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Host-race specificity in the endemic pygmy mistletoe *Korthalsella salicornioides* (Viscaceae)

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ABSTRACT

Korthalsella Tiegh. is a genus of stem hemiparasites in the family Viscaceae, represented in New Zealand by three endemic species: *K. clavata*, *K. lindsayi*, and *K. salicornioides*. The most host-specific is *K. salicornioides* as it parasitizes two main host genera *Leptospermum* (Myrtaceae) and *Kunzea* (Myrtaceae), while the other two species are considered generalists parasitizing a wider range of host species. *K. salicornioides* is naturally uncommon and sparse, although it can be locally abundant on occasion. Mistletoe populations are at risk primarily due to habitat destruction and subsequent loss of hosts. Cross-infection experiments in *K. salicornioides* provided some insight into the presence of putative host races, as better mistletoe seedling establishment success rates were apparent when the maternal and recipient hosts were the same. However, because previous molecular sequence data (nuclear internal transcribed spacers and chloroplast *trnQ-rps16*) for *K. salicornioides* were not informative about specific host-races, more rapidly evolving molecular markers might be expected to detect host races.

In this study, next generation sequencing was used to develop novel microsatellite markers for *Korthalsella*. Eleven markers were reliably amplifiable and the most polymorphic for *K. salicornioides* were used to genotype 272 *K. salicornioides* individuals from 16 populations. Across all populations few alleles were identified, and within-population assessment of genetic variation indicated that many populations have low levels of genetic diversity and high proportions of homozygotes. Despite the presence of few alleles, a high degree of genetic differentiation between most populations was detected and was found to reflect host species and geography.

The findings of this study that *Korthalsella salicornioides* populations have low levels of genetic variation but host-specific races, has important conservation implications. The main conservational focus should be maintaining and increasing host *Leptospermum* and *Kunzea*

populations. The spread of mistletoe seed on hosts within or between populations may also increase the chances of continued survival. However, it is imperative that genetic material comes from the same host species, and consideration should also be given to the geographic area, especially in the Wairarapa. This study provides insights into the population structure within and between the different host populations and suggests several interesting areas of future study.

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ABBREVIATIONS

%P -percentage of polymorphic loci

A -number of alleles

A_S -allele size

A_T -total number of alleles

AFLP -Amplified Fragment Length Polymorphism

AMOVA -analysis of molecular variance

BLAST -basic local alignment search tool

bp -base pairs

cpDNA -chloroplast DNA

CASS -cheaply amplified size standard

CTAB -hexa-decetylammonium bromide

DNA -deoxyribonucleic acid

dNTP -deoxyribonucleotide triphosphate

DOC -Department of Conservation (New Zealand)

ER -Ecological Region

F_{IS} -component of Wright's (1921) fixation index, used to define within population structure by calculating the average observed heterozygosity of an individual relative to the expected heterozygosity of individuals in the population it belongs to

F_{ST} -component of Wright's (1921) fixation index, used to define between population structure by comparing the expected heterozygosity of individuals within a subpopulation to the total expected heterozygosity of individuals across all populations

H_E -expected heterozygosity

H_O -observed heterozygosity

IBD -Isolation by Distance

ITS -internal transcribed spacer

LnP(D) -mean posterior probability

MA	-Million years
MPN	-Dame Ella Campbell Herbarium
mtDNA	-mitochondrial DNA
N	-number of individuals
N_A	-number of alleles
N_E	-effective number of alleles
PCR	-Polymerase Chain Reaction
RAPD	-Randomly Amplified Polymorphic DNA
SNP	-Single Nucleotide Polymorphism
Tm	-melting temperature
VNTR	-Variable Number of Tandem Repeat loci

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Chapter 1

Introduction

1.1 Parasitic Flowering Plants

Parasitic plants comprise about one per cent of the flowering plants (angiosperms) (Heide-Jorgensen, 2008). The parasitic habit has independently evolved across twelve distinct angiosperm lineages belonging to 276 genera in 27 families (Nickrent, 2015). Parasitic plants have haustoria (modified roots) that invade either the host plant's roots or stems and connect to the xylem and/or phloem (vascular tissues) to extract nutrients and water from the host. Plants of a parasitic nature can be classified as either holoparasites or hemiparasites depending on their ability to photosynthesize or not. Holoparasites lack chlorophyll, and are therefore non-photosynthetic. They rely entirely on their host plant for carbon, water and other essential nutrients and thus function as heterotrophs. Hemiparasites however have chlorophyll when mature and photosynthesize, but still draw nutrients and water from the host (Smith & Smith, 2011). Stem parasites occur in several families (Table 1.1), and are mostly hemiparasites, while root parasites are more common, found in a number of families and are either holoparasites or hemiparasites.

Members of the Orobanchaceae (Broomrapes) have non-photosynthetic fleshy stems, e.g., the genera *Conopholis, Boschniakia*, and *Orobanche*. Other non-photosynthetic root parasites are found in the families *Lennoaceae*, *Hydnoraceae*, *Rafflesiaceae* (e.g., *Bdallophyton* and *Mitrostemon*), and *Balanophoraceae*. Parasitic species in Krameriaceae (the ratony family) and Santalaceae (the sandalwood family) have photosynthetic leaves (Heide-Jorgensen, 2008). The majority of the Santalaceae are root parasites but stem parasites are present as well. Stem parasites occur in several families,

and pathogenic members include dodder (*Cuscuta* and *Cassytha*) and some mistletoes from the Santalaceae.

Cuscuta (dodder) is a genus of 170-200 species, all of which live as stem holoparasites that parasitize diverse lineages, including green algae, ferns, gymnosperms and angiosperms (Kaiser *et al*., 2015). They occur worldwide and infect a range of agricultural and horticultural species, such as flax, clover, potatoes, ivy and petunias. This parasitic ability suggests that dodders have a range of adaptive mechanisms to attach to their hosts (Vaughn, 2002). Dodder has thin stems but neither leaves nor chlorophyll and produces haustoria that insert themselves into the vascular system of the host to draw nutrients (Lee, 2008).

The broomrape genus (*Orobanche*: Orobanchaceae) are root-holoparasites with species diversity centred in the Mediterranean basin (Barker, Press, Scholes, & Quick, 1996). There are over 1500 species with host-specificity known in some taxa, for example in *Orobanche amethystea* (Heide-Jorgensen, 2008). A number of *Orobanche* species have also shifted from their native hosts to crop species (Román *et al*., 2007), and are now infesting important food crops such as legumes and vegetables, thereby threatening the livelihood of many nations (Kaiser *et al*., 2015). *Orobanche minor* is known to infect hundreds of species ranging from the Poaceae to the Ranunculaceae, but has a clear preference for the Fabaceae and Asteraceae (Rumsey & Jury, 1991). Although *O. minor* parasitizes this taxonomically diverse host range, particular strains of the parasite have been found to infect specific hosts (Musselman & Parker, 1982). The extent of host-race preference of this species has not yet been determined however.

Table 1.1: The 12 orders of parasitic angiosperms with example families and genera. Santalales contains 20 families, seven examples of both root and shoot parasites are shown. Modified from Nickrent (2002b, 2015).

Order/Family	Number genera	Number species	Example genera	Parasitism type
Boraginales				
– Lennoaceae	2	4	*Lennoa, Pholisma*	Root, holo.
Cucurbitales				
– Apodanthaceae	3	23	*Apodanthes, Pilostyles*	Stem, holo.
Ericales				
– Mitrastemonaceae	1	2	*Mitrastema*	Root, holo.
Lamiales				
– Orobanchaceae	95	ca. 1950	*Castilleja, Epifagus, Euphrasia, Pedicularis, Orobanche, Striga*	Root, hemi. & holo
Laurales				
– Lauraceae	1	ca. 20	*Cassytha*	Stem, hemi.
Malpighiales				
– Rafflesiaceae	3	ca. 30	*Rafflesia, Rhizanthes, Sapria*	Stem & root, holo.
Malvales				
– Cytinaceae	2	9-11	*Bdallophyton, Cytinus*	Stem & root, holo.
Piperales				
– Hydnoraceae	2	15-18	*Hydnora, Prosopanche*	Root, holo.
Saxifragales				
– Cynomoriaceae	1	1-2	*Cynomorium*	Root, holo.
Solanales				
– Convolvulaceae	1	ca. 200	*Cuscuta*	Stem, hemi & holo.
Zygophyllales				
– Krameriaceae	1	18-23	*Krameria*	Root, hemi.
Santalales				
– Balanophoraceae	14	ca. 40	*Balanophora, Corynaea, Scybalium, Thonningia*	Root, holo.
– Loranthaceae	74	ca. 900	*Amyema, Peraxilla, Psittacanthus, Tapinanthus*	Stem & root, hemi.
– Misodendraceae	1	8	*Misodendrum*	Stem, hemi.
– Opiliaceae	10	32	*Agonandra, Opilia*	Root, hemi.
– Santalaceae	11	ca. 75	*Comandra, Santalum, Thesium*	Stem & root, hemi.
– Viscaceae	7	ca. 570	*Arceuthobium, Ginalloa, Korthalsella, Viscum*	Stem, hemi.
– Mystropetalaceae	3	4	*Dactylanthus, Hachettea*	Root, holo.
Total for Santalales:	**178**	**ca. 2,412**		
Grand Total:	**285**	**ca. 4,755**		

Root parasites, such as the New Zealand native woodrose (*Dactylanthus taylorii*), are generally capable of parasitizing a wide range of unrelated hosts. Woodrose is suspected of parasitizing the roots of about 30 species of native hardwood trees and shrubs found in podocarp-hardwood forests (Ecroyd, 1996) and is the only fully parasitic

angiosperm endemic to New Zealand (Holzapfel, 2001). The woodrose is “Nationally Vulnerable” and is ranked by the Department of Conservation as a threatened species of highest conservation priority in NZ. Endangered endemic short-tailed bats (*Mystacina tuberculata*) as well as introduced ship-rats (*Rattus rattus*) pollinate woodrose (Holzapfel, 2001). Habitat destruction, collection of woodrose specimens and browsing of flowers by the introduced brush tail possum (*Trichosurus vulpecula*) have seemingly caused a decline in its natural distribution (McLay, Tate, & Symonds, 2012).

In contrast to the generalist parasitic habit of woodrose, members of the genus *Rafflesia* (Rafflesiaceae) are specialist root parasites. *Rafflesia* contains 17 species, among which are the largest known flowers in the world, reaching up to 1 m in diameter (Nais, 2001). Pollination is mediated by carrion (bluebottle) flies of the genera *Chrysomy* and *Lucilia* but the *Rafflesia* plants are extremely rare because of infrequent pollination as nearby male and female flowers must synchronously bloom (Beaman, Decker, & Beaman, 1988). Each of the *Rafflesia* spp. are restricted to one or two host species of the 95 species from the genus *Tetrastigma* (Vitaceae) found in south-east Asia (Barcelona, Pelser, Cabutaje, & Bartolome, 2008; Nais, 2001). Speciation within *Rafflesia* may have been driven by firstly, isolation of populations on host vines with differing distributions and ecologies, and secondly, genetic divergence. Host-associated habitat isolation has caused insect species divergence as well (Thorogood, Rumsey, & Hiscock, 2009). However similar studies of host-driven speciation in parasitic plants are rare as many parasitic plants have a wide potential range of hosts (Press & Graves, 1995), although host generalists have distinctive patterns of host specificity at a local level.

1.2 Mistletoes

Mistletoes are obligate stem hemi-parasites, found on trees worldwide, belonging to five families and represented by approximately 1,600 species in 88 genera (Sultan, 2014) in the Santalales. Families include the Amphorogynacae, Loranthaceae, Misodendraceae, Santalaceae, and Viscaceae, with the Loranthaceae and Viscaceae with 973 and 573 species, respectively, the most speciose mistletoe families (Nickrent, 2015). There are seven genera within the Viscaceae including: *Arceuthobium, Dendrophthora, Ginalloa*, *Korthalsella, Notothixos, Phoradendron* and *Viscum.* Molecular phylogenetic work by Nickrent (2002a) demonstrated that aerial parasites evolved independently five times in Santalales. The first santalalean lineage to evolve the mistletoe habit was *Misodendrum* (80 million years (Ma)), followed by Viscaceae (72 Ma), eremolepidaceous mistletoes in Santalaceae (53 Ma), the tribe Amphorogyneae in Santalaceae (46 Ma), and lastly Loranthaceae (28 Ma) (Vidal-Russell & Nickrent, 2008).

Mistletoes parasitize a range of different hosts depending on the species. *Nuytsia floribunda* (the Australian Christmas tree) parasitizes grasses and almost any host plant, while *Tristerix aphyllus*, another member of Loranthaceae, is only known to parasitize the two cacti *Echinopsis chilensis* and *Eulychinia acida* (Heide-Jorgensen, 2008). *Viscum album* ssp. *album* has been found to parasitize numerous hosts (Zuber & Widmer, 2000), while *V. minimum* has only been recorded on succulent *Euphorbia* species (Heide-Jorgensen, 2008). The host specificity in these two cases may be due to the co-evolution of mistletoes and their hosts over time. The stability of host availability through time and space had been found to be the key factor in host specific patterns. This was found from Norton and de Lange (1999) who examined the scope of host specificity of New Zealand's mistletoes in the Loranthaceae. They found that host specificity is advantageous in

homogenous communities as the mistletoes are able to utilize the most abundant tree species. However, parasitism of a wide range of hosts has been found in mixed forests with high tree species diversity, while open forests show high host specificity as there is low species diversity (Baas, Kalkman, & Geesink, 1990). Thus, host specificity may be the cause of genetic divergence and therefore an underestimated driver of speciation of parasitic plants (Thorogood *et al*., 2009).

1.3 *Korthalsella* in New Zealand

New Zealand's pygmy mistletoes belong to the *Korthalsella* genus. *Korthalsella* (Viscaceae) has an unconventional, discontinuous primarily Pacific distribution that extends from Malesia to Japan in the north; to Hawaii, the Marquesas and Henderson Islands in the east; Ethiopia and Madagascar in the west; and Australia and New Zealand in the south (Molvray, 1997). The Australian and New Zealand species are specialised local endemics and they are the southernmost representatives of Viscaceae. The family appears to have reached the limits of its distribution in the south and probably originated in Malesia in the north (Calder & Bernhardt, 1983; Molvray, Kores, & Chase, 1999). There are nine mistletoe species in New Zealand including five genera with six species in the Loranthaceae and three species in *Korthalsella* (Viscaceae) (Calder & Bernhardt, 1983).

Korthalsella in New Zealand, also known as Pygmy Mistletoes (Aiken, 1957), is represented by three species: *K. clavata* Cheeseman, *K. lindsayi* Engl., and *K. salicornioides* Tiegh. Pygmy mistletoes are aerial hemi-parasites, scale-leaved, have flattened internodes with flowers borne at the tip of internodes in the axils of rudimentary leaves or on specialised inflorescence branches (Henderson, Sultan, & Robertson, 2010). They are diminutive in size, approximately 5 cm with flowers ranging from 0.4 to 0.7 mm across, hence the name (New Zealand Plant Conservation Network, 2013). *Korthalsella*

salicornioides is the most widespread of the New Zealand *Korthalsella* species, ranging from North Cape to Invercargill and extending to off-shore Islands including Great Barrier, Little Barrier, Mayor, Kapiti, D'Urville, Adele, Codfish, Stewart and Big South Cape Island. All three species are missing from the Raukumara and East Cape Ecological Regions (ER), as well as from Egmont through Taranaki to King Country ERs (Sultan, 2014).

Korthalsella clavata host species include 42 taxa found across 11 families in 13 genera. The primary host genus is *Coprosma* (Rubiaceae), followed by *Aristotelia* (Elaeocarpaceae) and other genera including *Muehlenbeckia* (Polygonaceae), *Melicope* (Rutaceae), and three species from the Myrsinaceae (*Discaria*, *Olearia*, *Myrsine*). Hosts recorded for *K. lindsayi* include 45 taxa found across 15 families in 20 genera. The dominant host genus for *K. lindsayi* is also *Coprosma*, followed by *Melicope*, *Lophomyrtus* (Myrtaceae), *Myrsine*, *Muehlenbeckia* and *Sophora* (Fabaceae). The primary hosts of *K. clavata* and *K. lindsayi* are *Coprosma propinqua* and *Melicope simplex,* respectively. *K. salicornioides* parasitizes 26 taxa from six genera in five families but mainly parasitizes members of the genera *Leptospermum* (Myrtaceae) and *Kunzea* (Myrtaceae) (Figure 1.1). The primary host is *Leptospermum scoparium* J.R.Forst. & G.Forst., the secondary host is *Kunzea robusta* de Lange & Toelken, and the tertiary host is *Kunzea amathicola* de Lange & Toelken. Gymnosperms and monocots are not parasitized by the New Zealand's pygmy mistletoes. *Korthalsella clavata* and *K. lindsayi* are generalist species with Shannon-Wiener index values of 2.95 and 2.83, respectively. *Korthalsella salicornioides* is the most host-specific among the three species as it has a Shannon-Wiener index value of 1.17 (Sultan, 2014). *Korthalsella salicornioides* only occasionally uses the main hosts of the other two species while *K. clavata* and *K. lindsayi* share hosts at the tertiary level only (Figure 1.2) Despite this minor host overlap, the New Zealand *Korthalsella* species

demonstrate taxonomic host partitioning by utilising available flora which essentially eliminates interspecific competition among the three species.

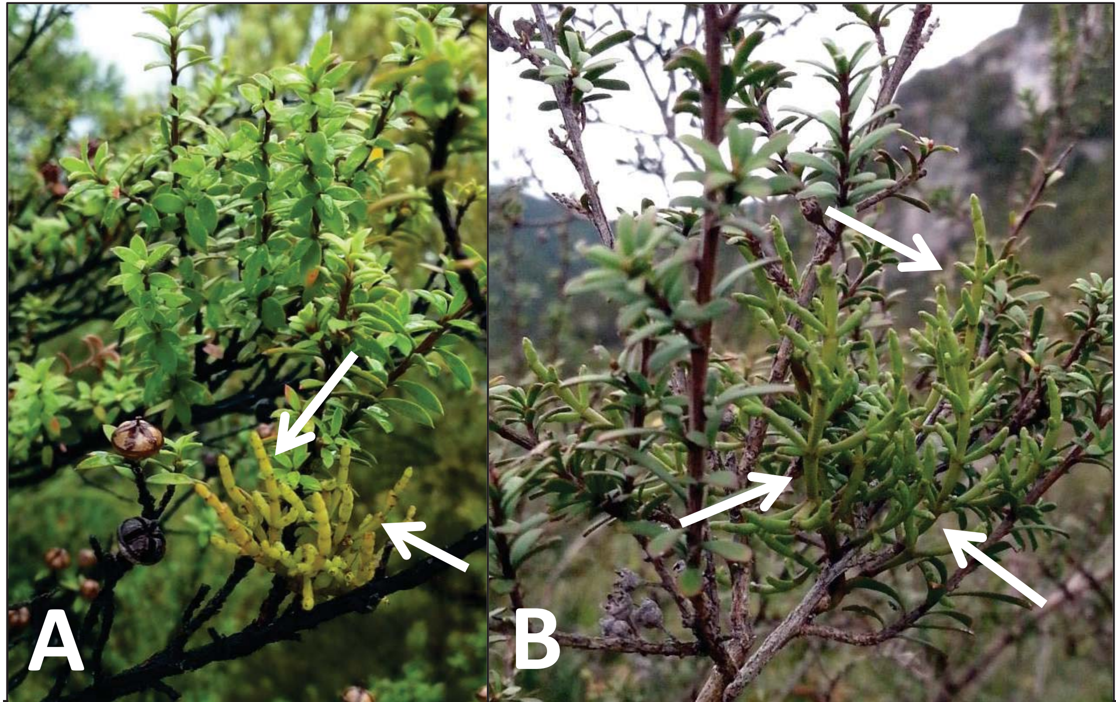

Figure 1.1: *Korthalsella salicornioides* Tiegh. (Viscaceae) parasitic on *Leptospermum scoparium s.l.* J.R.Forst. & G.Forst. (Myrtaceae) (A) and *Kunzea tenuicaulis* de Lange (Myrtaceae) (B). Arrows indicate mistletoe on the respective host.

