Genetic diversity and relationships of New Zealand totara (*Podocarpus totara)*

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

Master of Science
in
Genetics

at Massey University, Manawatu,
New Zealand.

Christina Whitney Marshall
2013

E kore te totara e tu noa ki te parae, engari me tu ki roto I te wao.

A totara is not found growing out in the open country, but in the heart of the forest

ABSTRACT

Totara (*Podocarpus totara*) is an iconic and endemic New Zealand species and its use as a timber for carving is still highly preferred by Tohunga whakairo (Māori carving experts). Current mature totara timber resources are scarce and mass replanting of totara is very costly. The ability to distinguish between species (especially *P. totara* and *P. hallii*) - identifying species from seed and seedlings – would be of much interest for nursery and restoration projects. Existing methods relying on bark characteristics, seed classification, and needle morphology are inadequate for this purpose. Hybridization can also make problematic species designations. This thesis reports the successful development of ten High Resolution Melting DNA markers that can differentiate New Zealand totara species. The chloroplast genome sequence of *P. totara* x *P. hallii* was completed and annotated, providing a further resource for developing additional molecular markers. The findings of this thesis will help ensure the "true" totara species (*P. totara*) is retained as a resource in perpetuity for Māori, conservationists, foresters and home gardeners.

ACKNOWLEDGEMENTS

The production of this thesis was not a sole effort on my part but a collection of the input from many people. I wish to express my deepest appreciation and thanks to all the wonderful people who have provided support, guidance and encouragement throughout my Masters project.

Firstly, I would like to acknowledge and thank my supervisors Peter Lockhart and Phillip Wilcox. Many thanks to Pete, for guiding me through the marathon of writing the thesis; for sharing your unique sense of humour; and for playing your banjo tracks to me. Thanks to Phill for his manaakitanga and making me feel welcome in his whare. Thanks for providing me with a decent beer and kai after a long day in the lab, and for being a caring soundboard, tēnā rawa atu koe.

Many thanks to my "unofficial supervisors" Nick Roskruge and David Chagne. Thanks to Nick for helping with Iwi consultation and always being available for a korero and cup of tea. Thanks to David for spending great deals of time teaching me how to analyse all the HRM data. All the one-on-one sessions and comments towards the results section of the thesis are appreciated.

Thanks to all the people of Level 5, Science Tower D, IMBS. Thanks to Trish for all your help with lab work dilemmas, and the tedious task of annotating the chloroplast genome. Thanks to the many people who helped with queries towards lab procedures, data analysis, DOC permits, paper assignments, IT, and thesis writing. With special thanks to Patrick Biggs, Robin Atherton, Simon Cox, Barbara Schoenfeld, Ibrar Ahmed, Matthias Becker, Nicole Grunheit and Oliver Deusch.

I would also like to thank the staff of Scion especially to Greg Steward, Heidi Dungey and David Bergin. Many thanks to Greg for providing help with DOC permits, sample gathering and thesis editing. Also to David for his support in sampling, and being open with his knowledge of totara. Thanks to Mark Morris for sample gathering and to the South Park lab members and other associated staff for all the help and friendly support.

Thanks must also go to all the other people that helped in one way or another. Thanks to John McCullum for assistance with the Galaxy pipeline. Thanks to Jimmy Schuster and James Rickard for help with traditional Maori knowledge and site selection. Also, many thanks to Takarei Te Tamaki, Jackie Aratema, Paul Horton, Maikara Tapuke, George Mutu, and Tom Myers for giving their permission to collect samples.

Special thanks must be given to all the trusts and organisations that provided financial support. To Future Forest Limited (Scion) for funding this project. To Wi Pere Trust for the Education Grant. Also to Kahungunu Iwi Inc., Wairoa Waikaremoana Trust Board, Te Whanau-a-Taupara Trust, Whakatohea Trust, Whakapaupakihi Trust 6 & 7 and Massey University for the education grants and scholarships.

Most importantly I would like to thank my whanau and friends. For without their support I would not have had the strength to persevere through the past two years. Special thanks go to my parents Terry and Jewell Marshall and grandparents Haina and Trevor Christie. Thanks to my sisters Alannah, Shannen and Terri and to all my other extended whanau. I sincerely appreciate the phone calls, text messages, emails and long talks when I came back home. For it is these things that made me feel loved and gave me perspective when times were rough. I am extremely grateful for the financial support you provided, along with Mum and Nan always ensuring I had a package of food to take back to Palmy – Me te arohanui ki a koutou katoa.

Last but not least I would like to thank Jeff, who entered my life towards the end, and unfortunately most stressful part of the project. I am greatly indebted to you for being such an understanding, supportive, and loving person. I thank you for your efforts to get me out of bed in time for the bus, rides you gave me to Massey, and many many meals you provided. Thank you for being there I truly appreciate it - Taku arohanui ki a koe.

Ehara taku toa, he taki tahi, he toa taki tini

My success should not be bestowed onto me alone, as it was not individual success but success of a collective

MY POSITION WITHIN THIS RESEARCH PROJECT

Ko Takitimu me Horouta te waka

Ko Whakapunake rāua ko Okahuatiu te maunga

Ko Te Wairoa-Hopupu-Honengenenge-Matangirau raua ko Waipaoa te awa

Ko Ngāti Kahungunu rāua ko Te Aitanga-a-Māhaki te iwi

Ko Ngāi Te-Apatu rāua ko Whanau a Kai te hapu

Ko Takitimu rāua ko Te Rongopai te marae

Ko Wi Pere te tipuna

Ko Christina ahau

Tihei Mauri Ora!

Previous to the commencement of this study I completed a summer project at Massey University where I investigated the taxonomy of the New Zealand kamokamo or *C. pepo*. The project involved the use of molecular techniques such as PCR, Sanger sequencing, and also networking with local Māori growers. At the conclusion of the project it became clear that a research field that used such research skills and also provided some benefit to Māori would bring utmost satisfaction to me. As a Māori science student I also felt obliged to partake in a research project that would be of some significance to my whanau, iwi, and I. By coincidence I was briefed on the commencement of my summer project about the Totara, Forestry project at Scion. I was very interested in being part of a project where modern research techniques could be utilised to gain knowledge and help solve the issue of declining timber resources for Tohunga whakairo (carving). This thesis is written in hope that the future resources of totara will be sustained in a manner that allows the use of timber for whakairo by Māori throughout New Zealand.

TABLE OF CONTENTS

LIST OF TABLES

APPENDICES

LIST OF FIGURES

CHAPTER 1 INTRODUCTION

1.1 GENERAL INTRODUCTION

Totara (*Podocarpus totara*) is an iconic and endemic New Zealand species. Its use as a timber for carving is highly preferred by Tohunga whakairo (Māori carving experts). The commercial potential of totara as a softwood timber has also been recognised by various organisations in New Zealand due to its durability, resistance to marine borer and ability to be easily worked (McConchie, 1999; NZ Wood, 2010; Tanes Tree Trust, 2010). Totara timber quality is influenced by various factors including the site, age, silviculture and particularly genetics. Species level differences influence timber quality as *P. totara* is known to have characteristics that favour its use over other *Podocarpus* species (grows taller, more durable). However, discriminating *P.totara* seedlings from closely related *Podocarpus* species (*P. hallii*, *P. acutifolius*, *and P. nivalis)* can be difficult and this is important when (re)establishing new totara woodlots. Ultimately, furthering our genetic knowledge will improve understanding of overall timber quality, and also protection of totara as a resource for both contemporary and traditional Māori uses.

Current mature totara timber resources are scarce and mass replanting of totara is very costly (Bergin, 2000). There have been reports of totara seedlings purchased actually being another species (usually *P. hallii*) A. Laurie (personal communication, 2010). An ability to distinguish between species (especially *P. totara* and *P. hallii*) will help by correctly identifying both seed and seedlings. Existing methods such as studying bark characteristics, needle and seed morphology can be imprecise because of hybridisation. Until now there has been no historical need to identify totara from seedlings and hence there is no mātauranga Māori (traditional Māori knowledge) to assist.

This project aims to identify DNA markers that will differentiate New Zealand *Podocarpus* species to aid *nursery growers in assuring seedling identity.* This will ensure that representative "true" species can be retained as a resource in perpetuity for Māori, conservationists, foresters and home gardeners. This thesis reviews the origin, natural distribution, biology, uses, and species delimitation of *P. totara.* It also reviews molecular methods currently available for species differentiation. It reports the application of novel molecular markers for identifying and distinguishing New Zealand *Podocarpus* species.

1.2 NATURAL HISTORY OF *PODOCARPUS*

1.2.1 ORIGINS OF NEW ZEALAND PODOCARPS

The earliest recorded appearance of a New Zealand podocarp macrofossil was in the lower Triassic (before the separation of New Zealand from Gondwana; 248 million years B.P.). This was in the form of a Canterbury leaf fossil, identified as *Mataia podocarpoides* (Townrow, 1967). Fossil pollen from *Podocarpus* first appeared in the upper Triassic around 200 million years B.P Mesozoic era (Fleming, 1975). However, it was not until the Cretaceous period (100 million years B.P) that *Podocarpus* fossils became abundant.

Pollen flora of the Cretaceous period was dominated by *Podocarpus* species despite the emergence of angiosperms in the fossil record. By this time, land connections between early New Zealand and other continents had already been broken (Fleming 1975; Mildenhall et al. 2004). Early angiosperm pollen was identified in the Eocene circa (40 million years B.P) and the first lowland totara-like fossil was identified in the lower Miocene (24 million years B.P.). In addition, molecular clock data suggests that Podocarpoid-Dacrydioid lineages separated 60-94 Million years ago (MYA) in the mid to late Cretaceous period (Biffin, Conran, & Lowe, 2011). Currently there are four endemic species of *Podocarpus* present in New Zealand, commonly referred to collectively as "totara" including *P. totara*, *P. hallii*, *P. acutifolius*, and *P. nivalis*. Other

endemic Podocarpaceae genera in New Zealand include *Dacrycarpus*, *Dacrydium*, *Lagarostrobus*, *Halocarpus*, *Lepidothamnus*, *Manoao* and *Prumnopitys* totalling 17 species.

There is still debate around the origin of present day totara species. The first possibility is that present day totara species are direct descendents of fossil lineages (Fleming, 1975). The second is that present day totara is a result of transoceanic dispersal (Biffin, Hill, & Lowe, 2010). A recent study using chloroplast molecular clock data found totara to originate 40 MYA (Leslie et al., 2012). This recent study has revealed that the lineage of New Zealand and Australian Podocarps, which includes *P. alpines* and *P. lawrencii,* diverged from other podocarp species prior to but near the paleogene boundary approximately 65 MYA. This is subsequent to the breakup of Gondwanaland and hence suggests present day totara in New Zealand have arrived by transoceanic dispersal.

1.2.2 NATURAL DISTRIBUTION AND FOREST ECOLOGY

Pleistocene glacial-interglacial climate changes are thought to have played an important role in determining the current distribution patterns of New Zealand flora. Wardle (1963) argued that many plant species have been unable to reoccupy their previous habitats since the last glacial period. Wardle suggested that areas in the North Island and northern regions of South Island were places where species survived the Pleistocene glacial conditions. In particular, it has been suggested that the distribution of *P. acutifolius* can be explained by considering the predicted ice cover and available habitat in the Pleistocene (Wardle, 1972). The recently discovered Waihoensis variant (*P. totara* var. waihoensis) is speculated to have arisen as a consequence of the arrival of warmer conditions. The rising temperatures are also thought to have given a competitive advantage to drought-tolerant podocarps such as matai and *P. totara* leading to a wider interglacial distribution (McGlone, 1988).

Dwindling *P. totara* is apparent when comparing the current distribution of *P. totara* with previous records where it once was a main component of lowland conifer forest

in New Zealand. Although reasonably large totara forests can still be found near Whirinaki, Te Urewera, Puketi, Waipoua, and Pureora, it mostly exists now as small stands or as isolated trees in farming areas (Bergin, 2003). The rapid decline in totara numbers occurred in the early 1800s, and like many other native trees, is thought to be a result of the harvesting of materials for building, bush fires, and forest clearing to make way for agriculture and exotic forestry projects (Bergin, 2003; Best, 2005a; McConchie, 1999). Reasonably large totara forests can still be found near Whirinaki, Te Urewera, Puketi, Waipoua, and Pureora. However, *P. totara* is known as one of the few native tree species that can grow out in the open, such as in paddocks, and is frequently regarded as a weed by farmers (Bergin, Kimberley, & Low, 2008).

While some species are grown in very distinct areas, *P. totara* and *P. hallii* are not as clearly delimited. *P. totara* and *P. hallii* occur throughout most of New Zealand with an important difference being the altitude at which they are found (Salmon, 1998). *P. totara* is normally found in lowland areas and lower montane forest, generally up to 600m above sea level. However, *P. hallii* is more commonly found in subalpine forest and is located in a wider range of sites including wetter, colder and less fertile soils (Bergin, 2003). Although the two species are believed to be of differing altitudes there are exceptions. For example: hybrids of *P. totara* and *P. hallii* are thought to occur at lowland sites such as Banks Peninsula (South Island, New Zealand). In addition, Bergin and Kimberly (1992) have reported that *P. totara* and *P. hallii* 'overlap' at Pureora Forest. Hence altitude cannot be solely used for discriminating *P. hallii* from *P. totara*.

Other species of New Zealand *Podocarpus* are more easily identified because of their distinct distributions and morphologies. Both *P. acutifolius* and *P. nivalis* have an allopatric distribution (Salmon, 1998). *P. nivalis* is commonly found in subalpine areas, such as the New Zealand North Island Tongariro region, and upper forest margins. While *P. acutifolius* is found in montane and lowland forest in north Westland of the New Zealand South Island (Allan, 1961; D. Bergin, 2003). Hybrids of *P. acutifolius* and *P. totara* (*P. totara var. waihoensis*) can be found along the west coast of the New Zealand South Island (Wardle, 1972). Hybrids are present in areas where these distributions overlap between all four totara species. Flavonoid data provides support

for interpretation of these morphological forms (Allan, 1961; Webby, Markham, & Molloy, 1987).

1.2.3 REPRODUCTIVE BIOLOGY

P. totara is a dioecious plant that is wind pollinated. Reproductive maturity is reached from ten years of age based on observations of viable seeds (Bergin, Kimberley, & Low, 2008). The reproductive cycle begins with the growth of yellow-green catkins on male trees that release pollen in spring. This is followed by production of cone like glaucous ovules on female plants in October-November. The cones are fertilised following 2-3 months of wind pollination, and ripening of the fruit occurs in autumn (Bergin, 2003; Wilson & Owens, 1999).

Like most Podocarps, totara seed is spread via birds after consumption of the fruit. The *P. totara* berries serve as a food source for many native birds including bell birds (*Anthornis melanura*), kereru (*Hemiphaga novaeseelandiae*), and tui *(Prosthemadera novaeseelandiae*). It has been suggested that an increase in *P. totara* numbers would eventually increase the numbers of birds that disperse totara, as more food source would become available (Beveridge, 1964). Bird-mediated dispersal has presumably helped to overcome geographical barriers, such as altitude, and has facilitated hybridisation between totara species (particularly *P. totara* and *P. hallii*).

1.3 USES OF TOTARA

1.3.1 TOTARA AND MĀORI COSMOLOGY

To understand the importance of totara to Māori one must first understand the Māori view of creation. The magazine Te Ao Hou (New Zealand Government, 1965) discusses that “the nature of the world and its inhabitants was primarily determined by the events that took place during its creation”. It is said that Ranginui (sky god) rested upon Papatuanuku (land mother) in the beginning, and that their children were born

between them in darkness. The children then decided to separate their parents to allow light to come into the world. The children of Rangi and Papa then became gods to different parts of the natural world including Tāne-Mahuta who became god of the forest. It is important to note that different Māori tribes (iwi) have their own versions of the creation story (Erlbeck & Waipara, 1998; New Zealand Government, 1965; Winitana & Reisinger, 2010). The word Tāne means 'male' and is thought to be the source of strength (and mana - prestige) for all things born into this world. Tāne is the ancestor of all trees in the forest as he took Mumuwhango and by her created the totara tree. Also, as canoes are made from trees of the forest for this reason they are known as 'the trunk of Tāne". Thus the story of creation is significant in understanding the value of totara to Māori because it recognises the connection between the gods and a being on earth – totara (Government, 2012; Grace, Grace, & Potton, 2006; New Zealand Government, 1965; Te Rito, 2007). Totara was considered to have special mythical origin and was hence regarded as an 'rākau rangatira' or wooden chief and the saying "kua hinga he totara I roto I te wao nui a Tāne" a totara has fallen in the great forest of Tāne. There are many other quotes signifying the qualities of totara for example: "E kore te totara e tu noa ki te parae, engari me tu ki roto i te wao – a totara is not found growing out in the open, but in the heart of the forest (Best, 2005a, p. 106). Totara trees were held with the utmost prestige and great chiefs were often likened to totara (Hamlin, 1962).

Understanding mātauranga Māori is also essential when dealing with a New Zealand taonga (prized possession) such as totara. Mātauranga Māori is the "knowledge or understanding of everything visible and invisible existing in the universe" (Mohi, 1993) For species differentiation of totara seedlings there is no known mātauranga Māori. From discussions with Tohunga whakairo (Māori carving experts) there was no need for Māori to differentiate *P. totara* (desired wood species for carving) from *P. hallii* as juveniles because there was always a surplus supply of rākau (wood). It is known that Māori were ahead of their time in a conservation perspective, as elders would put a tapu (cordon) on areas of the forest after a mature tree was felled to allow for natural regeneration of the forest (Best, 2005a).

1.3.1.1 TRADITIONAL MĀORI USES

P. totara was used extensively by Māori. Traditionally totara wood was used for carving, canoes, musical instruments, toys, and containers (Best, 2005b; Francis, 1956; Riley & Enting, 1994). Such uses arose from the totara wood characteristics such as length, lightweight, durability and ability to be easily worked. *P. totara* is more favoured as a timber species than *P. hallii* as it has a smaller stature and tapered boles. Totara was found in abundant numbers throughout New Zealand, so was often the more common choice for construction of ocean war canoes, or waka. *Agathis Australis* or kauri is the only native tree that can be considered a more suitable timber source.

Totara played a vital role in carvings (whakairo) for Māori historically and also currently as it is widespread, easily worked, and was durable (Bergin, 2003; Best, 2005a; Walker, 2008). Carvings were a manner by which Māori recorded their whakapapa (geneology), karakia (prayer), cosmology (origin of the universe) and methodologies, thus the role of carving was vital to the history and culture of Māori. Many items which have traditional carvings, and have been retained through history, are held with the utmost prestige and respect by Māori including marae, waka huia, and taiaha. Carvings cannot be 'read' in the Europeans sense as they are often details of tribal affairs and pay deepest respect to history, ancestors, and the people for whom they were prepared for.

Totara was also used medicinally by the Māori. For example: bark was used as splints for broken bones; 'paipai' or skin complaints were treated with the smoke from burning wood; the boiling of the inner bark gave rise to an oil used to reduce fever (Bennett & Cambie, 1967; Phillips & Wadmore, 1950; Riley & Enting, 1994; Williams, 1996). *P. totara* has not been commonly used as extensively as a medicine since the introduction of more convenient western medicines.

1.3.1.2 HISTORICAL EUROPEAN USES

Early European settlers also exploited the qualities of totara. Early reports show that Captain Cook used an infusion of totara leaves with manuka leaves to treat crewmen

for scurvy (Williams, 1996). Other uses include the use of timber for buildings, fence posts, railway sleepers and bridges. The natural residues within totara made it an excellent timber source for early wharf construction as it is resistant to marine borer and is very durable. As totara resources became exhausted, other exotic species such as pine (with preservative treatment) and Douglas-fir became more dominant timber resources. However, there is still considerable demand for a perpetual long-term resource of *P. totara* as its demand for cultural and economic applications is growing(Bergin, 2000).

1.4 APPLICATION OF DIFFERENTIATING TOTARA SEEDLINGS

Currently there are few supplies of mature *P. totara* stands appropriate for timber usage. The majority of natural stands are situated in conservation reserves and this has raised concerns with various interested parties (*iwi* – Māori tribal group, the forestry sector, and landowners). However, to develop a sustainable management system for the planting of *P. totara*, correct identification of seedling is essential (Bergin, 2000).

P. totara has been shown to freely hybridise with other species, such as *P. hallii* and *P. acutifolius,* and this can result in areas where morphology of species overlap and hence species identification can be difficult. Morphological characteristics are imprecise for seed and juvenile *P. totara* hybrids, and waiting till they mature is time consuming for slow growing trees. Understanding the genetic relationship between *P. totara* and other species with which it hybridises will assist in discriminating species at seedling level, and hence benefit revegetation programmes for *P. totara*. Ultimately this will ensure there is a future and enduring resource of *P. totara* timber.

1.4.1 COMMERCIAL TIMBER RESOURCE

Totara stands have potential financial value if managed appropriately (Bergin, 2000; Clifton, 1990; McConchie, 1999; NZ Wood, 2010). As the use of imported decorative

timber becomes less acceptable the value of native plantations, such as totara, increases. Totara can grow on a range of sites throughout New Zealand and wood characteristics of *P. totara* favours its management over other native *Podocarpus* species. Establishment of totara stands requires mass planting of seedlings, of which species identity needs to be certain. Alongside seed selection, other practices can be carried out (Eg. use of a manuka nurse, minimal silviculture input). Such practices can reduce establishment costs and provide possible avenues for commercialising totara timber.

As *P. totara* is a slow growing species, efforts into genetic improvement of the species have been little pursued. *P. totara* takes twice as long to grow to an equal volume of *Pinus radiata* in planted stands but 4x longer in natural stands (Bergin, 2003; McConchie, 1999). Slow growth, along with high costs of plantation establishment are key reasons that totara is often disregarded as a commercial plantation species. Also lack of sustained market for totara, low proportion of heartwood in first 50-100 years, and competition with imported tropical timber.

1.4.2 CARVING TIMBER FOR MĀORI

P. totara has always been held in high regard as a carving timber by Māori, and is still of considerable cultural value (Bergin, 2003; Mead, 1995; Phillips, 1997; Walker, 2008). The selection of timber for carving requires that specific 'totara' species are retained. *P. totara*, or true totara, is highly sought for as it has a straight grain, can be easily worked, is durable, and grows taller and wider than any other Podocarp species in New Zealand. The value of totara timber for such uses cannot be easily quantified but is known to reward owners in many other ways (McConchie, 1999).

1.4.3 PHARMACEUTICAL COMPANIES

More recently, *P. totara* was discovered to contain a useful extract which has been given the trade name 'totarol' (Jaiswa et., 2007; Kubo, Muroi, & Himejima, 1992). Totarol is extracted from dead *P. totara* wood, or old fence posts and displays antibacterial activity (Mende Biotech Ltd, 2006). Interestingly, the New Zealand based

company Red Seal has a toothpaste branded with the Totarol extract called Taradent and the toothpaste description states that "Totarol provides for a natural mouthwash effective against bacteria" (Red Seal Natural Health, 2010). Businesses, such as Red Seal, rely on the existence of a perpetual *P. totara* resource. As recycled resources are diminishing there is an increasing demand for an effective totara management plan.

1.4.4 NURSERY GROWERS

Identification of New Zealand *Podocarpus* species at seedling stage is not straight forward thus a difficult task for nursery growers. The four *Podocarpus* species can be distinguished on the basis of wood microstructural properties: ray height, ray parenchyma walls, tracheid length, and growth rings according to an identification key stated in Patel (1967). Morphology boundaries overlap, especially leaf and seed characteristics, and precise identification of species via seedlings can be difficult. It is thought that hybridisation has commonly occurred across mountain transects where the lower altitude *P. totara* sympatrically introgresses with the higher altitude *P. hallii* (Bergin, Kimberley, & Lowe, 2008; Webby, Markham, & Molloy, 1987). Bergin (2003) described the two distinct seed morphologies of *P. totara* and *P. hallii* as well as a range of morphologicalintermediates (Figure 1). Thus, any genetic information that can help delimit these species would be of use when selecting seed required by nursery growers.

Figure 1: Long narrow seed of *P. hallii* (Left) and ovoid seed of *P. totara* on the right with intermediate forms suspected to be of hybrid origin also shown in between (from Bergin, 2003).

1.4.5 ECORESTORATION

Prior to the arrival of humans, lowland and montane New Zealand was composed mostly of beech and podocarp forests with trees such as kahikatea, matai and totara all being dominant trees. However, since the colonisation by Europeans approximately 150 years ago, little of these forests remain. As stated before, forests were largely cleared for productive exotic pastoral or forestry land use and the restoration of these forests has been limited (Bergin, 2003; Best, 2005a).

Restoration of native podocarp forests is desirable if we are to maintain landscape continuity between relict stands of old forest (Bergin, 2000). However totara in the wild has received little interest compared to other podocarp species (Ogden, Stewart, & Allen, 1996). It has become apparent that for a sustainable resource of native timber, whether it is totara, rimu or kauri, we need to have management plans in place to replant the species. Such a plan would again, require genotyping sources of totara. A DNA marker system that is cost effective for screening seedlings or juveniles to determine species identity would be useful in replanting, and hence aid in eco-restoration projects Bergin, 2000; McConchie, 1999).

1.5 SPECIES DELIMITATION

The Flora of New Zealand Vol. I. recognises four native *Podocarpus* species including *Podocarpus totara* G. Benn. Ex D. Don (Allan, 1961). An important point of note is that *Podocarpus* species native to New Zealand are commonly known by their Māori name '*totara*' including *Podocarpus totara* (true totara/totara) and three other closely related relatives, *Podocarpus hallii* (Hall's totara), *Podocarpus acutifolius* (needle-leaved totara) and *Podocarpus nivalis* (snow totara) (Bergin, 2003). Thus the name "*totara*" does not always necessarily distinguish between species. The New Zealand *Podocarpus* species are similar in appearance with the strongest morphological overlap seen between *P. totara* and *P. hallii.* However, the genetic basis of such morphological characteristics is poorly understood.

Currently New Zealand *Podocarpus* species are delimited by morphology descriptors that include bronze to dark green coloured leaves with an adaxial surface that is dark green and the abaxial surface yellowish green. Leaves are linear-lanceolate, acute, apex, pungent, straight to slightly falcate, sessile near narrow base, and they also have a distinct to obscure mid-vein (Figure 2). The adult leaves range from 1.5-3 cm × 3-4 mm, and juveniles range from 20 × 1-2 mm in size (Allan, 1961; Salmon, 1998). *P. totara* can grow up to 30m in height and 2m in diameter and have a thick reddish brown, deeply furrowed bark (Figure 3) (Metcalf, 2002).

