the species to be widely distributed (Schmidt-Adam *et al*., 2002). The species has several effective pollinators, including New Zealand native honeyeaters (Meliphagidae) and other native and introduced birds and bees (Anderson, 2003; Schmidt-Adam *et al*., 2000). Natural populations of *M. excelsa* are mosaic, consisting of both self-compatible and self-incompatible individuals (Schmidt-Adam *et al*., 1999). Seeds produced through outcrossing are typically larger and more vigorous than those produced through self-pollination (Schmidt-Adam *et al*., 2000).

M. robusta

M. robusta (Northern rata) is also a tree growing up to 25m with firm bark and numerous broom-like branchlets (Allan, 1961; de Lange 2004b). Individual trees usually start life as an epiphyte and later develop a hollow trunk of up to 2m diameter with many interlocking roots (Beddie, 1953). Unlike *M. excelsa*, *M. robusta* has more open flower heads and compact clusters of smaller leaves with notches at the tips (Haines & Wilcox, 2007). *M. robusta* is endemic to New Zealand, where it is widely distributed from Te Paki southwards to Wellington, Marlborough, Nelson and Westland. *M. robusta* is a key species in the forest ecosystems of the Wellington region (McKessar & Sawyer, 1999).

Although currently the species is not considered endangered, *M. robusta* is believed to be in decline (Sawyer & McKessar, 2007) and its previous wide range has contracted, especially in the lower parts of the North Island. Major threats to this species include possums and hybridisation with *M. excelsa*, which is establishing well beyond its natural range (Sawyer & McKessar, 2007). The decline of native pollinators (lizards and birds) has also been implicated as a threat in some places (Burns, 1996). Project Crimson, a national non-profit organization, is currently promoting the protection and conservation of *M. excelsa* and *M. robusta* (http://projectcrimson.org.nz/).

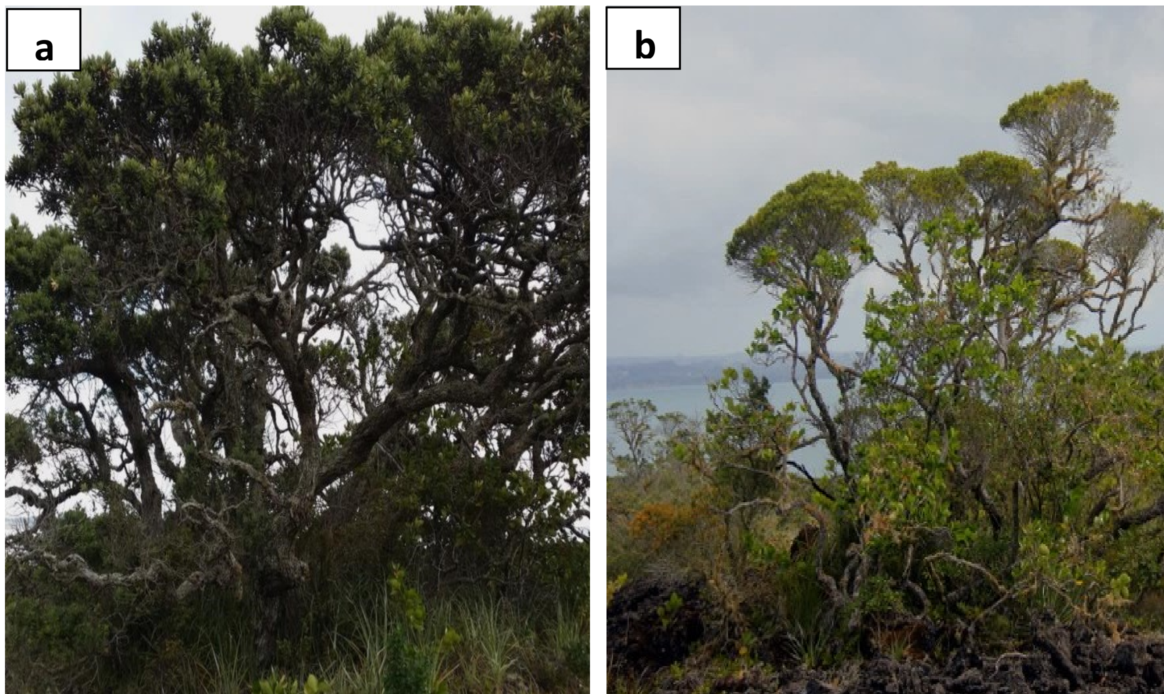

Figure 4.1 *M. excelsa* (a) and *M. robusta* (b) on Rangitoto Island.

4.2.2 Description of study site (Rangitoto Island)

Rangitoto Island is an ideal place to study hybridisation between *M. excelsa* and *M. robusta* because they grow sympatrically and are the dominant tree species on this island. Rangitoto is a 600-year-old volcanic island located 8km from Auckland city (20 to 30 minutes by ferry). It was the site of the largest and youngest volcanic eruption in the last 150,000 years in the Auckland region (Wilcox, 2007; Needham *et al*., 2011). After its eruption, the island is thought to have been first colonized by vegetation from the nearby Motutapu Island, which survived the devastating eruption (Miller *et al*., 1994). With an area of 2321ha, the island has

a distinct ecology that is registered by the Department of Conservation (McEwen, 1987). Despite the prevailing harsh conditions, Rangitoto is home to about 200 native plant species and more than 40 fern species (Miller *et al*., 1994).

Figure 4.2 Rangitoto Island

Because the island is still young, the soil is thin and not well-developed, primarily consisting of humus produced by decaying leaves and other plant matter (Wilcox, 2007). In contrast, neighbouring Motutapu Island, which is millions of years old, has fertile soil conducive to cultivation (Bade, 2011). However, Motutapu Island does not have the same natural values as Rangitoto, which has a distinct volcanic landscape dominated by *M. excelsa* (Bade, 2011). The mean annual temperature and rainfall on Rangitoto Island is 16°C and 1185mm respectively and Summit and McKenzie Bay are the coolest areas on the island (Wilcox, 2007).

The history of Rangitoto Island´s vegetation and the date of the first colonization of the dominant *M. excelsa* trees are debated and not fully known (Haines *et al*., 2007). Based on tree ring counts and historical accounts, Millener (1953) suggested that the island has been forested for no more than 200 years. Julian (1992) also suggested that the current vegetation is the result of a more rapid development on lava flows from a later eruption in the late

nineteenth century. On the other hand, Nichol (1992) suggests that because the island experienced fire at different times and because trees were cut for Auckland´s firewood market, the current forest cover may not be the original forest on the island. Hence, the first colonization of the island by vegetation might have happened 600 years ago on lava flows following the initial eruption of the island 600 years ago.

4.2.3 Extraction of DNA and amplification of markers

Leaf samples for genomic DNA extraction were collected in plastic bags containing silica gel from a total of 95 randomly selected trees of *M. excelsa* (37), *M. robusta* (29) and suspected putative hybrids (29) of these two species on Rangitoto Island, where natural hybridisation is believed to have taken place. Parental and putative hybrid individuals were sampled based on leaf morphology observed in the field. Individuals were not, however, distinguished by species for hybridisation analysis. Genomic DNA was extracted from 20mg silica gel-dried leaves using the NucleoSpin® Plant II DNA Extraction Kit according to the manufacturer's protocol.

The molecular detection of hybrids was performed using AFLP and SSR markers. Chapter 3 gives a detailed explanation of these two markers. Hybrid individuals in several plant species have been identified using both AFLP (e.g., Shasany *et al*., 2005; Koerber *et al*., 2013; Avila-Flores *et al*, 2016) and SSR markers (Iqbal *et al*., 2010; Mohan *et al*., 2013; Nadeem *et al*., 2014). The AFLP reactions were performed as described by Vos *et al*. (1995). The specific reaction mixtures and conditions used for both AFLP and SSR reactions were the same as those described in Chapter 3 for the genetic diversity study of *M. bartlettii*.

Before running the AFLP reactions, 16 randomly drawn samples (7 *M. excelsa*, 5 *M. robusta* and 4 suspected hybrids) were used to screen 14 AFLP primer combinations. Four primer combinations (JOE E*co*RI-AGG + M*se*I-CT; JOE E*co*RI-AGG + M*se*I-CG; JOE E*co*RI-AGG + M*se*I-CTG; and NED E*co*RI-ACC+ M*se*I-CG) showed high polymorphism and reproducibility and therefore were selected for the analysis. For the SSR analysis, the same 16 samples were also used to screen and test the transferability of 15 SSR primers that were developed for *M. polymorpha* by Crawford *et al*. (2008). Nine of these tested primers were polymorphic and reproducible. A list of AFLP and SSR primers that were used in the study is given in Table 4.1.

Table 4.1 AFLP and SSR primers used in this study (n = 95 individuals).

AFLP			SSR			
Primer combination	**No. of bands**	***P* (%)**	**Locus**	**Primer sequence (5' to 3')**	***K***	**Size (bp)**
JOE E*co*RI-AGG + M*se*I-CT	54	38 (70.4%)	MePo501	M13F: TCTTTCGCCGGATTACTT R: AGTGCCTTATTCATGCTATGT	11	118-242
			MePo502	M13F: ATGAGGAGGATTACGTATTA R: CGTATTTACTCCCAATTATCA	13	126-247
JOE E*co*RI-AGG + M*se*I-CG	51	45 (88.2%)	MePo503	M13F: CTCACATCGCTTGTCTA R: CCAAATTAAGAACGATACAT	7	192-215
			MePo506	M13F: ATCCCCTCACGATTATAG R: ATGGGCTGACGAATATA	7	164-224
JOE E*co*RI-AGG + M*se*I-CTG	42	37 (88.1%)	MePo508	M13F: ACTTATTGAGTGTGACCTATAC R: TAGAGCGAAGCAAATATAT	3	146-154
			MePo511	M13F: ACATAGCCTCAAGCGTAAGT R: AAGGAGAGCCCCATTTAC	4	208-242
NED E*co*RI-ACC+ M*se*I-CG	59	52 (88.1%)	MePo513	M13F: CAGAACTGGTATCCTGATATA R: AATGAGTTGGGATTTAGAG	12	164-236
			MePo514	M13F: GCTTTCCTGCAACAGAGTAG R: GTGCATCAACCGCTACAT	15	160-216
			MePo515	M13F:GAAGAGATAAACGATTTCTATT R: CGATGATTTAGCTTAGAT	16	202-260
Total	**206**	**172(83.50%)**			**88**	

P = Proportion of polymorphic loci at 5% level; *K* = Total number of alleles

4.3 Data analysis

Raw AFLP data were scored using GeneMarker V.2.6.4. AFLP bands were scored automatically as present (1) or absent (0). The automatic scoring was also inspected visually to ensure that the software had scored correctly. Raw codominant SSR data were also scored for each locus across all individuals using GeneMarker V 2.6.4. The scored AFLP and SSR markers were then used to detect interspecific hybridisation between *M.excelsa* and *M.robusta* using NEWHYBRIDS V. 1.1 (Anderson and Thompson, 2002) and STRUCTURE V. 2.3.4 (Pritchard *et al*., 2000) programs. These Bayesian-based methods have been used to detect plant hybrids in several previous studies (e.g., Wallace, 2006; Hoban *et al*., 2009; Tovar-Sanchez *et al*., 2012; Balao *et al*., 2015).

