Microsatellite Genotyping in New Zealand's Poplar and Willow Breeding Program: Fingerprinting, Genetic Diversity and Rust Resistance Marker Evaluation

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ABSTRACT

Poplars (*Populus spp.*) and willows (*Salix spp.*) are the most employed sustainable soil stabilisation tools by the pastoral sector in New Zealand. The poplar and willow breeding program at the New Zealand Institute for Plant & Food Research Limited (PFR) supports and improves the versatility of poplars and willows as soil stabilisation tools. Good germplasm management involves being able to accurately and objectively identify breeding material and characterise genetic diversity and relationships. Poplar rust is the primary pathogen of concern for the breeding program and evaluating candidate resistance markers could improve selection efficiency. We employed microsatellite markers to fingerprint and characterise the genetic diversity of the germplasm collection. In addition, we also evaluated SSR marker 'ORPM277's potential usefulness as a molecular marker to screen for rust resistance in New Zealand. We found that most microsatellite markers utilised in this study were moderately to highly polymorphic with Polymorphic Information Content values averaging 0.482 and 0.497 in the poplar and willow collections respectively. A DNA fingerprinting database was generated that differentiated between 95 poplar accessions and 197 willow accessions represented by 19 and 55 species groups respectively. Genetic variation was high and very similar in both poplar and willow groups, with the main source of variation coming from within genotypes. No correlation was found between phenotypic and genotypic rust resistance data using the ORPM277 microsatellite marker.

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ABBREVIATIONS

AFLP™ – amplified fragment length polymorphism

AMOVA – Analysis of Molecular Variance

CO1 – cytochrome oxidase

cpDNA – chloroplast DNA

CTAB – cetyltrimethylammonium bromide

DBH – diameter at breast height

DNA – Deoxyribonucleic acid

ETA – emissions trading scheme

F_{IS} or F – Sewell Wright's F-statistic. F_{IS} is an inbreeding coefficient that is the proportion of the variance in a subpopulation that is within in an individual.

F_{ST} – Sewell Wright's F-statistic used for codominant data. F_{ST} is a measurement of the proportion of variance between groups relative to the total variance.

GWA – Giant Willow Aphid (*Tuberolachnus salignus*)

H_{exp} – Nei's Gene Diversity

H_O – The proportion loci that are observed to be heterozygous relative to the Hardy-Weinburg Equilibrium

HS-PCR – heat-soaked polymerase chain reaction

Mer – *Melamspora* resistant locus

MPI – New Zealand Ministry for Primary Industry

N – Number of Accessions

Na – Number of Alleles

NBS-LRR - nucleotide-binding site leucine-rich receptor

NGS – next generation sequencing

nrDNA – nuclear deoxyribonucleic acid

NZD – New Zealand dollars

PCR – polymerase chain reaction

PFR – Plant and Food Research

PFR – Plant and Food Research New Zealand Ltd.

PVR – Plant Varietal Rights

R genes – disease resistant genes in plants

RAPD – random amplification of polymorphic DNA

rDNA – ribosomal deoxyribonucleic acid

RFLP – Random fragment length polymorphism

SNP – short nucleotide polymorphism

SSR – Simple Sequence Repeat

TOPLESS – transcriptional corepressor

UPGMA – Unweighted Pair Group Method with Arithmetic Mean

ϕ_{ST} – F_{ST} analogue used for binary presence/absence marker or sequence data.

Measures the proportion of variance between groups relative to the total variance.

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1 Introduction

Willows (*Salix spp.*) and poplars (*Populus spp.*) are the two main genera in the Salicaceae family. Willows represent a very a diverse group of nearly 350 species divided into tree and shrub classifications with mixed ploidy levels. Poplars represent a smaller group of 25 diploid species that fit into six discreet sections with intersectional incompatibilities. Poplar domestication began with the traditional selection and breeding of superior individuals as a conservation project in 1914 at the Royal Botanical Gardens of Kew (Isebrands & Richardson, 2014). In contrast, willows have a much shorter breeding history that started with the improvement of basket willow production.

Poplars and willows were first used as soil stabilising tools in the 1950s on erosion-prone agricultural slopes in the North Island of New Zealand, before being widely adopted across the country for diverse uses including but not limited to: shelterbelt protection, waterway stabilisation, bioremediation, soil stabilisation, ornamental uses and carbon sequestration (Wilkinson, 1999). To support their development a breeding program was started on 20 March 1973 by Chris van Kraayenoord under the National Plant Material Centre (NPMC) with three primary objectives: i) maintain and enhance the genetic stock ii) breed new enhanced varieties iii) transfer the technology to regional councils and farmers (Wilkinson, 1999). With support from the International Poplar Commission (IPC) and International Union of Forest Research Organisation (IUFRO), additional poplar and willow germplasm was imported to New Zealand to enlarge the National Germplasm Collection and enhance the breeding programme. The germplasm collection has now grown to 95 poplar accessions (individual

samples) and 197 willow accessions, with three replicate trees per genotype. The collection is represented by a diverse range of wild pure species, wild interspecific hybrids, domesticated pure species and domesticated interspecific hybrids.

Simple Sequence Repeat (SSR) markers are non-coding tandem-repeat motifs that are ideal for genotyping studies due to their low-application cost combined with a high degree of polymorphism, Mendelian inheritance, neutral evolution, codominance and reproducibility. SSR markers have been proven useful for clonal differentiation, genetic characterisation, parental identification and marker assisted selection (MAS) for a wide range of plant species. SSR-based DNA fingerprinting, genetic characterisation and parental identification has been successfully employed in numerous tree breeding programs including poplars (Rajora & Rahman, 2003), willows (Singh, Singh, Naik, Thakur, & Sharma, 2013), eucalyptus (*Eucalyptus spp.*) (Lezar, Myburg, Berger, Wingfield, & Wingfield, 2004), *Acacia spp.* (Le et al., 2017) and *Corylus spp.* (Freixas-Coutin et al., 2019). SSRs have also been successfully employed in marker-assisted resistance breeding to ink disease (*Phytophthora spp.*) in chestnuts (*Castania spp.*) (González, Cuenca, López, Prado, & Rey, 2011), apple scab (*Venturia inaequalis*) in apples (*Malus domestica*) (Patocchi et al., 2005) and *Puccinia psidii* rust in eucalyptus (Mamani et al., 2010).

The accurate identification of clones, accessions and parents is essential for germplasm management, the protection of plant varietal rights (PVR), maintaining accurate pedigree records, identifying elite breeding material and improving the overall efficiency of the breeding program. Presently, poplar and

willow clonal identification and parental identification in New Zealand is based on morphological characters. Morphological traits of poplars are highly susceptible to environmental and growth cycle factors which can make it difficult to distinguish between different clonal varieties (Peszlen, 1994). Misidentification issues can occur through labelling errors and genotype duplications. Moreover, there is a relatively low level of anatomical variation between clones of the same species or hybrid selections which can further hamper morphological-based identification efforts. Generally, full-sibling hybrid pedigrees are known due to controlled pollination processes. However, cross-contamination and mislabelling can occur during the breeding process resulting in pedigree record discrepancies. With the advent of low-cost genotyping, there are now highly accurate and efficient DNA marker-based methods to elucidate unknown parents and identify germplasm. However, this requires either a reference sample or a genotype database.

One of the primary objectives of New Zealand's poplar and willow breeding program is to improve the genetic diversity of clonal cultivars. Genetic diversity is important in agricultural crops because it increases the probability of progeny inheriting desirable allele frequencies (O. Frankel, 1984). Understanding the genetic diversity of germplasm is a crucial starting point to increase the genetic diversity of breeding populations and released cultivars. To date, there has been no assessment of the genetic diversity or relationships of New Zealand's poplar and germplasm collection. This has limited the breeders' ability to effectively manage germplasm material and effectively select progenitors to achieve the breeding objective.

As an improvement to the phenotypic system of clonal identification of New Zealand's poplar and willow germplasm collection, the aim of this study is to (1) produce and evaluate a genotype-based identification system, and (2) use the genotype-based identification system to analyse the genetic diversity and relationships among and within germplasm accessions.

Poplar rust (*Melampsora larici-populina*) is the primary pathogen of concern to New Zealand's poplar breeding program. Rust disease causes widespread defoliation of poplar trees and reduces the durability and usability of clones. Phenotypic selection methods are used in the program, but no marker evaluation has been conducted as a selection approach in poplar breeding in New Zealand. The *Mer* locus was shown to control resistance against three pathotypes of poplar rust in *P. deltoides* (M. T. Cervera et al., 1996; J. Zhang et al., 2001). Mapping studies later revealed that an SSR marker 'ORPM-277' was present in the same linkage group as the *Mer* locus (V. Jorge, Dowkiw, Faivre-Rampant, & Bastien, 2005b). While the authors speculated that ORPM-277 could serve as a qualitative poplar resistant marker in MAS, no published marker evaluations have been conducted. This study will determine whether ORPM277 is linked to phenotypic rust resistance in a *P. deltoides* × *nigra* breeding population.

1.1.1 Research Objectives

The objectives of this study were to: 1) Develop a DNA fingerprinting database of New Zealand's poplar and willow germplasm collection. 2) Estimate the level of genetic diversity that exists in the germplasm collection. 3) Evaluate genetic relationships between individual accessions 4) Evaluate whether the 'ORPM277' microsatellite marker confers resistance to poplar rust in New Zealand.

2 Literature Review

2.1 Introduction

New Zealand's poplar (*Populus spp.*) and willow (*Salix spp.*) breeding program based at Plant and Food Research (PFR), Palmerston North focuses on improving the genetic diversity, usability and durability of clonal cultivars across the country. New Zealand's poplar and willow germplasm serves as the fundamental collection of genetic diversity that is used to improve clonal cultivars. This genetic diversity is crucial for improving resistance to increasing environmental pressures like poplar rust (*Melamspora spp.*). However, a low level of anatomical variation exists within species groups, making phenotypic identification very challenging. Furthermore, the level of genetic variation that exists is poorly understood and the exploration of genotyping in the program is limited. To improve the management of genetic material and better inform the breeding program, this study uses microsatellite-based DNA fingerprinting for genetic characterisation. Moreover, this study will also evaluate the usefulness of microsatellite-based genotyping for poplar rust in New Zealand. To understand the importance of this study, a broad literature review has been conducted. This review begins with an overview of poplar and willow taxonomy and reproductive systems, including the biology and impacts of poplar rust, discusses the breeding program and importance of poplars and willows in a New Zealand context, and reviews the role of microsatellite-based genotyping in poplar and willow germplasm.

2.2 The genus *Populus*

2.2.1 Origin and Distribution

The *Populus* genus is well documented in fossil records from the Paleocene (66-55mya) onwards and is thought to have arisen from wetland and riparian ecological zones in North America (Collinson, 1992). It is hypothesised that species of *Populus* were dispersed from North America to the rest of the North Hemisphere via the Bering Land Bridge and the North Atlantic Land Bridge (X. Liu, Wang, Shao, Ye, & Zhang, 2017). Subsequently, the *Populus* genus now inhabits a wide range of ecological zones from sub-arctic boreal to sub-tropical (Eckenwalder, 1996). The richest centres of species diversity are Eastern Asia (14 species) and North America (9 species) with other centres of diversity including Europe (3 species), Central Asia (2 species) and Africa (2 species) (Hamzeh, Périnet, & Dayanandan, 2006).

2.2.2 Description and Classification

Populus is a deciduous flowering tree genus within the *Salicaceae* family. Compared to the *Salix* genus, the *Populus* genus is relatively small and includes 29 species divided into six ecologically and morphologically distinct sections (Table 2.2-1) (*Populus, Tacamahaca, Leucoides, Abaso, Turanga* and *Aigero*) (Eckenwalder, 1996). Understanding each section in the *Populus* genus is important in a breeding context due to the prevalence of incompatibilities between members in different sections (Willing & Pryor, 1976). Historically, the placement of species in these sections were based on morphological characters which have subsequently been generally supported by recent genetic studies (Z. Wang et al., 2014). However, some chloroplast DNA (cpDNA) and nuclear DNA

(nrDNA) studies have re-allocated species to different sections (Stettler, Bradshaw, Heilman, & Hinckley, 1996).

Table 2.2-1 Summary of Populus Sections

Section	*Populus* Species	Centre of Origin
Abaso	*P. guzmantlensis, P. mexicana*	Mexico
Aigeros	*P. deltoides*[1], *P. fremontii, P. nigra*[1]	North American and Europe
Leucoides	*P. heterophylla, P. lasiocarpa, P. wilsonii*	North America and Asia
Tacamahaca	*P. angustifolia, P. balsamifera, P. cathayana, P. koreana, P. laurifolia, P. maximowiczii*[1], *P. simonii*[1], *P. suaveolens, P. szechuanica*[1], *P. trichocarpa*[1], *P. tristis, P. ussuriensis, P. yunnanensis*[1]	North America and Asia
Turanga	*P. euphratica, P. ilicifolia*	Africa and Asia
Populus	*P. adenopoda, P. alba*[1], *P. davidiana, P. grandidentata, P. sieboldii, P. tremula, P. tremuloides*[1]	Northern Hemisphere

2.3 The genus *Salix*

2.3.1 Origin and Distribution

The *Salix* genus is represented in the fossil record from the Early Eocene (56mya) which is slightly later than the *Populus* genus (Collinson, 1992). Both the *Salix*

[1] Species of commercial importance to New Zealand

genus and the *Populus* genus share a similar evolutionary history, occupying riparian habitats in North America before distribution occurred throughout the North Hemisphere via the Bering Land Bridge and the North Atlantic Land Bridge (Elias, Short, Nelson, & Birks, 1996). However, despite a high level of genomic similarity, it is hypothesised that *Salix spp.* have evolved faster than *Populus spp.* due to uneven selection pressures (Hou, Wei, Pan, Zhuge, & Yin, 2019). The *Salix* genus is now distributed in cold and temperate regions with high soil moisture across the Northern Hemisphere (Figure 2.3-1) (Verwijst, 2001). Furthermore, some members of the *Salix* genus are also represented in the Southern hemisphere in South-East Asia, Central/Southern America and Africa (Figure 2.3-1) (Argus, 1999). The richest centres of diversity for the *Salix* genus are China and Russia (Verwijst, 2001).

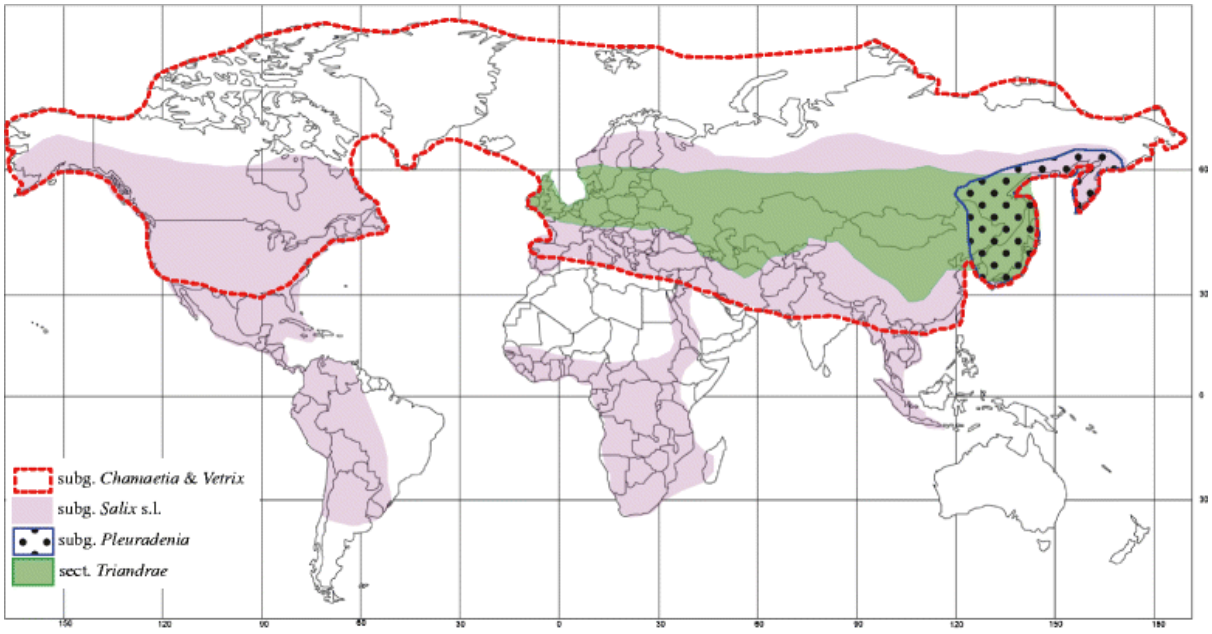

Figure 2.3-1 - Global distribution of *Salix spp.* Source : J. Wu et al. (2015)

2.3.2 Description and Classification

The *Salix* genus is the most diverse in the *Salicaceae* family consisting of 330-500 species of deciduous flowering trees and shrubs and 200 naturally occurring hybrids (Argus, 1997). The *Salix* can be broadly divided into two main groups that each has important commercial characteristics : i) tall growing tree willows which includes the weeping willow (*Salix babylonica*), the white willow (*Salix alba*) and black willow (*Salix nigra*) ii) shrubby willows which includes the purple willow (*Salix purpurea*), broadleaf willow (*Salix glaucophylloides*) and the basket willow (*Salix viminalis*) (Wilkinson, 1999). The infrageneric classification of the *Salix* genus is complex and highly disputed due to the wide plasticity of phenotypes, commonality of natural hybridisation and broad species diversity. Early attempts to infragenerically classify the *Salix* genus resulted in the description of two subgenera, *Protitea* and *Euitea* (Kimura, 1928). Tutin et al. (1964) later divided the *Salix* genus into three subgenera, *Salix*, *Chamaetia* and *Caprisalix* based on two morphological values: stamen number and bud scale.

However, recent attempts to classify the subgenera of *Salix* using phylogenetic cpDNA and nrDNA data has proved inconclusive due to the low resolution of clades and differing classifications between studies (Azuma, Kajita, Yokoyama, & Ohashi, 2000; Chen, Sun, Wen, & Yang, 2010). Subsequently, based on the two aforementioned robust morphological characters the *Salix* genus has now been divided into five widely accepted subgenera: *Chosenia*, *Pleuradenia*, *Longifoliae*, *Protitea* and *Salix* (J. Wu et al., 2015).

2.4 Poplar Rust (*Melampsora larici-populina*)

2.4.1 Origin of the *Melamspora* genus

The *Melampsora* genus is the basal ancestral genus in the *Puccinales* (*syn. Uredinales*) group. Historical phylogenetic analyses were indicative of fern rusts (*Uredinopsis spp.* and *Milesina spp.*) occupying the basal ancestral position in the *Puccinales* group phylogeny (Leppik, 1965). However, 18S ribosomal DNA (rDNA) sequences of *Puccinales* species with a high level of confidence (100% bootstrap support) showed that fern rusts did not occupy the basal phylogeny in the rust fungi group (Sjamsuridzal, Nishida, Ogawa, Kakishima, & Sugiyama, 1999). Later rDNA phylogenies which included *Melamspora spp.* were conducive of the *Melamspora* genus occupying the basal position in the rust fungal group (Aime, 2006). The broad host specificity of the *Melamspora* genus is further evidence to support its position as the basal ancestor for the entire rust group (Pei & McCracken, 2005).

2.4.2 Description, Classification and Identification of Poplar Rust

All poplar rusts are members of the *Melampsora* genus that consists of several extinct and extant species, molecular identification methods are available to distinguish genotypes at both the species and individual level. Poplar rusts are

members of the *Melamsporaceae* family that belong to the rust fungi *Puccinales* group. Numerous species of poplar rust exist including the commercially significant *Melamspora larici-populina, Melampsora alii-populina, Melampsora occidentalis, Melamspora medusae* and the naturally occurring hybrid *Melamspora medusae x larici-populina*. Species of rust fungi are often differentiated by distinguishing morphological characteristics of urediniospores (Swertz, 1994). However, in the absence of molecular identification data, poplar rusts are challenging to identify due to echinulate commonalities of the urediniospores (Pascal Frey, Gérard, Feau, Husson, & Pinon, 2005). Tian, Shang, Zhuang, Wang, and Kakishima (2004) developed a taxonomic identification protocol utilising a combination of teleospore and urediniospore morphologies along with host specificity to interspecifically differentiate poplar rusts. However, DNA barcoding and fingerprinting methods have proven to be more effective compared with conventional morphological identification methods in identifying poplar rust at a species and individual level respectively. Reference DNA barcodes have been successfully developed for all *Melamspora spp.* by utilising the cytochrome oxidase 1 (CO1), rDNA 28S and the internally transcribed spacer regions (ITS) (Feau et al., 2009). Interestingly, DNA fingerprinting has been conducted on poplar rust to confirm hybridisation lineages of various pathotypes in France (P Frey, Gatineau, Martin, & Pinon, 1999). Furthermore, Spiers (1998) indicated the need for poplar rust DNA fingerprinting in New Zealand to accurately identify the extent of hybridisations and population dynamics, which could serve as the basis for future studies.