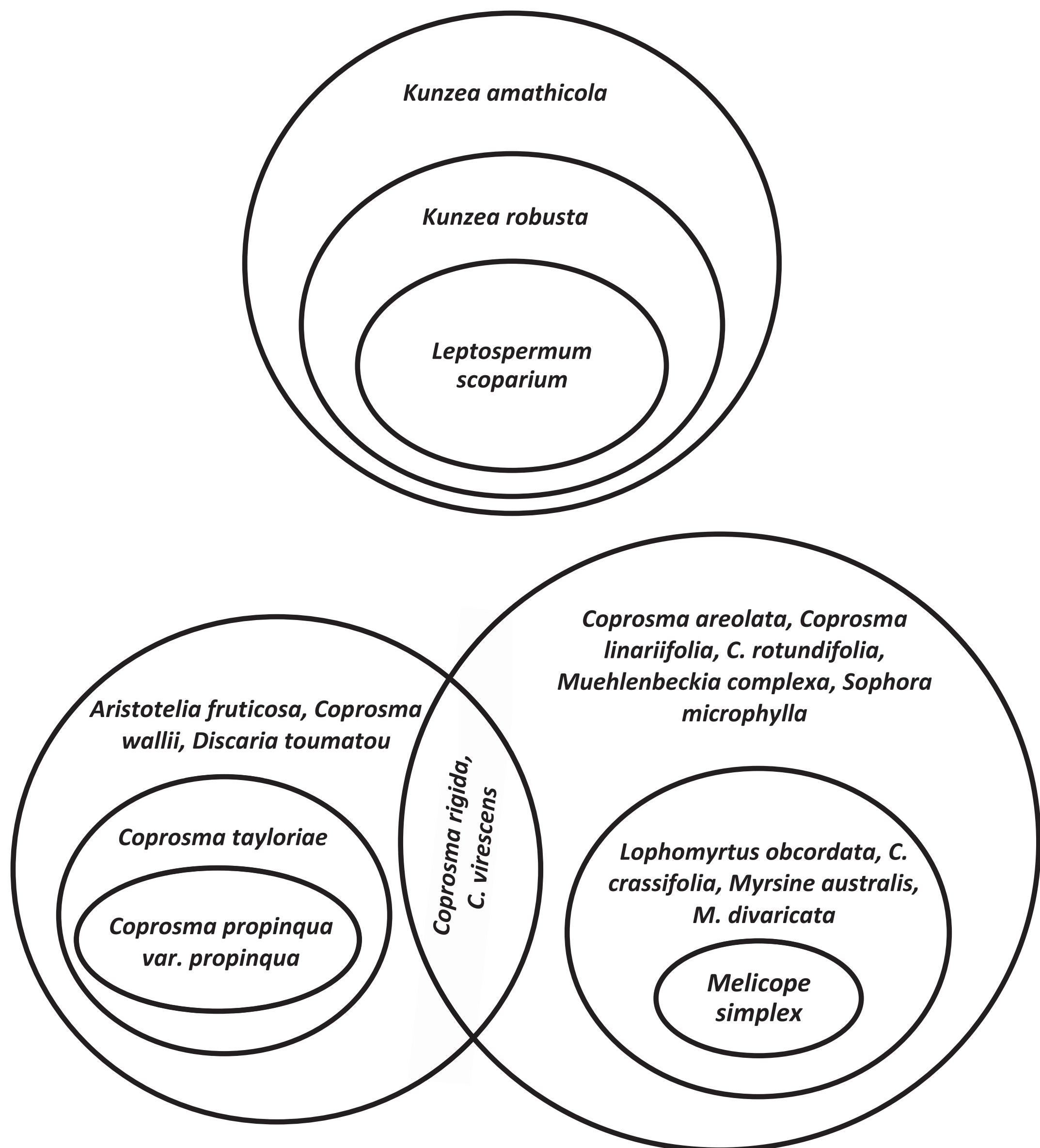

Figure 1.2: Primary (inner most circle), secondary (middle circle) and tertiary host (outer most circle) of *Korthalsella salicornioides* (top), *K. clavata* (left) and *K. lindsayi* (right) showing very little overlap in main hosts-modified from Sultan (2014).

1.3.1 Conservation of *Korthalsella* and its hosts

Forest dominated New Zealand below the alpine treeline 3,000 years ago (McGlone, 1989) but since the arrival of Maori about 1000BP and Europeans in the early 1800s, the forests have undergone widespread destruction (Ewers *et al*., 2006). Approximately three-quarters of indigenous forests were burned, cleared and logged, reducing cover from 82% to 23% of the land surface area. Non-anthropogenic factors such as volcanic activity, climate change and natural fires also are factors driving Holocene vegetation change in New Zealand (Fleet, 1986; McGlone, 1989). Habitat loss has extreme repercussions, such as loss of biodiversity, and can lead to species extinction.

Mānuka (*Leptospermum scoparium* J.R.Forst. & G.Forst. Myrtaceae), commonly known as tea tree, has in the past, been widely regarded as a major native weed in New Zealand (Gardiner, 1953; Sewell, 1949). In recent years, its colonising role as one of New Zealand's pioneer plants in the natural succession from cleared land to climax forest has changed this view. Since European settlement, the number of mānuka and kanuka (*Kunzea* spp. Myrtaceae) plants in the country has dramatically reduced (van Epenhuijsen, 2006). From the 1940s to 1960s a disease, colloquially called 'mānuka blight,' killed large areas of mānuka in New Zealand. Mānuka blight was first noted around 1937 around Canterbury, South Island. The mānuka appeared fire-blackened and dies within several years of the onset of the attack (Hoy, 1961; Miller, 1971). The growth of a sooty mould fungus (*Capnodium walteri* Sacc.) feeding on the honeydew excreted by the felted scale insect (*Eriococcus orariensis* Hoy) was the cause (Hoy, 1954, 1959, 1961; Mulcock, 1954). Mānuka was regarded as a major pasture weed at the time of the mānuka blight discovery because of its prevalence as a result of attempts to reduce the area of land under bracken (Guthrie-Smith, 1953). Farmers across New Zealand bought infected mānuka and as a

result the disease was taken to the North Island where it quickly spread (Sewell, 1953). More recently, studies have shown that sooty mould on ornamental and wild mānuka is mainly caused by *E. leptospermi* rather than *E. orariensis* (van Epenhuijsen, Henderson, Carpenter, & Burge, 2000). Today, some mānuka populations are still infected by the scale insect and sooty mould and result in the decline of individuals.

Kunzea ericoides (A.Rich) Joy Thomps. populations are known to have exceptional morphological and genetic variability (Cheeseman, 1906; de Lange & Murray, 2004; de Lange *et al*., 2005). There have been many attempts over the years to describe the variation found (Kirk, 1889, 1899; Simpson, 1945), but the complex as a whole had not been critically analysed until recently. A combination of hybridisation experiments, morphological, cytological, and DNA sequence data were used to examine the differences within the members of the New Zealand *K. ericoides* complex (de Lange, 2006; de Lange, 2007; de Lange & Murray, 2004; de Lange *et al*., 2005; de Lange *et al*., 2010). These papers concluded that *K. ericoides* was not a single species. The most recent paper by de Lange (2014) describes ten endemic species with seven of these new. The North Island of New Zealand supports four endemic species, *K. linearis, K. salterae*, *K. tenuicaulis*, and *K. toelkenii*, while the South Island has one, *K. ericoides*. Three species extend across the two main Islands including, *K. amathicola*, *K. serotina*, and *K. robusta*. One species, *K. sinclairii*, is endemic to Great Barrier Island (Aotea) and another species, *K. triregensis*, is endemic to the Three Kings Islands.

Mānuka and kanuka plants can act as an important tool for re-vegetating bare and eroded slopes. They create shade and shelter from the wind and provide an excellent nursery for other slower growing native seedlings. Once these plants grow taller and overtop them, the mānuka and kanuka become overshaded and die away. They are not

typically eaten by browsing animals present in New Zealand such as sheep, cattle and goats; therefore they are favourable for restoration projects. Mānuka flowers are an important source of pollen and nectar for native bees, moths, beetles and geckos (Department of Conservation, 2015). The honey produced from mānuka plants is becoming world-renowned for its high antioxidant and anti-bacterial properties. However, the pygmy mistletoes are more nutritious than their hosts (Bannister, 1989) and are food resources for frugivorous birds and larval forms of insects.

Populations from all three New Zealand species of *Korthalsella* are declining due to habitat transformation caused by fire and vegetation clearance (Sawyer & Rebergen, 2001). *Korthalsella salicornioides* is the most at-risk species of the three and is classified as "uncommon/sparse" under the New Zealand threat classification series for New Zealand's indigenous vascular plants (de Lange *et al*., 2012). The lack of natural regeneration and death of hosts caused by natural senescence is causing a decline in mistletoe populations in the Wairarapa Conservancy. The cutting of *Leptospermum* and *Kunzea* scrub for firewood is a threat to already declining *K. salicornioides* populations. On Kapiti Island, the replacement of *Kunzea* hosts by vegetation succession of broadleaved forests may possibly lead to the local extinction of *K. salicornioides* (Sawyer & Rebergen, 2001). Host range studies will be an important tool for devising conservation management plans.

1.3.2 Dispersal mechanisms and life history traits

Endangered plants typically suffer from the impact of small population size, such as inbreeding, genetic drift and reduced gene flow due to isolation. As a consequence these factors will decrease genetic variation within a population while increasing variation amongst populations, thereby reducing the evolutionary potential of the species or population (Ellstrand & Elam, 1993). *Korthalsella salicornioides* populations are only found

in scattered occurrences from its likely range and while the species is naturally uncommon and sparse, it can be locally abundant on occasion. The distribution of *K. salicornioides* tends to be in dense localised patches which are presumed to help ensure effective pollination as pollinators such as Diptera have a small foraging area (Henderson *et al*., 2010). *Korthalsella salicornioides* is an ambophilous species (relying on both wind and insects for pollination) and is fully self-compatible but shows a low rate of autonomous selfing and is therefore dependent on pollen vectors (Sultan, 2014). Flexible pollination ecology is advantageous when temporal and spatial variations affect the relative abundance of pollinators (Culley, Weller, & Sakai, 2002).

Explosive seed discharge occurs in *K. salicornioides,* with dispersal distances generally 1.3 to 2.3m up to 7m depending on direction and height of host canopy. Seed dispersal is mostly abiotic which allows seed to spread throughout populations randomly, but the small sticky seed possibly allows occasional long-distance dispersal via birds (Sultan, 2014). Clonal growth and vegetative reproduction by proliferation of the mistletoe was also found by Sultan (2014). He found that the number of seedlings/sprouts and juvenile/small mistletoes was substantially higher compared to adult/large mistletoes across different sites. Clonally reproducing plants are found to have more abundant adult populations compared to plants that lack vegetative reproduction which shows more abundant seedling populations (Forbis, 2003). Hence *Korthalsella salicornioides* has a suite of ambophily, self-compatibility and some selfing, and clonal growth and therefore a flexible reproductive biology.

1.3.3 Evidence for host-race specificity

It has been noted that in populations with both *Leptospermum* and *Kunzea* present, *K. salicornioides* will only parasitize one of the hosts throughout that population.

Furthermore, in multiple mixed *Leptospermum* and *Kunzea* populations that are geographically close, *K. salicornioides* parasitizes one host in one population but the other host in the other. Very rarely does the mistletoe parasitize both host genera when they co-occur. The degree of host-parasite specificity was studied in the New Zealand *Korthalsella* species (Viscaceae) by Sultan (2014). Nuclear ITS (internal transcribed spacer) and chloroplast *trnQ-rps16* sequences were used to assess the molecular variability of the three mistletoe species across their geographic range on specific hosts. Chloroplast *trnQ-rps16* results showed a geographically based genetic structure to the haplotypes rather than a host-based parasitism structure. Distinct North and South Island haplotypes were identified in *Korthalsella salicornioides* but distinct haplotypes in Northland, Coromandel and Christchurch populations were also found. ITS sequence variability was concentrated in the North Island, perhaps due to a longer presence in the North Island compared to South Island (Figure 1.3). There was overlap in the distribution of two sequence types in the central North Island which corresponded to a mixed *Kunzea* and *Leptospermum* population. The genetic variation was insufficient to determine the presence of host-specific races, possibly because these DNA marker regions were not rapidly evolving and so the sequence data were not sensitive enough to detect different genotypes.

However, the reciprocal transplant studies conducted by Sultan (2014) support the hypothesis that ecotypes that are adapted to different host types exist within *Korthalsella salicornioides*. He found that by comparing successful establishment of *K. salicornioides* seedlings on *Leptospermum* and *Kunzea* hosts, in reciprocal and in corresponding maternal and seed hosts, that there is potential for host-adapted races in *K. salicornioides*. The seeds had a statistically significantly better success rate of seedling establishment when the maternal and recipient hosts were the same, despite the low percentages of germination. Seeds collected from *Leptospermum* mistletoes and planted onto

Leptospermum hosts (*Leptospermum* × *Leptospermum*) had a success rate of 4.74% compared to a 1% success rate in *Kunzea* × *Leptospermum* seed plantings. Similarly, the *Kunzea* × *Kunzea* seed plantings had a success rate of 8.65% compared to the miniscule success rate in *Leptospermum* × *Kunzea* of 0.58% thus indicating the presence of potential *Leptospermum* and *Kunzea* specific races. The regions used in the molecular study were variable among species but the little within-species diversity showed that they were too conserved to detect population-level differences and host-associated genetic diversity. This result suggested that more rapidly evolving regions with greater genetic variation, such as microsatellite markers, may help resolve the presence of host races in different ecological regions in *K. salicornioides*.

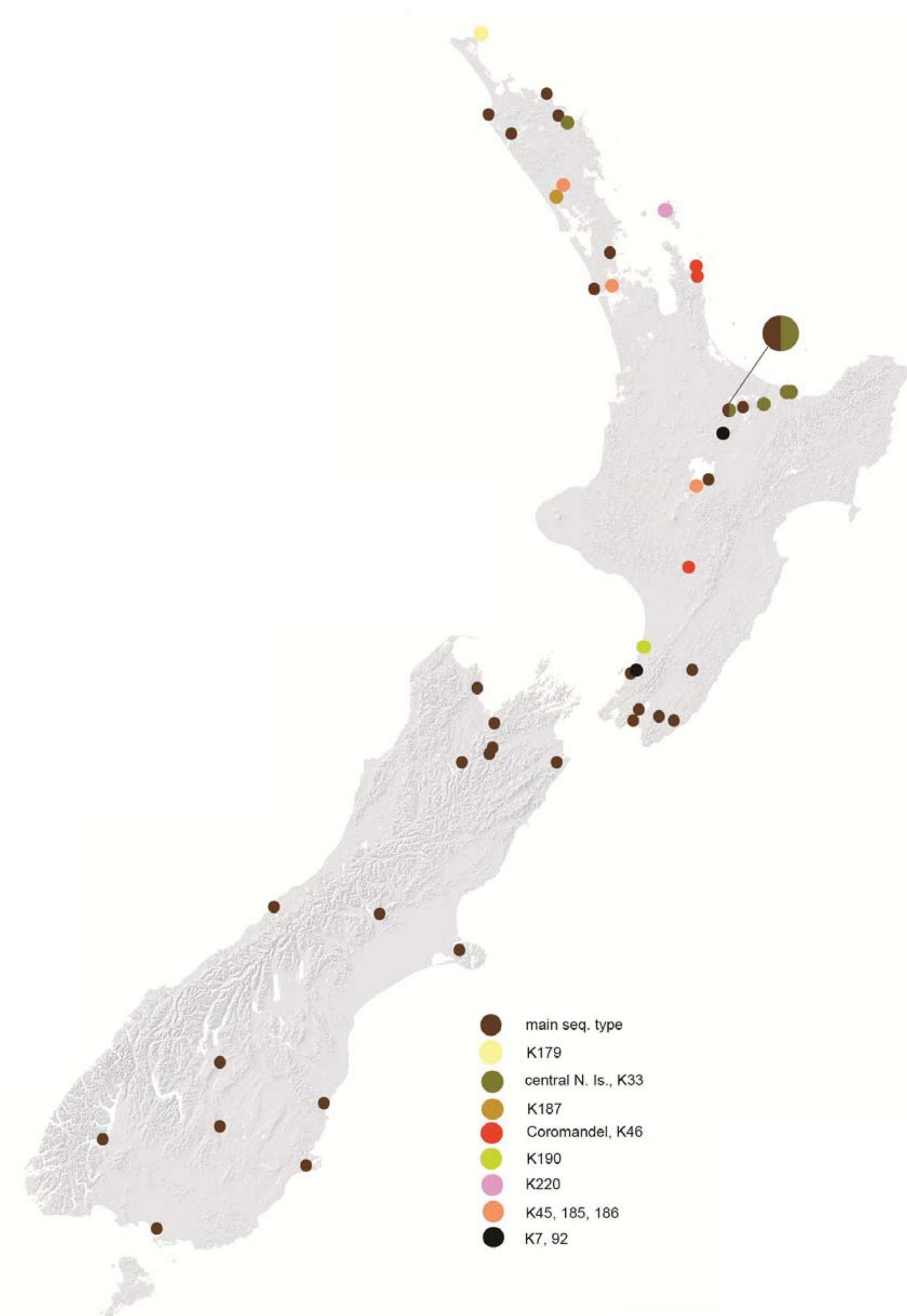

Figure 1.3: ITS sequence type diversity in *Korthalsella salicornioides*. Most of the North and South Island populations had identical ITS sequences (main sequence type). Sequence variation is concentrated in the North Island. Symbol with two colours shows the presence of more than one sequence type at a particular location (Sultan, 2014).

1.4 Markers to Assess Genetic Variation

Genetic markers consist of essentially two types – protein and DNA (molecular). Protein molecular markers (allozymes) were the first molecular markers to evaluate genetic variation in populations (Hubby & Lewontin, 1966). Allozymes are based on enzyme variation in individuals but largely have been replaced by DNA markers. Plant molecular markers can be categorized into three classes: mitochondrial DNA (mtDNA), chloroplast (cpDNA) and nuclear DNA markers (Wan *et al.*, 2004). Mitochondrial markers are inherited maternally, show high rates of mutation, but are non-recombining so they only have one-quarter of the genetic effective population size (Ne) of nuclear markers (Ballard & Whitlock, 2003). Chloroplast DNA has the smallest genome size (120-170kb) compared to mtDNA (200-2500kb) and the nuclear genome (60Mbp-150 000 Mbp). It is useful in resolving phylogenetic relationships at varying taxonomic levels as it is considered to be conserved in its evolution in terms of nucleotide substitution with very few rearrangements (Patwardhan, Ray, & Roy, 2014). Nuclear DNA markers such as amplified fragment length polymorphisms (AFLPs), random amplified polymorphic DNA (RAPDs), single nucleotide polymorphisms (SNPs), and variable number of tandem repeat loci (VNTRs: minisatellites, microsatellites) are biparentally inherited.