As described by the developers, the model in STRUCTURE allows individual samples to have a mixed ancestry, where various proportions of their genomes are derived from different subpopulations or species. Hence, the STRUCTURE program yields an estimate of the proportion of a genome of a given individual originating from one or more genetic populations. Chapter 3 gives a detailed discussion of STRUCTURE, as the software was also used to analyse the genetic structure of *M.bartlettii*.

NEWHYBRIDS has a model that computes the posterior probability that a given sample belongs to all six genotypic classes (i.e., two parental and four hybrid classes: F1, F2, F1 x Parent_1 backcross, F1 x Parent_2 backcross). Unlike the NEWHYBRIDS model, the STRUCTURE model does not differentiate recent hybrids such as F1 and F2, as both these hybrid classes have on average 50% of their genomes from each parental species (Anderson, 2008).

As described in Anderson and Thompson (2002), the NEWHYBRIDS method is similar to the STRUCTURE method in that the program uses a Bayesian model-based clustering approach to analyse the structure of populations. Like the STRUCTURE method, it uses Markov Chain Monte Carlo simulations to calculate the posterior probability that a given individual belongs to each of the six genotypic classes. The program, however, differs from other programs that use Bayesian methods in that it assumes the analysed samples comprise pure parental species and recent hybrids of these parental species, and it therefore uses an inheritance model defined in terms of genotype frequencies corresponding to different hybrid categories.

The NEWHYBRIDS method does not require prior knowledge of allelic frequencies of parent species, nor does it require the availably of a separate sample consisting of only pure parent species. Moreover, although a high genetic differentiation between hybridizing species is helpful, the NEWHYBRIDS method does not require parental species to have species-specific (private) alleles (Vaha & Primmer, 2006).

Initially, the NEWHYBRIDS program was developed to analyse codominant markers such as microsatellites, but later the program was modified to analyse dominant markers such as AFLPs. Anderson (2008) describes a detailed mathematical description of the modification of the program. The NEWHYBRID software uses a genotype frequency class input text file to specify the six classes to which a given sample may belong. The file has the following format (Anderson, 2003):

6	A_1A_1	A_1A_2	A_2A_1	A_2A_2
Pure_A	1.00000	0.00000	0.00000	0.00000
Pure_B	0.00000	0.00000	0.00000	1.00000
F1	0.00000	0.50000	0.50000	0.00000
F2	0.25000	0.25000	0.25000	0.25000
A_Bx	0.50000	0.25000	0.25000	0.00000
B_Bx	0.00000	0.25000	0.25000	0.50000

The first character (6) specifies the number of classes. Pure_A is the class name for one of the pure parents. Pure_B is the class name for the other pure parent. F1 and F2 are class names for the F1 hybrid and F2 hybrid respectively. A_Bx and B_Bx refer to class names for Population A back crosses (resulting from crossing an F1 individual and an individual from pure parent population A) and population B backcrosses (resulting from crossing an F1 individual and an individual from pure parent population B), respectively. The four remaining columns specify the expected proportions of the four possible genotypes in each class as follows:

A_1A_1 = both A_1 alleles coming from parent population A

A_1A_2 = allele A_1 coming from parent population A and allele A_2 coming from parent population B

A_2A_1 = allele A_2 coming from parent population A and allele A_1 coming from parent population B

A_2A_2 = both A_2 alleles coming from parent population B

In this study, the posterior probability for belonging to the six classes was computed without prior information on hybrid status of the sample individuals and without prior allele frequency information. For program settings, the default Jeffery´s prior was used on π (mixing proportions parameter) and Θ (allele frequencies parameter). For comparison purposes, the Uniform prior was tested and gave similar results. 100,000 MCMC generations and a burn-in period of 20,000 steps were used for the analysis after longer MCMC generations and burn-in periods were tested and found to yield similar results.

For the STRUCTURE analysis, for K varying from one to ten, ten runs were performed using a 100,000 steps burn-in period followed by 500,000 MCMC repetitions, which was long enough to obtain consistent results. The best value of K was determined based on the rate of change in probability (ΔK) between successive K values, following the work of Evanno *et al*. (2005; see Chapter 2). Results of the structure analysis and implementation of Evanno's (2005) method were performed using the Structure Harvester program (Earl & vonHoldt, 2012), which is available at http://taylor0.biology.ucla.edu/structureHarvester/.

Following the STRUCTURE and NEWHYBRIDS analyses, the next step was to decide a threshold value for treating individuals as pure parents. Unfortunately, the exact point or level of introgression at which hybrids are recognized as members of either parental species is unclear. Thus, an arbitrary threshold value of $P \geq 0.90$ was used both for the posterior probability of membership (for NEWHYBRIDS) and for the proportion of genome inherited from either parent (for STRUCTURE) to assign a given individual to one of the parental classes. In other words, if an individual's genotypic frequency for the parental class was lower than 90% or if the individual inherited less than 90% of its genome from either parent, then it was identified as a hybrid. For comparison, results obtained based on other P values were also examined.

$P \geq 0.90$ was chosen as a threshold value because it was also used by several previous authors (e.g., Grant *et al*., 2004; Ortego *et al*., 2014; Balao *et al*., 2015; Certner *et al*., 2015; Li *et al*., 2016). Moreover, a hybrid detection efficiency test of these Bayesian-based methods by Vaha and Primmer (2006) showed that the highest efficiency for identifying hybrids under various scenarios of hybridisation was obtained when this threshold value was used. However, other studies have used different threshold values (e.g., Pierpaoli *et al*., 2003 used 0.80; Hoban *et al*., 2012 used 0.75; Avila-Flores *et al*., 2016 used 0.95; Abraham *et al*., 2011 and Backs *et al*., 2016 used 0.85). Thus, for comparison, the numbers of hybrids obtained using other threshold values ($P \geq 0.80$ and $P \geq 0.95$) were also analysed. The F1 hybrids in STRUCTURE were identified using an arbitrary range of 40-60% ($0.40 < P < 0.60$) probability of being a member of one of the pure parental classes. This range was used by Ortego *et al*. (2014) and similar values ($0.45 < P < 0.55$) were used by Avila-Flores *et al.* (2016).

Once parental and hybrid classes were identified, all individuals were grouped into three populations (pure *M. exclesa*, pure *M. robusta* and hybrids) for principal coordinate analysis (PCoA) and estimation of population-level genetic diversity and structure. The PCoA is a

visually appealing, distance-based clustering method (Kindt *et al*., 2009) that complements the results obtained with STRUCTURE and NEWHYBRIDS.

To estimate genetic diversity (i.e., proportion of polymorphic loci, *P%* at the 5% level and expected heterozygosity, H_e), the AFLP binary data matrix was analysed using AFLP-SURV 1.0 (Vekemans, 2002). For each population, allelic frequencies were computed using a Bayesian method with non-uniform prior distribution of allele frequencies (Zhivotovsky, 1999), assuming no deviation from Hardy Weinberg Equilibrium. Chapter 2 gives a brief description of this method. For the SSR data, the genetic diversity within populations was calculated using GenAlex 6.5 (Peakall & Smouse, 2012). The estimated genetic diversity parameters included the number of alleles per locus (N_a), number of effective alleles per locus (N_e), observed heterozygosity (H_o) and expected heterozygosity (H_e).

4.4 Results

4.4.1 Hybrid detection with AFLP markers

The four AFLP primer combinations yielded a total of 206 alleles (Table 4.1). 172 of the 206 AFLP markers (83.5%) were polymorphic. The AFLP patterns aligned with no difficulty, suggesting *M. excelsa* and *M. robusta* are closely related.

Using the $P \geq 0.90$ threshold probability of belonging to a parental class, the AFLP NEWHYBRID analysis identified 64 individuals as a pure parental type (35 pure *M. excelsa* and 29 pure *M. robusta*) and 31 individuals as hybrids (Table 4.2 and Figure 4.3a). Although two of the 31 hybrids (trees 46 and 66) had their highest posterior probability for parental classes (tree 46 had $P = 0.77$ for being pure *M. excelsa* and tree 66 had $P = 0.83$ for being pure *M. robusta*), both trees were counted as hybrids because their probability values were below the threshold value ($P \geq 0.90$). All the remaining 29 hybrid trees had probabilities lower than 0.90 for either of the parental classes and were therefore easily identified as hybrids.

Based on the highest *P* values for a given hybrid class, the 29 hybrids were assigned to different classes as indicated in Table 4.3. The table shows that, using the criteria $P \geq 0.60$, which left no hybrid individual unassigned, there were seven F1 hybrids, three F2 hybrids, 12 *M. excelsa* backcrosses, and seven *M. robusta* backcrosses. The other criteria ($P \geq 0.95$, $P \geq 0.90$ and $P \geq 0.80$) gave similar results. The difference in the number of *M. excelsa* backcrosses and *M. robusta* backcrosses was five in all cases but one ($P \geq 0.95$) where the

difference is four, suggesting the presence of a bidirectional but biased introgression towards *M. excelsa*.

Table 4.2 Comparison of the number of parental and hybrid individuals detected by the NEWHYBRIDS and STRUCTURE analyses of AFLP markers.

Criteria based on probability of Parental class	**AFLP - NEWHYBRIDS**			**AFLP - STRUCTURE**		
	M. excelsa	*M. robusta*	Hybrids	*M. excelsa*	*M. robusta*	Hybrids
$P \geq 0.80$	36	30	**29**	40	32	**23**
$P \geq 0.90$	35	29	**31**	38	31	**26**
$P \geq 0.95$	35	28	**32**	36	29	**30**

Table 4.3 Different hybrid classes detected by the NEWHYBRIDS analysis based on AFLP markers.

Hybrid Classes							
Criteria based on probability of hybrid class	**F1 hybrids**	**F2 hybrids**	***M. exclesa* backcross**	***M. robusta* backcross**	**Unassigned hybrids**	*** hybrids assigned as parental type with $P < 0.90$**	**Total**
$P \geq 0.95$	5	1	10	6	7	2	**31**
$P \geq 0.90$	5	2	11	6	5	2	**31**
$P \geq 0.80$	6	2	12	7	2	2	**31**
$P \geq 0.60$	7	3	12	7	0	2	**31**

* Two samples (tree ID 46 and 66) were identified as parental type with $P = 0.77$ (tree ID 46) and $P = 0.83$ (tree ID 66). Because these probabilities are lower than the threshold value required for falling into a parental class ($P \geq 0.90$), they were counted as hybrids.

In the STRUCTURE analysis, the ΔK test (Evanno *et al.*, 2005) showed that the highest ΔK value was found for $K = 2$ both with AFLP (Figure 4.3b) and SSR (Figure 4.4b) markers. This suggests the presence of two distinct genetic populations (pure *M. excelsa* and pure *M. robusta*) with the hybrid population consisting of admixture individuals whose genomes are drawn from the two parental species.