2.4.3 Life Cycle

The poplar rust life cycle is heteroecious and macrocyclic, completing its biological reproductive cycle on two separate hosts in five different spore stages (Figure 2.4-1). During summer periods the uredinia produce dikaryotic urediniospores every 14 days (Van Kraayenoord, Laundon, & Spiers, 1974). These urediniospores are highly mobile in air currents and continue to asexually infect other neighbouring and long distance hosts (Smith, Blanchette, & Newcombe, 2004). Due to the deciduous nature of poplars, urediniospore production ceases post-leaf drop in autumn (Van Kraayenoord et al., 1974). However, the telial stage can occur on fallen leaves, whereby, telia overwinter and produce teliospores (Hacquard et al., 2013). Subsequently, these teliospores can germinate on conifers (*Pinus spp.*) to produce basidiospores (Figure 2.4-2) (Vialle, Frey, Hambleton, Bernier, & Hamelin, 2011). Moreover, basidiospores produce spermagonium to complete the sexual phase of the poplar rust cycle. Aeciospores are then dispersed onto poplars to complete the reproduction cycle. However, in the absence of a suitable secondary conifer host, poplar rusts will continuously asexually reproduce with urediniospores, resulting in reduced genetic variation (Van Kraayenoord et al., 1974).

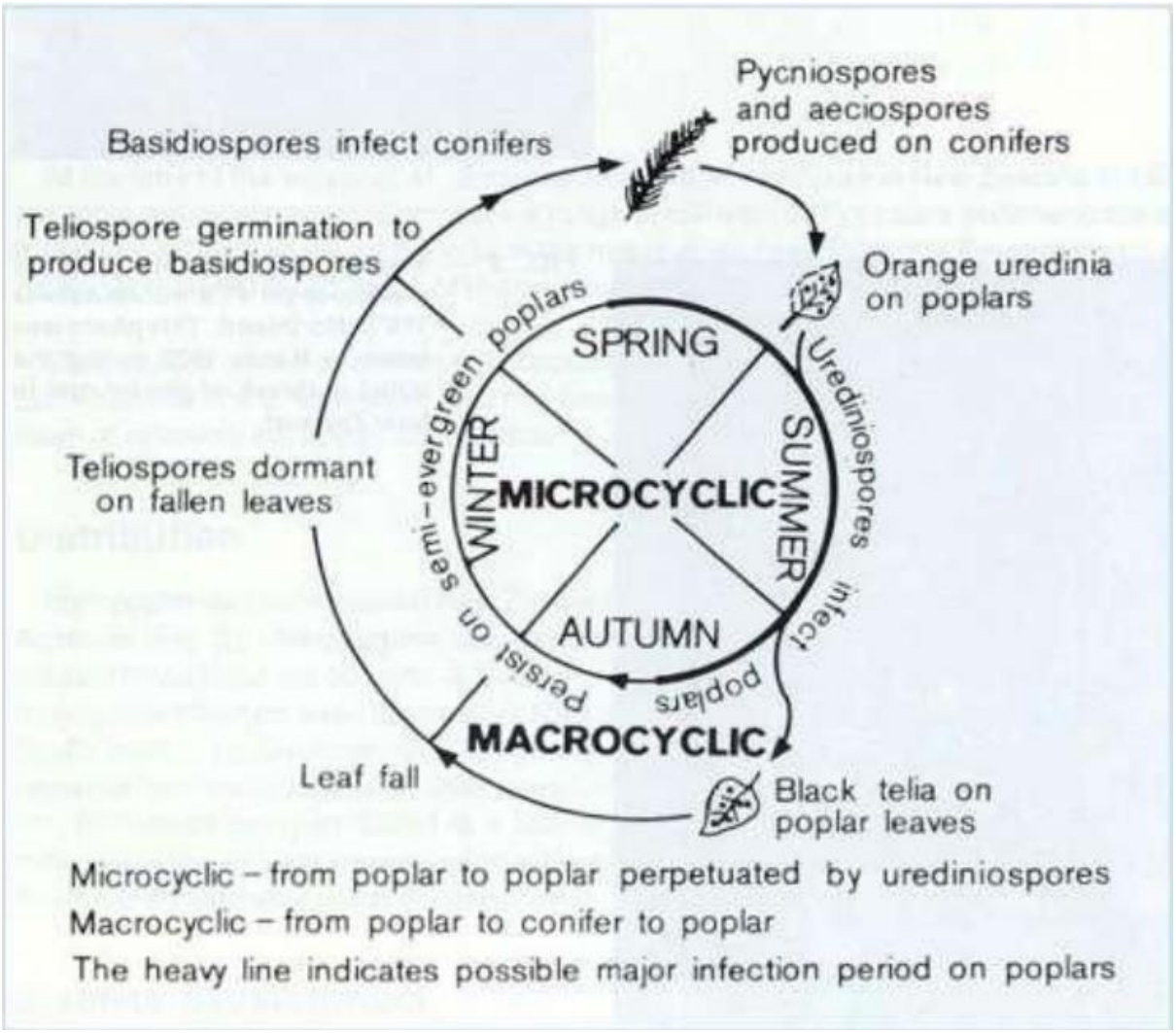

Figure 2.4-1 – Overview of the Life Cycle of Poplar Rust, Source:

(Sivakumaran & Mclvor, 2010)

Figure 2.4-2 - Detailed reproductive life cycle of rust. Source: (Vialle et al., 2011)

2.4.4 Host Effects

Poplar rust is an obligate biotrophic pathogen that can have a significant impact on poplar hosts. Typical symptoms include the presence of bright orange uredia on the underside of the leaf, stunted root/shoot growth, early defoliation and dieback (Figure 2.4-3). Similar to other plant-pathogen systems, growth stunting symptoms are often caused by the diversion of resources and energy from normal processes into defence responses. Transcriptome analyses suggests that infected susceptible clones experience significant changes in defence pathways concurrently with reductions in carbon/carbohydrate metabolism and net

photosynthetic rates (Major, Nicole, Duplessis, & Séguin, 2010). Comparisons of inter-clonal variation in transcriptome analyses also reflects the varying degrees of clonal response to poplar rust infection (Germain & Séguin, 2011).

Figure 2.4-3 - Severe *M. larici-populina* infection on 'Blanc de Garonne' (*P. nigra*). Location: National Poplar Germplasm Center, Palmerston North, New Zealand

2.4.5 Impact of Poplar Rust in New Zealand

Poplar rust had a significant early impact in New Zealand due to the narrow genetic base of planted material and the lack of a robust resistance breeding program. Around March 1973 poplar rust was identified in New Zealand having arrived from Australia via Long Distance Dispersal (LDD) of spores in the atmospheric currents (Spiers, 1989). Since its arrival, poplar rust quickly spread

in both the North and South Islands, causing widespread dieback of the highly susceptible and widely used 'Lombardy' (*P. nigra*) clones (Wilkinson, 1999). Research suggests that the early dieback of poplars in New Zealand was likely aided by the very narrow genetic base that existed at the time (Spiers, 1989). Consequentially, a rust-resistance breeding program was established at the National Plant Materials Centre in Palmerston North in 1973 and the initial actions of the program was to import and evaluate novel rust resistant genetic material. This initially led to the replacement of susceptible *P. nigra* clones with rust tolerant varieties like 'Veronese' and 'Tasman' (*P. deltoides x nigra*). However, further breeding efforts replaced these clones with varieties like 'Weraiti' (*P. deltoides x nigra*), 'Gus' (*P.maximowiczii x nigra*) and 'Androscoggin' (*P. maximowiczii x trichocarpa*) that had a higher degree of rust tolerance.

2.4.6 Distribution and Genetic Variation in New Zealand

The distribution of poplar rust is widespread in New Zealand with very low genetic diversity. Original reports of poplar rust arrival indicated only two species of poplar rust, *M. medusae* and *M. larici-populina* had established in New Zealand (Van Kraayenoord et al., 1974). Later reports indicated that *M. medusae* failed to persist due to the limited presence of secondary-host conifers (Wilkinson & Spiers, 1976). However, a naturally occurring hybrid, *M. medusae x populina*, was identified and described in March 1991 (Spiers & Hopcroft, 1994). It is hypothesised that this hybridisation occurred in Australia before being distributed via-LDD once again to New Zealand. Despite the re-introduction of this hybrid, it is believed to have failed to overwinter and establish in New Zealand. The recent National Poplar Rust Survey confirmed that the only poplar rust species still

present in New Zealand is *M. larici-populina* (Sivakumaran & Mclvor, 2010). The survey also concluded that *M. larici-populina* in New Zealand was likely to have a narrow genetic base due to the lack of secondary-host conifers necessary for sexual reproduction. However, the survey did not analyse the genetic diversity of *M. larici-populina* or its virulence diversity. Despite this, sexual reproduction limitations compounded with a founders effect has likely resulted in very narrow genetic variation of the pathogen (Barrès et al., 2008). Therefore, despite the high level of potential damage that poplar rust poses, the limited number of species and considered low genetic diversity of poplar rust in New Zealand makes breeding poplars for resistance more durable.

2.4.7 Poplar-Poplar Rust Interactions

To effectively control the outcome of poplar rust, it is important to understand the key determinants of the poplar-poplar rust interaction. The completion of the *M. larici-populina* and *P. trichocarpa* genome sequences has provided a model foundation for exploratory analyses of pathosystem gene families, transcriptomes, and proteomes in tree-microbe interactions. Effector proteins are small molecules produced by a pathogen to negatively regulate the cellular processes of the infected host to allow further infection. The diversity of effector proteins play an important role in determining the virulence strains of fungal pathogens like *M. larici-populina*. Non-host effectoromics conducted between *M. larici-populina* and *Nicotiana benthamiana* reveals that a major candidate effector 'MLP124017' in *M. larici-populina* interacts with PopTPR4, a transcriptional corepressor (TOPLESS) gene found in poplars (Petre et al., 2015). However, the significance of this molecular interaction has yet to be elucidated. Most plant resistant genes (*R* genes) encode nucleotide-binding site leucine-rich receptor (NBS-LRR)

proteins, which recognise pathogen-derived metabolites and initiate a type of controlled cellular death known as a hypersensitive response (Shao, Xue, Wang, Wang, & Chen, 2019). To date, two major-qualitative NBS-LRR coding genes, *Mer* and R_1 have been finely genetically mapped on the *P. deltoides* linkage group 19 in a region that is densely clustered with other NBS-LRR coding genes (Bresson et al., 2011). In the same study, a major quantitative resistance gene that strongly influences uredinia size, R_{US}, was finely mapped to the peritelomeric region of the *P. trichocarpa* linkage group 19. It is notable that both *Mer* and R_1 have both been overcome by *M. larici-populina* virulences 7 and 1 respectively (V. Jorge, Dowkiw, Faivre-Rampant, & Bastien, 2005a). After the virulence 7 strain arose in France, widespread devastation occurred in monoculture plantings of 'Beaupré' (*P. deltoides x trichocarpa*) cultivars which carried the *Mer* gene (Dowkiw et al., 2012). Speculatively, the ability for *M. larici-populina* to overcome poplar defences is likely enhanced by monoculture plantings of poplar clones globally. Therefore, an evaluation of rust resistance markers in the breeding program is needed in combination with the encouragement of polyculture plantings by end-users.

2.5 Reproductive Systems

2.5.1 Vegetative Reproduction

Poplar and willow species are both highly capable of effective and rapid asexual vegetative reproduction which is beneficial for the breeding program. Breeding programs often target rooting ability in their selections and there are very limited cases of cultivars being released with poor rooting ability (du Cros, 1984). The ease of vegetative reproduction in poplar and willows is beneficial to the breeding program because clonal varieties are homogenous removing the need for

expensive or time-consuming homogenisation that is experienced in other non-clonal breeding programs. Vegetative reproduction occurs in two distinct ways: propagation via branch material and clonal colonies. It is hypothesised that this high degree of successful vegetative propagation can be attributed to the conserved function of auxin biosynthesis genes in the Salicaceae family (Haissig, 1970; Zhao et al., 2014). Willows and some poplars produce clonal colonies through the differentiation of meristematic tissue in adventitious roots (Haissig, 1972). This provides an evolutionary advantage, whereby, establishing populations have the ability to quickly recolonise riparian zones after a flood event (Krasny, Zasada, & Vogt, 1988). In poplars, clonal colony reproduction occurs through basal shoots that arise from root apical meristematic tissue on a 'mother plant' which subsequently differentiates into clonal suckers (Ahuja, 1987). Moreover, under certain environmental conditions, massive clonal colonies can form. As an example, in Utah, United States, an 80,000 year old 'Pando' colony of *Populus tremuloides* extends over 43.6ha and consists of an estimated 47,000 clonal stands, making it both the oldest and largest organism by mass (DeWoody, Rowe, Hipkins, & Mock, 2008).

2.5.2 Willow Pollination

Willows are a dioeciously enforced outcrossing group with mixed pollination systems. Willows' floral organs consist of long and narrow flower spikes called catkins. In New Zealand, willows produce pollen from June to November and fruit maturity is reached approximately one month post-pollination (Newstrom-Lloyd, 2013). Willows have a rare generalist bimodal pollen transmission system, consisting of wind and insect vectors (Fox, 1992; L. L. Wang et al., 2017). Consequentially, to avoid cross-contamination in a breeding context, the

combination of outcrossing and a bimodal pollen transmission system makes flower isolation critical (R. Frankel & Galun, 2012). Entomophily is the primary pollen transmission vector and achieved via the production of chemical attractants, moderate nectar quantities and adhesive pollen (Dötterl, Füssel, Jürgens, & Aas, 2005; Dötterl, Glück, Jürgens, Woodring, & Aas, 2014). In contrast, anemophily is a secondary vector and seed-set success rates in different species range between 30% (*Salix elaeagnos*) and 10% (*Salix triandra*) (Karrenberg, Kollmann, & Edwards, 2002). Furthermore, wind pollination success rates also significantly vary between years, populations and ecotypes (Elmqvist, Ågren, & Tunlid, 1988; Tamura & Kudo, 2000). Subsequently, this unique bimodal pollination system has aided the widespread distribution and diversity of willow species.

2.5.3 Poplar Pollination

Poplars are an anemophilic and dioecious outcrossing group. Like willows, poplars' floral organs are catkins. However, in contrast to willows, poplar catkins have larger numbers of stamens conglomerated into the floral bud (Cronk, Needham, & Rudall, 2015). Consequentially, the larger number of stamens produce greater amounts of pollen which compensates for the wasteful process of wind pollination. In New Zealand, pollen production occurs during September and October with fruit maturity occurring two months after successful pollination (Wratt & Smith, 2015). Some interspecific incompatibilities exist, which are often caused by heterogenous cytochemical and structural characteristics of pollen and stigmas in the *Populus* genus (Willing & Pryor, 1976). *P. alba* and *P. nigra* experience gametophytic incompatibility, whereby pollen tube arrest occurs in the style (Villar, Gaget, Said, Knox, & Dumas, 1987). Often these interspecific

incompatibilities are distinctly intersectional, for example, species in the *Populus* section are incompatible with other sections, whereas, species in the *Tacamahaca* and *Aigeiros* section are compatible with the majority of other sections (Willing & Pryor, 1976). In wild populations, intersectional incompatibilities could theoretically reduce the frequency of spontaneous interspecific hybrids which reduces the transfer of rust resistant genes between genera and increases genetic variation between incompatible sections. Due to the theoretical increased genetic variation between incompatible sections, domestic hybridisation between two incompatible sections should theoretically result in a high level of genetic diversity. To overcome pollination barriers in the breeding program, organic solvents with low dielectric constants, such as, n-hexane or ethyl acetate can be applied to the stigmas to remove species-specific rejection factors (Jain & Shivanna, 1988; Williams, Clarke, & Knox, 2013).

2.6 Soil Erosion in New Zealand

2.6.1 Overview

Soil erosion is one of the largest environmental issues facing New Zealand. New Zealand is a small, hilly and mountainous country in the south-west Pacific, renowned for unpredictable weather, high winds, geological events and fertile soil. According to Basher (2013), four types of erosion exist in a New Zealand context:

- surface erosion
- hill erosion
- mass-movement erosion
- waterway erosion.

While New Zealand accounts for less than 0.1% of the world's surface, it is responsible for 200MT or 2% of the world's soil erosion (Hicks, Hill, & Shankar, 1996). New Zealand's soil erosion costs an estimated 24 million New Zealand Dollars (NZD) to control, while the damage caused by soil erosion is estimated to cost over $NZ103 million (Basher, 2013). Soil erosion is typically a natural process, however, widespread deforestation events combined with environmental effects has resulted in a significant increase in the frequency of landslides and soil erosion (Derose, Trustrum, & Blaschke, 1993; Glade, 2003; Wilmshurst, 1997). Furthermore, the effects of climate change and extreme weather events are expected to accelerate soil erosion in the future.

2.6.2 Agriculture and Soil Erosion

The majority of soil erosion in New Zealand is from agricultural land, particularly sloping agricultural land. This has led to significant loss in agricultural production. In New Zealand, 20% of the gross domestic product (GDP) relies on the top 150mm of soil (Mackay, 2008). The majority of New Zealand's agricultural industry is pastoral-based, consisting of primarily of dairy production followed by beef, sheep and lamb (Bascand, 2012). While, pastoral agriculture accounts for a large proportion of New Zealand's economy, it is also responsible for 44% of New Zealand's soil erosion problem (Basher, 2013). Agricultural-related soil erosion has predominantly been caused by a combination of poor management practices, overgrazing of pasture, deforestation and high intensity rainfall (Wilkinson, 1999). The effects of soil erosion on agricultural production are widespread in New Zealand, with a significant loss of mobile nutrients from the topsoil which, in turn, increases the demand for fertiliser inputs and leads to waterway contamination with nitrogen, phosphorus and sediment and negatively

impacts the global image for New Zealand's agricultural products (Haygarth & Jarvis, 1999).

2.6.3 Soil Erosion's Impacts on Infrastructure

While the loss of agricultural production is a primary effect of soil erosion, the damage to infrastructure, increased waterway pollution, disrupted communications and the increased severity of flood events are also major concerns. In New Zealand, over ten thousand landslide events extending over 20,000km^2 per-annum result in extensive damage to road and rail infrastructure while stretching emergency response services, particularly during storm events (Crozier, 2005). Soil erosion can result in significantly increased levels of nutrients and suspended sediment in waterways, which negatively impacts biological community structure, increases the mortality of native freshwater species and causes toxic-algal blooms (Dorich, Nelson, & Sommers, 1984; Ryan, 1991). The severity of flood events can also be increased by the large volumes of sediment causing localised damage downstream in river systems and tributaries (Mike Marden, 2004). Therefore, significant investment into soil erosion control is required in New Zealand to curb its widespread negative impacts.

2.6.4 Poplars and Willows for Erosion Control

2.6.4.1 Poplars

The sustainability and reversal of soil erosion in New Zealand's pastoral production systems relies on increasing the durability and frequency of tree plantings. In New Zealand, erosion control on agricultural hill country is the primary use for poplar trees, one of few countries to do so (Isebrands & Richardson, 2014). This exploits poplars' rapid root and height growth, moreover, the roots systems are extensive and act as large underground nets to stabilise large

areas of soil (Wilkinson, 1999). Poplars planted against the prevailing wind can also reduce wind erosion rates on loose soil (Kort, Collins, & Ditsch, 1998). A study conducted on treed and untreed pastoral hill sites showed that poplars reduced landslide frequency by 95% on planted areas compared to non-planted controls, while landscape scarring was also significantly reduced (G. Douglas et al., 2013). However, cultivar and species vary in their erosion-control suitability, depending on the environment. *P. deltoides x yunnanensis* and *P. maximowiczii x P. nigra* were found to have significantly higher root biomass, a key soil stabilisation trait, compared to four other species/hybrids in a multi-site trial (G. B. Douglas, McIvor, & Lloyd-West, 2016). Significant variation in erosion-control suitability is also found within species groups. For example, *P. maximowiczii x P. nigra* clonal poplar trials conducted on numerous unstable pastoral hill country sites revealed significant variation in diameter at breast height (DBH) between-sites which is a measurement correlated to root size and erosion control ability (McIvor, Hedderley, Hurst, & Fung, 2011). Furthermore, the same study also highlighted varying degrees of survivability and clone-site suitability, which impacts soil erosion control.