Microsatellite markers are commonly found throughout the genome (Selkoe & Toonen 2006).They are being utilized more frequently because they are codominant so estimates of heterozygosity can be made. Microsatellite markers consist of tandemly repeating mono-, di-, tri- and tetra-nucleotide units with repeat sizes of 1-6 bp repeated several times flanked by regions of non-repetitive unique DNA sequences which are distributed throughout the genomes of most eukaryotic species (Tautz, 1989). Microsatellite markers are highly polymorphic, co-dominant, undergo Mendelian

inheritance, easy to genotype, capable of detecting differences among closely related species and typically require a small number of loci, which makes them ideal for use in population genetic, conservation biology, and evolutionary biology studies (Ashley & Dow, 1994; Sunnucks, 2000). Most microsatellite loci are non-coding, thus variation is independent of natural selection. Different alleles at a locus are characterized by the different number of repeat units. They give the same kind of information as allozymes (distinguishable loci with co-dominant alleles), but they are more variable and generally neutral compared to allozymes. Additionally, microsatellite markers allow the use of degraded or minute amounts of DNA (Queller, Strassmann, & Hughes, 1993). The advantage of co-dominant markers is the ability to differentiate between heterozygotes and homozygotes, which is important in population genetic studies. Microsatellite markers are generally more species-specific than other markers and can provide information on the genetic structure of populations and on species delimitation (Duminil & Di Michele, 2009). Therefore they are excellent marker system for use in a population genetics approach to determining if host-race specific populations exist.

Korthalsella salicornioides has certain life history traits including selfing, insect pollination and seed dispersal by gravity or animal attachment that correlate with low heterozygosity in populations (Hamrick, Linhart, & Mitton, 1979; Loveless & Hamrick, 1984). Combining these traits with the distribution pattern of isolated populations, we expect to find low genetic diversity within *K. salicornioides*. According to a survey of genetic variability and life history traits for 110 species of seed plants (angiosperms) by electrophoresis studies (Hamrick *et al*., 1979), the genetic diversity within *Korthalsella* populations is at the lower end of the scale. Based on allozyme data (Molvray, 1990), *Korthalsella* populations outside of New Zealand have an average of 19.2% polymorphic loci with a mean of 1.2 alleles per locus. ITS and chloroplast markers were able to

distinguish North and South Island sequence types as well as clades grouping in the South Island (Sultan, 2014). Therefore, with the sensitivity of microsatellite loci, we expect to find a greater genetic diversity within and among populations which will aid in the determination of host specific races.

1.5 Analysis Methods for Population Structure

Like many rare plant species, *Korthalsella salicornioides* exists in small geographically isolated populations in fragmented habitats (Young, Boyle & Brown, 1996), and is locally abundant on occasion. Species with this distribution pattern often show low levels of genetic diversity which can restrict their ability to respond to evolutionary pressures (McLay *et al*., 2012). Random processes such as unpredictable catastrophic events and demographic, environmental and genetic stochasticity are high risk with small isolated populations (Ouborg, Vergeer & Mix, 2006). Repercussions of small population size include inbreeding, genetic drift and decreased gene flow due to isolation which leads to reduced genetic variation within a population and increased variation between populations (Ellstrand & Elam 1993). Inbreeding increases homozygosity in a population and may lead to a build-up of deleterious alleles (Lopez *et al*., 2009). Alternatively, in locally adapted populations it may be beneficial to inbreed rather than outcross (Hereford, 2010).

F statistics are commonly used to estimate and interpret the genetic structuring of populations (Wright, 1921, 1951). F_{IS} (F) is a “within population” statistic that estimates levels of inbreeding, heterozygote/homozygote excess and gene flow within a population by comparing levels of allele fixation, hence the name “fixation index” (Aegisdottir *et al.,* 2009). Values range from -1 to +1, where a negative value indicates a heterozygote excess while a positive value indicates a homozygote excess (heterozygote deficit). When all

individuals in a population are homozygous for the same allele at a particular locus (i.e., both H_O and H_E are 0), F cannot be calculated as there needs to be variation at the locus (more than one allele). Its calculation is shown in equation 1, where H_E is the expected heterozygosity and H_O is the observed heterozygosity. Allele fixation can be used to indicate the probability or occurrence of inbreeding (Lopez *et al*., 2009).

$$Equation\ 1: \quad (F) = F_{IS} = \frac{H_E - H_O}{H_E}$$

F_{ST} compares the H_E within subpopulations to the H_E among all populations, collectively treated as one population. Values of 0 represent a group of populations with no genetic differentiation (perfectly mixed); while values of 1 indicate high levels of genetic variation and the populations are differentiated. If populations are not differentiated (a value of 0), then there is no genetic divergence and thus there may be gene flow between these populations. It is calculated by equation 2, where H_{ET} is the total expected heterozygosity and H_{ES} is the sub-population expected heterozygosity. Actual values are seldom 0 or 1 and so require interpretation to be biologically comprehensible. From allozymes, Wright (1978) suggests that values from 0-0.05 indicate little variation, 0.05-0.15 moderate variation, 0.15-0.25 great variation, and values above that indicate very great variation.

$$Equation\ 2: \quad F_{ST} = \frac{H_{ET} - H_{ES}}{H_{ET}}$$

STRUCTURE (Pritchard *et al*., 2000) provides another form of analysis to calculate genetic similarity. It does this by calculating the proportion of the genome derived from hypothetical ancestral populations calculated independently of the genome assignment of individuals. The K value determines the number of theoretical ancestral populations which

are compared to determine the optimal K value by calculating the likelihood of a model fitting the data.

1.6 Focus of this Research

High host specificity found in *Korthalsella salicornioides* and ecological transplant data suggests host-specific races. *Korthalsella salicornioides* is currently classified as Naturally Uncommon/Sparse on the New Zealand threatened plant list. Populations are found throughout the North and South Islands, but many are geographically isolated in fragments of mānuka/kanuka scrub. Habitat fragmentation, population size, and avaliability of host plants would have contributed to the vulnerable status as well as affecting the genetic variation of the species. The goal of this study is to use a population genetic approach to assess genetic diversity and structure of populations and to investigate if host-specific races can be distinguished.

Objective 1: Design novel *K. salicornioides* microsatellite markers using genomic sequence.

Objective 2: Utilise the microsatellite markers to assess genetic variation and structure of populations throughout the North Island of New Zealand.

Objective 3: Use the information from the genetic study to determine the presence of host races to aid future management plans for the species.

These aims will be addressed in the following two chapters. Chapter 2 was written following the 'primer note' guidelines for *Applications in Plant Sciences*. Chapter 3 investigates the genetic variation and host-specificity of populations throughout the North Island, and Chapter 4 summarises the results from the previous chapters and and includes a conclusion.

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The following Chapter has been written as a manuscript for publication following the 'primer note' guidelines for *Applications in Plant Sciences*. It is a revised version drafted by Megan van Etten. GenBank accession numbers will be deposited into GenBank prior to publication.

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Chapter 2

Microsatellite Markers for *Korthalsella* (Viscaceae) Species in New Zealand

2.1 Abstract

Premise of the study: Microsatellite markers were developed for New Zealand species of *Korthalsella* (Viscaceae) for population genetic studies.

Methods and Results: From sequencing a total genomic DNA library (using Illumina MiSeq), we identified and developed 16 microsatellite markers for *Korthalsella*. The primer pairs amplified di- tri- and tetra-nucleotide repeats with 1-4 alleles per locus. We tested these markers on four *K. salicornioides* populations and six individuals each of *K. clavata* and *K. lindsayi*. Seven markers were polymorphic among populations of *K. salicornioides;* six markers differed among species; five markers were monomorphic across all three species; and four markers amplified in *K. salicornioides* but not for the other species screened. Average observed heterozygosity was very low, ranging from 0-0.020, however average F_{ST} values show differentiation among populations.

Conclusions: Despite low levels of heterozygosity, the new primers will provide an important resource for population genetic studies in the genus *Korthalsella*.

2.2 Introduction

Korthalsella Tiegh. (Visaceae) is a genus of leafless obligate stem-hemiparasites distributed mainly around the Pacific, but also occurring in Ethiopia and Madagascar (Danser, 1937). In New Zealand, the three endemic mistletoe species (*K. clavata* Cheeseman, *K. lindsayi* Engl., and *K. salicornioides* Tiegh.) show varying degrees of host-specificity (Sultan, 2014). Of these, *K. salicornioides* tends to be the most host-specific, primarily parasitizing *Leptospermum scoparium* J. R. Forst & G. Forst s. l. and *Kunzea* spp. (A. Rich.) Joy Thomps. s. l. (both Myrtaceae) (Sultan, 2014). *K. salicornioides* populations are at serious risk due to the clearance of its host species for firewood and agricultural purposes (Sawyer & Rebergen, 2001). This species is currently classified as "uncommon and sparse" under the New Zealand threat classification system (de Lange *et al*., 2013), so any further loss of habitat could be detrimental to the species. We developed microsatellite loci from *K. salicornioides* for future studies aimed at examining population differentiation. We also tested these markers in the other two endemic *Korthalsella* species in New Zealand.

2.3 Methods and results

One *Korthalsella salicornioides* individual was selected from an *Erica arborea* L. host (Dunedin, New Zealand; Table 2.1) as the source DNA for marker development. Genomic DNA was extracted from fresh leaf/stem material using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). A DNA library was prepared using the Illumina TruSeq Library Preparation Kit (Illumina, San Diego, California) according to the manufacturer's protocol. The indexed library was pooled with three other libraries in equal concentration and sequenced using the paired-end 250 bp chemistry on an Illumina MiSeq (Illumina) by the New Zealand Genome Services (Palmerston North, New Zealand). The resulting 3.6 million

sequences were trimmed of low quality results using a 0.01 quality cutoff in DynamicTrim in SolexaQA (Cox, Peterson & Biggs, 2010). The remaining sequences were assembled using Velvet (Zerbino & Birney, 2008) with *k*-mer lengths between 21 and 81 tested to maximize the N50, which was for a *k*-mer length of 51 bp. From both the assembled and unassembled reads, putative chloroplast and mitochondrial sequences were removed by performing a BLAST search against related chloroplast and mitochondrial sequences obtained from GenBank [chloroplast sequences from *Helianthus annuus* L. (Asteraceae) and *Fagopyrum esculentum* Moench. (Polygonaceae), and mitochondrial sequences from *Beta macrocarpa* Guss. (Amaranthaceae)]. The remaining 3.6 million sequences were analysed for perfect and imperfect microsatellite regions using Phobos (di- to hexanucleotide repeats with a length of ≥6 repeat units; Mayer, 2010), resulting in 140,216 repeat regions. To reduce the number of microsatellite regions from which to design primers, we discarded sequences that did not meet certain criteria thought to affect microsatellite mutation or primer design. We removed sequences if there was more than 1 repeat region within 150 bp, had mononucleotide repeats ≥6 bp within 70 bp of the microsatellite region, or the microsatellite was imperfect (containing mutations including substitution, insertion and deletions), had >11 repeat units or was close (<70 bp) to the beginning or end of the sequence. The remaining 3,985 sequences were imported into Geneious 6.0 (Biomatters, Auckland, New Zealand) to examine the assemblies. Further filtering of the sequences were done based on sequences aligning to >1000 sequences, having low pairwise identity, or overlapping only in the repeat region. The best quality assemblies were selected and primers designed within Geneious using Primer3 (Rozen and Skaletsky, 2000) with default settings used except: product size = 100–300 bp; primer size = 17 (minimum)–19 (optimal)–21 (maximum); melting temperature (Tm) = 52–55–58°C; GC content = 40–50–60%; maximum Tm difference = 5°C; GC clamp = 1;

maximum poly x = 4. An M13 tag (Boutin-Ganache *et al*., 2001) was added to the 5' end of the forward primer (CACGACGTTGTAAAACGAC) and a PIG tail to the 5' end of the reverse primer (GTTTCTT) to promote non-template (A) addition (Brownstein, Carpten & Smith, 1996).

For reasons of practicality, 45 primer pairs were chosen to trial a range of: uninterrupted number of repeats, types of microsatellites (e.g., di-, tri-, tetra-, penta-, and hexa-), and PCR product sizes. These 45 were initially trialled on six populations from across the three species' ranges (Table 2.1): two *K. salicornioides* from Dunedin Cemetery (one from an *Erica arborea* host and one from a *Kunzea sp.* host) and one from Ahipara (*Leptospermum scoparium* host), two *K. lindsayi* from Cole's Bush (*Melicope simplex*) and Peel Forest (*Myrsine australis*), and two *K. clavata* from Oporua (*Coprosma propinqua*) and Dean Burn Forest (*Melicope simplex*). Plant tissue was field-collected and silica gel-dried and extracted using a modified CTAB procedure (Doyle & Doyle, 1987). The 10 µL PCR cocktail contained 1 µL of 1:10 dilution DNA:H_2O (5–50 ng), 0.02 µM forward primer, 0.45 µM reverse primer, 0.45 µM M13 primer (labelled with FAM, NED, or VIC), 1.5 mM MgCl2, 1x buffer BD (Solis BioDyne, Tartu, Estonia), 250 µM of each dNTP, and 0.5 U Firepol Taq polymerase (Solis BioDyne). The PCR cycling program had an initial denaturation of 95°C for 3 minutes; 35 cycles of 95°C for 30 seconds, 53°C for 40 seconds, and 72°C for 1 minute; and a final extension at 72°C for 10 minutes. PCR products (0.14–1.25 µL) of 2–3 loci with differing fluorophores were pooled and added to 9 µL Hi-Di formamide (Applied Biosystems, Carlsbad, California, USA) and 1 µL CASS ladder (Symonds & Lloyd, 2004) for subsequent fragment sizing on an ABI 3730 Genetic Analyzer (Applied Biosystems) by Massey Genome Service at Massey University (Palmerston North, New Zealand). Alleles were visualized and scored using GeneMapper version 3.7 (Applied Biosystems). Of the 45 primer pairs trialled, 16 did not amplify and three were unscorable.

Of the rest, 10 worked in only one species (usually *K. salicornioides*), five were monomophic across all three species, five were polymorphic across species but not within, and six were polymorphic within a species. No heterozygotes were found.

To further test for polymorphism, 16 markers (Table 2.2) were chosen to score on another individual from Dean Burn forest and Cole's Bush, as well as two individuals from two more populations of both *K. clavata* and *K. lindsayi* (Table 2.1). Five loci had weak or inconsistent amplification and were not scored further. The remaining 11 markers were selected for further investigation using four populations of *K. salicornioides* to demonstrate the utility of the markers in a population genetic framework. The four *K. salicornioides* populations were distributed across the North Island of New Zealand and varied in host species (Table 2.3): Kerikeri, Marton and two populations around Lake Wairarapa. One mistletoe plant per host individual was collected and preserved in silica gel. DNA was extracted, PCRs performed and genotypes scored as above. For these four populations, the number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities were determined using GenAlEx (Peakall & Smouse, 2006). Seven loci amplified reliably for all three species, six of which differed amongst them (*K. salicornioides* usually different from *K. clavata* and *K. lindsayi*; Table 2.2). Within *K. salicornioides* populations, seven loci were polymorphic with 1-4 alleles found (Table 2.3). Observed heterozygosity was very low, ranging from 0-0.020. In fact, in one of the populations (Kerikeri) no heterozygotes were found and of the 40 *K. salicornioides* individuals genotyped only 7 were heterozygous at a particular locus. This low heterozygosity correlates with certain life history traits such as selfing, insect pollination and seed dispersal by animal attachment or gravity which has been observed in *Korthalsella* (Loveless & Hamrick, 1984). Despite low genetic diversity within populations, the average F_{ST} of 0.691 shows the markers are

detecting population structure in *K. salicornioides* and this may be related to host-specific genotypes.

2.4 Conclusions

Using whole-genome Illumina sequencing we designed and tested 45 primer pairs to amplify microsatellite loci. Of these, 11 markers amplified consistently and were further tested on *K. salicornioides* individuals from four populations and individuals from three populations each of *K. clavata* and *K. lindsayi*. Very low rates of heterozygosity within populations suggests little outcrossing in *K. salicornioides*. However these loci identify population structure within and among species suggesting they will be useful for population genetic studies.

Table 2.1: Locations and herbarium voucher information for *Korthalsella* populations used in this study.

Species	Population	Host species	Voucher specimen accession no.	Latitude, longitude
K. clavata	Dean Burn Forest	*Melicope simplex*	MPN 47869	-45.919619, 167.633103
	Dean Burn Forest	*Coprosma rotundifolia*	MPN 47870	-45.919619, 167.633103
	Broken River	*Aristotelia fruticosa*	MPN 47871	-43.198517, 171.746039
	Broken River	*Discaria toumatou*	MPN 47872	-43.198517, 171.746039
	Rappahannock River	*Coprosma propinqua*	MPN 47873	-42.163297, 172.277566
	Rappahannock River	*Coprosma tayloriae*	MPN 47874	-42.163297, 172.277566
	Oporua	*Coprosma propinqua*	MPN 47875	-41.271771, 175.272936
K. lindsayi	Cole's Bush	*Melicope simplex*	MPN 47876	-40.288522, 175.467033
	Cole's Bush	*Coprosma rigida*	MPN 47877	-40.288522, 175.467033
	Otanerito Bay	*Coprosma crassifolia*	MPN 47878	-43.841720, 173.055806
	Otanerito Bay	*Melicope simplex*	MPN 47879	-43.841720, 173.055806
	Aramoana	*Coprosma crassifolia*	MPN 47880	-45.776946, 170.697611
	Aramoana	*Myrsine australis*	MPN 47881	-45.776946, 170.697611
	Peel Forest	*Myrsine australis*	MPN 47882	-43.892629, 171.262342
K. salicornioides	Ahipara	*Leptospermum scoparium*	MPN 47887	-35.208878, 173.146003
	Dunedin Cemetery	*Erica arborea*	MPN 47888	-45.861581, 170.525353
	Dunedin Cemetery	*Kunzea sp.*	MPN 49589	-45.861581, 170.525353
	Lake Wairarapa 1	*Leptospermum scoparium*	MPN 47884	-41.28219, 175.15151
	Lake Wairarapa 3	*Kunzea robusta*	MPN 47886	-41.237806, 175.165556
	Kerikeri	*Leptospermum scoparium*	MPN 49555	-35.224768, 174.007003
	Marton	*Kunzea robusta*	MPN 49556	-39.987695, 175.364047

***Note*: MPN = Dame Ella Campbell Herbarium at Massey University, Palmerston North, New Zealand.**

Table 2.2: Primer sequences and characteristics of 16 microsatellite loci developed from *Korthalsella salicornioides*.