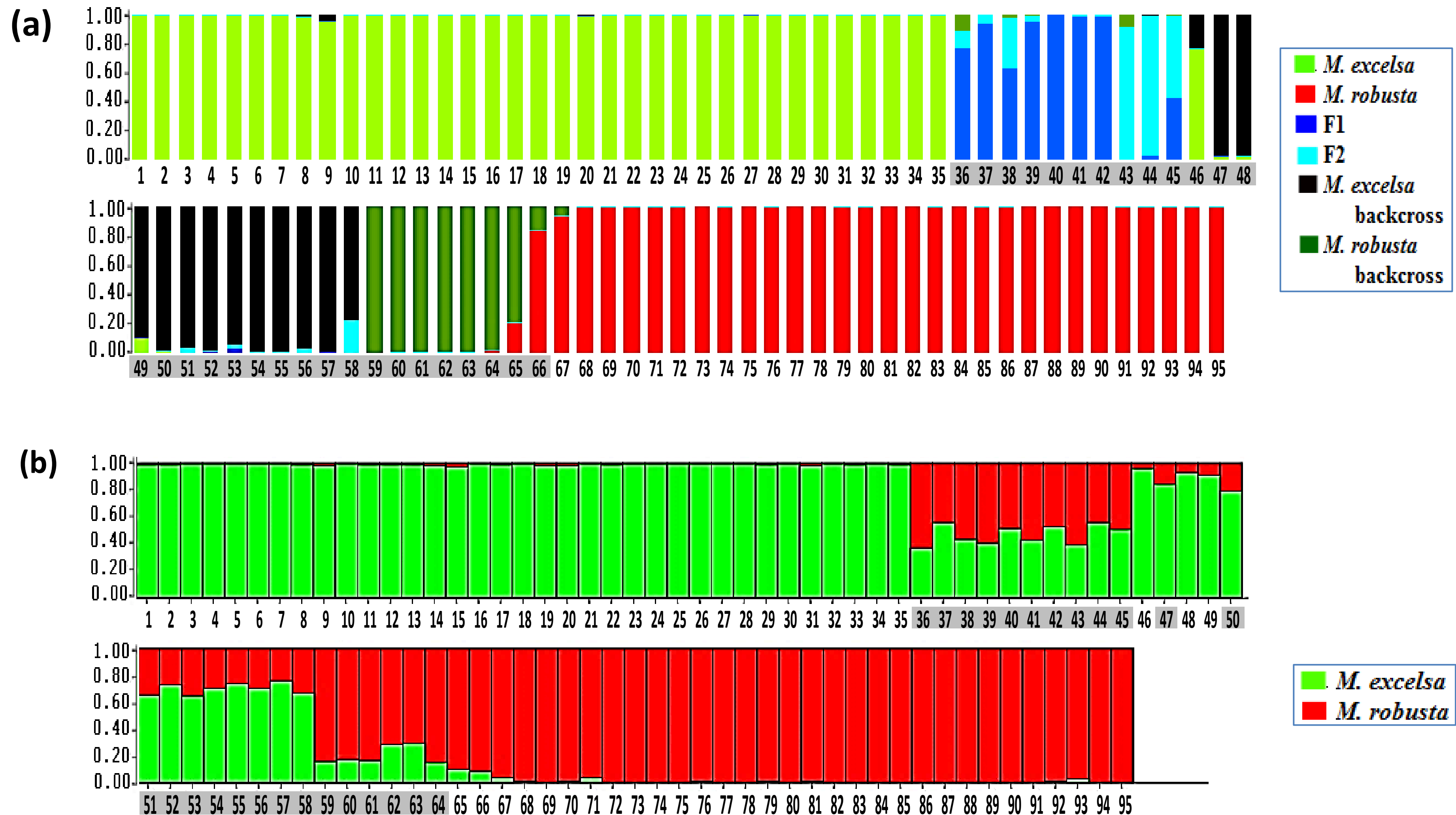

Figure 4.3 Genotype class assignments of trees by NEWHYBRIDS (a) and clustering analysis by STRUCTURE (b) based on AFLP markers. Each individual tree is represented by a vertical bar. Numbers on the x-axis refer to individual tree ID and numbers on the y-axis refer to the posteriori probability of trees belonging to parental and various hybrid classes (for NEWHYBRIDS) or probability of membership of trees to the inferred class (for STRUCTURE). Shaded tree IDs are hybrids (based on $P \geq 0.90$ threshold).

The STRUCTURE analysis of the AFLP markers identified a similar number of hybrids to the NEWHYBRIDS analysis. The difference between the two programs in the total number of hybrids detected was very small, especially when using the $P \geq 0.95$ threshold value (Table 4.2, Figure 4.3b). Using all three threshold criteria, all parental types that were identified by the NEWHYBRIDS analysis were also identified as such by the STRUCTURE analysis. The slightly lower number of hybrids detected by the STRUCTURE analysis was due to the identification of few individuals as parental types, which NEWHYBRIDS identified as hybrids.

As expected, all F1 hybrids detected in the NEWHYBRIDS analysis had probabilities between 0.4 and 0.6 in the STRUCTURE analysis. Because all F2 hybrids also had probabilities within this range, the STRUCTURE program failed to distinguish F1 and F2 hybrids. In the STRUCTURE analysis, *M. excelsa* backcrosses and *M. robusta* backcrosses had a major portion of their genomes derived from pure *M. excelsa* and pure *M. robusta* respectively.

4.4.2 Hybrid detection with SSR markers

The nine SSR primers gave a total of 88 alleles (i.e., 9.77 alleles per locus; Table 4.1). Using the $P \geq 0.90$ threshold probability, the SSR NEWHYBRID analysis identified 67 individuals as parental type (37 pure *M. excelsa* and 30 pure *M. robusta*) and 28 individuals as hybrids (Table 4.4 and Figure 4.4a).

Based on the highest P values for a given hybrid class, these 28 hybrids were assigned to different classes as indicated in Table 4.5. The table shows that, using the criteria $P \geq 0.50$, which left only one hybrid unassigned, there were seven F1 hybrids, four F2 hybrids, 11 *M. excelsa* backcrosses, five *M. robusta* backcrosses and one unassigned individual (tree ID 65). This tree was identified as a *M. excelsa* backcross with the lowest probability of all *M. excelsa* backcrosses identified with the AFLP NEWHYBRIDS analysis. The other threshold values ($P \geq 0.95$, $P \geq 0.90$ and $P \geq 0.80$) did not give similar results. Unlike in the case of AFLP markers, very few hybrid individuals had probabilities greater than 0.90 for any hybrid class. Most hybrid individuals had probabilities ranging from 0.5 to 0.8.

Based on $P \geq 0.50$ and $P \geq 0.80$ (Table 4.5), which left few individuals unassigned, the difference between the number of *M. excelsa* backcrosses and *M. robusta* backcrosses was

six, supporting the bidirectional but biased introgression towards *M. excelsa* that was revealed by the AFLP markers.

Table 4.4 Comparison of the number of parental and hybrid individuals detected by the NEWHYBRIDS and STRUCTURE analyses of SSR markers.

Criteria based on probability of Parental class	SSR - NEWHYBRIDS			SSR - STRUCTURE		
	M. excelsa	***M. robusta***	**Hybrids**	***M. excelsa***	***M. robusta***	**Hybrids**
$P \geq 0.80$	37	30	**28**	43	34	**18**
$P \geq 0.90$	37	30	**28**	38	30	**27**
$P \geq 0.95$	36	26	**33**	37	30	**28**

Table 4.5 Different hybrid classes detected by the NEWHYBRIDS analysis based on SSR markers.

Criteria based on probability of hybrid class	F1 hybrids	F2 hybrids	*M. exclesa* backcross	*M. robusta* backcross	Unassigned	Total
$P \geq 0.95$	0	0	1	0	27	**28**
$P \geq 0.90$	1	2	3	0	22	**28**
$P \geq 0.80$	5	2	9	3	9	**28**
$P \geq 0.50$	7	4	11	5	1	**28**

In the STRUCTURE analysis, as for the AFLP markers, the ΔK test (Evanno *et al.*, 2005) showed that the highest ΔK value was found for $K = 2$ (Figure 4.4b), suggesting the presence of two distinct genetic populations (pure *M. excelsa* and pure *M. robusta*) with the hybrid population consisting of admixed individuals from these two parental populations. The STRUCTURE analysis identified a very similar number of parental and hybrid classes as the NEWHYBRIDS analysis, especially when using $P \geq 0.90$ and $P \geq 0.95$ threshold values (Table 4.4, Figure 4.4b). The two programs showed a wider discrepancy in the number of *M. excelsa* and hybrids when the lower threshold value $P \geq 0.80$ was used to distinguish parental and hybrid classes.

As found with the AFLP markers, all parental types that were identified by the NEWHYBRIDS analysis were also identified by the STRUCTURE analysis as parental types at all the three threshold values. The lower number of hybrids detected by the STRUCTURE analysis was due to the identification by this program of a few individuals as parental types

that were identified by the NEWHYBRIDS program as hybrids. Once again, all F1 hybrids that were detected in the NEWHYBRIDS analysis had probabilities between 0.4 and 0.6 in the STRUCTURE analysis. Like in the AFLP analysis, the STRUCTURE program failed to distinguish F1 and F2 hybrids as individuals of both classes had probabilities between 0.4 and 0.6. *M. excelsa* backcrosses and *M. robusta* backcrosses had a major portion of their genomes derived from pure *M. excelsa* and pure M. robusta respectively.

Generally, the results from NEWHYBRIDS and STRUCTURE showed that there is high agreement between these two programs using both AFLP and SSR markers.