2.6.4.2 Willows

Tree and shrub willows are used principally for streambank erosion control in New Zealand due to their flooding resistance, adaptations to a riparian habitat, long fibrous root system, rapid growth, high evapotranspiration rates and ease of propagation. Willows have the ability to contribute to riverbank stability by maintaining its course, reducing river speeds near the rivers' edge, encouraging silt deposits and preventing stock from accessing the riverbank (Isebrands & Richardson, 2014). Willows have fine fibrous roots that have a high binding

effect in coarse soils and riverbanks, causing a large stabilisation effect (Wilkinson, 1999). Experiments conducted using river hydrology wave modelling suggests that 100m of *Salix alba* riverbank plantings can attenuate wave heights in extreme conditions by around 70% compared to 100m of unplanted riverbanks (De Oude et al., 2010). This wave attenuation effect significantly reduces the level of energy applied to a riverbank and, therefore, reduces the rate of soil erosion. Willows also act as silt traps, building up silt in planted areas so reversing soil erosion and reduce the negative impacts of sediment yield in downstream catchment areas (Rey & Burylo, 2014).

2.7 Applications of Poplars and Willows in New Zealand

2.7.1 Growth Habits and Suitability

Poplars and willows have numerous growth habits that make them particularly suited to New Zealand's environment and conditions. Poplar and tree willows can be established from bare poles in the presence of grazing livestock, which reduces the level of input required to successfully establish plantings (Wilkinson, 1999). Compared to other temperate species like eucalypts (*Eucalyptus spp.*), poplars and willows have a rapid early growth rate, which allows for faster establishment and greater usability (R. Ceulemans, McDonald, & Pereira, 1996). Poplars and willows are also highly suited to a silvopastoral environment where the narrow canopy form of some cultivars allows for greater light permeability to the forage understory compared to other temperate tree species (McIvor & Douglas, 2012).

2.7.2 Livestock Shade

Poplars and willows provide essential shade and for livestock in New Zealand. During drought conditions in New Zealand, temperatures can regularly exceed 30C° and with the advent of climate change these temperatures are likely to

increase (Plummer et al., 1999). Due to historical deforestation practices and the outdoor nature of New Zealand's agricultural system many pastoral farms lack natural shading. This has consequences on livestock production and welfare where excessive temperatures and thermal radiation are known to reduce milk and beef productivity and negatively impact livestock physiology (Bohmanova, Misztal, & Cole, 2007; Finch, 1986; Schütz, Rogers, Poulouin, Cox, & Tucker, 2010). Poplar and willow trees planted on New Zealand farms have reduced understory temperatures by 3C° and increased the provision of shade to livestock positively impacting productivity, animal welfare and public perception of farm practices (McGregor, Mackay, Dodd, & Kemp, 1999).

2.7.3 Livestock Fodder

Poplars and willows are nutritious and palatable trees for livestock which contributes to their role in a silvipastoral environment. Separate studies on the effects of poplar and willow supplementation on ewes during a drought both highlighted that crude protein intake was significantly higher in the supplemented group which resulted in lowered live weight loss and greater reproductive rates compared to the un-supplemented group (McWilliam, Barry, Lopez-Villalobos, Cameron, & Kemp, 2004; Pitta, Barry, Lopez-Villalobos, & Kemp, 2005). Micronutrient deficiencies like cobalt (Co) are relatively common in New Zealand pasture and cause muscular dystrophy and starvation symptoms in ruminants (Graham, 1991). Poplars and willows accumulate micronutrients at significantly higher ratios compared to pastoral plants and can alleviate some micronutrient deficiencies in agricultural ruminants (Robinson et al., 2005). However, certain cultivars are more effective in supplementing fodder nutrition. For example, 'Yeogi' (*P. alba x glandulosa*) and 'Crow's Nest' (*P. x*

euramericana x nigra) accumulate minimum amounts of toxic cadmium (Cd) and higher amounts of zinc (Zn), copper (Cu) and Co compared to other cultivars (Robinson et al., 2005). Therefore, breeding applications could be used to improve the nutritional content of poplar and willow varieties.

2.7.4 Bioremediation

Poplars and willows both play a role in the bioremediation of New Zealand soils. There is research to suggest the suitability of poplars and willows as pioneering trees for the bioremediation of barren and contaminated ex-timber sites (Robinson & McIvor, 2013). In agricultural systems, riparian willows can be used to prevent livestock from entering and contaminating waterways while also providing biological barrier for contaminants and runoff from pastoral farms (Robinson & McIvor, 2013). Cadmium (Cd) is a heavy metal trace element that has risks for New Zealand's agriculture. Cd, as a contaminant in super-phosphate fertiliser, can accumulate to high levels in pastoral soils. Subsequently, Cd can be passed through the food chain and can impact both animal and human health (Waalkes, 2000). If Cd reaches toxic levels in localised soils, willows and poplars present a useful bioremediation option. Research suggests that both poplars and willows have a high Cd accumulation coefficient and can significantly reduce localised Cd levels through cellular compartmentalisation (Robinson et al., 2005). However, Cd bioaccumulation-rate varies within clones of poplars and willows, with most willow varieties outperforming poplar varieties in Cd bioaccumulation rates (Kuzovkina, Knee, & Quigley, 2004; Robinson et al., 2005). Due to the variation of inter-clonal bioremediation-capabilities that exist in the germplasm collection and the increasing demand for phytoremediation, future breeding

initiatives could focus on improving the suitability of poplars and willows in remediating New Zealand's soils.

2.7.5 Shelterbelts

New Zealand's oceanic climate and geography subjects it to high wind conditions all-year round. Because wind has a significant depressing effect on agricultural production, its management is essential. Poplars planted in shelterbelts are a common wind control strategy because of their rapid establishment, high wind tolerance, form and height. Since the advent of poplar rust (*Melampsora larici-populina*) in New Zealand the 'Lombardy' poplar has been replaced in windbreaks by *Populus x euramericana* cultivars and *Salix matsudana x alba* willow cultivars (Wilkinson, 1999). In the horticultural industry, wind can have a significant impact on fruit quality, with some estimates suggesting a 20% increase in kiwifruit rejection rates if wind is uncontrolled in an orchard (McAneney, Judd, & Trought, 1984). Therefore, the capacity for wind damage in New Zealand's 1.6 billion kiwifruit industry is high. Furthermore, windbreaks can also improve orchard production practices by reducing spray drift and irrigation inputs.

2.7.6 Apiculture

Willows are a valuable spring fodder source for honeybees (*Apis mellifera*) in New Zealand's rapidly expanding $NZ328M p/a apiculture industry (Plant & Food Research, 2017). During the post-winter period, bee colonies undergo a rapid eight-fold expansion in number, this dramatically increasing the hive demand for high-protein pollen to support young-nurse bee growth (Haydak, 1970). Subsequently, hives that have access to high-protein pollen during the spring-time experience significant two-fold increases in honey production during

the year due to improvements in young-nurse bee immunity, metabolism and survivability (Di Pasquale et al., 2013). Compared to other pollen sources, willow pollen is considered to be highly nutritious with a high protein content and a complete amino acid profile (Day, Beyer, Mercer, & Ogden, 1990). A nationwide survey of New Zealand's honey composition revealed that more than half of the samples tested contained significant quantities of *Salix spp.* pollen (Moar, 1985). Furthermore, during the spring-time honey bees have a high-level of preference for willow species along with Rosaceae fruit trees, oak (*Quercus spp.*), white clover (*Trifolium repens*) and dandelion (*Taraxacum officinale*) (Keller, Fluri, & Imdorf, 2005). Therefore, the abundance of willows in New Zealand's agricultural landscape helps to support a vulnerable period in the honey production cycle.

2.7.7 Carbon Sequestration

Poplar and willow plantings are a part of the solution for New Zealand's transition to a low emissions economy. Global warming is a growing international problem that is anthropogenically escalated by excessive levels of atmospheric carbon (Cox, Betts, Jones, Spall, & Totterdell, 2000). The Kyoto Protocol outlines the need for afforestation of agricultural land as part of the solution for reducing atmospheric carbon (McCarl & Schneider, 2001). Poplar and willows are ideal for carbon storage in New Zealand's agricultural environment due to their rapid growth, high rate of carbon sequestration and silvopastoral suitability (McIvor & Douglas, 2012; Rytter, 2012). To support tree plantings, in 2017 New Zealand's Ministry for Primary Industries (MPI) introduced their 'One Billion Trees Programme' with the aim of planting one billion trees by 2028 manage climate change (by extracting and storing atmospheric carbon), promote

biodiversity, optimise land use and boost public perception (M Marden, Lambie, & Rowan, 2018). Poplar and willow are likely to play a role in the one billion tree initiative due to their rapid growth, easy propagation, low maintenance and transportability in the hill country. Furthermore, MPI has also implemented the Emissions Trading Scheme (ETS) that encourages agricultural land owners to create planted areas on pastoral land that exceed one hectare and 30% canopy cover in return for carbon credits.

2.8 Breeding Program Overview

2.8.1 Introduction and Background

New Zealand's poplar and willow breeding program is based in Palmerston North which is located in the temperate Manawatu region. Poplar and willow trees were advanced in the 1950s to stabilise agricultural slopes and riverbanks in the North Island of New Zealand. To support this application, a poplar and willow breeding program was created in the 1960s by Chris van Kraayenoord under the National Plant Materials Centre (NPC) with three primary objectives: i) maintain and enhance the genetic stock ii) breed new improved varieties iii) transfer of technology to regional councils and farmers (Wilkinson, 1999). Since the implementation of the breeding program, numerous successful clonal varieties have been released with a high degree of suitability in New Zealand's demanding climate.

2.8.2 Germplasm

Germplasm consists of imported materials that undergo a strict quarantine process adhere to New Zealand's biosecurity regulations. The national poplar and willow collection centre is located in the Manawatu and holds the majority of clonal accessions. However, numerous accessions are also held nationwide on privately

owned land. The core willow germplasm collection contains 203 accessions, consisting of 38 pure species, 18 interspecific hybrids and one intraspecific hybrid. The core poplar collection contains over 100 accessions consisting of 12 pure species and 12 interspecific hybrids from the *Populus*, *Aigeros* and *Tacamahaca* sections. While the germplasm accession is extensive, there has been no research into germplasm genotyping, which provides an opportunity for this study.

2.8.3 Hybridisations and the Breeding Cycle

The majority of hybridisations in poplar and willows are interspecific in order to increase the genetic diversity of cultivars. Between August-October a small number of crosses are made, depending on the species and flower/pollen maturity times (Figure 2.8-1). Pollination is usually controlled using sterilised equipment and isolation bags. Seeds are collected and germinated in November-December before being transplanted to root trainers. Juvenile seedlings are exposed to poplar rust (*Melampsora spp.)* at an early development phase, this increases the duration of exposure and removes extremely susceptible genotypes from the breeding population at an early stage (Steenackers, 1972). F_1 Populations (n=200-800) are established on private land using a variety of trial designs and after two years a selection pressure of 10% is applied. Selected individuals are clonally bulked before being exposed to multi-site and multi-year trials lasting approximately 15 years. Subsequently, after this trial process, high quality clones are selected for varietal release and may be registered for Plant Varietal Rights (PVR) and released clonally as cultivars. However, the low level of morphological variation between released cultivars means PVR protection is challenging. Therefore, genotyping could be applied to identify in-use cultivars.

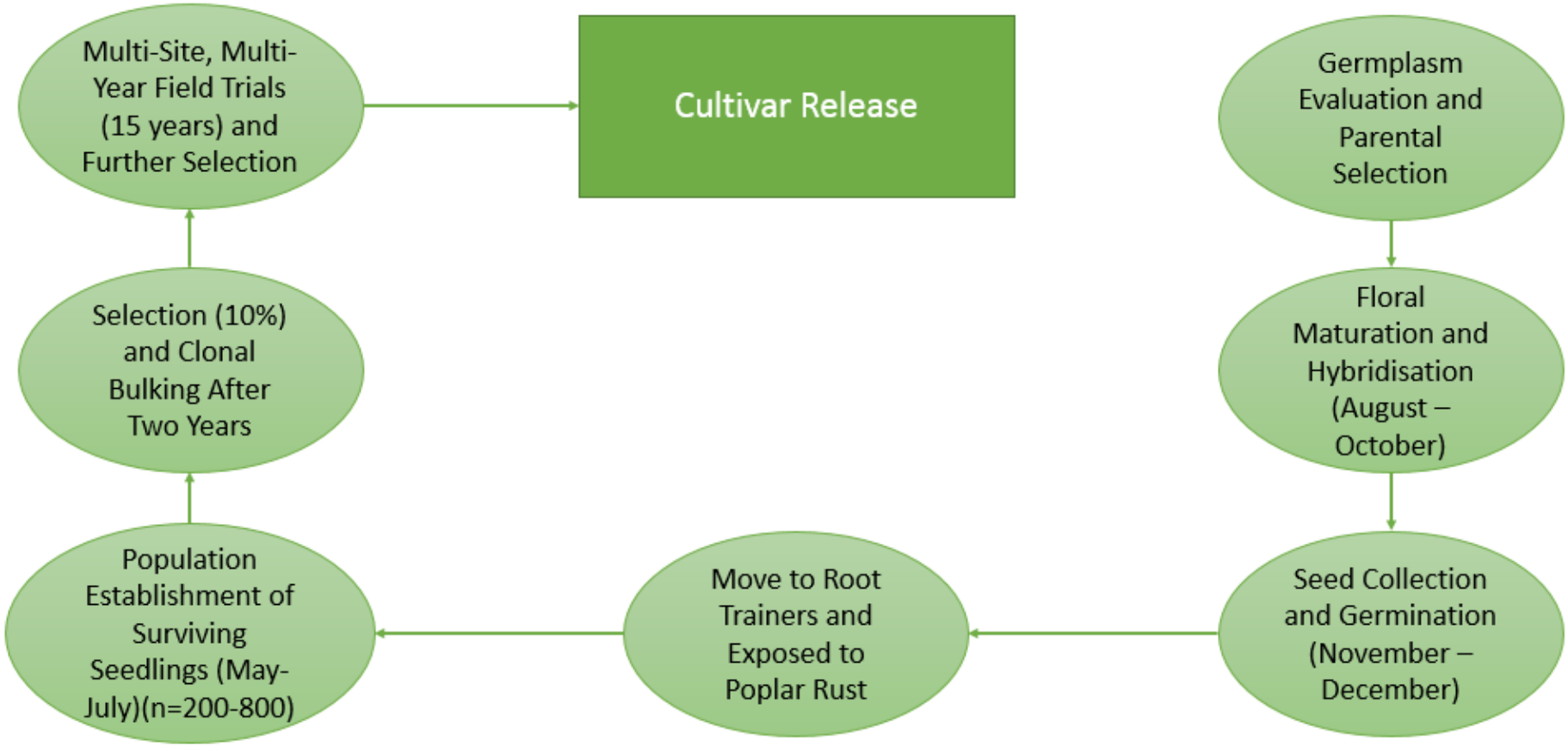

Figure 2.8-1 - Visual Overview of Poplar and Willow Breeding Cycle

2.8.4 Primary Trait Selection Criteria

2.8.4.1 Vigour

Vigour is an important trait in poplars and willows because clones must be able to establish quickly from vegetative cuttings to provide soil erosion control and avoid unwanted grazing by livestock. Therefore, vigour at a juvenile stage is particularly important and selected for early in the breeding cycle. Vigour is a complex quantitative trait that is controlled by numerous gene-environment interactions (Hu et al., 2018). Vigour can be phenotyped in poplars and willows by measuring DBH and height growth over time (McKown & Guy, 2018). Because clones are often established in hill countries with dry soils and high erosion conditions, early root establishment and root biomass accumulation under drought conditions is vital to ensure survivability (G. B. Douglas et al., 2016). Because vigour is highly influenced by environmental factors and complex gene-environmental interactions, careful site selection is important in multi-site trials (Wilkinson, 1999).

2.8.4.2 Tree Architecture

A straight stem is important in commercial poplar and willow clones because it improves the transportability of poles and also the usability of the clones in a silvipastoral environment. A considerable level of variation in form, branching angle and tree architecture has been reported across different poplar species and clones (Reinhart Ceulemans, Stettler, Hinckley, Isebrands, & Heilman, 1990). In New Zealand, pastoral farmers have a preference for upright and narrow trees that minimise shading of the pasture understory. Branch lateral spread is determined by using branch-angle measurements and visual appraisal.

2.8.4.3 Wind Resistance

Breeding for wind resistance in New Zealand is important because clones planted in high erosion zones are often subject to strong wind conditions. In high wind zones, poplar and willow branches have a high degree of leverage and trees are subject to branch breakages or uprooting (Mitchell, 2013). Therefore, effective breeding for wind resistance has two selection components, wind-resistant branch architecture and strong root systems. Wind resistance is a highly complex trait and cultivars are evaluated during extensive multi-year, multi-site field trials nationwide. While poplars and willows can be selected for wind resistance, the use of pollarding, which involves canopy removal, is reported to be another effective wind-protection technique (McIvor & Douglas, 2012).

2.8.4.4 Drought Tolerance

As global warming increases, New Zealand is often subject to prolonged periods of drought, particularly in the eastern hill country where poplars and willows are located. To improve clonal durability, drought tolerance is an important selection trait for New Zealand poplar and willow breeders. Drought tolerance is a highly complex trait controlled by numerous functional and regulatory genes (Shinozaki & Yamaguchi-Shinozaki, 2006). The mechanism behind drought tolerance is usually avoidance through stomatal regulation, biomass ratio modifications and leaf abscission, all of which can be phenotyped in controlled environmental trials (Pallardy & Kozlowski, 1981). Drought tolerance is phenotyped in New Zealand's breeding program through controlled environment expriments using biomass growth, stomatal conductance, water use efficiency and water potential measurements as indicators. These phenotyping methods have revealed a good degree of drought tolerance in some hybrid poplar germplasm, which is promising for future improvements (T. Jones, McIvor, &

McManus, 2016). There are also reports in the literature of numerous willow varieties that are highly drought tolerant and used as fodder supplementation during periods with low-rainfall (G. Douglas, Bulloch, & Foote, 1996).

2.8.4.5 Pest and Disease Resistance

2.8.4.5.1 Possums

Possums (*Trichosurus vulpecula*) are New Zealand's most destructive herbivory pest and the defoliation of poplar and willow clones reduces their usability, which makes possum-resistance an important trait of focus for the breeding program. Possums in New Zealand preferentially seek shelter and nutrition from willow and poplar trees (Cowan, 2001). They can damage poplar and willow trees through bark damage in winter, bud removal during spring and defoliation during summer (Thomas, Warburton, & Coleman, 1984). Selection for possum resistance involves observations of possum damage during field trials and no genotyping or advanced phenotyping has been reported in the literature. To increase the durability of poplar and willow clones, breeders have selected low palatable species groups like *Salix matsudana* and hybridised them with other resistant species groups like *Salix pentandra* (Wilkinson, 1999). The phenolic glycoside, salicin, is believed to be the primary compound responsible for possum-resistance in poplars and willows. Quantitative Trait Loci (QTL) mapping has been conducted on salicin in *Salix purpurea* using RAPD markers and could serve as a basis for genotyping for possum-resistance in the future (Sulima, Przyborowski, & Załuski, 2009).

2.8.4.5.2 Giant Willow Aphid

Giant Willow Aphid (GWA) (*Tuberolachnus salignus*) is the most important pest of current concern to New Zealand's willow plantations. GWA is a sap-sucking aphid that infests willow trees and occasionally some poplar trees. GWA has dispersed throughout

the North and South Island of New Zealand since its arrival in 2013 (Sopow, Jones, McIvor, McLean, & Pawson, 2017). The damage potential for willows is high since a single GWA can assimilate saccharides produced by 5-20 cm^2 of leaf area per day (Mittler, 1958). Consequentially, the flow of solutes is hindered, resulting in significantly reduced growth and tree health. Presently, no constitutively GWA resistant willow cultivars exist in New Zealand due to the lack of host specificity exhibited by GWA (Sopow et al., 2017) However, there appears to be a wide range of phenotypic host preferentiality between clones in the literature with a wide degree of environmental influence, indicating potential polygenic resistances (Collins, Fellowes, Sage, & Leather, 2001). Therefore, future breeding efforts could include QTL mapping and further phenotypic germplasm screening for resistances.

