A Population Genetics Investigation of the New Zealand Endemic *Lophomyrtus bullata* (Myrtaceae), a Species of Conservation Concern Due to the Threat of Myrtle Rust

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Amy Leigh Macdonald

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Abstract

Lophomyrtus bullata Burret (Myrtaceae) is a shrub or small tree species endemic to New Zealand. The species is a member of the Myrtaceae family and is one of two species in the *Lophomyrtus* genus. The conservation status of *L. bullata* was raised to "Threatened – Nationally Critical" following the establishment of the fungal pathogen *Austropuccinia psidii* (G. Winter) Beenken 2017 in New Zealand in 2017. Repeated infections of the fungal disease can lead to extensive loss of foliage, premature dropping of fruits and plant death. Infected *L. bullata* exhibit all these symptoms, and rapidly declining regional populations indicate a high potential for localised extinction. The regional disappearance of *L. bullata* could isolate remaining populations, decreasing gene flow and connectivity. Additionally, rapidly decreasing population sizes could increase the potentially harmful effects of inbreeding and genetic drift, reducing the species' fitness and increasing its risk of extinction.

An understanding of the genetic variation and structure of *L. bullata* populations could help determine its vulnerability to such genetic effects. To this end, Illumina sequencing was used to develop microsatellite markers for *L. bullata*. From the 1,351,112 successfully paired and merged sequences, 55 microsatellite loci were isolated, and primer pairs were designed. After an initial screening of the 55 primer pairs, 12 were identified as polymorphic and amplified consistently. These 12 microsatellite markers were genotyped across 452 samples representing 18 populations of *L. bullata,* one population of *L. obcordata,* and two populations of *L. bullata* x *L. obcordata* putative hybrids.

The 18 populations of *L. bullata* showed low genetic differentiation, low expected heterozygosity and very few private alleles. *Lophomyrtus bullata* populations in the upper North Island region had the highest expected heterozygosity, and there is evidence of a correlation between expected heterozygosity and decreasing latitudinal distance. High genetic differentiation was observed between the populations of *L. bullata* and *L. obcordata* and between the two hybrid populations and the *L. bullata* populations. The population structure results suggest the presence of two to five genetic clusters within *L. bullata.* The clusters reflect the geographic location of the populations, in addition to a potential North versus South cline. These patterns could have formed due to past climatic events such as glacial cycling and volcanic activity, isolation by distance, or some combination of these.

These results suggest that *L. bullata* is potentially vulnerable to the negative effects of genetic stochasticity. The continued spread and infection of *Austropuccinia psidii* could significantly exacerbate these effects. However, the higher genetic diversity and differentiation of the putative hybrid populations suggest a possible avenue for the species to acquire new adaptive variation, though this would heavily depend on the hybridisation mechanics of the species, for which there is little information. Additionally, the significant population structure of *L. bullata* identified in this study can be used as a guide for conservation practices, including seed banking, population management and re-vegetation projects.

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List of Abbreviations

%P	percentage polymorphic loci
AMOVA	analysis of molecular variance
BIC	Bayesian information criterion
bp	base pairs
CASS	cheap as size standard
CTAB	cetyltrimethylammonium bromide
DA	discriminant analysis
DAPC	discriminant analysis of principal components
ddH2O	doubled distilled water
DNA	deoxyribonucleic acid
dNTP	nucleoside triphosphate
e.g.	for example
F_{IS}	Wright's fixation index
F_{ST}	measure of population differentiation
GBS	genotyping-by-sequence
He	expected heterozygosity
Ho	observed heterozygosity
HWE	Hardy-Weinberg equilibrium
IBD	isolation by distance
IUCN	International Union for Conservation of Nature
Ma	million years
mm	millimetres

Mya	million years ago
n	chromosome number in a haploid
N	sample size
Na	number of alleles per locus
Ne	number of effective alleles per locus
NGS	next generation sequencing
Pa	number of private alleles
PCA	principal component analysis
PCR	polymerase chain reaction
RADseq	restriction site-associated DNA sequencing
Rxy	correlation coefficient of the Mantel test
SNP	single nucleotide polymorphism
SSR	simple sequence repeat
STR	short tandem repeat
TBE	tris (HCl), boric acid and EDTA
QTL	quantitative trait locus

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Chapter 1

Introduction

1.1 Conservation biology

The field of conservation biology was created with the specific objective of protecting and preserving the world's biological diversity (Meine et al., 2006). This mission-driven field was formed in direct response to the current mass-extinction event primarily attributed to anthropogenic causes, such as habitat destruction and fragmentation, overharvesting, and the introduction of exotic species (Beggs, 2001; Craig et al., 2000; Diamond, 1989). Determining the best practices for slowing the ever-increasing rate of populations declining to extinction is a vital focus for conservation biologists, and the need for conservation biology to be multi-disciplinary in its approach was evident from its inception. The wide-ranging field incorporates ideas and tools from a variety of related areas of study, including resource management, ecology, wildlife biology and genetics (Frankham et al., 2002). These disciplines combine to examine what the International Union for Conservation of Nature (IUCN) has identified as the three main components of biodiversity: ecosystem diversity, species diversity and genetic diversity (Schatz, 2009).

Acknowledgment of the role genetics plays in the conservation of a threatened species has grown over the past decade (Anderson et al., 2021) and has led to the expansion and increased visibility of the field of conservation genetics (DeSalle & Amato, 2004; Frankel & Soule, 1981). The main goal of conservation genetics is to identify the level of genetic diversity present within a species and undertake steps to stabilise and preserve that diversity (Frankel & Soule, 1981). Such steps typically involve minimising the impact of destructive forces that can cause genetic erosion (e.g., genetic drift, inbreeding and outbreeding depression) by restoring gene flow and diversity within and among isolated declining populations (Frankel & Soule, 1981; Mijangos et al., 2015). Preserving genetic diversity in a species is essential as it reflects its capacity to adapt to environmental changes (Frankel & Soule, 1981; Holsinger & Falk, 1991). If such environmental changes are irreversible, the persistence of a species depends on its potential to adapt, which, in turn, is dependent upon genetic diversity (Chevin et al., 2010;

Reynolds et al., 2012). Therefore, a species (or population) with low genetic diversity may fail to adapt and have a higher probability of extinction (Holsinger & Falk, 1991).

Conservation genetics studies can employ a range of different methodologies to obtain information on the genetic diversity of a species. This thesis uses a population genetics approach to investigate the genetics of a species of conservation concern: the New Zealand endemic *Lophomyrtus bullata* Burret (Myrtaceae). The species is threatened by the establishment of the pathogenic fungus *Austropuccinia psidii* (G. Winter) Beenken 2017 (myrtle rust) and requires urgent conservation intervention (Smith et al., 2020; Sutherland et al., 2020; Toome-Heller et al., 2020). The following section provides an overview of the methods utilised in population genetic studies and how the information gained through those methods can be applied to the conservation and preservation of biodiversity in New Zealand.

1.2 Population genetics

Population genetics involves characterising the level and distribution of genetic diversity within and among populations. In this context, a population refers to a group of interbreeding individuals. The measures of genetic diversity are typically determined by estimating allele frequencies at a set of loci (Kimura & Ohta, 1971). Changes to allele frequencies depend on how various evolutionary mechanisms are acting on a population. Such mechanisms include mutation, natural selection, genetic drift/draft, and gene flow. Divergent natural selection and genetic drift/draft can increase divergence between populations, while gene flow typically counteracts it by facilitating the movement of genetic material between populations (Casillas & Barbadilla, 2017; Hamilton, 2021). Population genetics analyses can potentially determine to what degree each of these forces influences a population's genetic structure and the genetic divergence between populations of the same or different species.

1.2.1 Molecular markers used in population genetics

To obtain estimates of how variable alleles are at a particular locus, population genetic studies employ the use of molecular markers (Schlötterer, 2004). The first molecular markers developed were allozymes (Schlötterer, 2004). These were based on protein variations in enzymes, specifically differences in size and charge (Schlötterer, 2004). These have largely been replaced with the development of DNA-based molecular markers, particularly after the inception of the Polymerase Chain Reaction (PCR) technique. DNA molecular markers

typically used today include microsatellite loci and several methods that detect and differentiate SNPs (Single Nucleotide Polymorphisms).

Microsatellites are short, tandem repeat sequences (STRs) or simple sequence repeats (SSRs) that are frequently found throughout prokaryotic and eukaryotic genomes and are assumed to (typically) evolve neutrally (Li et al., 2002). The repeats of microsatellites can range from one or two bp (mono- and dinucleotides, respectively) up to six bp (hexanucleotides). The length of these repeats is highly variable between individuals and species (Selkoe & Toonen, 2006) due to a mechanism called replication slippage, which happens during the process of DNA replication (Ellegren, 2004). Essential characteristics of microsatellites, such as a high mutation rate, codominance, and high levels of polymorphism (Guichoux et al., 2011), have led them to be used in a wide range of applications, including molecular forensics, genome mapping, plant breeding, conservation genetics and population genetics (Hodel et al., 2016; Kalia et al., 2011).

Despite the high versatility of microsatellites, there are a few noted issues with their use. The most discussed is the proneness of the replication slippage mechanism to cause back mutations, which can lead to homoplasy (Viard et al., 1998). This can make it difficult to determine whether trends observed in allele frequencies are due to homology or homoplasy (Hodel et al., 2016). Other notable issues include the possible suppression of F-statistic estimates if there are very high or very low frequencies of the most common alleles (Gautier et al., 2013; Jakobsson et al., 2012). The tendency for microsatellite markers to have a higher number of alleles per locus can also potentially result in falsely increasing F-statistics (Zimmerman et al., 2020).

In the past decade, there has been an increasing trend to use SNPs for genetic analysis in population genetics and other disciplines. The use of SNPs was initially out of favour due to cost and time constraints, but the increased improvement of Next Generation Sequences (NGS) techniques has made this a viable option (Elshire et al., 2011). SNPs are single base pair mutations that occur widely throughout the eukaryotic genome. SNPs involve the presence of at least two different nucleotides at a given position in the genome, resulting in two alternative alleles (Arif et al., 2010). Compared with microsatellites, SNPs have lower levels of homoplasy (Morin et al., 2004; Rafalski, 2002) and are also better able to detect linkage disequilibrium and haplotypes compared to other markers (Rafalski, 2002). The most common SNP-based approaches are genotyping-by-sequence (GBS) and restriction site-associated DNA sequencing (RADseq), which both involve restriction enzymes and unique barcoded adaptors used to assign bulked sequences to individual samples (Elshire et al., 2011; Miller et al., 2007).

SNP-based methods allow a considerable amount of sequencing data to be produced, which can be used to assess genetic variation across the whole genome (Hodel et al., 2016), and due to the large amount of data produced, there is less concern for genotyping errors (Gautier et al., 2013). However, the cost and complexity of these methods are still much higher compared to microsatellites, especially if data from many individuals are required for the study (Hodel et al., 2016; Zimmerman et al., 2020). There have also been several issues with these SNP-based methods, including the fact that since SNPs are typically only biallelic, allelic diversity estimates have been suggested to be lower compared to microsatellite loci, which can often have many alleles (Zimmerman et al., 2020).

There is currently a debate about which of these two marker systems, SNPs or microsatellites, is the most adequate in terms of both practicality and estimates of genetic diversity (Coates et al., 2009; Hodel et al., 2016; Tsykun et al., 2017). Some have even predicted that the methods used to detect the potentially non-neutral SNPs will largely replace neutral markers like microsatellites (Brumfield et al., 2003; Zimmerman et al., 2020). Some comparative studies between microsatellites and SNPs have observed similar levels of diversity and population structure estimates (Heenan et al., 2023; Lemopoulos et al., 2019; Zimmerman et al., 2020). However, in other cases, there were significant differences between the marker systems (Fischer et al., 2017; Van Inghelandt et al., 2010). The majority of the comparisons seem to suggest that choosing a suitable marker type depends on the study species and the questions the research wishes to answer.

1.2.2 Population genetics analytical methods

The data obtained through molecular markers can be applied to various analyses to determine the population structure of a species, most notably the application of the Hardy-Weinberg Equilibrium (HWE). The Hardy-Weinberg equilibrium, developed separately by Hardy (1908) and Weinberg (1908), is typically employed in population genetics studies as a null model of evolution (Wakeley, 2005; Wright, 1949). This null model assumes a theoretical population that has no evolutionary forces acting on it, i.e., no migration, mutation, genetic drift or natural selection, and random mating is occurring. In population genetic studies, HWE analysis typically involves detecting whether a population conforms or deviates from the expectations of HWE. Deviations can indicate the potential influences of various evolutionary mechanisms, such as natural selection or random genetic drift.

F-statistics are population-based metrics developed by Wright (1949) for use in population genetic studies. They include F_{IS}, F_{ST} and F_{IT}, though F_{IT} is rarely used and will not be discussed further. F_{IS} measures the frequency of heterozygotes observed relative to the expected under the null model of HWE. The resulting value can range from a negative one to a positive one, with a negative value indicating heterozygote excess, a positive value indicating homozygote excess, and values closer to zero indicating the population is at HWE. Heterozygote excess in a population could be due to selection for heterozygotes, asexual reproduction, high amounts of outcrossing or hybridisation (Balloux, 2004; Wu et al., 2020). In contrast, inbreeding, null alleles or selection for homozygotes can lead to homozygotes excess in a population (Robertson & Hill, 1984). F_{ST} measures the amount of genetic differentiation between two different populations, with the resulting value ranging from zero to one. If a value closer to zero is obtained, this would indicate that the populations are more genetically similar, whereas a value closer to one would indicate that the populations are more genetically different.

There are several ways to visually identify and observe patterns in a dataset. A few of the most commonly used methods include STRUCTURE (Pritchard et al., 2000) and Neighbour-net (Bryant & Moulton, 2004). STRUCTURE is a program used by population geneticists to analyse different genetic groups based on the genetic composition of individuals (Pritchard et al., 2000). It does this by applying a systematic Bayesian clustering algorithm to the data, which clusters individuals into groups that conform to the assumptions of HWE (Pritchard et al., 2000). Neighbor-net uses distance matrices to develop a complex network that can similarly be used to analyse the genetic distance between individuals and populations (Bryant & Moulton, 2004). These analytical methods can provide information on the patterns of relationships between populations that differ in characteristics such as geographical location, phenotype, and ecological behaviour.

Data also can be observed visually through multivariate analysis methods such as Principal Component Analysis (PCA) or Discriminant Analysis (DA). A PCA scales or centres the data and then plots the data in multidimensional space, starting with the two data points that have the maximal amount of variance (Reich et al., 2008). This can provide information on how different populations are grouped and how similar or dissimilar they are compared to other populations. Discriminant Analysis (DA) is similar to PCA, except it requires groups of genotypes to be identified beforehand and then plots the data by increasing the variance between groups (Jombart et al., 2010). Discriminant Analysis of Principal Components (DAPC) is a form of DA that first transforms the data into principal components, then identifies

genetic clusters within the transformed data, and plots them using the DA method (Jombart et al., 2010). Generally, most population genetic studies will use multiple approaches to verify that the patterns observed in the data are a reflection of what is actually occurring in the populations rather than a product of the type of analysis used.

1.2.3. A population genetics approach to conservation genetics

The knowledge gained from population genetic methods can be directly applied to conservation management practices. Applying a population-level approach as opposed to a species-level approach to conservation management allows for a greater understanding of the genetic structure and connectivity of a species. For example, the total genetic diversity of a species may only be represented by a few highly diverse populations, with the remaining isolated and declining. Failing to recognise these populations that are in need of conservation management could result in their localised extinction and subsequent loss of genetic diversity for the species. Additionally, if the genetics of two populations of a species differ greatly, implementing the same conservation practices on both populations could be more harmful than helpful, and instead, the populations should be managed as separate units (Chapple et al., 2012; Crandall et al., 2000).

Understanding how the genetic variation of a species is partitioned across a landscape can also reveal different patterns of relationships. These patterns can be geographical, e.g., populations in one particular region are all genetically very similar compared to other more distantly distributed regions, or environmental; e.g., the presence of rare soil types increasing fitness for individuals with sui

genotypes (Aguirre-Liguori et al., 2019). This information can be used in conservation practices to capture and preserve the entire range of local diversity present for a threatened species, increasing efficiency by reducing costs and the number of sites required for preservation (Edwards et al., 2021; Neel & Ellstrand, 2003; Wu et al., 2020). Similar principles can be applied to seed banking rapidly declining species to ensure that collections are from populations that cover the entire range of the species diversity (Schoen & Brown, 2001).

Information on the distribution of genetic variation in a species can be incorporated into its restoration efforts, which can greatly increase their success (Mijangos et al., 2015). This includes determining the need for eco-sourcing, especially if populations are highly differentiated across different regions (McKay et al., 2005). In this case, eco-sourcing might

be required to prevent the introduction of potentially unsuitable genotypes, increasing the genetic load and decreasing fitness (Willi et al., 2022). Alternatively, if high levels of inbreeding are observed in a population, then collecting seed or sourcing individuals for restoration can be avoided, as individuals are likely highly inbred and contain deleterious alleles (Mijangos et al., 2015). The probability of outbreeding depression can also be determined, which can be useful in ascertaining the outcomes of translocating individuals between populations to restore gene flow and increase genetic diversity (Frankham et al., 2011).

However, the current reliance on neutral molecular markers for analysing genetic diversity has been a point of contention. Specifically, it has been argued that neutral genetic diversity measured by neutral markers does not predict the adaptive potential of a species, and instead, there should be a greater focus on the measurement of functional genetic diversity (Teixeira & Huber, 2021). There is compelling evidence both for and against this argument (Allendorf et al., 2010; García-Dorado & Caballero, 2021; Kramer & Havens, 2009; Ouborg et al., 2006; Storfer, 1996; Teixeira & Huber, 2021; Willi et al., 2022). Studies indicate that the relationship between neutral genetic diversity and adaptive potential is complex, with many supporting the association between neutral genetic diversity and effective population size (N_e), which can be a helpful indicator of population viability (Frankham, 2015; Kardos et al., 2021; Mittell et al., 2015; Santos et al., 2012), but with most other associations appearing weaker (Reed & Frankham, 2003). However, many agree that neutral genetic diversity can still be useful in relation to conservation (DeWoody et al., 2021; García-Dorado & Caballero, 2021; Willi et al., 2022), especially when used in tandem with quantitative genetic variation (Kramer & Havens, 2009). The information provided by neutral diversity has aided the conservation of many species (e.g., Armstrong and de Lange (2005), Barnaud & Houliston (2010), Coates et al. (2015), Edwards et al. (2021), Hutchison et al. (2017), Szczecinska et al. (2016)).

It should also be noted that due to the effects of genetic draft, linked selection/selective sweeps, and background selection, no locus evolves independently, and even 'neutral' variation can be affected by selection (Berry et al., 1991; Charlesworth et al., 1993; Gillespie, 2000, 2001; Smith & Haigh, 1974). Additionally, variation for non-adaptive loci may also become adaptive in the future (Harrisson et al., 2014). Therefore, rather than simply focusing on functional genetic diversity, a small highly conserved region of the genome, for threatened species, it is more beneficial to focus on whole-genome diversity, including neutral variation (DeWoody et al., 2021). Studies have shown that increased genome-wide genetic variation positively

correlates with increased fitness and population viability (Leimu et al., 2006). Many population genetic studies use high mutation rate markers like microsatellites as an assumption that they will better reflect the whole genome genetic diversity rather than functional genetic diversity, which is difficult to detect and will adapt more slowly (unless there is strong selection pressure). Furthermore, suppose little genetic variation is observed in a species through the use of highly variable markers like microsatellites that measure neutral genetic diversity (theoretically the most variable regions of the genome). In that case, this strongly indicates little variation is present, which is extremely useful information for a species of conservation concern.

Though the use of neutral markers is recognised as imperfect, recent technological developments could provide the solution. For instance, whole genome sequencing and RADseq allow for a large number of SNPs to be identified and screened, achieving a more in-depth analysis of the variation in the genome (Willi et al., 2022). QTL mapping and genome-wide association studies can contribute to a more detailed screening of adaptive or functional genetic diversity, which can be used in tandem with whole-genome diversity (Caballero & García-Dorado, 2013; Willi et al., 2022). However, the methods described are expensive and a monumental amount of work that would be impractical for most small-scale conservation schemes. In comparison, traditional population genetic methods can be relatively inexpensive, and the information obtained can still provide significant insights into the population dynamics, effective population size, effects of inbreeding, genetic drift, and patterns of genetic variation, all of which can greatly contribute to the preservation of a threatened species.

1.3 Threats to native species

The number of flora and fauna species under threat has substantially increased over the last few decades, with over 42,000 species worldwide currently facing extinction (IUCN, 2022). Such an extreme decline in species is primarily due to humans significantly modifying environments, both on large scales, such as climate change and on more minor scales, such as introducing new threats into environments in the form of invasive species. The current level of biological invasions occurring from the introduction of exotic species is unprecedented in earth's history (Ricciardi, 2007) and is a significant contributor to the current magnitude of species facing extinction (Pyšek et al., 2020). The increased transportation of people, animals, wood and live plants has allowed for the expansion and invasion of exotic species to new environments (Kueffer, 2017; Ridley et al., 2000). These invasions have severe consequences

for native biota, including the extinction of affected native species, decline in genetic diversity of native populations, and altering of species richness and communities, which can affect ecosystem functioning and services (Blackburn et al., 2019; Pyšek et al., 2020; Suarez & Tsutsui, 2008). In particular, the introduction of invasive microorganisms into new environments has caused major biodiversity losses worldwide (Brasier & Buck, 2001; Rigling & Prospero, 2018; Scheele et al., 2019), and specific concern has been raised about the increasing threat of invasive fungal species (Fisher et al., 2020; Fisher et al., 2012). In New Zealand, such a threat is already realised with the effects of the pathogenic fungus known as Kauri dieback disease (*Phytophthora agathidicida*) causing tree mortality and decreased population numbers of the endemic host species *Agathis australis* (Araucariaceae) (Bradshaw et al., 2020). The establishment of a new fungal disease, myrtle rust, has the potential to cause similar, if not more harmful, effects to the native species of New Zealand.