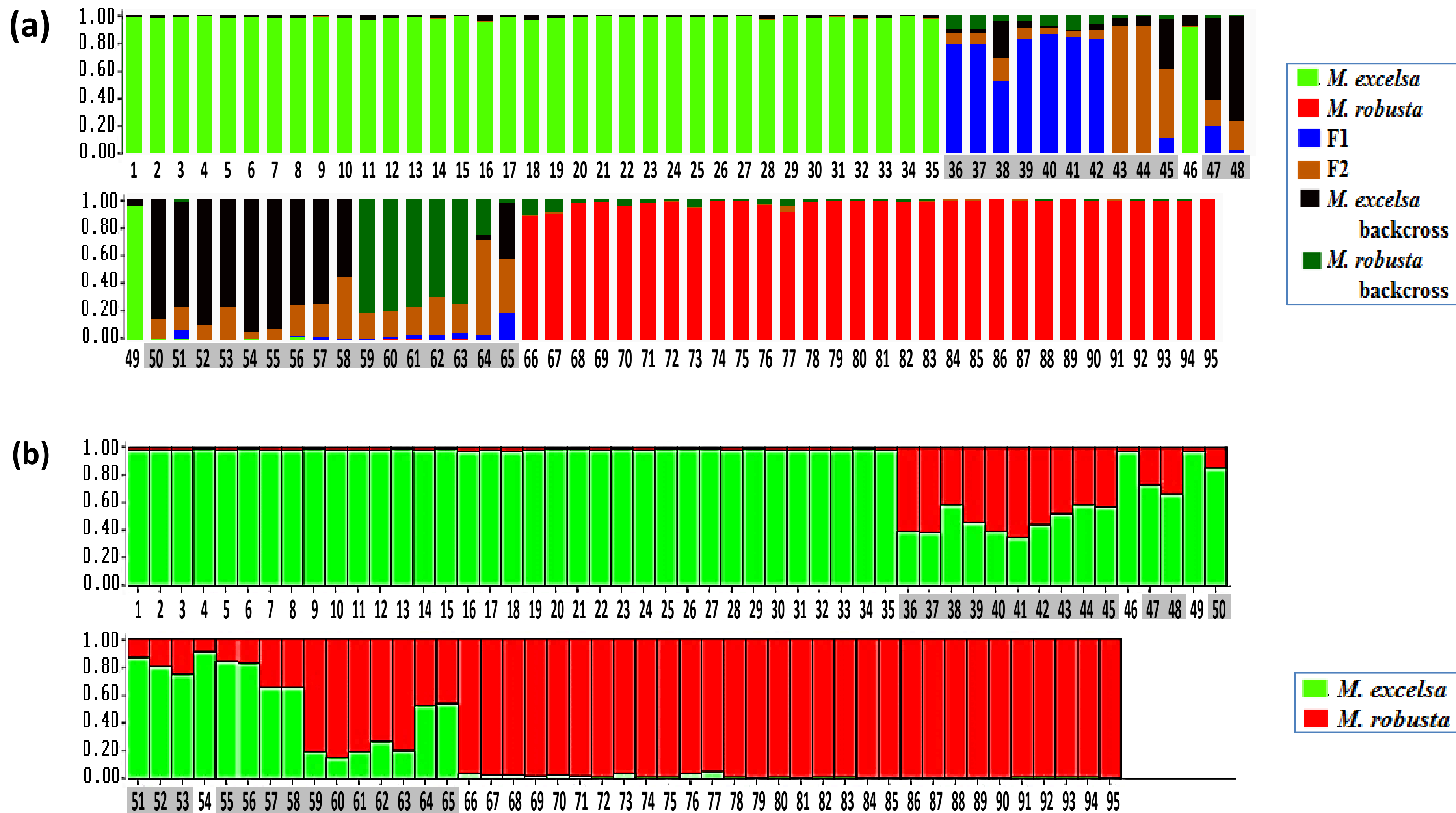

Figure 4.4 Genotype class assignments of trees by NEWHYBRIDS (a) and clustering analysis by STRUCTURE (b) based on SSR markers. Each individual tree is represented by a vertical bar. Numbers on the x-axis refer to individual tree ID and numbers on the y-axis refer to the posteriori probability of trees belonging to parental and various hybrid classes (for NEWHYBRIDS) or probability of membership of trees to the inferred class (for STRUCTURE). Shaded tree IDs are hybrids (based on $P \geq 0.90$ threshold).

A comparison of tree class (parental or hybrid) based on observation of leaf morphology with the tree class revealed from the NEWHYBRIDS analysis is given for some individuals in Table 4.6. For morphological identification in the field, a tree was identified as pure *M. exclesa* if the leaves were elliptical / oblong with slightly rolled edges, with white dense tomentose pubescence beneath and larger and dark greener than *M. robusta* leaves. A tree was identified as pure *M. robusta* if the leaves were ovate-shaped, with a distinctly notched apex, and were smaller, flatter and thinner than *M. excelsa* leaves. A tree was identified as a "*M. excelsa*-like" hybrid if the leaves were slender, with little or no tomentose beneath, an acute rather than notched apex, and larger than the typical leaves of *M. robusta*. A tree was identified as a "*M. robusta*-like" hybrid if the leaves were typical *M. robusta* leaves but with an unnotched apex or with a less ovate shape and a larger leaf size than the typical leaves of *M. robusta*. Table 4.6 shows there were eight cases where morphology-based hybrid identification did not accurately identify tree classes.

Table 4.6 List of samples showing a mismatch between tree class (parental or hybrid) based on leaf morphology observed in the field and the corresponding class as revealed by the molecular markers. The *M. excelsa*-like and the *M. robusta*-like hybrids represent *M. excelsa* backcrosses and *M. robusta* backcrosses, respectively.

Tree ID	**Tree Class based on leaf morphology**	**Tree class based on NEWHYBRIDS analysis**	
		AFLP	**SSR**
9	More *M. excelsa*-like hybrid	pure *M. excelsa*	pure *M. excelsa*
46	pure *M. excelsa*	*M. excelsa* backcross	pure *M. excelsa*
53	pure *M. excelsa*	*M. excelsa* backcross	*M. excelsa* backcross
55	pure *M. excelsa*	*M. excelsa* backcross	*M. excelsa* backcross
62	pure *M. roubsta*	*M. robusta* backcross	*M. robusta* backcross
66	pure *M. roubsta*	*M. robusta* backcross	pure *M. robusta*
67	More *M. roubsta*-like hybrid	pure *M. roubsta*	pure *M. roubsta*
77	More *M. roubsta*-like hybrid	pure *M. roubsta*	pure *M. roubsta*

After assigning individual trees to three populations based on AFLP NEWHYBRIDS analysis, bands which were shared by hybrids and only one of the parental species (*M. excelsa* or *M. robusta*) were identified. The list and frequencies of these bands are given in Table 4.7. The results show that hybrid individuals had 53 bands that distinguished the two parental species. The majority of these bands (30) were shared with *M. excelsa* while 23 were shared with *M. robusta*.

Table 4.7 Frequency of AFLP bands which are shared by hybrids and only one of the parental species.

Amplifying AFLP Primer combination	Band size (bp)	Band frequency in *M. excelsa* population	Band frequency in *M. robusta* population	Frequency of shared parental band in hybrid population
NED EcoRI-ACC+ MseI-CG	69	0.43	0.00	0.42
	83	0.00	0.76	0.48
	121	0.00	0.10	0.03
	165	0.43	0.00	0.23
	193	0.00	0.07	0.16
	196	0.00	0.17	0.13
	198	1.00	0.00	0.90
	245	0.00	0.24	0.10
	324	0.00	0.31	0.39
	346	0.31	0.00	0.13
	349	0.49	0.00	0.19
	355	0.54	0.00	0.48
	397	0.00	0.17	0.06
	412	0.00	0.31	0.32
	418	0.49	0.00	0.19
	434	0.00	0.07	0.03
	465	0.60	0.00	0.32
	476	0.60	0.00	0.26
	481	0.57	0.00	0.19
	494	0.00	0.31	0.13
JOE EcoRI-AGG + MseI-CTG	102	0.60	0.00	0.39
	116	0.83	0.00	0.58
	196	0.00	0.38	0.32
	198	0.34	0.00	0.06
	286	0.00	0.07	0.19
	310	0.57	0.00	0.42
	357	0.00	1.00	0.71
	399	0.03	0.00	0.03
	411	0.11	0.00	0.10
	415	0.97	0.00	0.10
	435	0.29	0.00	0.16
	440	0.63	0.00	0.29
JOE EcoRI-AGG + MseI-CG	79	0.54	0.00	0.32
	85	0.00	0.10	0.03
	158	0.00	0.10	0.03
	169	0.03	0.00	0.03
	175	0.40	0.00	0.23
	209	0.00	0.17	0.10
	268	0.00	0.31	0.19
	375	0.00	0.52	0.32
	554	1.00	0.00	0.65
JOE EcoRI-AGG + MseI-CT	103	0.69	0.00	0.42
	117	0.83	0.00	0.58
	162	0.00	0.10	0.10
	167	0.00	0.28	0.19
	228	1.00	0.00	0.74
	310	0.60	0.00	0.42
	341	0.37	0.00	0.29
	357	0.00	1.00	0.74
	360	0.89	0.00	0.74
	383	0.00	0.10	0.13
	386	0.26	0.00	0.16
	392	0.00	1.00	0.77

Some bands were not private to one of the parental species, but they showed a substantial difference in frequency (≥0.70) between the two parental species. Table 4.8 gives a list and the frequencies of these bands. The table shows that hybrid individuals shared 25 high-frequency bands from both parental species (14 bands from *M. excelsa* and 11 bands from *M. robusta*).

Table 4.8 AFLP bands that have substantial frequency difference (≥ 70%) between parental species.

Amplifying AFLP Primer combination	**Band size (bp)**	**Band frequency in *M. excelsa* population**	**Band frequency in *M. robusta* population**	**Frequency of shared parental band in hybrid population**	**Band frequency difference between *M. excelsa* and *M. robusta* (≥0.70)**
NED EcoRI-ACC+ MseI-CG	90	0.91	0.03	0.55	0.88
	141	1.00	0.24	0.90	0.76
	179	0.03	1.00	0.71	0.97
	251	1.00	0.24	0.74	0.76
	258	0.91	0.07	0.71	0.85
	271	0.03	0.93	0.61	0.90
	311	1.00	0.30	0.81	0.70
JOE EcoRI-AGG + MseI-CTG	104	0.03	1.00	0.74	0.97
	142	0.11	1.00	0.90	0.89
	156	0.11	0.97	0.55	0.85
	223	0.06	0.93	0.58	0.87
	485	0.11	0.90	0.65	0.78
JOE EcoRI-AGG + MseI-CG	89	0.89	0.19	0.55	0.70
	95	1.00	0.17	0.81	0.83
	121	0.03	0.97	0.61	0.94
	127	0.97	0.24	0.84	0.73
	132	1.00	0.24	0.87	0.76
JOE EcoRI-AGG + MseI-CT	137	0.03	0.83	0.77	0.80
	209	0.30	1.00	0.84	0.70
	223	0.06	0.97	0.58	0.91
	248	0.97	0.03	0.84	0.94
	286	1.00	0.14	0.81	0.86
	318	0.86	0.07	0.81	0.79
	372	1.00	0.07	0.74	0.93
	497	0.89	0.10	0.55	0.78

4.4.3 Genetic diversity and structure

The genetic diversity and structure analyses were conducted after grouping individuals into three populations based on the results from the NEWHYBRIDS analysis. Table 4.9 gives estimates of the genetic diversity of the three populations based on both AFLP and SSR markers. These results show that the proportion of polymorphic loci (*P %*) ranged from 68.90% for the *M. robusta* population to 89.30% for the hybrid population, with an average of 77.2%. Both of the genetic diversity estimates (expected heterozygosity, H_e and the

proportion of polymorphic loci, *P*%) showed that the hybrid population had the highest genetic diversity (*P%* = 89.3%, H_e = 0.28) and the *M. robusta* population had the lowest genetic diversity (*P%* = 68.90%, H_e = 0.17), with the *M. excelsa* population having an intermediate diversity (*P%* = 73.7%, H_e = 0.22). As expected, the SSR markers gave a much higher estimate of genetic diversity (H_e) for all populations (0.65 for *M. excelsa*, 0.55 for *M. robusta* and 0.71 for the hybrid population) than the AFLP markers.

Table 4.9 Genetic diversity within populations based on AFLP and SSR markers

Population	***N***	**AFLP**			**SSR**						
		P %	H_e	F_{st}	N_a	N_e	N_{pa}	N_{pa} **excluding hybrid population**	H_o	H_e	F_{st}
M. excelsa	35	73.7	0.22	0.24*	6.89	3.69	11	42	0.63	0.65	0.12**
Hybrids	31	89.3	0.28		7.11	3.52	3	-	0.78	0.71	
M. robusta	29	68.9	0.17		4.67	2.54	10	22	0.57	0.55	
Mean		**77.2**	**0.22**		**6.22**	**3.25**	**8**		**0.66**	**0.63**	

N – Population size; *P%* - proportion of polymorphic loci; H_e - expected heterozygosity; N_a - Number of alleles per locus; N_e - Effective number of alleles per locus; N_{pa} – Number of private alleles; H_o - Observed heterozygosity; F_{st} – Wright´s Fixation index interpreted as genetic differentiation among populations (Significant deviations from zero are indicated by asterisks (*P < 0 .000, **P < 0 .001, n = 9999 random permutations)).