2.8.4.5.3 Poplar Rust

Poplar rust (*Melampsora spp.*) is the most important pathogen of concern to New Zealand's poplar plantations. There is a wide range of variability of poplar rust resistant genotypes in the National Poplar Germplasm Collection, ranging from highly susceptible clones like 'Lombardy' (*P. nigra*) through to completely resistant clones like 'SV72' (*P. deltoides*). Rust resistance is screened using phenotyping methodology developed by Schreiner (1959). This involves manually scoring each individual twice in the growing season to give a score for the average area of uredinia coverage area on the leaf, number of infected leaves and total estimate of tree infection. However, this phenotyping method is time consuming and subjective. Therefore, this study will explore the potential of a faster and more efficient genotyping method that could provide an objective indication for the presence of rust resistance in both breeding populations and germplasm accessions (2.9.6).

2.9 Genotyping with Microsatellites

2.9.1 Advantages

Simple Sequence Repeats (SSRs) are tandem repeat (2-10 nucleotides) microsatellite markers that are highly beneficial in genotyping studies due to their selective neutrality, Mendelian inheritance, high polymorphism, codominance and reproducibility. SSRs are flanked by highly conserved flanking regions that allows for the amplification of the repeating region through Polymerase Chain Reaction (PCR). Because SSRs are located non-coding regions of the genome, they are selectively neutral. When a mutation occurs in a microsatellite region it results in the loss or gain of an entire repeating motif (2-10 nucleotides) which leads to higher rates of mutation and polymorphism compared to other regions of the genome (Chakraborty, Kimmel, Stivers, Davison, & Deka, 1997). SSRs are codominant markers that can distinguish between heterozygotic and homozygotic loci, this allows for the determination of heterozygosity rates, genetic diversity and marker usefulness. Despite small differences in allele sizing, studies suggest that SSRs are one of the most reproducible markers (C. Jones et al., 1997).

2.9.2 Marker Comparisons

Short Nucleotide Polymorphisms (SNP) and SSRs were the two primary marker types considered for this study. SNPs are a common marker type that consists of variation in a single base pair position within a genome (Brookes, 1999). SNPs have some inherent advantages over SSRs including: their abundance in a genome and the fact that they do not rely on fragment size allows for greater standardisation across multiple studies and laboratories (Nybom, Weising, & Rotter, 2014). However, the main difference between SNPs and SSRs is in their mutation rate, which is much greater in SSRs compared to SNP (Van Inghelandt, Melchinger, Lebreton, & Stich, 2010). Therefore, over ten-times the amount of data is required to achieve the same resolution in SNPs compared to

SSRs, which can make data analysis and management more time consuming (Yang et al., 2011). Because this study is analysing a large of genotypes (n = 299) in a relatively short time period with limited resources, a smaller dataset is preferred, warranting the use of SSRs over SNPs.

2.9.3 DNA Fingerprinting

2.9.3.1 History

The discovery of the power of microsatellites to discriminate between individual genotypes is one of the greatest discoveries in modern biological science. Alec Jeffreys and his team first discovered DNA 'fingerprinting' at the University of Leicester in 1985 when they found that "a probe based on a tandem-repeat of the core sequence can detect many highly variable loci simultaneously and can provide an individual-specific DNA 'fingerprint'" (A. J. Jeffreys, Wilson, & Thein, 1985, p. 67). Their discovery led to the modernisation of forensics and provided the basis for the molecular identification of suspects or victims crimes across the globe (Roewer, 2013). Jeffreys team's discovery later translated from forensic science into animal science and orthinology in 1987 when the first DNA fingerprinting studies of dogs, cats and birds were first published (Burke & Bruford, 1987; A. Jeffreys & Morton, 1987). Interestingly, DNA fingerprinting was first applied in agricultural and botanical science in 1988 when branches from the same *P. deltoides* tree were shown to have identical DNA fingerprints, highlighting the somatic stability of microsatellites (Rogstad, Patton, & Schaal, 1988). In the same year, Dallas and Rykov successfully discriminated between different rice and barley genotypes respectively in two separate studies (Dallas, 1988; Ryskov, Jincharadze, Prosnyak, Ivanov, & Limborska, 1988). DNA fingerprinting was adopted by agriculture in the mid-1990s for germplasm identification in soybeans (Rongwen, Akkaya, Bhagwat, Lavi, & Cregan, 1995), *Phaseolus spp.* (Hamann, Zink,

& Nagl, 1995) and *Brassica spp.* (Bhatia, Das, Jain, & Lakshmikumaran, 1995). The use of microsatellites in DNA fingerprinting for cultivar identification are still widely published in a variety of species including mango (Surapaneni et al., 2019), sesame (Bhattacharjee et al., 2020) and potato (Duan et al., 2019).

2.9.3.2 Poplar Germplasm

The publishing of the poplar genome in 2004 caused a surge in highly successful SSR-based DNA fingerprinting projects involving poplar germplasm. The earliest poplar DNA fingerprinting study was non-microsatellite based and successfully generated the first random amplified polymorphic DNA (RAPD) fingerprint of 32 elite poplar cultivars to protect breeders' rights (Castiglione et al., 1993). However, RAPD markers are less informative and more time consuming in their application compared to SSRs, which resulted in the demand for SSR identification in poplars (Powell et al., 1996). Later, 12 SSRs were successfully isolated and characterised in trembling aspen (*Populus tremuloides*) (Dayanandan, Rajora, & Bawa, 1998). Early DNA fingerprinting studies on poplar germplasm were primarily concerned around the validity of markers, and proved to be highly successful (Rahman, Dayanandan, & Rajora, 2000). Rahman and Rajora (2002) first used 12 SSR markers to distinguish the identity of 17 *Populus canadensis* cultivars. The US Department of Energy's Joint Genome Institute published a very high quality 520mbp (megabase pair) *P. trichocarpa* genome arranged in 1446 scaffolds in 2004 (Tuskan et al., 2004). Consequently, this enabled a huge resource for SSR mining, leading to the widespread characterisation of novel microsatellites. Since the genome project, SSR-based DNA fingerprinting studies in poplars have been conducted across the globe on germplasm in Italy (Fossati et al., 2005), China (Gao et al., 2006; H. Liu et al., 2016) and Serbia (Galović et al., 2010; Saša et al., 2009). DNA fingerprinting has also been reported in ex-situ conservation germplasm collections in

Europe (Storme et al., 2004). Each study has consistently differentiated between genotypes, identified clonal duplicates and highlighted that SSR markers are highly informative in poplars. Despite the success of DNA fingerprinting studies in poplar, there has been no published literature post-2016. While the application of DNA fingerprinting studies in poplar germplasm has been validated, there has been no application of the technique in New Zealand's poplar breeding program. Therefore, a study is warranted to evaluate the usefulness of DNA fingerprinting on poplars and willows and its application in New Zealand's breeding program.

2.9.3.3 Willow Germplasm

Compared to poplars, the use of DNA fingerprinting in willows was very limited until their development as a woody biomass crop. Similarly to poplars, early studies on willows utilised non-microsatellite markers like random amplified polymorphic DNA (RAPD) (Chong, Yeh, Aravanopoulos, & Zsuffa, 1995), restriction fragment length polymorphisms (RFLP) (Lin, Hubbes, & Zsuffa, 1994) and amplified fragment length polymorphisms (AFLP™) (J. H. Barker et al., 1999) on very small sample sizes (<15) (Chong et al., 1995; Lin et al., 1994). However, RAPD, RFLP and AFLP markers lack the efficiency and informativeness of SSRs and are less efficient in their application (Powell et al., 1996). Rahman et al. (2000) identified 12 SSR markers in poplars and highlighted that they were also conserved and highly informative in willow species, paving the way for future studies. The poplar genome project (Tuskan et al., 2004) also served as a rich source for microsatellites in willows due to the high level of macrosynteny between the two groups (Dai et al., 2014). Despite the availability of SSRs, DNA fingerprinting studies in willows have been very limited compared to poplars until their uptake as a valuable biomass crop (Volk et al., 2011). Following this emergence, microsatellite-based DNA fingerprinting has been successfully conducted

on novel willow biomass germplasm in Canada (Ngantcha, 2010), India (Raja, Singh, & Bhat, 2018; Singh, Joshi, Choudhary, & Sharma, 2013) and China (Q. Wu, Liang, Dai, Chen, & Yin, 2018) to distinguish between clones and assess the genetic relationships. Despite the success of DNA fingerprinting in willow germplasm collections across the globe, the technology has never been implemented in New Zealand's willow breeding program. Therefore, this study intends to evaluate the usefulness and application of DNA fingerprinting in New Zealand's willow breeding program.

2.9.4 Genetic Diversity

Improving the genetic diversity of released clonal varieties is one of the key objectives in New Zealand's poplar and willow breeding program. Greater genetic diversity is seen as important to withstand changing environmental pressures. The fundamental goal of durable plant breeding is to maximise the frequency of desirable alleles in released cultivars. In order to attain this and improve the management of genetic resources, it is important to understand and characterise the genetic diversity of germplasm collections (O. Frankel, 1984). The use of microsatellite markers has been touted as the 'gold standard' for assessing the genetic diversity among and within groups, populations, individuals or species; this is because of their low application cost, high polymorphism and codominance (Hoshino, Bravo, Nobile, & Morelli, 2012). SSRs have successfully been used extensively to characterise germplasm diversity in various tree crops including English walnut (*Juglans regia*) (Bernard, Barreneche, Lheureux, & Dirlewanger, 2018), hazelnut (Gökirmak, Mehlenbacher, & Bassil, 2009) and apple (Testolin et al., 2019). Numerous SSR-based genetic diversity analyses have now been conducted on poplar and willow germplasm banks globally, with studies consistently indicating high levels of diversity among genotypes (Singh, Singh, et al., 2013; Ukwubile, Ahuchaogu, & Tajudeen, 2014). To improve the information available to

breeders, this study will attempt to characterise the genetic diversity and relationships among and within New Zealand poplar and willow accessions.

2.9.5 Parentage Analysis

Accurate pedigree records are an important part of any control pollinated breeding program, however, contamination during cross pollination is common. Compared to other PCR-based markers, SSRs have the highest degree of accuracy when analysing parental lineages (Gerber, Mariette, Streiff, Bodenes, & Kremer, 2000). Therefore, SSRs are the most common marker used in parentage analysis across different fields including forensics, ecology and anthropology. SSRs have also been used in numerous breeding programs to identify or confirm pedigrees with a high degree of success (Bernardo et al., 2000; X. Zhang et al., 2002). This is due to their codominance and high polymorphism combined with Mendelian segregation and conserved inheritance (Zietkiewicz, Rafalski, & Labuda, 1994). Therefore, this study endeavours to evaluate the use of SSR-based parentage analysis and elucidate any pedigrees that exist within the germplasm, existing breeding populations and released clonal cultivars.

2.9.6 Poplar Rust

In recent decades, microsatellites have played an extensive role in genotyping for disease resistant genes. With the growing concern around poplar rust, numerous studies have been conducted to elucidate potential molecular markers linked to major rust resistant genes in poplar germplasm. Early studies identified the *Melamspora* resistance (*Mer*) locus that was responsible for qualitative rust resistance in *P. deltoides* (M. T. Cervera et al., 1996). The *Mer* locus was later identified as a NBS-LRR gene that induces a hypersensitive response in poplars when compatible strains of *M. larici-populina* urediniospores are present (Laurans & Pilate, 1999). When more microsatellites became available after the poplar genome project, researchers began

looking for markers linked to the *Mer* locus (Tuskan et al., 2004). A French team screening for qualitative and quantitative resistances in poplar found that the ORPM277 microsatellite marker is closely linked with the *Mer* locus on linkage group 19 of a *P. deltoides* parent in a *P. deltoides x trichocarpa* population (V Jorge, Dowkiw, Faivre-Rampant, & Bastien, 2005). However, ORPM277 has not been field tested or validated in any further studies. To improve the efficiency of the breeding program, this study will utilise a *P. deltoides* x *P. trichocarpa* population to validate ORPM277 as a candidate microsatellite marker for resistance to *M. larici-populina*, with the view of genotyping qualitative resistance in poplar germplasm.

2.10 References

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3 Microsatellite Genotyping in New Zealand's Poplar and Willow Breeding Program: Fingerprinting, Genetic Diversity and Rust Resistance Marker Evaluation

3.1 Method

3.1.1 Plant Material

Leaf samples were collected from the core collection of the National Poplar and Willow Germplasm Centre located at the Massey University Plant Growth Unit and Plant and Food Research (PFR) in Palmerston North, New Zealand (40°22'S 175°36'E). The collection consists of single-row tree plantings with three representative clones per accession (1m spacing between clones). Poplar samples included 95 clonal accessions from 20 groups consisting of seven pure species and 13 domesticated hybrids from three different sections (*Populus, Aigeiros* and *Tacamahaca*) (Appendix 1). Willow samples include 195 accessions from 52 groups consisting of 19 pure species and 33 domesticated hybrids (Appendix 6). Three healthy juvenile leaves per sample were picked and transferred into O-ring tubes. Leaf material was preserved in either -80° freezers or silica.

3.1.2 DNA Extraction, Quantitation and Normalisation

DNA was extracted from leaves using a modified cetyltrimethylammonium bromide (CTAB) buffer protocol (Appendix 2) (Procunier, Xu, & Kasha, 1990). Two treatments of RNAse A were used as follows. The initial volume and concentration were 57.5µL and 10 mg/mL (50 µg/mL) respectively, which was later increased to 230µL and 10

mg/mL (50 µg/mL) due to excessive electropherogram stutter. DNA was quantitated through a fluorometric assay using either the Qubit® 2.0 fluorometer (Life Technologies, Carlsbad, California, US) or the SpectraMax Gemini fluorometer (Molecular Devices, Sunnyvale, CA, USA). DNA was subsequently normalised on a Janus® Automated Workstation to 5ng/µL using water as the diluent.

3.1.3 Primer Selection

Primers were selected based on mapping success, linkage group coverage (M.-T. Cervera et al., 2001), polymorphic content and quality in previous poplar (Fossati et al., 2005; Liesebach, Schneck, & Ewald, 2010; Rathmacher et al., 2009) and willow SSR-based studies (Lauron-Moreau, Pitre, Brouillet, & Labrecque, 2013; Singh, Joshi, et al., 2013). Primer pairs were sourced from Sigma-Aldrich™ (St Louis, MO) and re-suspended to a stock solution concentration of 100µM before being diluted to a working solution of 10µM. In total, 12 primers were used for the poplar collection (Table 3.1-1) selected on and 17 primers were used for the larger willow collection (Table 3.2-2). Notably, 'WPMS' and 'PMGC' series markers were originally identified in poplars, however, their reported conservation in many willow species justified their use on the willow collection (Takeshi Hoshikawa, Satoshi Kikuchi, Teruyoshi Nagamitsu, & Nobuhiro Tomaru, 2009).

Table 3.1-1 – Characteristics of Primers used on the Poplar Collection

Loci	Repeat Motif	Forward Primer (5'- 3')	Reverse Primer (5'- 3')	Size Range (bp)
PMGC014[2]	(CTT)	TTCAGAATGTGCATGATGG	GTGATGATCTCACCGTTTG	210
PTR2[3]	$(TGG)_8$	AAGAAGAACTCGAAGATGAAGAACT	ACTGACAAAACCCCTAATCTAACAA	204-225

[2] *Poplar Molecular Genetic Cooperative* database (http://poplar2.cfc.washington.edu/pmgc/)

PTR7[3]	$(CT)_5AT(CT)_6$	ATTTGATGCCTCTTCCTTCCAGT	TATTTTCATTTTCCCTTTGCTTT	200-230
WPMS09[4]	$(GT)_{21}(GA)_{24}$	CTGCTTGCTACCGTGGAACA	AAGCAATTTGGGTCTGAGTATCTG	246-298
WPMS16[5]	$(GTC)_8$	CTCGTACTATTTCCGATGATGACC	AGATTATTAGGTGGGCCAAGGACT	139-184
PMGC2163[2]	(GA)	CAATCGAAGGTAAGGTTAGTG	CGTTGGACATAGATCACACG	220
ORPM_30[6]	$(TC)_9$	ATGTCCACACCCAGATGACA	CCGGCTTCATTAAGAGTTGG	222-238
WPMS20[5]	$(TTCTGG)_8$	GTGCGCACATCTATGACTATCG	ATCTTGTAATTCTCCGGGGCATCT	210-222
PMGC2020[2]	(GA)	TAAGGCTCTGTTTGTTAGTCAG	GAGATCTAATAAAGAAGGTCTTC	150
PTR 5[3]	$(TG)_7$	CTTCTCGAGTATAAATATAAAACACCA	TCACATCACCCTCTCAGTTTCGC	200-230
PMGC2392[2]	(GA)	TAAGGCTCTGTTTGTTAGTCAG	GAGATCTAATAAAGAAGGTCTTC	192
WPMS18[5]	$(GTG)_{13}$	CTTCACATAGGACATAGCAGCATC	CACCAGAGTCATCACCAGTTATTG	219-248
ORPM277[6]	$(GA)_4$	CTTTGGATTGCTTGCGTTTT	TTACCATTGCTGCCATTTCA	201

Table 3.1-2 - Characteristics of Primers used on the Willow Collection

Loci	**Repeat Motif**	**Forward primer (5'-3')**	**Reverse Primer (5'-3')**	**Size Range (bp)**
GSIMCT024[7]	$(CT)_{10}$	TCATTTGCTCGATGAGGTTG	GTGGTAGTTGCAAAAGGGGA	300
CHA475[8]	$(GT)_{12}(GA)$	AGGGAATGAGAGATGGTAGAGT	GGGAAGGTAAGTTGGTGTTG	151-176
WPMS16[9]	$(GTC)_8$	CTCGTACTATTTCCGATGATGACC	AGATTATTAGGTGGGCCAAGGACT	139-184

[3] (Rahman & Rajora, 2002)
[4] (Van der Schoot, Pospíšková, Vosman, & Smulders, 2000)
[5] (Smulders, Van Der Schoot, Arens, & Vosman, 2001)
[6] (Tuskan et al., 2004)
[7] (Stamati, Blackie, Brown, & Russell, 2003)
[8] (T. Hoshikawa, S. Kikuchi, T. Nagamitsu, & N. Tomaru, 2009)
[9] (Smulders et al., 2001)

WPMS18[9]	$(GTG)_{13}$	CTTCACATAGGAC ATAGCAGCATC	CACCAGAGTCATCACCA GTTATTG	219-248
SB88[10]	$(ACCGCC)_5ACCGC$	TATTGCTTTGATGG CGACTGC	CAGCAACGGAAATAGCA ACAG	93–110
SB199[10]	$(TG)_{11}CG(TG)_6$	CTATTTGGTCTCAA TCACCTT	CTTTACCTCAGAAAATCC AGA	102–140
SB38[10]	$(TG)_{27}$	CCACTTGAGGAGT GTAAGGAT	CTTAAATGTAAAACTGAA TCT	116-144
SB85[10]	$(CCG)_5$	CTCAGCAACTCAAT CCAACTA	GTTTGTTAGGGGAGGTAA GAA	81-87
PMGC2020[11]	(GA)	TAAGGCTCTGTTTG TTAGTCAG	GAGATCTAATAAAGAAG GTCTTC	150
WPMS15[9]	$(CCT)_{15}$	CAACAAACCATCA ATGAAGAAGAC	AGAGGGTGTTGGGGGTG ACTA	201-219
SB80[10]	$(TC)_{21}$	TAATGGAGTTCAC AGTCCTCC	ATACAGAGCCCATTTCAT CAC	115-143
WPMS20[9]	$(TTCTGG)_8$	GTGCGCACATCTAT GACTATCG	ATCTTGTAATTCTCCGGG GCATCT	222-252
CHA464[8]	$(CA)_{10}$	GTGGCCCTCTGAA GGTTGA	GCGGCTTATAATGTGATT TAGT	189-197
sb194[10]	$(CA)_{14}$	TGTGAGATAAGAT TTGTCGGT	CCATAAATAAAAAACGT GAAC	108-152
sb243[10]	$(GCC)_3ATCATTCCCC(GCC)_4$	ATTCCTTTCTTCAT CAGTAGC	GACAACGCCATTCACATG ACC	102-113
sb201[10]	$(CT)_4CC(CT)_3(CA)_{22}$	CCTCTTTTTCTATT GTGGTCT	GGCATGTATTTTTACTCC AAC	193-244
PTR2[3]	$(TGG)_8$	AAGAAGAACTCGA AGATGAAGAACT	ACTGACAAAACCCCTA	204-225

[10] (J. Barker, Pahlich, Trybush, Edwards, & Karp, 2003)
[11] *Poplar Molecular Genetic Cooperative* database (http://poplar2.cfc.washington.edu/pmgc/)

3.1.4 PCR amplification

PCR amplification was performed with 2.5µL of normalised DNA (5ng/µL) in a volume of 8.85µL of water, 1.5µL each of 10x buffer and dNTPs (2mM) (Invitrogen, Carlsbad, CA), 0.45µL $MgCl_2$ (50mM), 0.1µL of Platinum® Taq Polymerase (Invitrogen, Carlsbad, CA), 0.0195µL (10µM) of M13-tailed forward primer, 0.3µL of reverse primer and 0.3µL of dye-labelled M13 primer (13nM) (FAM/VIC/NED/PET). PCR amplification was attained by using a touchdown protocol on an Eppendorf Master Cycler Pro as follows: i) 94°C for 2 minutes 49 seconds, ii) 20 cycles of 94°C for 55 seconds, 65°C for 55 seconds and 72°C for 1 minute 39 seconds iii) 20 cycles of 94°C Linkusfor 55 seconds, 55°C for 55 seconds and 72°C for 1 minute 39 seconds iv) 72°C hold step for 10 minutes.