1.3.1 Myrtle rust

Myrtle rust, also known as guava rust or eucalypt rust, is a disease caused by the pathogenic fungus *Austropuccinia psidii*(Sphaerophragmiaceae), which exclusively affects members of the Myrtaceae family (Glen et al., 2007). The fungus was first described by Winter (1884) in South America, where it was infecting the local guava (*Psidium guajava*). To date, a total of nine different strains have been identified, with the strain known as the pandemic biotype being the most widespread (da S Machado et al., 2015; Sandhu et al., 2016; Stewart et al., 2018). The highly invasive pathogen has spread globally, including to the USA, Japan, China, South Africa, and the Myrtaceae-species-rich Australia, where it has caused extensive damage at both a species and ecosystem level, with many endemic species at severe risk of localised extinction (Carnegie et al., 2016; Fernandez-Winzer et al., 2020; Pegg et al., 2017). There are limited effective treatments for myrtle rust (Pathan et al., 2020), and susceptibility and resistance to the disease vary widely depending on the species. There have even been instances of variation in susceptibility according to geographic location occurring within the same species (Yong et al., 2019).

The fungus spreads primarily through airborne asexual urediniospores that land on and infect plant tissue (Beresford et al., 2020). Ideal conditions for infection include a temperature range of 18-28° C, high humidity, and moisture (Beresford et al., 2020; Hunt, 1968; Tessmann et al., 2001). When the described conditions occur, the result can be massive disease outbreaks (Tessmann et al., 2001). The infection of myrtle rust causes the appearance of pustules, or

clusters, of yellow spores on young leaves, shoot tips, fruits and flowers (Pegg et al., 2014). Repeated infection of myrtle rust can cause shoot tip dieback, tissue necrosis, defoliation, premature dropping of fruits and flowers, and eventually plant death (Carnegie et al., 2016; Pegg et al., 2014; Sutherland et al., 2020). The large loss of foliage and photosynthetic damage caused by myrtle rust can decrease the rates of photosynthesis (Bilgin et al., 2010; Carnegie et al., 2016; Gonçalves et al., 2022). This can lead to depleting carbohydrate stores, potentially resulting in developmental changes, such as a subsequent decline in the size of new emergent leaves (Carnegie et al., 2016). Loss of fruits (and subsequently seeds) affect fecundity and decreases recruitment of the next generation (Pegg et al., 2014). This, combined with targeting young leaf tissue, which prevents the regeneration of seedlings, can result in a rapid decline in population size (Carnegie et al., 2016). There are also potential ecosystem-level effects caused by myrtle rust, such as species composition changes and decreases in the richness and structure of insect and plant communities (Carnegie et al., 2016; Pegg et al., 2017).

1.3.2 Myrtle rust in New Zealand

In New Zealand, the Myrtaceae family consists of four tribes (Myrteae, Leptospermeae, Syzygieae, and Metrosidereae), comprising 28 species (Allan, 1961; de Lange, 2014; de Lange & Schmid, 2021), although this number will decrease if the most recent treatment of *Kunzea* is recognised (Heenan et al., 2023). Myrtaceae species are found in diverse forest types, with many species serving important roles in the early succession of natural and regenerating forests (Wardle, 1991; Wiser & De Cáceres, 2013; Wiser et al., 2011). They are used in the production of food sources, such as the New Zealand species *Leptospermum scoparium* (Mānuka), which is utilised in the production of mānuka honey (Clearwater et al., 2021). Many species also have cultural significance to the indigenous people of Aotearoa, including *Metrosideros excelsa* (Pōhutukawa), which holds deep cultural significance to Māori (Hiroa, 1949).

Myrtle rust was detected in New Zealand for the first time in May 2017, potentially arriving via wind currents from Australia (Beresford et al., 2018; Du Plessis et al., 2019; Ho et al., 2019). The pathogen has since spread throughout the North and South Island, infecting 17 native species, resulting in the conservation status of all native Myrtaceae species being elevated to 'threatened – nationally critical' (de Lange et al., 2018). The disease has been found to severely impact the two endemic species in the *Lophomyrtus* genus (Beresford et al., 2020; Sutherland et al., 2020). One of the species, *Lophomyrtus bullata,* has been observed to be highly susceptible to the disease, with no perceived natural resistance present in the species

(Smith et al., 2020; Sutherland et al., 2020; Toome-Heller et al., 2020). The extent of infection in *L. bullata* is severe, with the leaves, stems, developing fruit, and regenerating seedlings of the species all heavily affected. This suggests there is potential for myrtle rust to cause the localised extinction of *L. bullata* (Sutherland et al., 2020; Toome-Heller et al., 2020), with the death of mature trees already reported (Beyond-Myrtle-Rust, 2020). The situation suggests an urgent need for detailed research into both the ecological and genetic aspects of *L. bullata* to support its conservation.

1.4 *Lophomyrtus bullata*

The only New Zealand members of the largest tribe, Myrteae, are two genera *Lophomyrtus* and *Neomyrtus*. *Lophomyrtus* consists of two endemic species, *Lophomyrtus bullata* and *Lophomyrtus obcordata* (Raoul) Burret (rōhutu). *Lophomyrtus obcordata* is distributed throughout the North and South Island of New Zealand (Allan, 1961), and where the two sister taxa co-occur, they can form fertile hybrid offspring (de Lange, 2022a, 2022b). The hybrid name of the hybrid forms is *Lophomyrtus* x *ralphii* (de Lange, 2022a). The taxonomic history of *Lophomyrtus bullata* is complex, with the species originally published by Allan Cunningham (1839) under the name *Myrtus bullata* Sol. ex A. Cunn *nom. illeg.* In 1941, the species was transferred to the new genus *Lophomyrtus* erected by Burret (1941) and therefore renamed *Lophomyrtus bullata* Burret.

Lophomyrtus bullata is a perennial shrub or small tree species that generally grows approximately 6-10 meters in height (Allan, 1961) and has a chromosome count of $2n$=22 (Dawson, 1987). The leaves are typically about 2-5 mm long, bullate (covered in rounded swellings), and green in colour when young and purple/red pigmented at maturity (Fig. 1.1) (Allan, 1961). They have white flowers approximately 12 mm in length, with five petals and several stamens. The hypanthium surrounds several ovaries that contain 2-3 locules (de Lange, 2022a). They produce berries that are typically 4-8 mm long, dark red to black and have many seeds (Allan, 1961).

The species is distributed across the North Island and the northern part of the South Island (Fig. 1.2) (Allan, 1961) and is generally found in coastal and lowland podocarp forests but also sub-alpine shrubland up to an altitude of 600 m (Allan, 1961; Cheeseman, 1906). *Lophomyrtus bullata* is typically found on the margins of forests or in canopy gaps, as they tend to prefer

open and light areas (Salmon, 1980). However, they can also grow in denser forests and wetlands and form an essential part of successional regenerating shrubland (de Lange, 2022a).

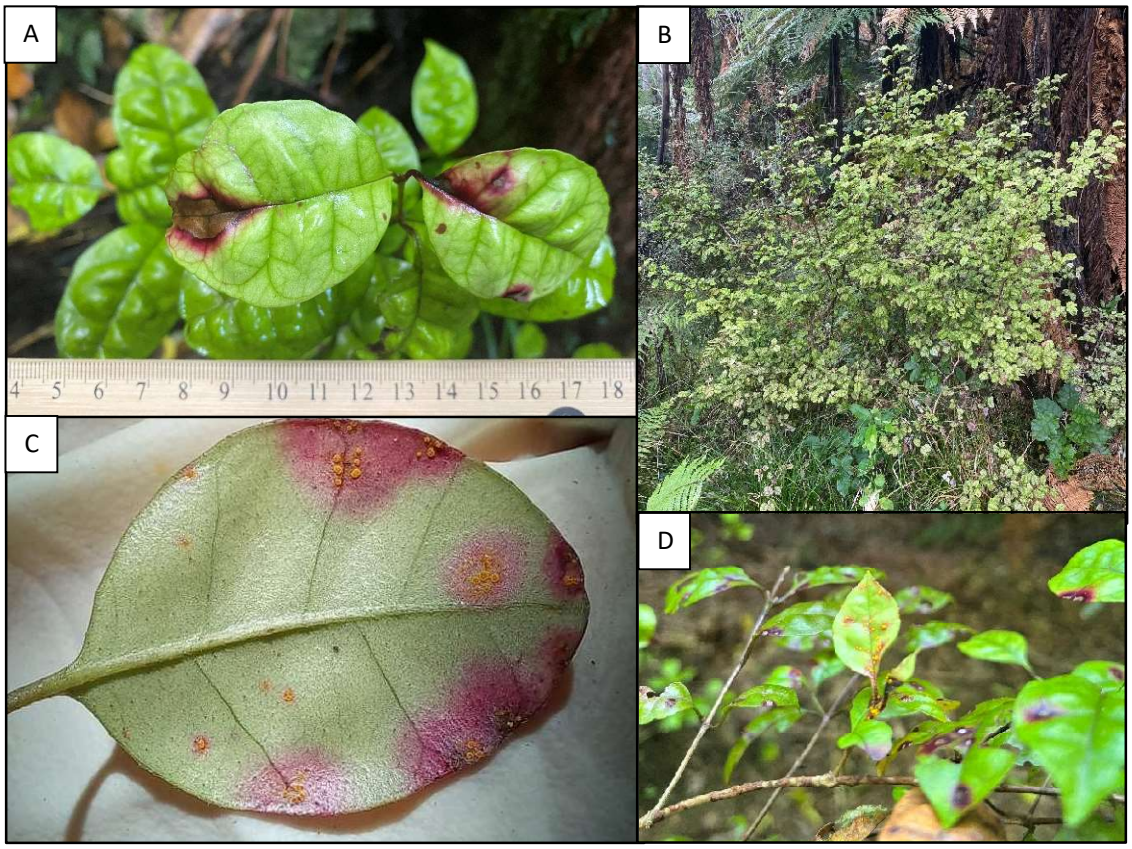

Figure 1.1. Example morphology of *Lophomyrtus bullata* **A**, Leaves of *L. bullata*, **B**, Mature *L. bullata* tree, **C**, a close up of the underside of a *L. bullata* leaf infected with myrtle rust spores, **D**, *L. bullata* individual heavily infected by myrtle rust.

Traditionally, Māori used the ripe berries of *L. bullata* as a food source (Best, 1942; Cambie & Ferguson, 2003). The berries contain anthocyanins (Lowry, 1976), which are thought to have beneficial properties, such as acting as antioxidants, improving eyesight, anti-inflammatory properties and potential inhibitory effects against the growth of cancerous cells (Hagiwara et al., 2001; Harborne & Williams, 2000; Youdim et al., 2000). The wood of *L. bullata* was traditionally used to make poles by canoeists of the Whanganui tribes and was also used to craft humming tops (Best, 1925a, 1925b).

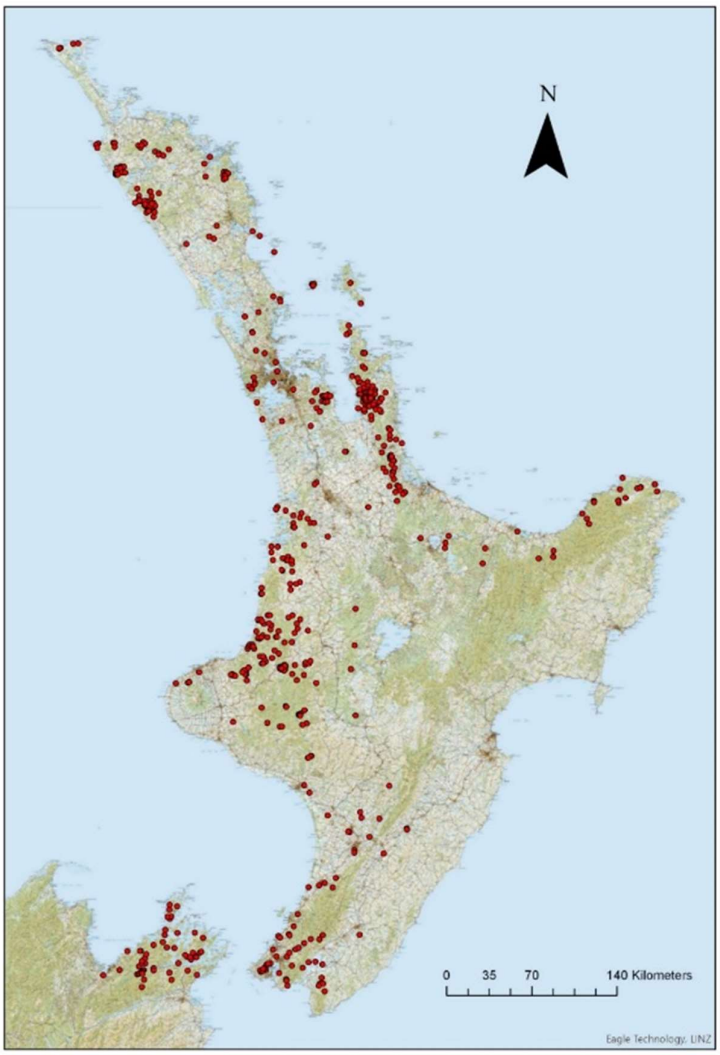

Figure 1.2. Distribution of *Lophomyrtus bullata.* Compiled from NZ Nation Vegetation Survey database, and Australasian Virtual Herbarium.

The leaves of *L. bullata* have medicinal properties and have been traditionally used by Māori to treat cuts and bruises (Riley, 1994). Studies have shown that the leaves of *L. bullata* have significant properties such as cytotoxic activity, antimicrobial effects, and antifungal activity (Larsen et al., 2005; Woollard et al., 2008). Chemical distillation performed on the leaves of *L. bullata* resulted in the isolation of the rare natural compound bullatenone, which has been suggested to have potential uses in applications such as sunscreens and insect repellents (Amaike, 1990; Muta, 1991). *Lophomyrtus bullata* is currently the only known natural source of this compound (Brandt et al., 1954; Parker et al., 1958).

Myrtaceae species are important contributors to the ecosystem. They provide resources such as sugar-rich flowers and berries benefited by a range of invertebrates and vertebrates, and their high wood density makes them excellent and lasting sources of carbon storage (Affeld et al., 2009; Clearwater et al., 2021; Jo et al., 2022; Schmidt-Adam et al., 2000). However, little is known about the specific ecological function *L. bullata* serves in its environment. A recent survey of biota associated with *L. bullata* carried out on herbarium specimens found the presence of 176 taxa, including bryophytes, pteridophytes, spermatophytes, and mycobiota, all of which could potentially be impacted by the disappearance of *L. bullata* from the ecosystem (Prasad et al., 2022). Similarly, there is little knowledge of the interactions between invertebrate species and communities, their dependence on *L. bullata*, or how the increasing spread of myrtle rust could potentially change these interactions. For instance, honeybees have been observed harvesting myrtle rust spores and even appeared to be actively choosing to harvest spores over nectar (Schmid et al., 2021). Additionally concerning is that research shows spores can remain viable after harvest for up to nine days (Pattemore et al., 2018). There are also potential impacts from the feeding of fungal spores by insects (Kolesik et al., 2021) and other fungal species (Beyond-Myrtle-Rust, 2022).

L. bullata seeds are dispersed by birds, most notably by Tui, NZ Bellbird and Kereru (Kelly et al., 2010; Westphal, 2019), and is cross-pollinated by bees, with a flowering time from November to February. It is unclear how efficient pollination is or if it has any self-pollination or self-compatibility capabilities. Some Myrtaceae species, such as Eugenia species, have been noted to be self-pollinating or self-compatible (da Silva & Pinheiro, 2009). Understanding the efficiency of seed dispersal, pollination, and whether *L. bullata* can self-pollinate or is self-compatible could determine how efficient its reproduction strategies are. Such information is especially important considering that self-incompatible species are greatly affected by decreasing population size and loss of diversity, which can cause pollen limitation, resulting in reduced seed set and fecundity (Aizen & Harder, 2007; Wagenius, 2006).

1.5 Focus of this research

Lophomyrtus bullata is currently threatened by the highly invasive pathogenic fungus myrtle rust, which is causing a rapid decline in population numbers throughout its geographical range. There is currently no information on the genetic structure of the endemic plant species, and its high susceptibility and little resistance to the fungal disease suggest the potential for significant losses of genetic diversity and eventual extinction. A population genetic study could provide an understanding of the genetic variation currently present, as well as the effects of gene flow and inbreeding on the population structure of the species, all of which could aid its future conservation. In addition, it would provide a preliminary analysis to track changes in population structure and diversity over time to assess the full impact of myrtle rust on *L. bullata*.

Therefore, this study aims to:

1. Develop microsatellite markers for *Lophomyrtus bullata.*

2. Investigate the distribution of genetic variation of *L. bullata* across its geographical range.

3. Infer historical and biogeographical influences on the patterns of variation in *L. bullata* and determine its implications for its future conservation.

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Chapter 2

Marker Development of *Lophomyrtus bullata* (Myrtaceae)

2.1 Abstract

The New Zealand endemic plant species *Lophomyrtus bullata* is currently experiencing a rapid decline in population numbers across its geographical range. This is due to the establishment of the highly invasive 'myrtle rust' fungus *Austropuccinia psidii* in New Zealand. A direct consequence of this decline could be a paralleled decrease in the genetic diversity of the species, which could have concerning implications for its future conservation. Microsatellite markers were developed for the purpose of evaluating the present, and potential future, genetic variation and population structure of the species. Twelve new microsatellite markers were developed for *L. bullata* from the initial isolation of 55 microsatellite loci. Primers were screened on 24 individuals from four different populations. The markers were found to be polymorphic with an average of 3.71 (SE = 0.26) alleles per locus and an average observed and expected heterozygosities of 0.46 (SE = 0.04) and 0.47 (SE = 0.03), respectively. The newly developed markers were tested for transferability to the congener, *Lophomyrtus obcordata*, and were found to amplify successfully in this species. These 12 new microsatellite markers will facilitate research into the population genetics of the threatened species and have the potential for use in other related taxa.

2.2 Introduction

Lophomyrtus bullata (ramarama) is a long-lived perennial shrub or small tree species endemic to New Zealand. The species has green and red pigmented leaves that are characteristically bullate or 'bubble-like' in appearance and are typically found in the understorey of coastal and lowland podocarp forests (Allan, 1961; Cheeseman, 1906). The species is distributed throughout the North Island and the Marlborough region of the Northern South Island (Allan, 1961). Where the species co-occurs with its widespread sister taxa, *Lophomyrtus obcordata*, the two species are able to form hybrids, with many hybrid forms developed into ornamental cultivars (de Lange, 2022a).

The establishment of *Austropuccinia psidii* (Sphaerophragmiaceae) in New Zealand has resulted in severe consequences for *L. bullata* (Ho et al., 2019; Toome-Heller et al., 2020). The species' high susceptibility and little to no natural resistance has led to declining population numbers and a high risk of localised extinction (Sutherland et al., 2020; Toome-Heller et al., 2020). Localised extinction could result in the loss of regional genetic variation, decreasing genetic diversity within the species. Such a loss of diversity essentially reduces the long-term evolutionary potential of the species (Wayne & Morin, 2004), resulting in it being more vulnerable to any future environmental changes (Ellstrand & Elam, 1993).

The spread of myrtle rust in Australia has already resulted in dire consequences for local endemic flora. Several myrtle species have been severely impacted by the disease, with large and rapid declines in population sizes. In one native species, *Rhodomyrtus psidioides*, populations have declined by over 50% in the five years after the establishment of myrtle rust (Carnegie et al., 2016). It is predicted that these species will face localised extinction in the very near future (Pegg et al., 2017). Therefore, to combat this same threat in New Zealand, sufficient knowledge of the species' ecological roles, genetic structure, and genetic diversity is paramount to developing successful conservation and recovery strategies.

To assess the genetic diversity across the species' populations, molecular markers that are selectively neutral and highly variable are required (Hodel et al., 2016; Selkoe & Toonen, 2006). One of the most common marker types used is microsatellites. Microsatellite loci are short tandem repeat sequences (STRs) of DNA that can range from one to six base pairs long (hexanucleotides). The length of the region can be highly variable among individuals (due to differing numbers of repeats), making them valuable tools in the analysis of genetic diversity

between different populations of the same or sometimes closely related species (Haasl & Payseur, 2011). In this study, through the use of Illumina-based sequencing, 12 microsatellite markers were developed for *Lophomyrtus bullata* for the purpose of investigating the population structure and genetic diversity of the species for its future conservation management.

2.3 Methods

2.3.1 Illumina sequencing and primer design

DNA sequences of *L. bullata* were obtained in an Illumina Miseq run prior to the start of this research project. The method used will be briefly described in this paragraph. Young leaf tissue was collected from individuals of *L. bullata* located on the Manawatū campus of Massey University, Palmerston North, New Zealand. Genomic DNA was extracted from these individuals using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle & Doyle, 1987). The genomic DNA was then sheared into 200-500 bp sections using a sonicator, after which Illumina sequencing adapters and individual-specific sequence tags were ligated to the ends. Sequencing was then carried out on one half of a Miseq run (Illumina), resulting in 8,815,516 raw reads.

The reads were paired and merged using Geneious software (version 9.1.8) (Kearse et al., 2012), with 1,351,112 of these sequences successfully merged. The sequences were then uploaded as a Fastq file to the Galaxy web platform (Afgan et al., 2018) and repeat regions were identified using the STR detection tool (Fungtammasan et al., 2015). The parameters for repeat detection were as follows: dinucleotide repeats with a minimal length of seven bp and a 50 bp buffer on either side of the repeat regions. Partial motifs were not considered, and a hamming threshold of zero was applied. From the search, 22,742 dinucleotide repeats were detected, ranging from 7 – 104 repeat units.

A range of microsatellite loci that differed in repeat length (from 7 up to 11) were selected, and the Geneious add-in Primer3 (Rozen & Skaletsky, 2000) was used to design flanking primer pairs. The default criteria were used to design the primers, which included a product size range of 120-300 bp, a primer size of 17-21 (bp) with 19 selected as the optimum, a melting temperature between 52-58 ºC, a 50% GC content and a GC clamp of one. Primer pairs were discounted if more than five bp mononucleotide repeats were present within the region between the primers and the microsatellite locus or within the primer region itself. A 19 bp tail was

added to the 5' end of the forward primer, and a pigtail sequence was added to the 5' end of the reverse primer (Brownstein et al., 1996).