The Pair-wise F_{ST} matrix (Table 4.10) based on both data sets shows that the hybrid population had a lower genetic differentiation from the *M. excelsa* population than it had with the *M. robusta* population, suggesting a more frequent (biased) introgression of hybrids with *M. excelsa*.

Table 4.10 Pair-wise F_{ST} estimates of genetic divergence between populations obtained with SSR (above diagonal) and AFLP (below diagonal) markers

	M. excelsa	Hybrids	*M. robusta*
M. excelsa		0.05	0.19
Hybrids	0.11		0.07
M. robusta	0.43	0.16	
All values are significantly different from zero at P < 0.001 for SSR and at P < 0.000 for AFLP based on 9999 permutations			

The principal coordinate analyses based on both AFLP (Figure 4.5a) and SSR data (Figure 4.5b) showed a clear separation of the three populations, with hybrids filling the gap between the parental species. The first and second axes explained 44.48% and 3.59% of the variation respectively for the AFLP data and 36.43% and 8.46% of the variation respectively for the SSR data.

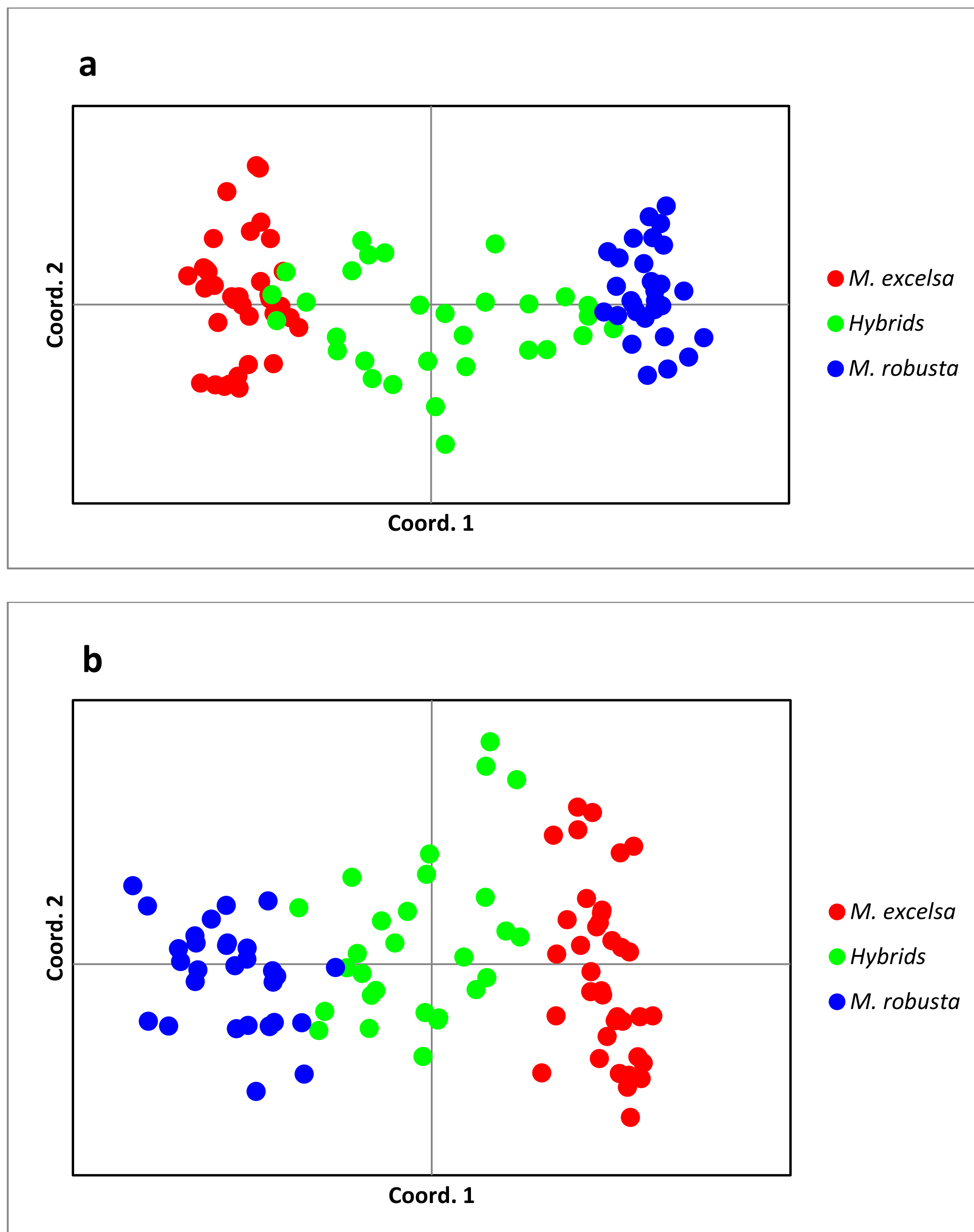

Figure 4.5 Principal component analyses of 95 individuals of the study populations based on AFLP markers (a) and SSR markers (b). The first and second axes explained 44.48% and 3.59% of the variation, respectively for the AFLP data and 36.43% and 8.46% of the variation, respectively for the SSR data.

4.5 Discussion

4.5.1 Molecular detection of hybridisation

Because of their ability to discriminate closely related genomes, AFLP and SSR markers have been successfully used to confirm the presence or absence of hybridisation between species (e.g., Mohan *et al*., 2013; Koerber *et al*., 2013; Nadeem *et al*., 2014; Avila-Flores *et al*, 2016). A review on the genetic studies of hybridisation in New Zealand showed that these two markers are among the most common markers used in New Zealand (Morgan-Richards *et al*., 2009). The AFLP makers and chloroplast markers in particular have been used repeatedly (e.g., Perrie *et al*., 2003; Gardner *et al*., 2004; Smissen & Heenan, 2007; Smissen *et al*., 2007).

Simpson (2005) suggested that natural hybridisation has probably been an important factor in the evolution of pohutukawa and rata. A phylogeographic study of five New Zealand endemic *Metrosideros* species (*M. excelsa, M. robusta, M. bartlettii, M. umbellata and M. kermadecensis*) also identified hybridisation (Gardner *et al*., 2004). The authors hypothesised that hybridisation might have been initiated by the coexistence of these species in the same refugium during the Pleistocene glacial period, which caused a maximum contraction of the range of these species. There are also numerous other reports of hybridisation between *M. excelsa* and *M. robusta* (e.g., Cooper, 1954; Allan, 1961; Julian, 1992; Wilcox, 2007; Dawson *et al*., 2010), although until now, no molecular work had been done to confirm this. Hybrids of these two species have been referred to as *Metrosideros excelsa* Sol. ex Gaertn. x *M. robusta* A. Cunn. (Julian, 1992).

This study offers the first genetic confirmation for the occurrence of hybridisation between *M. excelsa* and *M. robusta* on Rangitoto Island. Both distance-based (PCoA) and model-based (STRUCTURE and NEWHYBRIDS) cluster analysis methods confirmed the presence of interspecific hybridisation between these two species on the island. Given the molecular phylogenic study of genus *Metrosideros* (Chapter 2), these two species probably belong to one monophyletic clade with a position furthest from the root of the tree, suggesting their recent evolutionary origin. Because most island endemic species have a recent evolutionary origin, they usually have a weak postzygotic barrier and tend to hybridize without much difficulty even when they have morphological and ecological differences (Levin *et al*., 1996; Francisco-Ortega *et al*., 2000).

Based on the AFLP NEWHYBRID analysis, which detected the maximum number of hybrids, 31 individuals (33% of the total sample) were hybrids using the $P \geq 0.90$ threshold value (Table 4.2). The number of individuals falling under the various hybrid classes (Table 4.3) using the $P \geq 0.60$ threshold value, which left no individual unassigned, showed approximately 71% of these 31 hybrids (22 individuals) were advanced generation hybrids (i.e., 12 *M. excelsa* backcrosses, seven *M. robusta* backcrosses and three F2 hybrids). Because of their proximity to parental classes, the two individuals that were assigned as parental types with $P < 0.90$ can be considered backcrosses. With this assumption, the total number of backcrosses would be 24, or 77% of the total hybrids detected, suggesting the presence of a considerable introgression between *M. excelsa* and *M. robusta* on Rangitoto Island.

The STRUCTURE analysis using this same marker (AFLP) gave comparable results (26 hybrids detected using the $P \geq 0.90$ threshold value). The only difference was that the STRUCTURE program identified five individuals as parental types while the NEWHYBRIDS program identified them as hybrids. The STRUCTURE analysis showed that two of these five individuals (tree ID 65 and tree ID 66) had probabilities which fell on the border separating hybrids from parental types (tree ID 65 had $P = 0.90$ and tree ID 66 had $P = 0.91$). If these two individuals are considered as hybrids, then the difference in the number of hybrids detected by the two programs is just three. At threshold $P \geq 0.95$, the difference was just two (tree ID 46 and tree ID 67).

Generally, both programs detected a comparable number of hybrid individuals. This supports Vaha and Primmer (2006), who assessed the performance of these two clustering methods under various hybridisation scenarios (i.e., varying number of loci and F_{st} values of hybridising taxa) and found out that both methods have similar hybrid detection efficiency. Other studies that used both programs to detect hybridisation found similar results (e.g., Streiff *et al*., 2005; Wallace, 2006; Hoban *et al*., 2009).

However, although the two programs gave comparable results, the NEWHYBRIDS program is preferred because of its ability to identify different hybrid classes with a given probability. Also, the NEWHYBRIDS program has been reported to be more accurate than the STRUCTURE program in certain cases (Burgarella *et al*., 2009). Vaha and Primmer (2006) also showed that NEWHYBRIDS performs slightly better than STRUCTURE when F1 hybrids and backcrosses of both parents are present, which is the case in this study.

The NEWHYBRIDS analysis of SSR markers (Table 4.4) unexpectedly detected a smaller number of hybrids than the AFLP markers (28 hybrids compared with AFLP´s 31 detected hybrids). Moreover, Table 4.5 shows that using three of the four threshold values ($P \geq 0.95$, $P \geq 0.90$, and $P \geq 0.80$), the SSR markers detected fewer backcrosses than the AFLP markers, although under the lowest threshold values ($P \geq 0.50$ and $P \geq 0.60$) both markers produced similar results. This suggests that the SSR markers were not as efficient as AFLP markers in distinguishing backcrosses from the parental classes. Although this seems surprising given the assumed higher variability of SSR markers, it could be explained by the few loci used (nine) and the moderate number of alleles per locus (9.77) obtained in this study.