Locus	Primer sequences (5’-3’)a	Fluorescent dye (pooling group)	Repeat motif	Size range in *K. salicornioides* (bp)	Size range in *K. lindsayi* (bp)	Size range in *K. clavata* (bp)	Ta (°C)
Kor-1	F: GTCACACAGATATCCCTGG R: ACAGGTTTGTTCCATCCAG	FAM (5)	(AAT)7	171	171	171	53
Kor-2	F: TCACTACTCAACATACCCC R: TTAAGGAGGGTTTGACCAC	FAM (4)	(TA)6	357-359	355	355	53
Kor-4	F: CCACCACTACTCAACACTC R: CTGGTTTCCATTCGTTGTG	VIC (4)	(TA)7	333-335	318	318	53
Kor-7	F: TCAATCCTCAAACATATGGG R: TTGGTGACTTTGTGTAGTC	FAM (5)	(TA)7	339	339	339	53
Kor-12	F: ATGGGGATGAGGTTTTACC R: TGCCACTAGAAATAAAGGAG	VIC (3)	(CTT)6	141	-	-	53
Kor-13	F: AAGTTAGCAGCTTCTCCAC R: CGTATGATGGCTTAGGGTC	FAM (1)	(AATA)7	262-282	270	266	53
Kor-16	F: ATGTACTGGTTGGTCAAGG R: CAGGATCAGAAGCTCACAG	VIC (2)	(TA)7	282	282	282	53
Kor-18	F: GCCCACATAGTGTCCTAAC R: GGCTCTATTCAATTTGCCAC	FAM (4)	(TC)8	188-190	171	171	53
Kor-21	F: AGTTGGGATTTGTCCTTGG R: TATGGGAAGAAACGCTCTG	FAM (3)	(TG)11	179-181	-	-	53

Locus	Primer sequences (5’-3’)a	Fluorescent dye (pooling group)	Repeat motif	Size range in *K. salicornioides* (bp)	Size range in *K. lindsayi* (bp)	Size range in *K. clavata* (bp)	Ta (°C)
Kor-23	F: TAGGGCCTAAAAGACTGGC R: GCATTGTTTCCTGGGTTTC	VIC (5)	(AT)8	235	235	235	53
Kor-26	F: TTCCATGACCCACACATAG R: CCCTTTAAAACCCAACATTC	FAM (6)	(AT)9	239	239	239	53
Kor-28	F: ATGCCACCTAAACCATCTG R: GCTTCACGCTTCATTAGTG	VIC (1)	(ACC)8	246-249	249	240-249	53
Kor-37	F: CCTTGGGTAATAGACTCTCC R: TGATGTGTCATGCTAGACG	VIC (2)	(ACC)7	149	-	-	53
Kor-39	F: CAAAACTTTGGAACCTCTCC R: TGGCTTGATATGAACTTGG	VIC (1)	(AG)6	166-168	164	164	53
Kor-42	F: CATTCAACGCCTACAAACC R: AACCGGCTAGGATCAAATG	VIC (6)	(ATT)6	188	188	188	53
Kor-45	F: ACCAACTAAGTGTCTCCTC R: CGCGAACGATGACATTCTC	VIC (3)	(TA)10	216-222	-	-	53

***Note*: Ta = annealing temperature used in PCR.**
a M13 tail (CACGACGTTGTAAAACGAC) added to the 5’ end of each forward primer and a PIG tail (GTTTCTT) added to the 5’ end of each reverse primer.

Table 2.3: Genetic properties of the newly developed 11 microsatellite loci across four North Island populations of *Korthalsella salicornioides*.

Host	*Leptospermum scoparium*						*Kunzea robusta*						
	Wairarapa 1 (*n*=10)			Kerikeri (*n*=10)			Wairarapa 3 (*n*=10)			Marton (*n*=10)			Total (*n*=40)
Locus	A_S	H_O	H_E	A_S	H_O	H_E	A_S	H_O	H_E	A_S	H_O	H_E	A_T
Kor-2	359	0.000	0.000	357	0.000	0.000	357, 359	0.000	0.320	357	0.000	0.000	2
Kor-4	335	0.000	0.000	335	0.000	0.000	335	0.000	0.000	333, 335	0.100	0.095	2
Kor-12	141	0.000	0.000	141	0.000	0.000	141	0.000	0.000	141	0.000	0.000	1
Kor-13	274,282	0.100	0.375	262	0.000	0.000	274, 282	0.000	0.500	274, 282	0.100	0.095	3
Kor-16	282	0.000	0.000	282	0.000	0.000	282	0.000	0.000	282	0.000	0.000	1
Kor-18	188, 190	0.000	0.420	190	0.000	0.000	188, 190	0.100	0.255	190	0.000	0.000	2
Kor-21	179	0.000	0.000	179	0.000	0.000	179, 181	0.000	0.180	179, 181	0.100	0.255	2
Kor-28	249	0.000	0.000	249	0.000	0.000	249	0.000	0.000	249	0.000	0.000	1
Kor-37	149	0.000	0.000	149	0.000	0.000	149	0.000	0.000	149	0.000	0.000	1
Kor-39	166, 168	0.100	0.455	166	0.000	0.000	166, 168	0.200	0.320	166, 168	0.000	0.480	2
Kor-45	216, 218, 220	0.100	0.405	216	0.000	0.000	216, 218, 220	0.200	0.515	216, 218, 222	0.000	0.460	4
Average		0.027	0.150		0.000	0.000		0.045	0.190		0.027	0.126	

***Note*: n = number of sampled individuals; A_S = allele sizes; A_T = total number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity.**

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Chapter 3

Population genetics and host-race specificity in *Korthalsella salicornioides* (Viscaceae)

3.1 Abstract

The parasitic habit has independently evolved across twelve distinct angiosperm lineages belonging to 276 genera in 27 families. Co-evolution of parasite and host can lead to specialised relationships between species. Host-specificity is important in biology to provide insight into evolution and the speciation process. *Korthalsella salicornioides* (Viscaceae) is a stem hemiparasite endemic to New Zealand which is threatened due to habitat destruction. Eleven polymorphic microsatellite loci were used to analyse populations of *K. salicornioides* in the North Island of New Zealand to assess the genetic diversity within the species, determine how this variation is distributed to evaluate the genetic structure of populations, and determine if host-races of the parasite exist between the two main host genera. Across all populations a low number of alleles were detected (1.14). On average each population had a few alleles per locus (2.1), low expected heterozygosity (H_E=0.04) and a high F_{IS} (0.84). A high degree of genetic differentiation between populations was found (F_{ST}=0.640) and differentiation correlated with host genera and not geographical distance. Populations were found to fall into two, three or four genetic clusters which largely reflected host-specificity. These results suggest that populations have low genetic variability within populations but due to *K. salicornioides* selfing habit, promoting outcrossing may hinder local adaptation and the formation of host-races that is occurring. Instead, increasing the mistletoes habitat and dispersing seed within populations onto potential host trees will promote recruitment and increase the chances of continued survival. This study provides important insight into

host-specific races found within *Korthalsella salicornioides* and has revealed interesting possibilities for future study.

3.2 Introduction

Host-parasite co-speciation is an intricate interaction in which temporal association, ecological factors and phylogenetic history may be involved (Hoberg, 1997). Host-parasite relationships are often stable, non-random associations that demonstrate a lengthy evolutionary history (Hoberg, 1997; Olivier *et al*., 1998). Studies on parasite-host interactions, biogeography, and co-speciation indicate that, in cases of highly developed specialisation, the phylogeny of the parasites mirrors that of their hosts (Johnson *et al.*, 2003; Page, 2003). The distribution of such specialised parasites is dependent on host availability (Sultan, 2014), thus these species can be quite range restricted (Garcia-Franco & Rico-Gray, 1996). Although generalist parasitic plants can potentially parasitize a diverse range of hosts (Kelly, Venable & Zimmerer, 1998; Press & Graves, 1995), many parasites vary in their host specificity (Norton & Carpenter, 1998). When a single species of plant utilizes two or more host species over an extensive period of time, genetic changes that favour its development on one particular host species (Norton & Carpenter, 1998) may result in high genetic similarity (assuming gene flow) between populations of the parasite on the same host (Linhart *et al*., 2003). Consequently, parasites may evolve different races on different host species that could eventually diverge into new species (Glazner, Devlin & Ellstrand, 1988; Linhart *et al.*, 2003). Speciation via adaptation to different hosts is therefore an important process in parasite evolution (e.g., Olivier *et al*., 1998; Jerome & Ford, 2002; Diegisser, Seitz & Johannesen, 2006).

Most of the literature on processes of parasite speciation and the formation of genetic races primarily have been demonstrated with parasitic animals on plants, for

example phytophagous insects (Diegisser *et al*., 2006; Feder, Chilcote & Bush, 1988; Feder *et al*., 2003; Syed, Guerin & Baltensweiler, 2003), lice (Barker & Close, 1990; Hafner & Page, 1995) and ticks (McCoy, Boulinier & Tirard, 2005; McCoy *et al*., 2001). Parasitic plants share many characteristics with parasitic animals, and although they are ecologically and economically important (Press & Phoenix, 2005), the evolutionary processes in parasitic angiosperms have still been inadequately explained, with only a few genera, such as *Arceuthobium* (Nickrent & Stell, 1990; Jerome & Ford, 2002; Linhart *et al*., 2003) and *Viscum* (Zuber & Widmer, 2000), that have been examined in detail. In many of these studies (excluding Nickrent & Stell, 1990), distinct host races were geographically separated. Therefore, an interesting issue arises of whether differentiation occurs, or can be maintained, in populations where two or more host species grow adjacent to one another (sympatric) in mixed stands.

Mistletoes of the families Loranthaceae and Viscaceae (order Santalales) are the most frequent groups of stem parasites on angiosperms and present the opportunity to study host specialisation (Norton & Carpenter, 1998). Pygmy mistletoes of the genus *Korthalsella* Tiegh. (Viscaceae) often use more than one host species. The entire life cycle is completed on their angiosperm host trees. They are chlorophyllous and photosynthetic, yet obtain all of their water and nutrients from their host. For that reason, they are classified as obligate stem-hemiparasites (Nickrent, 2011). Three species of *Korthalsella* are found in New Zealand and show varying levels of host specificity (Sultan, 2014). *K. salicornioides* is the most host-specific out of the three and mainly parasitizes Myrtaceous species but is known also to parasitize some *Coprosma* (Rubiaceae), *Erica* (Ericaceae), *Melicope* (Rutaceae) and *Sophora* (Fabaceae) species. *Leptospermum scoparium* (mānuka) J. R. Forst & G. Forst (Myrtaceae) is the primary host, *Kunzea robusta* de Lange & Toelken is the secondary host and *Kunzea amathicola* (all *Kunzea* spp. referred to as kanuka) de

Lange & Toelken is the tertiary host for *K. salicornioides* (Sultan, 2014). Mānuka and kanuka (tea trees) are natural, 'seral' (mid-stage) successional species in New Zealand and provide an environment for native seedlings to establish. Mānuka and kanuka can be found growing in mixed stands throughout the country (Department of Conservation, 2015). *K. salicornioides* has rarely been found to parasitize both host genera when they are sympatric. In Sultan's (2014) study, seven out of eight North Island populations had both *Leptospermum* and *Kunzea* present, but *K. salicornioides* was parasitic on one of the hosts throughout that population. In only one case, *K. salicornioides* parasitized both hosts in one population. This observed parasitic preference raises the issue of whether host preferences are occurring in *K. salicornioides*.

Reciprocal transplant studies conducted by Sultan (2014) suggest that there is potential for host-adapted races in *K. salicornioides*. He compared successful establishment of *K. salicornioides* seedlings on *Leptospermum* and *Kunzea* hosts, in reciprocal experiments; i.e., *K. salicornioides* seeds collected from *Kunzea* were placed on both *Kunzea* and *Leptospermum*, and *K. salicornioides* seeds from *Leptospermum* were placed on both *Kunzea* and *Leptospermum*. The seeds had a significantly better success rate of seedling establishment when the maternal and recipient hosts were the same, despite the overall low percentages of germination. Thus the hypothesis that ecotypes that are adapted to different host types exist within *Korthalsella salicornioides* was supported.

To further test for host specificity, Sultan (2014) used nuclear ITS (internal transcribed spacer) and chloroplast *trnQ-rps16* sequences to assess the molecular variability of *K. salicornioides* across the geographic range and on specific hosts. Chloroplast *trnQ-rps16* results showed a geographically based genetic structure to the

haplotypes rather than a host-based parasitism structure. Distinct North and South Island haplotypes were identified in *Korthalsella salicornioides* but distinct haplotypes in Northland, Coromandel and Christchurch populations were also found. ITS sequence variability was concentrated in the North Island, perhaps due to a longer presence in the North Island compared to South Island. Interestingly two ITS sequence types were discovered in the same mixed population of *Kunzea* and *Leptospermum* in the central North Island. Only one sample from each host was collected and sequenced, therefore more samples would be required to further investigate genetic differences at this population. The genetic variation in the markers sequenced was insufficient to determine the presence of host-specific races throughout New Zealand, as most of the collections from different hosts had identical ITS sequences, and the chloroplast *trnQ-rps16* region mainly showed different North and South Island haplotypes. This is possibly because these DNA marker regions did not evolve rapidly enough to detect host-specific differences. Although the markers were sensitive enough to detect a difference in the one sympatric population, more samples of both mānuka-host mistletoes and kanuka-host mistletoes in this sympatric population and across the geographic range of the species are required to examine the extent of host-specificity.

The regions used by Sultan (2014) in the molecular study were variable among *Korthalsella* species, but the little within-species diversity showed that they were too conserved to investigate population-level differences and host-associated genetic diversity. This result suggested that more rapidly evolving regions with greater genetic variation, such as microsatellite loci, may help resolve the presence of host races in different ecological regions in *K. salicornioides*. Microsatellite loci are used more frequently in population genetic studies as they have a far larger number of alleles than allozymes and a higher mutation rate than ITS and chloroplast sequences (Gao *et al*.,

2002; Takahashi, Takahashi & Maki, 2011). Microsatellite loci are co-dominant molecular markers that allow for calculations of population genetic parameters, such as F_{IS} and F_{ST}, which are useful for characterising and comparing populations. The high mutation rate and typically selective neutrality of microsatellite markers also allows interpretation of gene flow and population structure (Selkoe & Toonen 2006; Semagn, Bjornstad & Ndjiondjop, 2006).

In this study, eleven polymorphic microsatellite loci were used to analyse populations of *Korthalsella salicornioides* in the North Island of New Zealand to: (1) assess genetic diversity within the species, (2) determine how this variation is distributed to evaluate the genetic structure of populations, and (3) determine if host-races exist between the two main host genera. Results indicate the presence of a low level of genetic variation within populations, but the genetic structure between populations revealed specific genotypes relating to host species. Host-specific trends are discussed, as well as how the results of this study could be used to guide future conservation management of the species.

3.3 Material and Methods

3.3.1 Sample collection

A total of 318 individuals were sampled from 16 populations of *Korthalsella salicornioides* throughout the North Island of New Zealand (Figure 3.1, Table 3.1 and Table 3.2). Populations were selected based on geographical and host-species composition from the regional host patterns reported in Sultan (2014). Samples were collected between 2014 and 2015 (Table 3.2) under the Department of Conservation (DOC) permit 43101-FLO, Auckland Council Research Permit, and permission from the Whakatane District Council and Kawerau District Council. Land area covered by host was used to estimate relative

host population size. The exact number of infected trees was difficult to determine in some populations due to the size of the trees and the cryptic nature of the mistletoe but was estimated (Table 3.1) based on how many trees were collected from. The number of mistletoe plants in each population was not estimated due to the clonal nature of *Korthalsella salicornioides* and although binoculars were used, it is not possible to determine the exact number of mistletoes in every tree.

Table 3.1: Relative *Korthalsella salicornioides* population size information based on the estimated number of host trees parasitized for locations used in this study.

Population	Location	Relative mistletoe population size
1	Coromandel	Large 50+
2	Lake Wairarapa 1	Large 50+
3	Lake Wairarapa 2	Large 50+
4	Lake Wairarapa 3	Large 50+
5	Waikanae	Small <20
6	Hokio Beach	Very small <10
7	Manukau Domain	Small <20
8	Paihia	Small <20
9	Kerikeri	Large 50+
10	Motuoapa	Large 50+
11	Monika Landham	Medium 20-50
12	Te Kopia	Large 50+
13	Kohi Point	Medium 20-50
14	Waitakere Ranges	Small <20
15	Marton	Large 50+
16	Te Puia	Large 50+

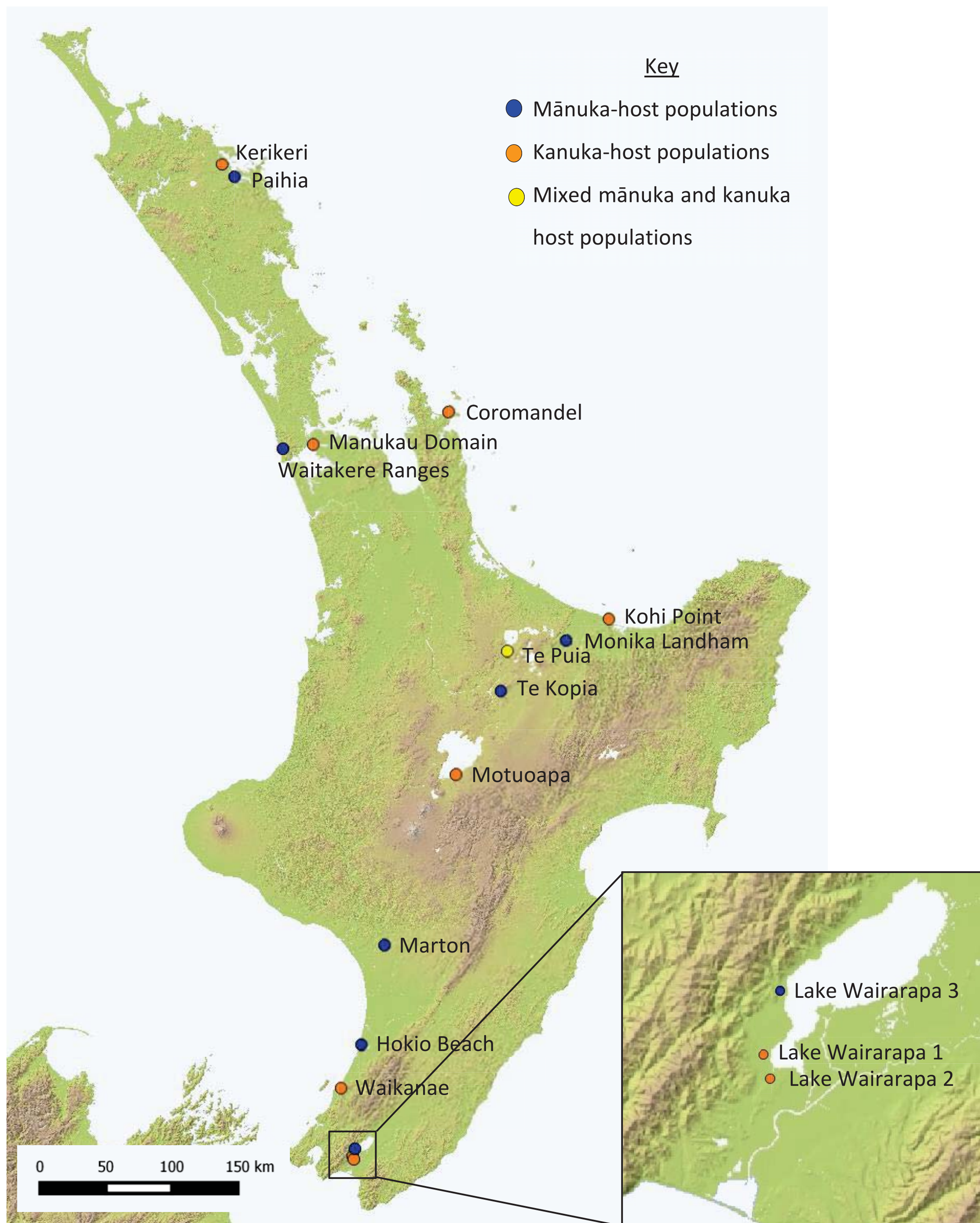

Figure 3.1: Distribution map of the populations of *Korthalsella salicornioides* sampled for this study. Maps were created by QGIS v2.12.3-Lyon (2015). Dots represent populations as indicated in Table 3.2. Mānuka host populations in orange, kanuka host populations in blue and mixed mānuka and kanuka host population in yellow (Key). Bottom inset shows distribution of populations in closer detail for Wairarapa area.