2.3.2 Microsatellite marker trialling and statistical analysis

In total, 55 primers were designed, and through the initial screening of eight individuals of *L. bullata* from a Wellington population, 36 were identified as amplifying well with the approximately correct product size visible on a 1.5% agarose gel. These 36 primers were then screened on 15 individuals of *L. bullata* from five different sites: Northland, Tauranga, Taranaki, Te Kuiti, and Wellington. PCR was carried out with each reaction having a total volume of 10 µL. This contained: 3.7 µL of H_2O, 1x BD buffer (Solis Biodyne, Tartu Estonia), 2.5 µM of $MgCl_2$, 10 µM of each dNTPs, 2.5 µM of forward primer, 4.5 µM of reverse primer, 4.5 µM of M13 tail primer labelled with either VIC, NED or FAM (depending on the marker), 0.5 U of FIREPol *Taq* DNA Polymerase (Solis Biodyne, Tartu, Estonia) and 1 µL of 1:10 diluted genomic DNA template. PCR was performed with cycling conditions as follows: 3 minutes at 95° C, 30 cycles of 30 minutes at 95° C, 40 minutes at 52° C, 40 minutes at 72° C and then a final extension for 20 minutes at 72° C.

The PCR products were then checked on a 1.5% agarose gel stained with ethidium bromide. For successfully amplified loci, 3 µL of the PCR products from four different loci were pooled, and 1.25 µL of that pooled solution was added to 9 µL of Hi-Di formamide (Applied Biosystems) and CASS ladder (Symonds & Lloyd, 2004). The samples were separated and sized by capillary electrophoresis on an Applied Biosystems 3130XL genotype analyser at the Massey Genome Service (Palmerston North, New Zealand). Allele scoring was completed using GENEMAPPER (version 4.0) (Applied Biosystems).

Of these 36 markers, several were discounted because they were monomorphic or showed evidence of amplification of more than one locus. Twelve of the 36 markers were selected for subsequent trialling based on consistent and clean amplification and relatively moderate levels of variability (Table 2.1). Using the above methods, the markers were then trialled on a further 24 individuals from four different populations; Northland, Auckland, Taranaki, and Wellington. The 12 microsatellite markers were also successfully checked for cross-amplification in the closely related species *Lophomyrtus obcordata,* using 12 individuals from one South Island population. The number of alleles per locus per population, observed heterozygosity, expected heterozygosity, and Hardy-Weinberg equilibrium (HWE) were calculated using GenAlEx software (version 6.5) (Peakall & Smouse, 2006, 2012).

Table 2.1. Characteristics of 12 microsatellite markers developed for *Lophomyrtus bullata.*

Locus	Primer Sequence (5'-3')	Repeat motif	Allele size range (bp)	M13 dye
LB55	F: TCTACGGCCAAGGAAAGTGC R: TGCCAGTTGATTAAGGGTAAC	AT	274-296	FAM
LB44	F: CGCCCTTTGATTGATCGCTG R: TGCCACGTGTAAGGTTGGAC	TC	313-335	VIC
LB54	F: TCGTCACACTTTATGGCCGC R: GAAGCCTTTCCCCCTCGATC	GA	244-256	NED
LB42	F: ATCACTGATAAGGCCACCGG R: ACAGACAATCCCCGGAAGAC	TG	262-272	FAM
LB25	F: GACCAAGTCATCGGTCTTC R: TCAGATTCCAACAGGACAAG	AG	285-290	VIC
LB31	F: AGGGTATCAGGTTTTGGGCC R: TGTTCAGATGGTAGTCTCGC	CT	353-365	NED
LB41	F: TTTTTGACTGGGGTGGGTCC R: TAGTGGGTGTGATGGATGGC	CA	219-229	FAM
LB33	F: GCTCAGGCCAGGAAAATCATG R: GACACAGGGCTTGCATTTGG	CT	265-291	VIC
LB30	F: CATGCACAAGTACATCCCCG R: TTACATATCCTGGCGGCTGC	GA	339-346	NED
LB47	F: CATTTGCCTGCCTCATCCAC R: TTCATGGGTCACAGAAGCCC	CT	194-205	FAM
LB40	F: CATGACGCCTTGTGTTCTGTG R: AATTCCAGTTACCCTCGCCG	CT	280-290	VIC
LB39	F: GCTTTGTGTTCTTCCTTGGCC R: ACGGTGAGGTGGTTTTGGG	CA	287-303	NED

2.4 Results

A total of 64 alleles were identified across the 12 loci using 94 individuals from four different populations (Table 2.2). The number of alleles per locus ranged from three to eight, with an average of 3.71 (SE = 0.26), and private alleles were observed at 14 separate loci. The observed heterozygosity ranged from 0 to 0.91, and the expected heterozygosity ranged from 0 to 0.83. The total mean for the observed and expected heterozygosities across all populations and loci was 0.46 (SE = 0.04) and 0.47 (SE = 0.03), respectively. Four loci showed significant deviations from the HWE due to heterozygosity deficiency (Table 2.2).

Table 2.2. Results of initial primer screening in four populations of *Lophomyrtus bullata.* Column labels are: number of samples (N), number of alleles (NA), observed heterozygosity (Ho), expected heterozygosity (He). *Significant deviation from HWE ($P< 0.01$).

Locus	*Northland*				*Auckland*				*Taranaki*				*Wellington*			
	N	**Na**	**Ho**	**He**	**N**	**Na**	**Ho**	**He**	**N**	**Na**	**Ho**	**He**	**N**	**Na**	**Ho**	**He**
LB55	21	4	0.19	0.47*	23	5	0.48	0.57	23	2	0.13	0.12	22	1	0.00	0.00
LB44	20	8	0.85	0.83	23	9	0.91	0.76	24	6	0.67	0.71	23	4	0.39	0.42
LB54	22	4	0.64	0.53	21	5	0.38	0.59*	24	3	0.50	0.52	23	4	0.74	0.62
LB42	23	3	0.65	0.53	22	2	0.09	0.09	24	2	0.04	0.04	23	2	0.13	0.12
LB25	23	3	0.61	0.51	22	2	0.14	0.13	24	2	0.33	0.50	23	2	0.48	0.47
LB31	21	6	0.57	0.73*	23	4	0.65	0.66	24	4	0.63	0.69*	23	5	0.74	0.60
LB41	22	6	0.55	0.51	22	5	0.46	0.42	24	3	0.50	0.44	23	2	0.09	0.08
LB33	21	6	0.57	0.77	22	8	0.91	0.77	24	4	0.63	0.64	23	5	0.70	0.71
LB30	20	2	0.35	0.35	22	3	0.23	0.21	24	1	0.00	0.00	23	2	0.04	0.04
LB47	22	3	0.68	0.61	22	3	0.41	0.46	24	2	0.50	0.5	23	3	0.39	0.52
LB40	22	3	0.27	0.24	21	4	0.62	0.54	23	3	0.48	0.44	23	2	0.48	0.5
LB39	21	5	0.71	0.68	22	4	0.68	0.57	24	4	0.38	0.61	23	3	0.48	0.55
Mean	21.50	4.42	0.55	0.56	22.10	4.50	0.50	0.48	23.80	3	0.4	0.43	22.9	2.92	0.39	0.3
SE	0.29	0.51	0.06	0.05	0.19	0.62	0.08	0.07	0.11	0.39	0.07	0.07	0.08	0.38	0.08	0.073

2.5 Discussion

A total of 55 microsatellite loci were isolated and developed into markers for the endemic New Zealand species, *Lophomyrtus bullata*. From these initial 55, it was noted that 12 markers had consistent and clean amplification, and further screening found that these loci had moderate levels of polymorphism. An average of 3.71 alleles per locus and a total mean expected heterozygosity of 0.47 was observed in four populations of 24 individuals of *L. bullata*. These values are similar to another endemic New Zealand Myrtaceae species, *Metrosideros bartlettii,* which had an expected heterozygosity of 0.48 (Melesse, 2017). These species have similar life history traits, but *M. bartlettii* has a significantly smaller geographical range (de Lange, 2022b). Only three populations of *M. bartlettii* survive, and all are located in the upper Northland, with most only having a few individuals per population (Melesse, 2017). However, the variation observed in the microsatellite markers developed for *L. bullata* contradicts the general trend of higher expected heterozygosity in plant species with similar life history traits (Nybom, 2004). For example, long-lived perennial species have a mean expected heterozygosity of 0.68, and outcrossing species have a mean of 0.65 (Nybom, 2004). Similarly, the microsatellite markers developed for the endemic New Zealand species *Pseudopanax crassifolius* had a higher expected heterozygosity compared to these markers (Shepherd et al., 2008).

The average number of alleles per locus observed for the developed markers are also lower than that of other long-lived, woody Myrtaceae species. For example, *Myrtus communis* (5.91 alleles across two populations) (Albaladejo et al., 2010), *Pimenta pseudocaryophyllus* (5.42 alleles across two populations) (Morgante et al., 2012), and *Eucalyptus leucoxylon* (12.3 alleles across two populations) (Ottewell et al., 2005). However, the overall low variability observed in these markers could potentially be due to the low amount of diversity present in populations of *L. bullata* rather than the markers themselves.

The twelve microsatellite markers developed in this paper could be applied to other applications. Microsatellites have been used to construct genome maps of many plant species, including spinach (*Spinacia oleracea*) (Khattak et al., 2006), eggplant (*Solanum melongena*) (Stàgel et al., 2008), *Quercus robur*, and *Castanea sativa* (Barreneche et al., 2004). They have additionally been employed in the construction of quantitative trait loci (QTL) maps for highly valued traits in plants, such as disease resistance (Lagat et al., 2008; Suwabe et al., 2006). This is especially relevant to these markers developed for the myrtle rust-threaten *L. bullata.* This is because if a link is established between a developed marker and possible myrtle rust

resistance, this could aid future restoration projects by potentially using marker-assisted selection to breed in natural resistance to myrtle rust. Such microsatellite markers have been developed and employed for Barley (*Hordeum vulgare*), where a microsatellite locus tightly linked to a locus responsible for resistance to the barley yellow virus was identified (Tyrka et al., 2008). Similarly, microsatellite markers linked with resistance to a fungus (*Erysiphe pisi*) were identified in the pea species *Pisum sativum* (Ek et al., 2005). The successful transferability of these markers to *L. obcordata* means they could also potentially be employed in a similar population genetic study focusing on the species. This is necessary because *L. obcordata* is similarly threatened by myrtle rust (Sutherland et al., 2020), and a population genetic study could provide important information for the conservation of the species.

2.6 Conclusions

A set of 12 microsatellite markers were successfully developed for *Lophomyrtus bullata* and will be suitable for investigating the population genetics of the species. These markers will provide information on the genetic diversity, patterns of gene flow, and population dynamics of *L. bullata*. This knowledge will help to guide the conservation management of *Lophomyrtus bullata*.

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Chapter 3

Population genetics of *Lophomyrtus bullata* (Myrtaceae)

3.1 Abstract

The conservation of threatened endemic species is vital to preserving a region's biodiversity. *Lophomyrtus bullata* is an endemic woody shrub or small tree species threatened by the arrival of the pathogenic fungus *Austropuccinia psidii* in New Zealand. High susceptibility to the disease has resulted in populations declining at an alarming rate. This decline in numbers could also be reflected in a decrease in genetic diversity, which could limit the species' ability to respond to evolutionary pressures, thus potentially increasing its extinction risk. Twelve microsatellite markers were used to assess the genetic variation and structure of 18 populations of *L. bullata.* In addition, one population of the similarly threatened *L. obcordata* and two putative *L. bullata* x *L. obcordata* hybrid populations were also included in the analysis. Across populations of *L. bullata,* a total of 97 alleles were detected, with very few private alleles. A low expected heterozygosity (He = 0.44) and a low genetic differentiation between populations (F_{ST} = 0.14) were additionally observed. Populations were found to cluster into five distinct genetic groups corresponding to the populations' geographical distribution. A distinct genetic separation of the upper and the lower North Island sites occurring along the biogeographical pattern known as the Taupo line was also observed. These results could be useful as a guide for seed banking collections, population management, and re-vegetation projects, all of which can help contribute to the successful long-term conservation of *Lophomyrtus bullata*.

3.2 Introduction

Understanding the genetic diversity and population structure of a species is an important aspect of conservation biology (DeWoody et al., 2021; Kardos et al., 2021). The level of genetic diversity determines a species' ability to adapt to environmental changes, with low diversity typically associated with inbreeding and reduced fitness (Charlesworth & Willis, 2009; Frankel & Soule, 1981; Kardos et al., 2021). How genetic variation is distributed across a species' geographical range often reflects connectivity among populations, and the degree of gene flow, which can additionally be used to make inferences about life history (Aguilar et al., 2008; Hamrick & Godt, 1996; Nybom, 2004). This knowledge of a species can be readily applied to conservation practices, including identifying high-priority sites for conservation, determining the need for eco-sourcing in restoration projects, and ensuring germplasm collections are made from viable populations that represent the entire genetic range of the species (Frankel & Soule, 1981; Schoen & Brown, 2001). Application of such information has contributed to the conservation of many threatened species around the world (e.g., Coates et al. (2015); Hindrikson et al. (2017); Maki (2003); Szczecinska et al. (2016)), as well as to the conservation of several New Zealand species (e.g., Barnaud and Houliston (2010); Broadhurst et al. (2008); Smissen et al. (2011); Young et al. (2001)).

New Zealand has a high frequency (approximately 83-85%) of endemic vascular plant species (McGlone et al., 2001; Millar et al., 2017), which have a rich and complex history. Many New Zealand plant species would have survived numerous widescale geological changes since the Miocene, including submersion, glaciation, and volcanism, resulting in severe population bottlenecks and subsequent radiations (Fleming, 1979; Lockhart et al., 2001; McGlone et al., 2001; Meudt et al., 2015). More recently, endemic species have been impacted by the introduction of exotic pests and diseases and human-mediated habitat destruction and fragmentation (Beggs, 2001; Bradshaw et al., 2020; Craig et al., 2000). This has contributed to approximately 45% of the New Zealand indigenous vascular flora being listed as 'at-risk' or 'threatened' under the NZ classification system (de Lange et al., 2018). The potential extinction of these at-risk species contributes to a global loss of biodiversity and leads to the disruption of ecosystem services and functions on which many species, including humans, depend (Balvanera et al., 2006; Cadotte et al., 2008; Ceballos et al., 2017; Daily, 1997; Quijas et al., 2010). Therefore, protecting and conservating New Zealand's endemic plant species are critical foci.

The conservation status of the endemic New Zealand species *Lophomyrtus bullata* (ramarama) was elevated to 'threatened – nationally critical' following the establishment of the invasive fungal pathogen *Austropuccinia psidii* (Sphaerophragmiaceae) (Beresford et al., 2018; de Lange et al., 2018; Du Plessis et al., 2019; Ho et al., 2019). *Lophomyrtus bullata* forms a shrub or small tree at maturity and is one of the 28 Myrtaceae species found in New Zealand (Allan, 1961; de Lange, 2014). It has characteristic bullate or 'bubble-like' leaves that are green with an occasional red pigmentation and flowers with five white petals and numerous stamens (Allan, 1961). The species is insect pollinated (mainly by bees), and the seeds are encased in red berries that are bird dispersed (Allan, 1961; Kelly et al., 2010; Westphal, 2019). Traditionally, Māori used the fruits of *L. bullata* as a food source and the leaves for medicinal purposes (Best, 1942; Cambie & Ferguson, 2003; Riley, 1994). The species is distributed around the North Island and the north-eastern corner of the South Island and is generally found in lowland podocarp forests, where it forms an integral part of the forest understory (Allan, 1961; Cheeseman, 1906; Jo et al., 2022). In habitats where *L. bullata* is found in sympatry with its congener, *L. obcordata,* the two species often produce hybrid offspring, with the hybrids referred to as *Lophomyrtus* x *ralphii* (de Lange, 2022). *Lophomyrtus obcordata* and the hybrids are similarly threatened by *Austropuccinia psidii* (Beresford et al., 2020; de Lange et al., 2018), in addition to numerous other native and endemic New Zealand Myrtaceae species (Smith et al., 2020).

The fungal pathogen *Austropuccinia psidii,* known as myrtle rust, was first described in South America (Winter, 1884) and exclusively effects members of the Myrtaceae family (Carnegie & Pegg, 2018). Infection occurs through airborne spores that cause the appearance of clusters of yellow urediniospores on young tissue, mainly shoots and leaves (Beresford et al., 2020; Pegg et al., 2014). However, flowers and fruits of susceptible species can also be impacted, and repeat infections can cause extensive loss of foliage and plant mortality (Carnegie et al., 2016; Pegg et al., 2014; Sutherland et al., 2020). *Lophomyrtus bullata* is highly susceptible to the disease, and, to date, there have been no observations of any natural resistance (Sutherland et al., 2020; Toome-Heller et al., 2020). Instead, populations of *L. bullata* are rapidly declining, with infection causing shoot-tip dieback, premature dropping of fruits, and mortality of both seedlings and adult plants (Beyond-Myrtle-Rust, 2020; Sutherland et al., 2020; Toome-Heller et al., 2020), all of which can heavily impact the natural regeneration of populations. A similarly severe level of infection was reported for many native Australian Myrtaceae species, several of which are now facing extinction (Carnegie et al., 2016; Pegg et al., 2017). This

indicates *L. bullata* is at serious risk of localised extinction in New Zealand (Sutherland et al., 2020). This myrtle-rust-driven extinction of native species will hugely impact plant communities and ecosystems. Such extinctions could also lead to changes in forest composition and structure, increasing the ease with which exotic species can invade (Carnegie et al., 2016; Parker et al., 1999). Additionally, extensive loss of individuals can lead to a decrease in photosynthesis, productivity, and nutrient cycling in forests (Bilgin et al., 2010; Carnegie et al., 2016), potentially negatively impacting other native flora and fauna.

There is currently no information on the genetic diversity or population structure of *Lophomyrtus bullata.* To address this knowledge gap and to support conservation efforts, polymorphic microsatellite loci were developed to analyse populations across the species' natural geographic range. *Lophomyrtus obcordata* and putative *L. bullata* x *L. obcordata* populations also were included in the analysis to assess the potential effects of hybridisation on the species. The patterns observed here are used to investigate possible biogeographic influences on the distribution of genetic variation and should inform and support the future conservation management of the species.

3.3 Methods

3.3.1 Sample collection

A total of 406 individuals of *L. bullata* were collected from 18 sites covering the geographical range of the species (Fig. 3.1). A range of 20-25 individuals were sampled from each site, with an average of 21.3. A further 20 individuals were collected from each of the two *L. bullata* x *L. obcordata* putative hybrid sites, and a further 20 individuals (12 were used in the current study) of *L. obcordata* were collected from a South Island site. To avoid repeated sampling of the same individual and to ensure an unbiased representation of the population, individuals were randomly sampled along a transect line. The sampled individuals' spacing varied to accommodate both the site and population size. For each individual, two to three leaves were collected and placed in zip-lock plastic bags containing silica gel to desiccate and preserve the tissue until further use. A photo voucher for each population was made, and GPS coordinates (Supplementary Table 1) and other relevant observations were noted.

Collections were made between March - October 2022. The collection sites included Department of Conservation (DoC) land, privately and iwi-owned land, and land under the control of the Auckland regional council (Fig 3.1). Approval for sampling in the Auckland regional council site was obtained through the Manaaki Whenua-Landcare Research, Beyond Myrtle Rust program and was granted by the owner or iwi for privately or iwi-owned land. For the DoC reserve sites, permission was granted utilising the Manaaki Whenua-Landcare Research Global concession (permit no. CA-31615-OTH). Local iwi was contacted for each site, with consultation and approval sought for sampling.

Precautions were taken when collecting to limit the spread of myrtle rust between different sites and individuals within the same site. These precautions included: the wearing of overalls and hats that were removed and bagged before leaving each site and washed between site visits, all equipment and footwear were cleaned after use at each site with 70% ethanol (or isopropyl alcohol), and gloves were worn when collecting on site and cleaned with 70% ethanol (or isopropyl alcohol) between individual collections. Great care was also taken to avoid the collection of infected material. The approximate population size and associated flora were also noted for each site, as well as any evidence of either current or past infection of myrtle rust.

3.3.2 DNA extraction and microsatellite amplification

Samples were left to dry in the silica gel for at least one week. DNA was extracted from the sampled material using a modified DNeasy Plant mini kit protocol (Qiagen). Step 11 of the protocol was not repeated, resulting in the final volume of 100 µL of eluted DNA. A volume of 2 µL of the DNA samples were then run on a 1% agarose gel (comprised of agarose and TBE buffer, which contained: Tris (HCl), Boric acid and EDTA) at 75V for 45 minutes, and the gel was then stained with ethidium bromide and visualised using UV light. This was to check for the concentration and quality of the DNA samples. Samples that were deemed to be of poor quality were not used in subsequent analyses. All individuals were screened at the twelve microsatellite loci developed for *L. bullata* following the same PCR and genotyping methods described in Chapter 2.