Although the number of alleles obtained per locus was comparable to that obtained for Hawaiian *Metrosideros polymorpha* (10.77), for which these markers were developed (Crawford *et al*., 2008), this number is smaller than other studies that have used SSR markers for hybridisation analysis (e.g., Hoban *et al*., 2012 - 12 SSR loci gave 29.7 alleles per locus; Li *et al*., 2016 - 10 SSR loci gave 14.4 alleles per locus, Backs *et al*., 2016 – eight SSR loci gave 12.63 alleles per locus). Conversely, although AFLP markers are dominant markers, the large number of loci recovered with these markers (206 markers) must have compensated for the bi-allelic nature of AFLP markers and offered a better resolution than the multi-allelic but few SSR loci.

The SSR markers not only revealed fewer hybrids but also smaller probabilities of class assignments and more unassigned individuals than the AFLP markers. This suggests that a limitation of the SSR markers is that it is difficult to effectively assign individuals to a particular class with a low number of loci and moderate number of alleles per locus. Similar observations were made in a hybridisation analysis of the native North American butternut (*Juglans cinerea*) and the introduced Japanese walnut (*J. ailantifolia*; Hoban *et al*., 2009). This study used eight microsatellite markers, resulting in four unclassified individuals and 13 individuals with low probabilities ($0.5 \leq P \leq 0.8$) out of the 55 hybrids that were detected using the $P \geq 0.50$ threshold value. In several other studies that used a small number of SSR markers (e.g., Lepais *et al*., 2009; Penaloza-Ramirez *et al*., 2010; Cullingham *et al*., 2012), the markers were good at broadly classifying individuals as pure parental types or hybrids rather than at differentiating different hybrid classes.

Although recent hybridisation can be detected using a few SSR loci, an accurate and efficient detection of all hybrid classes (F1, F2 and backcrosses) may require as many as 24 to 48 SSR loci (Vaha & Primmer, 2006). Epifanio and Philip (1997) stated that a high hybrid detection

error could be involved if few loci are available, even with the presence of species-specific diagnostic loci. Unfortunately, the majority of genetic studies use fewer markers. A survey of published literature by Koskinen *et al*. (2004) showed that 90% of genetic studies used less than 10 microsatellite loci. In the present study, we used nine SSR loci. Therefore, although the present study successfully detected a good number of hybrids, the actual number of hybrids may be higher.

Although the SSR markers produced slightly lower estimates of hybridisation than the AFLP markers, both markers detected a number of first and advanced generation hybrids, confirming a significant level of introgression between *M. excelsa* and *M. robusta* on Rangitoto Island. The detection of all hybrid classes in this study agrees with Allan (1961), who stated that *M. excelsa* and *M. robusta* hybrids constituted a series of polymorphic forms between the extreme parental types rather than a single intermediate type. Carse (1926) also observed that in some hybrids the calyx and capsule looks like those of *M. excelsa* (then known as *M. tomentosa*), whereas other hybrids looked more like *M. robusta*, which suggests the hybrid populations consist of both *M. excelsa*-like and *M. robusta*-like hybrids. This is supported by this study, which found a number of *M. excelsa* and *M. robusta* backcrosses. Cooper (1954) and Julian (1992) also believed that Rangitoto Island contains some hybrids that are indistinguishable from the two parental species and others that are intermediate between the parental species. The authors also mentioned that most of the hybrids looked like *M. excelsa* more than *M. robusta.*

The fact that the hybrid population possesses bands that are diagnostic to both parental species (Table 4.7) is another genetic confirmation of hybridisation between *M. excelsa* and *M. robusta*. The hybrid population had 23 bands that are present in the *M. robusta* population but missing in the *M. excelsa* population and 30 bands also present in the *M. excelsa* population but missing in the *M. robusta* population. In total, 53 bands that distinguish the two parental species were found in the hybrid population, suggesting that the genome of hybrid individuals has been drawn from both *M. excelsa* and *M. robusta* through hybridisation. Although they were not exclusively found only in one of the parental species, 25 bands also showed a substantial difference in frequency (≥ 70%) between the parental species (Table 4.8). These 25 bands are not as strongly diagnostic as the 53 bands listed in Table 4.7, but the fact that the hybrid population shared these high frequency bands from both parental species suggests extensive introgression between *M. excelsa* and *M. robusta*.

Another strong line of evidence for hybridisation between *M. excelsa* and *M. robusta* is the pattern of SSR private alleles (Table 4.9). The table shows that *M. excelsa* and *M. robusta* had 11 and 10 private alleles respectively, whereas the hybrid population had only three private alleles which might have resulted from increased mutation rates or intragenic recombinations between divergent parental alleles in hybrids (Golding & Strobeck, 1983; Hoffman & Brown, 1995). This suggests that the genetic makeup of the hybrid population was derived from the parental populations through hybridisation. When private alleles were computed for the parental populations excluding the hybrid population, *M. excelsa* and *M. robusta* had 42 and 22 private alleles, respectively. This implies the hybrid population combines 31 (42-11) private alleles of *M. excelsa* with 12 (22-10) private alleles of *M. robusta*, indicating extensive introgression between *M. excelsa* and *M. robusta*. The PCoA cluster analysis based on both markers (Figure 4.5) placed individuals in two distinct parental groups (*M. excelsa* and *M. robusta*) with hybrids in between these parental species on the ordination space.

All the above findings confirm Cooper (1954)'s report of successful natural hybridisation between these two species on Rangitoto Island and of fertile hybrids that can reproduce with the parental species. Whiting (1986) found a 19% germination rate of seeds collected from a wide range of *Metrosideros* hybrids growing on Rangitoto Island, which confirms the fertility of the hybrid individuals. Although a 19% germination rate seems low, Dawson (1968) found only a very low proportion of seeds (9%) of *M. excelsa* and *M. robusta* contained embryos and he found that low fertility is also a characteristic of other species belonging to the Myrtaceae family.

Higher fertility of hybrids has also been reported for other New Zealand species. For instance, F1 hybrids of New Zealand *Chionochloa* species showed as high as 90% pollen fertility (Connor, 1967). In this species, hybrids were more vigorous than either parental species. A hybridisation study of the New Zealand *Kunzea ericoides* complex also showed that none of the hybrid plants had a reduced pollen fertility or seed viability (de Lange *et al*., 2005).

In this study, both AFLP and SSR markers showed that F2 hybrids make up the smallest portion of the sample. This cannot be attributed to the limitation of the markers to detect these second-generation hybrids since both markers detected a good number of advanced generation backcrosses. It could be that F2 hybrids have a lower viability than the other

hybrid classes. A low frequency of F2 hybrids has also been reported for other species (e.g., Lepais & Gerber, 2011; Ortego *et al*., 2014).

Arnold (1997) mentioned that two species should overlap in time and space for hybridisation to take place. Natural hybridisation between *M. excelsa* and *M. robusta* is therefore expected to occur in areas where distributions and flowering times of these two species overlap. Dawson *et al*. (2010) mentioned that the flowering time of these two species usually overlaps towards the end of flowering of *M. excelsa* and the start of flowering of *M. robusta.* The flowering period for *M. robusta* may range from October to February but mainly from November to January, whereas that of *M. excelsa* may range from August to March but mainly from November to December (Allan, 1961).

Apart from having a flowering time overlap, the outcrossing nature of these two species and the presence of generalist pollinators might also have facilitated hybridisation between *M. excelsa* and *M. robusta*. A number of effective pollinators are known for *M. excelsa* and *M. robusta*, including native New Zealand honeyeaters (Meliphagidae), several introduced birds, native and introduced bees, lizards and bats (Anderson, 1997; Sawyer & McKessar, 2007). The fact that these two species are pollinated by birds and dispersed by wind also helps the transport of pollen and seed over large distances to ensure opportunities for hybridisation.

4.5.2 Patterns of hybridisation

The introgression between two hybridising taxa can be bidirectional (i.e., F1 hybrids backcross to both parental species) or unidirectional (to only one of the parental species). In this study, both *M. excelsa* and *M. robusta* backcrosses were detected, indicating bidirectional introgression. However, the fact that both molecular markers revealed more *M. excelsa* backcrosses than *M. robusta* backcrosses (Table 4.3 and Table 4.5) suggests that the bidirectional introgression is biased towards *M. excelsa*. This agrees with Cooper (1954) and Julian (1992), who reported that most hybrids looked more like *M. excelsa* than *M. robusta.*

The genetic structure analysis (Table 4.10) and the PCoA analysis (Figure 4.5) also showed that the hybrid population is genetically more similar to *M. excelsa* than to *M. robusta*. In addition, the hybrid population shares more bands with the *M. excelsa* population than with *M. robusta* population (Table 4.7), suggesting a relatively more frequent introgression towards *M. excelsa*. Hence, although introgression of F1 hybrids is not limited to only *M.*

excelsa or *M. robusta*, the results from the various analyses seem to suggest a more biased introgression towards *M. excelsa*.

Asymmetric introgression is common in plants (Tiffin *et al*., 2001), and a bidirectional but biased introgression has been reported for several species (e.g. Burgess *et al*., 2005; Thompson *et al*., 2010b; Hoban *et al*., 2012). For example, a hybridisation analysis between two Mexican species (*Tithonia tubaeformis* and *T. rotundifolia)* showed a more frequent introgression of hybrids towards *T. rotundifolia* (Tovar-Sanchez *et al*., 2012).

Asymmetric introgression can be caused by asymmetric hybrid viability (Ellison *et al*., 2008), by differences in relative abundance or dispersal ability of the hybridising taxa (Burgess *et al*., 2005; Lepais *et al*., 2009), or by differences in the reproductive system of the species involved in the hybridisation process (Field *et al*., 2011; Natalis & Wesselingh, 2012). On Rangitoto Island, a difference in dispersal ability of the parental species is unlikely since both species coexist sympatrically. Moreover, *Metrosideros* species are known for their efficient dispersal (Wilson, 1996). Differences in floral structure, flowering time and mating system are also unlikely an issue for *M. excelsa* and *M. robusta*, since there are no significant differences in in these characteristics between the two species. The most likely explanation is the relative abundance of these two species, as *M. robusta* is rare compared with the abundant *M. excelsa* on Rangitoto Island.

The high abundance of *M. excelsa* on Rangitoto could be due to the large size of the founding population or a repeated dispersal from nearby Moutapu Island. A difference in the abundance of parental species has been suggested as a cause of asymmetrical hybridisation between two endemic New Zealand species, *Helichrysum lanceolatum* and *Anaphalioides bellidioides* (Smissen *et al*., 2007). The relative abundance of parental species has also been suggested as the cause of a biased introgression in several species (e.g., Burgess *et al*., 2005; Lepais *et al*., 2009; Neophytou *et al*., 2011; Hoban *et al*., 2012).

Julian (1992) thought that the lower abundance of *M. robusta* on Rangitoto Island could be attributed to the great competitive ability of *M. excelsa* in the coastal forest environment, and the lower number of *M. robusta* seeds in seed rain given it is uncommon or absent on surrounding islands. Regardless, the lower abundance of *M. robusta* is likely to result in a lower number of pollen donor individuals as shown in other studies (e.g., Tovar-Sanchez *et al*., 2012; Sork *et al*., 2002). Another explanation could be related to the fitness of *M. robusta* or *M. robusta*-like hybrids. Although *M. robusta* can establish on open ground, it is not as

successful as *M. excelsa* in such conditions and instead prefers an epiphytic life on branches of tall trees (Julian, 1992). *M. excelsa*, on the other hand, has roots and leaves superbly adapted to open and harsh conditions such as drought and salt (Simpson, 1994).