Table 3.2: Locations, host tree and herbarium voucher information for *Korthalsella salicornioides* populations used in this study.

Population (Location)	Geographical Co-ordinates	Host Tree Parasitized	MPN Accession #	# samples Collected	Collector	Date Collected
1 (Coromandel)	36°42'39.2"S, 175°44'36.3"E	*Leptospermum scoparium* s.l.	MPN 47883	23	A.W. Robertson (AWR)	1 Jan 2014
2 (Lake Wairarapa 1)	41°16'55.9"S, 175°09'05.4"E	*Leptospermum scoparium* s.l.	MPN 47884	25	J.A. Tate (JAT) & A.W.R	1 Feb 2014
3 (Lake Wairarapa 2)	41°17'57.1"S, 175°09'28.9"E	*Leptospermum scoparium* s.l.*	MPN 47885	24	J.A.T, A.W.R & S.M. Pearson	14 Apr 2014
4 (Lake Wairarapa 3)	41°14'16.1"S, 175°09'56.0"E	*Kunzea robusta*	MPN 47886	23	Tate *et al.*	14 Apr 2014
5 (Waikanae)	40°52'14.1"S, 175°02'48.5"E	*Leptospermum scoparium* s.l.*	MPN 49870	14	J.A.T & S.M. Pearson (S.M.P)	25 Aug 2014
6 (Hokio Beach)	40°35'54.6"S, 175°11'57.2"E	*Kunzea amathicola*	MPN 49562	7	S.M.P & L.M. Sivyer	8 Mar 2015
7 (Manukau Domain)	36°56'00.1"S, 174°43'12.7"E	*Leptospermum scoparium* s.l.	MPN 49563	13	S.M.P	13 Mar 2015
8 (Paihia)	35°17'55.6"S, 174°06'05.0"E	*Kunzea linearis**	MPN 49564	13	S.M.P & A.G.B. Reed	15 Mar 2015
9 (Kerikeri)	35°13'29.1"S, 174°00'25.2"E	*Leptospermum aff. scoparium* (a)	MPN 49555	20	S.M.P & A.G.B. Reed	20 Nov 2015
10 (Motuoapa)	38°55'46.3"S, 175°52'53.2"E	*Leptospermum scoparium* s.l.	MPN 49571	22	S.M.P	17 Nov 2015
11 (Monika Landham)	38°05'22.2"S, 176°41'57.4"E	*Kunzea tenuicaulis*	MPN 49565	21	C. Flanigan & S.M.P	7 Apr 2015
12 (Te Kopia)	38°24'36.3"S, 176°12'33.5"E	*Kunzea tenuicaulis*	MPN 49566	22	P.B. Cashmore & S.M.P	8 Apr 2015
13 (Kohi Point)	37°56'40.4"S, 177°01'11.7"E	*Leptospermum scoparium* s.l.	MPN 49567	20	S.M.P	9 Apr 2015
14 (Waitakere Ranges)	36°57'31.7"S, 174°29'33.8"E	*Kunzea robusta**	MPN 49568	9	S.M.P	24 Apr 2015
15 (Marton)	39°59'15.8"S, 175°21'51.0"E	*Kunzea robusta*	MPN 49556	15	N.J.D. Singers & S.M.P	8 Nov 2015
16 (Te Puia)	38°09'57.1"S, 176°15'01.9"E	*Kunzea tenuicaulis* *Leptospermum scoparium* s.s.	MPN 49570, MPN 49569	47	P.B. Cashmore & S.M.P	8 Apr 2015

***Note:* * indicates alternative host tree present in population but not parasitized. #, number.**

Stem/leaf material was randomly sampled throughout a population to maximise the representation across the distribution of the population. The New Zealand pygmy mistletoes can clonally reproduce by sprouting and suggests that individuals present on one host can be treated as identical (Sultan, 2014). Therefore only one mistletoe plant was sampled from each individual host tree. Sample sizes ranged from 7 to 47 individuals per population (Table 3.2). The initial aim was to sample a minimum of ten individuals per population, but in some cases this was not possible due to a small population size. One herbarium sample per population was also collected from host trees for identification purposes and to serve as a voucher to be deposited in the Dame Ella Campbell Herbarium at Massey University (MPN). Tissue of *K. salicornioides* was collected fresh and preserved in silica gel.

3.3.2 DNA extraction and genotyping

DNA was extracted from dried tissue, after removing fruits, using a modified CTAB method (Doyle & Doyle, 1987) and then stored at -20°C until needed. A minimum of 10 individuals per population were extracted to screen the genetic diversity in populations except where the population size did not permit. DNA quantity and purity was assessed using gel electrophoresis. Samples that did not produce a single clear high molecular weight band were re-extracted. If the second extraction did not yield good quality DNA, then another individual was chosen for extraction.

A total of 272 individuals were screened at eleven microsatellite loci described previously (Chapter 2). From the original 318 samples, 46 individuals (mainly from populations 9 to 13, inclusive) were not screened due to poor DNA quality. PCR amplification was performed in a volume of 10µL with 1× buffer BD (Solis BioDyne, Tartu, Estonia), 1.5 mM $MgCl_2$, 250 µM of each dNTP, 0.5 U Firepol Taq polymerase (Solis

BioDyne), 0.02 µM forward primer, 0.45 µM reverse primer, 0.45 µM M13 primer (labelled with FAM or VIC), and 1 µL of 1:10 diluted DNA:H_2O (5-50 ng). Amplification by PCR included an initial denaturation at 95°C for 3 minutes; then 35 cycles at 95°C for 30 seconds, 53°C for 40 seconds, and 72°C for 1 minute followed by a final extension at 72°C for 10 minutes.

One of two fluorescent dyes, FAM or VIC (NED was not used as it did not amplify reliably), was incorporated into each amplification and 2-3 loci were pooled for genotyping. PCR products (0.7-1.8µL, depending on strength, which was determined by visualizing on agarose gel) were co-loaded and added to 9µL Hi-Di formamide (Applied Biosystems, Carlsbad, California, USA) and 1µL CASS ladder (Symonds and Lloyd, 2004) for subsequent fragment sizing on an ABI 3730 Genetic Analyzer (Applied Biosystems) by Massey Genome Service at Massey University (Palmerston North, New Zealand). Alleles were visualized and scored manually in GeneMapper version 4.0 (Applied Biosystems).

3.3.3 Assessing genetic variation

Individuals with data missing for four or more microsatellite markers were removed from the data set (7-2, 7-13, 16-2). Data were organised in GenAlEx v6.502 (Peakall & Smouse, 2012) and data were exported in formats appropriate for analyses in STRUCTURE 2.3.4 (Pritchard, Stephens, & Donnelly, 2000) and MSAnalyzer (MSA) 4.05 (Dieringer & Schlotterer, 2003). SPLITSTREE4 (Huson, 1998) was used to create a population NeighborNet from a distance matrix of proportion of shared alleles generated with MSA. GenALEx was used to assess each microsatellite locus for observed and expected heterozygosity (H_O and H_E), and the total number of alleles (A_T). The genetic variation was assessed using GenAlEx which calculated Weir and Cockerham's (1984) F-statistics (F_{ST} and F_{IS} analogues). Genetic diversity for each population was assessed across all loci in

GenALEx using the expected heterozygosity (H_E), observed heterozygosity (H_O), observed number of alleles (N_A), the effective number of alleles (N_E), F_{IS} and the percentage of polymorphic loci (%P). All loci were used for subsequent analyses but genetic diversity (H_E, H_O, N_A, N_E, F_{IS} and %P) was also analysed independently using only the seven polymorphic loci (Appendix 1).

3.3.4 Resolving genetic structure and differentiation

The distribution of genetic variation was assessed in STRUCTURE (Pritchard *et al.*, 2000) which identifies the most likely number of genetic clusters within the *Korthalsella salicornioides* data set. Bayesian analysis, using multi-locus genotype data, constructs “ancestral” populations and partitions and assigns ‘individuals’ genotypes to those populations. No prior information relating to the geographic or host origin of the individual was included in the analyses. Parameters used for STRUCTURE analyses were: 15 replicates run for each K (putative ancestral population) value from 1-10, assumed admixture, infer lambda, and 100,000 iterations of burn-in and 1,000,000 iterations of data collection. The K value with the best fit to the data was determined using the ΔK method following Evanno, Regnaut, & Goudet (2005) and the mean posterior probability (LnP(D)) was plotted.

Isolation by distance (IBD) was assessed using a Mantel test between pairwise F_{ST} values obtained from GenAlEx v6.502 and transformed into $F_{ST}(1-F_{ST})$ and tested against the natural log of the geographic distance between two populations (km) in Isolation By Distance Web Service v3.23 (Jensen, Bohonak, & Kelley, 2005). Mistletoe populations were then separated into mānuka-host and kanuka-host groups and the Mantel test was performed on each of the two host genera separately. AMOVA (analysis of molecular variance) was calculated in GenAlEx v6.502 to determine how genetic variation was

partitioned within and between populations for all 16 populations, and between the host-races identified using the most supported K-value identified by STRUCTURE results (K=4).

3.4 Results

3.4.1 Genetic Variation

Eleven markers were genotyped in 272 *Korthalsella salicornioides* individuals. Success rate of marker amplification ranged from 95.6-100% across individuals (Table 3.3). Across all populations a total of 23 alleles were observed from the eleven loci with a mean of 2.1 alleles per locus. The number of alleles per locus ranged from one to four (Table 3.3). A total of five private alleles were observed, with an average of 0.31 private alleles per population. Marton possessed two of the private alleles, while Manukau Domain, Kerikeri, and Te Puia each possessed one of the private alleles. The mean number of alleles per locus within-populations was 1.14 (range 0.91 to 1.64), with the number of effective alleles per locus averaging at 1.06 (range 0.91 to 1.29). With both N_A and N_E, Lake Wairarapa 3 represented the highest value and Kohi Point represented the lowest (Table 3.4).

Polymorphic loci per population (%P) ranged from 0 to 54.55%, with an average of 12.5%. Populations that showed polymorphic loci were clustered in the lower North Island (excluding Waikanae), but also including the mixed (both *Leptospermum* and *Kunzea* present) population in Te Puia. Observed heterozygosity (H_O) was very low, with an average of 0.004 (range 0 to 0.02). Twelve out of the 16 populations were monomorphic at all loci. The only populations to have any heterozygotes were located in the lower North Island, and included the three Lake Wairarapa populations (0.011, 0.015, and 0.02) and the Marton population (0.019). The range of expected heterozygosity (H_E) was 0.000 in 10 populations and 0.173 at Lake Wairarapa 3, with an average of 0.04 (Table 3.4). In all

cases, H_E was greater than H_O, which is commonly observed in microsatellite data sets (Nybom, 2004). This also correlates with the life history of *K. salicornioides* (Sultan, 2014). F_{IS} values ranged from 0.511 at Marton to 1.000 at Hokio Beach. No populations showed a significant heterozygote excess, as in all cases H_E was greater than H_O. Mean F_{ST} for all populations was 0.640, showing a high degree of differentiation between populations (Table 3.3).

Table 3.3: Characteristics of eleven microsatellite loci for 272 samples of *Korthalsella salicornioides*.

Locus	A	Size Range (bp)	H_O	H_E	F_{IS}	F_{ST}	% amplification
Kor-02	3	357-361	0.000	0.010	1.000	0.970	100
Kor-04	2	333-335	0.004	0.004	-0.037	0.034	95.6
Kor-12	1	141	0.000	0.000	N/A	N/A	100
Kor-13	3	262-282	0.012	0.068	0.822	0.820	99.6
Kor-16	1	282	0.000	0.000	N/A	N/A	100
Kor-18	3	188-192	0.008	0.091	0.913	0.586	100
Kor-21	2	179-181	0.004	0.046	0.903	0.673	100
Kor-28	1	249	0.000	0.000	N/A	N/A	100
Kor-37	1	149	0.000	0.000	N/A	N/A	100
Kor-39	2	166-168	0.008	0.114	0.930	0.772	100
Kor-45	4	216-222	0.008	0.119	0.934	0.640	95.6
Mean	**2.1**		**0.004**	**0.041**	**0.781**	**0.640**	**99.2**

***Note*: A, number of alleles per locus; size range (bp), range of allele sizes at microsatellite loci; H_O, observed heterozygosity; H_E, expected heterozygosity; % amplification, percentage amplification; F_{IS}/F_{ST}, estimates of Wright's fixation index for all microsatellite markers; and N/A, observed and expected heterozygosity are both zero and therefore F_{IS} cannot be calculated.**

Table 3.4: Population information and genetic diversity estimates for 16 populations of *Korthalsella salicornioides*.

Pop	Location	N	P_A	N_A	N_E	%P	H_O	H_E	F_{IS}
Pop 1	Coromandel	23	0	1.000	1.000	0.00%	0.000	0.000	N/A
Pop 2	Wairarapa 1	25	0	1.455	1.229	36.36%	0.011	0.134	0.904
Pop 3	Wairarapa 2	24	0	1.182	1.099	18.18%	0.015	0.064	0.763
Pop 4	Wairarapa 3	23	0	1.636	1.287	54.55%	0.020	0.173	0.874
Pop 5	Waikanae	12	0	1.000	1.000	0.00%	0.000	0.000	NA
Pop 6	Hokio Beach	7	0	1.091	1.087	9.09%	0.000	0.045	1.000
Pop 7	Manukau Domain	11	1	1.000	1.000	0.00%	0.000	0.000	N/A
Pop 8	Paihia	12	0	1.000	1.000	0.00%	0.000	0.000	N/A
Pop 9	Kerikeri	16	1	1.000	1.000	0.00%	0.000	0.000	N/A
Pop 10	Motuoapa	14	0	1.000	1.000	0.00%	0.000	0.000	N/A
Pop 11	Monika Lanham	12	0	1.000	1.000	0.00%	0.000	0.000	N/A
Pop 12	Te Kopia	12	0	1.000	1.000	0.00%	0.000	0.000	N/A
Pop 13	Kohi Point	12	0	0.909	0.909	0.00%	0.000	0.000	N/A
Pop 14	Waitakere Ranges	9	0	1.000	1.000	0.00%	0.000	0.000	N/A
Pop 15	Marton	14	2	1.545	1.193	45.45%	0.019	0.114	0.511
Pop 16	Te Puia	46	1	1.364	1.229	36.36%	0.000	0.128	1.000
Mean		**17**	**0.31**	**1.14**	**1.06**	**12.5%**	**0.004**	**0.04**	**0.84**

***Note*: N, sample size genotyped and used in this study; P_A, number of private alleles; N_A, number of alleles per locus; N_E, number of effective alleles per locus; %P, percentage of polymorphic loci; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, fixation index score; N/A, observed and expected heterozygosity are both zero and therefore F_{IS} cannot be calculated.**

3.4.2 Genetic Structure

The K value with the best fit to the data was determined using the ΔK method (Figure 3.2) following Evanno *et al.* (2005) and the mean posterior probability (LnP(D)) was plotted (Figure 3.3) to further assess host trends. The ΔK method for evaluating the most likely value of K from STRUCTURE likelihood results revealed that K=4 provided the best fit (Figure 3.2). Two clusters in K=4 reveal a host pattern where the majority of mānuka-host populations cluster together and the majority of kanuka-host populations cluster together (Figure 3.4B). Two kanuka-host populations in the lower North Island (Lake Wairarapa 3 and Marton) group together and two mānuka-host populations also in the lower North Island (both in the Wairarapa region) group together. The *K. salicornioides* growing on both mānuka and kanuka in Te Puia are separated into two different clusters (kanuka-host

individuals grouped with the two lower North Island kanuka-host populations, while mānuka-host individuals grouped with the main kanuka cluster).

ΔK also showed high probability scores for K=3 and K=2 (Figure 3.2). Under K=2, the populations are mostly separated based on host without any geographic trends (Figure 3.4A). The only exception to this pattern is found in Kohi Point, which groups with the kanuka cluster instead of the mānuka cluster. Hokio Beach individuals are also divided between the two main host clusters for all three K values (K=2, K=3, and K=4). When K=3 (not shown), the mānuka/kanuka trend continues from K=2 but the two mānuka Lake Wairarapa populations cluster together as shown in Figure 3.4B but the separation of kanuka-host populations in the lower North Island (Lake Wairarapa 3 and Marton) and separation of Te Puia no longer occurs.

To further assess host-trends, the mean posterior probability (LnP(D)) was calculated from 15 iterations per cluster (K) from STRUCTURE analyses and then plotted (Figure 3.3). Although ΔK provides an indication of the strongest pattern of genetic differentiation, higher K values can often resolve further partitioning. Therefore the K=7 graphs were constructed and used to further assess geographic patterns and investigate population structuring of the mistletoe based on which species it is parasitizing within the *Kunzea* genus (Figure 3.4 C).

STRUCTURE results from K=7 show some trends when comparing between the species of *Leptospermum* and *Kunzea* (Table 3.5). Four *Kunzea* species (*K. robusta, K. amathicola, K. linearis,* and *K. tenuicaulis*) and three varieties of *Leptospermum* (*Leptospermum scoparium s.l., Leptosperum aff. scoparium (a),* and *Leptospermum scoparium s.s.*) were hosts in this study (Table 3.2).

There does not appear to be host-specificity in *Korthalsella salicornioides* when comparing between the *Kunzea* spp. based on K=7. *K. robusta* was the host species for three populations in Marton, Lake Wairarapa 3 and Waitakere Ranges. All three of these populations are split up into different clusters with the Lake Wairarapa 3 population consisting of a mixture of almost all clusters. A single *K. linearis* population was sampled from Paihia as well as a single *K. amathicola* population near Levin (Hokio Beach). Both of these clustered in the main "Kunzea" grouping as well as the *K. tenuicaulis* population from Te Kopia and Waitakere Ranges. Two *L. scoparium* populations (Kohi Point and the *L. scoparium* host individuals found in the mixed population in Te Puia) also clustered with this grouping. Three *K. tenuicaulis* populations were sampled from Te Puia, Monika Landham and Te Kopia. All of these populations were found in separate clusters. Interestingly, *K. salicornioides* growing on *K. tenuicaulis* at Te Puia was separated into two clusters and was divided spatially and genetically by the *K. salicornioides* growing on *L. scoparium*.