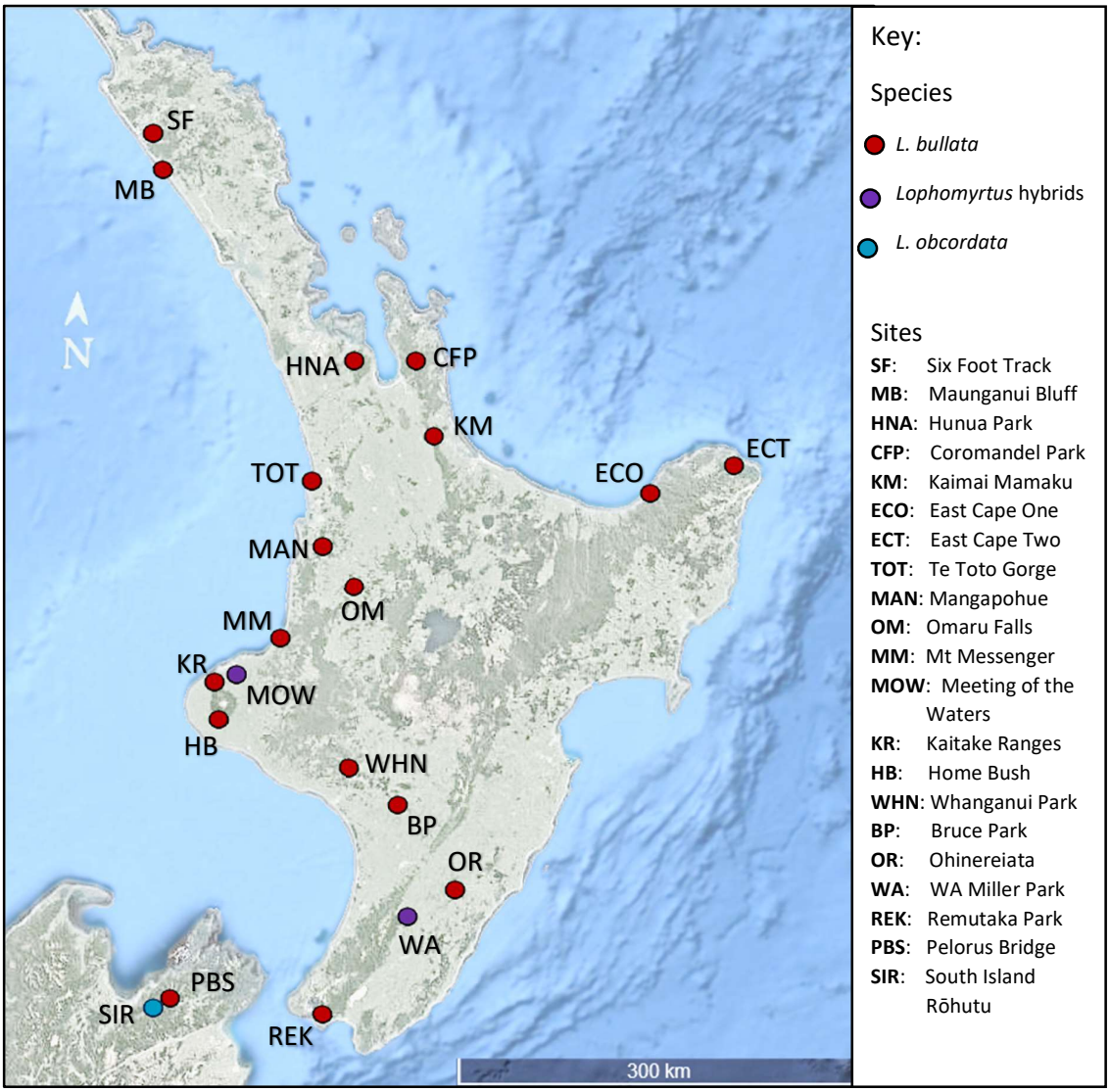

Figure 3.1 Map of sampling sites for *Lophomyrtus bullata, L. obcordata,* and putative hybrids. Each coloured circle indicates the GPS coordinates taken for a particular site.

3.3.3 Data processing and genetic diversity analyses

Individuals with missing data for more than three loci (a total of six individuals) were removed from the analysis, leaving a total of 452 individuals for further analysis. The data was formatted for GenAlEx software (version 6.5) (Peakall & Smouse, 2006, 2012) and checked for null alleles and possible sequencing errors using MICRO-CHECKER software (version 2.3.3) (Van Oosterhout et al., 2004). For all following analyses, the dataset was split into two different

datasets; dataset *L.bull* which contained only the 18 *L. bullata* sites, and dataset *L.all,* which contained the same 18 *L. bullata* sites in addition to the two putative hybrid sites (Meeting of the Waters and WA Miller Memorial Park), and twelve individuals of *L. obcordata*. This partitioning allowed for separate analyses to be completed on the data both with and without the influence of hybridisation. The number of alleles, number of effective alleles, number of private alleles, observed and expected heterozygosity, Wright's fixation indices (Wright, 1931), deviations from Hardy-Weinberg Equilibrium (HWE), and pairwise F_{ST} distance matrices were all calculated using GenAlEx (version 6.5). Regression analyses between expected heterozygosity and latitudinal distance and the number of alleles per locus (Na) and latitudinal distance were additionally calculated in Excel.

3.3.4 Genetic structure analyses

The datasets were then exported separately into STRUCTURE (Pritchard et al., 2000) to identify genetic groups. The parameters used for each analysis were: 150,000 iterations of burn-in and 1,000,000 Monte Carlo Markov Chain iterations, with the admixture model used and allele frequencies set to correlated. *K*=1-10 were analysed with ten replicate runs. The resulting output files were uploaded to STRUCTURE HARVESTER (version 0.6.94) (Earl & VonHoldt, 2012) to test for the mean likelihood of *K* values using the Evanno method (Evanno et al., 2005). The STRUCTURE results were additionally uploaded to CLUMPAK (Kopelman et al., 2015), which uses a Markov clustering algorithm to cluster replicate runs for the same and across different *K* values. This allowed for the identification of the optimal alignment of the replicate runs for each *K* and for comparisons between different *K* values to be made.

For further analyses, the two datasets were separately imported into R studio (RStudio-Team, 2020). The *provesti.dist* function from the R package poppr (Kamvar et al., 2014) was employed to generate a population-level genetic distance matrix. The distance matrices were then separately converted into the Nexus format and exported to SplitsTree5 (Huson & Bryant, 2006) to create a population-level Neighbor-net spilts graph.

The R package adegenet was then employed to produce a centred PCA for both dataset *L.bull* and dataset *L.all*. The datasets were scaled using the *scaleGen* function, and the *zero* method was used to replace missing data. The first two principal components were retained for analysis. The datasets were then analysed using Discriminant Analysis of Principal Components (DAPC) (Jombart et al., 2010). The *find.clusters* function in the R package adegenet (Jombart,

2008) was used first to transform the data into principal components, then cluster the data into a specified number of groups. The Bayesian information criterion (BIC) identified a *K* of ~7 as the most supported number of groups for the dataset *L.bull* and a *K* of ~12 for the dataset *L.all*. However, *K*-means of 2-12 for both datasets were investigated and were also visualised utilising the *compoplot* function. For all DAPC analyses, 40 Principal Components (PCs) were retained, and the first two were plotted.

Due to the continuous nature of the genetic variation present in these datasets, the clustering algorithm of DAPC did attempt to identify groups that are structured in a gradient. This results in groupings that contain a large degree of overlap, which makes it difficult to interpret. The authors of the software acknowledge such problems with continuous data (Jombart et al., 2010), and they suggest using the scatterplot function. This allows the interpretation of any genetic structure across such gradients (Jombart et al., 2010). This method for visualisation was applied to these datasets. Original DAPC plots can be found in the Supplementary Information (Supplementary Figs 2, 4).

An AMOVA test was performed in GenAlEx (version 6.5) to determine how the variation present is partitioned within and among populations. The test was completed using 999 permutations and was performed on both datasets, *L.bull* and *L.all*, and on additional datasets where the 18 populations were grouped based on their STRUCTURE and Neighbor-net clusters. The latter was used to compare genetic differentiation among genetic groups identified using those methods; these datasets had a total of seven clusters, including Northland (SF and MB), Bay of Plenty (HNA, CFP, and KM), East Cape (ECO and ECT), Waikato (TOT, MAN, OM), Taranaki (MM, KR, HB, WHN, PBS), Lower North Island (BP, OR and REK), and putative hybrid and *L. obcordata* (MW, WA, and SIR) populations.

Only dataset *L.bull* was used to test for isolation by distance via the Mantel test due to Meeting of the Waters and WA Miller Memorial Park populations containing putative hybrids. For the Mantel test, GENEPOP software (version 4.7.5) (Raymond, 1995; Rousset, 2008) was used to transform pairwise F_{ST} values into $F_{ST}(1-F_{ST})$ (Rousset, 1997), which was tested against the geographical distance and the natural log of geographical distance in GenAlEx (version 6.5). A Mantel test using both pairwise F_{ST} values against the geographical distance was also calculated in GenAlEX.

3.4 Results

3.4.1 Microsatellite diversity

All twelve microsatellite loci amplified successfully, with a total of 97 alleles identified. There was an average of 3.4 alleles per locus and a range of 1-9 (Table 3.1). MICRO-CHECKER identified potential null alleles at one locus (LB47), though this is due to an excess of homozygosity, which could be due to reasons other than the presence of null alleles. Most loci showed significant deviations from HWE for at least one population due to heterozygosity deficiency. Despite these observations, the data were unmodified for future analyses, as removing these loci would likely decrease the resolution of genetic structure, impacting the interpretation of results (Dharmarajan et al., 2013).

3.4.2 Genetic diversity

The summary statistics for each population are reported in Table 3.1. The mean observed heterozygosity across all populations and loci was 0.43; the lowest was in the KR population (0.35), and the highest was in the SF population (0.56). The mean expected heterozygosity was 0.44, with 0.36 (OR) the lowest and 0.57 (SF) the highest. The F_{IS} values ranged from -0.08 (MM) to 0.08 (ECT) (Table 3.2). The population with the greatest number of private alleles in the dataset *L. bull* was equally SF and HNA with three private alleles. In the dataset *L.all,* SIR had the greatest number of private alleles with nine (Table 3.1); however, this is not quite a fair comparison as the SIR site is an *L. obcordata* population, and it is expected that comparison between this population and populations of a different species would lead to such a result. The analysis between expected heterozygosity and latitudinal distance for the *L. bullata* populations showed a correlation with expected heterozygosity decreasing with latitudinal distance. The regression analysis for this correlation was significant ($P < .00005$, $R^2 = 0.72$) (Supplementary Fig 5). The analysis between the number of alleles per locus and latitudinal distance showed a similar correlation, with the number of alleles per locus decreasing with latitudinal distance and was additionally significant (*P value:* 0.00002, R^2: 0.66) (Supplementary Fig 6).

Table 3.1. Population genetic statistics for populations of *L. bullata*, *L. obcordata*, and *Lophomyrtus* hybrids. Column labels are: number of samples (N), number of alleles (NA), number of effective alleles (Ne), number of private alleles (PA), percentage of polymorphic loci (%P), observed heterozygosity (Ho), expected heterozygosity (He), and fixation index (F_{IS}).

Site	Site Code	N	Na	Ne	Pa	%P	Ho	He	F_{IS}
Six Foot Track	**SF**	22	4.42	2.75	3	100	0.56	0.57	0.01
Maunganui Bluff	**MB**	18	4.33	2.80	2	100	0.46	0.53	0.07
Hunua Park	**HNA**	22	4.42	2.30	3	100	0.51	0.47	-0.06
Coromandel Park	**CFP**	22	3.92	2.54	2	100	0.49	0.52	0.04
Kaimai Mamaku	**KM**	22	3.67	2.23	0	100	0.48	0.48	-0.01
East Cape One	**ECO**	20	3.17	2.37	2	83.33	0.47	0.49	0.04
East Cape Two	**ECT**	20	3.00	1.97	0	83.33	0.38	0.40	0.08
Te Toto Gorge	**TOT**	24	3.8	2.11	1	100	0.42	0.43	0.03
Mangapohue	**MAN**	23	3.92	2.27	1	100	0.51	0.47	-0.05
Omaru Falls	**OM**	24	3.10	2.06	0	100	0.40	0.42	0.05
Mt Messenger	**MM**	22	2.83	1.88	0	83.33	0.42	0.39	-0.08
Meeting of the Waters	**MOW**	20	3.92	2.71	1	100	0.54	0.56	0.03
Kaitake Ranges	**KR**	24	2.67	1.85	0	75	0.35	0.37	0.06
Home Bush	**HB**	24	3.00	2.05	0	91.67	0.4	0.43	0.04
Whanganui Park	**WHN**	23	2.75	1.94	0	83.33	0.41	0.42	0.04
Bruce Park	**BP**	24	3.25	2.06	1	83.33	0.42	0.42	0.02
Ohinerata	**OR**	24	2.83	1.75	1	83.33	0.38	0.36	-0.03
WA Miller Park	**WA**	20	2.92	2.28	0	91.67	0.59	0.51	-0.16
Remutaka Park	**REK**	23	2.83	1.90	1	83.33	0.38	0.38	-0.01
Pelorus Bridge	**PBS**	19	2.92	1.91	1	91.67	0.38	0.39	0.07
South Island Rōhutu	**SIR**	12	3.83	2.42	9	91.67	0.44	0.42	-0.06

3.4.3 Genetic structure of Lophomyrtus bullata

The F_{ST} value for all populations of *L. bullata* was 0.14, suggesting low to moderate levels of genetic differentiation among populations (Table 3.2). The highest difference in pairwise F_{ST} was for ECT and KR (0.18), and the lowest difference in pairwise F_{ST} values was equally for BP and PBS (0.03) and HNA and TOT (0.03) (Table 3.2). Using the Evanno method, the STRUCTURE analyses for the dataset *L. bull* showed that *K*=2 had the strongest support (Fig. 3.2, Supplementary Fig 1). However, the Evanno method cannot determine a Delta *K* value for *K*=1. It has been suggested that if a Delta *K* value for *K*=2 is >53, then there is good support for the solution (Cullingham et al., 2020), suggesting that the *K*=2 results for *L. bullata* are well supported. The results for *K*=2 were also plotted according to geographical location to determine if the results made biological sense (Fig.3.3).

The next strongest support was at *K*=5. Both the Evanno method and the STRUCTURE mean likelihood method indicated support for this group, and higher *K* values showed increased instability in groupings. The groupings at *K*=5 correspond to the geographic locations of the sites (Fig. 3.4). However, there appears to be a high degree of admixture. At *K*=2, all upper North Island sites (SF, MB, HNA, CFP, KM) and the East Cape sites (ECO, ECT) grouped together, and all sites south of 38^{o} latitude grouped together (MM, KR, HB, WHN, BP, OR, REK, PBS), with the three Waikato populations (TOT, MAN, OM) appearing as a stratified gradient between the North and South groups (Fig.3.2). At *K*=3, the East Cape populations (ECO, ECT) are clustered together in a separate group from the original north-south groupings. However, there is an overlap with the Tauranga population (KM). At *K*=4, the Waikato (TOT, MAN, OM), Taranaki (MM, KR, HB), Whanganui (WHN), and South Island populations (PBS) are clustered together, though this is subdivided at *K*=5 into two groups, one containing all Waikato populations (TOT, MAN, OM), and the other containing the remaining populations.

Table 3.2. Pairwise F_{ST} between populations of *L. bullata*, *L. obcordata*, and *Lophomyrtus* hybrids.

	SF	MB	HNA	CFP	KM	ECO	ECT	TOT	MAN	OM	MM	MOW	KR	HB	WHN	BP	OR	WA	REK	PBS
MB	0.04																			
HNA	0.05	0.04																		
CFP	0.07	0.05	0.03																	
KM	0.05	0.06	0.03	0.04																
ECO	0.08	0.08	0.05	0.05	0.06															
ECT	0.11	0.12	0.07	0.08	0.08	0.05														
TOT	0.06	0.05	0.03	0.05	0.04	0.07	0.10													
MAN	0.06	0.06	0.05	0.06	0.05	0.09	0.13	0.03												
OM	0.06	0.05	0.04	0.04	0.03	0.09	0.11	0.03	0.04											
MM	0.09	0.09	0.07	0.08	0.09	0.14	0.15	0.05	0.05	0.04										
MOW	0.07	0.09	0.06	0.06	0.07	0.11	0.12	0.07	0.07	0.06	0.08									
KR	0.1	0.11	0.09	0.09	0.09	0.16	0.18	0.06	0.05	0.06	0.05	0.08								
HB	0.09	0.09	0.06	0.07	0.07	0.13	0.14	0.06	0.04	0.04	0.03	0.07	0.03							
WHN	0.07	0.06	0.06	0.07	0.07	0.12	0.17	0.04	0.03	0.05	0.05	0.08	0.04	0.04						
BP	0.08	0.1	0.07	0.09	0.08	0.15	0.17	0.07	0.04	0.06	0.05	0.08	0.04	0.03	0.04					
OR	0.08	0.1	0.07	0.11	0.09	0.15	0.17	0.06	0.06	0.06	0.07	0.08	0.04	0.06	0.05	0.04				
WA	0.09	0.08	0.07	0.08	0.11	0.12	0.15	0.08	0.07	0.08	0.10	0.06	0.11	0.09	0.09	0.10	0.11			
REK	0.09	0.12	0.07	0.10	0.09	0.14	0.14	0.08	0.07	0.06	0.05	0.08	0.05	0.04	0.06	0.03	0.04	0.11		
PBS	0.08	0.09	0.06	0.09	0.07	0.14	0.15	0.06	0.05	0.04	0.03	0.08	0.04	0.03	0.04	0.03	0.05	0.10	0.03	
SIR	0.23	0.23	0.25	0.23	0.27	0.27	0.31	0.26	0.24	0.27	0.29	0.16	0.29	0.27	0.27	0.28	0.30	0.18	0.29	0.30

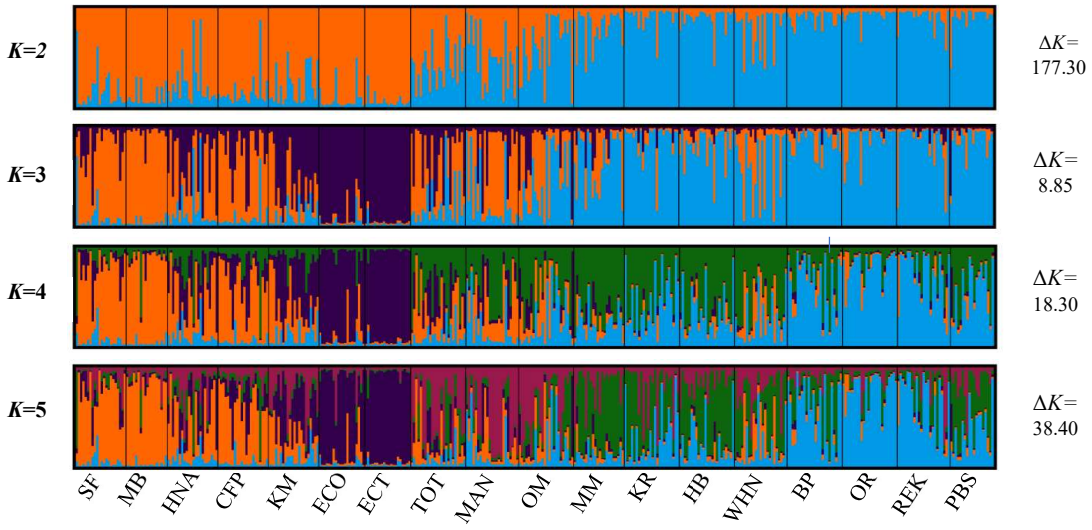

Figure 3.2 STRUCTURE plots for *L. bullata* only. Sites are labelled by site code (bottom), *K* value (left), and Delta *K* value (right).

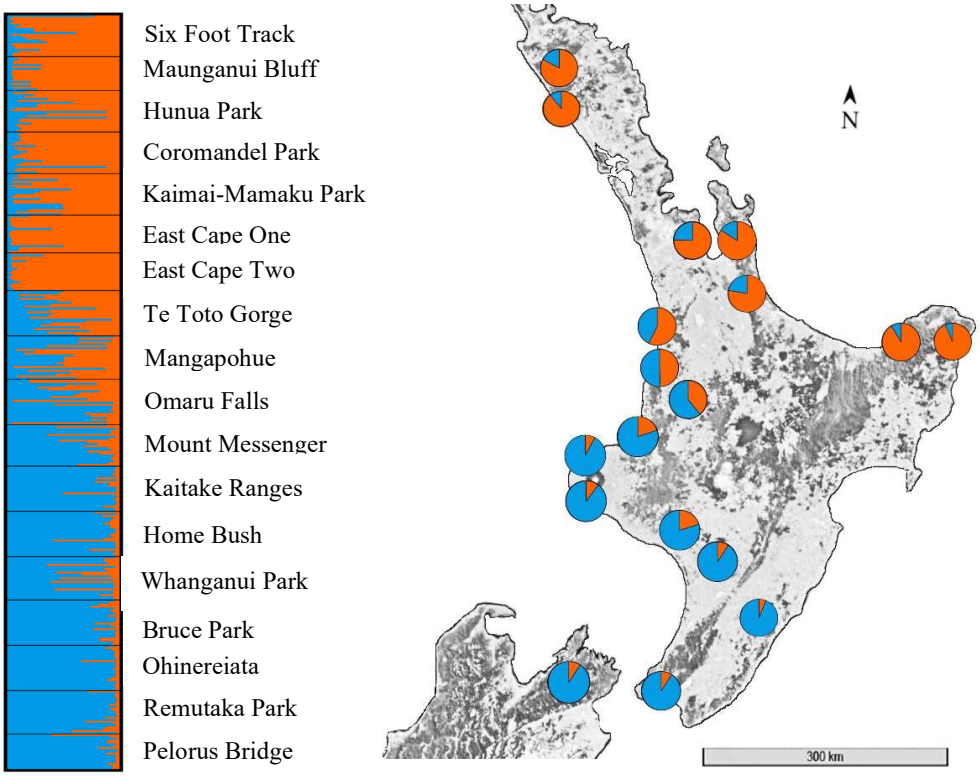

Figure 3.3 STRUCTURE plot results for *K*=2 in relation to the *L. bullata* population's geographical locations. Each pie chart represents a sample site, with the colours representing the proportion of that populations' membership to the genetic clusters identified by STRUCTURE.