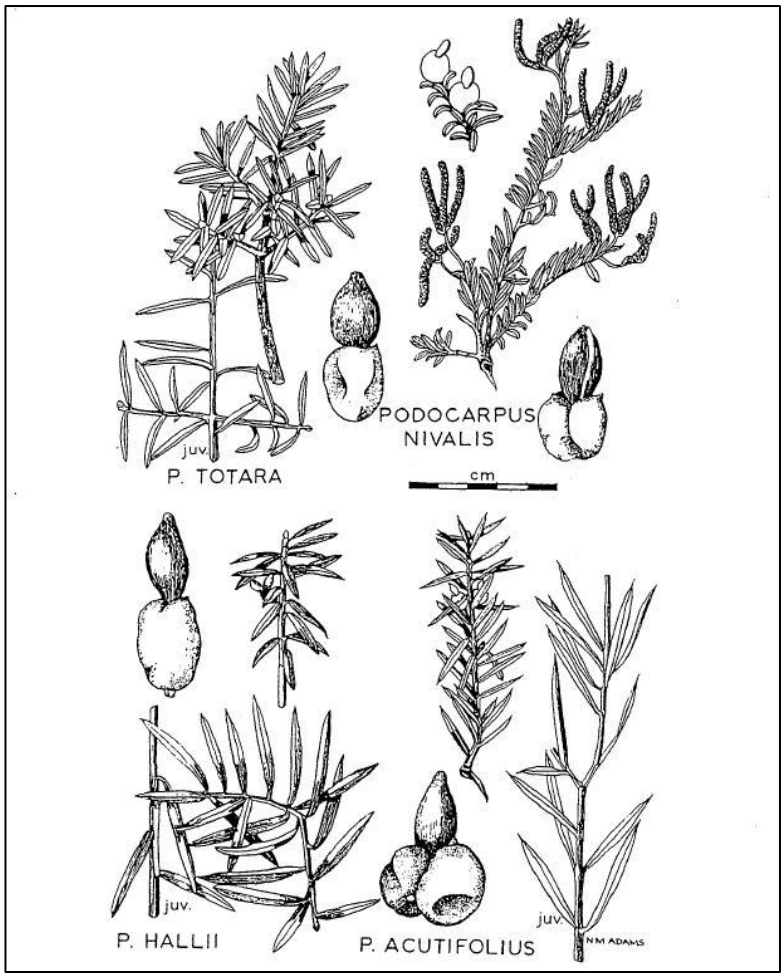


Figure 2: Leaf morphology of *P. totara*, *P. hallii*, *P. acutifolius*, *P. nivalis* (Eagle, 2006).

P. hallii is very similar to *P. totara* morphologically. Allan (1961) states that *P. hallii* is characterised by brownish green coloured leaves that range from 2.5-5cm × 4-5cm when juveniles, and 2-3cm × 3-4mm when adult. Leaves have an evident midvein, are pungent, linear-lanceolate, and are rigid. *P. hallii* can grow up to 20m in height and 1.25m in diameter (Allan, 1961; Metcalf, 2002). *P. hallii* can be easily distinguished from other species, by its unique thin papery bark (Figure 3). Bark thickness is regarded as a good means of determining whether a tree is *P.totara or P.hallii* (Matsui, et al., 2004).

P. acutifolius is characterised by a more shrub-like appearance with distinctive prickly leaves (Figure 2). Its height can vary from 1 to 9m tall and grows up to 4m in diameter (Allan, 1961; Collins, 1979). *P. nivalis*, another close relative of *P. totara*, grows as a prostrate shrub (Collins, 1979). *P. nivalis* can grow up to 3m in height; it is wide branching with leaves closer together and arranged spirally (Figure 2).

The *Podocarpus* species in New Zealand are thought to freely hybridise and such hybrids display a wide range of leaf, seed, and tree morphologies. A distinct variety of totara, *P. totara* var. Waihoensis has been identified and morphological and flavonoid marker studies strongly suggest it to be a hybrid between *P. totara* and *P. acutifolius* (Webby, Markham, & Molloy, 1987) . Commonly found "golden totara" (*P. aurea*) is thought to be a hybrid between *P. hallii* × *P. totara*, and the "Pendulus" variety is regarded as a *P. acutifolius* × *P. nivalis* hybrid (Allan, 1961; Wardle, 1972; Webby, Markham, & Molloy, 1987). DNA data would help to test these hypotheses.

P. totara is easily worked, cuts smoothly across the grain and is very amenable to all machining operations including jointing. *P.totara* wood has a moderate density of 480kg/m3. It is not suited for large beam or joint construction. Heartwood is very durable with a modulus of rupture equal to 62 MPa and a modulus of elasticity of just 6.4 GPa. Thus it is very durable in the ground; a 50 x 50mm stake would last at least 25 years, equivalent to the H4 Hazard class. The natural durability is due to extractives such as totarol in the wood.

P. totara sapwood is also resistant to both fungal and insect attack, including marine borers, hence sapwood is suitable for marine environments. The wood of *P. totara* is considered mature from 75-100 years depending on whether tree is from a natural or planted stands, due to differences in growth rates and parameters used to class a tree as mature (Bergin & Ecroyd, 1987; NZ Wood, 2010).

P. hallii wood properties are not known to the same detail as to they are for *P. totara*. Differences include bark being much thinner and the timber not being as smooth grained (Matsui et al., 2004). Timber is usually sourced from trees of a smaller mean height and diameter than *P. totara*, and has tapered boles, due possibly to higher elevation growing environments (Hinds & Reid, 1957).

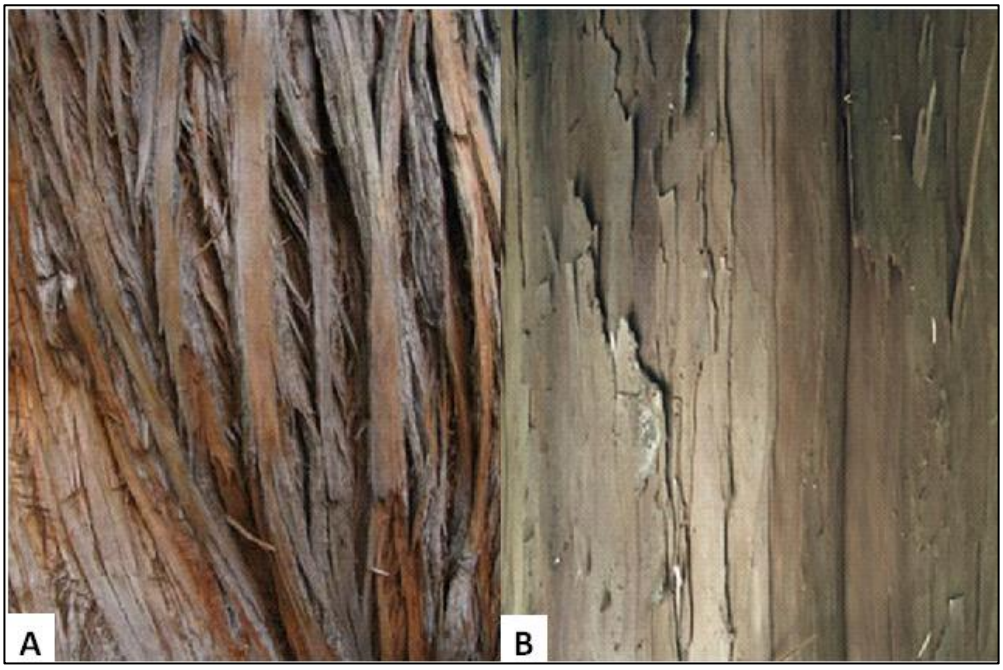


Figure 3: Deeply furrowed bark of *P. totara* (A), thin paper bark of *P. hallii* (B)(NZ Plant Conservation network, 2012)

1.5.1 PROVENANCE VARIATION

Early growth of *P. totara* seedlings were found to be highly correlated with the rate of germination after sowing in a study conducted by Bergin & Kimberley (1992). The height in 2 year old *P. totara* seedlings grown in a common garden experiment from

seed collected from various locations was measured and height was found to be negatively correlated with provenance latitude. In this study it was suggested that genetic factors were linked with the mean summer temperature of the seed source locality. The authors also found that variation seen in branch length and stem form were not correlated to provenance site variation; and that no differences between provenances were seen in leaf size and foliage colour (Bergin & Kimberley, 1992).

Previous to this, Hawkins (1988) carried out arguably the most substantial study on provenance variation in *P. totara*. The study presented data for seedling nutrition, temperature, and isozyme analysis experiments. The study found no correlation between optimal growing temperature and climate of seed origin, but correlation was found between the frost tolerance of *P. totara* and climate of seed origin. These findings were supported by a subsequent isozyme study where frost hardiness of *P. totara* seedlings was found to be positively correlated with the altitude of provenance origin (Hawkins et al., 1991). The variation was suggested to be determined more by genetic factors rather than environmental factors, as they demonstrated allele frequencies at one locus that were significantly correlated with provenance frost hardiness (Bergin, Kimberley, & Low, 2008).

In one study, using random amplified polymorphic DNA markers (RAPD), almost 90% of the genetic variation identified was found within individual *P. totara* populations whilst only 10% of the variation was partitioned between populations (T. Richardson, unpublished). These markers while useful for studying genetic variation within the *P. totara*, were not tested on other totara species, thus their general applicability in closely related species was undetermined.

In another study, isozyme analysis of *P. totara* found populations to have a genetic differentiation of 36.2% greater than the conifer average of 6.8%. It was noted that 33.8% of the diversity was due to differences between *Waihoensis* and *totara* varieties (Billington, 2005). These authors also found *P. hallii* to exhibit the highest mean number of alleles per locus and percentage polymorphism. The greater genetic variability seen in *Podocarpus* species is mostly due to the degree of inbreeding,

differences in chromosome number, and amount of hybridisation (Hawkins, 1998). However, the extent of hybridisation and its evolutionary significance is still unclear.

1.5.2 GENOMIC CHARACTERISTICS

As adults, the four totara species can be quite distinct, but as juveniles their appearance is very similar. Conifer genomes are generally stable and large in size. *P. totara* in particular has a nuclear genome size of 22.5 2C (2 copy) DNA, with *P. hallii* having a nuclear genome size approximating to 17.4 2C DNA. There is little genetic information available, but *P. totara*, *P. hallii* and *P. acutifolius* have a chromosome number of 2n=34, while *P. nivalis* has 2n=38. Heterochromatin distribution has also been investigated in studying the evolution and distribution of New Zealand Podocarps (Davies, Brien, & Murray, 1997; Khoshoo, 1960).

1.6 DEFINING SPECIES LIMITS

1.6.1 MOLECULAR MARKER DEVELOPMENT

The development of molecular markers serves as a primary tool for assessing the evolutionary and population history of species (Seeb et al., 2011). Numerous studies have utilised molecular markers such as RAPD, RFLPs, microsatellite, and single-nucleotide polymorphisms (SNPs) to assess genetic relationships in plants (Arif et al., 2010; Nybom, 2004; Sudheer et al., 2009; Naresh et al., 2009) . Prior to development of these DNA-based techniques, allozymes were the most commonly used genetic markers and they were exploited to address questions relating to the biology of plant population. However, with recent molecular marker advancements, “point mutations” (single nucleotide polymorphisms: SNPs) are becoming more frequently studied as they are the most common type of genetic variation in the genome. They occur in coding and non-coding regions, and are becoming progressively more cost-effective to assay (Cruzan, 1998; McCouch et al., 2010). SNPs can also address plant population

add historical questions that were not practical to address with other types of molecular markers.

Before the arrival of "next-generation" (i.e., high-throughput) sequencing technologies, the marker strategies used were often limited to factors such as marker characteristics – type, number, and variability, and available resources - time, money, and equipment (Liao & Lee, 2010). The two main methods for deducing genetic relationships based on DNA sequence differences in plants were fingerprinting and Sanger sequencing. In fingerprinting, small regions of the genome are amplified and polymorphisms are scored on the basis of a peak being present or absent. In comparison, Sanger sequencing (Sanger, Nicklen, & Coulson, 1977) uses sequence data or primers from studies of closely related species to amplify regions of interest. Sanger sequencing can generate targets up to 1000 bp in size and this can be achieved very cheaply However, Sanger sequence is limiting in that it is slow to generate large numbers of overlapping sequences. This method, and most methods of DNA fingerprinting, are limited by the length of DNA they can characterise as amplification is performed by polymerase chain reaction (PCR). Because of this shortcoming, multiple genome regions are often targeted. For example, one recent study involving rice utilised multiple SNP markers to generate a cost-affordable genome wide assay (McCouch et al., 2010). Such an approach overcomes the limitation of trying to amplify large regions, and provides for analysis of genetic variation across the genome. Ideally, the more sequence data available, the easier it is to find genetic variation, and hence methods that provide greater genome coverage are being increasingly employed for plant population studies (Bräutigam & Gowik, 2010; Imelfort et al., 2009).

1.6.2 NEXT-GENERATION (HIGH-THROUGHPUT) SEQUENCING

As single nucleotide polymorphisms (SNPs) are the most sought after molecular marker, owing to their high abundance, a technology that can generate data rich in SNPs is most favourable (Eckert et al., 2008; Seeb et al., 2011; Zeng et al., 2010). For this purpose, new sequencing technologies, from a variety of companies (Illumina,

Roche, ABI etc) are increasingly being utilised for the development of DNA markers. These new methods can produce high sequence coverage with high accuracy relatively cheaply in comparison to traditional Sanger sequencing. Of particular interest to researchers is that the new sequencing technologies can create massive amounts of data for non-model organisms which can aid in identification of species relationships, and investigation of hybridisation. In one recent high-throughput sequencing study, Hohenlohe et al. (2011) found thousands of SNPs for assessing the extent of hybridisation between two trout species. High-throughput sequencing was attractive for the present project, in which there was a need to develop SNP markers for addressing questions of genetic diversity, and the extent of hybridisation between species.

1.6.3 CHLOROPLAST MARKERS

In many plant studies chloroplast DNA (cpDNA) is considered ideal for molecular marker studies (Cruzan, 1998). CpDNA is inherited paternally and maternally (uniparental) in different plant species, and the gene organisation is conservative. CpDNA also have regions that are known to be highly variable, or 'hotspot regions', and these present an avenue for high resolution marker development (Ahmed et al., 2013; Kim et al., 2009). However, cpDNA markers are not typically linked to phenotypic traits of interest, and for such studies genome wide analyses nuclear genome SNPs are often used. Conifers chloroplasts are typically inherited paternally and thus they are moved by both seed and pollen dispersal (Hamza, 2010). Chloroplast markers often provide greater insight into the geographic structure of populations as they are haploid and so are fixed by genetic drift more rapidly than nuclear DNA. SNPs can be developed for nuclear and cpDNA, provided that sequence data is available for the organism of interest.

1.6.4 NUCLEAR EST MARKERS

Nuclear markers also allow the identification of hybrid and clonal individuals (Hamza, 2010). Nuclear markers differ in that they are bi-parentally inherited and hence are useful for resolving spatial structure and genetic diversity of species (In this case *P.*

totara, P. hallii, P. acutifolius, and *P. nivalis*). Eckert et al. (2008) performed a high-throughput genotyping study for *Pinus taeda* where various SNPs were mapped throughout the nuclear genome to help deduce how traits such as drought tolerance, wood quality, and disease resistance were inherited. Thus the combined analysis of these different marker types is expected to provide the greatest possible insight into species structure and genetic diversity of totara forest stands.

1.6.5 LOW COST SCREENING METHODS FOR NUCLEAR DNA

There are various techniques available for assessing the genetic diversity in nuclear DNA of plants. As mentioned previously, DNA fingerprinting (e.g, AFLP, RFLP, ISSRs, and RAPDs) has commonly been used to study intraspecific variation. However these types of data can be noisy and are not ideal for studies involving inter-specific relationships. These methodologies are likely to be superseded in the future by high-throughput sequencing methods (Meudt & Clarke, 2007).

SNP mapping via High Resolution Melting (HRM) provides an alternative low cost method for screening nuclear DNA. Such advantages of HRM and hence reasons why preferred by some researchers is because of its ease of use, sensitivity, specificity and non destructive nature. It can also assay large numbers of regions simultaneously and is ideal method for low cost applications such as species differentiation that require only a few discriminating loci. HRM can be implemented effectively when coupled with high-throughput sequencing and is considered by some authors to be the first method of choice when screening large primers sets across many accessions (Rouleau et al., 2009; C. F. Taylor, 2009; Vossen et al., 2009)

1.7 HYPOTHESES AND WORK UNDERTAKEN

a) In the study reported in this thesis, nuclear molecular markers were developed for New Zealand *Podocarpus* species after mapping NGS (Illumina) sequence reads to conifer ESTs. This approach proved more successful than transfering nuclear molecular markers previously developed for *Pinus radiata*. The markers developed for *Podocarpus* were used to screen *P. totara* and closely related species using an HRM protocol. Of particular interest was determining whether transects made across a heterogeneous landscape would show evidence of introgression between *Podocarpus* species. This hypothesis has been suggested from patterns of morphological variation, and was evaluated here.

b) The chloroplast genome of *P. totara* which had previously been sequenced but which was unfinished, was assembled into contigs, gaps filled, completed and annotated. From the determined genome sequence, hypervariable regions of substitutions were identfied through prediction from the distribution of chloroplast oligonucleotide repeat sequences as recently described and reported in Ahmed et al. (2013). These predicted regions of sequence variation were then evaluated by mapping to the reference genome a mixed population of totara Illumina reads.

c) The potential of the nuclear and chloroplast markers developed in the present study for species identification and future phylogeographic studies in New Zealand has been discussed.

CHAPTER 2 MATERIALS AND METHODS

2.1 MĀORI CONSULTATION

Working with Totara – A New Zealand taonga

Before conducting research on New Zealand *Podocarpus* "totara" it was appropriate to seek support and relevant information from local iwi. A requirement of the DOC (Department of Conservation) permit for collection of tissues was that relevant iwi also be consulted (Table 1). In doing so, this project has recognised that the tangata whenua or Māori have a significant role as kaitiaki (guardian) within all the different iwi and hapū of New Zealand. Seeking approval from Māori was not always an obligation but an important part of the process required to achieve the aims of the research project. Consultation allowed an opportunity for iwi to voice any issues pertaining to risks or benefits of the proposed research. Consultation also ensured that the research did not affect the relationship that tangata whenua have with their whenua (land), water, wahi tapu, taonga species, natural resources or mahinga kai. Ultimately the process allowed the iwi representative to discuss the research project and agree to conditions of consent for sampling.

Iwi consultation was sought for tissue collection from the following sites: Mount Pureora, Mamaku Ranges, Makatiti Dome, and Mount Taranaki. Initial contact was made by either email or phone call. Efforts were made to meet with appropriate iwi representatives for each sampling site. Details of these meetings were passed onto DOC. We also met with a carver from Te Puia Arts and Crafts Center and Historic Places Trust to confirm that our project aims would be useful (Table 1).

As part of the consultation process we also spoke with James Rickard (Tohunga whakairo – carving expert) and James Schuster (Historic Places Trust). Project methods and aims were described, and use of projects resources discussed. This allowed the input of end-users to the collection of samples for project and use of markers after

completion of project. Neither of these parties were representing their iwi but were both important stakeholders.

Table 1: Contacts for Māori consultation.

Person	Iwi Affiliation	Role	Area for Sampling
James Rickard	Ngāti Porou	Te Puia Māori Arts and Crafts Centre Tutor	n/a
Jimmy Schuster	Ngāti Tarawhai, Ngāti Pikiao	Member of New Zealand Historic Places Trust	n/a
Takarei Te Tamaki	Ngāti Tura, Ngāti Te Ngakau	Chair of Ngāti Tura Ngāti Te Ngakau Hapu Trust	Mamaku Area
Jackie Aratema	Ngāti Whakaue, Ngāti Tarawhai	Chairman Rotoititi 15 Block	Makatiti
Paul Horton	Rangitaane O Manawatu (ROM)	ROM representative	Massey University
Maikara Tapuke	Te Atiawa	Te Atiawa representative	Taranaki
George Mutu	Ngāti Tuteata	Rangatira of Ngāti Huranga-a-te-rangi hapu within whose ancestral boundaries Scion's main campus in Rotorua is located.	Scion - Rotorua

2.2 OTHER CONSULTATION – DOC

The majority of samples were collected on land which DOC managed (Pureora, Mamaku, Taranaki) and appropriate collection forms sought and protocols followed. For this study a "Low Impact, Research and Collect Permit" was submitted for each of the sites. As required by DOC, local iwi were contacted and notified of the project. Minimal samples were collected to reduce impact on trees, and protocols specified by DOC were abided by.

2.3 SAMPLING

Effective sampling is important to ensure meaningful estimates of genetic diversity across natural populations when no previous genetic variation information is available. To achieve this in the present study, samples sites were identified which likely covered the range of morphological variation for the two main *Podocarpus* species used for timber (*Podocarpus totara, Podocarpus hallii*).

2.3.1 SITE SELECTION

The main target populations were for *P. totara* and *P. hallii* species while less extensive sampling was made for *P. acutifolius* and *P. nivalis*. *P. totara* samples were collected from 20 year-old planted provenance trial located at Tapapakanga (near Miranda Bay, Coromandel) and Tiniroto (near Gisborne). The latter was established by the Gisborne District Council on a conservation and wildlife reserve and involved fewer seed lots than the Tapapakanga site which had been established by the Forest Research Institute. To test whether variation in morphology at some localities could be explained by hybridisation between species, samples were collected across altitude transects located in the middle of the North Island at Mount Pureora, Mount Taranaki, the Mamaku Range, and Makatiti Dome for *P. totara* and *P. hallii*. Additional samples from all four *Podocarpus* species as well as *P. aurea* and *P. lawrencii* were collected from the Dunedin Botanical Garden (DBG) and/or Scion's Rotorua campus. Samples that were collected from Department of Conservation (DOC) scenic reserves were collected under DOC permit numbers BP-29552-FLO and TW-32225-FLO. In some cases, samples were also collected on private land where landowner's permission was sought. We chose sites that represented sources of *P. totara* from around the country that were also easily accessible for resampling if needed (Table2). Permission from DBG (Dunedin Botanical Garden) was sought (and received) via Mr Tom Myers, an employee at DBG.

Table 2: Sites chosen based on presence of totara species. Target populations of *P. totara* and *P. hallii* present at 7 sites.

	P. totara	*P. hallii*	*P. acutifolius*	*P. nivalis*	Other [1]
Location					
Scion	√	√	√	√	√
Mount Pureora	√	√			
Mount Taranaki	√	√			
Mount Makatiti	√	√			
Mamaku Ranges	√	√			
Tapakanga[2]	√				
Tiniroto[3]	√				
DBG[4]		√	√	√	√

Footnotes:
1 – Other = Other *Podocarpus* species and includes: *P. aurea*, *P. lawrencii* and *P. nivalis* 'Bronze king")
2 – 23 seed sources represented in Tapapakanga trial
3 – 5 seed sources represented in Tiniroto trial
4 – Dunedin Botanical Gardens

2.3.2 SAMPLE COLLECTION

For collecting in scenic reserves, individual trees adjacent to walking tracks were sampled by collecting small amounts of easily accessible foliage. An exception was Mount Pureora where a shotgun, operated by a licensed member of Scion, was used only on individuals where branches were out of reach. Pureora Forest is old growth relatively undisturbed forest with most trees over 30m in height. Extendable pruners were used where necessary to obtain samples. Effort was made to record GPS location, DBH (Diameter-Breast-Height), height, and sex of each individual sampled. A maximum of 30 randomly chosen samples of *P. totara* and *P. hallii* were taken per site to adequately sample variation present in each of the totara populations (Table 3).

Where possible we sampled from mature trees showing no sign of pest attack or disease. Fresh young leaf material (softer, lighter in colouring) was used for DNA extraction. Sample collection information consisted of small 3-7cm^2 pieces of needle material placed immediately in a clean pre-labelled plastic ziplock bag. At the end of each day, samples were stored in a -20˚C freezer. Sample details are provided in Table 1 of the Appendix.

Table 3: Details of planned totara sample collection

	P. totara	*P. hallii*	*P. acutifolius*	*P. nivalis*	Other	Total
Location						
Scion	8	8	6	1	3	26
Mount Pureora	30	30				60
Mount Taranaki	20	20				40
Mount Makatiti	30	30				60
Mamaku Ranges	30	30				60
Tapapakanga site	46					46
Tiniroto site	14					14
DBG[1]			5	10	5	20
						326

[1]Dunedin Botanical Gardens

2.4 GENOMIC DNA EXTRACTION

Genomic DNA was extracted from either fresh or -20°C stored leaf tissue from 278 samples of the various totara species. DNA was extracted with a Machery-Nagel NucleoSpin® 96 Plant II kit following a modified version of the manufacturers protocol (Telfer et al., 2012). Electrophoresis using a 0.7% agarose gel confirmed the isolation of high molecular weight genomic DNA. The Invitrogen Quant-iT PicoGreen kit (BMG Labtechnologies) was used to quantify DNA samples and this was visualised via Fluoro-star Galaxy software. Extracted DNA was stored in miliQ-purified water at -80˚C.

2.5 MOLECULAR MARKER DEVELOPMENT

Molecular markers in the present study were developed as a primary tool for detecting sequence differences between *P. totara* and closely related species *P. hallii*, *P. nivalis, P. acutifolius* (Everett, Grau, & Seeb, 2011). With technologies such as the Illumina GAIIx platform, sequence data for independent gene loci in non-model organisms can be easily obtained and used to aid in identification of hybrid species and introgression - genes of one species present in another(Everett, Grau, & Seeb, 2011; Liao & Lee, 2010). In this study, Illumina short read (75 bp) sequence data was utilised to develop molecular markers that would identify both chloroplast and nuclear polymorphisms (Figure 4).

2.5.1 PLANT GENOMES

There are three genomes in plants: nuclear, mitochondrial and chloroplast. Nuclear and organellar genomes often show different evidence for population structure because of their different modes of transmission. Chloroplast genomes are unique in that they typically follow a uniparental mode of inheritance. Whilst angiosperm chloroplasts are most often maternally inherited, conifer chloroplasts (as well as mitochondria) are typically inherited paternally and are thus moved by both seed and pollen dispersal (Cato & Richardson, 1996; Hamza, 2010; Wilson & Owens, 1999b). Paternal inherited markers that are haploid often provide greater insight into historical changes in the geographic structure of populations because effective population size for haploid genomes is smaller than is the case for nuclear encoded markers. In contrast, nuclear markers being bi-parentally inherited and typically unlinked, provide additional insight into potentially complex relationships between species (Hamza, 2010) as might be the case with *P. totara, P. hallii, P. acutifolius,* and *P. nivalis*. Thus the combined analysis of these different marker types is expected to provide the greatest future insight into the evolutionary and population histories of totara forest stands.