3.1.5 Genotyping

Genotyping was performed by diluting 4µL of each PCR product from four different dye labelled M13 primers (FAM/VIC/NED/PET) with 80µL of water. Subsequently, 9µL of PCR-diluted product was mixed with 0.1041µL of GeneScan™ - 500 (Applied Biosystems, Warrington, UK) and 8.33µL of HiDi™ Formamide. The multiplex product was denatured using heat-soaked PCR (HS-PCR) at 95°C for five minutes and multiplexed in a capillary electrophoresis machine (Applied Biosystems Hitachi 3500 Genetic Analyser).

3.1.5.1 Genotyping for Poplar Rust Resistance

Rust resistant PN 909 (*P. deltoides*) and rust susceptible PN 874 (*P. nigra*) parents were used to establish an F_1 population (n=30) in 2018 at the Massey University Plant Growth Unit. Progeny were scored for rust susceptibility using a protocol developed by

Schreiner (1959) in the middle of the growing season and before leaf senescence (Figure 3.1-1). Samples were taken from the parents and the entire F_1 population and the candidate rust resistance marker 'ORPM277' was amplified and analysed. Samples were genotyped for the presence or absence of ORPM277 and compared to the phenotypic observations.

Estimate of leaf infection		Estimate of infected leaves on tree		Estimate tree infection
Descriptive rating	Numerical rating	Percent	Numerical rating	Numerical rating
Light	1	Less than 25	1	1
		25 - 50	2	2
		50 - 75	3	3
		More than 75	4	4
Medium	5	Less than 25	1	5
		25 - 50	2	10
		50 - 75	3	15
		More than 75	4	20
Heavy	25	Less than 25	1	25
		25 - 50	2	50
		50 - 75	3	75
		More than 75	4	100

Figure 3.1-1 - Table Used to Score and Describe Rust Resistance for a Given Poplar Tree. Source: Schreiner (1959)

3.1.6 Data Analysis

3.1.6.1 Allele Scoring

Alleles peaks were scored using GeneMarker v2.2.0 (SoftGenetics LLC, Pennsylvania State College, PA). Manually-scored raw fragment sizes were statistically converted into binned allele classes based on the length of the motif repeats and allele distributions using the software FLEXIBIN (Amos et al., 2007). For the poplar collection, processed fragment data were exported in an integer format to GenAlEx 6.5 (Peakall & Smouse,

2006) which is based on Microsoft Excel (Microsoft Corporation, Redmond, WA) scripts. Due to the mixed ploidy nature of the willow collection, processed fragment data were organised in Microsoft Excel and imported into the specialised polyploid microsatellite R 3.5.3 (R Core Team, 2013) package POLYSAT (Clark & Jasieniuk, 2011).

3.1.6.2 Frequency and Distance Based Analysis

GenAlEx was used to organise the poplar data and calculate all frequency-based analyses. POLYSAT was used to organise the polyploid willow data and calculate frequency-based analyses. Due to unknown and mixed ploidy levels in willows, co-dominant microsatellite data were converted to dominant binary presence/absence microsatellite data to perform frequency-based analyses.

Wright's F_{ST} statistic was used to estimate the level of genetic variation of codominant microsatellite data, while ϕ_{ST} is a derivative of the F_{ST} statistic and can be used to estimate the level of genetic variation of binary microsatellite data (Wright, 1949). Values range on a scale between 0 (no differentiation) and 1 (complete differentiation). Polymorphic Information Content (PIC) was used to estimate the polymorphic content and informativeness of each marker. Botstein, White, Skolnick, and Davis (1980) organised the interpretation of PIC values into three categories: i) if the PIC value is greater than 0.5 it represents a high level of genetic diversity or marker informativeness ii) if the PIC value is between 0.25 and 0.5 it represents a moderate level of genetic diversity or marker informativeness iii) if the PIC value is below 0.25 it represents a low level of genetic diversity or marker informativeness.

Raw data was exported to the R-based (R Core Team, 2013) POPPR (Kamvar, Tabima, & Grünwald, 2014) package. Analysis of Molecular Variance (AMOVA) was used to

determine the source of variation among and within the poplar and willow collections. AMOVA was calculated using the 'ade4' method (Dray & Dufour, 2007) in conjunction with the farthest neighbour algorithm. Nei's genetic distance was used to determine the diversity between species groups (Nei, 1972).

To generate a dendrogram, Jaccard's similarity coefficient (Jaccard, 1908) was used to generate a distance matrix which was organised into a dendrogram at an accession-level using the Ward's method (Ward, 1963) based on the unweighted pair group method with arithmetic mean (UPGMA). Dendrograms were visualised and adjusted in R using the DENDEXTEND package (Galili, 2015). To estimate the distortion between the distance matrix and the dendrogram a cophenetic correlation coefficient was generated using the R-based cophenetic function which is based on the formula proposed by Sokal and Rohlf (1962).

3.2 Results

3.2.1 Markers

Overall, the success of marker scoring was varied. Some markers produced excessive stutter or completely failed to amplify, while others produced clear well-resolved fragments with limited stutter or false peaks. Consequently, some markers needed to be re-analysed multiple times. Sixteen markers were originally screened against 199 willow samples. However, low polymorphism and amplification rates in the *Salix alba* and *Salix matsudana* × *alba* groups required an additional marker (PTR2) to differentiate between accessions. Two markers (SB201 and WPMS20) failed to amplify despite re-analysis. Twelve markers were screened against 95 of the poplar germplasm samples (N = 95). Three of the 12 markers (PTR5, PTR 7 and WPMS09) completely failed to amplify despite re-analysis. ORPM30 consistently amplified in two distinct

size ranges and was subsequently separated into two separate loci (ORPM30_1 and ORPM30_2).

Overall, the markers used in this study were moderately informative (PIC 0.25-0.5) in both the poplar (Table 3.2-1) and willow (Table 3.2-2) collections. Amplification rates were higher in the poplar collection (83%) (Table 3.2-1) than in the willow collection (60.2%) (Table 3.2-2). However, the mean number of alleles per marker (n = 14) were identical in both poplars and willows. In the poplar collection, PMGC2163 and the WPMS series were the most informative markers based on high allelic richness, good amplification rates and high PIC values (Table 3.2-1). While PMGC2392 was the least informative due to low amplification rates (52%) and PIC values (PIC = 0.157), allelic richness was very high (N Alleles = 16) (Table 3.2-1). In the willow collection, gSIMC024 (PIC = 0.487) and CHA475 were highly informative markers while WPMS18 and CHA464 were the least informative due to monomorphism and poor amplification rates respectively.

Table 3.2-1 - Marker Characteristics of 10 successfully amplified SSRs used on the Poplar Collection

Marker	N Alleles	PIC	H_o	Amplification %
PMGC2163	25	0.405	0.541	100
PTR2	10	0.359	0.497	96.9
PMGC2392	16	0.157	0.019	52
WPMS16	15	0.529	0.737	94.8
PMGC2020	16	0.553	0.808	85.7
PMGC014	13	0.398	0.497	65
ORPM30_1	8	0.236	0.236	75.5

ORPM30_2	16	0.344	0.334	72.4
WPMS_20	7	0.349	0.572	94.8
WPMS_18	11	0.415	0.576	93.8
MEAN	**14**	**0.375**	**0.482**	**83**

PIC = Polymorphic Information Content, H_O = Observed Heterozygosity, N Alleles = Number of Alleles Identified

Table 3.2-2 Marker Characteristics of 15 successfully amplified SSRs used on the Willow Collection

Marker	N Alleles	PIC	Amplification %
gSIMC024	24	0.485	65.8%
CHA475	32	0.460	94.5%
WPMS16	6	0.444	95.0%
WPMS18	1	N/A	26.6%
SB88	7	0.234	96.5%
SB199	25	0.457	64.8%
SB38	4	0.584	20.1%
SB85	7	0.625	79.4%
PMGC2020	22	0.477	26.6%
WPMS15	12	0.427	68.8%
SB80	26	0.505	73.9%
CHA464	4	0.631	5.0%
SB194	20	0.507	35.7%
SB243	12	0.515	60.8%
PTR2[12]	4	0.611	89.6%

[12] Only used on *S. alba* and *S. alba x matsudana* clones

MEAN	**14**	**0.497**	**60.2%**

3.2.2 DNA Fingerprinting

This study successfully distinguished between 93% of the poplar and 99% of the willow collections and generated unique distinguishable fragment patterns for future use, undistinguishable accessions are considered clonal duplicates. Two willow samples were identified as being identical (Probability = 99.99%), but they were sourced from separate clones (Table 3.2-3). Six poplar samples were identified as being identical (Probability = 99.99%), though they were sourced from separate clones in the collection (Table 3.2-3). Private alleles (unique alleles found in one species group) were found in 10 of the 22 species groups in poplar (Appendix 3) and 26 of the 52 species groups in willow and could tentatively be identifiers for those species groups (Appendix 4). Only a small number of loci were needed to differentiate between accessions (Figure 3.2-1). Between species groups, the probability of identifying genotypes exceeded a significance level of 99% when three or more loci combinations were used (Figure 3.2-1). However, within species groups the probability of identifying genotypes to a 99% significance level required five or more loci combinations.

Table 3.2-3 – Identical Samples Sourced from Separate Clones

Matching Sample Pair	**Accession ID**	**Species**	**Source**
A	PN 302 Balana	*Salix* × *calodendron (caprea* × *viminalis)*	Massey University Collection Row 2 / Number 34
	PN 325 Black Willow SCCB		Massey University

			Collection Row 2 / Number 37
B	‘PN 031 GB’	*Populus szechuanica*	Aokautere Collection, Manawatu
	‘PN 941 Nepal’		
C	Chaneys Forest, Cpt 2/20	*Populus deltoides* × *nigra*	Matariki Forest Stands, Canterbury
	Hanmer Forest, Cpt 8/3		
D	Lowmount Forest, Cpt 4/23	*Populus maximowiczii* × *trichocarpa*	Matariki Forest Stands. Canterbury
	PN 040 Androscoggin		Aokautere Collection, Manawatu

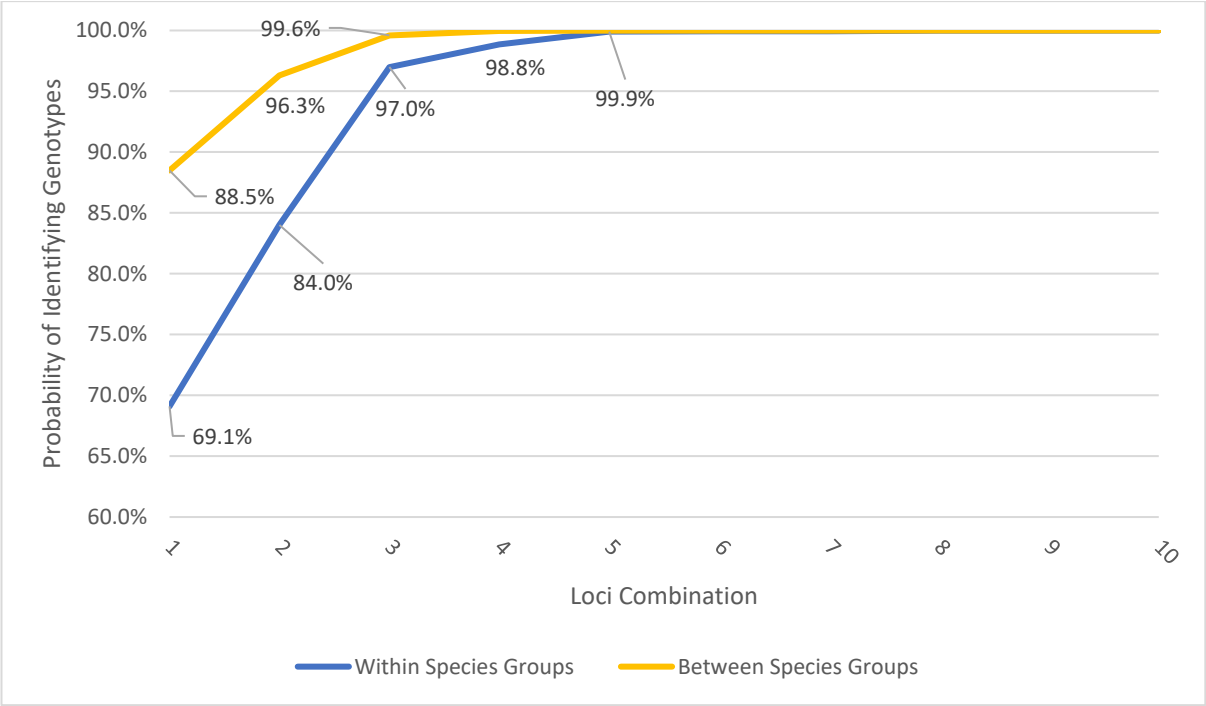

Figure 3.2-1 - Probability of Excluding a Genotype with Additive Loci Combinations

3.2.3 Genetic Variation

Except for the unique polyploid 'Toa' accession, the typic diploid nature of the poplar collection allowed for a more in-depth genetic diversity analysis, utilising co-dominant microsatellite data. In contrast, the complex mixed aneuploid/alloploid ploidy system of the willow germplasm collection required allele sizes to be converted into binary presence/absence data. This prevented the calculation of heterozygosity values and fixation indices for willows, so genetic distance data and diversity indices were used to quantify genetic diversity as has been used in other polyploid germplasm studies (dos Santos et al., 2012).

Overall, this study determined that a high level of genetic variation exists across the poplar and willow germplasm collection. When codominant data were converted to binary data, ϕ_{ST} was high and very similar in the poplar (ϕ_{ST} = 0.345) and willow (ϕ_{ST} =

0.342) collections. In contrast, the poplar group's codominant F_{ST} score (F_{ST}=0.557) was greater than the binary ϕ_{ST} score (ϕ_{ST} = 0.345). The Shannon Information Index (I) was high and gave a similar level of diversity in poplars (I = 1.803) and willows (I = 1.492).

To confirm the extent of genetic variation between species groups, Nei's genetic distance was used (Nei, 1972). Genetic variation was high with values ranging between low 0.226 (*Salix alba, Salix alba* × *matsudana*) and very high 3.626 (*Salix fragilis, Salix cinerea* × *viminalis*) in the willows with an average of 1.326. Poplar germplasm accessions expressed a similar variation in genetic distance ranging between low 0.201 (*Populus deltoides* × *trichocarpa* / *Populus deltoides* × *szechuanica*) and very high 4.039 (*Populus angustifolia* × *Populus simonii*) with an average of 1.186. Jaccard's dissimilarity matrix was used to estimate genetic variation at an accession level. Willow accessions ranged between low (*J* = 0.111) and very high (*J* = 1.0), with an average of 0.833. At an accession level, genetic variation in poplars ranged between moderate (*J* = 0.444) and very high (*J* = 1.0) with an average of *J* =0.863. Analysis of Molecular Variation (AMOVA) suggests that the source of this genetic variation occurs predominantly within accessions as opposed to among accessions for both poplars and willows (65%) (Table 3.2-4).

Table 3.2-4 – Summary Table of an Analysis of Molecular Variance (AMOVA) to Estimate the Source of Genetic Variation in the Poplar and Willow Germplasm Collection

Source	Degrees of Freedom	Sum of Squares	Mean Squares	Estimated Variance	% of Molecular Variance
POPLARS					

Among Accessions	77	1095.000	4.420	3.14	34.55%
Within Accessions	95	192.000	5.97	5.97	65.44%
Total	172	699.718	8.70	9.12	100%
WILLOWS					
Among Accessions	56	944.58	1.86	3.25	34.25%
Within Accessions	140	875.22	6.25	6.25	65.74
	196	1820.41	9.28	9.51	100%

Further genetic diversity analysis of the poplar accessions revealed that heterozygosity was high (Table 3.2-5). Furthermore, inbreeding was low across all species groups, which is suggested by a low mean fixation index value (inbreeding coefficient) and only a small difference between the observed and expected heterozygosity values. Pure species groups had significantly lower levels of heterozygosity and gene diversity compared to hybrid species groups ($p < 0.05$). While the fixation index is relatively low in both hybrid and pure species groups, it was significantly higher in the hybrids when compared to pure species ($p < 0.05$). The groups with the greatest level of genetic variation were *P. maximowiczii* × *nigra* and *P. deltoides* × *nigra*. Both groups expressed a high level of heterozygosity and gene diversity with low levels of inbreeding. In contrast, *P. trichocarpa*, *P. yunnanensis* and *P. deltoides* expressed the lowest levels of heterozygosity, while *P. yunnanensis* expressed the lowest levels of gene diversity. *P. yunnanensis* is a pure species represented by a very small collection of samples imported from China and likely contributed to it having the lowest level of gene diversity.

Table 3.2-5 - Summary Statistics of Genetic Variation across Major Poplar Species and hybrid Groups

***Populus* Group**	**N**	**Na**	**H_o**	**H_{exp}**	**F**
P. deltoides	9	39	0.379	0.487	0.175
P. deltoides × *ciliata*	3	24	0.500	0.422	-0.086
P. deltoides × *nigra*	31	60	0.536	0.630	0.130
P. maximowiczii × *nigra*	11	40	0.588	0.600	0.025
P. maximowiczii × *trichocarpa*	11	40	0.453	0.583	0.360
P. nigra	4	43	0.642	0.502	-0.242
P. szechuanica	4	26	0.533	0.499	-0.078
P. trichocarpa	6	31	0.295	0.608	0.540
P. trichocarpa × *nigra*	6	34	0.413	0.604	0.318
P. yunnanensis	2	21	0.350	0.363	0.040
Hybrid MEAN	**-**	**39.6**	**0.498**	**0.568**	**0.150**
Pure Species MEAN	**-**	**32**	**0.440**	**0.492**	**0.087**
Total MEAN	**-**	**3.545**	**0.469**	**0.530**	**0.118**

N= Number of Accessions, Na = Number of alleles, H_O = Observed Heterozygosity, H_{exp} = Nei's Gene Diversity, F = Fixation Index (inbreeding coefficient)

3.2.4 Genetic Relationships

3.2.4.1 Poplars

Figure 3.2-2 shows an unweighted pair group method with arithmetic mean (UPGMA) dendrogram that was produced using Jaccard's similarity coefficient. The cophenetic correlation between the Jaccard's distance matrix and the dendrogram was 0.77, suggesting a high goodness of fit. Seven distinct clusters were identified and comprised

93 of the 95 accessions analysed. Hierarchical clusters appear to be influenced by species groups and parental relationships.

Cluster I is predominantly comprised of *Tacamahaca* accessions with the exception of *P. alba* × *glandulosa* and 'Blanc de Garonne' (*P. nigra*). Interestingly, 'Blanc de Garonne' is distant from the remaining *P. nigra* accessions in Cluster V and appears in the same cluster as its progeny 'NZ 07-006-014' (*P. trichocarpa* × *nigra*). It is evident that Cluster II is dominated by *P. deltoides* accessions and *Tacamahaca* members that have been hybridised with *P. deltoides*. We found that *P. deltoides* accessions in Cluster II are quite distant from their *P. nigra Aigeros* section counterparts in Cluster V. Cluster III is the smallest cluster and mainly consists of *P. trichocarpa* accessions including 'NZ 87-007-001' (*P. trichocarpa*) which was the progenitor to the 'NZ 07' (*P. maximowiczii x trichocarpa*) series accessions in the same cluster.