Biased introgression can also happen when pollinators prefer one of the parental species over the other (Dorit *et al*., 2007). For instance, a hybridisation analysis of two Swiss species, *Silene dioicia* and *Silene latifolia,* showed a biased introgression towards *S. dioicia* due to a clear preference of the pollinating bee (*Bombus terrestris*) for hybrids and *S. dioicia* compared to *S. latifolia* (Minder *et al*., 2007). *M. excelsa* is known for setting large inflorescences and on average each flower produces about 46 µL of nectar per day (Schmidt-Adam *et al*., 1999). Although *M. robusta* is reported to have high nectar output (Sawyer & McKessar, 2007), it has fewer flowers than *M. excelsa*. Hence, nectar-feeding pollinators may prefer to visit *M. excelsa* more often than *M. robusta*, resulting in a biased introgression towards *M. excelsa*. Larger numbers of flowers also mean larger pollen production. Flower number and pollen size can have effect on the pattern of plant hybridisation (Buggs & Pannell, 2006; Lepais *et al*., 2009), and differences in pollen viability have been shown to cause a biased introgression (Dorit et al., 2007).

Another possible reason for the biased introgression could be that *M. robusta* backcrosses have a lower adaptation to Rangitoto Island than *M. excelsa* backcrosses. Although there could be other historical and demographic reasons, the wide occurrence of *M. excelsa* on Rangitoto Island and the fact that individuals are taller and more well-established than *M. robusta* tress may suggest that *M. excelsa* has genotypes that are better adapted to the conditions of Rangitoto Island than the genotypes of *M. robusta*. If that is the case, hybrids with a greater portion of their genome derived from *M. excelsa* would be expected to survive better than hybrids with more of their genome derived from *M. robusta.* This would lead to a higher frequency of *M. excelsa* backcrosses and lower frequency of *M. robusta* backcrosses, as found in this study.

Further studies are required to confirm the presence of biased introgression with greater certainty and to identify which of these factors best explain the biased introgression. The direction of introgression needs to be studied further with larger sample sizes and markers that can cover a wider genomic region (e.g., SNPs, which can be discovered rapidly for larger sample sizes using the new RAD-sequencing technology; Miller *et al*., 2007; Baird *et al*., 2008). Controlled cross-pollination and germination experiments could also compare the relative performance of backcrosses of both species to determine if a significant performance

difference might dictate the pattern of hybridisation on Rangitoto Island. Such experiments would also help to determine if there is a significant difference in pollen performance (percentage of pollen germination, length of pollen tube, etc.) between *M. excelsa* and *M. robusta*.

4.5.3 Morphology vs. molecular-based hybrid detection methods

Results from the molecular work demonstrate that the identification of parental and hybrid trees based on the observation of leaf morphology is not always a reliable approach. Cases where morphologically identified hybrids were classified by genetic data as parental types have also been reported for other species (e.g., Kronforst *et al*., 2006; Blair & Hufbauer, 2010). Table 4.6 shows eight cases where the identification based on leaf morphology differed from the results of genetic data. Five of these nine cases show the limitation of the morphological approach in detecting hybrid individuals, reinforcing that morphology-based detection of hybrids may lead to underestimation of the actual level of introgression between species and that genetic markers are more reliable hybrid detection tools (Lopez-Caamal & Tovar-Sanchez, 2014).

In the other three cases, trees identified in the field as hybrids were found to be parental individuals based on the molecular work. This was not surprising, as it was difficult to determine whether these three samples were hybrids or parental types. In tree 67 and tree 77, some leaves showed clearly observable notches, while other leaves of the same tree appeared to have no notches. Moreover, the size and shape of the leaves could have been indicators of introgression with *M. excelsa* or just a manifestation of intraspecific genetic variation. Local edaphic factors rather than hybridisation might have influenced the morphology of some of the leaves on those trees.

The difficulty in identification of tree 9 in the field was associated with the small amount of tomentose on the underside of the leaves. I identified this tree as a *M. excelsa*-like hybrid because pure *M. excelsa* plants have dense rather than sparse tomentose. However, the fact that the molecular work identified this tree as pure *M. excelsa* suggests that the sparse tomentose I observed was either at the early stage of its development or that internal leaf conditions and/or external soil conditions might have prevented the full expression of this trait on this tree.

There may be a continuum of leaf morphology on Rangitoto Island due to successful introgression of fertile F1 hybrids, which could make it difficult to determine if leaf morphology is caused by hybridisation or not. Another explanation could be that those three trees are hybrids but the markers failed to detect them as such. This might happen if the diagnostic leaf morphological traits are controlled by a few genes within which none of the randomly distributed and picked AFLP fragments are located. It could also occur if these trees are the result of excessive backcrossing that ultimately erased the hybridisation signal in the AFLP markers, thereby identifying them as pure individuals rather than hybrids.

Nonetheless, the above observations show that morphology-based hybrid identification can be difficult and unreliable. It is not uncommon to see an absence of correlation between molecular and morphological traits (e.g., Hardig *et al*., 2000; Lihova *et al*., 2007; Blair & Hufbauer, 2010). Moreover, the task of detecting hybrids based on morphological observations alone becomes especially difficult beyond the F1 generation since the genetic distinction between hybrids and pure parental species becomes smaller and smaller as the number of generations of introgression grows.

Therefore, morphological traits are not helpful for a thorough analysis of hybridisation although they might help us to locate hybrid zones. Moreover, morphological traits do not differentiate between hybrid classes, which are crucial in the management of species (Allendorf *et al*., 2001). For instance, knowing that there are many individuals in the parental classes with few backcrosses would suggest that populations have not become a hybrid swarm and that pure parental species could be recovered by eliminating hybrid individuals if needed.

In summary, the primary objective of the present study was to genetically confirm the presence or absence of hybridisation between *M. excelsa* and *M. robusta* on Rangitoto Island. To this effect, although the number of different hybrid classes obtained with AFLP and SSR markers varied, both markers successfully detected a comparable, high number of hybrid individuals, suggesting that significant interspecific hybridisation is occurring between these two species on Rangitoto Island.

4.5.4 Implications of hybridisation between *M. excelsa* and *M. robusta*

Because conservation is based on the assumption that species are discontinuous pure groups, efforts often focus on species and not hybrids, which are usually considered valueless, unfit

or pollutants of genetic purity. Hybrids are not included in national and international conservation acts, including the IUCN (Fitzpatrick *et al*., 2015) and the US Endangered Species Act (Gompert & Buerkle, 2016). In cases of human-mediated hybridisation, greater value may be given to local genotypes instead of the introduced genotypes or their hybrids. However, in cases of natural hybridisation the attachment of a greater value to one species over another or over hybrids is less clearly justified since divergence and remixing of genotypes have always been parts of natural evolutionary processes (Allendorf *et al*., 2001; Fitzpatrick *et al*., 2015).

The conservation of hybrids is a controversial issue. Some argue that hybrids should not be protected if they pose a threat of genetic swamping to an endangered species (e.g., Vila *et al*., 2000; Lopez-Pujol *et al*., 2012). Others argue that hybrid populations deserve conservation because they are sources of immense genetic variation (e.g., Anderson & Stebbins, 1954; Thompson *et al*., 2010a). Still others judge the conservation value of hybrids based on whether the hybridisation is caused naturally or by anthropogenic factors (Allendorf *et al*., 2001).

Perhaps the best strategy is to protect the whole threatened habitat containing both parental and hybrid populations without worrying too much about losing the genetic integrity of the threatened and less abundant species. This species' alleles can be found in hybrid individuals and its survival can be enhanced by the introgression of adaptive genes of the more common congener through hybridisation. In other words, we should develop a gene-centred view rather than a species-centred view when decisions are made for conserving species (Petit, 2004). This means hybrids should be viewed as storehouses of genetic material of both parental species rather than as threats to their purity. Hybrid individuals are endowed with more variability than parental species and may therefore withstand future environmental challenges better than parental species. Hence, protecting genetic variation that can be generated through hybridisation should be given priority over conservation efforts that exclusively focus on maintaining the purity of species.

Conservation strategies may be successful if they embrace a more inclusive approach and focus on the ecological consequences of hybridisation rather than on its effect on the genetic or taxonomic purity of specific species (Fitzpatrick *et al*., 2015). The aim of conservation in relation to hybridisation should be to preserve the current ecosystem functioning and interactions among members of the ecosystem, rather than focusing on a narrow species-based approach. Hybridisation is important for ecosystem functioning as it represents a

biological interaction that can have broad ecological consequences (Ellstrand & Schierenbeck, 2000; Fitzpatrick *et al*., 2015). Several authors (e.g., Ruiz-Jaen & Aide, 2005; Fitzpatrick *et al*., 2010; Lester *et al*., 2010) have also emphasized that the ecological integrity of an entire ecosystem is more important than the taxonomic purity of specific species.

The value of the hybrid population of *M. excelsa* and *M. robusta* should therefore be based on its potential contribution to the Rangitoto ecosystem (i.e., boosting genetic diversity of the ecosystem, offering ecosystem services, and providing economic, cultural and aesthetic values). Conservation efforts should focus on boosting hybrid populations rather than eliminating them, so that favourable genotypes can disseminate.

The spread of genes from one species into another depends on fitness and adaptability of hybrid individuals (Meirmans *et al*., 2009). In this sense, although it is difficult to predict the future consequences of hybridisation, some elementary experiments can be conducted to assess the fitness and potential future conservation values of hybrid individuals. The relative performance of hybrid and parental genotypes can be tested and determined under a range of experimental or natural conditions, such as examining the relative regeneration capacity of parental and hybrid genotypes following fire. Similar experiments have been conducted for *Eucalyptus melanophloia* × *E. crebra* hybrids and parental individuals (Drake, 1981) and for *E. risdonii* × *E. amygdalina* hybrids and parental individuals (Potts & Reid, 1985). In conducting such experiments, genetic confirmation of hybrid vs. parental individuals is extremely important. Information on the relative fitness of hybrids and parental individuals under an artificially introduced environmental stress would also help to predict the future dynamics and composition of the Rangitoto forest under the most likely threats the population might face, thereby helping with future management plans.

Crossing experiments should also be done to learn about the relative success of backcrossing between F1 hybrids and the two parental species. This could help describe the pattern of introgression, which is crucial to predict the outcomes of hybridisation. If introgression has a symmetric (unbiased) pattern, then it will most likely lead to the collapse of the two parental taxa into a single hybrid swarm. If the introgression is asymmetrical, then the phenotypic differences between the parental species will be maintained. Such experiments can also determine whether adaptive alleles of one parental species will be introgressed into another species, which is important especially for the less abundant *M. robusta* to increase its genetic diversity and adaptability.

Although the present study revealed a bidirectional introgression that is biased towards *M. excelsa*, the pattern of hybridisation can be described in more detail using a larger sample size and more powerful markers such as SNPs, which can be quickly analysed using recent DNA sequencing technologies. If a symmetrical introgression and hence a potential collapse of the two parental taxa into a single hybrid swarm is the most likely outcome in the future, it may be preferable to view the hybrid swarm as a source of rich genetic diversity and adaptive potential for the entire ecosystem and for the evolution of new species in the future.