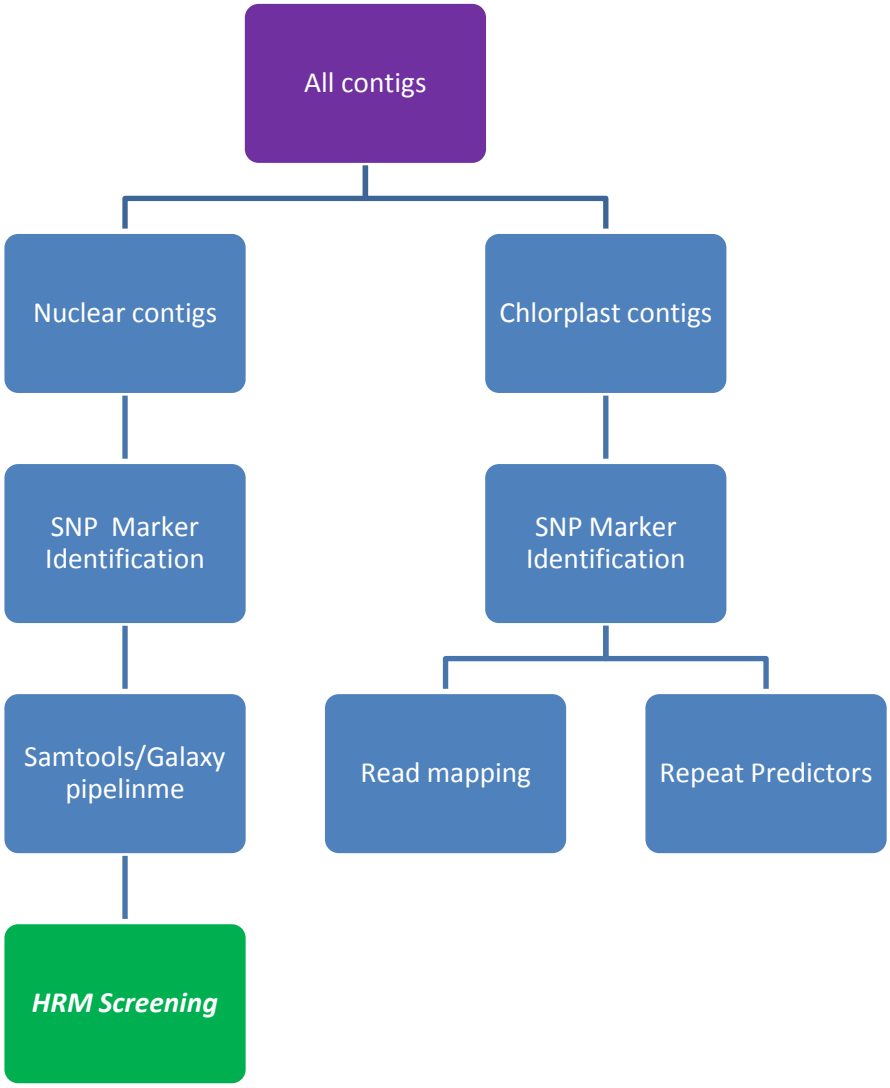


Figure 4: Diagram summarising the process used to detect interspecific SNPs.

2.5.1.1 NUCLEAR MARKER DISCOVERY

2.5.1.1A PRIMERS FROM *PINUS RADIATA*

Two methods were utilised to identify nuclear DNA markers in the present study. The first approach to identifying interspecific genetic variation was to use primers that were known to amplify polymorphic regions in *Pinus radiata.* In a previous study of loblolly pine (*Pinus taeda*), the transferability of such markers was shown to have a

success rate of 80% for amplification and 40% success rate for SNP transferability (Brown et al., 2001; Chagné et al., 2004). In this study twelve primers previously used in screening *Pinus radiata* (by colleagues at Scion, Rotorua, New Zealand*)* were used to screen intraspecific accessions of totara for the presence of SNPs. These primers had been designed for nuclear DNA regions which were expressed during xylogenesis. In *Pinus radiata*, these primers amplify DNA fragments 80-100 bp in length (Table 2 Appendix).

2.5.1.1B GAIIX READS MAPPED TO CONIFER ESTS

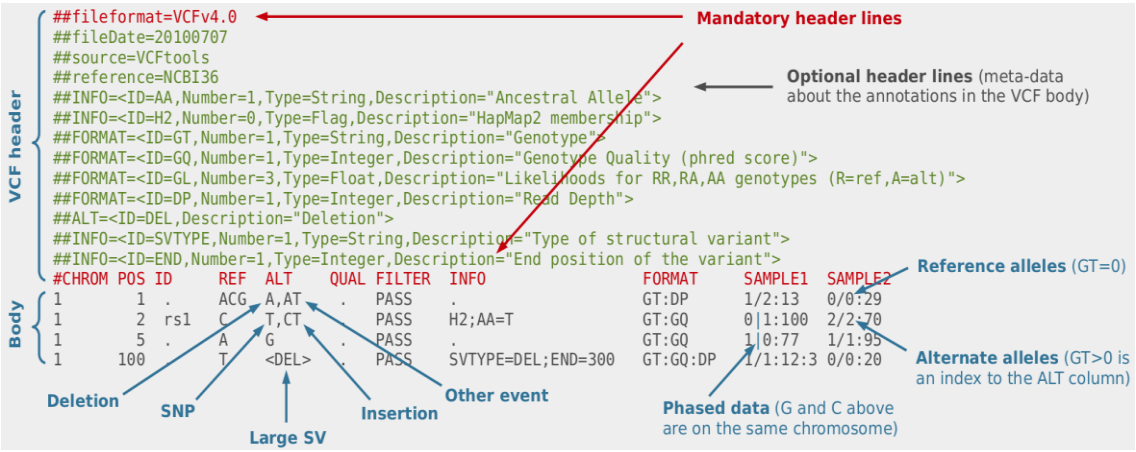


Figure 5: Representation of a typical VCF file with each line representing a SNP call and the information to it shown in tabular format.

Prior to commencement of this study, GAIIx sequence data was generated for a mixed sample of ten *P. totara x P. hallii* plants from Turitea Campus, Massey University, Palmerston North, New Zealand. These plants were identified as possible hybrids (*P. totara*, *P. hallii*) by herbarium curator Chris Ecroyd from Scion, Rotorua, New Zealand. As the sequence data could possibly originate from two species (*P. totara, P. hallii*) we were interested to bioinformatically determine whether SNPs occurred within nuclear genes for these data.

Dr. Oliver Deusch assisted in assembly of contigs, where DynamicTrim (Cox, Peterson, & Biggs, 2010) was used to filter out short reads of sub-optimal quality so as to obtain the longest contiguous sequences with less than a 0.05 error probability of error. Filtered reads were then assembled with Velvet software (Zerbino & Birney, 2008) using a 23-63 sized k-mer range. The initial set of contigs was then filtered for nuclear data by blasting against four EST datasets using a cut-off e value of 10^{-10} (*Pinus taeda, Picea glauca, Picea sitchensis, Crypotomeria japonica*). The resulting contigs were blasted against chloroplast data, and contigs that were not homologous to known cpDNA were blasted against Conifer GenBank sequences.

To identify SNPs the original nuclear contigs data set was blasted against the EST database. Orthologues were found and then SNPs among orthologous reads were identified bioinformatically. To do this, the reads were first sorted by length using LengthSort so that any reads shorter than 60bp were discarded before analysis. Bowtie and Samtools were then employed to create a SAM file of reads that mapped to the nuclear contig dataset (Langmead et al., 2009; Li et al., 2009). SNPs within this multiple sequence alignment were then called using bcftools (Li et al., 2009) and some of the data were manually checked manually using Tablet to assess validity of the SNPs before running through a Galaxy pipeline. A VCF file (Figure 5) containing the SNP calls was then put through the Galaxy (Rozen & Skaletsky, 1999)-based analytical pipeline to identify polymorphic regions and design corresponding HRM primers (Baldwin et al., 2012). This pipeline produced a file which contained details for the SNPs and suggestions for HRM specified primers (Table 4).

Using Excel, the SNP regions in this file were filtered so that transversion SNPs (A-T and G-C) were excluded as these are known to show weak HRM signal differences (Dang et al., 2012). A SNP read depth minimum of 7 was used so that SNPs that are more likely to be true SNPs were screened first. The origin of each SNP was noted by blasting the contigs of each SNP to Blast2GO and a total of 120 primers from a range of genes were then screened (Table 3 Appendix).

Table 4: Parameters used to design HRM primers

	Minimum	Maximum
Product size	50	150
Mispriming	12	24
Primer size	18	27
Prime Tm	57.0	63.0
Primer GC%	40	55
Max. Self	3.0	1.0

2.5.1.2 HIGH RESOLUTION MELTING (HRM) SCREENING

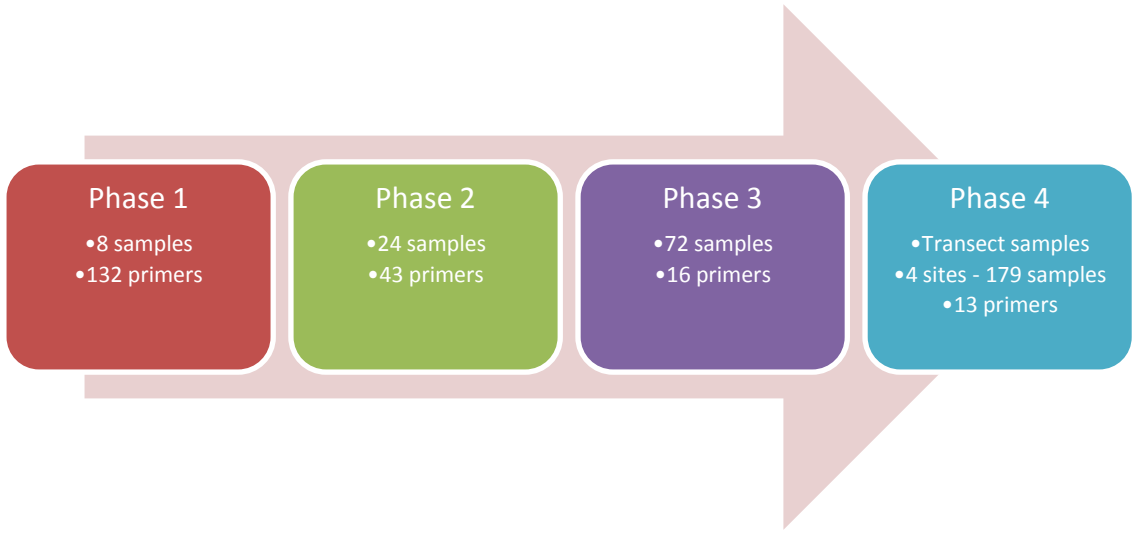


Figure 6: Summary of screening process for marker development. Phase 1 was carried out on all primers that were designed. Successful primers were further tested on Phase 2 and so on.

A High Resolution Melting (HRM) PCR protocol was used to screen primers. 132 primers were tested on a subset of eight *Podocarpus* accessions for initial screening using a 96 well plate format. PCR was performed in a total volume of 7µL containing 20ng of template DNA, 2 mM $MgCl_2$, 140 nM forward and reverse primer and 0.7 x HRM master mix (Roche Applied Science). PCR amplifications were performed on a Roche LightCycler® (Roche Applied Science). PCR parameters were as follows: 95°C for 5 min followed by 40 cycles at 95°C for 10 sec, 50°C for 30 sec and 72°C for 15 sec.

The final melting step involved heating products to 95°C for 1min and then reducing temperature sample to 40°C for 1min. Melting curves were generated with continuous fluorescence acquisition during a final ramp from 65°C to 95°C (ramp rate of 1.1°C/sec).

Initial screening of primers was performed on 2 samples (Table 5) from each of the four totara species (2x *P. totara* 2x *P. hallii*, 2x *P. acutifolius*, 2x *P. nivalis*). The primers which produced polymorphic results for phase 1 via HRM were than further tested on a larger set of samples (six of each species). Primers which successfully demonstrated polymorphism for phase 2 were then carried through to the next stage of screening which involved validating the identity of 72 samples. These 72 samples were collected over a larger distribution for all four totara species, and included: *P. totara P. hallii*, *P. acutifolius*, *P. nivalis*, and *P. aurea*, *P. lawrencii* and three synthetic hybrids (mix of DNA from *P. totara* and *P. hallii*). Successful primers in phase 3 where than taken through to analyse transect samples (Figure 6). Samples used for phase 1 screening were used as a positive control for phase 2, 3 and 4 screening. Full details of samples used for screening are summarised in Table 4 of the Appendix.

Table 5: Summary of samples used for screening (seed source)

	Phase 1	Phase 2	Phase 3	Phase 4
Total # Samples	**8**	**24**	**72**	**179**
P. totara	2 (2)	6 (6)	29 (27)	57 (6)
P. hallii	2 (2)	6 (3)	23 (5)	111 (4)
P. acutifolius	2 (1)	6 (2)	11 (2)	2 (1)
P. nivalis	2 (1)	6 (2)	6 (2)	3 (1)
P. aurea				1
P. lawrencii				2 (1)
Synthetic hybrids			3 (3)	3 (3)

2.5.1.3 HIGH RESOLUTION MELTING (HRM) ANALYSIS

Software algorithms have a huge influence on interpretation of data generated by HRM. The LightCycler® 480 Gene Scanning Software was utilised for HRM analysis as it

has been specifically developed to provide accurate analysis. For HRM analysis, the melting curves were normalised and then temperature shifted so that each sample could be compared. Curves were modified using the LC480 software in the melting curve genotyping module (version 1.5; Roche). The normalised and temperature shifted melting curves correspond to the final melting peak curve after normalisation. When a primer produced sequence variation, the normalised and temperature-shifted melting curves had a relative fluorescence difference of 3.0 or greater for at least two of the four totara species. Normalisation was manually adjusted and for normal routine framework normalisation ranges were filled in automatically. By default the sensitivity was set to 30% for all amplicons to avoid false negative amplicons. Successful primers (markers) were assessed as positive if relative fluorescence difference was observed between all four of the totara species or it showed different melting peak curves for the totara species. Successful primers were deemed as candidate markers and tested on a further range of samples (Figure 6).

2.5.1.4 ANNOTATION OF NUCLEAR MOLECULAR MARKERS

To analyse the nature of nuclear molecular markers, screened via HRM, the gene identities were sought using Blast2GO (Conesa et al., 2005). In some cases this provided identification of the gene regions from which the markers were derived.

2.5.1.5 CLUSTER ANALYSIS

Cluster analysis is a common method used to interpret molecular data by differentiating and grouping individuals. For this study, Neighbor-Joining was used to produce bifurcating trees in which the branch lengths indicated genetic distinctiveness between accessions. Neighbor-Net splits graphs were also constructed as these give an indication of contradictory signals of relationship present in the data. Graphs were built for molecular marker screening of the four transects. HRM results for 178 accessions (Table 4 Appendix) were converted into Nexus-formatted files for analysis software so that clustering could be visualised. Neighbor-Net analyses of accessions was carried out using SPLITSTREE *v.* 4.12.18 (Huson & Bryant, 2006). Data from *e*ach

site was analysed separately and then together with representatives for other species (*P. lawrencii*, *P. aurea*). Neighbor-Joining trees were exported from SPLITSTREE and visualised using Dendroscope (www-ab.informatik.uni-tuebingen.de/software/dendroscope).

2.6 THE CHLOROPLAST GENOME

2.6.1 ASSEMBLY AND ANNOTATION OF CHLOROPLAST GENOME

The reads from mixed accessions of cultivated *Podocarpus* were assembled into a reference genome that was later used for mapping Illumina reads. The de novo assembler, Velvet (Zerbino, Birney 2008) was used for the initial assembly of the chloroplast reads into contigs (contiguous sequences). Velvet uses the De Bruyn algorithm to generate large contigs from the short read sequences that were generated by the GAIIx. Contigs were further assembled using the Geneious *v.* 5.5 assembler (Drummond et al., 2011) and contigs did not circularise as gaps were present. Chloroplast primers were then designed to close the gaps in the *P. totara* chloroplast genome (Table 6). These were designed using primer3 software (Rozen & Skaletsky, 1999).

Table 6: Details of primers used to fill *P. totara* chloroplast gaps

Primer name	Primer sequence (5' to 3')
TOT9F	TTAAGCGGATCAGATTTCGCA
TOT68R	ACGGGGTTTCTGAAGACGGGGTT
TOT68F	ACGGGGCTCGAACCCGTAAC
TOT46R	GGTGACGTAGCGCCCTTGTTG
TOT46F	TCCCCCTCATCTCACCTATGC
TOT17R	AGGCCAATAGGGTTGGTGCG
TOT15R	GTGGAAAGCCGTATTCGACG
TOT15F	TATCTCGGGGCCCTCGCTCTT
TOT17F	AGGCCAACGTTAGTTCAAATCCAGT
TOT4R	TCCGTCTTGACAGGGCGGTACT
TOT4F	TGGGTCGATGCCCGAGTGGT
TOT26R	CGGCACCTGTGCTAGCGACTC
TOT26F	CCAAGGTCCGGAACCATGTGCT
TOT22R	TAGGTCCACCGGTATGGCGA

TOT22F	GGGGTAAAAGTTGTCGCAGATCCCA
TOT9R	GGACTGTTCTCTCCTTGTTTTCCGC

PCR reactions were carried out in 20µL volume reactions containing 2µL of 10 x PCR buffer (10mM Tris-HCl, 50mM KCl, pH 8.3; Roche Applied Science, Auckland) 20M betaine, 5mM dNTPs, 10µM of each primer, 0.2 U Taq polymerase (Roche Applied Science, Auckland) and 50ng of template DNA. PCR amplification was performed using the protocol from (Shaw, et al., 2007).

Unincorporated primers were removed from the solution containing PCR products using SAP-EXO (2U Shrimp Alkaline Phosphatase (*SAP*) 1 and 1U Exonuclease (EXO1)[2]) reagent (USB Corporation, Cleveland, OH) prior to sequencing with an ABI Big Dye cycle sequencing protocol. The sequencing reaction was carried out in Biometra thermal cycler as follows: 37˚C for 30mins, 80˚C for 15mins with a 60˚C hold. Sequencing reactions were cleaned up using the Agencourt CleanSEQ protocol to remove unincorporated fluorescent dNTPs. DNA sequencing products were subjected to capillary electrophoresis (CE) at the Massey University Genome Service (MUGS, Massey University, Palmerston North) on a 3730 Genetic Analyzer (Applied Biosystems) using a 50cm array using the BigDye™ Terminator Version 3.1 Ready Cycle Sequencing kit with data collected using Run 3730 Data Collection *v3.0* software (Applied Biosystems).

The chloroplast genome was initially annotated using Dogma (Wyman, Jansen, & Boore, 2004). Regions that were unresolved were manually refined by comparison to genes of more closely related species such as *Pinus thunbergii*. Geneious *v5.5* was used to construct a figure depicting annotations of the *P. totara* chloroplast genome, and SEQUIN *v12.21* was utilised to submit annotations to GenBank.

2.6.2 IDENTIFYING POLYMORPHIC "HOTSPOT" REGIONS IN THE CHLOROPLAST GENOME

The co-occurrence of indel mutations and nucleotide substitution are features of plant genomes, and the location of direct repeats have previously been used to predict polymorphic regions suitable for potential marker development (Lockhart et al., 2001; McLenachan et al., 2000). Recently in Ahmed et al. (2013) the genome-wide association between four repeats types (direct, inverted, idels, substitutions) was calculated for taro (*Colocasia esculenta*). A circos plot was used to visually display this. The figure has been reproduced here as Figure 7. It shows the striking genome-wide correlation between oligonucleotide repeats in taro, indels and substitutions in closely related species. A similar plot was also constructed for our *P. totara* reference chloroplast genome using a chloroplast map script written by Dr. Patrick Biggs (Massey University) for Reputer (Kurtz et al., 2001). This gave us a circular map where repeat sizes of at least 15bp (the largest being 275 bp) could be visualised. The association between repeats and mutations among ten accessions of *P. totara x P. hallii* were then investigated.

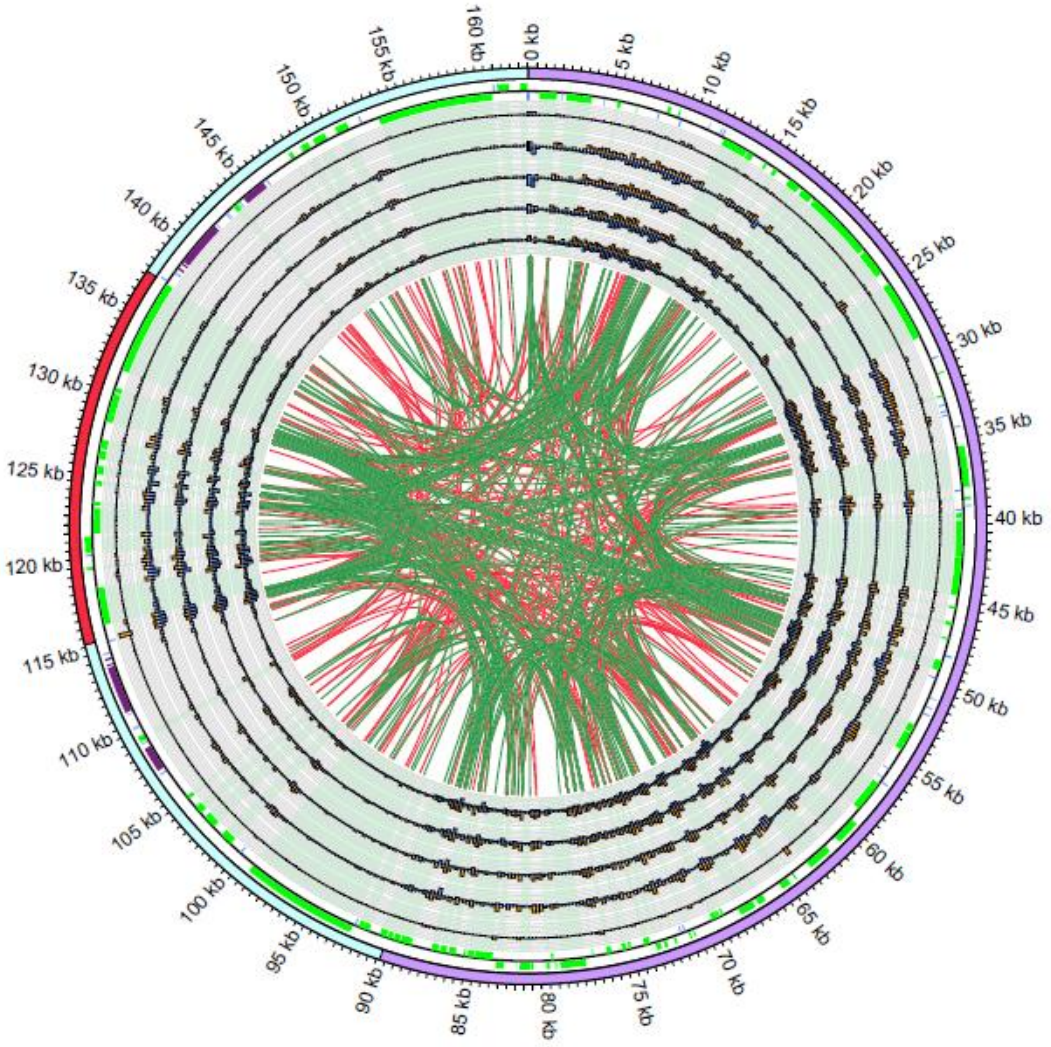


Figure 7: Circos plot of taro (*Colocasia esculenta*) var. RR from Ahmed et al. (2013) showing the relationship between short repeats within the chloroplast (cp) genome and distribution of indels and SNPs in pairwise comparisons of taro var. RR cp genome with other aroid cp genomes. Two ends of a red line mark the two locations of the forward (direct) repeats, while those of a green line mark the two locations of the reverse (inverted) repeats on the genome. The plot indicates the striking genome wide correlation between repeats, indels and substitutions.

2.6.2.1 SNPS DISCOVERED BY VISUALISATION OF MAPPING

To investigate whether similar relationship of repeats, SNPs and indels occurred in *Podocarpus*, the GAIIx reads from the sequencing of ten *P. totara* x *P. hallii* accessions

was mapped to the chloroplast reference genome. This allowed data comparison of putative SNP regions between the chloroplast genomes of *C. esculenta* and the P. *totara x P. hallii* reference. Reads were aligned to the chloroplast sequence using bowtie (Langmead et al., 2009). The SAM file was then visualised using TABLET and putative SNPs identified by eye (Milne et al., 2010). Putative SNPs were called if a highlighted base was seen for a column of reads, thus indicating there was a difference to the consensus (Figure 8).

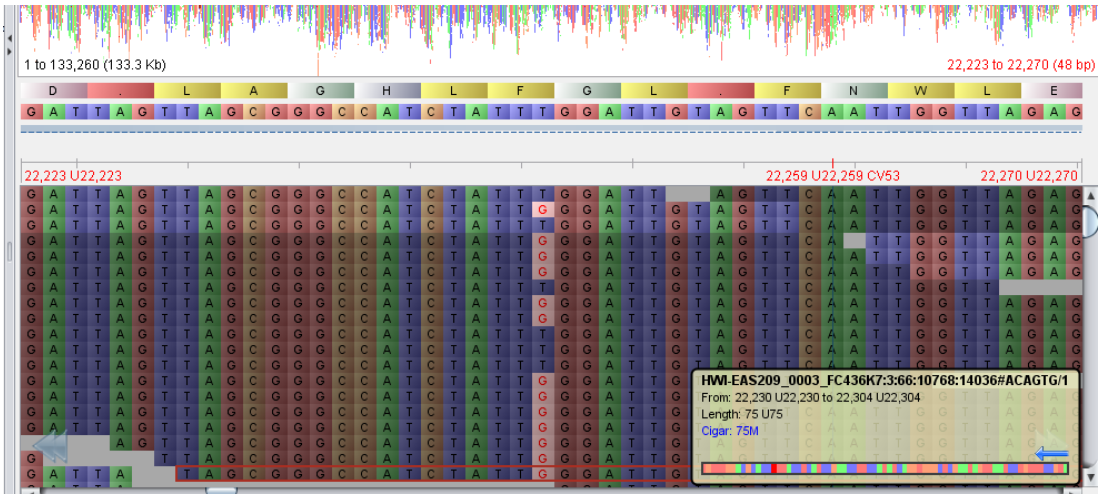


Figure 8: Example of a putative SNP of the chloroplast genome visualised using Tablet. The SNP "G" is highlighted in pink and is positioned in the central column. The text box shows the length of the read and coordinate details from the Illumina cell.