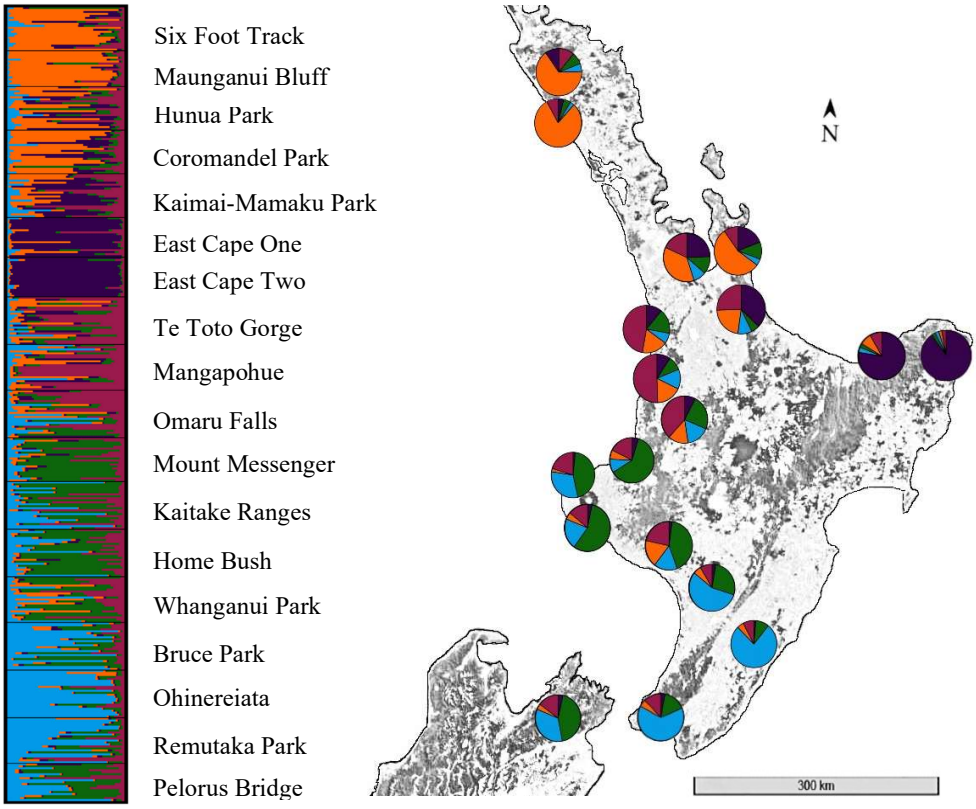

Figure 3.4 STRUCTURE plot results for *K*=5 in relation to the *L. bullata* population's geographical locations. Each pie chart represents a sample site, with the colours representing the proportion of that populations' membership to the genetic clusters identified by STRUCTURE.

The patterns observed in the population-level Neighbor-net analyses for the dataset *L.bull* are similar to the STRUCTURE analyses (Fig. 3.5). The largest split observed in the analysis separates the two East Cape populations (ECO, ECT) from the rest of the sites with the next longest distinguishing the two Northland populations (SF, MB). In each case, the two populations are further separated by long branches, suggesting further divergence within each region. The next longest split was between the Taranaki (MM, KR, HB), lower North Island (WHN, BP, OR, REK), and South Island (PBS) sites versus all sites North thereof. A smaller branch separates the Waikato sites (TOT, MAN, OM) from the rest of the upper North Island sites (SF, MB, HNA, CFP, KM), placing them in the middle of the network. The lower North Island and South Island sites are all clustered tightly together with short branches but are separated into two distinct groups: the Taranaki, Whanganui, and South Island sites (MM, KR, HB, WHN, PBS), versus the Manawatū, and Wellington sites (BP, OR, REK).

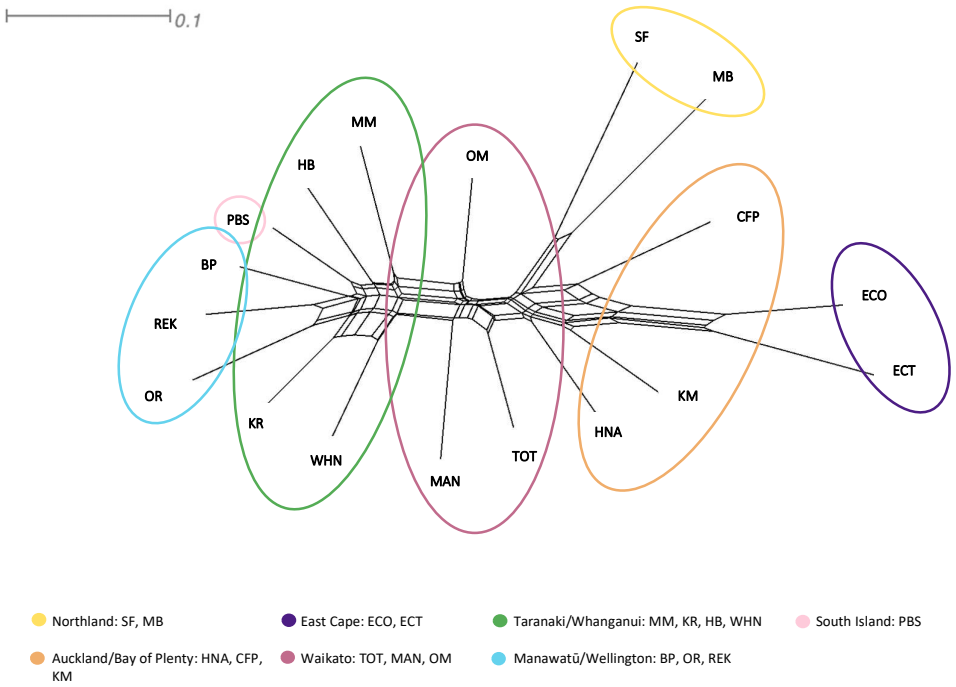

Figure 3.5 Population-level Neighbor-Net analyses of *L. bullata* populations. Populations are labelled by site code, with coloured spheres indicating geographic locality.

The PCA results for the *L.bull* dataset show a north to south positioning among sites along the x-axis (Fig 3.6a). This north to south cline is most evident in the PCA with an orange-to-blue colour gradient applied (Fig 3.6b), the populations on the orange scale of the gradient represent the upper North Island sites, and populations on the blue scale of the gradient represent the lower North Island and South Island sites. The East Cape sites are coloured grey to avoid those sites interrupting the north to south pattern observed across the rest of the distribution. There is a high degree of overlap between sites, with the greatest overlap typically occurring between sites within the same region. Additionally, the Waikato, Taranaki, Whanganui, lower North Island, and South Island sites are more tightly clumped together (Fig 3.6a, b), suggesting these sites are more genetically similar. The first PC explains approximately 4.8% of the variation in the data, with the second PC explaining 3%. The PC explaining only a small amount of the variation may be due to the minimal variation within the species. The scatterplot of the DAPC for *K*=7 shows the same north to south cline, with the East Cape (ECO, ECT) individuals showing a moderate degree of separation from the other sites (Fig. 3.7a). Again, the north to south pattern is most obviously visible in the DAPC with the orange-to-blue colour gradient

applied (Fig 3.7b). Though this correlation is weaker in this analysis, it shows greater discrimination between the Taranaki sites and the lower North Island compared to the PCA.

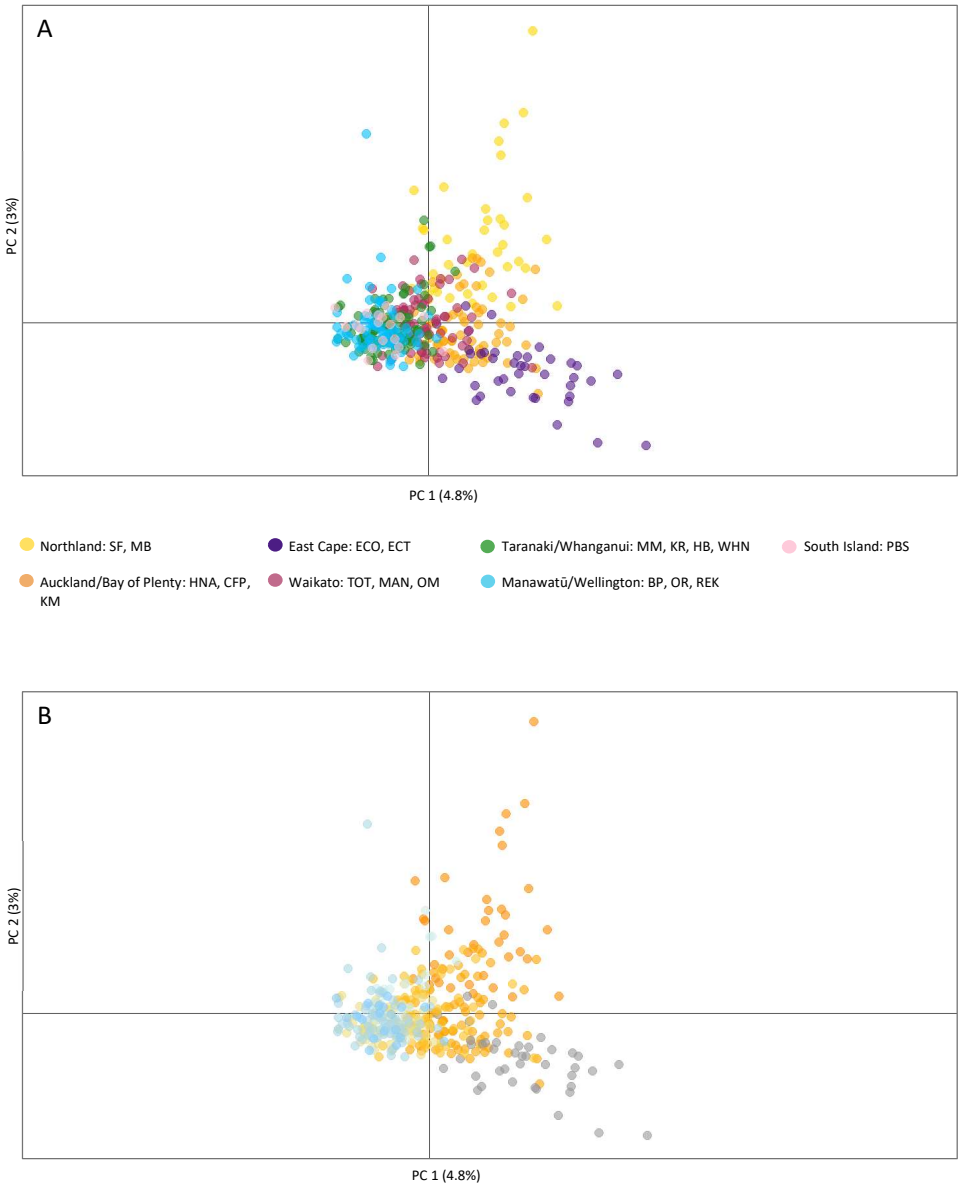

Figure 3.6 PCA plots of *L. bullata* populations. **A**, Pattern of variation represented by geographic locality. **B**, Pattern of variation showing north to south colour gradient, orange scale indicates northern populations, blue scale indicates southern populations, and grey indicates East Cape populations.

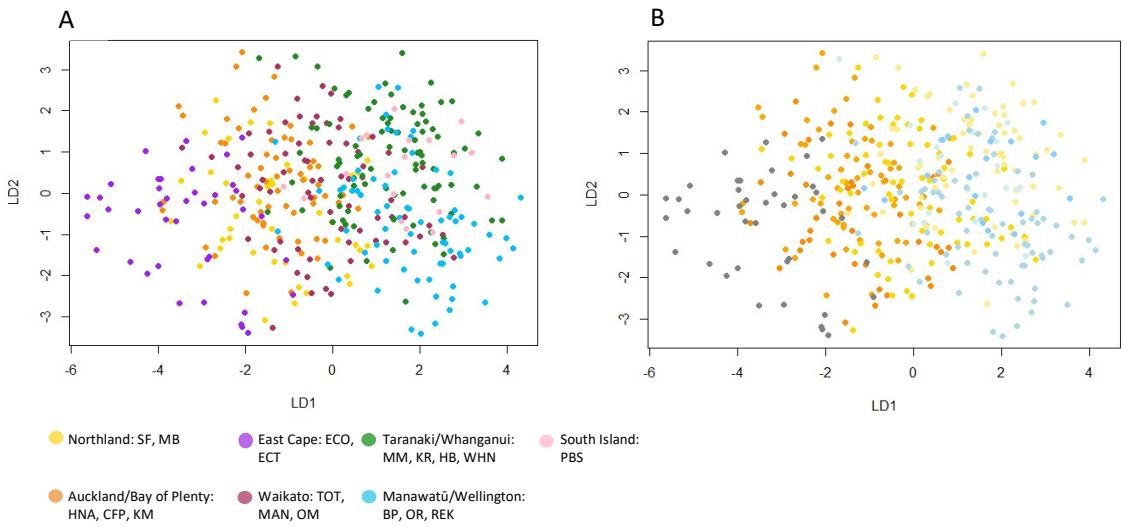

Figure 3.7 DAPC scatterplots of inferred *L. bullata* clusters. **A**, Pattern of variation represented by geographic locality**. B**, Pattern of variation showing north to south colour gradient, orange scale indicates northern populations, blue scale indicates southern populations, and grey indicates East Cape populations.

3.4.4 Genetic structure of the putative hybrids and Lophomyrtus obcordata sites

The pairwise F_{ST} values for the *L. obcordata* population suggest significant genetic differentiation from the *L. bullata* populations, ranging from 0.23 to 0.31 (Table 3.2). The highest pairwise F_{ST} values are between ECT and SIR (0.31). As expected, the pairwise F_{ST} values for the *L. obcordata* population and the putative hybrid populations (MOW, WA) were considerably lower (0.16, 0.18), suggesting that they are more genetically similar. The results of the STRUCTURE analyses for the dataset *L.all* showed support at *K*=3, 4, and 6 (Fig.3.8). Similar to the *L. bull* dataset runs, there were high amounts of admixture among populations. The groups identified for *K*=2 were the same two groups identified for the *L.bull* dataset, in addition to the hybrid populations (MOW, WA) and the *L. obcordata* population (SIR) being grouped with the Northern sites (SF, MB, HNA, CFP, KM, TOT, MAN, OM) (Fig 3.2). At *K*=3, the hybrid (MOW, WA) and *L. obcordata* (SIR) populations are clustered in a separate group that is retained in the results of all higher *K* values. The following *K* values (4-6) split all other populations into the same five groups as described for the *L.bull* dataset.

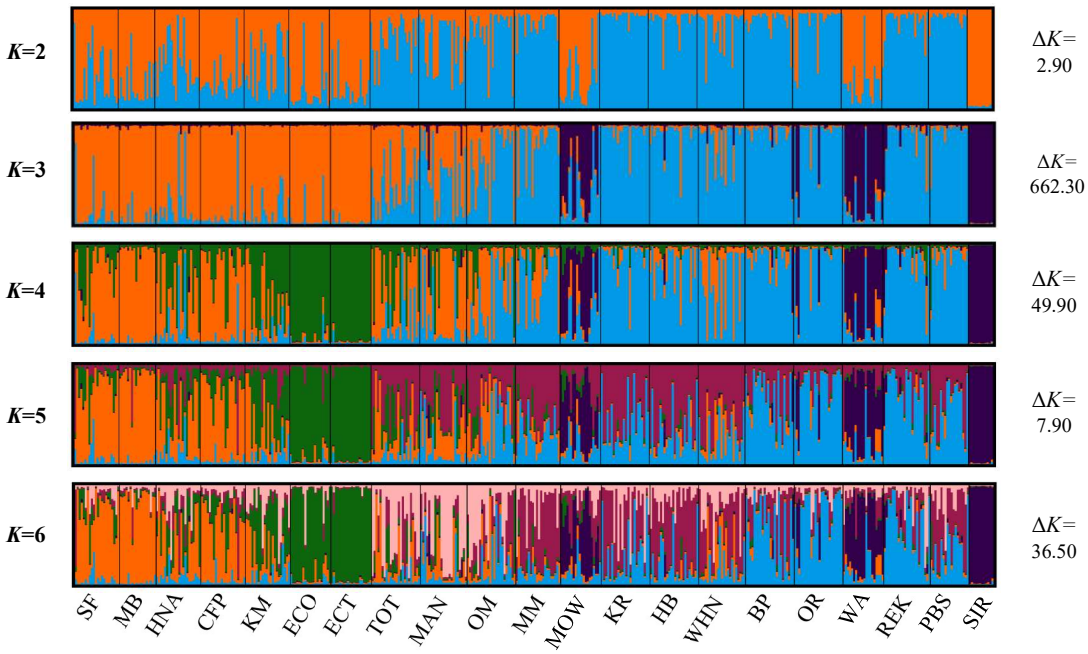

Figure 3.8 STRUCTURE plots for *L. bullata, L. obcordata* (SIR), and putative hybrid (WA, MOW) populations. Sites labelled by site code (bottom), *K* value (left), and Delta *K* value (right).

In the Neighbor-net analysis for the dataset *L.all* (Fig. 3.9a), the two putative hybrid populations WA and MOW are grouped together and are separated from the *L. bullata* sites by a long branch extension. The two putative hybrid populations are further separated, indicating that the hybrid populations are also relatively genetically different from each other. The *L. obcordata* site (SIR) is further split from the *L. bullata* and the putative hybrid populations by a long branch. Similar to all other analyses, the PCA for dataset *L.all* found that the greatest genetic distance is between the two species (Fig. 3.9b). The first PC explains 9.4% of the variation in the data, with the second PC explaining 3.8%. The DAPC revealed the same patterns as the PCA (Fig. 3.10). The *L. bullata* sites are clumped together on one side of the x-axis, with the sole *L. obcordata* population positioned on the opposite and the two putative hybrid sites spaced in between (Fig. 3.10).

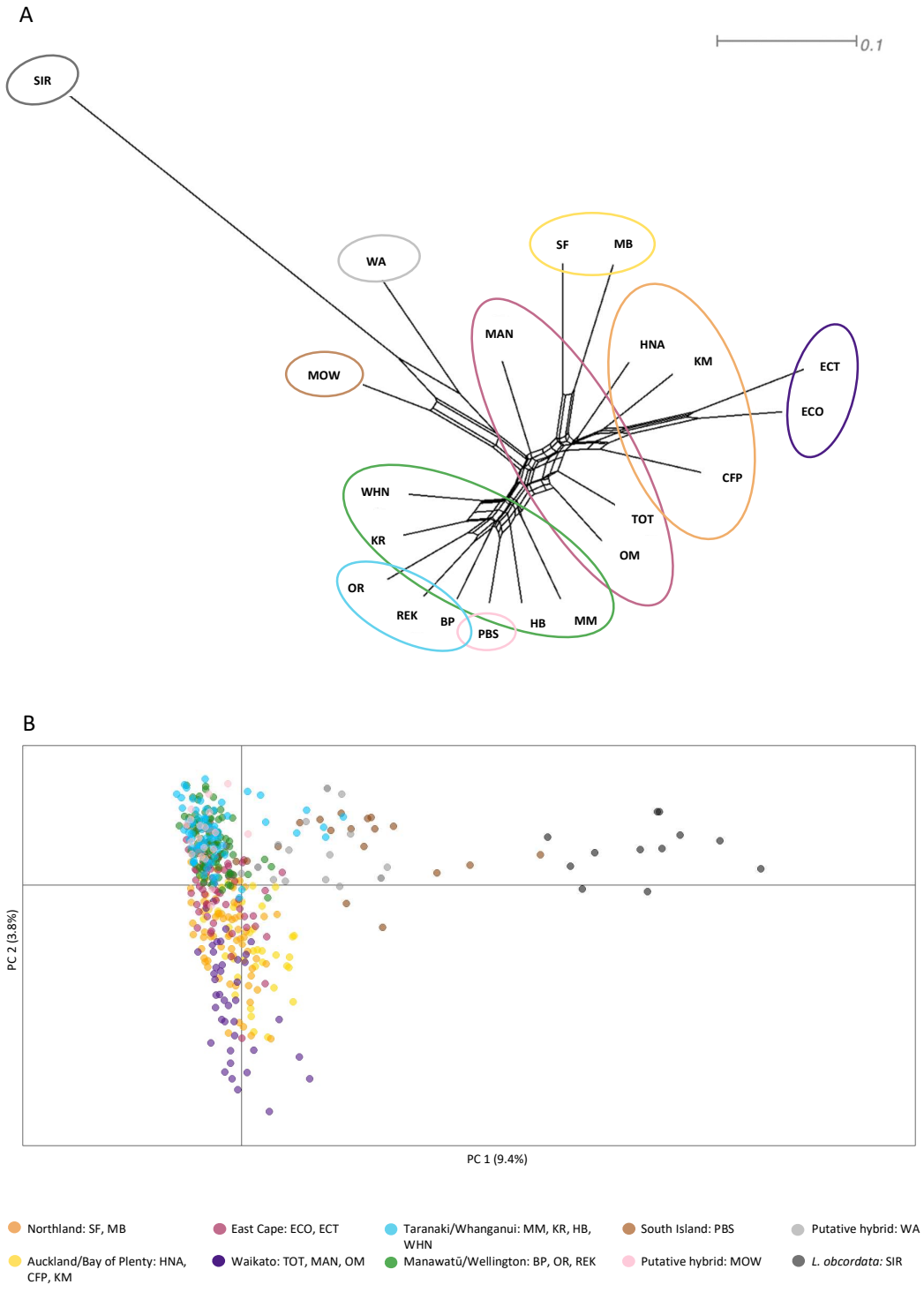

Figure 3.9. Spatial analyses of *L. bullata, L. obcordata,* and the putative *L. bullata* x *L. obcordata* hybrid sites. **A**, Population-level Neighbor-Net analyses. **B**, PCA analysis plot. Colouration indicates geographic locality of populations.