Future evolutions of new species can happen on Rangitoto Island via sympatric speciation. Although such speciation is not as common as allopatric speciation, mathematical models and empirical evidence indicate that the evolution of new species can happen without geographical isolation (Dieckmann & Doebeli, 1999; Savolainen *et al*., 2006; Bolnick & Fitzpatrick, 2007). In fact, recent reviews and empirical evidences (e.g., Kulathinal *et al*., 2009; Pinho & Hey, 2010; Arnold *et al*., 2012) show that sympatric speciation is not as rare as was previously assumed.

Although the extensive introgression of hybrids with *M. excelsa* and *M. robusta* suggests the absence of reproductive isolation and thus a low likelihood of hybrid speciation, sympatric speciation could occur in the future if edaphic and other ecological factors cause phenological differences. For instance, it has been reported that sympatric speciation created two palm species on Lord Howe Island (*Howea forsteriana* and *H. belmoreana*) because of reproductive isolation resulting from a six-week difference in peak flowering time (Savolainen *et al*., 2006). The authors suggest that a difference in soil substrates might have triggered the difference in flowering. Sympatric speciation has also been reported in other species (e.g., Schliewen *et al*., 1994; Filchak *et al*., 2000).

In New Zealand, there are reports of new plant species and hybrid lineages created through hybridisation (e.g., *Podocarpus totara var. waihoensis* – Wardle, 1972; *Anaphalioides hookeri* - Breitwieser *et al*., 1999; *Ranunculus nivicola* – Carter, 2006). The potential implications of hybridisation in the evolution of organisms is so far largely unknown (Abbott *et al*., 2013), but recent DNA sequencing technologies and powerful molecular markers can provide a better understanding of hybrid speciation by revealing the genetic, morphological and ecological patterns associated with the formation of new species via hybridisation.

If crossing experiments and new sequencing technology show the presence of a biased or more frequent introgression of F1 hybrids with *M. excelsa* than with *M. robusta*, then the

hybridisation between *M. excelsa* and *M. robusta* will likely serve to boost the genetic diversity of *M. robusta* rather than causing it to collapse into a hybrid swarm. Although rare and numerically inferior species can be genetically swamped by a more common related species, this may not occur if gene flow from hybrids to the rare species is moderate or low (Lepais & Gerber, 2011). Instead, it could enhance the genetic diversity of the hybridising species without losing its identity (Fitzpatrick *et al*., 2010; Becker *et al*., 2013). A moderate level of introgression has been reported to boost the genetic diversity of rare or genetically less diverse hybridizing taxa in other species (e.g., East Pacific black mangroves - Nettel *et al*., 2008; California oak – Ortego *et al*., 2014; genus *Ligularia* – Yu *et al*., 2014).

Therefore, a moderate or low level of mating between F1 hybrids and *M. robusta* could help to save rare alleles of *M. robusta* in the hybrid population, boost its genetic diversity, allow the exchange of some alleles that may have evolutionary significance, and enhance its adaptive potential on Rangitoto Island. The distinct clusters of *M. excelsa* and *M. robusta* revealed in this study suggest that genetic swamping of *M. robusta* population is not currently occurring. Similar observations were made for oak species despite the notorious hybridisation that is evident within this group (Song *et al*., 2015; Backs *et al*., 2016). Backs *et al*. (2016) found no evidence of genetic swamping despite the presence of hybridisation between sympatric oak species. Song *et al*., (2015) similarly found that natural hybridisation between two other sympatric oak species resulted in the coexistence of the parental species with hybrid individuals remaining distinct.

Genetic swamping of *M. robusta* probably will not happen if one or a combination of the following factors are present: (1) if *M. robusta* has a considerable self-compatibility for self-pollination, which is unlikely as the species is predominantly outcrossing; or (2) if there is a biased or more frequent backcrossing of hybrids with *M. excelsa* rather than with *M. robusta*, which can be tested using a crossing experiment and hybridisation analysis with more samples, powerful markers such as SNPs and new DNA sequencing technologies. On the other hand, genetic swamping may still be happening, but adequate time has not yet passed for it to take effect and be detected by the present study.

In this study, the hybrid populations showed a higher genetic diversity than the parental populations, which is similar to several other studies (e.g., Caraway *et al*, 2001; Tovar-Sanchez *et al*., 2008; Tovar-Sanchez *et al*., 2012; Bzdega *et al*., 2016). As mentioned above, the high genetic diversity contained within the hybrid population could boost the genetic diversity of *M. robusta*, enabling it to colonize new and a wider range of habitats, which is

important given the current rates of global climate change (Lindsey *et al*., 2013). If species do not possess a good level of genetic variation, they may fail to adapt to novel environmental conditions and ultimately face extinction. Compared to mutation, hybridisation provides a higher genetic diversity and faster adaptation, as alleles that can be useful in a new environment are immediately available at frequencies higher than that can be obtained through mutation (Hedrick, 2013).

The introgression of adaptive genes into *M. robusta* could help minimize its risk of extinction and to recover despite a changing environment, which is difficult to achieve in a small population by relying only on current and future mutation-driven genetic variations (Baskett & Gomulkiewicz, 2011; Carlson *et al*., 2014; Hamilton & Miller, 2016).

An experiment designed to compare the evolutionary performance of parental yeast (*Saccharomyces*) genotypes and their interspecific hybrids under various rapid and increasingly harsh environments showed that hybrid populations managed to survive longer and grow in harsher environmental conditions better than parental population (Stelkens *et al*., 2014). This suggests hybridisation can play a role in boosting genetic diversity to facilitate evolutionary rescue. The success of the weedy species *Hieracium pilosella* in the South Island has been attributed to the high genetic variability that the species acquired through hybridisation with *H. praealtum* (Morgan-Richards *et al*., 2004). Hybridisation has also been reported to help Darwin´s finches (Grant & Grant, 2008) and sunflowers (Rieseberg *et al*., 2003) to adapt to new niches. Hybridisation has been used to rescue rare and small populations in plants (e.g., Willi *et al*., 2007) and animals (e.g., Pimm *et al*., 2006).

A broader view of conservation enables us to appreciate the role of hybridisation in the evolution of new species and enrichment of genetic diversity, which might be useful for the functioning of the entire ecosystem. However, a narrow species-based conservation approach might be justifiable for highly endangered species (Ruiz-Jaen & Aide, 2005). Therefore, it is important to consider how many populations and locations of the parental (especially *M. robusta*) populations exist in other parts of New Zealand and how many of these locations have natural hybridisation.

Neither *M. excelsa* nor *M. robusta* is a nationally endangered species and both are extensively found in other parts of New Zealand, so their natural hybridisation should not be a concern. Moreover, there are no other known locations where these two species naturally grow side by side and undergo hybridisation. Hence, the natural hybridisation on Rangitoto Island should

be seen as a unique opportunity to understand how hybridisation can enhance genetic diversity.

Moreover, while allowing natural hybridisation to continue on Rangitoto Island, the *M. robusta* populations can be checked if individuals are becoming hybrids and pure parental populations are disappearing. At that point, the remaining parental genotypes could be conserved by transplanting trees to another location where hybridisation with *M. excelsa* is not possible. Once a widespread introgression happens, it will be difficult to isolate pure parental individuals from hybrids for founding a new population (Allendorf *et al*., 2001), so seeds should be collected from pure-bred trees for future genetic rescue. The local extinction of *M. excelsa* is unlikely at least soon given its current large population size on Rangitoto Island.

M. robusta is a key species in the Wellington region, where it grows in mixed coastal, lowland and montane forest communities. It protects understory vegetation, serves as a host tree for a number of epiphytes, maintains a stable forest hydrology because of its water holding capacity, feeds indigenous bird and insects, and is valued for its aesthetic and cultural significance (McKessar & Sawyer, 1999; Sawyer & McKessar, 2007). Here, this species is reported to be threatened by hybridisation with *M. excelsa* (Sawyer and McKessar, 2007), but unlike the case on Rangitoto, the hybridisation in Wellington has been caused by the indiscriminate planting of *M. excelsa* outside its natural range (Sawyer & McKessar, 2007). Allendorf *et al.* (2001) recommended the conservation of naturally admixed populations but also suggested that such populations that are created by human-induced hybridisation do not deserve conservation effort unless there are only few or no pure populations left.

However, human-induced hybridisations may sometimes boost genetic diversity and biodiversity via hybrid speciation and transgressive segregation (Crispo *et al*., 2001). Human-induced hybridisation can help rare and endangered species through the introduction of alleles that can mask deleterious recessive alleles in smaller populations (Fraser *et al*., 2010). These might otherwise be expressed due to the high chance of mating between closely related individuals in small populations. Hence, rather than treating all naturally caused hybridisation as good and anthropogenic hybridisation as bad, it would be better to understand and assess the consequences of any hybridisation event on a case-by-case basis before conservation actions are taken.

In Wellington, the long-term effect of the hybridisation between *M. robusta* and *M. excelsa* depends on the fitness, biology and ecology of the hybrid individuals in that particular region. Hence, extensive data needs to be gathered on the nature and fitness of hybrids prior to any management action. Probably, the hybridisation between these species will lead to either the enhancement of the genetic diversity of the *M. robusta* population or the genetic assimilation of this species in the long run. It is therefore important to prioritise the current need – improving genetic diversity of the local *M. robusta* population – as opposed to protecting the species' genetic identity.

On the other hand, protecting *M. robusta*'s identity may be a priority if the species is not viewed as endangered enough for there to be concerns about boosting its genetic diversity. Moreover, its genetic diversity could be increased without hybridisation by establishing new populations in areas where the species existed previously. A much higher genetic diversity could be achieved through hybridisation with *M. excelsa* but that level of genetic diversity may not be required right now given the species is not endangered. Hence, there is an argument that effort should be dedicated to the maintenance and expansion of the pure *M. robusta* population, while preventing its hybridisation with *M. excelsa* in the Wellington region. Because hybridisation of the two species in the region is most likely in its infancy, further hybridisation can be stopped or slowed by removing hybrid individuals and/or *M. excelsa* trees.

When the establishment of new *M. robusta* populations is required, threats such as possums should be dealt with prior to the restoration effort. Restoration efforts may involve not only revegetation but also the restoration of its habitat for plants and animals that directly or indirectly affect the survival of the species. For revegetation, effort should be made to establish *M. robusta* seedlings from locally sourced material. Locals should also be educated and discouraged from growing *M. excelsa* outside its natural range where *M. robusta* populations naturally exist. When planting is done, care should also be taken to avoid hybridisation with *M. excelsa*. For example, Forest and Bird (Lower Hutt branch) is planting *M. robusta* on Matiu/Somes Island, where the species originally occurred, and is only planting on the eastern side of the island where *M. excelsa* does not grow (McKessar & Sawyer, 1999; Sawyer & McKessar, 2007). Other initiatives to protect and recover the species over large parts of its range include Project Crimson (http://projectcrimson.org.nz/), the Native Forest Restoration Trust, Karori Wildlife Sanctuary Trust, Otari Native Botanic

Garden, Greater Wellington Regional Council, Queen Elizabeth II National Trust and the Department of Conservation (McKessar & Sawyer, 1999; Sawyer & McKessar, 2007).

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