CHAPTER 3 RESULTS

3.1 MĀORI CONSULTATION

Before conducting research on totara it was appropriate to obtain support and relevant information from local iwi for the regions from which the research was being conducted. A requirement of our DOC permit was that relevant iwi also be consulted. Therefore face-to-face meetings were organised and attended with different iwi/hapu representatives for areas where sampling was being undertaken. All iwi representatives approached were happy to support the agenda of the research project and they gave permission to sample from land specified in DOC permits (permit numbers BP-29552-FLO and TW-32225-FLO). In some instances information was provided that assisted in the project design. For example: we received information from Jimmy Schuster that Makatiti was abundant in regenerated *P. hallii* forest (Figure 9) and through consultation with Jackie Aratema we were allowed access to this otherwise inaccessible site. One request made by iwi and DOC was that all material not used by the conclusion of this study be returned to its source of origin. Through consultation with the iwi we discovered valuable information which helped planning of the project. Discussions with James Rickard, and Jimmy Schuster also gave insight into how totara had been traditionally used and how it is now currently used in whakairo (carving). A summary of consultation details is given in Table 1 in Chapter 2.

Figure 9: Photograph of Makatiti Dome.

3.2 SAMPLE COLLECTION

A total of 277 individual plants were sampled from 33 different populations around New Zealand (Table 7). A total of 118 *P. totara* from 31 sites, and 120 *P. hallii* from six sites were collected. The origins of all samples are shown in Figure 10. The location of each individual was determined using a Garmin GPS60 handheld unit (Figure 11-14) Additional sample details are given in Table 5 of the Appendix.

Table 7: Number of totara samples collected (original planned estimates shown in brackets).

	P. totara	*P. hallii*	*P. acutifolius*	*P. nivalis*	Other [1]	Total
Location						
Scion	(8) 3	(8) 5	(6) 3	(1) 2	(3) 13	26
Mount Pureora	(30) 30	(30) 30				60
Mount Taranaki	(20) 11	(20) 20				31
Mount Makatiti	(30) 0	(30) 30				29
Mamaku Ranges	(30) 14	(30) 30				44
Tapapakanga[2]	(46) 46					46
Tiniroto[3]	(14) 14					14
DBG[4]		5	(5) 6	(10) 12	(5) 4	27
						277

Footnotes:
1 – Other = Other *Podocarpus* species include *P. aurea*, *P. lawrencii*, and a variation of *P. nivalis* 'Bronze king")
2 – 23 seed sources represented in Tapapakanga trial

3 – 5 seed sources represented in Tiniroto trial
4 – Dunedin Botanical Gardens

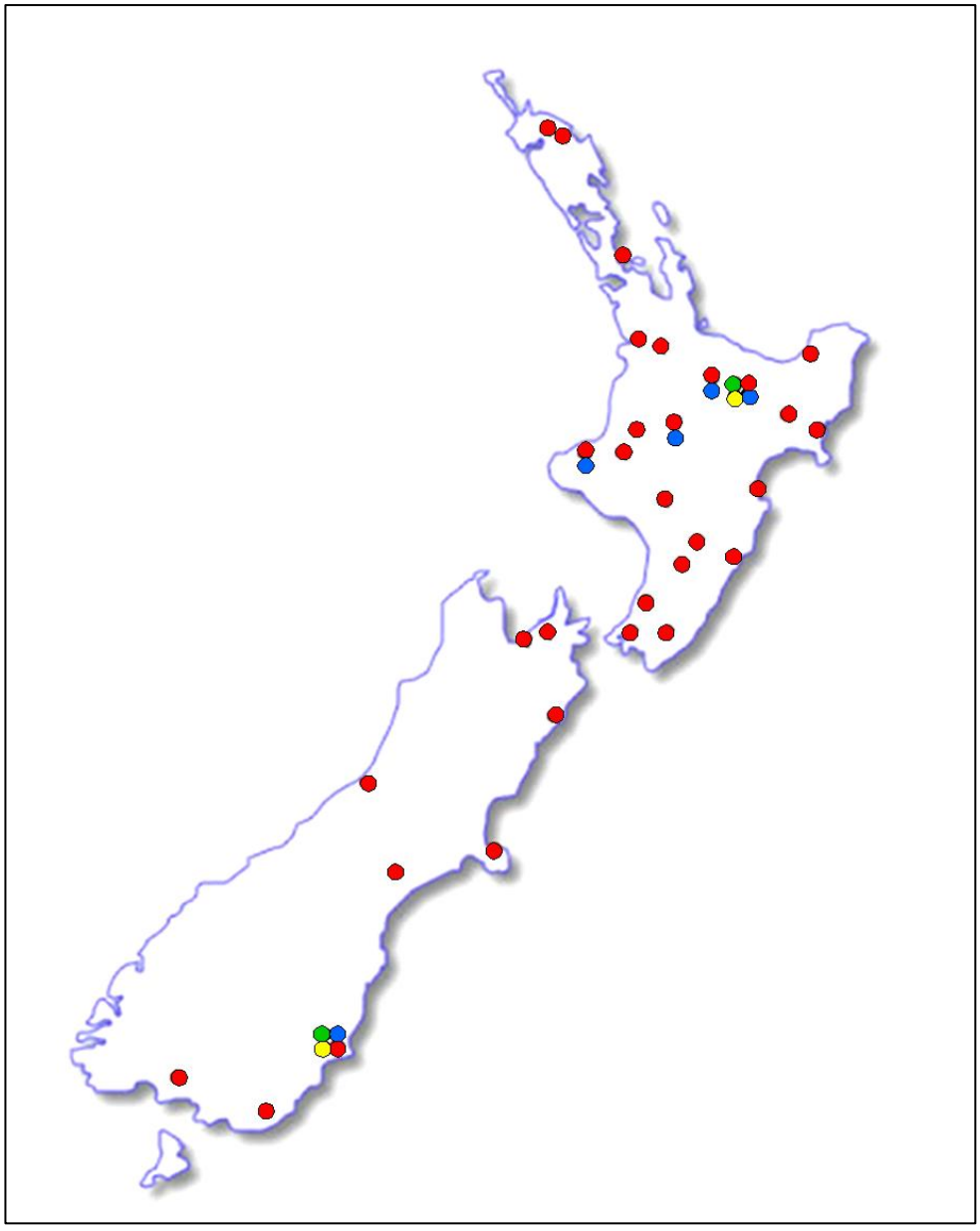


Figure 10: Geographic origin of *Podocarpus* samples collected, *P. totara* (red), *P. hallii* (blue), *P. acutifolius*, (green), *P. nivalis* (yellow). Most *P. totara* samples were sowed from 20 year-old provenance trial established near Thames and Gisborne.

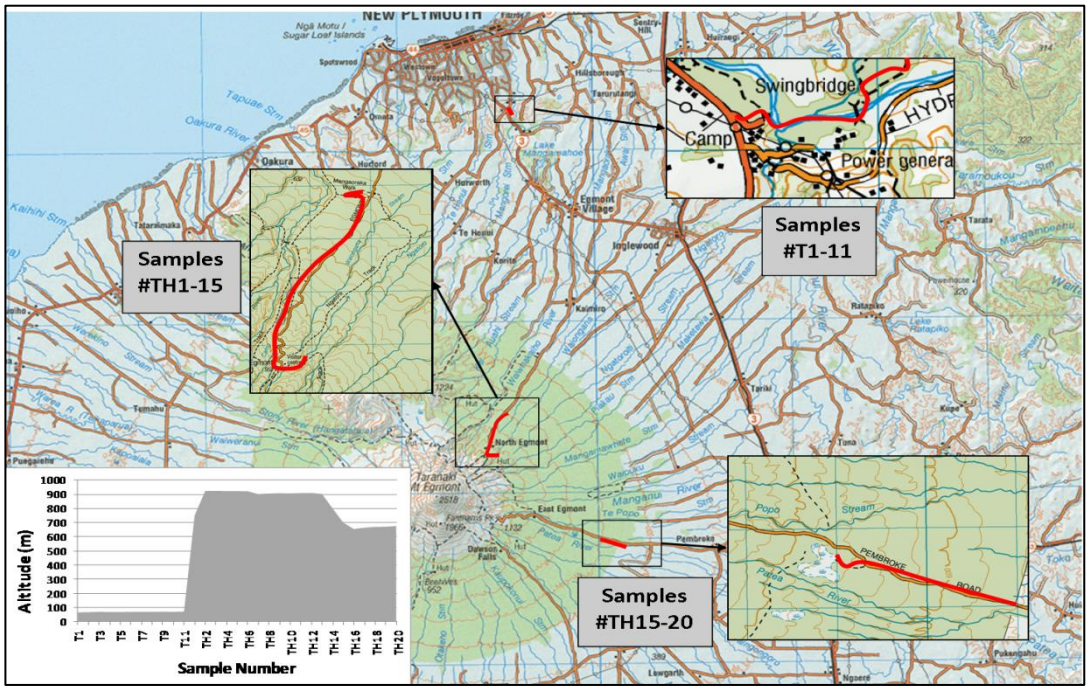


Figure 11: **Geographic origin of *Podocarpus* samples collected from the Taranaki site. Altitude at which samples have been collected is indicated (bottom left).**

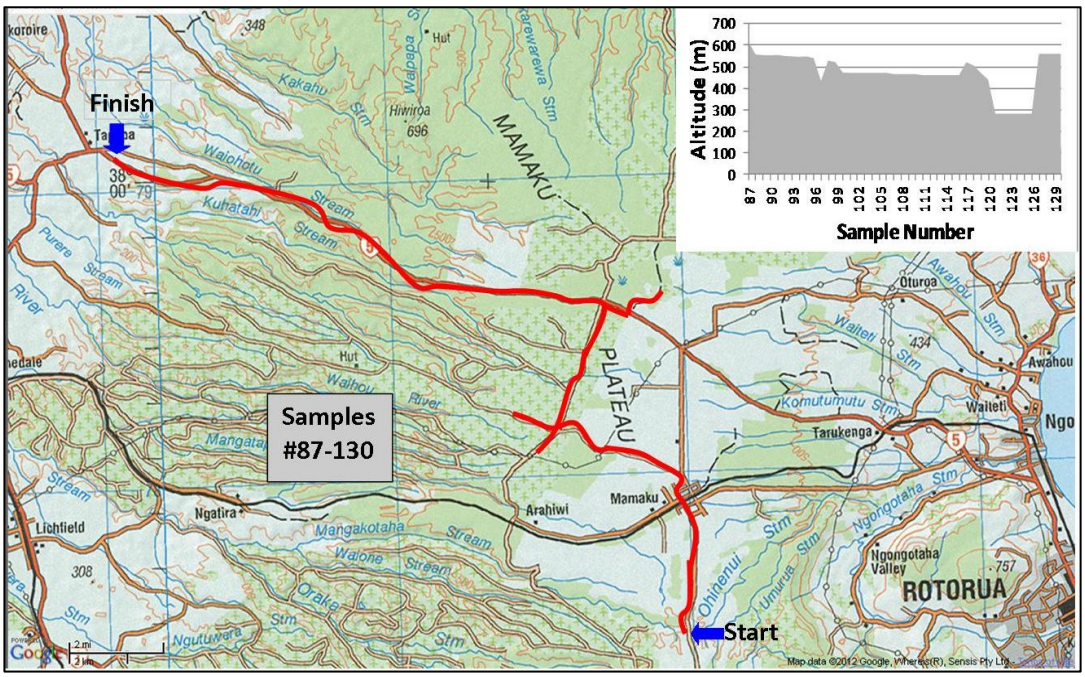


Figure 12: **Geographic origin of *Podocarpus* samples collected from the Mamaku site. Altitude at which samples have been collected is indicated (top right)**

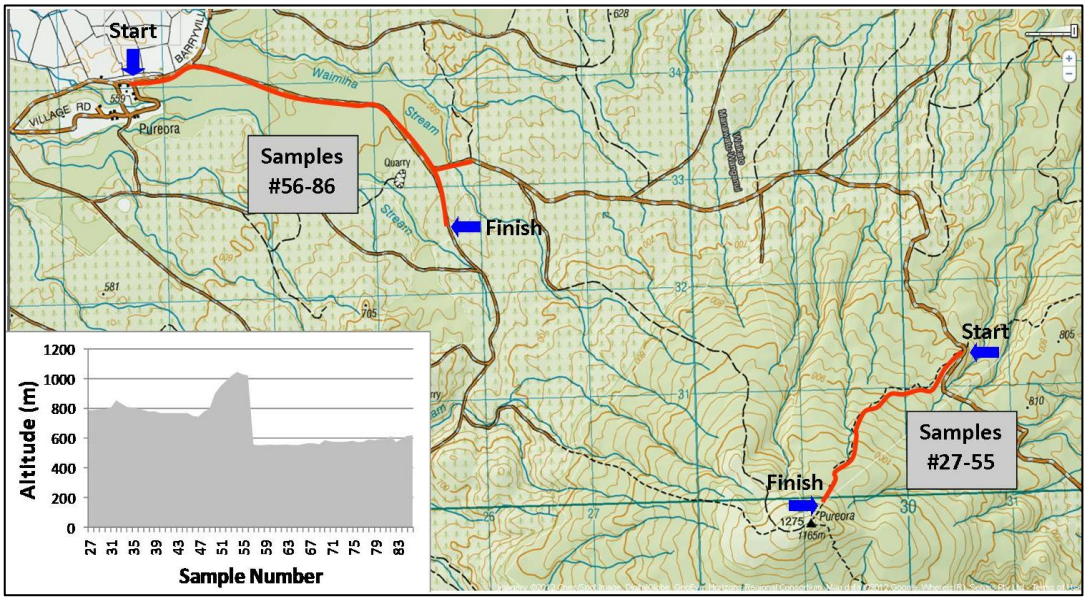


Figure 13: **Geographic origin of *Podocarpus* samples collected from the Pureora site. Altitude at which samples have been collected is indicated (bottom left).**

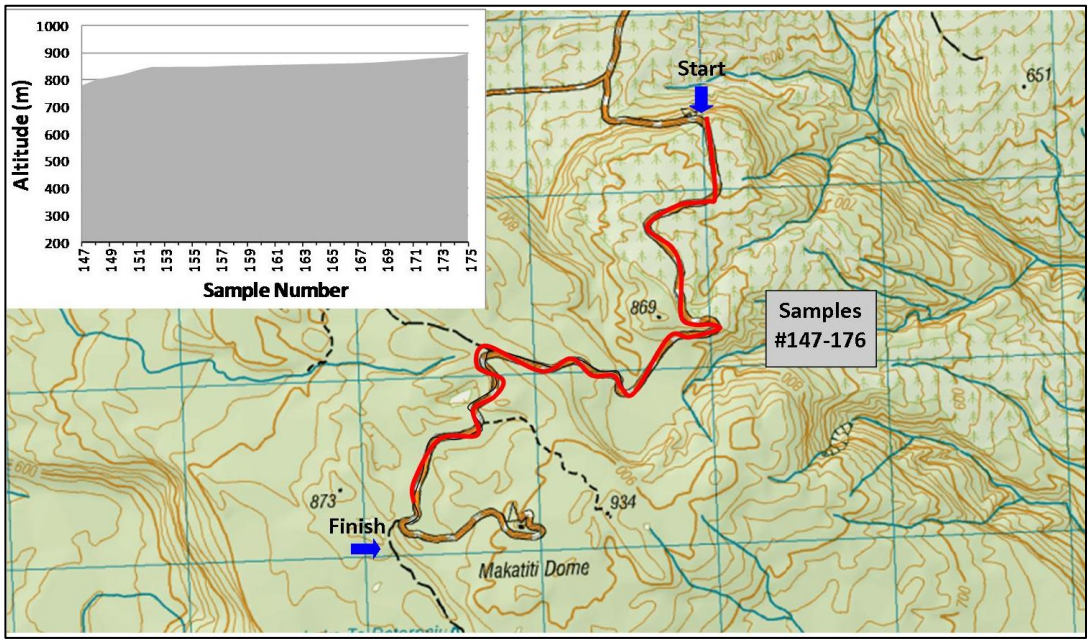


Figure 14: **Geographic origin of *Podocarpus* samples collected from the Makatiti site. Altitude at which samples have been collected is indicated (top left)**

3.3 GENOMIC DNA EXTRACTION

High molecular weight genomic DNA was isolated from 100g tissue from each of the 323 samples using a Machery-Nagel NucleoSpin® 96 Plant II kit. Electrophoresis using a 0.7% agarose gel confirmed the isolation of high molecular weight genomic DNA (Figure 15). DNA was estimated to be typically greater than 12Kb in size.

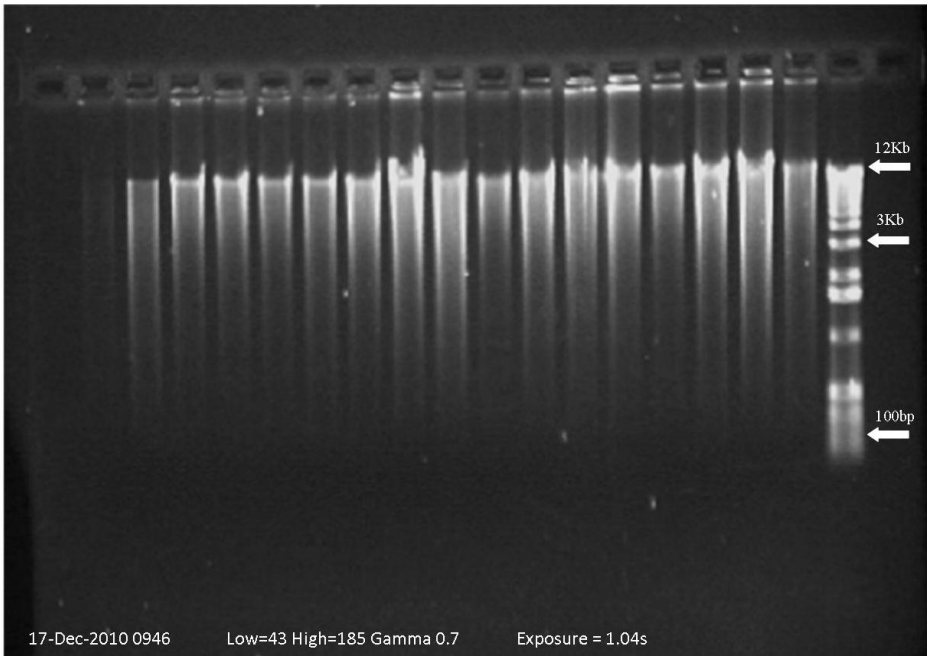


Figure 15: Electrophoresis of totara DNA extracts to confirm the presence of high molecular weight DNA

3.4 MOLECULAR MARKER DEVELOPMENT

3.4.1 NUCLEAR MARKERS

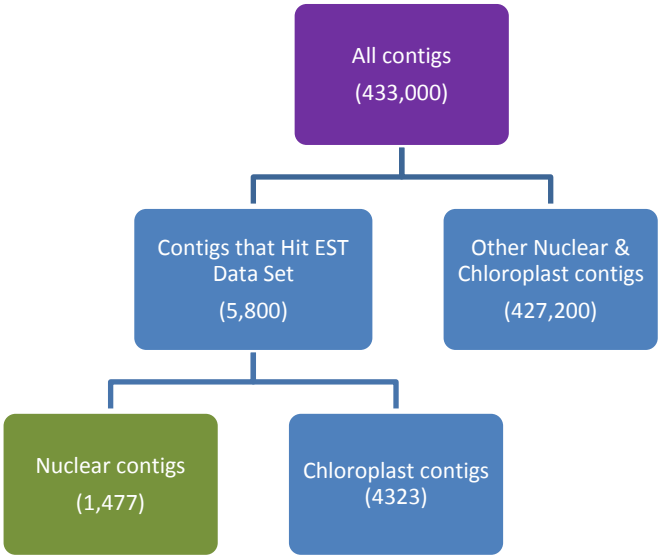


Figure 16: Summary of protocol for SNP identification of nuclear data from totara

High-throughput sequencing of nuclear DNA can provide a rich source of molecular markers for studying interspecific relationships in plants. Here we made use of Illumina 75bp reads obtained for a genomic prep of nuclear DNA (enriched for *P. totara* chloroplasts) previously sequenced at Massey University on an Illumina GAIIx. These reads had been assembled into contigs and mapped to conifer ESTs. PCR primers were then developed for a subset of these contigs (Figure 16). Further details are provided below.

3.4.1.1 GAIIX READS MAPPED TO CONIFER ESTS

As the Illumina sequenced DNA came from ten different plants (sourced from the Massey University Turitea campus), it was of interest to determine whether there was genetic variation among these accessions. To determine this, the reads were mapped to the nuclear dataset of 1,477 contigs and analysed using bcf tools (Li et al., 2009).

This produced a file containing 400 putative SNPs and these were visually assessed with Tablet (Figure 17). Five SNPs called with bcftools are shown in Table 8. The SNP file (VCF) was analysed using the HRM Galaxy pipeline (Baldwin et al., 2012) which identified HRM primers designed for 193 of the 400 SNP (Table 9). Further filtering, with the Galaxy pipeline, was then undertaken selecting for occurrence of transversions, a minimum read depth of 7, and a variety of gene origins. SNPs that had higher read coverage specified by the "MQ" value were chosen in priority to other SNPs. This filtering method gave 120 primers for screening via HRM. The details of these primers are shown in Table 3 of the Appendix.

Table 8: Example of five SNPs identified using bcftools software. Contig name refers to nuclear contig used. Pos: is the position of the SNP in the contig. Ref: is the reference base call, and Alt: is the alternative base call. Info refers to information about the SNP including DP: raw read depth, VDB: variant distant bias, and MQ: SNP calling quality.

Contig name	POS	REF	ALT	QUAL	INFO
totara_5_43.fna_NODE_133_length_4015_cov_34.873722	3593	A	T	3.54	DP=16;VDB=0.0437;AF1=0.4998;AC1=1;DP4=4,4,0,4;MQ=20;FQ=5.47
totara_5_39.fna_NODE_2063_length_3364_cov_19.570749	2911	C	T	19.1	DP=8;VDB=0.0288;AF1=0.5;AC1=1;DP4=4,0,1,3;MQ=20;FQ=13.6
totara_5_47.fna_NODE_210_length_3334_cov_25.470606	846	G	A	64	DP=35;VDB=0.0537;AF1=0.5;AC1=1;DP4=7,12,4,9;MQ=20;FQ=67
totara_5_47.fna_NODE_210_length_3334_cov_25.470606	1332	A	G	5.46	DP=17;VDB=0.0521;AF1=0.4999;AC1=1;DP4=7,3,5,0;MQ=20;FQ=7.8
totara_5_45.fna_NODE_101_length_3154_cov_30.715599	777	G	A	3.54	DP=15;VDB=0.0076;AF1=0.4998;AC1=1;DP4=4,7,2,2;MQ=20;FQ=5.47

Figure 17: Example of a putative SNP visualised using Tablet. The SNP "G" is highlighted in pink and is positioned in the central column. The text box shows the length of the read and coordinate details from the Illumina cell.

The Galaxy pipeline produces HRM primers designed to SNP calls from bcftools. In the present study two criteria, raw read depth and mapping quality were used to choose the best primers for screening. In Table 9 all five examples have the same read depth of 10, but the mapping quality of the reads is different. The MQ (mapping quality) indicates how well reads cover the region where primers are specific, and the first three results have a much higher MQ of 20, compared to the last two results of 02. For Table 9, the first three results would be screened first.

Table 9: Example of five results obtained with the Galaxy pipeline. Contig name refers to nuclear contig used. Forward and reverse primers are shown. Information used for filtering is shown in columns 4 and 5.

Contig	Forward Primer	Reverse Primer	DP	MQ
totara_5_33.fna_NODE_6159_length_172_cov_16.616280:SAMTOOLS:SNP:133	GTGTTAGATTTGGGATCAAAAGG	CGGTTCTTCCTTTCAGCAAC	10	20
totara_5_37.fna_NODE_1619_length_452_cov_35.639381:SAMTOOLS:SNP:239	CATGTTGATCATGTCCTCCTGT	CAGAATGGGTTGAGGAATGG	10	20
totara_5_47.fna_NODE_16_length_280_cov_87.292854:SAMTOOLS:SNP:74	TGGAGTACCATGCTCGAGGT	AGAGGATCCTAATCTGGTATGCAA	10	20
totara_5_45.fna_NODE_847_length_586_cov_27.542662:SAMTOOLS:SNP:281	TGGACTATGGCCTATGGTATCC	TACACATCCTGCCCAATCTG	10	02
totara_5_45.fna_NODE_848_length_602_cov_36.500000:SAMTOOLS:SNP:50	TCCCCCTTCATCAAAAATTG	AAGCTTACAAGTTCTATAATCCCAAGA	10	02

Key

DP –Raw read depth

MQ –Root-mean-square mapping quality of covering reads (How well reads map to that SNP)

3.4.2 HIGH RESOLUTION MELTING (HRM) SCREENING AND ANALYSIS

Four phases were used to evaluate HRM primers for screening totara samples. An overview of the protocol and results obtained at each phase are shown in Figure 18.

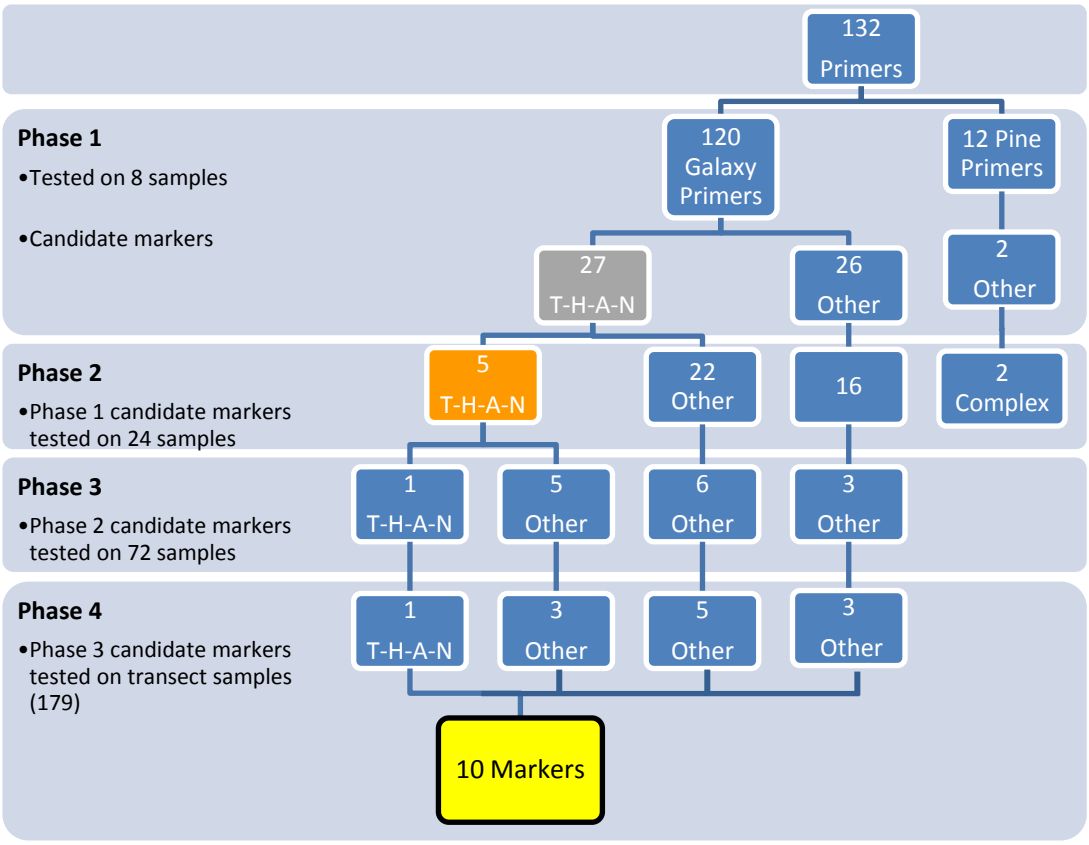


Figure 18: Schematic representation of screening results for primers. The grey box in Phase 1 corresponds to the results highlighted grey in Table 6 of the Appendix. Similarly for the orange box in phase 2. "Candidate Markers" refers to markers that were polymorphic and displayed melting curves for the *Podocarpus* species that were split into two or more groups; "Other" refers to other patterns of relationships suggested by observed splits. Table 10 describes the splits.