Cluster IV only contains *Tacamahaca* accessions and is dominated by *P. maximowiczii* hybrids that are a distant from *P. maximowiczii* × *nigra* hybrids located in neighbouring Cluster V. Cluster IV also contains 'NZ 87-007-004' (*P. trichocarpa*), which was the progenitor for the neighbouring 'NZ 07-005-079' (*P. maximowiczii* × *trichocarpa*) accession. Cluster V consists of *P. nigra* accessions on one side of the cluster and *P. maximowiczii* × *nigra* accessions on the other side. The unknown 'Manawatu Golf Course Poplar' is located in Cluster V. Its genotypic clustering confirmed its phenotypic form as likely *P. nigra* or a *P. nigra* hybrid. Interestingly, *P. deltoides* × *nigra* accessions clustered into two distinct clusters VI and VII rather than one cluster.

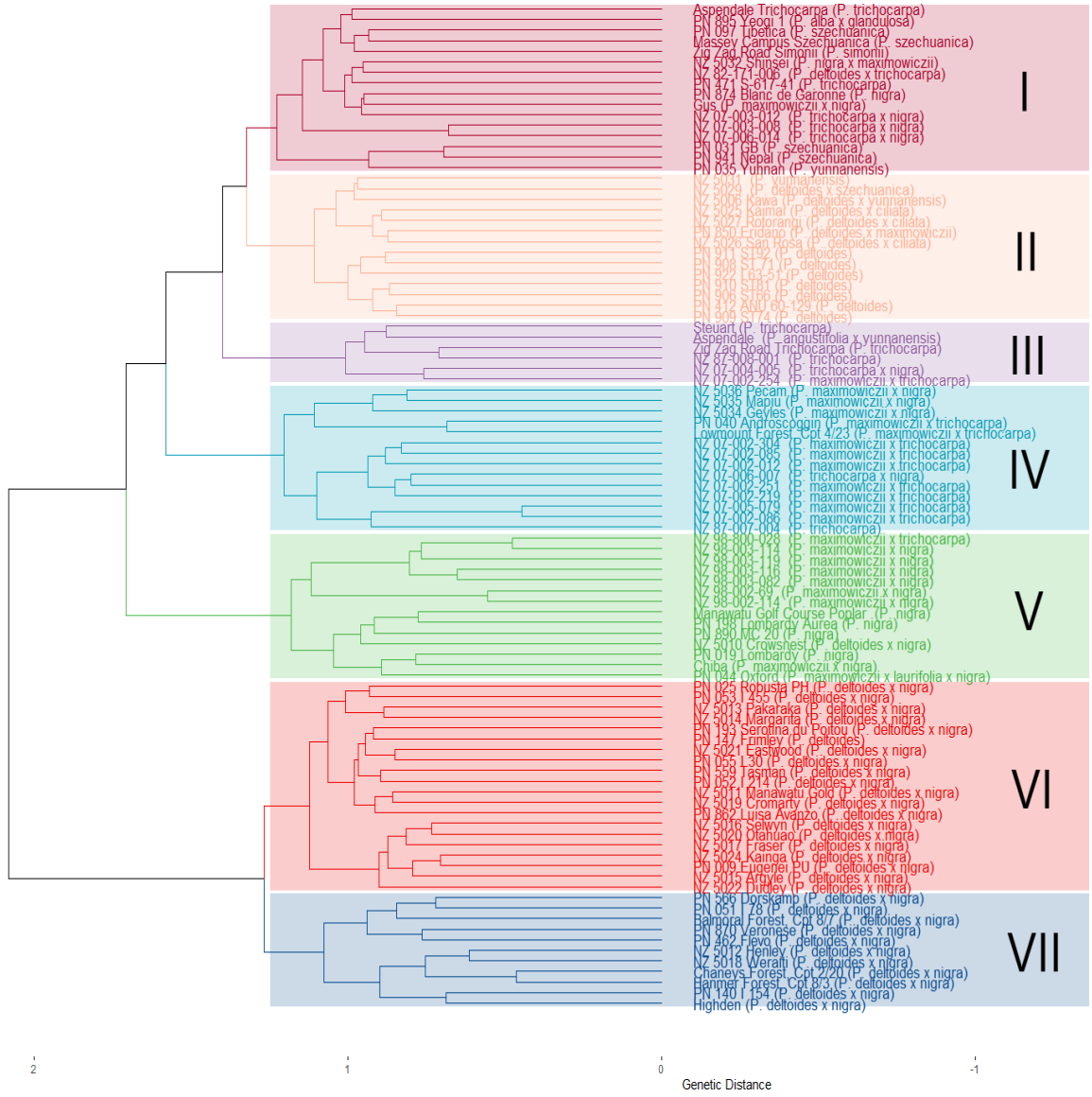

Figure 3.2-2 – UPGMA Dendrogram Showing the Genetic Relationships between Individual Poplar Germplasm Accessions

3.2.4.2 Willows

Figure 3.2-3 shows a UPGMA dendrogram of New Zealand's willow germplasm samples that was produced using Jaccard's similarity coefficient. Fourteen clusters (I-XIV) were identified and were categorised into three mega-clusters (A-C). The cophenetic correlation between the Jaccards's distance matrix and the dendrogram was 0.72, suggesting a high goodness of fit. Clusters appear to be predominantly structured based on species, natural geographic distribution and tree/shrub classification (Table 3.2-6). We found that 91% of tree willow accessions segregated into mega-clusters A and B, in contrast, 91% of shrub willow accessions segregated into mega-cluster C (Figure 3.2-3). Furthermore, 83% of the North American willow accessions were included in neighbouring clusters V and VI, while 97% of the Eurasian willow accessions were included in the remaining clusters.

Cluster I is comprised of various Eurasian tree and shrub willows; *S. alba* accessions in this cluster are surprisingly distant from the majority of other *S. alba* accessions contained in mega-cluster B (Table 3.2-6) (Figure 3.2-3). Cluster III contains half of the *S. matsudana* × *alba* hybrid accessions that likely share a closer genetic relationship to their *S. alba* parentage. Most *S. purpurea* accessions were grouped together in Cluster IV, which also includes two *S.* × *sordida* × *(cinerea* × *purpurea)* and *S. sordida* × *gracilistyla* accessions, this is likely because *S.* × *sordida* is a *S. purpurea* hybrid. Cluster V contains North American *S. nigra* tree willows, despite belonging to mega-cluster C which predominantly contains shrub willows. Cluster VI consists of North American North American shrub willows, except for *S. viminalis* which is Eurasian. Goat willows (*S. x calodendron, S. caprea, S. cinerea* and *S. reichardtii*) clustered close together in Cluster VII and Cluster VIII.

Cluster IX was the largest (n = 30) and consists of Eurasian species. Cluster IX contains outlying *S. purpurea* and *S. matsudana* accessions that are quite distant from their species group relatives in Clusters V and XIII respectively. We find it interesting that some outlying tree willow species like *S. matsudana* are present in Cluster IX, given that mega-cluster C is predominantly shrub willows. *S. daphnoides* clustered with *S. acutifolia* accessions in Cluster X which is confirms the view that that *S. acutifolia* is considered a subspecies of *S. daphnoides* (Rechinger, 1992). Cluster XI contains North American shrub willows *S. petiolaris, S. eriocephala, S. chilensis* that are genetically distant from the majority North American shrub willows in Cluster VI. Shrub willows in Cluster XI originate from central North America whereas shrub willows in Cluster VI originate from western North America (Argus, 2007). Cluster XII contains a variety of Eurasian tree willow species, though the presence of 'PN 735' (*S. nigra*) is of interest because it is North American and very distant from the remaining *S. nigra* accessions in Cluster V, including 'PN 734' (*S. nigra*). Furthermore, 'PN 735's phenotypic form is noticeably different too compared to the rest of the *S. nigra* accessions with much broader branching. The majority of *S. matsudana* accessions were grouped into Cluster XIII which also included numerous *S. matsudana* hybrids and a single *S. gooddingii* accession. Most of the *S. fragilis* accessions were grouped into Cluster XIV which also contains 'PN 211' (*S. alba*) which is very distant from the remaining *S. alba* accessions contained in mega-cluster B.

Table 3.2-6 – Summary of Hierarchal Clusters for the Willow Collection

Cluster	**Number of Accessions**	***Salix* Species/Hybrids Represented**	**Classification**	**Geographic Origin**
I	13	*S. triandra, S. fluviatalis, S. babylonica* × *fragilis, S. matsudana* × *lucida, S.*	Tree/Shrub	Eurasia

		alba and *S. lasiandra* × *pentandra*		
II	13	*S. alba*	Tree	Eurasia
III	26	*S. alba* and *S. matsudana* × *alba*	Tree	Eurasia
IV	15	*S. purpurea, S. sordida* × *(cinerea* × *purpurea), S. sordida* × *gracilistyla*	Shrub	Eurasia
V	6	*S. nigra*	Tree	North America
VI	11	*S. lasiandra, S. lasiolepsis, S. hookeriana, S. candida, S. viminalis, S. lasiolepis* × *viminalis, S. lasiolepis* × *opaca*	Shrub	North America
VII	3	*S. calodendron* × *(caprea* × *viminalis)*	Shrub	Eurasia
VIII	4	*S. reichardtii* × *(caprea* × *cinerea), S. caprea*	Shrub	Eurasia
I×	30	*S. repens, S. myricoides, S. udensis, S. schwerinii, S. sericans, S. gracilistyla, S lasiolepis* × *opaca, S. tetrasperma, S. matsudana, S. purpurea, S. daphnoides, S. balsatica, S. appenina, S. forbyana* × *(purpurea* × *viminalis), S reinii, S. cinereal, S. dichroa* × *(aurita* × *purpurea), S. auritoides, S. purpurea* × *viminalis, S. aegyptiaca*	Tree/Shrub	Eurasia
×	5	*S. daphnoides, S. acutifolia*	Shrub	Eurasia
×I	7	*S. purpurea, S. petiolaris, S. magnifica, S. eriocephala*	Shrub	Eurasia/North America

×II	10	*S. nigra, S. sepulcralis × (babylonica × alba), S. babylonica × fragilis, S. chilensis, S. matsudana*	Tree/Shrub	Eurasia/North America
×III	24	*S. matsudana, S. sepulcralis × (babylonica × alba), S. gooddingii, S. matsudana × lucida, S. alba*	Tree	Eurasia
×IV	21	*S. pentandra, S. matsudana, S. pentandra, S. fragilis, S. fragilis × alba, S. alba, S. rubens × (alba × fragilis), S. fragilis × pentandra, S. meyeriana*	Tree	Eurasia

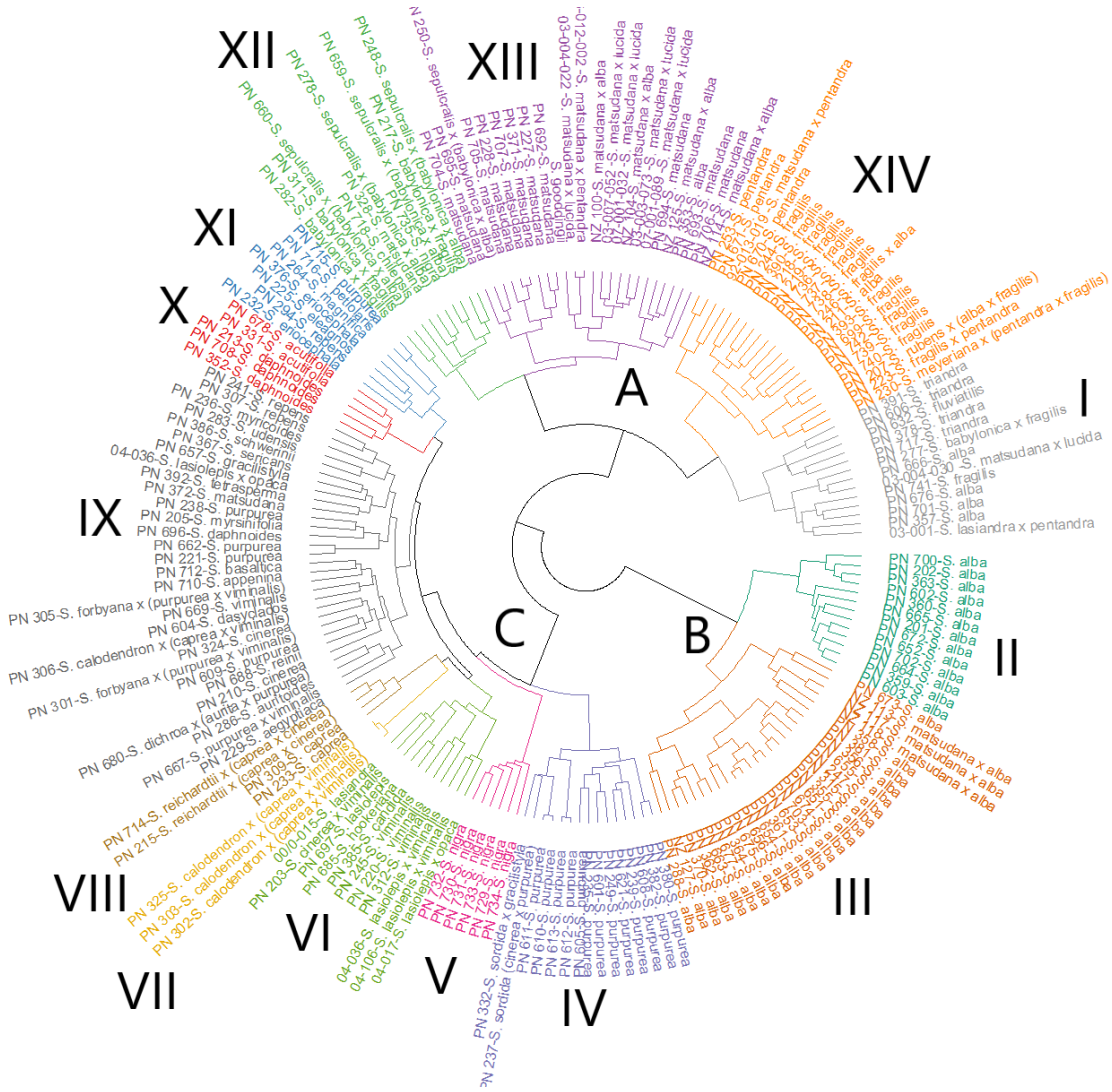

Figure 3.2-3 - UPGMA Dendrogram Showing the Genetic Relationships between Individual Willow Germplasm Accessions

3.2.5 Genotyping for Poplar Rust Resistance

Phenotyping the F_1 *P. deltoides x nigra* population revealed a 3:1 resistant to susceptible ratio of progeny. Designations for observed phenotypic rust infection scores described by Schreiner (1959) ranged from 0 (no infection) to 20 (medium) infection (Table 3.2-7). The ORPM277 marker was present monomorphically in the resistant maternal parent PN 909 (*P. deltoides*) and absent in the paternal parent PN 874 (*P. nigra*). ORPM277 segregated at a 1:1 presence/absence ratio among progeny and no significant

difference was identified in rust resistant scores between the presence and absence groups (Table 3.2-7).

Table 3.2-7 - Summary of Observed Phenotypic Rust Scores in Relation to Genotype

	Number of Individuals	**Mean Estimate of Rust Infection**	**Rust Score - Light (<5) (Number of Individuals)**	**Rust Score - Medium (5-25) (Number of Individuals)**
'ORPM 277' Marker Present	14	4.50	10	4
'ORPM 277' Marker Absent	13	4.07	10	3

3.3 Discussion

3.3.1 DNA Fingerprinting

This study has demonstrated that the accessions in the New Zealand poplar and willow germplasm collection could be uniquely identified based on their SSR fragment data. We present a full database of binned and raw fragment data now available on the Plant and Food Research database (New Zealand Plant and Food Research Limited, 2019) for use by plant breeders to manage and identify genetic material in the present and future breeding programmes. Numerous genotypes could be identified at a single locus, which is to be expected given the co-dominant and high polymorphic nature of SSRs in poplars and willows (Rahman & Rajora, 2002; Singh, Singh, et al., 2013). The propensity for clonal duplication in the poplar and willow breeding program was highlighted with the identification of four duplicate accessions. Duplications were likely due to a combination of factors including the low intraspecific anatomical variation between clones, mishandling of accessions and duplication of clonal varieties in different germplasm locations, or subsequent renaming of one source after commercialisation. Other DNA fingerprinting studies have also identified duplicate clonal accessions in germplasm collections accounted for as a result of human error (Bekkaoui, Mann, & Schroeder, 2003; Rajora & Rahman, 2003). It should be noted that we differentiated accessions that could not be identified based on their morphological characters alone. For example, ‘Highden’ (*P. deltoides x nigra*) clone has a phenotype that is indistinguishable from ‘Veronese’ (*P. deltoides x nigra*), however, we found that they had distinct though similar DNA fingerprints. We also found that two osier willow accessions ‘PN 302’ and ‘PN 325’ (*Salix × calodendron (caprea × viminalis)*) had identical fingerprints despite being identified historically as being unique in the

germplasm collection. This duplication could have occurred due to the mismanagement or mislabelling of the germplasm material.

Increasing ploidy levels, mixed intraspecific ploidy levels and high species diversity among willows likely enhanced the delimitation between accessions in this study. Cytological studies of willows show that ploidy levels vary both among and within species, ranging from diploids to dodecaploids (Guo, Hou, Yin, & Chen, 2016). Increasing ploidy levels and mixed intraspecific ploidy levels increases the polymorphic content of each loci, reducing the number of loci required to differentiate between accessions (Gulsen et al., 2009). It also likely that species richness positively contributed to the delimitation between accessions because species groups often contain unique alleles that improves interspecific differentiation. We detected a high frequency of private alleles at a species group level, which could explain why markers common to both willow and poplar collections had greater PIC values in the species-rich and polyploid willow collection.

The success of a DNA fingerprinting study depends on there being detectable inter-clonal variability of markers. Primer design is often species-specific, meaning poor cross-amplification rates across large genera or families can be a limiting factor (Šarac et al., 2015). As a result, willows had poor amplification rates and we consider this is due to the large number of species (n=55) sampled. Typically, poor amplification rates experienced in microsatellite studies requires employing more markers (Šarac et al., 2015). However, high species diversity in a germplasm collection often increases marker polymorphism and allelic diversity (Vellend, 2005). Consequentially, despite the poor amplification rates in willows, we found that the high polymorphic content of the markers was sufficient to differentiate the majority accessions. However, the *Salix*

alba species group had particularly low amplification rates, resulting in the need for more markers to delimit accessions. Twenty five of the 29 markers used on the poplar and willow collections can be recommended for future fingerprinting studies due to their allelic richness, high polymorphism, adequate amplification rates and moderate-high marker informativeness. Markers that were successful on poplar accessions were not necessarily successful in willow accessions like WPMS18 and PMGC2020. PMGC2020 had no amplification success while WPMS18 was monomorphic in all willow accessions where it successfully amplified, and so cannot be recommended as a marker for DNA fingerprinting of willows. In contrast, marker WPMS18 and PMGC2020 was highly polymorphic in all the poplar accessions and is recommended for future studies. Though marker PMGC2392 had poor amplification rates and low informativeness, its future use is still warranted purely for molecular identification purposes due to its high allelic richness and high frequency of private alleles. As experienced in other studies (Lexer, Fay, Joseph, Nica, & Heinze, 2005), ORPM30 consistently amplified in two distinct size ranges, which we found beneficial as the loci provided additional data. An explanation of the mechanism of this dual amplification has not been yet been reported in the literature or BLAST (Johnson et al., 2008).