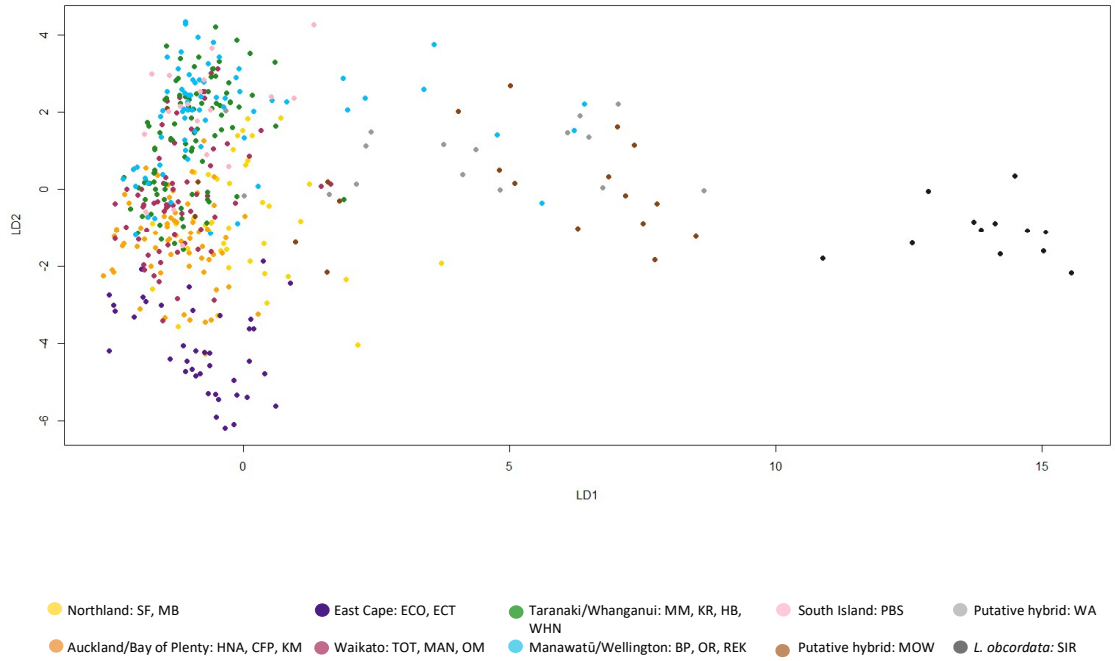

Figure 3.10. DAPC scatterplots of inferred *L. bullata, L. obcordata,* and the putative *L. bullata* x *L. obcordata* hybrid clusters. Colouration indicates geographic locality of populations.

3.4.5 AMOVA and IBD analyses

AMOVA analysis of dataset *L.bull* at the population-level AMOVA revealed that 81% of the variation originated within populations, with only 19% variation observed among populations. This pattern was also reflected in the analysis of dataset *L.all*, with 77% of the variation occurring within populations and 23% occurring among populations. The AMOVA results of the grouped populations followed similar patterns. Most of the variation originated within populations (85%), compared to among (15%) for the dataset excluding the hybrid and *L. obcordata* populations. In the dataset including the hybrid and *L. obcordata* populations as one group, the variation was also shown to have originated within populations (83%) compared to among populations (17%). The Mantel test showed significant isolation by distance for all genetic distance measures (Table 3.3, Fig 3.11).

Table 3.3. Mantel test results of populations of *L. bullata.* Rxy: correlation coefficient. P(rxy-rand>=rxy-data): the probability of a positive autocorrelation (one tailed t test)

Genetic Distance metric	Geographical distance (km)	Rxy	*P*(rxy-rand >= rxy-data)
$F_{ST}(1-F_{ST})$	Standard	0.57	0.001
Pairwise F_{ST}	Standard	0.61	0.010
$F_{ST}(1-F_{ST})$	Natural log	0.56	0.010

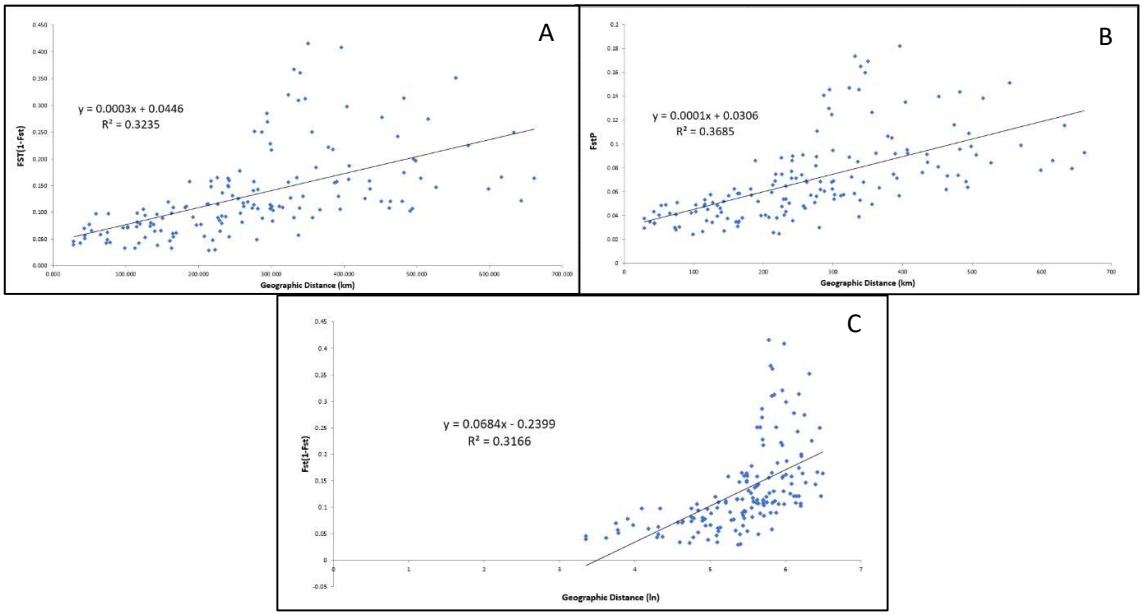

Figure 3.11 Mantel test results for *L. bullata* populations. **A**, $F_{ST}(1-F_{ST})$ against geographical distance. **B**, Pairwsie F_{ST} against geographical distance. **C**, F_{ST} $(1-F_{ST})$ against natural log of geographical distance.

Table 3.4. Population genetic studies of New Zealand and/or Myrtaceae plant species used to compare expected heterozygosity (H_e) with *Lophomyrtus bullata.* All cited studies were conducted using microsatellite markers.

Species	Family	Distribution	Growth form	Breeding system	H_e	Reference
Lophomyrtus bullata	Myrtaceae	Widespread, continuous	Shrub or tree at maturity	Outcrossing	0.44	This paper
Dactylanthus taylorii	Balanophoraceae	Rare, disjunct	Parasite	Outcrossing	0.46	McLay et al. (2022)
Pseudopanax ferox	Araliaceae	Widespread, disjunct	Shrub or tree at maturity	Outcrossing	0.48	Shepherd and Perrie (2011)
Metrosideros bartletti	Myrtaceae	Rare, disjunct	Tree at maturity	Outcrossing	0.50	Melesse (2017)
Pseudopanax crassifolius	Araliaceae	Widespread, continuous	Shrub or tree at maturity	Outcrossing	0.63	Gemmell et al. (2022)
Kunzea ericoides	Myrtaceae	Widespread, continuous	Shrub or tree at maturity	Primarily outcrossing	0.70	Heenan et al. (2022)
Corymbia calophylla	Myrtaceae	Regional, continuous	Tree at maturity	Outcrossing	0.71	Sampson et al. (2018)
Melaleuca alternifolia	Myrtaceae	Regional, disjunct	Shrub or tree at maturity	Outcrossing	0.72	Rossetto et al. (1999)
Kunzea robusta	Myrtaceae	Widespread, continuous	Shrub or tree at maturity	Primarily outcrossing	0.74	Heenan et al. (2022)
Eucalyptus gomphocephala	Myrtaceae	Regional, disjunct	Tree at maturity	Primarily outcrossing	0.75	Nevill et al. (2014)
Metrosideros polymorpha var. glaberrima	Myrtaceae	Widespread, disjunct	Shrub or tree at maturity	Outcrossing	0.76	Stacy et al. (2020)
Eucalyptus globulus	Myrtaceae	Widespread, continuous	Tree at maturity	Primarily outcrossing	0.82	Steane et al. (2006)

3.5 Discussion

The primary objective of this chapter was to investigate the genetic diversity and structure of the New Zealand endemic *L. bullata* across its geographical range. The species is threatened by the highly invasive fungus commonly known as myrtle rust and could face an extreme decline in population numbers and regional extinction. Information on the amount of genetic diversity present within *L. bullata* and how that variation is partitioned across its distribution is fundamental to ensuring the best possible conservation management outcomes for the species.

3.5.1. Genetic diversity of Lophomyrtus bullata

The genetic diversity of *Lophomyrtus bullata* at a species level was observed to be low, with an expected heterozygosity (He) of 0.44 and an average number of alleles per locus of 3.4 (Table 3.2). Comparing these findings to those of species with similar life history characteristics in a review of plant genetic diversity by Nybom (2004), *L. bullata* displays lower He than expected for long-lived perennials (0.68), outcrossing species (0.65), and species whose seeds are dispersed by ingestion (0.73). Comparisons with other New Zealand species or Myrtaceae species with similar life history traits to *L. bullata* also show that *L. bullata* has lower He (Table 3.4). For example, the similarly widespread species *Kunzea robusta* has a significantly higher He compared to *L. bullata* (Heenan et al., 2022). However, similar levels of genetic diversity were found in *Pseudopanax ferox,* which is widespread around New Zealand*,* but in contrast to *L. bullata*, *Pseudopanax ferox* populations are highly disjunct, and some contain only a few individuals (Shepherd & Perrie, 2011).

The low genetic diversity observed in *L. bullata* relative to other similar species is surprising, as it is typically expected that species with large populations, like *L. bullata*, contain greater genetic diversity than smaller populations as they are less vulnerable to stochastic environmental and genetic events (Frankham, 1996; Leimu et al., 2006). In cases where large populations have lower diversity than expected, other factors could be more important in explaining the species' current genetic diversity (Ellstrand & Elam, 1993). Among such factors are the historical influences on a species, e.g., the effects of glacial cycling during the Last Glacial Maximum (LGM) (Marske & Boyer, 2022). Such a factor could have influenced the

genetic diversity of *L. bullata,* as glacial cycling during the Last Glacial Maximum (LGM) heavily impacted the structure and range of the forests around New Zealand (McGlone et al., 2010; Newnham et al., 2013). Formerly widespread forest habitats contracted in size due to glaciation and would have been restricted to isolated areas termed glacial refugia. In New Zealand, regions north of the Bay of Plenty have often been proposed to be areas of glacial refugia (Marske & Boyer, 2022; Wallis & Trewick, 2009). These regions were thought to have consisted of podocarp-broadleaf and beech forests that otherwise could not have survived in the colder and more arid climates brought on by glaciation (Buckley et al., 2015; Marske & Boyer, 2022). Modern populations in regions of suspected former glacial refugia are sometimes observed to be more genetically diverse or distinct compared to other regions (Byrne, 2008; Hewitt, 2000). In this regard, distinct patterns of interspecific diversity in the upper North Island of New Zealand have been observed in plant species (McGlone, 1985; Gardner et al., 2004), and invertebrate species (Buckley et al., 2015;Ellis et al., 2015; Trewick et al., 2022).

At the population level for *L. bullata,* the Northland (SF, MB) and Coromandel (CFP) sites exhibit higher genetic diversity (He) than other sites, and the Northland (SF) and Auckland population (HNA) have the highest number of private alleles, though this is still low at three (Table 3.1). However, it is possible these could be areas of former glacial refugia for the species. If this is the case, *L. bullata* may have undergone a severe genetic bottleneck in the past, resulting in a loss of diversity. Additional effects of this bottleneck could have been an increase in genetic drift and subsequent inbreeding for some populations, which would have increased the genetic similarity between individuals and decreased the overall genetic diversity (Hedrick & Kalinowski, 2000). Additionally, if the Northland, Coromandel and Auckland regions were the sole source for recolonisation, then the genetic diversity of the newly colonised populations would have been impacted by founder effects, potentially contributing to the overall low genetic diversity observed in populations of *L. bullata* (Barton & Charlesworth, 1984). Such an effect could also explain the relationship between decreasing He, Na, and latitude, as there could have been a serial expansion of populations further south, with each wave of colonisations pioneered by ever-diminishing gene pools (Fig 3.12). This would have contributed to the overall low genetic diversity observed in populations of *L. bullata.*

It is unclear if these regions were the sole locations of populations of *L. bullata* during the Pleistocene glaciation. *L. bullata* is generally found in lowland forests and would have been

heavily restricted in its range during this period. It is possible that other populations survived in microrefugia in other regions of the North Island during the Pleistocene, and these regions also contributed to the expansion and recolonisation of new populations. The higher levels of diversity found in the Northland, Coromandel, and Auckland could instead be a reflection of these refugia being the most extensive and stable in terms of forest coverage during this period or from the increased dispersal of individuals from other refugia. Survival of species in multiple microrefugia throughout the Pleistocene has been suggested for other New Zealand species (e.g., Buckley et al. 2010), as well as for species in other regions of the world (e.g., Nevill et al. 2010; Shi et al. 2014).

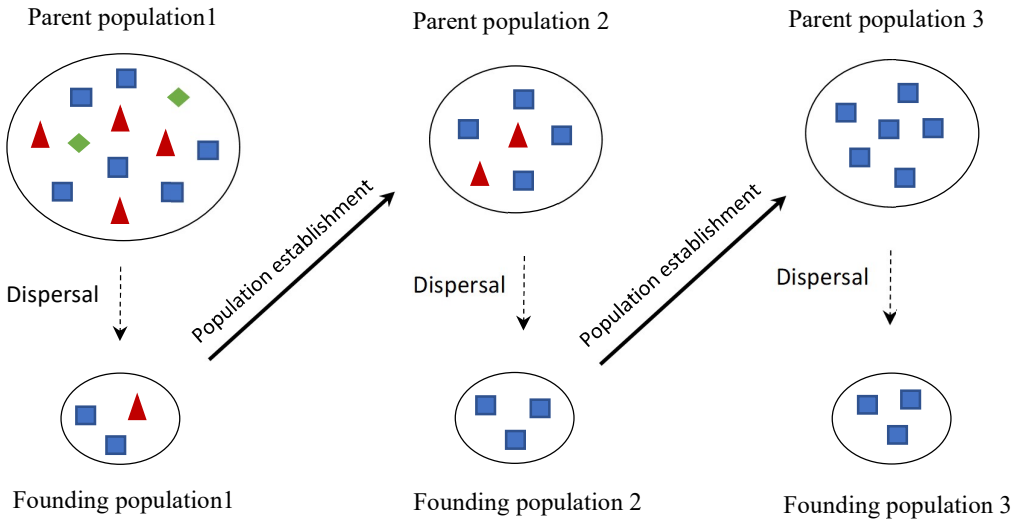

Figure 3.12 A simplified figure explaining how the founder effect contributes to lower genetic diversity in subsequent colonised populations.

The lower-than-expected genetic diversity present in the species also could be due to the effects of myrtle rust. Myrtle rust causes an increase in tree mortality, leading to a decrease in population size over time. This loss of individuals could potentially result in a loss of alleles and could increase the effect of genetic drift and inbreeding, decreasing the genetic variation of the population further. However, it is uncertain if myrtle rust has been established in New Zealand for a sufficient period of time for the effect on genetic diversity to be observable in the long-lived *L. bullata*. Myrtle rust was first detected in New Zealand in May 2017 (Beresford et al., 2018), approximately six years ago at the time of writing. The disease has spread rapidly

since then and is now distributed widely around New Zealand (Toome-Heller et al., 2020). An Australian study investigating the impact of myrtle rust on the native species *Rhodamnia psidioides* found that over half the trees surveyed died after only four years of exposure to the disease (Carnegie et al., 2016). It is possible that, in regions where the disease has been established for a more extended period of time in New Zealand (e.g., Taranaki), there would be more of a decrease in genetic diversity, though such a pattern is not evident in the current analysis. Comparing sites where myrtle rust has been detected versus the few where it has not been (Supplementary Table 2) does not seem to show any pattern of lower versus higher genetic diversity. However, the extent and severity of the infection for each site is not considered in this comparison, nor if there were any potentially resistant individuals present. This is partly due to the time of the year sampling took place at many sites (winter), which made observations on infection level difficult, as the most apparent signs of myrtle rust infection, the yellow urediniospores, are present on tissue mainly during the warmer months (December-April).

3.5.2. Genetic structure of Lophomyrtus bullata: North vs south patterning along the Taūpo line

One of the most supported trends observed in the data was a north to south pattern of genetic diversity, with two distinct genotypic clusters identified in the STRUCTURE analysis (Fig 3.2). However, instead of an abrupt change, there is a gradient between the two genetic groups that occurs across the populations situated at ~38°S latitude. This pattern is also evident in the Neighbor-net analysis, with one of the most notable splits occurring between the upper North Island and the lower North Island and South Island sites (Fig 3.5).

This pattern is consistent with a known biogeographic pattern called the Taūpo line. The pattern was originally described by Wardle (1963) as an area that divided the endemic species-rich northern North Island and the species-poor lower North Island. The pattern has also been reflected in phylogenetic analyses, with Heenan et al. (2017) identifying a significant decrease in both the phylogenetic diversity and the weighted phylogenetic endemism for vascular flora in the region below the Taūpo line. This pattern is repeated in invertebrates, and vertebrates, with higher levels of both inter- and intra-specific diversity reported for a variety of endemic species (Buckley et al., 2010; Buckley et al., 2009; Bulgarella et al., 2014; Chapple et al., 2009; Marske et al., 2011; Painting et al., 2017; Withers et al., 2021), as well as in plant species (Heenan et al., 2022).

The precise location and cause(s) of the trend around the Taūpo line have been debated (Buckley et al., 2015; Ellis et al., 2015). Many postulate that the formation of a sea strait during the Pliocene, caused by the combination of tectonic uplift and rising sea levels, is the source (Bunce et al., 2009; Suggate, 1978). Due to the formation of this sea strait (also known as the Manawatū Sea Strait), flora and fauna below 38° on the North Island would have faced near extirpation (Buckley et al., 2015; Marske & Boyer, 2022). Others suggest Pleistocene glacial cycling as the main cause, with forests persisting only north of 38° (Wood et al., 2017). Other causes, such as Pleistocene/Holocene volcanic activity, also have been suggested (Barrell et al., 2005). Some studies have identified lineage divergence in animal species that are Pliocene in origin or predate the Pleistocene (e.g., Marske et al. (2011), Chapple et al. (2009), Withers et al. (2021)), while others are believed to post-date it (e.g., Painting et al. (2017)). However, such trends in diversity in the North Island could be due to a combination of influences, having been first formed due to the Pliocene sea strait and then maintained through Pleistocene glacial cycling, where many species would have experienced large contractions in their natural ranges (Buckley et al., 2015).

The degree to which historical events would have an effect on a species is dependent on the evolutionary history of that species. The ancestor of *Lophomyrtus* is believed to have been present in New Zealand since at least the early Miocene, potentially colonising new emergent landmass after Oligocene drowning (Smissen et al., 2022; Vasconcelos et al., 2017). *L. bullata* and *L. obcordata* are believed to have diverged ~3.6-5 mya (Thornhill et al., 2015). Therefore, it is possible that both Pliocene marine retreat and Pleistocene glaciation had an effect on the distribution of the species. Marine or glacial retreat could be one possible explanation for the north to south split observed in *L. bullata*. The cline between the two genotypic clusters occurs along 38° to 38.9°S latitude, which fits with the explanation of recolonisation after marine or glacial retreat and suggests that current populations north of 38° represent either refugia (such as the Northland, Coromandel, and Auckland populations are suggested to be) or were more quickly recolonised. This would result in a gradient at 38° in latitude which acts as a boundary between older, more genetically diverse populations and younger, less diverse populations. The recolonisation of populations below 38° would have resulted in less genetic diversity within the new populations due to the founder effect and less diversity between the populations due to potentially more recently sharing a common ancestor. The PCA, DAPC, and Neighbor-net

analyses do suggest there is less genetic divergence between populations in the Taranaki (MM, KR, HB), Whanganui (WHN), Manawatū (BP, OR), Wellington (REK), and South Island (PBS) compared to the more Northern populations (Figs 3.5 - 3.7).

The north to south split between the two genetic groups could have additionally resulted from isolation due to volcanic activity in the central North Island. Due to the location of the populations comprising the gradient, this volcanic activity would most likely have been caused by the Alexandra Volcanic Group (AVG) and the Taūpo volcanic zone. The AVG is a chain of now-extinct volcanoes that consist of Mount Karioi (location of TOT population, Fig 3.1), Mount Pirongia, Kakepuku, Te Kawa, and the volcanic field known as the Okete volcanic formation (Briggs, 1983; Briggs et al., 1989; McLeod et al., 2022; Pittari et al., 2021). The AVG was considered to have been mainly active during the Pliocene-Pleistocene (2.74-1.60 Ma) (Briggs et al., 1989), though it has been suggested that the largest volcano, Mount Pirongia, may have been active later, between 1.6-0.9 Ma (Pittari et al., 2021). The Taūpo volcanic zone has been the site of repeated wide-scale eruptions for the last 2 Ma, with eruptions occurring as recently as a few hundred years ago (Barker et al., 2021; Wilmshurst & McGlone, 1996). Vegetation in the immediate surroundings of the eruptions would have been severely impacted by basaltic lava flows and tephra (Briggs & McDonough, 1990; Goles et al., 1996; McLeod, 2019; Wilmshurst & McGlone, 1996). Such effects would have eliminated much of the local populations of *L. bullata* and acted as a barrier to gene flow between the two genetic groups identified by STRUCTURE. Limited gene flow could have increased divergence between the two groups, and the gradient pattern observed in the Waikato populations (TOT, MAN, OM) could be the result of secondary contact after gene flow was re-established. Therefore, it is possible that the north-south pattern observed in *L. bullata* was first established by recolonisation after marine retreat and strengthened by the combined effects of glacial cycling and volcanic activity.