3.4.2.1 PHASE 1

Phase 1 involved screening PCR primer pairs on eight accessions representing morphotypes of four New Zealand *Podocarpus* species: two *P. totara*, two *P. hallii*, two *P. acutifolius* and two *P. nivalis*. Markers were deemed "candidates" for future screening if they exhibited a different melting curve for each of the four totara species. These markers were trialled in successive phases. Markers which were not phylogenetically informative were recorded as "monomorphic" if only one melting curve was observed for the 8 accessions (Figure 21A), and "complex" if the melting curves of individual accessions did not group with other individuals of the same species (Figure21B).

3.4.3.1a PCR primers transferability from Pinus radiata

A set of 12 primer pairs designed from *Pinus radiata* gene coding sequences were trialled on four *Podocarpus* species (*P. totara*, *P. hallii*, *P. nivalis*, *P. acutifolius*). Two did not amplify. Of those that did, three markers gave melting curve differences for the set of eight samples that were "complex". Therefore no pine markers were regarded as good candidates for successive phases.

3.4.3.1b Automatic PCR primer design using totara sequences using the Galaxy pipeline

Of a set of 120 primer pairs designed from the Galaxy pipeline, 113 (94%) successfully amplified and produced a PCR product. Fifty three (44%) primer pairs amplified PCR products with significant melting curve differences in the set of eight accessions tested. Twenty seven out of the 53 (50%) showed melting curves that were distinct for all four *Podocarpus* species, denoted T-H-A-N, (Figure 19) and the other 26 primers demonstrated differences that split the *Podocarpus* species into two or more groups (Table 10). Examples of HRM grouping are shown in Figure.

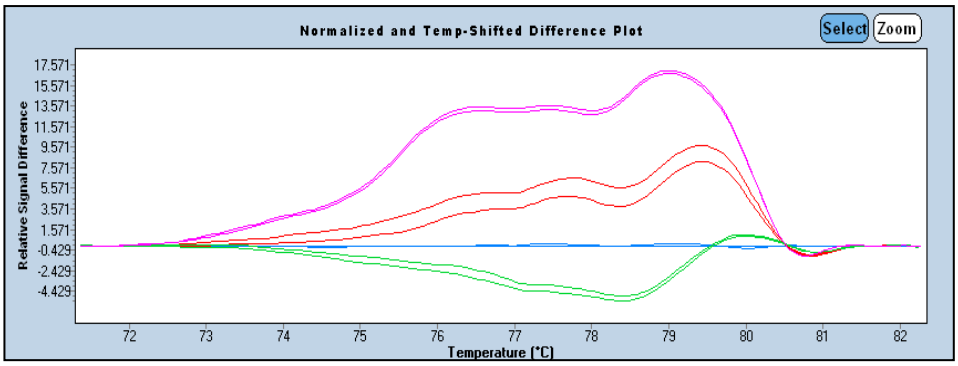


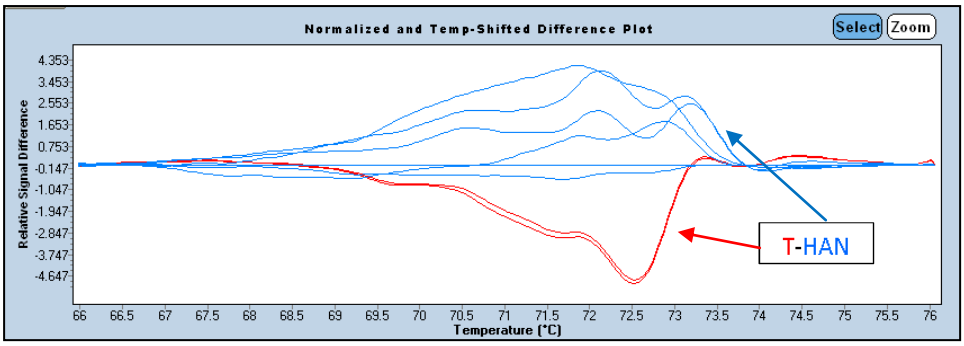
Figure 19: HRM profile of four *Podocarpus* species for marker GA90. Each PCR amplicon melting curve is shown as the relative signal difference between a reference melting curve (*P. totara*) and the curve for each of seven *Podocarpus* accessions. Relative signal difference for the melting curves are shown as follows: *P. totara* (blue), *P. hallii* (red), *P. acutifolius* (green), *P. nivalis* (pink).

Table 10: *Podocarpus* species splits obtained in HRM analysis.

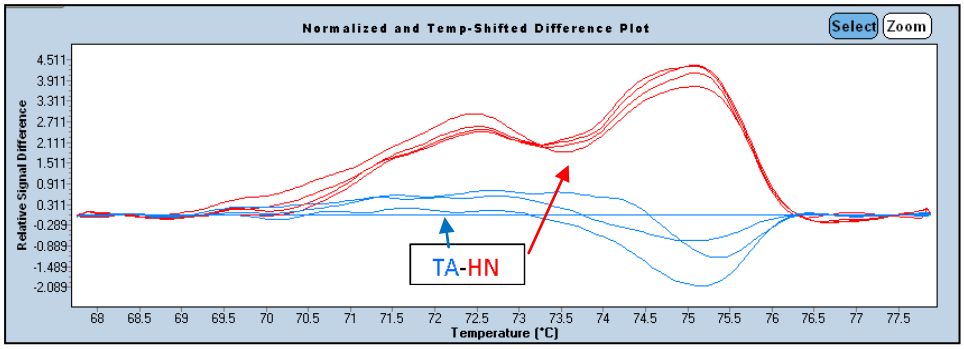
Key	Description	Melting Curves
TA-HN	Melting curves for T and A the same, but different from H and N.	2
TH-AN	Melting curves for T and H the same, but different from A and N.	2
T-HAN	Melting curves for H, A and N the same, but different from T.	2
THA-N	Melting curves for T, H and A the same, but different from N.	2
THN-A	Melting curves for T, H and N the same, but different from A.	2
TH-A-N	Melting curves for T and H the same. A has distinctly different melting curve. N has distinctly different melting curve.	2
TA-H-N	Melting curves for T and A the same. H has distinctly different melting curve. N has distinctly different melting curve.	3
TH-A-N	Melting curves for T and H the same. A has distinctly different melting curve. N has distinctly different melting curve.	3
T-A-HN	Melting curves for H and N the same. T has distinctly different melting curve. A has distinctly different melting curve.	3

Key:
T=*P. totara*
H=*P. hallii*
A=*P. acutifolius*
N=*P. nivalis*

GA44



GA77.



GA92.

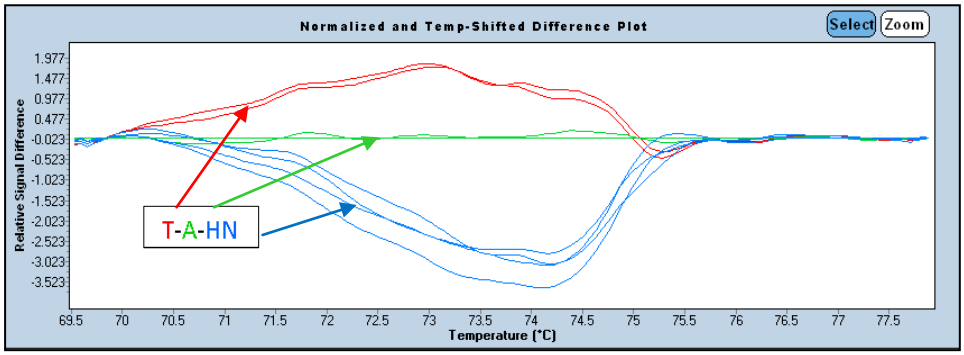
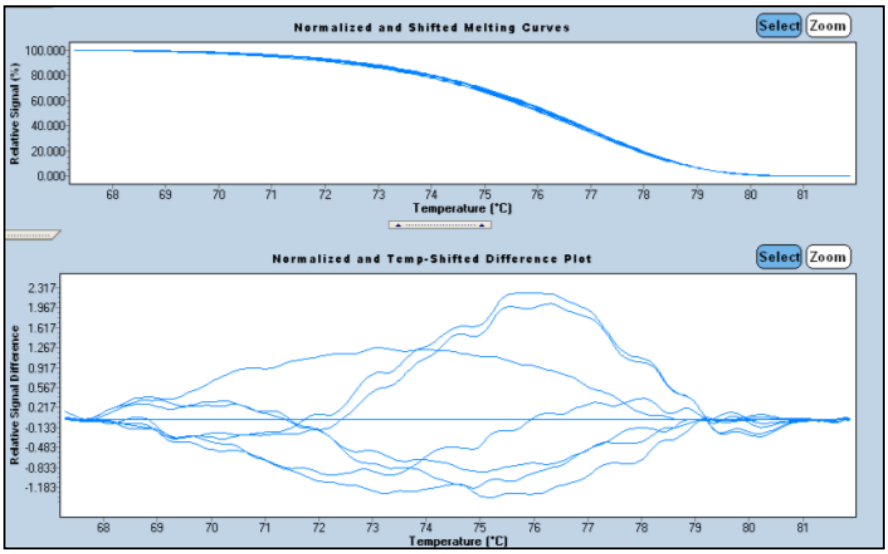


Figure 20: Examples of HRM profile for four *Podocarpus* species described in Table 10 using marker GA44, GA77, & GA92. Each PCR amplicon melting is shown as the relative signal difference between a reference melting curve (*Podocarpus totara* morphotypes) and the curve for each of the seven *Podocarpus* accessions. Relative signal differences for the melting curves are GA44: T-HAN grouping, with T (red) and HAN (blue). GA77: TA-HN grouping, with TA (blue) and HN (red). GA92: T-A-HN grouping, with T (red), A (green), HN (red).

A



B

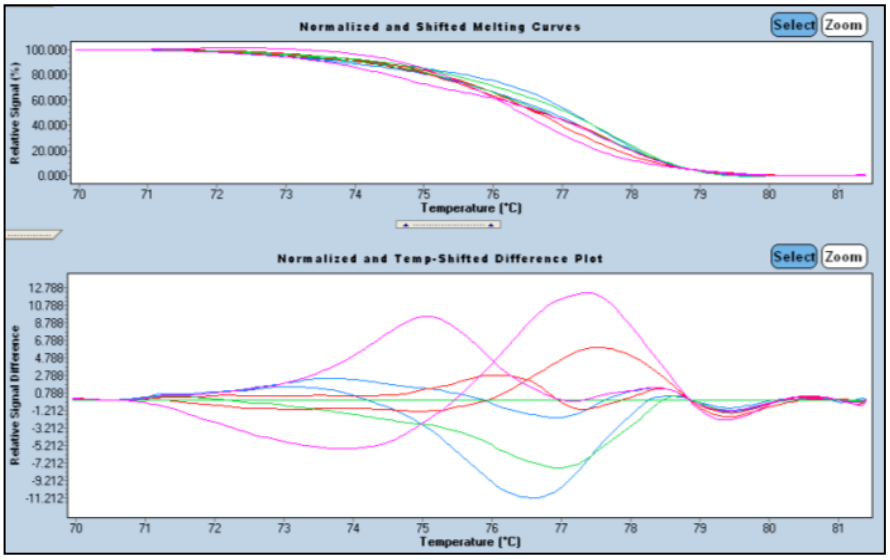


Figure 21: HRM profile of unsuccessful markers for four *Podocarpus* species. There are eight *Podocarpus* accessions (two accessions per morphotypes) represented in each figure. A: Marker GA37 produces a monomorphic melting curve (blue). B: Marker GA28 produced a complex profile (individuals from the same species are not grouped: *P. totara* (blue), *P. hallii* (red), *P. acutifolius* (green), *P. nivalis* (pink)).

3.4.2.2 PHASE 2

All markers that showed unique melting curves, i.e. one melting curve for each of the four *Podocarpus* species during phase 1, were chosen for phase 2 screening (Figure 17). In addition, 16 markers that split *P. totara* from other *Podocarpus* species, resulting in at least two groupings, were used for phase 2 screening. A total of 45 markers were tested on 16 new samples consisting of: four *P. totara*, four *P. hallii*, four *P. acutifolius* and four *P. nivalis*, along with the phase 1 samples that were used as reference controls.

Phase 2 screening resulted in 15 (33%) markers suitable for phase 3. Five markers showed melting curve differences between all four species, denoted T-H-A-N, (Figure) and the other ten markers had differences that allowed for two or more groupings of the *Podocarpus* species. HRM profiles that produced either conflicting results with phase 1 (Figure 24), or irreproducible markers that could not clearly differentiate between species were noted as “complex" and were excluded from further analysis (Figure 25) (Table 6 Appendix). Analysis of the melting peaks was taken into consideration when determining if differences were significant for some markers. For example, marker GA90 had a similar relative signal difference for *P. totara* and *P. nivalis* (Figure 22) but distinct melting peaks (Figure 23).

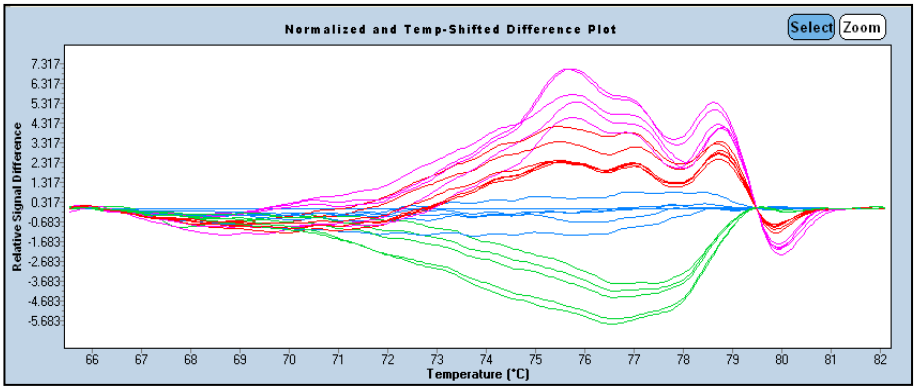


Figure 22: HRM profile of four *Podocarpus* species for marker GA90. Each PCR amplicon melting curve is shown as the relative signal difference between a reference melting curve (*Podocarpus totara*) and the curve for each of 23 *Podocarpus* accessions. Relative signal difference for the melting curves are as follows: *P. totara* (blue), *P. hallii* (red), *P. acutifolius* (green), and *P. nivalis* (pink).

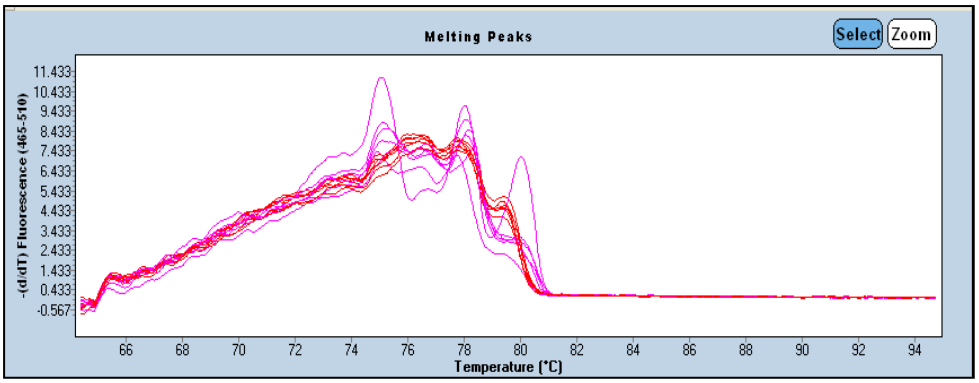
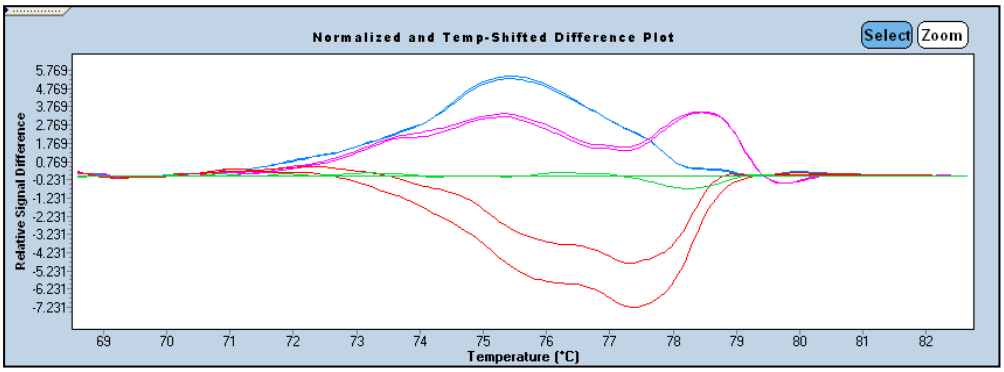


Figure 23: HRM melting peak analysis of four *Podocarpus* species for marker GA90. Each PCR amplicon melting curve is shown as fluorescence difference over temperature between a reference melting peak (*Podocarpus totara*) and the curve for each of 23 totara accessions in derivative plot format. This display is produced from superimposing normalised curves and plotting the fluorescence differences between samples (d/dT). Melting peaks for the melting curves are as follows: *P. totara* (red), and *P. nivalis* (pink).

A



B

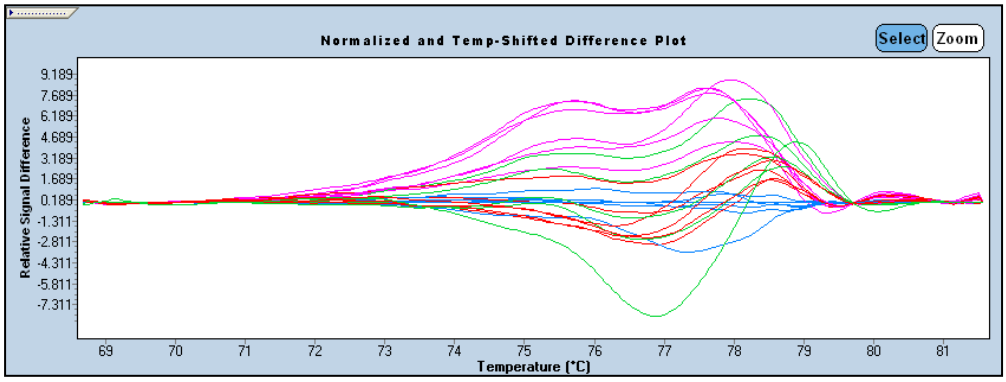


Figure 24: HRM profile of four *Podocarpus* species for marker GA42 phase 1 (A) and phase 2 (B). Each PCR amplicon melting is presented as the relative signal difference between a reference melting curve (*P. totara*) and the curve for either 7, or 23 accessions. Species are: *P. totara* (blue), *P. hallii* (red), *P. acutifolius* (green) *P. nivalis* (pink). (A) Shows distinct curves for each species, whereas in (B) where alternative accessions are used, "complex" relationships are apparent.

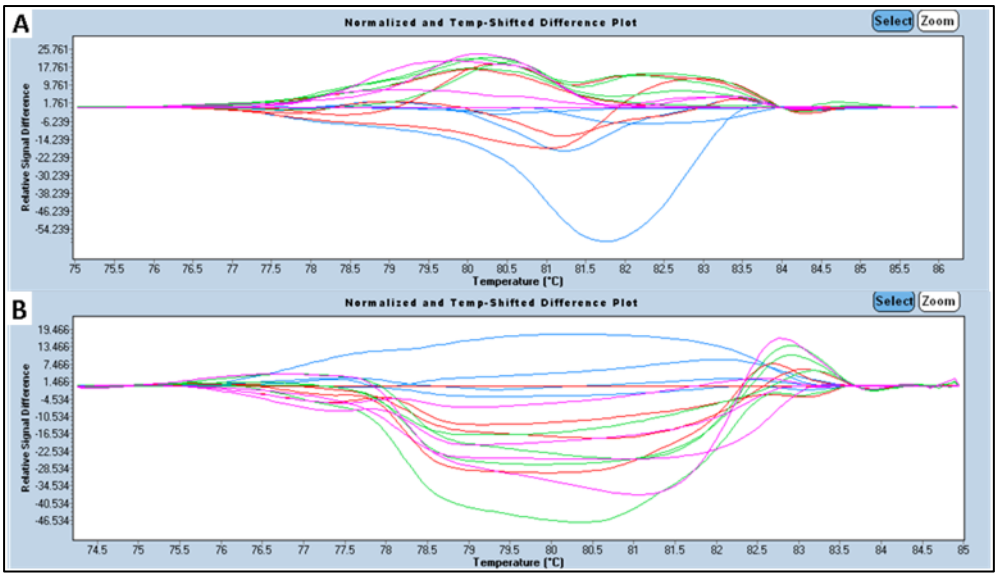


Figure 25: Example of complex HRM profile shown in phase 2 screening of markers (A) ATUB and (B) UAE. Each PCR amplicon melting is presented as the relative signal difference between a reference melting curve (*P. totara*) and the curve for each of 23 totara accessions. Species were: *P. totara* (blue), *P. hallii* (red), *P. acutifolius* (green) *P. nivalis* (pink).

3.4.2.3 PHASE 3

The 15 markers that yielded positive results from phase 2 were all used in phase 3 screening (Table 6 Appendix). Phase 3 screening consisted of testing a larger subset of the four species including three synthetic hybrid species. In total 72 samples were screened which included 29 *P. totara*, 19 *P. hallii*, four *P. acutifolius*, nine *P. nivalis*, three synthetic hybrids and the reference controls from phase 1. Twelve of the 15 markers produced species unique profiles, and were deemed candidates for the final stage of screening. Hybrids could be distinguished by an intermediate relative signal difference for two of the markers (GA99, GA113; an example is shown in Figure 26).

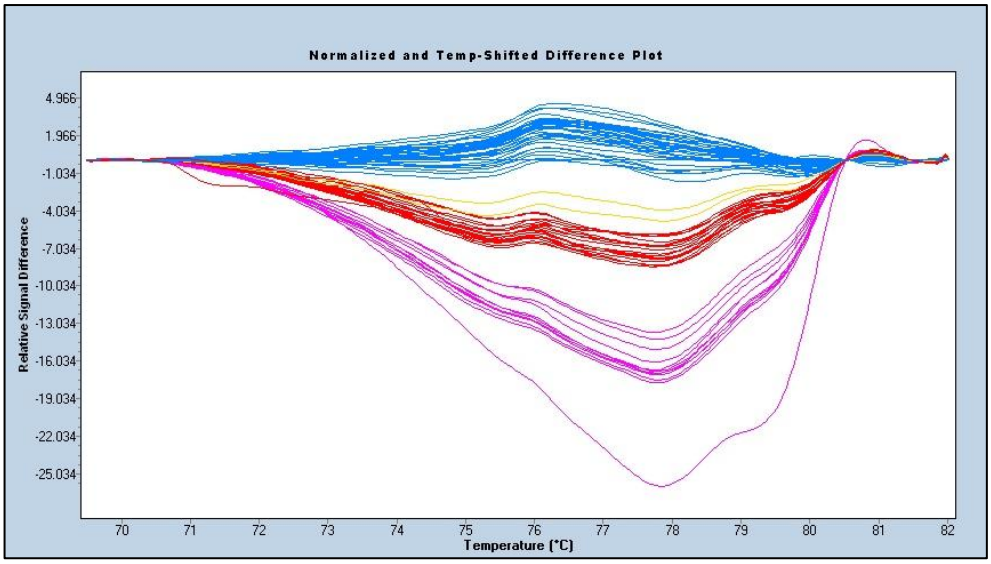


Figure 26: HRM profile of four species for marker GA113. Each PCR amplicon melting is shown as the relative signal difference between a reference melting curve (*P. totara*) and the curve for each of the 71 *Podocarpus* accessions. Relative signal difference for the melting curves are as follows: *P. totara + P. acutifolius* (blue), *P. hallii* (red), *P. nivalis* (pink), synthetic hybrid species (yellow).

3.4.2.4 PHASE 4

The 12 markers that yielded positive results for phase 3 were all used for phase 4 screening. Phase 4 screening consisted of testing the candidate markers from phase 3 on the four groups of transect samples (Mt. Pureora, Mt. Makatiti, Mt. Taranaki and Mamaku Ranges). A total of 179 samples were screened which included 55 *P. totara*, 109 *P. hallii*, one *P. nivalis* (bronze variety), two *P. lawrencii*, one *P. aurea* (golden totara), 3 synthetic hybrids and the reference accessions from phase 1. Ten of the 12 markers produced HRM profiles that split *Podocarpus* species into two or more groups with nine having results consistent with phase 3 screening. Synthetic hybrids (samples H1, H2, and H3) could be identified as having an intermediate relative signal difference for two of the markers (GA99, GA113; Table 6 Appendix).

Table 11: Phase 4 screening results for *P. aurea*, *P. lawrencii* and synthetic hybrid samples. "Synthetic hybrid" samples comprised *P. totara* and *P .hallii* DNA.