Leptospermum host populations clustered together based on K=7 despite the slight differences in varieties. There also seemed to be a geographical trend as the two Wairarapa populations (2 and 3) grouped together, while the Northern (Coromandel and Auckland) and central (Motuoapa) populations clustered together. The two other mānuka-host populations (Kerikeri and Waikanae) had genetically identical individuals, except for one locus. They cluster together despite the different variety found in Kerikeri and the geographic distance. Although the mānuka-host Kohi Point and Te Puia individuals grouped with the main kanuka cluster (Table 3.5), there appears to be a Bay of Plenty grouping for mānuka-host populations as well. From the STRUCTURE analyses, host-specificity in *Korthalsella salicornioides* extends to the host genus foremost.

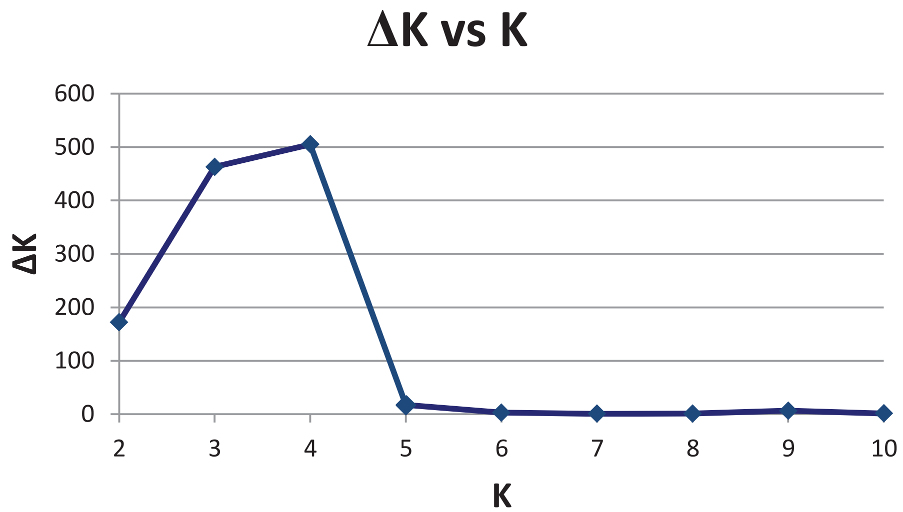

Figure 3.2: Plot of ΔK vs K for STRUCTURE results following Evanno *et al.* (2005) based on 15 replicates for each K value.

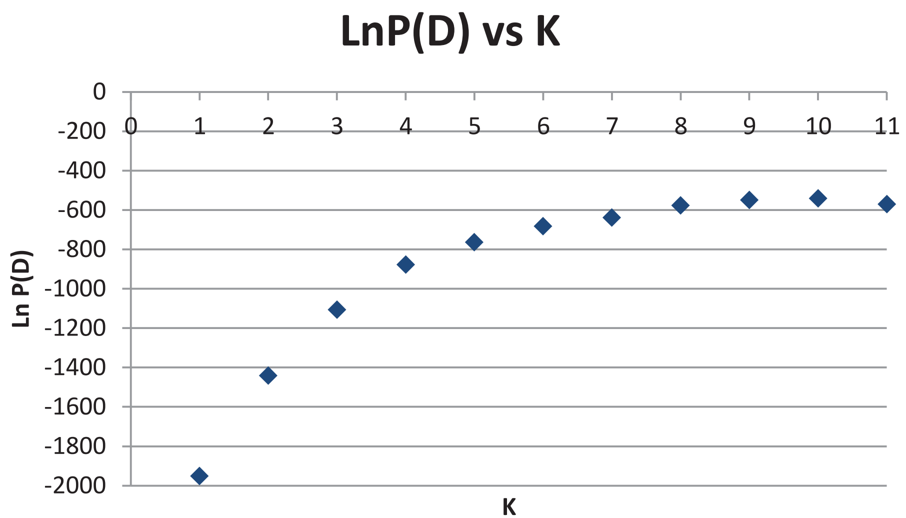

Figure 3.3: Plot of mean posterior probability (LnP(D)) values per cluster (K), based on 15 iterations per K from STRUCTURE analyses (Pritchard *et al.*, 2000).

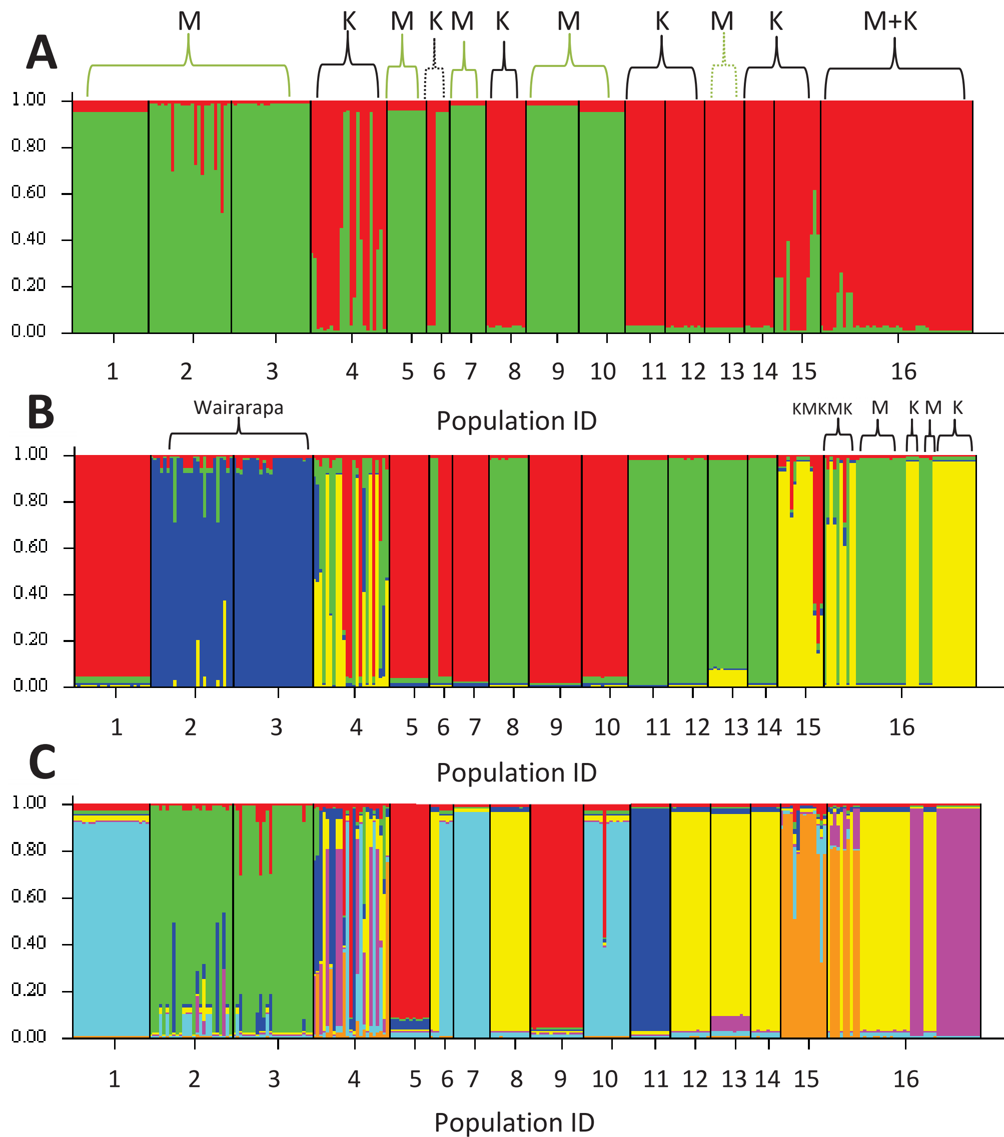

Figure 3.4: STRUCTURE cluster assignment of *Korthalsella salicornioides* individuals. A, K=2 STRUCTURE cluster plot for *K. salicornioides* individuals grouped by host. Two genetic clusters can be observed relating to host. *K. salicornioides* individuals collected on mānuka trees are represented in green, while *K. salicornioides* individuals collected on kanuka trees are represented in red. Two populations do not follow this trend (populations 6 and 13) shown by dashed lines. B, K=4 (best likelihood fit) The two Lake Wairarapa mānuka populations are grouped together (pop 2 and 3) based on geography and host as the Lake Wairarapa kanuka population (pop 4) is grouped with the other kanuka populations. Population 16 (Te Puia) is split into two clusters showing a host specific trend. *K. salicornioides* on kanuka is represented as yellow (clusters with pop 15), while *K. salicornioides* on mānuka is represented as green (clusters with the kanuka host populations). C, K=7. *Note:* colours between graphs do not correspond to the same clustering. Refer to Table 3.5 for corresponding population number and location information.

Table 3.5: Clustering of K=7 STRUCTURE results examining genetic structure within the *Kunzea* and *Leptospermum* genera. Colours correspond to those found in Figure 3.4C.

Cluster	Host	Location	Population number
Mixed	*K. robusta*	Lake Wairarapa 3	4
Orange	*K. robusta*	Marton	15
Orange	*K. tenuicaulis*	Te Puia	16
Dark blue	*K. tenuicaulis*	Monika Lanham	11
Pink	*K. tenuicaulis*	Te Puia	16
Yellow	*K. robusta*	Waitakere Ranges	14
Yellow	*K. linearis*	Paihia	8
Yellow	*K. tenuicaulis*	Te Kopia	12
Yellow	*K. amathicola*	Hokio Beach	6
Yellow	*L. scoparium*	Kohi Point	13
Yellow	*L. scoparium* (s.s)	Te Puia	16
Light blue	*L. scoparium*	Coromandel	1
Light blue	*L. scoparium*	Manukau Domain	7
Light blue	*L. scoparium*	Motuoapa	10
Green	*L. scoparium*	Lake Wairarapa 1	2
Green	*L. scoparium*	Lake Wairarapa 2	3
Red	*L. scoparium*	Waikanae	5
Red	*L. scoparium* (a)	Kerikeri	9

A mantel test showed that genetic differentiation between the populations did not correlate significantly with geographic distances (R^2 = 0.0104, p<0.1080, Figure 3.5). Mantel tests suggest there is no isolation by distance for both host groups (Figure 3.6), as both tests were not significant (p<0.358 and p<0.260). Using AMOVA to examine the among population differentiation for all populations found that the majority of the variation was partitioned among populations (66%), with the rest of the variation partitioned within populations (32%) and within individuals (2%) (Table 3.6). This indicates there is greater variation among populations than between them. Using the ΔK method (Evanno *et al.*, 2005) for evaluating the most likely value of K from STRUCTURE revealed that K=4 provided the best ad hoc fit (Figure 3.2). AMOVA results from comparing variation between the four STRUCTURE-derived K=4 groups identified the among region (4 groups) variation as 43%, among population within region variation as 33%, and the within population variation as 22% (Table 3.6).

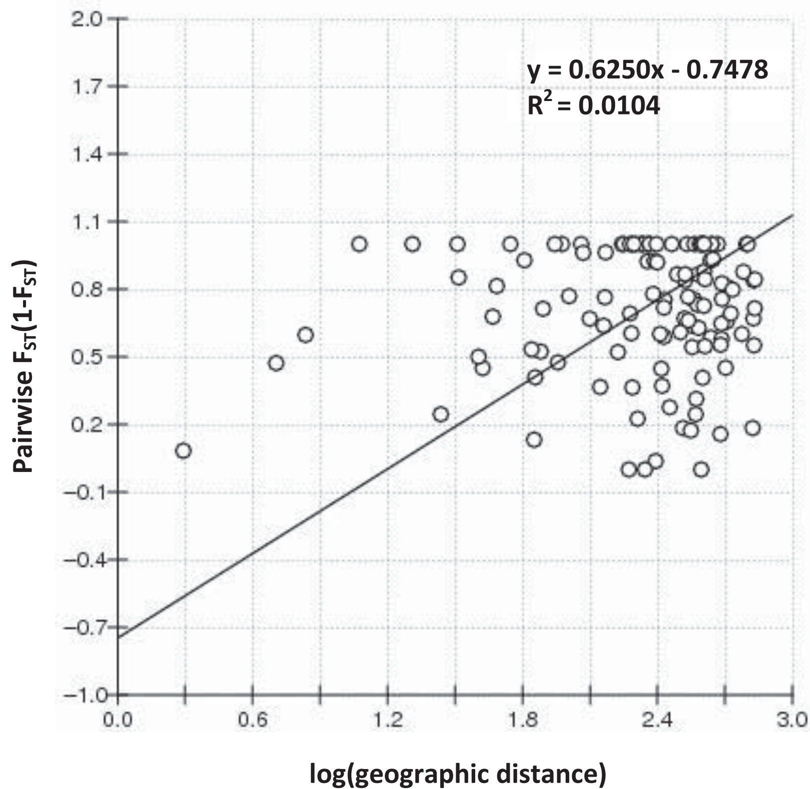

Figure 3.5: Mantel test results displayed in graph of pairwise $F_{ST}(1-F_{ST})$ against the natural log of geographic distance (km) for *Korthalsella salicornioides*. A positive, although not significant (*p* <0.1080), correlation between geographic distance and genetic distance is displayed.

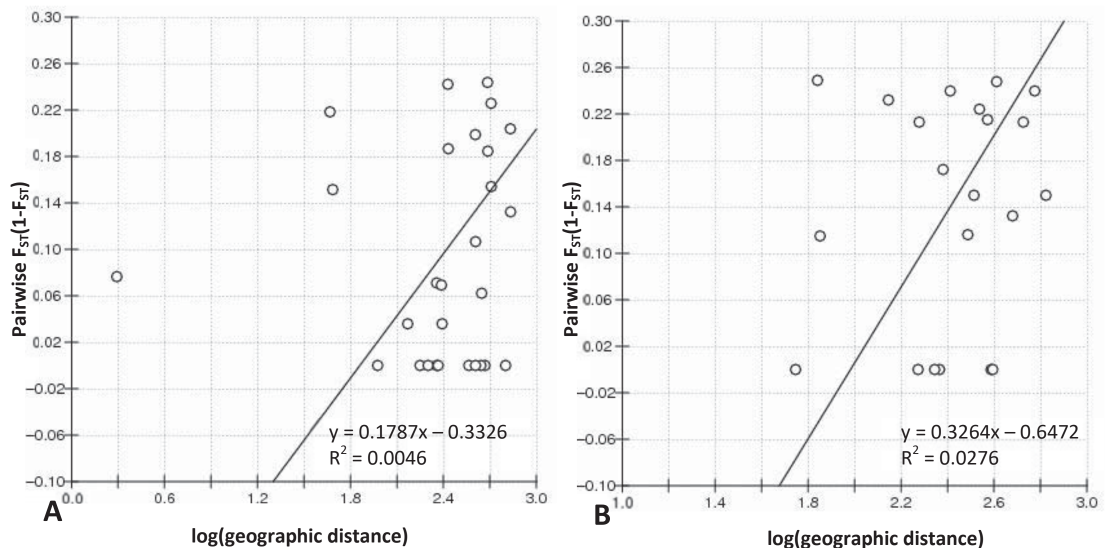

Figure 3.6: Mantel test results displayed in graphs of pairwise $F_{ST}(1-F_{ST})$ against the natural log of geographic distance (km) for *Korthalsella salicornioides* mānuka-host populations (A), and kanuka-host populations (B). Positive, although not significant (*p* <0.358 and *p*<0.260, respectively), correlation between geographic distance and genetic distance is displayed.

Table 3.6: AMOVA results for the partitioning of microsatellite variation in: A) all *Korthalsella salicornioides* populations, B) comparing between four K values (regions) identified in STRUCTURE. The degrees of freedom (d.f.), sum of squares, variance components and percentage variation. *p*<0.001.

	Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation
A)	**Among pop.**	15	397.733	0.773	66%
	Within pop.	256	195.724	0.368	32%
	Within indiv.	272	7.500	0.028	2%
	Total	543	600.958	1.169	100%
B)	**Among regions**	3	271.199	0.549	43%
	Among pop. within regions	13	173.274	0.423	33%
	Within pop.	255	148.985	0.278	22%
	Within indiv.	272	7.500	0.028	2%
	Total	543	600.958	1.278	100%

3.4.3 NeighborNet

Strong similarities were found in the patterns observed in the STRUCTURE analyses and the population-level NeighborNet (Figure 3.7). There is a main split that separates populations by host. *L. scoparium* host populations are positioned near each other with the exception of Kohi Point and the mānuka host mistletoes from Te Puia, which cluster with the *Kunzea* host populations on the other side of the division. The two mānuka host Lake Wairarapa populations (Pop 2 and Pop 3) cluster together and the Waikanae and Kerikeri populations are placed together as well. The Manukau Domain population is most closely related to the Coromandel and Motuoapa population which have the same genetic composition.

In the NeighborNet, the *Kunzea* host populations are positioned near each other as well, but show a few distinct groupings. The first is a cluster with Paihia, Te Kopia and Waitakere Ranges, with the *L. scoparium* host population Kohi Point positioned closely nearby, which is the same pattern observed in STRUCTURE. However, the Hokio Beach population clustered with this grouping in the STRUCTURE analysis but is positioned further away in the NeighborNet. The Monika Landham is more closely positioned to this

main *Kunzea* cluster while the lower North Island *K. robusta* population (Marton) is positioned further away.