3.3.2 Genetic Diversity and Relationships

Estimating genetic diversity relies on understanding and justifying various statistical measurements. Heterozygosity represents the allele diversity of a locus and is considered fundamental in estimating genetic diversity within germplasm collections, particularly when employing analyses, e.g. F-statistics, where observed and expected heterozygosity is often required (Gregorius, 1978). In diploid poplars, this is particularly useful as SSRs can be represented as co-dominant data and the analysis is straightforward. In polyploids, SSRs pose a unique challenge for estimating

heterozygosity, because accurate ploidy levels are required for R-packages (e.g. POLYSAT) to estimate heterozygosity and population-level allele frequencies (Clark & Jasieniuk, 2011). While many species have stable intraspecific ploidy, willows have mixed intraspecific ploidy levels, making reliable allele-frequency based ploidy level estimation in POLYSAT almost impossible (Ngantcha, 2010). Consequently, co-dominant willow data were converted to dominant binary/absence data which, when analysed with conventional statistics, are likely to underestimate allele dosages and genetic diversity (Cordeiro, Pan, & Henry, 2003; Pfeiffer, Roschanski, Pannell, Korbecka, & Schnittler, 2011). While this reduced the availability of heterozygosity-based statistics, we successfully employed distance-based metrics and frequency-based statistics such as the Shannon's Index and the modified F-statistic (ϕ_{ST}) to estimate genetic variation, as used successfully in other studies (Friedrich et al., 2017; Li et al., 2014). In our study F_{ST} scores in the codominant data set of poplars were greater than the dominant binary/absence ϕ_{ST} equivalent, indicating that ϕ_{ST} is an underestimate of the true level of diversity due to the loss of allele dosages and heterozygosity estimates. The ϕ_{ST} scores were still high in both poplar and willow groups and still serve as a representation of genetic variation between species groups, despite the potential underestimation of true diversity (Nybom & Bartish, 2000). The POLYSAT software can accurately model for heterozygosity using mixed ploidy codominant-based data by employing the de Silva method (Clark & Jasieniuk, 2011). However, exact ploidy levels are needed for each accession, which would have required flow cytometry to precede this study. There is evidence in other studies to suggest that SSRs are the best choice for DNA fingerprinting due to the higher per-locus information content of SSRs, whilst SNPs provide greater genetic variation inferences (García, Guichoux, & Hampe, 2018). To improve the efficacy of future polyploid studies it is recommended that both SNPs

and SSRs are utilised together and flow cytometry precedes mixed ploidy DNA fingerprinting and genetic characterisation, however, dominant binary presence/absence data can still effectively estimate genetic diversity if resources are limited.

Polyploidy has long been associated with increased genetic diversity due to increased allele dosages, mutations and reduced genetic drift. Theoretically, polyploid willows should contain a greater level of genetic diversity compared to poplars. However, this study revealed similar levels of genetic variation in both poplars and willows. The inheritance of allopolyploid willows could be disomic thereby reducing allele dosages and mutation rates to that of diploids. Both *S. alba* and *S. fragilis* segregate as allotetraploids, thereby reducing genetic variation in those species groups (Guo et al., 2016). For example, during meiosis an allopolyploid's chromosome segregates as bivalent homeologs, whereas autopolyploid's chromosomes segregate as multivalents (Meirmans & Van Tienderen, 2013). While the full extent of allopolyploidy is unknown in willows, it is considered to be fairly extensive (Fogelqvist et al., 2015).

Overall, the high estimated genetic diversity present in New Zealand's poplar and willow germplasm is considered to be principally due to three factors: i) the dioeciously enforced outcrossing nature of pure species present in the collection ii) interspecific breeding practices creating the domesticated hybrids present in the collection iii) the high frequency of different species groups. The first factor suggested is in accordance with Hamrick, Godt, and Sherman-Broyles (1992), who proposed that dioecious tree species have the highest levels of gene diversity due to very low self-pollination rates and therefore higher heterozygosity. In this study a high level of genetic variation was identified. The second factor is suggested by consideration of the largest source of genetic variation being within individuals and that the poplar interspecific hybrid

germplasm groups had significantly higher allele diversity compared to the pure species groups, i.e. interspecific breeding practices have increased the genetic diversity on the collections. The third factor is proposed because greater diversity of species present in a germplasm collection increases the likelihood of unique alleles and genetic variation being present (Warburton et al., 2006).

We consider that biogeography and tree/shrub or subgenera classification has significantly affected the genetic relationships between willow accessions and can explain certain anomalies. Our dendrogram revealed that tree and shrub willows were separated into different mega clusters. This has likely occurred because most tree willows belong to the *Salix* subgenus which is phylogenetically diverged from the *Vetrix* subgenus that contains most of the shrub willows (Argus, 1997). However, there are exceptions, like *S. triandra* which is a shrub willow that belongs to the *Salix* subgenus and this could explain why it clustered with tree willow accessions (Lauron-Moreau, Pitre, Argus, Labrecque, & Brouillet, 2015). We found that most Eurasian and North American accessions have great disparity between one another, as reported previously (J. Wu et al., 2015). We also found that North American shrub willows were divided into Central/Eastern and Western clusters based on their biogeographic origins. This could have occurred due to the mountain regions of North America acting as a geographic barrier between the species (Lauron-Moreau et al., 2015).

Dendrograms produced in this study revealed numerous incongruities in the poplar germplasm collection. Despite the high number of *P. deltoides x nigra* accessions, all of them clustered more closely to their *P. nigra* progenitors as opposed to *P. deltoides*. We suggest that this could have occurred due to a selection bias for *P. nigra* traits. We were surprised to find that *P. deltoides* accessions in Cluster II are quite distant from their

intrasectionally related *P. nigra* accessions in both the dendrogram and Nei's genetic distance analysis, despite being members of the same *Aigeiros* section. However, this is consistent with phylogenetic studies that place *P. deltoides* in a clade with other *Aigeiros* members and *P. nigra* in a distant clade with *Tacamahaca* members (Hamzeh & Dayanandan, 2004). This could explain why some *P. nigra* members like 'Blanc de Garonne' clustered with *Tacamahaca* members in this study. The large genetic distance between *P. nigra* and *P. deltoides* accessions reported in this study should be of great benefit to breeders because *P. deltoides x nigra* is the most common interspecific hybrid produced by the breeding program and this improves the chance of capturing high levels of genetic diversity in subsequent progeny. Consequently, this could explain why *P. deltoides x nigra* members had high heterozygosity and gene diversity and clustered into multiple groups with a large genetic distance between accessions.

3.3.3 Plant Breeding Implications

The DNA fingerprinting method used in this study was a fast, efficient, objective and accurate way of identifying clones at any maturity stage. We demonstrated that DNA fingerprinting is useful for identifying whether accessions are clonal duplicates. For example, prior to this study 'Highden' (*P. deltoides x nigra*) accession was suspected to be a clonal duplicate of 'Veronese' (*P. deltoides x nigra*). However, we found that while they are closely related, they are genotypically different. We also found that DNA fingerprinting can be used to identify clonal duplicates that were previously treated as separate accessions. For example, while 'Androscoggin' and 'Lowmount Forest Cpt 4/23' were originally treated as separate genotypes in the study, we showed that they are genotypically identical. Breeders can now utilise the database to refine accession identification by removing duplicates, query parental relationships of clones, protect and manage future and existing plant varietal rights and improve germplasm tracking

and management. Additionally, breeders can also identify clones at any maturity level. Breeding programs are continuously seeking novel sources of genetic material. By using the methodology described in this study, future germplasm accessions can be easily compared to existing accessions to determine relatedness or duplication.

This study generated two accession-level dendrograms to clarify genetic relationships in the respective germplasm collections, which will be a beneficial tool for the breeders. Without understanding genetic relationships, there is a potential for breeders to unknowingly increase inbreeding and fix deleterious alleles in offspring. In contrast, understanding genetic relationships allows breeders to select progenitors from distant cluster groups which increases the probability of capturing unique gene variants and increasing the genetic diversity of progeny. This is because the probability of capturing unique gene variants in progeny increases as the genetic distance between progenitors increases (Gregorius, 1978). We found that dendrograms are also useful for identifying or confirming accessions of unknown species origin which improves the management of genetic material. For example, the ‘Manawatu Golf Course’ poplar accession was always believed to be a *P. nigra* based on its morphology, and the clustering in this study placed this accession alongside other *P. nigra* accessions, confirming its morphological identification. Dendrograms can also identify genetic relationship patterns based on geographical origin or taxonomic status of accessions, which can be useful for breeders. For example, tree willows segregated into two distinct clusters, one containing *S. alba* accessions and the other containing *S. matsudana* accessions and the remaining tree willows. *S. matsudana* and *S. alba* are the most common interspecific cross parents in the willow breeding program. This segregation indicates to breeders that continued hybridisations between these two species groups will yield progeny with high genetic variation due to the distance between these two species groups.

High levels of genetic variation in a germplasm collection increase the probability of desirable gene variants being captured in future progeny and reduce the risk of inbreeding depressions. Classical breeding practices invariably produce a negative effect on genetic diversity, whereby, favourable allele combinations are selected at the expense of others (Chakravarthi & Naravaneni, 2006). In annual crops such as maize, wheat or sorghum the genetic diversity of the released cultivar is less important than a tree crop because the crop does not need to withstand changing environmental pressures over a period of decades (Manifesto, Schlatter, Hopp, Suárez, & Dubcovsky, 2001). While studying the extent of genetic diversity improves the management of genetic resources, understanding the source of genetic variation provides insights into the impact of the existing genetic diversity on future progeny. Because the largest source of genetic diversity in this study was contained within accessions rather than among accessions, the potential for increasing genetic diversity in subsequent progeny is lower compared to a collection with higher levels of variation among accessions (Singh, Singh, et al., 2013). However, the significant difference between hybrid and pure species groups is an indication that the level of genetic variation among accessions is enough to further increase the genetic diversity of future clones.

3.3.4 Genotyping for Poplar Rust Resistance

Despite being closely linked to the *Mer* resistance locus, this study strongly suggests the ORPM277 microsatellite marker is not suitable for marker assisted poplar rust resistance identification in New Zealand. Poplar rust diversity in New Zealand is considered very low, due to the lack of suitable secondary-host conifers for spores to complete their sexual reproductive cycle on, limiting the rust to constant cycles of asexual reproduction (Sivakumaran & Mclvor, 2010). Therefore, we suspect that a poplar rust bottleneck exists in New Zealand, limiting the pathogen's ability to

overcome plant defences and in turn improving the breeders' ability to integrate durable rust resistance into released cultivars. Earlier European studies inferred that the virulence 7 strain of *M. larici-populina* has overcome the *Mer* resistance locus which could explain the lack of correlation between genotypic and phenotypic data (Pascal Frey et al., 2005). To date, poplar rust virulence strains have not been investigated as poplar rust surveys have largely been centred on geographical locations, rust species present and evidence of their hybridisation with each other based on spore external morphology (Sivakumaran & Mclvor, 2010). However, complete phenotypic rust resistance in the ORPM277 *P. deltoides* positive parent as compared to complete phenotypic rust susceptibility in the ORPM277 *P. nigra* negative parent, could suggest that another quantitative or single trait loci is responsible for controlling rust resistance, or the linkage between ORPM277 and the *Mer* gene has been broken. It has been suggested that another quantitative trait locus could be responsible for 3 to 76% of phenotypic rust resistance variance in *P. deltoides* (V. Jorge et al., 2005b) which could provide a line of enquiry for future molecular resistance studies.

3.3.5 Future Directions

While the database created by this study is predominantly for the benefit of the breeding program it could also be used in further studies to elucidate the population structure of naturalised poplars and willows in New Zealand. Poplars and willows employed in New Zealand often form naturalised populations due to their vegetative reproduction mechanisms (Vegetative Reproduction 2.5.1). However, reduced genetic variation is typically found in introduced invasive species due to marked founder effects which can result in a diminished ability for populations to respond to increasing environmental pressures. This reduced genetic variation could be particularly concerning in areas where naturalised populations are effective in stabilising soil erosion. Employing the

database generated in this study could assess whether domesticated clones have played a role in establishing naturalised populations and whether these naturalised populations have experienced genetic drift from domesticated clones. In turn, this will aid in the management of these naturalised populations.

Future studies with a larger population size would likely provide a greater indication into whether ORPM277 is useful for marker assisted selection in the poplar breeding program. We also recommend analysing a larger population for QTLs as a starting point for quantitative marker identification. A greater understanding of poplar rust in New Zealand could also be achieved with molecular studies into the population genetics, geographical diversity and virulence identification in combination with further mapping and marker evaluation studies. Current phenotyping methods will need to be the mainstay of poplar rust screening in New Zealand until robust molecular screening methods can be developed and implemented.

3.4 Conclusion

With the advent of the molecular era, it is important for plant breeders to evaluate how novel molecular tools could be employed in their programs. SSR markers serve as an excellent starting point for introducing genotyping to small-scale breeding programs due to their low application cost, effective ability to characterise genetic material and capacity to be used in marker assisted selection.

We generated a DNA fingerprinting database consisting of 96 poplar and 197 willow accessions from New Zealand's poplar and willow germplasm resource. We conducted a genetic variation analyses on the database and our results suggest that a high level of genetic variation exists in both collections. AMOVA suggests that the source of this genetic variation is predominantly contained within accessions as opposed to among

accessions, which is characteristic of dioeciously enforced outcrossing species. The largest challenge faced by this study was the genetic variation analysis of polyploid SSR data which had to be converted from co-dominant data to dominant binary/absence data. We believe that this could have underestimated genetic diversity and allele dosages. As a future solution, we propose evaluating other marker types like SNPs alongside SSR markers when analysing the genetic variation of polyploid species if resources are available.

We discussed how effective poplar rust resistance is a challenge facing the poplar breeding program. As a potential solution, we analysed whether SSR markers could be used for MAS. We compared genotype data from a candidate qualitative rust resistant marker ('ORPM277') against phenotypic rust scores in a breeding population created by a rust susceptible and a rust resistant parent. Our results strongly suggest that the genotype data from the ORPM277 microsatellite marker is not correlated to the phenotypic rust scores and larger studies of both qualitative and quantitative markers will be needed to assess whether marker assisted selection is possible for poplar rust resistance in New Zealand. We also discussed the need for a greater understanding of the virulence, population structure and genetic diversity of poplar rust in New Zealand.

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4 Appendices

Appendix 1 – List of Poplar Germplasm Accessions

Accession ID	***Populus* Species**	**Germplasm Location**
PN 895 Yeogi 1	*P. alba x glandulosa*	Akura Nursery
Aspendale Angustifolia x Yunnanensis	*P. angustifolia x yunnanensis*	Aspendale
PN 909 ST74	*P. deltoides*	Aspendale

PN 147 Frimley	*P. deltoides*	Aokautere poplar collection
PN 909 ST74	*P. deltoides*	Aokautere poplar collection
PN 412 ANU 60-129	*P. deltoides*	Aokautere poplar collection
PN 922 I 63-51	*P. deltoides*	Aokautere poplar collection
PN 906 ST66	*P. deltoides*	Aokautere poplar collection
PN 908 ST 71	*P. deltoides*	Aokautere poplar collection
PN 910 ST81	*P. deltoides*	Aokautere poplar collection
PN 911 ST92	*P. deltoides*	Aokautere poplar collection
NZ 5027 Rotorangi	*P. deltoides x ciliata*	Akura Nursery
NZ 5026 San Rosa	*P. deltoides x ciliata*	Akura Nursery
NZ 5025 Kaimai	*P. deltoides x ciliata*	Akura Nursery
PN 850 Eridano	*P. deltoides x maximowiczii*	Aokautere poplar collection
Hanmer Forest, Cpt 8/3	*P. deltoides x nigra*	Hanmer Forest, Cpt 8/3
Balmoral Forest, Cpt 8/7	*P. deltoides x nigra*	Balmoral Forest, Cpt 8/7
PN 055 I 30	*P. deltoides x nigra*	Aokautere poplar collection
PN 051 I 78	*P. deltoides x nigra*	Aokautere poplar collection
PN 052 I 214	*P. deltoides x nigra*	Aokautere poplar collection
PN 053 I 455	*P. deltoides x nigra*	Aokautere poplar collection
PN 009 Eugenei PU	*P. deltoides x nigra*	Aokautere poplar collection
PN 462 Flevo	*P. deltoides x nigra*	Aokautere poplar collection

PN 025 Robusta PH	*P. deltoides x nigra*	Aokautere poplar collection
PN 559 Tasman	*P. deltoides x nigra*	Aokautere poplar collection
PN 870 Veronese	*P. deltoides x nigra*	Radio Nursery
Highden	*P. deltoides x nigra*	Akura Nursery
NZ 5017 Fraser	*P. deltoides x nigra*	Aokautere poplar collection
NZ 5020 Otahuao	*P. deltoides x nigra*	Aokautere poplar collection
NZ 5016 Selwyn	*P. deltoides x nigra*	Aokautere poplar collection
NZ 5018 Weraiti	*P. deltoides x nigra*	Aokautere poplar collection
NZ 5014 Margarita	*P. deltoides x nigra*	Aokautere poplar collection
NZ 5022 Dudley	*P. deltoides x nigra*	Aokautere poplar collection
NZ 5013 Pakaraka	*P. deltoides x nigra*	Aokautere poplar collection
NZ 5012 Henley	*P. deltoides x nigra*	Aokautere poplar collection
PN 862 Luisa Avanzo	*P. deltoides x nigra*	Aokautere poplar collection
PN 566 Dorskamp	*P. deltoides x nigra*	Aokautere poplar collection
NZ 5024 Kainga	*P. deltoides x nigra*	Aokautere poplar collection
NZ 5019 Cromarty	*P. deltoides x nigra*	Aokautere poplar collection
NZ 5021 Eastwood	*P. deltoides x nigra*	Aokautere poplar collection
NZ 5015 Argyle	*P. deltoides x nigra*	Aokautere demonstration planting
PN 140 I 154	*P. deltoides x nigra*	Aokautere demonstration planting

PN 193 Serotina du Poitou	*P. deltoides x nigra*	Aokautere poplar collection
NZ 5011 Manawatu Gold	*P. deltoides x nigra*	Roadside near Aokautere
NZ 5010 Crowsnest	*P. deltoides x nigra*	Radio Nursery
NZ 5029	*P. deltoides x szechuanica*	Aokautere Block 35
NZ 82-171-006	*P. deltoides x trichocarpa*	Aokautere demonstration planting
NZ 5006 Kawa	*P. deltoides x yunnanensis*	Akura Nursery
PN 044 Oxford	*P. maximowiczii x laurifolia x nigra*	Radio Nursery
NZ 5034 Geyles	*P. maximowiczii x nigra*	Aokautere poplar collection
NZ 5035 Mapiu	*P. maximowiczii x nigra*	Radio Nursery
NZ 5036 Pecam	*P. maximowiczii x nigra*	Radio Nursery
Chiba	*P. maximowiczii x nigra*	Radio Nursery
Gus	*P. maximowiczii × nigra*	Aspendale
NZ 98-002-114	*P. maximowiczii × nigra x nigra*	Radio Nursery
NZ 98-003-082	*P. maximowiczii x nigra x nigra*	Akura Nursery
NZ 98-003-114	*P. maximowiczii x nigra x nigra*	Akura Nursery
NZ 98-002-69	*P. maximowiczii x nigra x nigra*	Clyde
NZ 98-003-116	*P. maximowiczii x nigra x nigra*	Clyde
NZ 98-003-119	*P. maximowiczii x nigra nigra*	Clyde
Lowmount Forest, Cpt 4/23	*P. maximowiczii x trichocarpa*	Lowmount Forest
NZ 07-002-085	*P. maximowiczii x trichocarpa*	Clyde
NZ 07-002-304	*P. maximowiczii x trichocarpa*	Aokautere poplar collection

NZ 07-002-251	*P. maximowiczii x trichocarpa*	Radio Nursery
NZ 07-002-012	*P. maximowiczii x trichocarpa*	Radio Nursery
NZ 07-002-219	*P. maximowiczii x trichocarpa*	Aokautere poplar collection
NZ 07-002-254	*P. maximowiczii x trichocarpa*	Aokautere poplar collection
NZ 07-002-254	*P. maximowiczii x trichocarpa*	Aokautere poplar collection
PN 040 Androscoggin	*P. maximowiczii x trichocarpa*	Aokautere poplar collection
NZ 07-002-086	*P. maximowiczii x trichocarpa*	Aokautere poplar collection
NZ 07-005-079	*P. maximowiczii x trichocarpa*	Aokautere poplar collection
PN 198 Lombardy Aurea	*P. nigra*	Timaru
PN 890 MC 20	*P. nigra*	Aspendale
PN 019 Lombardy	*P. nigra*	Aokautere poplar collection
PN 874 Blanc de Garonne	*P. nigra*	Aokautere poplar collection
NZ 5032 Shinsei	*P. nigra x maximowiczii*	Aokautere Block 50
P. simonii	*P. simonii*	Zigzag Road, Manawatu
P. szechuanica	*P. szechuanica*	Massey campus
PN 941 Nepal	*P. szechuanica*	Aokautere poplar collection
PN 097 Tibetica	*P. szechuanica*	Aokautere poplar collection
PN 031 GB	*P. szechuanica*	Aokautere poplar collection
Steuart	*P. trichocarpa*	Radio Nursery
PN 471 S-617-41	*P. trichocarpa*	Aokautere poplar collection

Aspendale Trichocarpa	*P. trichocarpa*	Aspendale
NZ 87-008-001	*P. trichocarpa*	Aokautere poplar collection
NZ 87-007-004	*P. trichocarpa*	Aokautere poplar collection
Zig Zag Road Trichocarpa	*P. trichocarpa*	Zigzag Road, Manawatu
NZ 07-003-012	*P. trichocarpa x nigra*	Clyde
NZ 07-006-007	*P. trichocarpa x nigra*	Clyde
NZ 07-006-014	*P. trichocarpa x nigra*	Clyde
NZ 07-006-014	*P. trichocarpa x nigra*	Clyde
NZ 07-003-008	*P. trichocarpa x nigra*	Clyde
NZ 07-004-005	*P. trichocarpa x nigra*	Clyde
NZ 98-800-028	*Unknown*	Wairarapa Shelterbelt
Manawatu Golf Course Poplar	*Unknown*	Manawatu Golf Course
PN 035 Yunnan	*P. yunnanensis*	Aokautere poplar collection
NZ 5031	*P. yunnanensis*	Aokautere demonstration planting
Chaneys Forest, Cpt 2/20	*P. deltoides x nigra*	Chaneys Forest

4.1 Appendix 2

Appendix 2

CTAB DNA Extraction Protocol:

1. Add PVP and RNase A to an aliquot of 1.11 X stock CTAB extraction buffer sufficient for the number of samples being processed (see suggested volumes below). Warm buffer to aid solubility of PVP if required, mix well (vortex).