Name	Phenotypic Assignment	Species designation based on marker	GA15	GA48	GA54	GA58	GA78	GA84	GA90	GA92	GA99	GA113
21DBG	*P. aurea*	*P. aurea*	AuL	Au	H	SH	AuL	AuL	H	HN	SH	H
22DBG	*P. lawrencii*	*P. lawrencii*	AuL	N	L	AN	AuL	AuL	L	TA	TA	L
23DBG	*P. lawrencii*	*P. lawrencii*	AuL	N	L	AN	AuL	AuL	L	TA	TA	L
H1	*P. totara & P. hallii*	Synthetic hybrid	THA	TA	TA	TH	T	T	T	TA	SH	SH
H2	*P. totara & P. hallii*	Synthetic hybrid	THA	TA	TA	TH	T	T	T	TA	SH	SH
H3	*P.totara & P. hallii*	Synthetic hybrid	THA	TA	TA	SH	T	T	T	TA	TA	SH

Key

Au	*P. aurea*
L	*P. lawrencii*
SH	synthetic hybrid
H	*P. hallii*
T	*P. totara*
N	*P. nivalis*

All samples, except eleven Mamaku (individuals #117-123; 127-130) and three Makatiti (#155, 170, 171), had marker genotypes that matched with their phenotypic assignments. *P.* aurea (#DBG21) and *P. lawrencii* (#DBG22, DBG23) produced mixed results for the markers. Using the ten markers, in some cases HRM profiles grouped *P. aurea* with *P. hallii*, *P. acutifolius* and *P. totara*. In other cases *P. aurea* had a distinctly different melting profile. The two *P. lawrencii* samples produced results that grouped it with either P. totara or *P. nivalis* or separately with its own distinct curve (Table 11; Table 6 Appendix). More detail about each marker is described as follows:

Marker GA15 differentiated *P. totara* and *P. hallii* (Figure 27). This marker splits the *Podocarpus* species as THA-N. That is, *P. totara*, *P. hallii*, and *P. acutifolius* had the same melting curve while *P. nivalis* samples grouped separately. Samples from Mamaku (#105,106,116,118 and 120) that were phenotypically assigned as *P. totara*, displayed a similar HRM profile to *P. totara* but were distinct from other *P. totara* reference samples. Synthetic hybrids (samples H1, H2 and H3) could not be distinguished from *P. totara* and *P. hallii.* They all grouped together (blue). *P. lawrencii* and *P. aurea* samples had very similar melting curves.

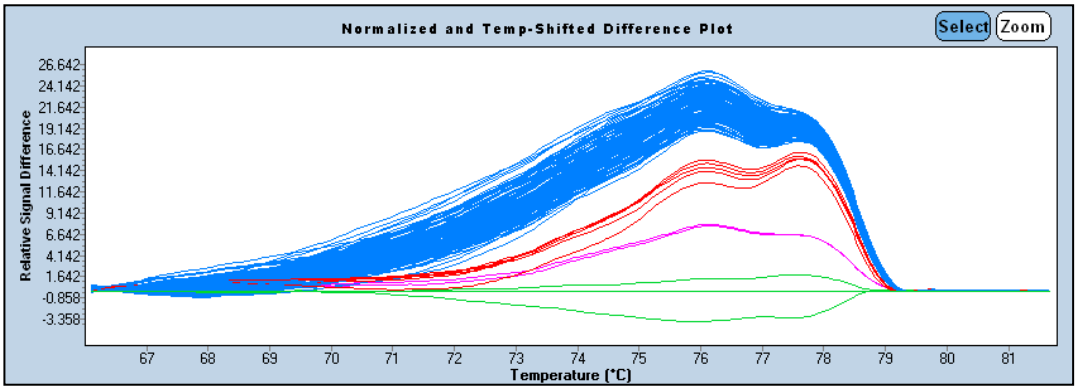


Figure 27: HRM profile of four species for marker GA15. Each PCR amplicon melting is shown as the relative signal difference between a reference melting curve (*P. nivalis*) and the curve for each of the 168 *Podocarpus* accessions. Relative signal differences for the melting curves are as follows: *P. totara* + *P. hallii* + *P. acutifolius* + synthetic hybrids (blue), Mamaku (#105,106,116,118 and 120) *P. totara* samples (red), *P. nivalis* (green), *P. lawrencii* + *P. aurea* (pink)

Marker GA48 differentiated *P. totara* and *P. hallii* (Figure 28). For this marker *P. totara* and *P. acutifolius* have the same melting curve while *P. nivalis* and *P. hallii* samples had distinct curves. *P. lawrencii* grouped with *P. nivalis* samples, whereas *P. aurea* was distinct. Synthetic hybrids (samples H1, H2 and H3) could not be distinguished from *P. totara* and *P. acutifolius*.

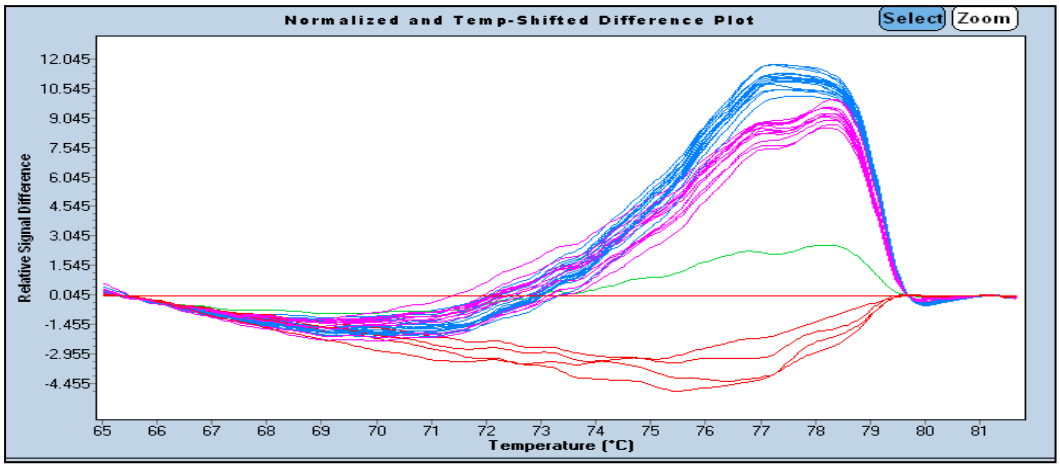


Figure 28: HRM profile of four species for marker GA48. Each PCR amplicon melting is shown as the relative signal difference between a reference melting curve (*P. nivalis*) and the curve for each of the seven 168 *Podocarpus* accessions. Relative signal difference for the melting curves are as follows: *P. totara* + *P. acutifolius* + synthetic hybrids (pink) *P. hallii* (blue), *P. nivalis* + *P. lawrencii* (red), *P. aurea* (green).

Marker GA54 differentiated *P. totara* and *P. hallii* (Figure 29). *P. totara* and *P. acutifolius* had the same melting curve while *P. nivalis* and *P. hallii* samples had distinct curves. *P. aurea* grouped with *P. hallii* samples, whereas *P. lawrencii* had a distinct curve. Synthetic hybrids (samples H1, H2 and H3) could not be distinguished from *P. totara* and *P. acutifolius*.

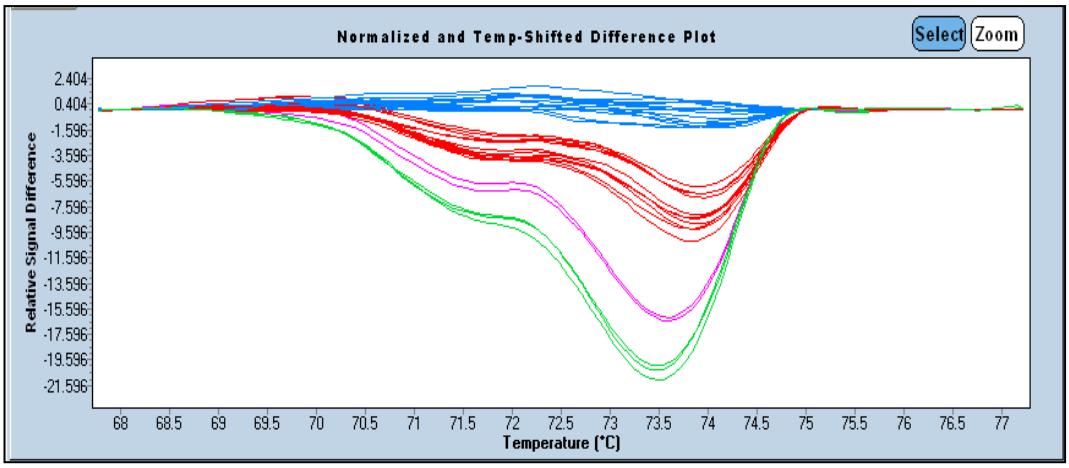


Figure 29: HRM profile of four species for marker GA54. Each PCR amplicon melting is shown as the relative signal difference between a reference melting curve (*P. totara*) and the curve for each of the 168 *Podocarpus* accessions. Relative signal difference for the melting curves are as follows: *P. totara* + *P. acutifolius* + synthetic hybrids (blue), *P. hallii* + *P. aurea* (red), *P. nivalis* (green), *P. lawrencii* (pink).

Marker GA58 was unable to differentiate *P. totara* and *P. hallii* samples (Figure 30). This marker split the *Podocarpus* species as TH-AN, where *P. totara* and *P. hallii* samples grouped together and where *P. nivalis* and *P. acutifolius* samples grouped together. *P. aurea* grouped with one synthetic hybrid (H3), with the other two hybrids (H1, H2) grouped with *P. totara* + *P. hallii.* *P. lawrencii* grouped with *P. nivalis* and *P. acutifolius.*

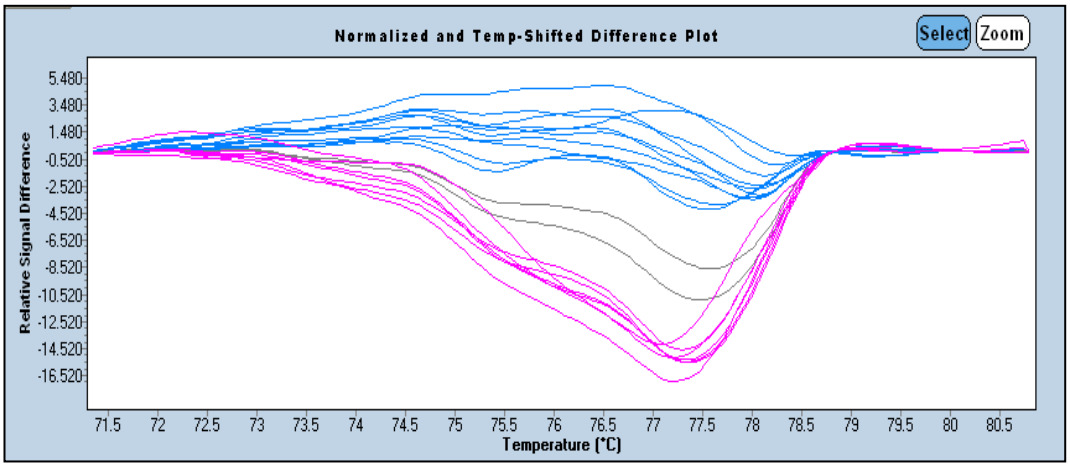


Figure 30: HRM profile of four species for marker GA58. Each PCR amplicon melting is shown as the relative signal difference between a reference melting curve (*P. totara*) and the curve for each of the 168 *Podocarpus* accessions. Relative signal difference for the melting curves are as follows: *P. totara* + *P. hallii* + two synthetic hybrids (blue), *P. nivalis* + *P. acutifolius* + *P. lawrencii* (pink), *P. aurea* + one synthetic hybrid (grey).

Marker GA78 differentiated *P. totara* and *P. hallii* samples (Figure 31). This marker showed relative signal differences with melting curves splitting *Podocarpus* species as T-A-HN, where *P. nivalis* and *P. hallii* display the same melting curve, while *P. totara* and *P. acutifolius* samples each displayed distinct melting curves. *P. aurea* samples grouped with *P. lawrencii.* Synthetic hybrids could not be differentiated because they grouped with *P. totara*.

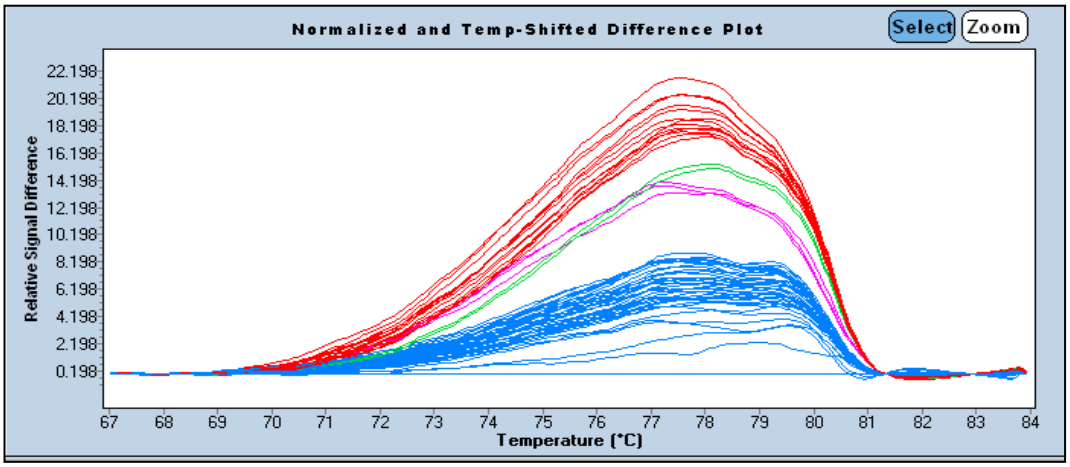


Figure 31: HRM profile of four species for marker GA78. Each PCR amplicon melting is presented as the relative signal difference between a reference melting curve (*P. hallii*) and the curve for each of the 168 *Podocarpus* accessions. Relative signal difference for the melting curves are as follows: *P. totara* + synthetic hybrids (red), *P. hallii* + *P. nivalis* (blue), *P. acutifolius* (green), *P. lawrencii* + *P. aurea* (pink).

Marker GA84 differentiated *P. totara* and *P. hallii* samples (Figure 32). This marker split the *Podocarpus* species as TA-H-N where *P. totara* and *P. acutifolius* had the same melting curve for relative signal differences but both *P. nivalis* and *P. hallii* samples had

distinct curves. The *P. aurea* samples grouped with *P. lawrencii.* Synthetic hybrids could not be differentiated. They grouped with the *P. totara* morphotype reference accessions.

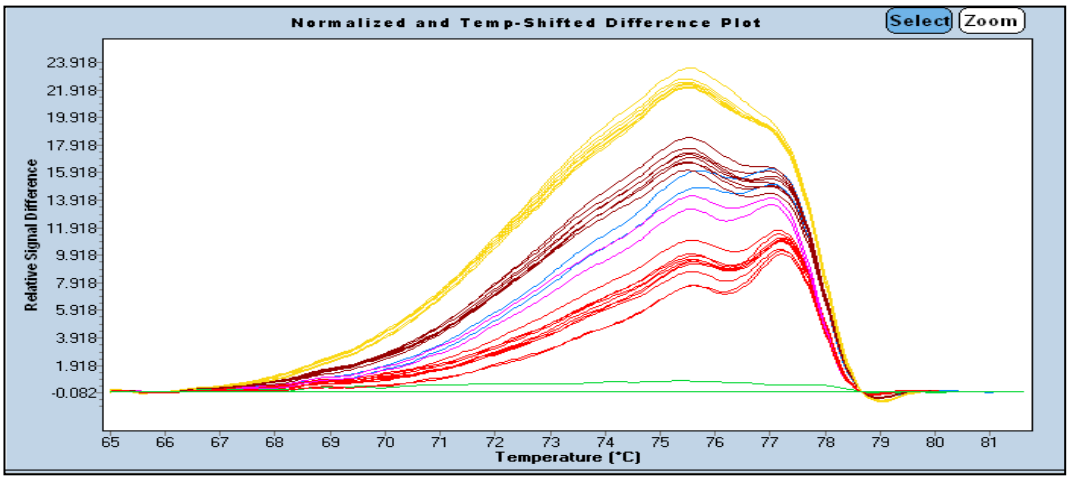


Figure 32: HRM profile of four species for marker GA84. Each PCR amplicon melting is shown as the relative signal difference between a reference melting curve (*P. nivalis*) and the curve for each of the 168 *Podocarpus* accessions. Relative signal difference for the melting curves are as follows: *P. totara*-control + synthetic hybrids (blue), *P. hallii*-Taranaki (red), *P. acutifolius* (pink), *P. nivalis* (green), *P. lawrencii* + *P. aurea* (grey), other *P. hallii* (brown), other *P. totara* (yellow).

Marker GA90 differentiated *P. totara* and *P. hallii* samples (Figure 33). This marker is the only marker that differentiated all *Podocarpus* species in all four phases, and is denoted as T-H-A-N. The melting curves were distinct for each morphotype. Also, the *P. totara* species could be broken into groupings with the Mamaku samples (individuals #105, 106, and 112-122) and Makatiti (#155, 170, 171) as one group (yellow line Figure 32) and all the other *P. totara* samples as another (brown line Figure 32). The *P. aurea* samples found to be grouped with *P. hallii. The P. lawrencii* samples had a distinct grouping. The synthetic hybrids could not be distinguished as they grouped with *P. totara*.

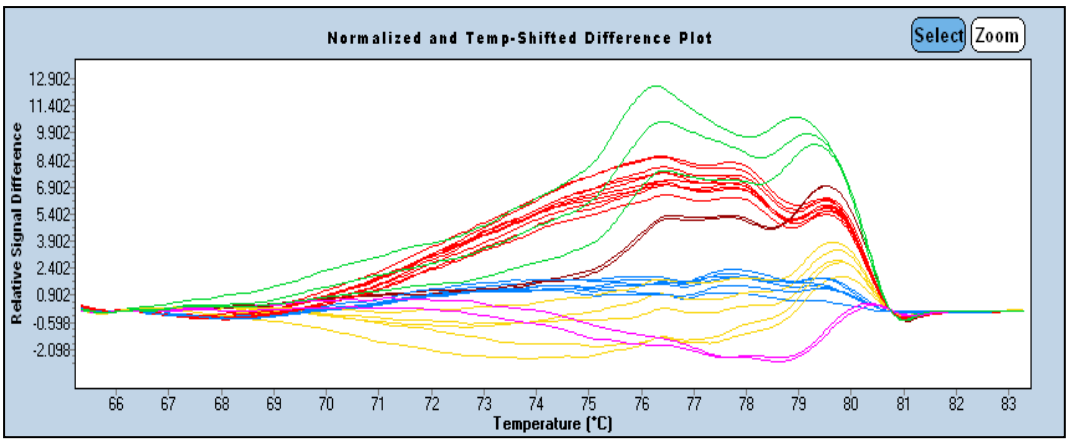


Figure 33: HRM profile of four species for marker GA90. Each PCR amplicon melting curve is shown as the relative signal difference between a reference melting curve (*P. totara)* and the curve for each of the 168 *Podocarpus* accessions. Relative signal difference for the melting curves are as follows: *P. totara* + synthetic hybrids (blue), *P. hallii* + *P. aurea* (red), *P. acutifolius* (pink), *P. nivalis* (green), *P. lawrencii* (brown), *P. totara* Mamaku samples (105, 106, and 112-122) and Makatiti (155, 170, 171) (yellow).

Marker GA92 differentiated *P. totara* and *P. hallii* samples (Figure 34). This marker split the *Podocarpus* species as TA-HN. The melting curves for relative signal differences were *P. totara* with *P. acutifolius,* and *P. hallii* with *P. nivalis.* The *P. aurea* samples are grouped with *P. hallii.* The *P. lawrencii* samples with *P. totara* and *P. acutifolius*. The synthetic hybrids could not be distinguished as they grouped with *P. totara* and *P. acutifolius.*

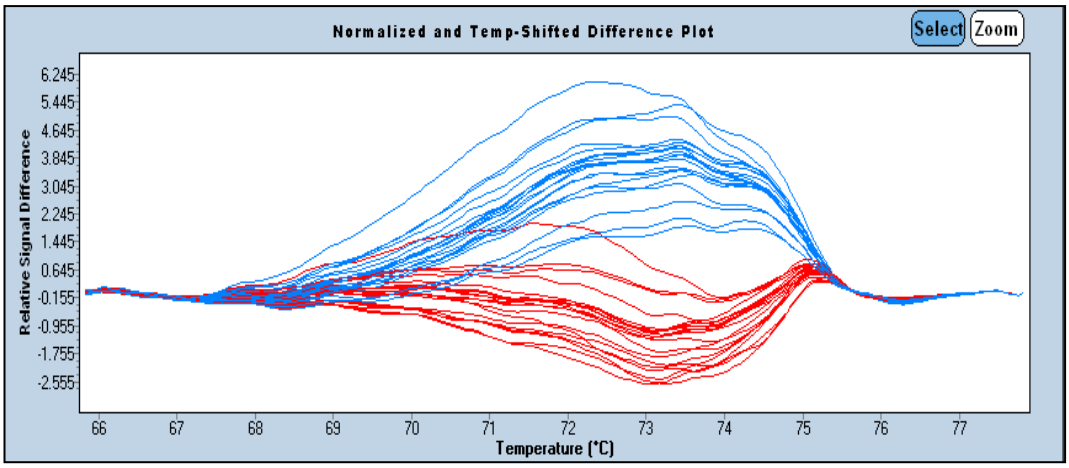


Figure 34: HRM profile of four species for marker GA92. Each PCR amplicon melting is shown as the relative signal difference between a reference melting curve (*P. hallii*) and the curve for each of the 168 *Podocarpus* accessions. Relative signal differences for the melting curves are as follows: *P. totara* + *P. acutifolius* + *P. aurea* (blue), *P. hallii* + *P. nivalis* + *P. lawrencii* (red).

Marker GA99 differentiated *P. totara* and *P. hallii* samples (Figure 35). This marker split the *Podocarpus* species as TA-H-N where *P. totara* and *P. acutifolius* had the same melting curve where *P. nivalis* and *P. hallii* showed a different curve. *P. aurea* has a curve similar to the two synthetic hybrids (H1 and H2), while *P. lawrencii* and synthetic hybrid H3 had a curve matching that of *P. totara*. Synthetic hybrids (samples H1 and H2) could be distinguished with marker GA99 as having an intermediate relative signal difference (Yellow-Figure).

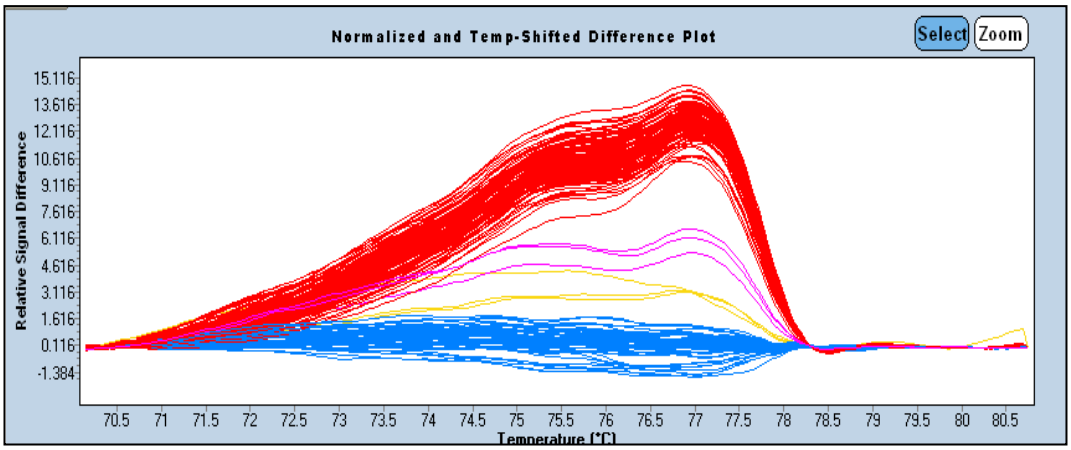


Figure 35: HRM profile of four species for marker GA99. Each PCR amplicon melting is shown as the relative signal difference between a reference melting curve (*P. totara*) and the curve for each of the 168 *Podocarpus* accessions. Relative signal difference for the melting curves are as follows: *P. totara* + 1x synthetic hybrids + *P. lawrencii* (blue), *P. hallii* (red), *P. nivalis* (pink), *P. aurea* + 2x synthetic hybrids (yellow).

Marker GA113 differentiated *P. totara* and *P. hallii* samples (Figure 36). This marker split the *Podocarpus* species as TA-H-N where *P. totara* and *P. acutifolius* have the same melting curve for relative signal differences but both *P. nivalis* and *P. hallii* samples were distinct. *P. aurea* grouped with *P. hallii* whereas *P. lawrencii* had its own distinct curve. Synthetic hybrids (samples H1, H2 and H3) had an intermediate relative signal difference (Pink- Figure).

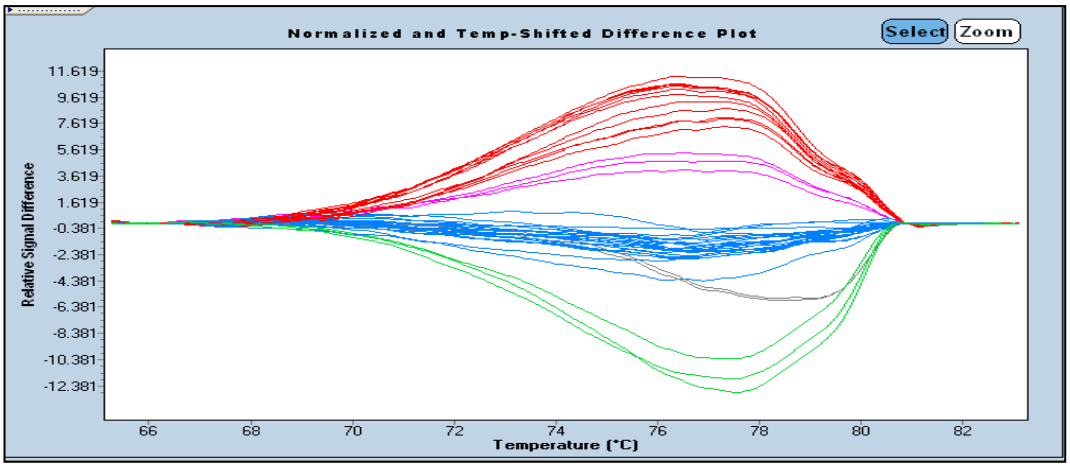


Figure 36: HRM profile of four species for marker GA113. Each PCR amplicon melting is shown as the relative signal difference between a reference melting curve (*P. hallii*) and the curve for each of the 168 *Podocarpus* accessions. Relative signal difference for the melting curves are as follows: *P. totara* + *P. acutifolius* (red), *P. hallii* + *P. totara aurea (blue), P. nivalis* (green), synthetic hybrids (pink), *P. lawrencii* (grey).