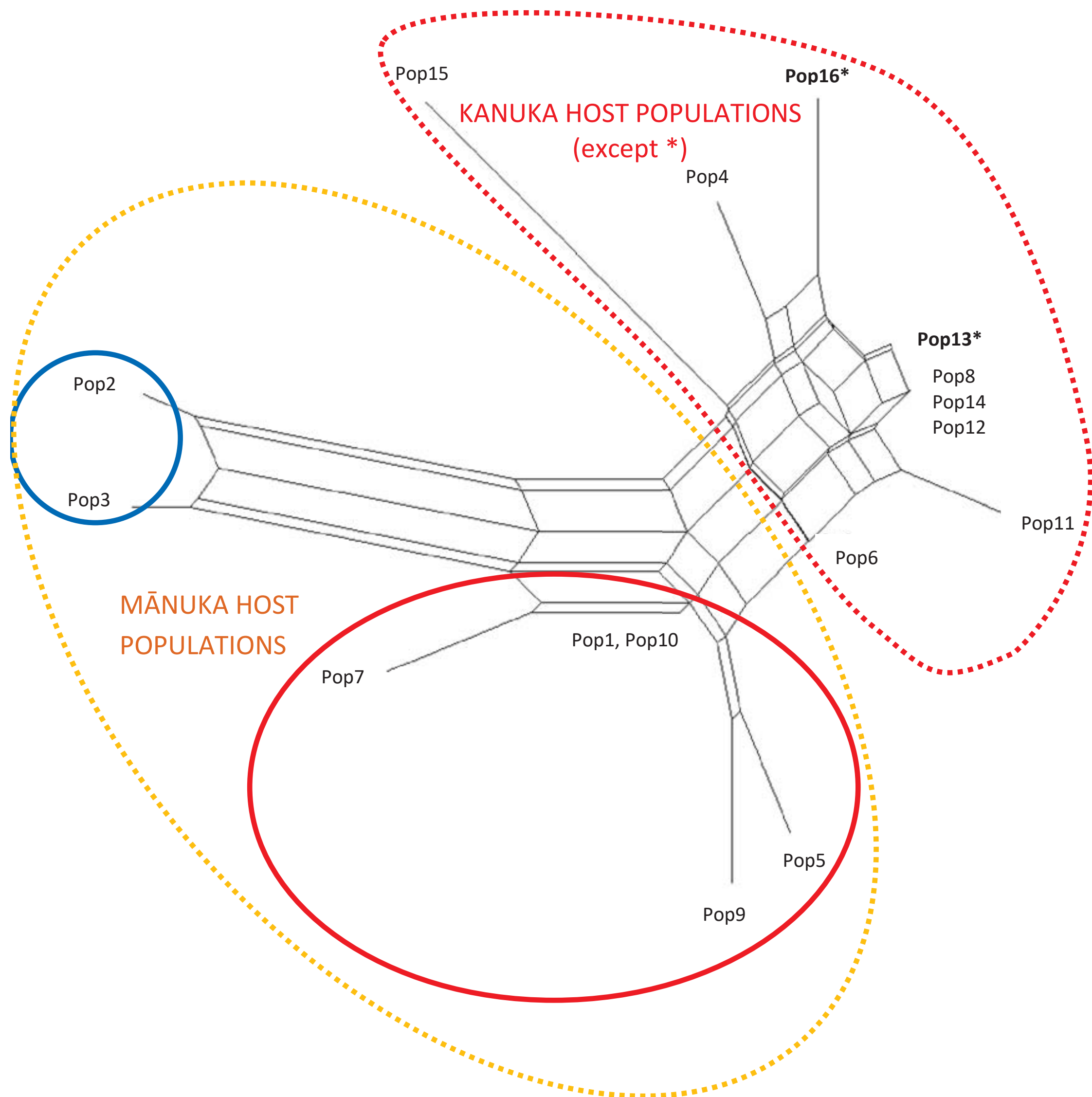

Figure 3.7: NeighborNet generated in SPLITSTREE4 based on pairwise genetic distances between all *Korthalsella salicornioides* populations. *Leptospermum* host *K. salicornioides* population lineages observed in STRUCTURE K=4 are indicated by the blue and red circles. Dotted orange circle shows grouping of mānuka-host populations on one side of the NeighborNet. Dotted red shape shows grouping of kanuka-host populations on the other side of the NeighborNet, populations with asterisks show exceptions to the main division. *Note*: refer to Table 3.5 for corresponding population number and location information.

3.5 Discussion

3.5.1 Genetic variation within Korthalsella salicornioides populations

Based on allozyme data, 28 *Korthalsella* populations from Hawaii, New Zealand, Australia and Taiwan have an average of 19.2% polymorphic loci with a mean of 1.2 alleles per locus (Molvray, 1990). According to a survey of genetic variability and life history traits for 110 species of seed plants (angiosperms) by electrophoresis studies (Hamrick, Linhart, & Mitton, 1979), the genetic diversity within *Korthalsella* populations (Molvray, 1990) is at the lower end of the scale. The microsatellite results here show similar results with just 12.5% polymorphic loci and 1.14 alleles per locus for 11 loci (Table 3.4). This low heterozygosity correlates with certain life history traits such as selfing or apomixis, and seed dispersal by animal attachment or gravity/explosive mechanisms which has been observed in *Korthalsella* (Hamrick *et al.*, 1979; Loveless & Hamrick, 1984; Sultan, 2014).

Private alleles

Compared to Nybom's (2004) meta-analysis of 307 studies using microsatellite markers for evaluating among and within-population diversity, the F_{ST} found in this study is higher than the average for all life history traits (life form, breeding system, seed dispersal and successional status). Many populations are completely isolated from each other; thus, limited gene flow between populations is expected. The model of isolation by distance predicts that measures of genetic differentiation at neutral loci will greatly increase with geographic distance (Wright, 1945). A number of theoretical (Holsinger & Weir, 2009; Slatkin, 1993) and empirical studies (Delmotte, Bucheli, & Shykoff, 1999; Tero *et al*., 2003) show strong correlations between geographic distance and pairwise F_{ST} values suggesting that if gene flow is occurring, it is mainly between neighbouring populations. Populations isolated by great distance and that are assumed to have been isolated for a long period of

time, are expected to have accumulated a greater number of private alleles due to mutation and restricted gene flow (Segarra-Moragues *et al*., 2005; Sosa *et al*., 2010). This is not the case for *K. salicornioides*, as there were few private alleles found and only four out of the sixteen populations had private alleles (Kerikeri, Manukau Domain, Te Puia and Marton). The low number of private alleles may just represent the overall low genetic variation found in *K. salicornioides*. However, the Marton population is the most geographically isolated population, not only from the populations sampled, but also from known populations throughout New Zealand. This population was the only one to have more than two private alleles, thus it is possible that the Marton population has had a higher number of generations than others without gene flow due to its isolation from other mistletoe populations.

The Isolation by Distance results in this study showed an insignificant correlation between population pairwise F_{ST} values and distance for all populations (Figure 3.5) and when separating by host genera (Figure 3.6). Theoretical research has suggested that greater differentiation between populations is due to founder effects and can be a result of colonisation history rather than migration or selection (Slatkin, 1977; Wade & McCauley, 1988). The lack of isolation by distance in *K. salicornioides* populations may be due to the colonization history, where population differentiation is based on the ancestral coloniser resulting in geographically close populations being highly differentiated due to potentially host-specific colonization effects. This founder effect with subsequent restricted gene flow between populations could result in divergence through adaptation to different host species. A lack of gene flow between close populations and reduced gene flow over large distances may also increase genetic distance and explain the lack of isolation by distance.

The loss of rare alleles can be a consequence of small population size, while heterozygosity is reduced significantly only after populations have been small for several generations (Barrett & Kohn, 1991). Expected heterozygosity, the number of alleles and polymorphic loci generally decrease with population size. This decrease tends to be greater for the number of alleles and polymorphic loci than expected heterozygosity (Leimu, Mutikainen, Koricheva & Fischer, 2006). This suggests that genetic drift, rather than directional or biparental inbreeding, causes the decline in genetic variation in small populations (e.g., Oostermeijer *et al*., 2003). It is further supported by F_{IS}, which estimates levels of inbreeding, and heterozygote/homozygote excess (Aegisdottir *et al.,* 2009), and is not related to population size (Leimu *et al*., 2006). Generally, there are no differences in population substructure between large and small populations. In this study, F_{IS} could only be estimated for 6 out of the 16 populations due to the low levels of genetic variation found within each population. For the six populations, five had large population sizes (50+ parasitized host trees) and one was considerably smaller (<10) (Table 3.4).

Seed dispersal and vector limitation of **Korthalsella salicornioides**

Stevenson (1934) suggested the *Korthalsella salicornioides* small seeds are not adapted to bird attraction and are dispersed by a weak explosive mechanism. Sultan (2014) measured the dispersal distances both in the field and in the laboratory. In the field, median ejection distance for *K. salicornioides* ranged from 1.3 to 2.3 m depending on the height and orientation of the host canopy (whether inward towards the forest or outwards towards the margin). Dispersal distances of 17 and 38 cm were recorded from a 22 cm height in the laboratory study. Thus, seed from *K. salicornioides* often infects the mother plant and potentially adjacent hosts. This has been described for a similar species by Zakaullah

(1988), who observed the mode of spread in *Korthalsella japonica* on oaks was from tree to tree. A different dispersal mechanism is therefore required for longer distances.

There is potential for both bird and wind dispersal in *K. salicornioides*. Sultan (2014) noted small insectivorous/omnivorous birds such as the grey warbler (*Gerygone igata*) and silvereye (*Zosterops lateralis*) visiting *K. salicornioides* – parasitized mānuka hosts. The sticky small seeds may be moved externally on bird feathers or feet as they visit mistletoe infected branches in search of insects, or internally after swallowing the fruits. The wind may also act as another dispersal agent, blowing the tiny seeds away from the parent plants. Mānuka/kanuka stands are often homogeneous in height and many mistletoe populations have little protection from the wind as there is no large canopy of a mature forest as shelter. Suitable hosts are often colonised by chance and therefore starting a new populations would require these rare events to occur. Thus the unreliable seed dispersal vectors can account for some of the current patchy distribution of *K. salicornioides*.

One of the populations visited at Hokio Beach is a fine example of patchy mistletoe distribution. The *K. salicornioides* population was relatively small as only seven *Kunzea amathicola* trees were infected. Three trees in close proximity (approximately 2-3m distance apart) were heavily parasitized by the mistletoe and were separated from the other four trees parasitized by approximately 20m. There were *K. amathicola* trees present between the two clusters but no trees were infected. Two of the four *K. amathicola* were heavily parasitized as well and separated by about 5-7m. The other two trees had been recently colonised as only one mistletoe was found on each of these trees (Figure 3.8). The *K. amathicola* population was vast but *K. salicornioides* was not established on many trees. Although binoculars were used, perhaps the trees between

the two clusters were parasitized and the mistletoe was illusive (i.e., higher up in the tree) due to its cryptic nature or tiny size. Interestingly though, all *K. salicornioides* individuals at this site were identical across all markers except for one. At the polymorphic marker, three individuals were homozygous for one allele while the other four were homozygous for another.

Figure 3.8: *Kunzea amathicola* at Hokio Beach. Mistletoes were collected from above the ladder's height in host tree canopy.

Lower North Island genetic "hot spot"

Overall, there was little heterozygosity observed in all *Korthalsella salicornioides* populations, but there appears to be a 'hot-spot' for mistletoe genetic variation centred in the lower North Island. Both observed heterozygosity and populations with polymorphic loci followed a lower North Island trend. All populations in the Lake Wairarapa area and the Marton population had polymorphic loci and individual heterozygotes. The Hokio

Beach population had polymorphic loci but no heterozygous individuals, possibly not detected due to a small sample size. The other lower North Island population (Waikanae) did not follow this trend but the mixed (both mānuka and kanuka parasitized) population in Te Puia (Rotorua) had multiple alleles per locus (4 of 11 loci), but all individuals were homozygous (Table 3.4). All other populations throughout the North Island were fixed at all loci.

The lower North Island had the greatest concentration of genetic variation found in *Korthalsella salicornioides*. The genetic data based on nuclear ITS from Sultan (2014) showed that sequence type variability was concentrated in the North Island compared to the South Island. Based on his inference that *K. salicornioides* has a longer presence in the North Island compared to the South Island, we can extend this further and theorise that *K. salicornioides* has had a longer presence in the lower North Island. Alternatively, the greater genetic diversity within these lower North Island populations may be linked to the large population sizes found here. Based on Leimu, Mutikainen, Koricheva & Fischer (2006) expected heterozygosity, the number of alleles, and the number or proportion of polymorphic loci significantly increases with population size. All three of the Lake Wairarapa populations and Marton population had large (50+ parasitized host trees) population sizes (Table 3.1) with many trees parasitized and *K. salicornioides* locally abundant on each tree. The Waikanae population consisted of 12 trees parasitized in a clumped area separated from other potential hosts by pasture. The lack of hosts and small host population size of this parasite population could potentially be why it does not fall into the lower North Island pattern. The Hokio Beach population (as mentioned before) was also relatively small with few hosts parasitized and showed a low percentage of polymorphic loci.

3.5.2 Host-races in Korthalsella

Although there was little variation within populations, the between population variation revealed interesting trends. The population wide F_{ST} was 0.640 (Table 3.4) suggesting high differentiation between populations which may not be surprising due to disparate populations, small dispersal distances, and if there are distinct host-races present in *Korthalsella salicornioides*. Populations were chosen strategically to evaluate host specificity and geographic genetic partitioning. If population structuring was mainly geographically based, we would observe populations in geographically close areas as more genetically similar than those further away. Based on both the STRUCTURE analyses and NeighborNet (Figure 3.4 and Figure 3.7), populations with the same host, at the level of genus, were foremost grouped together regardless of geographic proximity.

Te Puia was the only population sampled that had both host trees present and parasitized. In the other populations sampled that had both hosts present, *Korthalsella salicornioides* was only parasitizing one of the hosts. The markers used were sensitive enough to detect host-specific differences in *K. salicornioides* in the mixed Te Puia population. At K=4 (Figure 3.4B), all mānuka host *K. salicornioides* individuals and all kanuka host *K. salicornioides* individuals grouped separately. Furthermore, at K=7 (Figure 3.4C), the mistletoes parasitizing kanuka hosts were separated revealing a spatial trend in the population also. Mistletoes parasitizing kanuka hosts were genetically different on one side of the population compared to the other.

AMOVA results suggest there is greater variation among populations than within populations (Table 3.6) suggesting low diversity among the host-races and high differentiation between populations of mistletoe parasitizing both genera. Furthermore, comparing variation between the four STRUCTURE derived K=4 groups identified that the

greatest genetic variation was found among these groups (43%), with 33% found among these populations within the groups, and 22% within populations (Table 3.6). The significant amount ($p<0.001$) of genetic variance among the host groups shows that populations are genetically different and suggests there is limited gene flow between them.

Mechanisms of parasite differentiation

Parasite race formation involves genetic changes which are part of adaptation to enhanced fitness on a particular host. Host-specific parasite races are likely to form when the gene flow between parasite populations is reduced by factors such as geographic distance, limited range of dispersal, and patchy host populations. This limited gene flow over a substantial period of time is likely to lead to allopatric speciation (Norton & Carpenter, 1998). Isolation of the putative host races may simply result from physical separation because the two host species only occasionally occur together in equal abundance at a given site (Sultan, 2014). This spatial separation could limit gene exchange among mistletoe populations found on different hosts but would not explain how differentiation can occur and be maintained in sympatric populations. There are other restrictions to gene flow that may explain this. For example, as *Korthalsella* pollen and seed cannot travel very far, it is probable that, as in many plant species (e.g., Levin & Kerster, 1974), much of the pollen and seed are dispersed within individual host trees. Pollination among pygmy-mistletoe individuals occupying the same host tree is very likely, as is colonization by offspring of the same host tree that supports their seed parent. The likelihood of assortative mating and differentiation will therefore be enhanced. The homogenizing effects of gene flow over distances of a few metres can be overcome by

diversifying selection for host specificity and has been recorded on multiple occasions in parasitic plants (Linhart & Grant, 1996; Simms & Rausher, 1992).

3.5.3 Conservation implications for *Korthalsella salicornioides* and its hosts

The recent classification of *Kunzea* now shows that the ten species found in New Zealand are endemic (de Lange 2014), while *Leptospermum* dispersed from Australia and thus is not endemic (Allan 1961). Although the timing of the arrival of *Leptospermum* in New Zealand has not been established, the earliest record of its pollen type dates back to the upper Cretaceous and older Tertiary (Couper 1953, 1960). Fleming (1975) dated *Leptospermum* pollen to the Paleocene. It has been suggested that *Leptospermum* dispersal from regions of south-east Australia following the Miocene is most likely (Thompson, 1989). As scrub is thought to have dominated the interglacial periods, the opportunity for establishment by *Leptospermum scoparium* would have been limited to disturbed sites and coastal scrub (McGlone *et al.*, 2001). Since the arrival of human inhabitants, the widespread land clearance has greatly extended the range available to *L. scoparium*.

Both mānuka and kanuka life-history strategies are that of an '*r*-type' plant, adapted for colonisation, dispersal and fast population growth (Ogden, 1985). The typical characteristics of pioneer species include rapid growth rates, relatively short stature, ample seed production, high light demands, and short life cycle (Ogden, 1985; Mark *et al.*, 1989). Humans brought fires and cleared two thirds of the original forest cover across New Zealand (Cockayne,1928; Bellingham, 1956), creating many areas with low-nutrient status suitable for *L. scoparium* (Harris *et al.*, 1992) and *Kunzea*. Wardle (1991) recorded *L. scoparium* as the only New Zealand species to release seed after fire, a serotinous feature common in the Australian flora. The repeated fires, soil erosion and nutrient leaching

helped maintain *L. scoparium* cover in many areas where the plant community would eventually return to forest (Burrows, 1973).

Before the arrival of humans, *Korthalsella salicornioides* biogeography would have been affected mainly by the presence of suitable hosts in the environment. The more recent expansion of *Leptospermum* (its primary host) and the subsequent fragmentation of its distribution (due to elimination techniques such as spraying, burning, cutting and the introduction of mānuka blight into many populations) may have caused the isolated pockets of *K. salicornioides* we see today.

***Conservation of* Korthalsella salicornioides**

Despite the large number of endemic and threatened plants in New Zealand, there are fewer than fifteen published conservation genetic studies of the flora. The findings of this study that *Korthalsella salicornioides* populations have low levels of genetic variation but host-specific races, has important conservation implications. While the correlation between population size and genetic variation in *K. salicornioides* is unclear, there is theoretical (Ellstrand & Elam 1993) and observational (Leimu *et al.*, 2006) evidence that small populations have reduced genetic variation compared to large populations due to genetic drift and bottlenecks. For conservation purposes it may be useful to consider the species as four genetic clusters due to the genetic structure of *K. salicornioides*. Most populations are located in protected habitats; however the low density of host populations is of concern for the survival of individual populations and has long term consequences for the species persistence.

There are several management actions that need to be considered for the long term survival of *Korthalsella salicornioides*. First and foremost, the main conservation management action for *K. salicornioides* is to increase the available habitat (host trees) in

established populations. The loss of host trees is loss of habitat for the mistletoe, therefore increasing recruitment of host trees, looking after host and potential host trees, protection of hosts in unprotected sites, and maintaining the health of the environment will enable the species persistence over time.

Secondly, instead of attempting to increase the genetic variation in populations by introducing new alleles, attempts should be made to disperse seed sourced from the same population to promote recruitment and spread within a population. *K. salicornioides* natural dispersal is predominantly by the explosive mechanism but if potential host trees are too far away, it is often unlikely that *K. salicornioides* will be able to establish quickly. Spread of mistletoe seed around potential hosts within a population may increase the chances of continued survival. Trying to increase the genetic variation within populations by introducing novel genetic material may do more harm than good as *K. salicornioides* is an obligate selfer and therefore promoting outcrossing may hinder local adaptation (Hufford & Mazer 2003) and host specificity that is occurring. Conversely, maintaining low genetic variation in populations over time with no gene flow could adversely affect mistletoe populations. It is impossible to know which the best option is as relatively few studies provide empirical data on the issue. In the end it is likely to be both species and context specific.

Thirdly, new populations could be established with the goal of reducing isolation between populations as the observed population differentiation could be a result of fragmentation and small population effects rather than resulting from local selection and adaptation. For translocations of seed, results from STRUCTURE and NeighborNet suggest movement of genetic material should be within the two main host clusters. The spread of seeds is easy to do manually (Sultan, 2014) but often yields low success rates and

especially so if seed of one host type is placed on the wrong host. Thus it is imperative that seeds come from the same host type when seed is dispersed within and between populations. Consideration should also be given to the geographic area and the movement of genetic material between adjacent populations, especially in the Wairarapa. This will reduce the potential deleterious effects of outbreeding depression via coadapted gene complexes which has been recognised in translocations of rare species (Godefroid *et al.*, 2011). Initially, small scale translocations should be performed and monitored carefully for signs of reduced fitness due to outbreeding depression.