2. Use 900 µL of buffer per sample in a 2 mL o-ring tube. Mill leaves directly from frozen at 3.55 m/s for 1 X 1 min.

NB: Optimise milling down to a minimal speed to maximise gDNA integrity. 5 X 3 mm stainless steel ball bearings/ tube.

3. Add 100 µL 10 % n-lauroyl sarcosine. Mix by inversion.

NB: Added after milling as reacts with CTAB to produce viscous extraction buffer, thereby changing milling ball speed and efficiency.

5. Incubate at 65 ºC for 30 min., occasionally mixing during incubation.

6. Add 200 µL 24:1 Chloroform:Octanol. Mix by inverting several times.

7. Incubate on ice for 5 minutes to cool and mix separated layers occasionally.

8. Spin at 12K g for 8 minutes to separate layers.

9. Transfer top layer (800 to 900 µL) to a new 2ml tube. Add 2/3 vol. isopropanol (535 to 600 µL) to precipitate gDNA; incubate 5 min R/T. Mix by inversion.

10. Spin at full rpm speed for 10 min. Discard supernatant.

NB: Discard plant material pellet, unused supernatant and precipitate supernatant to waste bottle for proper disposal.

11. Wash pellet with 500 µL 76% ethanol with 10 mM NH_4OAc, spin 1 min, carefully decant ethanol (NB: pellet will be loose from tube wall).

12. Wash pellet with 70% ethanol (500 µL, spin 2 min).

13. Dry pellet in speed vac for 5 min. only.

14. Resus gDNA in 100 µL TE buffer. Do not vortex.

For 20 preps (in 2 mL tubes) [actually 23X]

20.7 mL 1.11 X Stock buffer

(2.3 mL 10 % n-lauroly Sarcosine)

230 µL 10 mg/mL RNase A (50 µg/mL)

0.23 g PVP

Appendix 3

List of Poplar Species Specific Alleles Identified:

***Populus* Species**	**Marker**	**Unique Allele**
P. deltoides	PMGC2392	158
P. deltoides	PMGC2392	168

P. deltoides	PMGC2392	171
P. deltoides	PMGC2392	173
P. deltoides	ORPM30_2	258
P. deltoides	WPMS_20	231
P. deltoides	WPMS_18	225
P. deltoides x nigra	PTR2	246
P. deltoides x nigra	PMGC2392	156
P. deltoides x nigra	PMGC2392	164
P. deltoides x nigra	PMGC2392	166
P. deltoides x nigra	PMGC2392	175
P. deltoides x nigra	PMGC2392	187
P. deltoides x nigra	PMGC2020	171
P. deltoides x nigra	ORPM30_2	242
P. deltoides x nigra	ORPM30_2	262
P. deltoides x trichocarpa	PMGC2163	235
P. maximowiczii x laurifolia x nigra	ORPM30_2	230
P. maximowiczii x nigra	ORPM30_1	211
P. maximowiczii x nigra	WPMS_20	267
P. maximowiczii x trichocarpa	ORPM30_1	208
P. maximowiczii x trichocarpa	WPMS_18	228
P. nigra	PTR2	217
P. nigra	WPMS16	158
P. nigra	ORPM30_1	197
P. simonii	WPMS16	183
P. simonii	WPMS16	195
P. simonii	WPMS_20	297
P. szechuanica	PMGC2163	201

P. szechuanica	PMGC2163	202
P. szechuanica	PTR2	224
P. szechuanica	PMGC014	222
P. szechuanica	ORPM30_2	260
P. trichocarpa	PMGC2163	217
P. trichocarpa	PMGC2163	224
P. trichocarpa	PMGC2163	228
P. trichocarpa	PMGC2163	230
P. trichocarpa	PMGC2163	233
P. trichocarpa	PMGC014	225
P. trichocarpa x nigra	PMGC2392	181
P. trichocarpa x nigra	PMGC2392	183
P. yunnanensis	PMGC2163	208
P. yunnanensis	PMGC2163	212
P. yunnanensis	WPMS16	146

Appendix 4

List of Willow Species' Specific Alleles Identified:

***Salix* species group**	**Marker Name – Allele**						
S. alba	PMGC2020-125	PMGC2020-127	PMGC2020-129	PMGC2020-131	PMGC2020-174	PMGC2020-182	PMGC2020-221
S. appenina	SB199-143						
S. candida	CHA475-195						
S. cinerea x viminalis	WPMS16-173						
S. daphnoides	SB80-163	SB80-165	SB194-132				

S. eleagnos	SB199-139						
S. hookeriana	SB199-103						
S. lasiolepis	SB38-175						
S. lasiolepis x opaca	gSIMC024-322						
S. lasiolepis x viminalis	CHA475-110	CHA464-196	SB243-110				
S. matsudana	SB199-153	PMGC2020-192	WPMS15-186	WPMS15-232	WPMS15-238	SB80-181	SB243-113
S. matsudana x alba	PMGC2020-184	PMGC2020-186	WPMS15-195				
S. matsudana x lucida	PMGC2020-145	PMGC2020-147	PMGC2020-161	CHA464-208			
S. myricoides	SB80-167						
S. nigra	SB88-97	PMGC2020-153	PMGC2020-157				
S. pentandra	gSIMC024-340	CHA475-133					
S. purpurea	gSIMC024-335	CHA475-207	CHA475-209	WPMS15-173	SB80-140	CHA464-214	SB243-104
S. schwerinii	SB199-163	SB199-165					
S. sepulcralis x (babylonica x alba)	SB38-176	PMGC2020-180	WPMS15-207				
S. sericans	SB199-145						
S. sordida (cinerea x purpurea)	CHA475-137						
S. tetrasperma	SB243-101						
S. triandra	SB85-110	WPMS15-170	WPMS15-180	SB80-171			

S. udensis	SB199-137	1	
S. viminalis	SB199-149	SB194-149	SB243-152
S. forbyana x (purpurea x viminalis)	SB194-114	SB194-116	

Appendix 6 – List of Willow Germplasm Accessions

Accession ID	*Salix* Species
PN 710	*Salix appenina*
PN 712	*S. basaltica*
PN 716	*S. petiolaris Sm.*
PN 685 Furry Ness	*S. hookeriana Barratt*
PN 392 Himalayas	*S. tetrasperma*
PN 604	*S. x dasyclados Wimm.*
PN 632	*S. fluviatilis*
PN 307	*S. repens*
PN 205	*S. myrsinifolia*
PN 688	*S. reinii Frach. & Sav. Ex Seem.*
PN 697 Salinas Bitter	*S. lasiolepis*
PN 286	*S. auritoides*
PN 264	*S. magnifica*
PN 385 Furry Ness	*S. candida Fluegge ex Willd.*
PN 283	*S. udensis*
PN 229	*S. aegyptiaca L.*
PN 241	*S. repens L. var. adscendens*
PN 367	*S. x sericans*
PN 236 Piperi	*S. myricoides*

PN 376 Americana	*S. eriocephala Michx. ?*
PN 294	*S. repens L.*
PN 386 Kinuyanagi	*S. schwerinii E. Wolf ?*
PN 225	*S. eleagnos Scop.*
PN 232 Nigra Longipes	*S. eriocephala Michx. ?*
PN 378 Brunette Noir	*S. triandra L.*
PN 233 N	*S. caprea L.*
PN 309 F	*S. caprea L.*
PN 215 Pussy Galore	*S. x reichardtii A. Kerner (caprea L. x cinerea L.)*
PN 714 Muscina	*S. x reichardtii A. Kerner (caprea L. x cinerea L.)*
PN 302 Balana	*S. x calodendron Wimmer (caprea L. x viminalis L.)*
PN 303 SM(=Balana)	*S. x calodendron Wimmer (caprea L. x viminalis L.)*
PN 306 Hybrida	*S. x calodendron Wimmer (caprea L. x viminalis L.)*
PN 325 Black Willow SCCB(=Balana)	*S. x calodendron Wimmer (caprea L. x viminalis L.)*
PN 220 Gigantea	*S. viminalis L.*
PN 245 NCCB	*S. viminalis L.*
PN 312 Bowles	*S. viminalis L.*
PN 669 Korso	*S. viminalis L.*
PN 301 Populifera	*S. x forbyana (purpurea L. x viminalis L.)*
PN 305 Sessilifolia	*S. x forbyana (purpurea L. x viminalis L.)*
PN 380 Abbeys	*S. purpurea L.*
PN 667 Pyramidalis	*S. purpurea L. x viminalis?*
PN 221 Rubra	*S. purpurea L.*
PN 235 Narrowleaf (Gracilis)	*S. purpurea L.*
PN 238 PMC	*S. purpurea L.*
PN 239 Eugenei	*S. purpurea L.*

PN 249 Booth	*S. purpurea L.*
PN 382 Links Dutch	*S. purpurea L.*
PN 601 Denmark	*S. purpurea L.*
PN 605 Holland	*S. purpurea L.*
PN 609 Green Dicks	*S. purpurea L.*
PN 610 Leicestershire Dicks	*S. purpurea L.*
PN 611 Lancashire Dicks	*S. purpurea L.*
PN 612 Schultz	*S. purpurea L.*
PN 613 Goldstones	*S. purpurea L.*
PN 621 Nana	*S. purpurea L.*
PN 715 Nicholsonii Purpurescens	*S. purpurea L.*
PN 680	*S. x dichroa (aurita L. x purpurea L.)*
PN 332	*S. x sordida x gracilistyla Miq.*
PN 237 Pontederana	*S. x sordida (cinerea L. x purpurea L.)*
PN 203 Argyracea	*S. cinerea L. x viminalis L.*
PN 324 Tricolor Mac	*S. cinerea L.*
PN 210 Oleifolia NCCB	*S. cinerea L. subsp. oleifolia (Sm.) Macreight*
PN 331 Taihape	*S. acutifolia*
PN 678 CZ	*S. acutifolia*
PN 213 G	*S. daphnoides Villars*
PN 352 QP	*S. daphnoides Villars*
PN 708 Otago	*S. daphnoides Villars*
PN 218 Russelliana (=NCCB)	*S. fragilis L.*
PN 219 Latifolia (=Gatungensis)	*S. fragilis L.*

PN 244 Tetrandra	*S. fragilis L.*
PN 390 Decipiens	*S. fragilis L.*
PN 393 Illertissen V10-30	*S. fragilis L.*
PN 699 AS (Aokautere School)	*S. fragilis L.*
PN 736 NL 435	*S. fragilis L.*
PN 737 NL 519	*S. fragilis L.*
PN 738 NL 543	*S. fragilis L.*
PN 739 NL 544	*S. fragilis L.*
PN 740 NL 576	*S. fragilis L.*
PN 741 NL 582	*S. fragilis L.*
PN 742 NL 640	*S. fragilis L.*
PN 223 Decipiens (=Hexandra)	*S. fragilis L. x ? (pentandra)*
PN 207 Bactrini	*S. x rubens Schrank (alba L. x fragilis L.)*
PN 246 Basfordiana	*S. fragilis L. x alba L. var. vitellina (L.) Stokes*
PN 217 Fargesii NCCB	*S. babylonica L. x fragilis L.*
PN 277 Fragilis U (Te Karaka)	*S. babylonica L. x fragilis L.*
PN 282 Pendulina	*S. babylonica L. x fragilis L.*
PN 311 Fragilis S (Gisborne)	*S. babylonica L. x fragilis L.*
PN 230	*S. x meyeriana Rostk. (pendandra L. x fragilis L.)*
PN 253 G	*S. pentandra L.*
PN 670 Dark French	*S. pentandra L.*
PN 671 Patent Lumley	*S. pentandra L.*
PN 729 AR 3	*S. nigra L.*
PN 730 AR 4	*S. nigra L.*
PN 731 AR 18	*S. nigra L.*

PN 732 AR 82	*S. nigra L.*
PN 733 AR 115	*S. nigra L.*
PN 734 Pryor 62-27	*S. nigra L.*
PN 735 Pryor 62-91	*S. nigra L.*
PN 718 Chilensis	*S. chilensis*
PN 227 Kew	*S. matsudana Koidz.*
PN 228 Tortuosa	*S. matsudana Koidz.*
PN 328 Pendula CBG	*S. matsudana Koidz.*
PN 371 YN 102	*S. matsudana Koidz.*
PN 372 YN 207	*S. matsudana Koidz.*
PN 372A YN 209	*S. matsudana Koidz.*
PN 692 CH2	*S. matsudana Koidz.*
PN 693 CH3A	*S. matsudana Koidz.*
PN 694 CH3B	*S. matsudana Koidz.*
PN 695A CH4A	*S. matsudana Koidz.*
PN 695B CH4B	*S. matsudana Koidz.*
PN 704 Tortuosa (Northland A)	*S. matsudana Koidz.*
PN 705 Tortuosa (Northland B)	*S. matsudana Koidz.*
PN 706 Tortuosa NP	*S. matsudana Koidz.*
PN 707 Tortuosa NG	*S. matsudana Koidz.*
NZ 1001 Cannock	*S. matsudana Koidz. x alba L.*
NZ 1002 Aokautere	*S. matsudana Koidz. x alba L.*
NZ 1003 Te Awa	*S. matsudana Koidz. x alba L.*
NZ 1040 Tangoio	*S. matsudana Koidz. x alba L.*
NZ 1130 Hiwinui	*S. matsudana Koidz. x alba L.*
NZ 1143 Adair	*S. matsudana Koidz. x alba L.*

NZ 1149 Wairakei	*S. matsudana Koidz. x alba L.*
NZ 1179 Makara	*S. matsudana Koidz. x alba L.*
NZ 1184 Moutere	*S. matsudana Koidz. x alba L.*
NZ 1254	*S. matsudana Koidz. x alba L.*
PN 248 Chrysocoma (Vitellina Pendula)	*S. x sepulcralis Simonk. (babylonica L. x alba L.)*
PN 250 Kemp	*S. x sepulcralis Simonk. (babylonica L. x alba L.)*
PN 278 Salamonii	*S. x sepulcralis Simonk. (babylonica L. x alba L.)*
PN 659 AR 131-25	*S. x sepulcralis Simonk. (babylonica L. x alba L.)*
PN 660 AR 131-27	*S. x sepulcralis Simonk. (babylonica L. x alba L.)*
NZ 1271	*S. alba L. x alba L.*
PN 201	*S. alba L.*
PN 202 Argentea C	*S. alba L.*
PN 211	*S. alba L.*
PN 315 Argentea CBG	*S. alba L.*
PN 355 I 4-58	*S. alba L.*
PN 356 I 1-59	*S. alba L.*
PN 357 I 2-59	*S. alba L.*
PN 358 I 3-59	*S. alba L.*
PN 359 I 4-59	*S. alba L.*
PN 360 I 6-59	*S. alba L.*
PN 361 I 8-59A	*S. alba L.*
PN 362 I 8-59B	*S. alba L.*
PN 363 Ulzio 1	*S. alba L.*
PN 364 Ulzio 2	*S. alba L.*
PN 370 Casale	*S. alba L.*
PN 602 Liempde	*S. alba L.*
PN 603 Belders	*S. alba L.*

PN 651 Barlo	*S. alba L.*
PN 652 Bredevoort	*S. alba L.*
PN 653 Drakenburg	*S. alba L.*
PN 654 Het Goor	*S. alba L.*
PN 655 Lichtenvoorde	*S. alba L.*
PN 656 Lievelde	*S. alba L.*
PN 663 R 202	*S. alba L.*
PN 664 R 203	*S. alba L.*
PN 665 Wantage Hall	*S. alba L.*
PN 672 NS 107-65-6	*S. alba L.*
PN 673 NS 107-65-7	*S. alba L.*
PN 674 I 16-63	*S. alba L.*
PN 675 I 2-61	*S. alba L.*
PN 676 I 12-61	*S. alba L.*
PN 677 I 75-62	*S. alba L.*
PN 700 NL 481	*S. alba L.*
PN 701 NL 489	*S. alba L.*
PN 702 NL 493	*S. alba L.*
PN 703 NL 503	*S. alba L.*
PN 666 Caerulea Foreman Essex	*S. alba L. var. calva G.F.W. Meyer*
PN 247 Britzensis	*S. alba L. var. vitellina (L.) Stokes*
PN 288 Chrysostella	*S. S. alba L. var. vitellina (L.) Stokes*
PN 353 Westhaven	*S. alba L. var. vitellina (L.) Stokes*
PN 381 Yelverton	*S. alba L. var. vitellina (L.) Stokes*
PN 696	*S. S. daphnoides Villars*
PN 657 Melanostachys	*S. gracilistyla Miq.*
PN 608 Irette	*S. purpurea L.*

PN 662 Japonica	*S. purpurea L.*
PN 377 Stone Rod	*S. triandra L.*
PN 391 Noir de Villaine	*S. triandra L.*
PN 606 Black Maul	*S. triandra L.*
PN 717 Semperflorens	*S. triandra L.*
04-017-OP002	*S. lasiolepis 106/3-017 × opaca*
04-036-OP011	*S. lasiolepis 116/1-036 × opaca (43/109/11 - Row/Stool Number/Clone Number, Aokautere block 44)*
04-106-026	*S. lasiolepis 106/3-017 × viminalis 'Gigantea' (40/26/26 - Row/Stool Number/Clone Number, Aokautere block 44)*
04-106-073	*S. lasiolepis 106/3-017 × viminalis 'Gigantea' (40/73/73 - Row/Stool Number/Clone Number, Aokautere block 44)*
04-036-035	*S. lasiolepis 116/1-036 × viminalis 'Gigantea' (43/14/35 - Row/Stool Number/Clone Number, Aokautere block 44)*
04-036-011	*S. lasiolepis 116/1-036 × viminalis 'Gigantea' (43/78/11 - Row/Stool Number/Clone Number, Aokautere block 44)*
03-003-073	*S. matsudana 'Kew' PN 227 × lucida spp. lasiandra 112/3-15*
03-004-022	*S. matsudana 'Kew' PN 227 × lucida spp. lasiandra 113/1-13*
03-004-030	*S. matsudana 'Kew' PN 227 × lucida spp. lasiandra 113/1-13*
03-007-052	*S. matsudana 'Kew' PN 227 × lucida spp. lasiandra unknown*
07-001-032	*S. matsudana 'Kew'' PN 227 × lasiandra 000/0-15*
07-001-089	*S. matsudana 'Kew'' PN 227 × lasiandra 000/0-15*
03-001-071	*S. lucida spp. lasiandra × pentandra 'Dark French' PN 670*
03-012-002	*S. matsudana 'Kew' PN 227 × pentandra 'Dark French' PN 670*
03-001-026	*S. lasiandra x pentandra 'Dark French' PN 670*
03-001-024	*S. lasiandra x pentandra 'Dark French' PN 670*
92-013-019	*S. matsudana 'Kew' PN 227 × pentandra 'Dark French' PN 670*
03-004-022	*S. matsudana 'Kew' PN 227 × lucida spp. lasiandra 113/1-13, PN sourced from Block B, Radio Nursery*
lasiandra 00/0-015	*S. lasiandra 00/0-015*

gooddingii	*S. gooddingii*