In summary, the ten markers produced definitive results (Table 12). For phase 4 screening, one marker (GA90) indentified all four species as genetically distinct. A further six markers (GA113, GA48, GA84, GA54, GA78, and GA99) split *Podocarpus* species into three groups (TA-H-N, T-A-HN) and three markers (GA15, GA92, and GA58) split *Podocarpus* species into two groups (TAH-N, TA-HN, TH-AN). Together these ten markers could identify 97% of the samples correctly (1731/1790 (179 samples x 10 markers). That is, in 97% cases phenotypic designations of species morphotypes matched specific genotypes (Table 6 Appendix). In contrast, synthetic hybrids exhibited mixed, but informative, signals.

Table 12: Summary of 10 markers developed and their phylogenetic resolutions. T=*P. totara*, H=*P. hallii*, A=*P. acutifolius*, N=*P. nivalis.*

Marker	GA15	GA48	GA54	GA58	GA78	GA84	GA90	GA92	GA99	GA113
Splits	TAH-N	TA-H-N	TA-H-N	TH-AN	T-A-HN	TA-H-N	T-H-A-N	TA-HN	TA-H-N	TA-H-N
Identifies hybrid nature	x	x	x	x	x	x	x	x	√	√
Differentiates *P.totara &P. hallii*	x	√	√	x	√	√	√	√	√	√

3.4.3 ANNOTATION OF NUCLEAR MOLECULAR MARKERS

The markers used for HRM screening were annotated using "Blast2GO". The results indicate the ESTs represented at different phases of screenings. Markers were found to be homologous to transposable elements, ribosomal proteins and genes of unknown function origin. Table 13 specifies the gene description of each of the ESTs for which markers were developed.

Table 13: Gene description for markers based on Blast2GO results. The 10 successful candidate markers are highlighted in red.

Marker Name	Gene Description	Amp	Phase 1 (8 samples)	Phase 2 (24 samples)	Phase 3 (72 samples)	Phase 4 (179)
Pine						
1-AGP	Arabinogalactin-like protein	X	X	x	x	x
2-DHN	Dehydrin	√	Complex	x	x	x
3-SAM	S-adenosylmethionine synthase	√	Mono	x	x	x
4-Ces4	Cellulose synthase	X	x	x	x	x
5-Btub	Beta-tubulin	√	Mono	x	x	x
6-C4H	Cinnamate 4-hydroxylase	√	Mono	x	x	x
7-Laccase	Laccase	√	Mono	x	x	x
8-Atub	Alpha-tubulin	√	TA-HN	Complex	x	x
9-Ces7	Cellulose synthase	√	Mono	x	x	x
10-Ces8	Cellulose synthase	√	Mono	x	x	x
11-CAD	Cinnamyl-alcohol dehydrogenase	√	Mono	x	x	x

12-UAE	-	√	TA-HN	Complex	x	x
Galaxy						
GA1	Unknown	√	Mono	x	x	x
GA2	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	Mono	x	x	x
GA3	ORF158 | DNA/RNA polymerases superfamily protein	√	Mono	x	x	x
GA4	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	Mono	x	x	x
GA5	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	Mono	x	x	x
GA6	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	Mono	x	x	x
GA7	Unknown	√	Mono	x	x	x
GA8	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	Mono	x	x	x
GA9	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	Mono	x	x	x
GA10	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	Mono	x	x	x
GA11	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	Mono	x	x	x
GA12	hAT transposon superfamily	√	TA-H-N	x	x	x
GA13	ORF170 | Reverse transcriptase (RNA-dependent DNA polymerase)	√	Mono	x	x	x
GA14	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	TA-HN	TA-HN	x	x
GA15	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	THA-N	THA-N	THA-N	THA-N
GA16	ORF158 | DNA/RNA polymerases superfamily protein	√	T-H-A-N	T-H-AN	Complex	x
GA17	Unknown	√	TA-HN	TA-HN	x	x
GA18	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	Mono	x	x	x
GA19	ORF158 | DNA/RNA polymerases superfamily protein	√	Mono	x	x	x
GA20	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	Mono	x	x	x
GA21	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	Mono	x	x	x
GA22	Unknown	√	Mono	x	x	x
GA23	ORF145A | Gag-Pol-related retrotransposon family protein	√	Mono	x	x	x

GA24	ORF240B | DNA/RNA polymerases superfamily protein	√	T-H-A-N	T-H-A-N	Complex	x
GA25	Gag-Pol-related retrotransposon family protein	√	Complex	x	x	x
GA26	ORF120 | Polynucleotidyl transferase, ribonuclease H-like	x	x	x	x	x
GA27	ORF158 | DNA/RNA polymerases superfamily protein	x	x	x	x	x
GA28	ORF120 | Polynucleotidyl transferase, ribonuclease H-like superfamily protein	√	Complex	x	x	x
GA29	ORF145A | Gag-Pol-related retrotransposon family protein	√	Mono	x	x	x
GA30	ORF158 | DNA/RNA polymerases superfamily protein	√	Mono	x	x	x
GA31	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	x	x	x	x	x
GA32	Unknown	x	x	x	x	x
GA33	Unknown	√	Complex	x	x	x
GA34	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	TA-HN	TA-HN	x	x
GA35	ORF158 | DNA/RNA polymerases superfamily protein	√	Mono	x	x	x
GA36	ORF240B | DNA/RNA polymerases superfamily protein	√	T-H-A-N	Complex	x	x
GA37	Unknown	√	Mono	x	x	x
GA38	Gag-Pol-related retrotransposon family protein	√	Complex	x	x	x
GA39	ORF158 | DNA/RNA polymerases superfamily protein	√	TH-AN	Complex	x	x
GA40	Unknown	√	T-H-A-N	TA-HN	Complex	X
GA41	hAT transposon superfamily protein	√	Mono	x	x	x
GA42	Unknown	√	T-H-A-N	Complex	x	x
GA43	Unknown	√	Complex	x	x	x
GA44	Unknown	√	T-HAN	T-HAN	x	x
GA45	Unknown	√	Complex	x	x	x
GA46	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	Mono	x	x	x
GA47	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	Mono	x	x	x
GA48	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	T-H-A-N	TA-H-N	TA-H-N	TA-H-N

GA49	Unknown	√	T-H-A-N	TA-HN	x	x
GA50	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	Mono	x	x	x
GA51	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	Mono	x	x	x
GA52	Unknown	√	Mono	x	x	x
GA53	ORF240B | DNA/RNA polymerases superfamily protein	√	TA-HN	TA-HN	x	x
GA54	Unknown	√	T-H-A-N	T-H-A-N	TA-H-N	TA-H-N
GA55	ORF158 | DNA/RNA polymerases superfamily protein	√	Complex	x	x	x
GA56	Unknown	√	Mono	x	x	x
GA57	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	Mono	x	x	x
GA58	ORF158 | DNA/RNA polymerases superfamily protein	√	T-H-A-N	T-H-A-N	TH-A-N	TH-AN
GA59	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	Mono	x	x	x
GA60	Unknown	√	Mono	x	x	x
GA61	Pentatricopeptide repeat (PPR) superfamily protein	√	T-H-A-N	TA-HN	TA-HN	Complex
GA62	Unknown	√	Mono	x	x	x
GA63	Tetratricopeptide repeat (TPR)-like superfamily protein	√	Complex	x	x	x
GA64	Unknown	x	x	x	x	x
GA65	hAT transposon superfamily	√	Complex	x	x	x
GA66	Unknown	√	Complex	x	x	x
GA67	hAT transposon superfamily	√	Mono	x	x	x
GA68	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	Mono	x	x	x
GA69	Unknown	√	Mono	x	x	x
GA70	ORF240B | DNA/RNA polymerases superfamily protein	√	Mono	x	x	x
GA71	ORF240B | DNA/RNA polymerases superfamily protein	√	TA-H-N	TA-HN	x	x
GA72	Polynucleotidyl transferase, ribonuclease H-like superfamily protein	√	T-H-A-N	TA-HN	x	x
GA73	ORF120 | Polynucleotidyl transferase, ribonuclease H-like	x	x	x	x	x

	superfamily protein					
GA74	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	Mono	x	x	x
GA75	Unknown	√	T-H-A-N	Complex	x	x
GA76	Unknown	√	Mono	x	x	x
GA77	Unknown	√	TA-HN	Complex	x	x
GA78	Unknown	√	T-H-A-N	TA-HN	TA-HN	TA-HN
GA79	ORF120 | Polynucleotidyl transferase, ribonuclease H-like superfamily protein	√	TA-HN	TA-HN	x	x
GA80	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	Mono	x	x	x
GA81	ORF158 | DNA/RNA polymerases superfamily protein	x	x	x	x	x
GA82	Unknown	√	Mono	x	x	x
GA83	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	TA-H-N	TA-HN	TA-HN	Complex
GA84	Unknown	√	T-H-A-N	TA-H-N	TA-H-N	TA-H-N
GA85	Unknown	√	TA-HN	TA-HN	x	x
GA86	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	TA-HN	TA-HN	x	x
GA87	ORF120 | Polynucleotidyl transferase, ribonuclease H-like superfamily	√	T-H-A-N	Complex	x	x
GA88	Unknown	√	Mono	x	x	x
GA89	ORF170 | Reverse transcriptase (RNA-dependent DNA polymerase)	√	T-H-A-N	TA-HN	x	x
GA90	ORF158 | DNA/RNA polymerases superfamily protein	√	T-H-A-N	T-H-A-N	T-H-A-N	T-H-A-N
GA91	ORF120 | Polynucleotidyl transferase, ribonuclease H-like superfamily protein	√	T-A-HN	TA-HN	x	x
GA92	Unknown	√	T-A-HN	TA-HN	TA-HN	TA-HN
GA93	ORF240B | DNA/RNA polymerases superfamily protein	√	Mono	x	x	x
GA94	ORF158 | DNA/RNA polymerases superfamily protein	√	TA-HN	Complex	x	x
GA95	Gag-Pol-related retrotransposon family protein	√	Mono	x	x	x

GA96	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	T-H-A-N	TA-HN	x	x
GA97	Tetratricopeptide repeat (TPR)-like superfamily protein	√	Complex	x	x	x
GA98	Unknown	√	T-H-A-N	Complex	x	x
GA99	ORF120 | Polynucleotidyl transferase, ribonuclease H-like superfamily protein	√	T-H-A-N	T-H-A-N	TA-H-N	TA-H-N
GA100	ORF158 | DNA/RNA polymerases superfamily protein	√	TA-HN	TA-HN	x	x
GA101	Gag-Pol-related retrotransposon family protein	√	TA-HN	x	x	x
GA102	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	T-A-HN	x	x	x
GA103	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	T-H-A-N	TA-HN	x	x
GA104	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	TA-HN	x	x	x
GA105	Unknown	√	TA-HN	x	x	x
GA106	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 10	√	TA-HN	x	x	x
GA107	ORF240B | DNA/RNA polymerases superfamily protein	√	T-H-A-N	TA-HN	x	x
GA108	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	Complex	x	x	x
GA109	Unknown	√	Complex	x	x	x
GA110	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	TA-HN	x	x	x
GA111	ORF158 | DNA/RNA polymerases superfamily protein	√	Mono	x	x	x
GA112	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	TA-HN	x	x	x
GA113	ORF158 | DNA/RNA polymerases superfamily protein	√	T-H-A-N	TA-H-N	TA-H-N	TA-HN
GA114	Unknown	√	T-H-A-N	TA-HN	x	x
GA115	Unknown	√	TA-HN	x	x	x
GA116	Tetratricopeptide repeat (TPR)-like superfamily protein	√	Mono	x	x	x
GA117	Unknown	√	T-H-A-N	TA-HN	x	x
GA118	Unknown	√	T-H-A-N	TA-HN	x	x
GA119	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	T-H-A-N	TA-HN	x	x
GA120	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8	√	TH-AN	Complex	x	x

3.4.4 CLUSTER ANALYSIS

3.4.4.1 PUREORA SITE

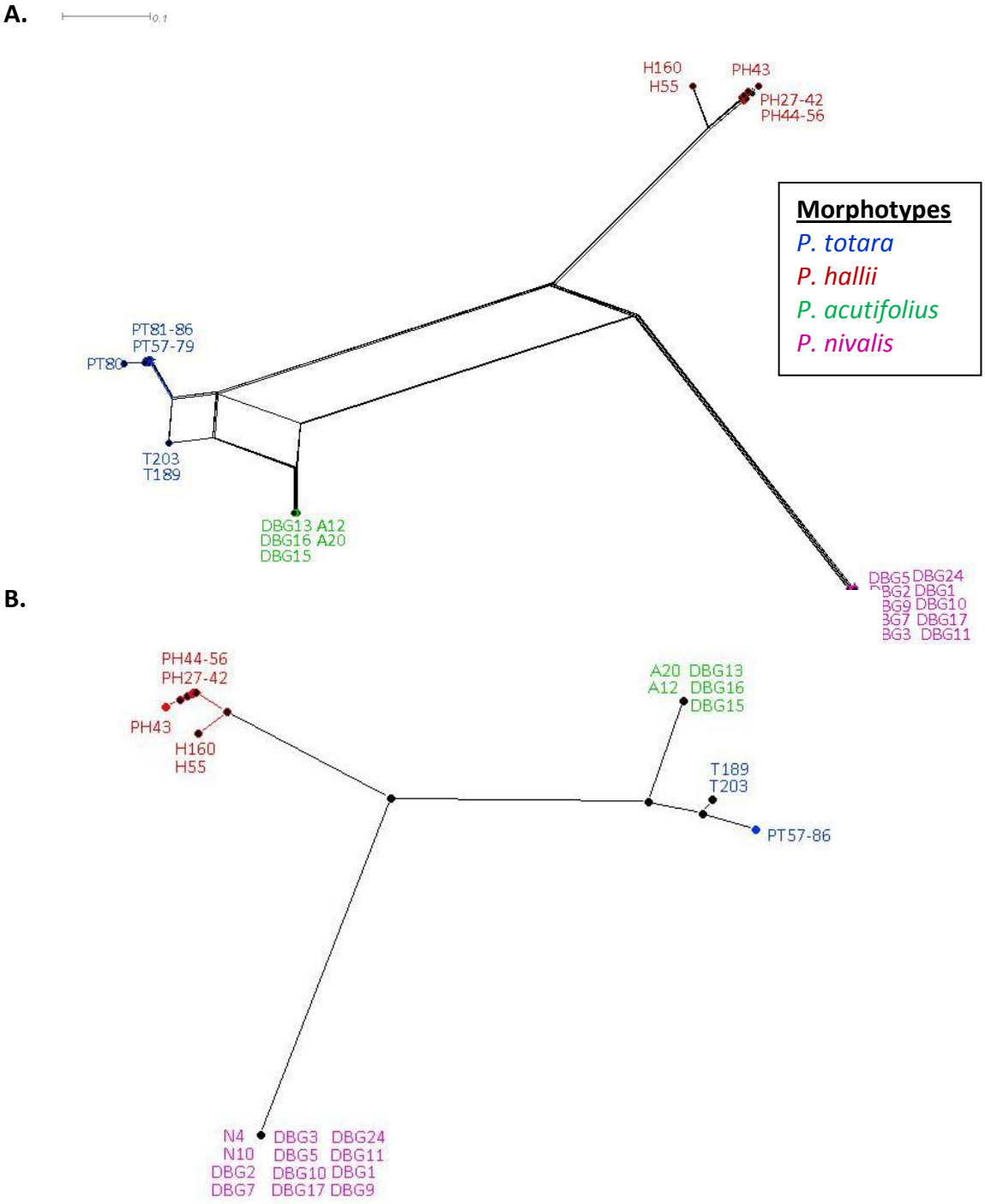


Figure 37: Neighbor-Net analysis (A) and Neighbor-Joining tree (B) for accessions from the Pureora Site. Species morphotypes are indicated by colour: *P. totara* (blue) *P. hallii* (red), *P. acutifolius* (green), and *P. nivalis* (pink).

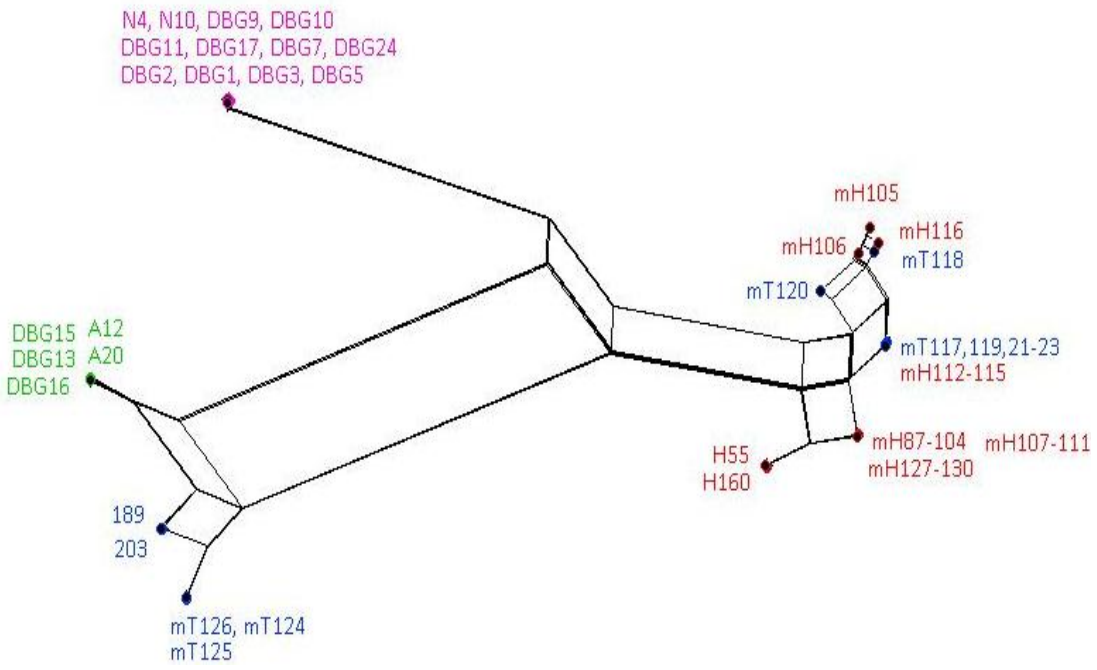
Neighbor-Nets were used to graphically represent the distances between nuclear genotypes for accessions sampled across four transects. For the Pureora site, the splits graph (Figure 37A) shows that *P. hallii* (red) is strongly split from *P. totara* (blue). Interestingly, *P. acutifolius* is more genetically similar to *P. totara* at this site than it is to the other two totara species (*P. nivalis, P. hallii*). *P. nivalis* is strongly split (genetically very distinct) from the other species.

The Neighbor-Joining tree again indicates that *P. totara* accessions are distinct from *P. hallii* (Figure 37B). *P. totara* is most genetically similar to *P. acutifolius*, with *P. nivalis* being genetically very different from all other species. The reference accessions for *P. totara* (T199, T203) and *P. hallii* (H55, H160) group closely with accessions exhibiting the morphotypes of their respective species.

While the Neighbor-Joining tree shows that the strongest signals in the data cluster accessions into their expected morphotypes, the Neighbor-Net graph also indicates a weak split (evidence in the data) linking T203 + T189 with *P. acutifolius*.

3.4.4.2 MAMAKU SITE

A.



B.

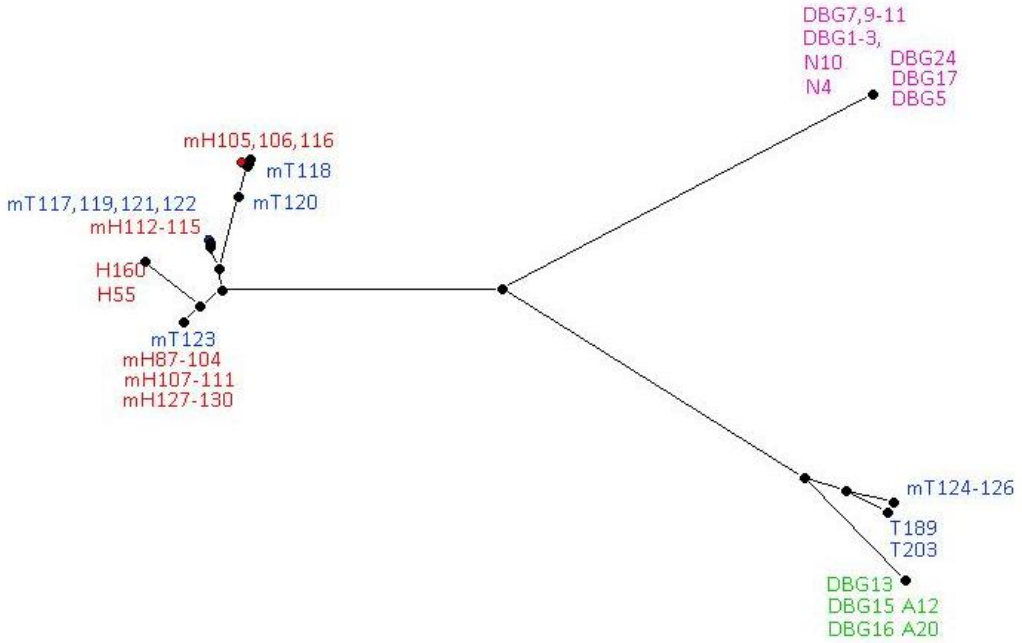


Figure 38: Neighbor-Net analysis (A) and Neighbor-Joining tree (B) for accessions from the Mamaku Site. Species morphotypes are indicated by colour: *P. totara* (blue) *P. hallii* (red), *P. acutifolius* (green), and *P. nivalis* (pink).

For the Mamaku site, the splits graph (Figure 38A) shows that *P. hallii* (red) is not strongly split from *P. totara* (blue). There are accessions (#117-123) that are phenotypically assigned as *P. totara* that group with *P. hallii*. Again, *P. acutifolius* is more strongly associated with *P. totara* at this site than to the other two totara species (*P. nivalis, P. hallii*). *P. nivalis* is strongly split from the other species.

The Neighbor-Joining tree also indicates that some *P. totara* accessions (#117-123) are indistinct from *P. hallii* (Figure 38B). *P. totara* is most strongly associated with *P. acutifolius*, with *P. nivalis* being most different from other species. The reference *P. totara* (T199, T203) and *P. hallii* (H55, H160) are distinct but, nevertheless still group closely with other accessions with similar morphotypes.

3.4.4.3 MAKATITI SITE

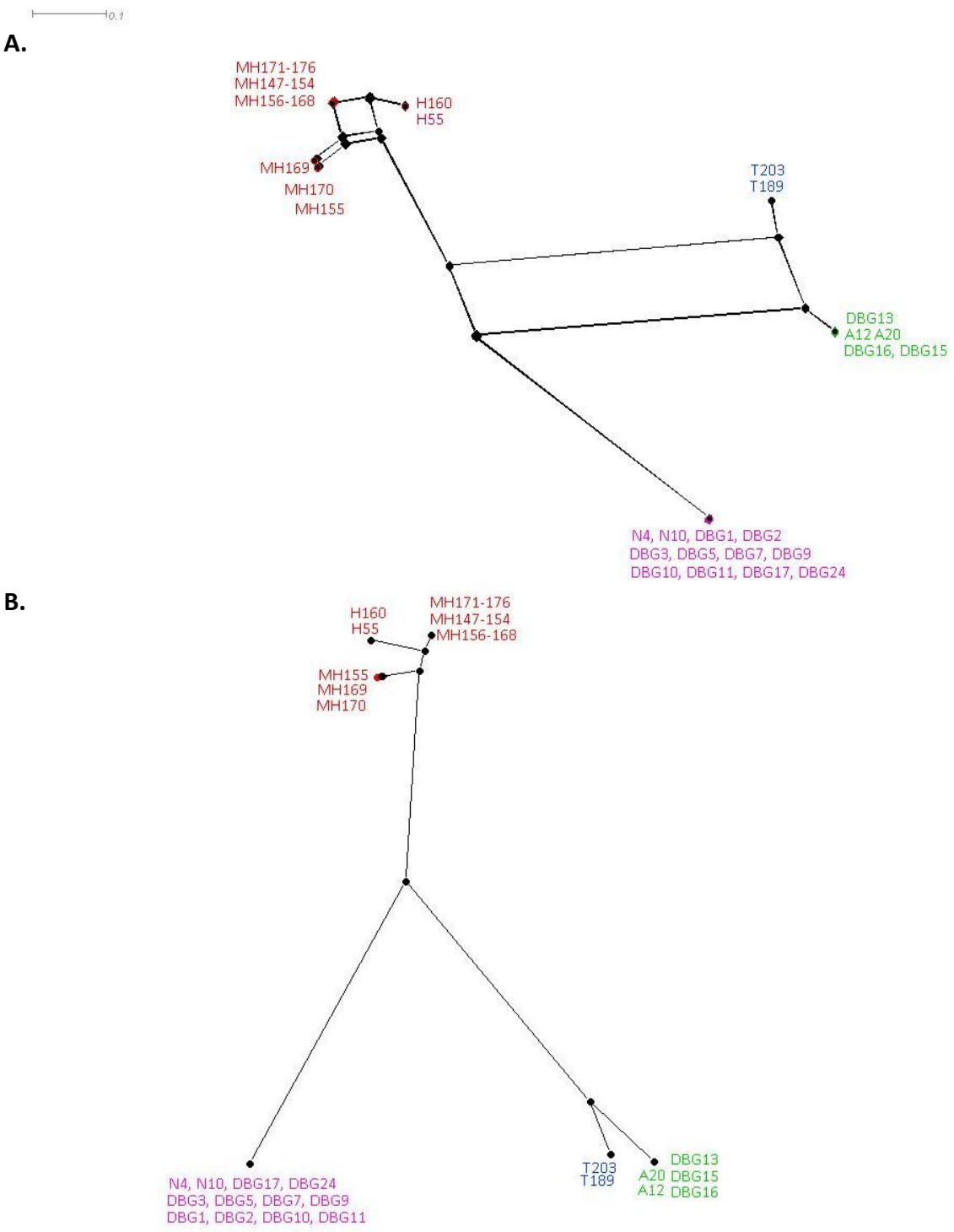


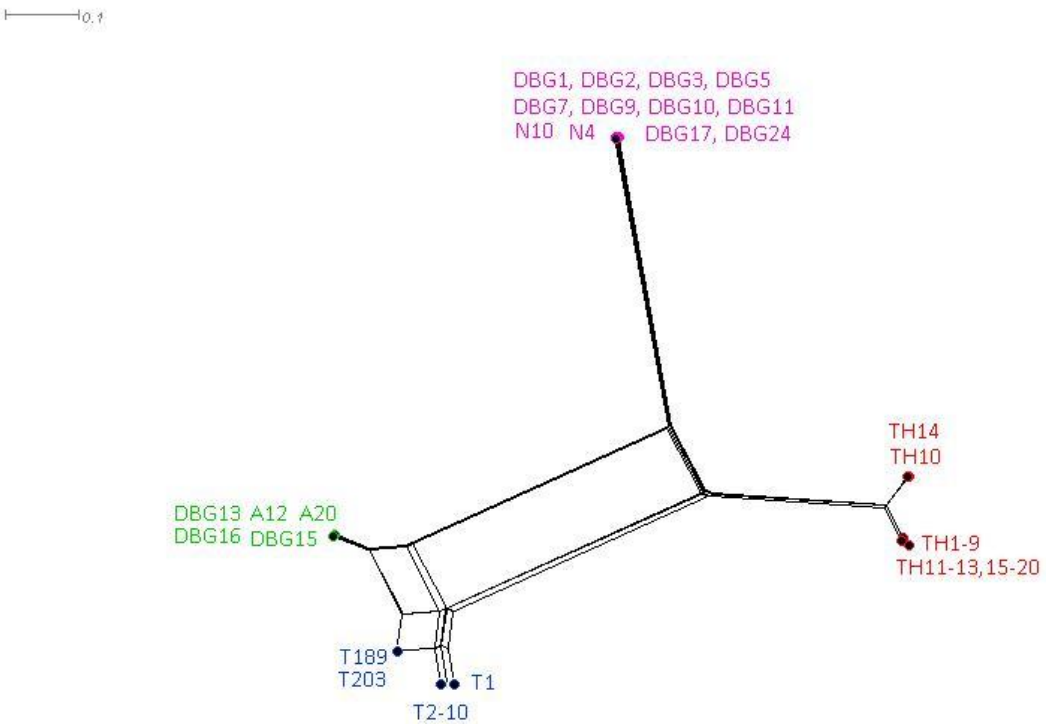
Figure 39: Neighbor-Net analysis (A) and Neighbor-Joining tree (B) for accessions from the Makatiti Site. Species morphotypes are indicated by colour: *P. totara* (blue) *P. hallii* (red), *P. acutifolius* (green), and *P. nivalis* (pink).