Host trees as economic benefits

Despite the historic general dislike of tea tree among farmers, some recognised the potential environmental consequences from the attempts to eradicate tea tree from the landscape. For example, Madden (1951) noted the risk of soil erosion, and Roberts (1957) noticed the supersedence by more undesirable plants and weeds. The negative perception of mānuka started to change in the 1970s and 1980s. Williams (1981) recognised the importance of mānuka and the morphologically similar kanuka, compiling a biography of articles and stressing the importance of considering “the value of existing vegetation for soil and water conservation, biological conservation and aesthetics.” Although mānuka and kanuka are occasionally still used as fire wood they are also valued for their ornamental and ethnobotanical use, as well as a source of honey and essential oils (Stephens *et al.*, 2005).

Tea tree oil was used in traditional Maori medicine for treating colds, inflammation and diarrhoea (Lis-Balchin and Hart, 1998). The bark and leaves were used for a range of medicinal purposes, with bark preparations used as sedatives, and leaf decoctions used to treat colds and reduce fever (Brooker *et al.*, 1987; Salmon, 1980). Nowadays, many

antibacterial medical studies of the oils have been undertaken (e.g., Weston *et al.*, 1999). Tea tree oils contain chemical compounds with antihelminthic (drugs that expel or destroy parasitic intestinal worms) and insecticidal properties (Brooker *et al.*, 1987; LisBalchin *et al.*, 2000), and were also shown to have antibacterial, antifungal and antioxidant properties (Lis-Balchin *et al.*, 2000).

Mānuka honey is now of great economic importance to New Zealand as it is considered to be of high quality, unique taste and many medicinal properties (Stephens *et al.*, 2005). It has been shown to have antibacterial effect against *Escherichia coli* (Mavric *et al.*, 2008), *Helicobacter pylori* (Somal *et al.*, 1994), *Staphylococcus aureus* (Allen *et al.*, 1991; Mavric *et al.*, 2008), methicillin-resistant *S. aureus*, and a number of vancomycin resistant and -sensitive *Enterococcus* strains (Cooper *et al.*, 2002). It is also an accepted topical treatment for wounds (Cooper 2004). Particular effectiveness is displayed by mānuka honey incorporated into wound dressings in treating burns, skin-grafts, ulcers, and skin or muscle infections containing antibiotic-resistant strains of bacteria (Molan & Betts, 2004).

Despite the anti-mānuka campaign and the intentional spread of mānuka blight (Madden, 1951; Roberts, 1957; Sewell, 1953), *L. scoparium* has manged to surpass the effects of the pathogen. The range and abundance of the original mānuka blight scale insect (*Eriococcus orariensis*) has been steadily reduced by a parasitic fungus, which has consequently resulted in the displacement of the less noxious scale insect (*E. leptospermi)* in recent years (van Epenhuijsen *et al.*, 2000). Mānuka blight now only seems to affect mānuka to a moderate degree, causing some branches and individual plants to die (Burrows and Lord, 1993). With regards to *K. salicornioides*, the loss of a few individual host trees may make quite an impact on the mistletoe population depending on the size.

In mānuka populations, spraying of winter oil may benefit any mistletoe present to keep the black sooty mould fungus from rising to a critical level (Derraik, 2008). Mānuka blight now appears to have little effect on the natural distribution of *L. scoparium* populations in the wild, thus the main threat of population decline is now human clearance for agriculture and firewood. The protection of tea tree can not only benefit the economy of New Zealand greatly, but preserve the biodiversity (such as endemic scale insects) found on these trees.

3.5.4 Future directions

The hypothesis that the natural classification of certain groups of parasites parallels that of their hosts was proposed by Fahrenholz in the late 1800's. Fahrenholz's work based on feather mites (Acarina) concluded phylogenetic parallelism of parasites and hosts, and later hypothesized that such a relationship held for the chewing lice (Mallophaga) and sucking lice (Anoplura). Eichler (1948, p.599) subsequently coined the term "Fahrenholz's Rule" which states that "in groups of permanent parasites the classification of the parasites usually corresponds directly [to] the natural relationships of the host." The basis of the hypothesis is the assumption that, at some point in the evolutionary history of host and parasite, the ancestral parasite enters a close association with the ancestral host, after which both evolve and speciate together. Thus, parasite phylogeny should mirror host phylogeny. The identification of new *Kunzea* species in New Zealand may parallel the genetic structure of *Korthalsella salicornioides* in years to come if populations become reproductively isolated on different host species. This raises the question of whether parasite divergence can be used to identify divergence occurring within the host. Is it possible to use data generated from parasites to generate hypotheses of host divergence among hosts with cryptic variation?

Associations between genetic variation, population size and fitness may be mediated by differences in the demographic structure of plant populations, e.g., if small plant populations only consist of old plants that are no longer contributing to offspring recruitment (Oostermeijer *et al*., 1994). Data on the demographic structure of these populations was not recorded, thus future studies could investigate the role of demographic population structure. Furthermore, due to the economic importance of the host species, and in particular *Leptospermum scoparium*, assessing whether *Korthalsella salicornioides* has any effect on the quality of honey produced and fitness of the trees parasitized. For example, does prolonged association of mistletoe and host have a negative effect on host growth and reproductive success?

It would be interesting to see if the host-specificity extends to populations that are greatly geographically isolated from other populations such as Kapiti Island and Great Barrier Island. Another question of interest is whether there is specificity not just to *Leptospermum* vs. *Kunzea*, but to particular *Kunzea* species, and in particular, are there races host-specific to the rarer species of *Kunzea*? This study showed no clear trends for specificity within the *Kunzea* genus but perhaps greater sampling from more of the species may show different patterns. Under de Lange's (2014) recent classification, one *Kunzea* species is endemic to the South Island (*K. ericoides*) and another species (*K. sinclairii*) is endemic to Great Barrier Island. Collecting mistletoe from both these hosts as well as the other *Kunzea* species that were not collected (*K. serotina*, *K. salterae* and *K. toelkenii*) would be of great interest and help elucidate this question. Sultan (2014) observed strong North and South Island differences in sequence type data. Therefore investigating whether host-specificity extends over both Islands or if there are geographically distinct genetic clusters from both the North and South Island would further our understanding.

Sampling from more known host species and reducing the geographic distance would provide more insight to the host specificity occurring within *K. salicornioides*. Ninety-six per cent of all records for *K. salicornioides* are from the two Myrtaceous genera (Sultan, 2014); therefore exploring the other four per cent may elucidate further on host-specificity in *K. salicornioides*. We may find a taxonomic hierarchy forming where mānuka and kanuka genotypes are more closely related than genotypes found on the more rarely parasitized species such as *Coprosma*, *Erica*, *Melicope* and *Sophora* species.

3.6 Conclusion

This study investigated host-specificity in the New Zealand endemic stem hemiparasite *Korthalsella salicornioides* with microsatellite markers using a population genetic approach. Our results suggest there is evidence for the existence of host-specific races in *K. salicornioides* between the two main host genera, *Leptospermum* and *Kunzea*. Although population genetic diversity within populations was low, *K. salicornioides* shows high differentiation between populations. High F_{IS} scores were observed in all populations with polymorphic loci indicating heterozygote deficiency, which could be caused by inbreeding between closely related individuals or clones. Considerable levels of genetic differentiation were found and the isolation by distance results suggest that if gene flow occurs it is not restricted to adjacent populations. Populations grouped in genetic clusters that reflect host-type and geography. Distinct genotypes were found in the sympatric population, indicating that host-specificity can occur when the two main hosts are sympatric and the mistletoe is parasitizing both. The low levels of genetic variation found in *Korthalsella salicornioides* correlate with the observed life history traits including selfing, pollination and seed disperal by gravity or animal attachment. The loss of habitat and limited dispersal onto potential host trees needs to be addressed. Sources of genetic

information for spread of seeds or pollen to increase numbers within populations should be based on the host-population type and population genetic structure results. The findings of this study are a significant advancement in the knowedge of host-specificity in *Korthalsella* and paves the way for further research into understanding host-specificity in New Zealand's endemic flora.

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Chapter 4

Conclusion

4.1 Introduction

All hemiparasitic pygmy mistletoes (*Korthalsella* spp.) can parasitize more than one species of angiosperm host. In New Zealand, *Korthalsella salicornioides* Tiegh. (Viscaceae) is the most host-specific out of the three endemic species. *K. salicornioides* has *Leptospermum scoparium* s.l. (mānuka) J.R.Forst. & G.Forst. (Myrtaceae) as one of its principal hosts and *Kunzea* spp. (kanuka) de Lange (Myrtaceae) as its secondary and tertiary hosts (Sultan, 2014). In mistletoe populations where the two main host genera are sympatric, *K. salicornioides* is found parasitizing one host and not the other in many occasions. This study attempted to investigate host-race specificity in *Korthalsella salicornioides*.

Certain life history traits found in *Korthalsella,* such as selfing, insect pollination and seed dispersal by gravity or animal attachment, correlate with low heterozygosity in populations (Hamrick, Linhart, & Mitton, 1979; Loveless & Hamrick, 1984). According to a survey of genetic variability and life history traits for 110 species of seed plants (angiosperms) by electrophoresis studies (Hamrick *et al*., 1979), the genetic diversity within *Korthalsella* populations is at the lower end of the scale. Based on allozyme data, *Korthalsella* populations have an average of 19.2% polymorphic loci with a mean of 1.2 alleles per locus (Molvray, 1990). Previously, the sequence variability of 50 *K. salicornioides* individuals from different populations was studied using nuclear ITS (internal transcribed spacer) and chloroplast *trnQ-rps16* markers. The genetic variability based on these markers in the mistletoe populations was geographically structured and

not host-associated. Cross-infection experiments in *Korthalsella salicornioides* provided more insight into the presence of putative host races, as better mistletoe seedling establishment success rates were apparent when the maternal seed source and recipient hosts were the same. Because the previous sequence data were uninformative, alternative molecular markers were developed to elucidate potential host-associated divergence.

Microsatellites allow for estimates of allele frequencies, and their high mutation rate can be used to detect more recent changes in the genetic structure of populations. These markers were chosen for this study due to the ease of genotyping (Ashley & Dow, 1994), relative faster evolution (Li *et al*., 2002), and their ability to determine heterozygosity due to their co-dominance (Duminil & Di Michele, 2009) making them informative for host-races within the species. Host-specificity is important in biology to provide insight into evolution and the speciation process. A population genetics approach was utilised with aims to:

1. Design novel *K. salicornioides* microsatellite markers using genomic sequence.
2. Utilise the microsatellite markers to assess genetic variation and structure of populations throughout the North Island of New Zealand.
3. Use the information from the genetic study to determine the presence of host races to aid future management plans for the species.

4.2 Findings

Eleven microsatellite markers were developed for the use in *Korthalsella salicornioides* to attempt to determine host races. The main findings were summarised within the previous chapters: Microsatellite markers for *Korthalsella* (Viscaceae) and Population genetics and host-race specificity in *Korthalsella salicornioides* (Viscaceae). This section will use these findings to address the aims of the study:

> Objective 1: Design novel *K. salicornioides* microsatellite markers using genomic sequence.

Next-generation Illumina MiSeq sequencing was utilized in sequencing *Korthalsella* DNA which was sorted into contigs and microsatellite primers were designed within Geneious 6.0 (Biomatters, Auckland, New Zealand) using Primer 3 (Rozen and Skaletsky, 2000). Forty-five primer pairs were initially trialled on samples of each of the three endemic *Korthalsella* species to identify reliable and polymorphic loci that could be used as markers for the second part of the study. Eleven microsatellite markers were developed that amplified consistently and were polymorphic. These markers were used to screen 272 samples of *Korthalsella salicornioides* collected from sixteen populations throughout the North Island. From the original 318 samples collected, forty-six individuals were not screened due to poor DNA quality. Most markers amplified 100% of the samples with only two markers amplifying 95% and one amplifying 99% of the samples, indicating these markers are reliable to use in a population genetics approach to delimiting host-races in *K. salicornioides*. Only seven markers were polymorphic with allele numbers ranging from 2 to 4 which may be low polymorphism but may be typical of the species.

Objectives 2 and 3: Utilise the microsatellite markers to assess genetic variation and structure of populations and use the information to determine the presence of host races to aid future management plans for the species.

Low within-population genetic diversity was found for the New Zealand endemic stem hemiparasite, *Korthalsella salicornioides*. Our results suggest that there is greater variation between populations than within them with only 32% of the variation attributed to within populations. Furthermore, there was greater variation among the four K values identified in STRUCTURE, indicating that populations with the same host are closely related. Although a low within population genetic diversity was found, *K. salicornioides* shows high differentiation between populations. High F_{IS} scores were observed in all populations with polymorphic loci indicating heterozygote deficiency, which could be caused by inbreeding between closely related individuals or clones. Considerable levels of genetic differentiation were found and the isolation by distance results suggest that if gene flow occurs it is not restricted to adjacent populations. Populations grouped in genetic clusters that reflect host-type and geography.

Our data indicate that in *Korthalsella salicornioides*, there is enough differentiation to imply host-race specificity. One mistletoe population was found parasitizing both sympatric hosts, and STRUCTURE results showed two distinct genetic clusters based on which host the mistletoe was parasitizing. These results will aid in the understanding of host-race specificty within *Korthalsella salicorniodes*. The identification of the distinct genetic clusters may be useful in future for conservation purposes. However further research is needed to investigate the extent of host-specificity. It may be helpful to investigate the genetic structure of mistletoe populations found on the rarer host species and to sample from both off-shore and South Island populations. Investigation into the

sympatric site might provide insights into the development of reproductive isolation, and identify environmental or genetic factors that are involved.

4.3 Limitations

For this study there are a number of limitations to be considered. The number of mistletoe plants in a population was not estimated as it is difficult to tell separate plants due to the clonal nature of *Korthalsella salicornioides* and although binoculars were used, it is not possible to determine the exact number of mistletoes in every tree. The number of trees infected was also difficult to determine exactly in some populations as the host trees were quite large and the mistletoes are cryptic and small in some instances. Sampling all the known host species for *Korthalsella salicornioides* was not obtainable for this study, as was sampling from more than one population with the same host species, especially in the *Kunzea* genus. Due to the conservation status of *K. salicornioides*, many populations are limited to DOC reserves. Permits to collect from more populations throughout New Zealand within the time constraints was not obtainable and therefore populations in the South Island and on different hosts (such as *Erica* and *Melicope*) will be collected once permits are attained. Despite the limitations within this study it provides an important assessment of population structure and host-specificity in *Korthalsella salicornioides*. Issues raised can be addressed in future with greater sampling of *K. salicornioides* on more *Kunzea* hosts and increased emphasis on sympatric populations.

4.4 Future Directions

Two other areas of research would be informative: Genotyping the host plants, both in parasitized populations and outside *K. salicornioides* range, and conducting ecological demographic work to determine parasite-host distribution within sympatric kanuka and mānuka populations. When mistletoe populations were sampled, host leaf material for every mistletoe sample, as well as leaf material from uninfected trees was collected. Illumina MiSeq data are in hand for the development of *Leptospermum scoparium* microsatellite loci, while *Kunzea* DNA needs to be run on the Illumina MiSeq platform and microsatellite primers designed.

Mānuka genetic data would be of great interest to many different areas of research. The morphology of some mānuka populations was considerably different compared to others (for example Kohi Point, Figure 4.1). Elucidating whether there is a genetic background to the differences or if it is due to environmental factors (exposed coastal cliff, Figure 4.2) is important and may also help explain the genetic differences found in *K. salicornioides* at this site. Furthermore, the development of microsatellite markers for *L. scoparium* may provide other research areas with important and informative tools (e.g., screening different mānuka cultivars and seed lines to detect plants that produce quality UMF honey).

With the recent classification of the *Kunzea* genus, microsatellite genetic data from all the newly described species would also be of great interest and a useful tool for examining hybridisation and introgression. De Lange (2014) suggested research using more discriminating molecular markers (such as microsatellite markers) is needed to determine the extent of introgression that has occurred between three *Kunzea* species in the Ahipara Plateau.

Figure 4.1: Leaf shapes of herbarium specimens of *Leptospermum scoparium* parasitized by *Korthalsella salicornioides*. Kohi Point: rounded and fleshy (A), Manukau Domain: typical mānuka leaf shape (B).

Conducting ecological demographic work within sympatric kanuka and mānuka populations is another important and interesting future research area. This would enable the determination of parasite-host distribution within these populations as well as help determine characteristic features of the host trees such as living status and age.

This study provides interesting areas for future study. Recently, a Massey University student, Katherine Murray, investigated mistletoe genotypes from the cross-infection experiments started by Amir Sultan (2014). Using six of the microsatellite markers developed in Chapter 2, she was able to observe genetic changes and host preference. Microscopy (dissecting, epifluorescence and confocal) was used to investigate how far the endophytic system extends into host branches and for evidence of adventitious sprouts. Once more robust sampling is completed (sampling from the other *Kunzea* species populations that were not collected, populations from other host genera, and from South and offshore Islands), as well as ecological demographic work, our understanding of host-race specificity in *K. salicornioides* will be greatly increased.

Figure 4.2: Two *Leptospermum scoparium* host populations at Kohi Point coastal habitat along walking track (A), and Kerikeri swamp habitat (B).

4.5 References

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Appendix 1: Population genetic diversity estimates for 16 populations of *Korthalsella salicornioides* based on the seven polymorphic loci

Appendix 1: Population information and genetic diversity estimates for 16 populations of *Korthalsella salicornioides* based on the seven polymorphic loci.

Pop	Location	N	P_A	N_A	N_E	%P	H_O	H_E	F_{IS}
Pop 1	Coromandel	23	0	1.000	1.000	0.00%	0.000	0.000	N/A
Pop 2	Wairarapa 1	25	0	1.714	1.360	57.14%	0.017	0.211	0.904
Pop 3	Wairarapa 2	24	0	1.286	1.156	28.57%	0.024	0.101	0.763
Pop 4	Wairarapa 3	23	0	2.000	1.451	85.71%	0.031	0.271	0.874
Pop 5	Waikanae	12	0	1.000	1.000	0.00%	0.000	0.000	NA
Pop 6	Hokio Beach	7	0	1.143	1.137	14.29%	0.000	0.070	1.000
Pop 7	Manukau Domain	11	1	1.000	1.000	0.00%	0.000	0.000	N/A
Pop 8	Paihia	12	0	1.000	1.000	0.00%	0.000	0.000	N/A
Pop 9	Kerikeri	16	1	1.000	1.000	0.00%	0.000	0.000	N/A
Pop 10	Motuoapa	14	0	1.000	1.000	0.00%	0.000	0.000	N/A
Pop 11	Monika Lanham	12	0	1.000	1.000	0.00%	0.000	0.000	N/A
Pop 12	Te Kopia	12	0	1.000	1.000	0.00%	0.000	0.000	N/A
Pop 13	Kohi Point	12	0	0.857	0.857	0.00%	0.000	0.000	N/A
Pop 14	Waitakere Ranges	9	0	1.000	1.000	0.00%	0.000	0.000	N/A
Pop 15	Marton	14	2	1.857	1.304	71.43%	0.031	0.180	0.511
Pop 16	Te Puia	46	1	1.571	1.360	57.14%	0.000	0.202	1.000
Mean		**17**	**0.31**	**1.214**	**1.102**	**19.64%**	**0.006**	**0.065**	**0.84**

***Note*: N, sample size genotyped and used in this study; P_A, number of private alleles; N_A, number of alleles per locus; N_E, number of effective alleles per locus; %P, percentage of polymorphic loci; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, fixation index score; N/A, observed and expected heterozygosity are both zero and therefore F_{IS} cannot be calculated.**