3.5.3. Genetic structure of Lophomyrtus bullata: Regional genetic structure

The most consistent genetic pattern observed was the clustering of populations into groups according to regional locality, which is most clearly represented in the *K*=5 STRUCTURE results (Fig 3.4). At *K*= 5, the three Waikato (TOT, MAN, OM) populations that coincide with the gradient pattern at *K*=2 form their own group, and the divide occurring along the Taūpo

line is still observed between this group and the neighbouring Taranaki/Whanganui group (Fig 3.4). Additionally, a separation occurring between the Whanganui population (WHN) and the Bruce Park population (BP) acts, to a small extent, as an east-west divide of the Taranaki/Whanganui group and the Manawatū/Wellington group (Fig 3.4). A similar pattern was noted for populations of *Dactylanthus taylorii* (McLay et al., 2022) and *Asplenium hookerianum* (Shepherd et al., 2007), with the patterns attributed to the effects of volcanic activity in the Taūpo zone, which could have limited gene flow between the east and west populations. On a finer scale, the divide between the Northland (SF, MB), Auckland (HUNA), and Coromandel (CFP) populations and the remaining populations correlates with a known biogeographic pattern termed the Kauri line (Fig 3.4), which represents the southern limit of the New Zealand endemic tree *Agathis australis* (Araucariaceae), known commonly as Kauri (Marske & Boyer, 2022; Wagstaff & Clarkson, 2012). However, such a division is less apparent in the analysis compared to the other patterns described above.

The majority of the regional groups identified by STRUCTURE contain a high degree of admixture. This admixture is most evident between populations on the borders of each group, which suggests gene flow between neighbouring regions. The significant result of the mantel tests (Table 3.3, Fig 3.11) suggests that distance may be a factor in limiting gene flow between populations, potentially driving the genetic differences between some of the regional groups. Since plants are sessile, gene flow in *L. bullata* occurs through bird dispersed seeds and bee-mediated pollination (de Lange, 2022). The latter has the potential to be more restricted by range compared to other mechanisms (e.g., wind pollination), which could result in more restricted gene flow (Wessinger, 2021). Populations that are closer in proximity would be more likely to have an overlap in pollinators, exchange genetic material and therefore be more genetically similar. More distantly distributed populations would be less likely to exchange genetic material, accumulate genetic differences, and become increasingly diverged (Rousset, 1997). However, this effect could be counteracted by the dispersal of seeds by birds as they are less restricted by range. This could result in a reduced population structure (Jesson, 2007), potentially explaining the high degree of admixture and low pairwise F_{ST} values (Table 3.2) between populations.

The pattern of regional genetic structure could be a reflection of the impacts of the founder effect. If the current populations were recolonised from glacial refugia, then each population

could have been established by different individuals from different source populations (Haase, 1993). This would result in each population having different allele frequencies, which would be based on the individuals that colonised that population (Haase, 1993). These allele frequencies could have been impacted further due to genetic drift occurring in the initially small founding populations. The differences could have been maintained due to barriers to gene flow, either geographical or genetic, preventing complete population homogeneity. However, if this was the case, the genetic differentiation between populations might be expected to be higher than what was observed in this analysis.

The *L. bullata* populations could be in the early stages of divergence, potentially accounting for the regional genetic structure observed. The increased divergence between populations could be due to increased habitat fragmentation from human-mediated forest destruction (Ewers et al., 2006), which would decrease population connectivity by limiting gene flow. If continued, this would eventually result in more genetically differentiated populations (Heenan et al., 2022). However, it is uncertain how likely this is given that *L. bullata* currently has a relatively continuous distribution, and it has been suggested that older biogeographical events tend to have more of an effect on the distribution of a species' genetic variation compared to more modern influences (Dussex et al., 2014; Withers et al., 2021). Although, such an issue could become more of a factor in the future due to the effects of myrtle rust causing localised extinctions. Population extinctions could result in large gaps in the distribution of the species, preventing gene flow and increasing genetic differentiation between regions.

3.5.4. Genetic structure of Lophomyrtus bullata: East Cape

Individuals from the two East Cape populations (ECO, ECT) consistently form a distinct genetic cluster across all analyses and have significantly higher pairwise F_{ST} values compared to all other *L. bullata* populations (Table 3.2). A similar genetic distinction in East Cape populations has been noted across many other species, including stick insects (Buckley et al., 2010), beetles (Marske et al., 2009), cicadas (Ellis et al., 2015), and frogs (Fouquette Jr, 1975). Several hypotheses have been suggested to explain the phenomenon, with the most supported suggesting that the northern East Cape acted as a glacial refugium during the Pleistocene (Buckley et al., 2010; Ellis et al., 2015; Marske et al., 2009). If this was the case for *L. bullata,* then it could potentially explain why the East Cape populations are significantly genetically

different from other populations. Alternatively, Gardner et al. (2004) suggested that a similar pattern found in *Metrosideros* trees resulted from the isolation of the East Cape populations from the Coromandel due to volcanic activity along the Bay of Plenty coastline during the Holocene. The STRUCTURE results of *L. bullata* indicate genetic similarity between the Tauranga (KM) population and the East Cape populations (Fig. 3.2), and it is possible that this could be the result of more recent gene flow between reconnected populations after similar isolation of the East Cape due to volcanic activity. One possible way to investigate this further would be to include *L. bullata* from sites located in Rotorua, as this area provides a bridge between the two aforementioned sites. Iwi approval for sampling was sought for sites in the Rotorua region but was declined.

3.5.5. Genetic structure of Lophomyrtus bullata: South Island

The results indicate a relationship between the Taranaki/Whanganui (MM, KR, HB, WHN) populations and the northern South Island population (PBS). The relationship is reflected in the low pairwise F_{ST} values (Table 3.2) and the STRUCTURE results, with the South Island population sharing a high proportion of membership to the Taranaki/Whanganui populations (Fig 3.2, 3.4). Similar patterns were observed in extinct Moa species (Bunce et al., 2009), species of skinks (Greaves et al., 2008; Greaves et al., 2007; Liggins et al., 2008; O'Neill et al., 2008), geckos (Nielsen et al., 2011), and plant species (Prebble et al., 2018). The pattern has been suggested to be due to the presence of a land bridge between the north-western South Island and Cape Egmont during the Pleistocene caused by lowered sea levels (Lewis et al., 1994; Trewick & Bland, 2012). The land bridge was suggested to have been exposed intermittently between glacial cycles to varying degrees (Lloyd, 2003). The continental shelf between Cape Farewell and Cape Egmont was the last region to be inundated by rising post-glacial sea levels (Lewis et al., 1994). The climate of the land bridge was cool and arid, like the majority of the lower North Island during this period, and therefore might have limited dispersal (Marske & Boyer, 2022). However, others have suggested that the land bridge was forested, at least at certain times between glacial cycles (Marra et al., 2009).

One possible explanation for this relationship between the Taranaki and South Island populations is that *L. bullata* was present in glacial refugia in the northern South Island (Heenan & Mitchell, 2003; McGlone et al., 2010). This suggests that the populations were reconnected

post-glaciation, possibly by the Pleistocene North-South Island land bridge. If this were the case, it would be expected to see more of a distinction between the South Island population and the lower North Island, considering, like the northern North Island, the northern South Island is a region observed to have greater endemism (McGlone, 1985; Wardle, 1963). However, this is not observed in the analyses, with the PCA, DAPC, and to some extent, Neighbor-net analyses suggesting that the South Island population and the lower North Island populations are all very genetically similar. This is also reflected in the pairwise F_{ST} values, with the South Island and lower North Island populations all having some of the lowest values (Table 3.2). Though, it should be noted that this could be impacted by the limited number of South Island populations included in the analysis. Therefore, it is more likely that either *L. bullata* was not present in the northern South Island due to glaciation and was repopulated post-glaciation either via the Taranaki land bridge or through dispersal across the Cook Strait or a combination of both.

3.5.6. Hybridisation as a survival mechanism

The patterns present in the *L.all* dataset indicated that although hybridisation occurs between *L. bullata* and *L. obcordata*, the two species are still genetically distinct. However, it was surprising that the initial STRUCTURE results did not separate species first, though this could be due to how the STRUCTURE software defines its ancestral groups by identifying possible HWE populations (Pritchard et al., 2000). As the lower North Island population may be a 'good' HWE population due to its high genetic similarity, all remaining populations may have been placed into the other genetic group. The low Delta *K* value for *K*=2 could be considered evidence of this (Fig 3.8, Supplementary Fig 3). All other analyses showed varying degrees of separation between the two species and their hybrids (Fig 3.9, Table 3.2). However, it should be noted that the patterns observed are potentially affected by the limited number of *L. obcordata* individuals included in the analysis.

Hybridisation between species can potentially act as a new source of genetic variation, perhaps even more so than mutation, if hybridisation frequently occurs (Abbott et al., 2013; Anderson, 1949; Arnold & Martin, 2009; Grant & Grant, 1994). The higher expected heterozygosity observed in the putative hybrid populations compared to the other *L. bullata* and *L. obcordata* populations (with the exception of the Northland *L. bullata* populations, which are suggested

to be glacial refugia) indicates potentially higher genetic diversity is present in hybrid populations (Table 3.1). This increase in diversity resulting from hybridisation could result in an increase in the species' adaptive potential, meaning it is potentially better able to adapt to rapid environmental change (Becker et al., 2013; Ogden, 1989; Rattenbury, 1962). This can make hybridisation an essential survival mechanism for many plant species. The mechanism behind hybridisation leading to increased adaptive potential has been termed adaptive introgression, with introgression described as when hybridising species transfer genetic material through repeated backcrosses (Anderson & Hubricht, 1938). Adaptive introgression can result in the transfer of selectively advantageous alleles from one parent species into another (Rieseberg et al., 1993) or in the development of new combinations of selectively favourable traits (Becker et al., 2013; Dittrich-Reed & Fitzpatrick, 2013). Examples of New Zealand plant species that have been suggested to adopt hybridisation as an evolutionary strategy include *Pachycladon* (Becker et al., 2013), *Nothofagus* (Ogden, 1989), *Sophora* (Shepherd & Heenan, 2021), and *Coprosma* (Rattenbury, 1962). However, the success of this strategy largely depends on the degree to which parts of the genome are being introgressed between populations (Abbott et al., 2013), if hybrids are able to backcross to both, one or none of the parent species, and that hybridisation results in an increase in adaptive potential, rather than a decrease in fitness (Becker et al., 2013). For context, it should be noted that in certain environmental conditions, it is possible for putative *L. bullata* x *L. obcordata* hybrid individuals to dominate and for *L. bullata* individuals to die out (de Lange, 2022), possibly indicating greater fitness for hybrids in certain environments.

Hybridisation between *L. bullata* and *L. obcordata* could provide new advantageous genetic combinations for myrtle rust resistance. Exchange and recombination of genetic material between individuals of *L. bullata* and *L. obcordata* could potentially allow for both species to acquire new traits for pathogen resistance through introgression. Interestingly, it has been observed that while populations of *L. bullata* are declining rapidly, populations of *L. obcordata* are still declining but currently at a slower rate in comparison (Prasad et al., 2022). Though there could be other causes for this slower decline, it is possible that *L. obcordata* could harbour some form of resistance against myrtle rust that is not present in *L. bullata* but could be shared through hybridisation. Examples of such an occurrence can be found in the New Zealand alpine species of *Pachycladon*, where past introgression from hybridisation led to the transfer of chemotypes of glucosinolate hydrolysis, which are used in herbivore and pathogen defence

(Becker et al., 2013; Voelckel et al., 2008). Additionally, the hybridisation of *L. bullata* and *L. obcordata* could lead to gene stacking for resistance, a method typically used in plant breeding programs. Gene stacking for resistance involves combining different resistance genes (R genes) into one plant, usually through hybridisation or genetic engineering, to allow for greater protection of those plants against pathogens (Rogozina et al., 2021). Both are possible scenarios where hybridisation with *L. obcordata* could potentially benefit the future preservation of *L. bullata.*

3.5.7. Implications for conservation

The establishment of myrtle rust in New Zealand has been hugely impactful on the endemic myrtle species *Lophomyrtus bullata.* The fast-spreading pathogen has already infected multiple populations of *L. bullata* from Northland to the Marlborough sounds. It has caused severe dieback of growth and death of juvenile and mature trees alike. Infection by myrtle rust and the corresponding effects on plant fecundity could act as an extreme selective pressure for *L. bullata*. The current low genetic diversity observed within populations of *L. bullata* suggests that the species is potentially limited in its ability to adapt to such pressure (Kardos et al., 2021). Tree mortality could cause population numbers to decline rapidly, increasing the effects of genetic drift and likely leading to the loss of rare alleles and the random fixation of others. This loss could decrease genetic variation and increase inbreeding, potentially resulting in reduced fitness for the species.

The population structure of *L. bullata* shows that while there are low to moderate levels of genetic differentiation present between populations, there is distinct regional genetic variation. Therefore, care should be taken that individuals from the appropriate regional locations are used for sourcing regeneration plantings. Regarding seed banking, priority should be given to Northland, Coromandel, and Auckland populations as they contain a higher degree of intraspecific genetic diversity (e.g., higher expected heterozygosity, and, though few were detected, a higher number of private alleles (Table 3.1)). It should also be ensured that all genetic regions are efficiently represented in the seed collections. Ongoing monitoring and preservation of *L. bullata* populations can potentially be limited to one or two populations from each locality to represent that region's genetic variation if required. It would also seem advisable to protect, monitor, and seed bank *L. bullata* x *L. obcordata* hybrid populations as

they potentially possess unique genetic compositions that may be important to conserving the *Lophomyrtus* lineage.

3.6 Conclusions

This study provides a detailed first estimate of the genetic variation within the threatened species *Lophomyrtus bullata*. The species has low levels of genetic diversity compared to other species with similar life histories. How this variation is partitioned across the geographic distribution of the species suggests it may at one time have been affected by a considerable reduction in range, possibly during the Last Glacial Maximum, which resulted in populations being restricted to the upper North Island. The subsequent range expansion of *L. bullata* from the upper North Island further south led to the recolonised populations harbouring lower genetic diversity compared to the source populations due to the founder effect. Such patterns could also have been influenced by volcanic activity isolating populations and increasing genetic differentiation. Significant regional genetic patterns observed could be attributed to the effects of past climatic events, isolation by distance, dispersal mechanisms of the species, or a combination of all of these factors. *L. bullata* may be threatened with extreme range reduction in the near future due to the effects of the pathogenic fungus myrtle rust, which is causing rapid population decline. This means *L. bullata* could potentially have additional decreases in its genetic diversity, increasing the species' risk of extinction further. The information described in this chapter is important for the future conservation and restoration efforts of this threatened species by helping to increase the efficiency and success of seed banking, population preservation, and regenerating plantings of *Lophomyrtus bullata.*

3.7 References

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Chapter 4

Conclusion

4.1 Introduction

The main focus of this thesis was to investigate the population genetics of *Lophomyrtus bullata.* A population genetics study can provide information on the connectivity, inbreeding, gene flow, life history and biogeography of a species. This information can provide a fuller resolution to the forces influencing a species' genetic structure. Understanding these forces is necessary to preserve genetic diversity and minimise genetic erosion. It is essential to conserve genetic diversity as it represents a species' ability to respond to new environmental threats (Frankel & Soule, 1981; Holsinger & Falk, 1991). If a species has low genetic variation, this could result in failure to adapt to changing environments and eventual extinction (Holsinger & Falk, 1991). Therefore, low diversity and genetic differentiation could have serious implications for the future conservation of *Lophomyrtus bullata.*

Lophomyrtus bullata (ramarama) is an endemic New Zealand species of the ecologically important Myrtaceae family (Jo et al., 2022). The species provides key resources such as sugar-rich nectar that is beneficial to bees (Jo et al., 2022), berries with antioxidant properties (Lowry, 1976), and a food source to a range of native bird species (Kelly et al., 2010; Westphal, 2019). According to one survey by Prasad et al. (2022), the trees of *L. bullata* provide a home to at least 176 taxa, including bryophytes, ferns, other seed plants, and mycobiota. Culturally, *Lophomyrtus bullata* is a significant species to Māori in New Zealand, with the berries, leaves and wood all traditionally utilised for food, medicine and crafting, respectively (Best, 1925a, 1925b, 1942; Cambie & Ferguson, 2003; Riley, 1994). However, due to the pathogenic fungus *Austropuccinia psidii* (myrtle rust), the population numbers of *L. bullata* are declining. This decline could lead to smaller population sizes that are more susceptible to genetic drift and other stochastic forces, potentially resulting in a further decline in genetic diversity and higher levels of homozygosity in populations (Leimu et al., 2006; Ouborg et al., 2006). Higher homozygosity rates could lead to inbreeding depression, resulting in a reduced fitness for the species and increasing its risk of extinction (Charlesworth & Willis, 2009; Wright et al., 2008).

The use of microsatellite markers enabled an investigation into the current population dynamics of *L. bullata.* This provided essential information that could be used to help inform the species' current conservation management and additionally help estimate the potential future impacts of myrtle rust on the genetic diversity of *L. bullata*. The aims of this study were to:

1. Develop microsatellite markers for *L. bullata.*

2. Investigate the distribution of genetic variation of *L. bullata* across its geographical range.

3. Infer historical and biogeographical influences on the patterns of variation in *L. bullata* and determine their implications for the future conservation of the species.

4.2 Findings

Twelve microsatellite markers were designed and employed in a population genetics study on the threatened species *Lophomyrtus bullata*. The main findings were presented in the previous chapters: Marker Development of *Lophomyrtus bullata* (Myrtaceae) and Population genetics of *Lophomyrtus bullata* (Myrtaceae). This section will use these findings to address the aims of the study.

1. Develop microsatellite markers for *L. bullata.*

Lophomyrtus bullata DNA sequences were obtained from an Illumina MiSeq run, which resulted in 1,351,112 reads successfully paired and merged using Geneious software (v9.1.8) (Kearse et al., 2012). From these sequences, 55 loci were detected using the STR detection tool (Fungtammasan et al., 2015) available on the Galaxy web platform (Afgan et al., 2018). Primers for these loci were then designed using the Geneious addin Primer3 (Rozen & Skaletsky, 2000) and were trialled on individuals of *L. bullata* from four separate populations and twelve individuals of *L. obcordata*. The twelve microsatellite markers selected for screening were polymorphic and amplified consistently in both species, indicating the successful transfer of the markers to *L. obcordata*. Four hundred individuals of *L. bullata*, 12 individuals of *L. obcordata*, and 40 *L. bullata* x *L. obcordata* putative hybrids were screened using the selected markers. A total of 97 alleles were detected, with the total number of alleles per marker ranging from 3-11. These findings suggest that there are moderate levels of polymorphism within the developed markers.

2. Investigate the distribution of genetic variation of *L. bullata* across its geographical range, and 3. Infer historical and biogeographical influences on the patterns of variation in *L. bullata* and determine its implications for the future conservation of the species.

The results presented in the previous chapters suggested that *L. bullata* has low to moderate levels of genetic diversity at the species level. The populations with the highest levels of diversity were the Northland, Coromandel and Auckland populations, with a linear decline of diversity for populations increasingly southward. One potential scenario for this trend is that these upper North Island areas acted as glacial refugia for *L. bullata* during the Pleistocene, with all or most of the remaining populations extirpated. Individuals from the upper North Island populations would have recolonised all other regions following glacial retreat. This scenario would explain the currently low diversity present within the species and the negative correlation of diversity with latitude because the founder effect would have impacted all recolonised populations. Therefore, it is likely that *L. bullata* underwent a range contraction and subsequent expansion during glacial cycling, potentially influencing the species' modern distribution of genetic variation.

The genetic differentiation between populations of *L. bullata* is low, suggesting all populations are genetically similar. This could be influenced by the already low genetic diversity present within the species and impacted by the majority of the populations potentially being repopulated by a few source populations after glacial retreat. However, there were distinct genetic differences between geographical localities, with populations in the same region appearing more genetically similar. This regional population structure could be due to the effects of isolation by distance, past climatic events, or a combination of both.

Common biogeographical patterns observed in other New Zealand flora and fauna are also present in *L. bullata*. A split between populations above and below ~38°S latitude is consistent with the biogeographical pattern known as the Taūpo line. This pattern is mainly thought to be due to the formation of the Manawatū Sea Strait during the Pliocene or the product of Pleistocene glacial cycling (Buckley et al., 2015; Marske & Boyer, 2022; Wood et al., 2017). One potential explanation for this pattern in *L. bullata* is recolonisation by the aforementioned glacial refugia populations. This would result in an upper and lower North Island split between older, more diverse populations and less diverse populations that have more recently been colonised. Alternatively, it could result from the isolation and divergence of the upper and

lower North Island populations due to volcanic activity occurring during the last 2 Ma (Briggs, 1983; Briggs et al., 1989; Wilmshurst & McGlone, 1996) or a combination of both factors. Other patterns observed included the genetic distinctiveness of the East Cape, with the sampled populations appearing very genetically different from all other populations analysed. This could be due to the unique soils and climate present in the East Cape driving divergence between the populations (Ellis et al., 2015; Molloy & Smith, 2002) or due to the northern East Cape being an area that was formerly a glacial refugium during the Pleistocene, as suggested by some (Buckley et al., 2015; Ellis et al., 2015; Marske et al., 2009). Additionally, the differences could have occurred from volcanic activity in the Bay of Plenty region during the Holocene, isolating the East Cape and leading to divergence (Gardner et al., 2004). Finally, the relationship between the South Island population and the Taranaki populations suggests potential dispersal and continuous gene flow between populations via the land bridge that was present between Taranaki and the north-western corner of the South Island during the LGM (Bunce et al., 2009; Lewis et al., 1994).

The knowledge obtained from the population genetic study of *L. bullata* could help improve the conservation management of the species. How the genetic variation of *L. bullata* is distributed across its geographical range can help ensure seed banking collections represent the total diversity present in the species. Collections could potentially consist of two or three populations per regional group, and populations located on the border of a regional group could be of particular interest as they have high amounts of admixture. This study's investigations into the effects of hybridisation on the genetic diversity of *L. bullata* suggest it could provide *L. bullata* with increased variation and potentially be a source of adaptive traits. Further research into this area would be required to determine if this was a possibility and the impacts this would have on the conservation management of both species.

4.3 Limitations

One of the main factors potentially impacting the results presented here is the high degree of missing data (13%). Missing data is a common occurrence in microsatellite studies, and the majority of studies typically aim for equal to or less than 10%. Increased levels of missing data can affect the ability to correctly identify population structure, resulting in fewer genotypic clusters recognised and more inaccurate clusters (Reeves et al., 2016). Missing data is typically

caused by either PCR amplification failure or uninterpretable peaks produced during genotyping (Reeves et al., 2016). Amplification failure can be due to many factors, including poor-quality template DNA, human-technical error, and the presence of null alleles (Reeves et al., 2016). In the case of this research, the issue most likely lies with the poor-quality template DNA extracted from the older leaves sampled, as they tend to result in more degraded and less clean DNA. The use of older leaves was the direct result of some of the sampling occurring during the winter when there was little new growth. However, technical error and the presence of null alleles are also possibilities. Null alleles are alleles that have mutations in the primer binding region which prevents successful amplification during PCR (Carlsson, 2008). This results in the removal of either one allele, leading the individual to be labelled homozygote for that locus, or both alleles, resulting in missing data (Carlsson, 2008; Reeves et al., 2016). The presence of null alleles can lead to an overestimation of homozygosity, resulting in lower measures of genetic diversity and potentially inflating measures of F_{ST} (Carlsson, 2008). For the results presented here, MICRO-CHECKER software (version 2.3.3) (Van Oosterhout et al., 2004) was used to detect null alleles, with the program indicating the potential presence of null alleles at one locus. This result could indicate that null alleles are affecting the results; however, the excess of homozygosity at this locus could also be due to other factors, e.g., selection for homozygosity or increased levels of inbreeding.

Sample size and sampling bias could be additional factors affecting the results. Though most populations had 20 or more individuals sampled, which is the targeted sample size for microsatellite analysis, some populations (notably the *L. obcordata* population) had fewer individuals than this. It is also possible sampling bias could have occurred even with random sampling. For some sites, sampling was limited to where it was geographically feasible, and a few of the populations had many individuals over an extensive range of space. This made ensuring that the individuals sampled were a fair representation of the entire population difficult at these sites.

4.4 Future directions

These results provide preliminary information on the genetic diversity and population structure of *Lophomyrtus bullata*, but further investigation is necessary to ensure the species' successful conservation. Additional sampling of populations in distribution gaps such as Rotorua,

Northern Auckland, or the Northern Marlborough Sounds would help to strengthen and verify the results. Sampling more isolated sites, such as Great Barrier Island or Cape Reinga, could also allow for a more detailed analysis of the degree of population connectivity and the limits of gene flow within *L. bullata.* A complementary population genetic study on the similarly myrtle-rust-threatened *L. obcordata* with additional hybridisation zones between the two species could provide a fuller resolution to the effects of hybridisation, i.e., whether it will provide new traits or lead to outbreeding depression. The use of chloroplast markers in tandem with these results could provide further insights into the biogeographical structure of both species. This is because when isolated populations that have diverged genetically undergo secondary contact, nuclear DNA can recombine and intermix, while chloroplast DNA is typically uniparentally inherited and will remain unchanged (Rogalski et al., 2015). Because of this, chloroplast markers are typically better at showing former patterns of geographic structure than nuclear DNA markers.

These results could also potentially be used to measure myrtle rust's effects on the diversity and population dynamics of *L. bullata*. Completing the same or similar population genetic study using the same microsatellite markers in five years would allow for direct comparisons between the species' current and future genetic diversity. This would be particularly relevant for populations where myrtle rust has only recently been established (e.g., MAN) and populations that currently have no observations of myrtle rust (e.g., OM) if myrtle rust was to establish there in the future. Additionally, populations where seeds have been collected in the past (e.g., ECT) could allow comparisons between past and current levels of genetic diversity. A similar approach was used to investigate *Banksia brownii ,* a woody shrub species endemic to Australia that has undergone a considerable reduction in numbers and localised extinction due to the plant pathogen *Phytophthora cinnamomi* (Coates et al., 2015). The study used comparisons between extant populations and seed collections from extinct populations to estimate the loss of genetic diversity following population extinction. It was observed that the species lost over a third of the total genetic diversity and 22 out of 88 private alleles due to *P. cinnamomi* (Coates et al., 2015).

The microsatellite markers developed for *L. bullata* could potentially be used in the construction of linkage maps and, subsequently, if a link is established, the mapping of quantitative trait loci potentially involved in resistance. Identification of resistance to myrtle

rust would be critical for the implementation of plant breeding resistance programs. These programs would allow for the production of myrtle-rust-resistant individuals that can be introduced into vulnerable populations. Freeman et al. (2019) conducted simulations to determine the probability of extinction of at-risk New Zealand Myrtle species, and found that the probability of survival for a population increased greatly even if only a small number of individuals that carried resistant genotypes were introduced.

More detailed knowledge of the hybridisation mechanics of *L. bullata* and *L. obcordata* would be informative for both species' conservation. For example, if one species is more preferred as the paternal or maternal line, if the hybrids can backcross into none, one, or both parent species, and the degree to which parts of the genome can be introgressed. Such information could be readily obtained and incorporated into plant breeding programs for *L. bullata* discussed above and in Chapter 2. This is especially relevant, considering hybridisation has been used as a key tool in breeding crop species (Katche et al., 2019). Additionally, the hybrid zones of *L. bullata* (geographical locations where the two species co-occur and form hybrids) can also be used to investigate biogeographical patterns. Hybrid zones have been suggested to be where once isolated lineages came into secondary contact after glacial retreat during the LGM (Hewitt, 2011). In other cases, hybrid zones are thought to be where two species were once isolated in the same microrefugia for an extended period, leading to interspecific gene flow between the species (Shepherd et al., 2022). Such a scenario was suggested to have occurred for the New Zealand species *Metrosideros* (Gardner et al., 2004) and *Pachycladon* (Becker et al., 2013), as well as for many plant species in other parts of the world (Hewitt, 1999).

Further investigation into the divergence between *L. bullata* and *L. obcordata* could also be an interesting area of research. A number of New Zealand species lineages have been suggested to be Pliocene in origin, with divergence occurring due to changes in landforms during this period rather than the product of glacial cycling during the Pleistocene (e.g., Marske et al. (2011), Chapple et al. (2009), Withers et al. (2021)). This could be the case for *L. bullata* and *L. obcordata,* as current research dates the species divergence between ~3.6-5 mya (Thornhill et al., 2015). The formation of the Manawatū Sea Strait during the Pliocene could have split the distribution of the ancestral *Lophomyrtus* species. This split resulted in *L. obcordata* evolving independently in the South Island while *L. bullata* evolved independently in the upper North Island. *L. obcordata* is more tolerant of colder climates, and is found further south. With

the retreat of the sea strait, *L. bullata* migrated further south and *L. obcordata* further north, either via dispersal over the Cook Strait or with the aid of the land bridge between the islands during the Pleistocene. Of course, the current distributions and traits of the species could be due to other factors, and this is just one possible explanation. The ideas discussed here are just a handful of many additional avenues of research provided from the significant patterns identified within *Lophomyrtus bullata*, all of which could aid the future conservation of the threatened species.

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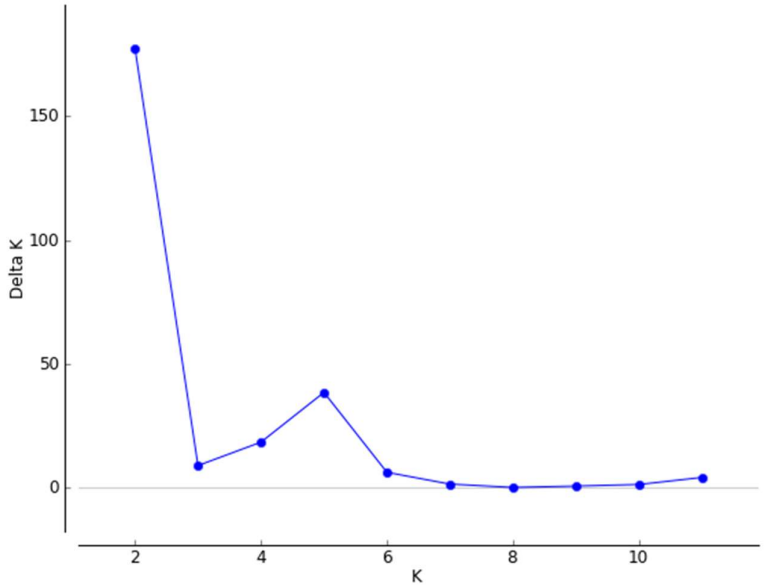
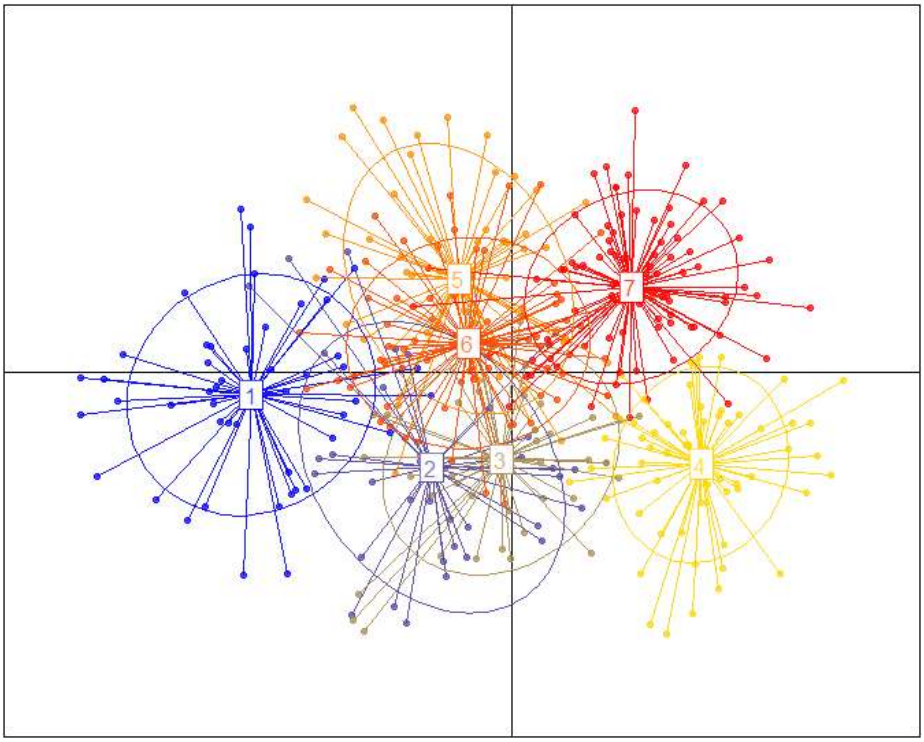
Supplementary Information

Supplementary Table 1. Geographic coordinates for each of the *L. bullata, L. obcordata* and *L. bullata* x *L. obcordata* sites.

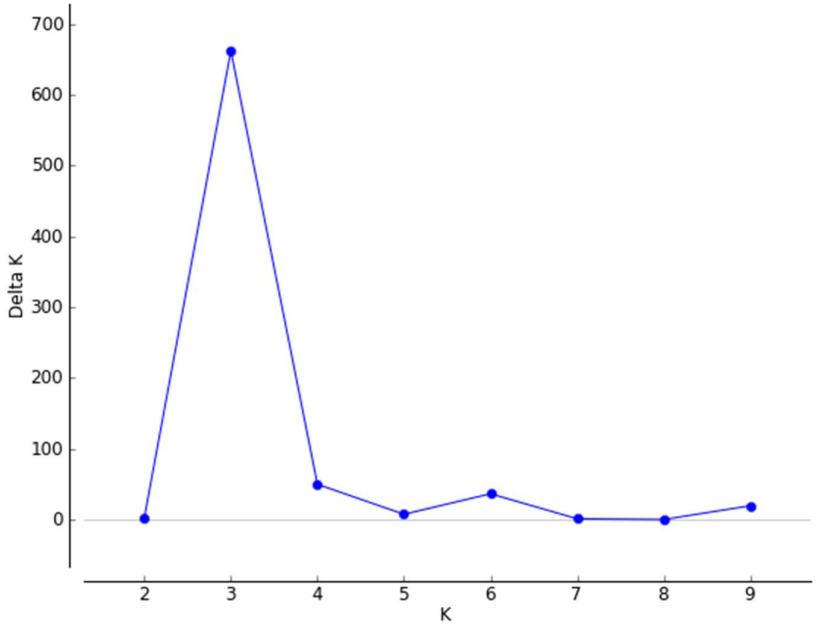
Location	Site code	Latitude	Longitude	Altitude (m)
Six Foot Track	SF	-35.512990	173.470381	437
Maunganui Bluff	MB	-35.757919	173.563243	446
Hunua Park	HNA	-37.040290	175.185480	467
Coromandel Park	CFP	-37.053889	175.673278	193
Kaimai Mamaku	KM	-37.492057	175.779379	215
East Cape One	ECO	-37.860846	177.619238	67
East Cape Two	ECT	-37.654444	178.297222	22
Te Toto Gorge	TOT	-37.87507	174.79671	420
Mangapohue	MAN	-38.260551	174.899487	179
Omaru Falls	OM	-38.522667	175.165917	299
Mt Messenger	MM	-38.90323	174.59384	55
Meeting of the Waters	MOW	-39.10249	174.12020	80
Kaitake Ranges	KR	-39.144986	173.980728	269
Home Bush	HB	-39.402518	174.015025	302
Whanganui Park	WHN	-39.726963	175.137986	39
Bruce Park	BP	-39.960917	175.532641	262
Ohinereiata	OR	-40.500609	176.050960	287
WA Miller Park	WA	-40.705977	175.653173	285
Remutaka Park	REK	-41.342184	174.947378	113
Pelorus Bridge/ South Island Rohutu	PBS/SIR	-41.297553	173.572722	37



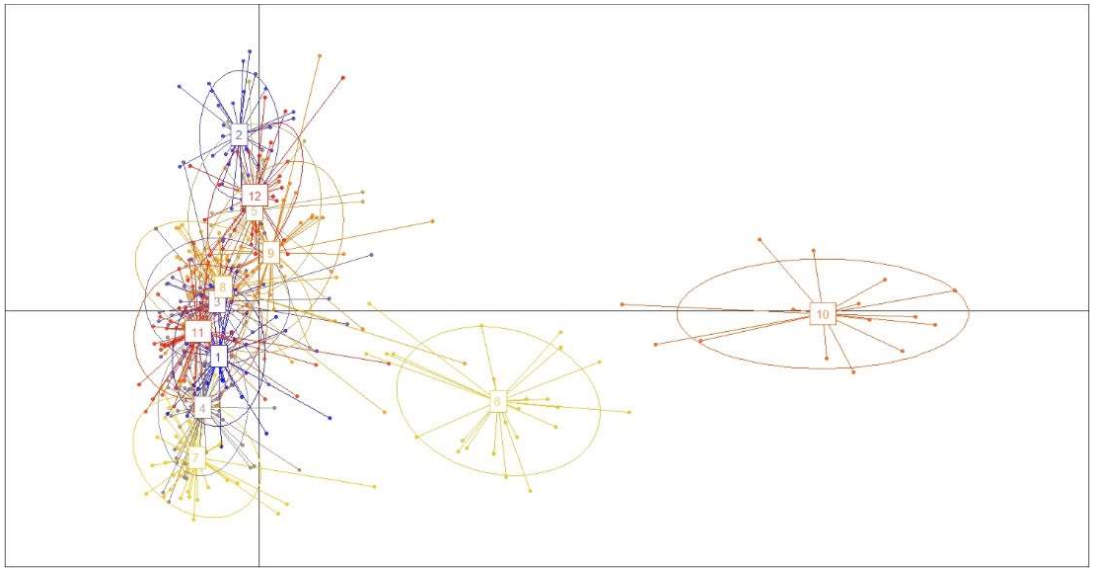
Supplementary Figure 1. Plot showing Delta K vs K results for the *L. bullata* sites. *K* values 2 and 5 were determined the best fit to describe the data.



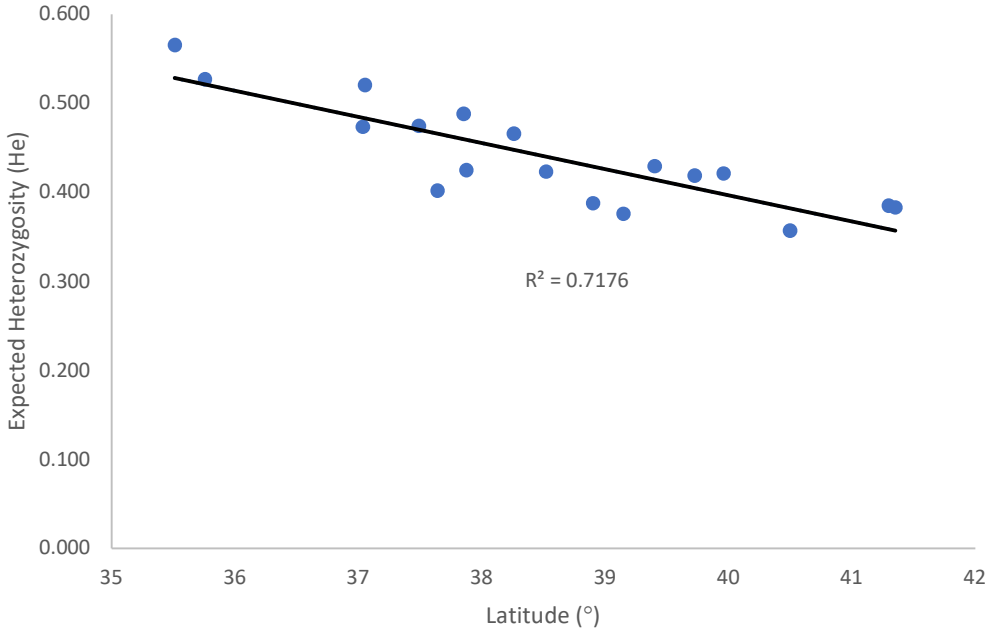
Supplementary Figure 2. Original DAPC clusters of *L. bullata* populations. Seven groups were identified.



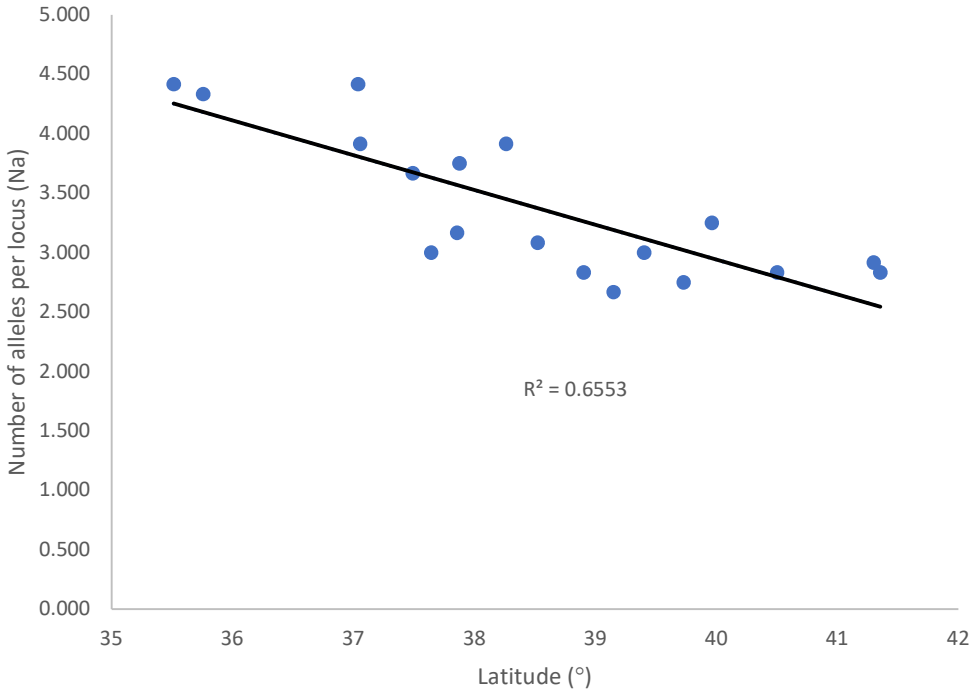
Supplementary Figure 3. Plot showing Delta K vs K results for the *L. bullata*, *L. obcordata,* and *L. bullata* x *L. obcordata* putative hybrid sites. *K* values 3 and 6 were determined as the best fit to describe the data.



Supplementary Figure 4. Original DAPC clusters of *L. bullata, L. obcordata,* and *L. bullata* x *L. obcordata* putative hybrid sites. Twelve groups were identified.



Supplementary Figure 5. Regression analysis of expected heterozygosity vs latitude for populations of *L. bullata.*



Supplementary Figure 6. Regression analysis of Number of alleles per locus vs latitude for populations of *L. bullata.*

Supplementary Table 2. Table indicating past and present detection of myrtle rust for each sample site of *L. bullata*, *L. obcordata*, and *L. bullata* x *L. obcordata* putative hybrids.

Site	Detection of myrtle rust	Source
Six Foot Track (SF)	Present	Myrtle rust reporter iNaturalist (2021)
Maunganui Bluff (MB)	Present	Myrtle rust reporter iNaturalist (2021)
Hunua Park (HNA)	Present	Myrtle rust reporter iNaturalist (2019), Personal obvs. (2022)
Coromandel Park (CFP)	Absent (though detected near)	-
Kaimai Mamaku (KM)	Present	Myrtle rust reporter iNaturalist (2019)
East Cape One (ECO)	Present	Personal obvs. (2022)
East Cape Two (ECT)	Present	Myrtle rust reporter iNaturalist (2020)
Te Toto Gorge (TOT)	Present	Myrtle rust reporter iNaturalist (2021)
Mangapohue (MAN)	Present	Myrtle rust reporter iNaturalist (2021), Personal obvs. (2022)
Omaru Falls (OM)	Absent	-
Mt Messenger (MM)	Present	Personal obvs. (2022)
Meeting of the Waters (MOW)	Present	Personal obvs. (2022)
Kaitake Ranges (KR)	Present	Personal obvs. (2022)
Home Bush (HB)	Present	Personal obvs. (2022)
Whanganui Park (WHN)	Absent	-
Bruce Park (BP)	Absent	-
Ohinereiata (OR)	Absent	-
WA Miller Park (WA)	Absent	-
Remutaka Park (REK)	Present	Myrtle rust reporter iNaturalist (2020)
Pelorus Bridge (PBS)	Present	Myrtle rust reporter iNaturalist (2018)