For the Makatiti site, the splits graph (Figure 39A) indicates that *P. hallii* (red) is strongly split from the reference *P. totara* (blue) accessions. Similar to what was observed at Pureora and Mamaku sites, *P. acutifolius* is more genetically similar to *P. totara* than it is to the other two *Podocarpus* species (*P. nivalis, P. hallii*). *P. nivalis* is again genetically very distinct.

The Neighbor-Joining tree also shows that *P. totara* accessions are distinct from P. *hallii* (Figure 39B). *P. totara* is most strongly associated with *P. acutifolius*, with *P. nivalis* being different to all other species. The reference, *P. hallii* (H55, H160), group closely with other *P. hallii* accessions.

3.4.4.4 TARANAKI SITE

A.



B.

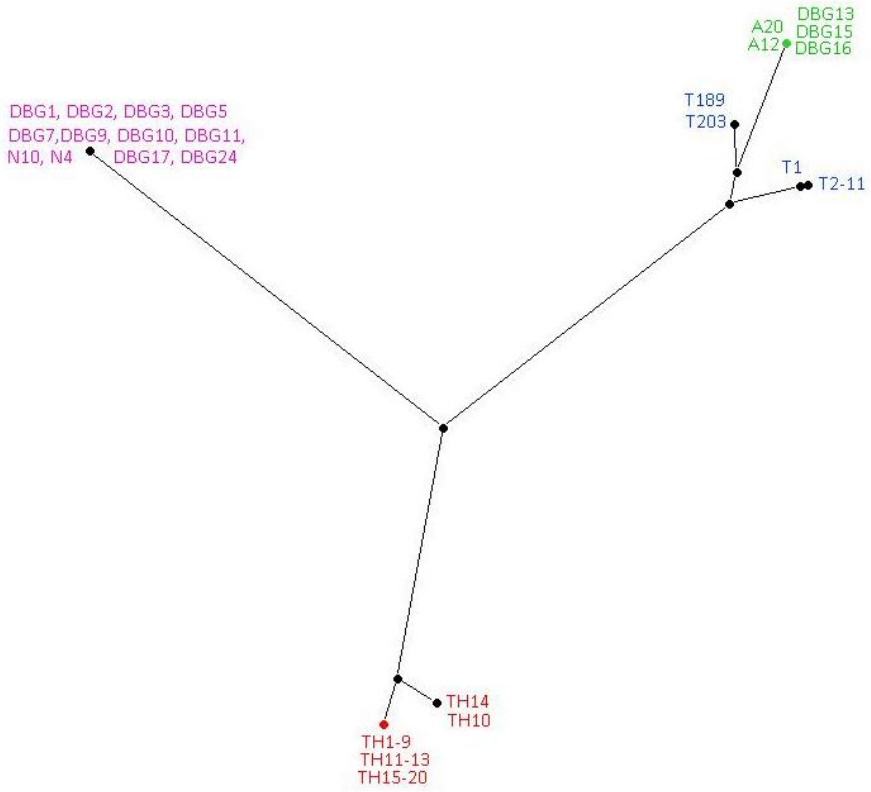


Figure 40: Neighbor-Net analysis (A) and Neighbor-Joining tree (B) for accessions from the Taranaki Site. Species morphotypes are indicated by colour: *P. totara* (blue) *P. hallii* (red), *P. acutifolius* (green), and *P. nivalis* (pink).

For the Taranaki site, the splits graph (Figure 40A) indicates that *P. hallii* (red) is strongly split from *P. totara* (blue). *P. acutifolius* is genetically more similar to *P. totara* than it is to the other two *Podocarpus* species (*P. nivalis, P. hallii*). *P. nivalis* is genetically distinct from the other species.

The Neighbor-Joining tree also indicates that *P. totara* accessions are distinct from *P. hallii* (Figure 40B). *P. totara* is most similar to *P. acutifolius*, with *P. nivalis* being distinct from all other species. The reference *P. totara* (T199, T203) and *P. hallii* (H55, H160) are distinct but similar to accession of their representative morphotypes.

An interesting observation is that in these graphs, and also the other graphs, the reference accessions of *P. totara* (203 and 189) always show the closest relationship with *P. acutifolius.*

3.4.4.5 ALL TAXA

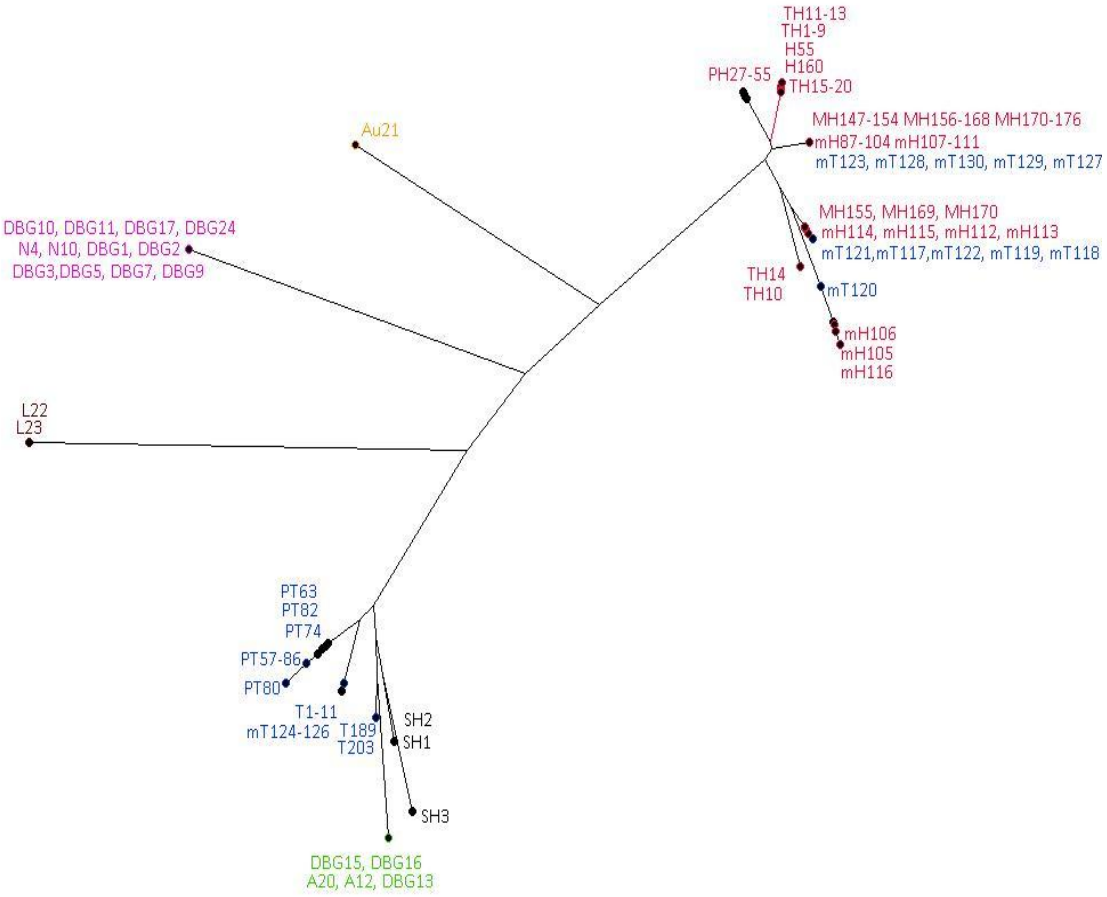


Figure 41: Neighbor-Joining tree for all accessions from Phase 4 screening including data from four transect sites (Pureora, Mamaku, Makatiti, Taranaki).

The Neighbor-Joining tree combining data from all sites (Figure 41) indicates strongly that *P. totara* accessions are typically distinct from *P. hallii* (red) accessions, with the exception of Mamaku accessions (# 117-123 and 127-130). *P. totara* (blue) is genetically most similar to *P. acutifolius* (green). Synthetic hybrids (black) associate most strongly with *P. acutifolius* accessions and the *P. totara* reference accessions (#189 and #203). *P. lawrencii* (brown), *P. nivalis* (pink), and *P. aurea* (gold) are distinct from all other species. *P. lawrencii* is genetically most similar to *P. nivalis* and *P. totara*. While *P. aurea* is most similar to *P. hallii* and *P. nivalis*. *P. hallii* displays the

most splits (genetic diversity) of all species. Similar results were obtained from the single site analyses of Pureora samples (#27-55), Taranaki (#1-20), Mamaku (#147-155) and Makatiti.

3.5 THE CHLOROPLAST GENOME

3.5.1 ASSEMBLY AND ANNOTATION OF THE TOTARA CHLOROPLAST GENOME

Chloroplast polymorphisms also provide a source of genetic variation for investigating and identifying source populations of totara seedlings and logged wood. Analyses were undertaken to investigate polymorphisms in the chloroplast genome among the mixed accessions previously sequenced. I also used Illumina reads from the mixed accessions to complete an assembly of contigs for the totara chloroplast genome. Contigs had already been assembled by Dr Oliver Deusch. However, he was unable to close gaps in the sequence. In the present work, primers were designed to sequence and close eight gaps between the chloroplast contigs, and successful PCR reactions occurred between the following primer pairs: TOT46F/TO9R, TOT9F/TOT15R, TOT15F/TOT68R, TOT68F/TOT26F, TOT22F/TOT26R, TOT4F/TOT22R, TOT17F/TOT4R, and TOT46R/TOT17R show in Figure 42.

Following gap closure, the *P. totara* complete chloroplast sequence length was found to be 133,259bp (Figure 43).The genome does not have inverted repeats characteristic of angiosperm taxa. Recent reports indicate that other conifers also lack an inverted repeat (e.g. Wu et al. 2011). Annotation of the genome showed it to be comprised of 81 genes, 4 rRNA (ribosomal RNA), and 35 tRNA (transfer RNA). The overall GC content across all contigs was found to be 37.2%.

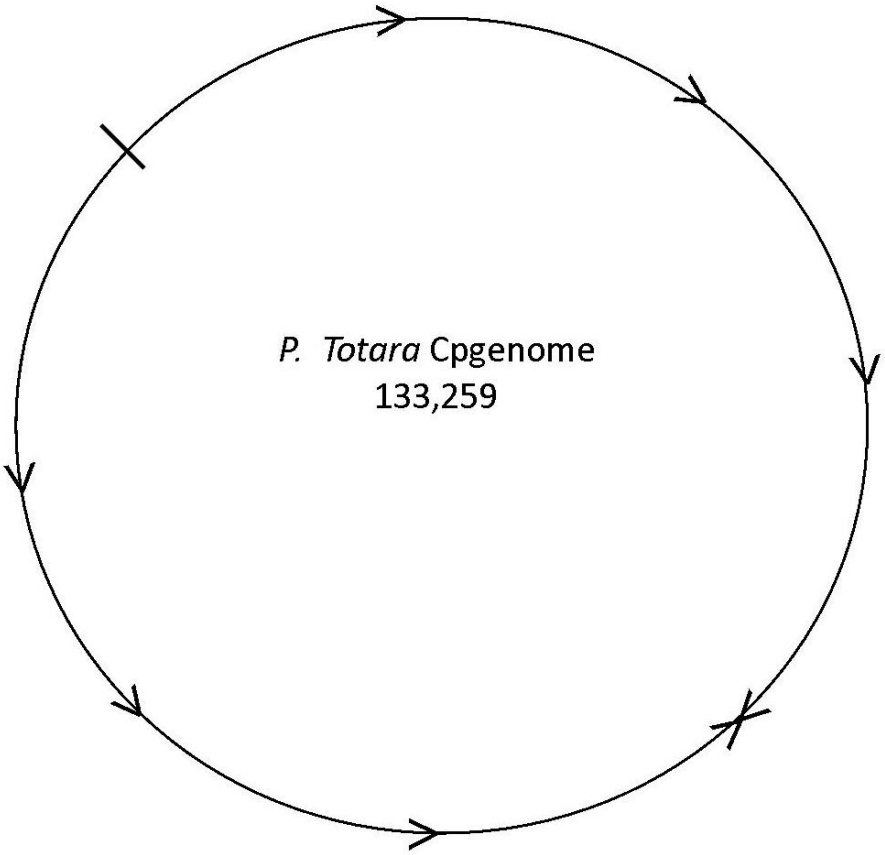


Figure 42: Chloroplast genome of *P. totara* with orientation and order of contigs deduced via PCR and Sanger sequencing.

3.5.2 IDENTIFYING POLYMORPHIC "HOTSPOT" REGIONS IN THE CHLOROPLAST GENOME

3.5.2.1 PREDICTION OF HOTSPOTS FROM LOCATION OF DIRECT REPEATS

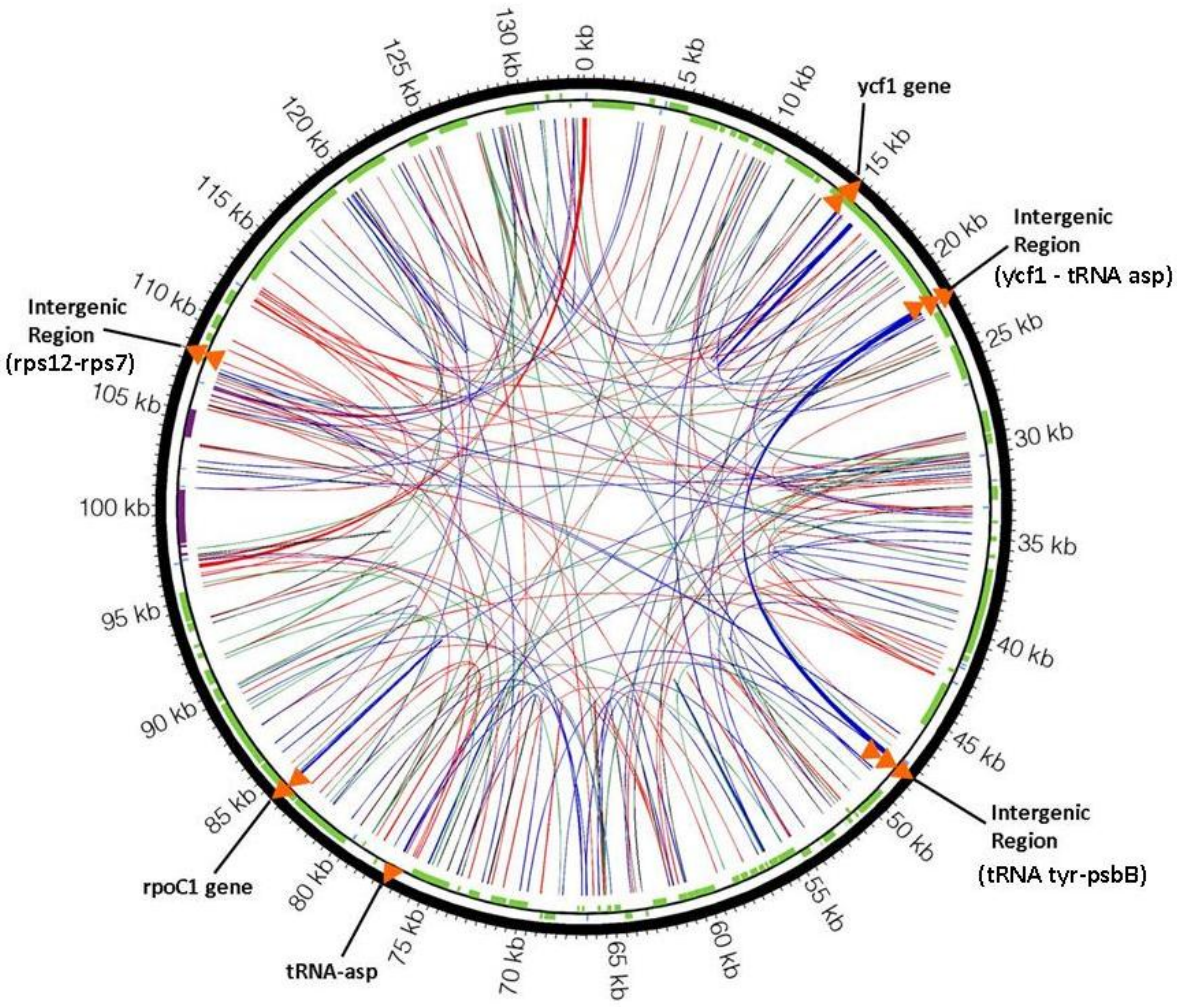


Figure 43: Circos plot of *P. totara* x *P. hallii*. The colours represent the 4 kinds of repeats red (palindromic), green (complement), blue (forward) and black (reverse) calculated by Reputer. Orange triangles locate SNPs discovered by mapping. Green blocks represent coding genes. Red blocks represent ribosomal genes.

Recent work by Ahmed et al. (2013) has suggested that there is a genome wide association between repeats, indels and substitutions in chloroplast genomes. I obtained a genome wide map for the location of repeats in the *P. totara* genome using a script written by Dr. Patrick Biggs. Lines on the graph connect the location of one oligonucleotide sequence in the genome with the location of its matching (repeat) sequence. Genome regions where repeats are most concentrated are predicted by Ahmed at al. (2013) to be regions where substitution hotspots are most likely to occur. For this figure, information has been plotted for repeats of length (15-275). The types of repeats considered are coloured red (palindromic), green (complement), blue (forward) and black (reverse). The repeats were identified using Reputer (Kurtz et al., 2001). This map is shown in Figure 43. Regions where repeats are concentrated include positions 22,000 blue (forward repeat) and 113,000 red (palindromic). To determine if there was evidence for these regions being hotspots for point mutations, the sequenced Illumina reads from cultivated plants at Massey were mapped to the assembled genome. The positions of putative SNPs were then marked on the Reputer map.

3.5.2.2 SNPS DISCOVERED BY VISUALISATION OF MAPPING

Shown on the chloroplast genome (Figure 43) are 13 putative SNPs identified in the chloroplast genome of 10 accessions of cultivated *P. totara.* Additionally, SNP details are given in Table 14. Figure 44 illustrates one of the putative SNPs visualised using Tablet. Some of the putative SNPs occur at regions, previously identified in Ahmed et al. (2013), where there appears to be a higher density of repeats. However, unlike the results for Aroid genomes (Ahmed et al., 2013) the repeats in *Podocarpus* are more evenly distributed across the chloroplast genome, making it visually more difficult to identify hotspot regions (compare Figure 43 and Figure 7 in Materials and Methods Chapter 3).

Table 14: Location of *P. totara* SNPs in putative hotspot regions of the chloroplast genome

Location	SNP	Annotation info
14,972	C→T	ycf1 gene
14,979	G→C	ycf1 gene
22,200	A→G	intergenic region (ycf1-tRNA asp)
22,201	G→A	intergenic region (ycf1-tRNA asp)
22,245	T→G	tRNA-Asp
47,851	A→G	intergenic region (tRNA tyr-psbB)
47,852	G→A	intergenic region(tRNA tyr-psbB)
47,896	T→G	intergenic region(tRNA tyr-psbB)
76,826	A→ G	tRNA-Asp
83,974	T→G	rpoC1 gene
83,985	C→A	rpoC1 gene
108,575	A→G	intergenic region (rps12-rps7)
108,577	T→G	intergenic region (rps12-rps7)

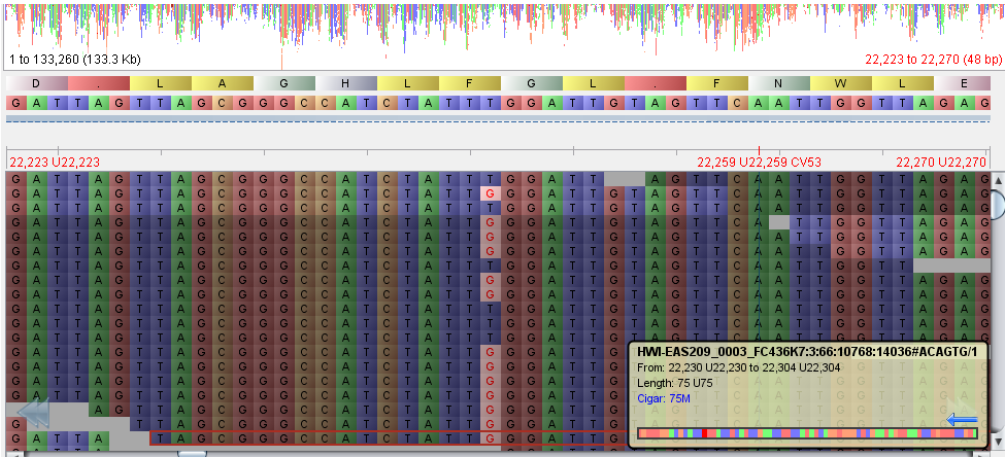


Figure 44: Example of a putative SNP in the chloroplast genome of *P. totara* visualised using Tablet. The SNP "G" is highlighted in pink and is positioned in the centre of the image. The text box shows the length of the read and coordinate details from the Illumina cell.

CHAPTER 4 DISCUSSION

4.1 CONSULTATION WITH MAORI

As a requirement of DOC, local iwi connected to sampling sites were consulted prior to sample collection. Prior to commencement of this project the WAI262 claim was still being argued in court and legal obligations regarding ownership of genetic data of indigenous flora were undetermined. In preparation for possible stringent requirements, extra effort was made to contact appropriate kaitiaki (guardians) prior to totara sampling, especially as totara is regarded as a taonga species by Māori. The collection of samples, for this project, are consistent with the practices of kaitiakitanga (or guardianship) where local iwi/hapu are recognised as guardians of forest species. Employing kaitiakitanga means samples gathered in this study will not be used for other applications besides what was described to stakeholders, and hence recognises that the manawhenua (or authority) is set with the iwi/hapu connected to site of collection. One requirement was stated upon consultation, where Tanenuiarangi-O-Manawatu requested that samples be destroyed after completion of study. Many research groups avoid consultation because consultation with Māori can seem difficult and time consuming. To assist others in future studies contact addresses of appropriate people found in this study have been included as a resource. Māori consultation carried out in this study proved helpful in a number of ways that have ensured success of the project. In particular, local knowledge was very helpful in identifying sample sites and phenotypic variants. Our efforts to consult more widely also revealed information that added to the validity of this study from a Māori – for example the impact of a dwindling extant resource for whakairo, the role that tools derived from Western Science could provide in addressing these impacts.

4.2 SAMPLE COLLECTION

Samples of plant tissue (young leaves) were collected to design DNA markers for differentiating the four New Zealand *Podocarpus* species *P. totara*, *P. hallii, P. nivalis, P. acutifolius*. Sample collection targets were 30 *P. totara* and 30 *P. hallii* for mountain transects with an exception being Mount Taranaki (20 *P. totara* and 20 *P. hallii*). This target was not reached for all sites for example: Mount Taranaki and Mount Mamaku had samples too close in range (less than 50m) hence 12 and 14 were only collected respectively. For example: Mount Makatiti no natural forests of *P. totara* species could be identified hence none were collected there. Ideally, samples should have been sampled across *a priori* identified putative hybrid zones for *P. totara*, and *P. hallii,* however disjunct patches of natural forest at most transect sites made recognition of such zones more difficult to identify than anticipated at the outset of the project. Despite sample numbers not being met, there were sufficient samples to test for evidence of introgression between species at the disjunct transect sites (118 *P. totara* from 31 sites, 119 *P. hallii* from six sites, eight *P. acutifolius* from two sites and 14 *P. nivalis* from 2 sites).

4.3 MOLECULAR MARKER DEVELOPMENT

4.3.1 NUCLEAR MARKERS

Previous to the commencement of this study Massey colleagues sequenced *P. totara x P. hallii* in a project to determine the sequence of the chloroplast genome. The extraction protocol for the GAII sequencing was enriched for chloroplast DNA, and while nuclear DNA comprised significant portions of the samples, relatively few regions showed homology to available conifer EST (Expressed Sequence Tag) libraries. Of the total 433,000 contigs of next-generation sequence data generated for 10 accessions of *P. totara* x *P. hallii* only 5,740 contigs were demonstrated to be of nuclear origin and suitable for SNP detection (based on our filtering methods described previously in

2.51). This meant that less than 1% of the reads were used for developing nuclear markers.

4.3.1.1 PRIMERS FROM PINUS RADIATA

Pinus radiata-derived primers provided an alternative approach for finding polymorphic gene regions in *P. totara*. The transferability of the primers was successful, in terms of amplification, but since these primers were chosen for regions of high intraspecific polymorphism this meant that they were unlikely to be ideal as markers for studying inter-species relationships. This proved to be the case, however such primers might be of use in future studies investigating intra-specific variation within *P. totara*.

4.3.1.2 GAIIX READS MAPPED TO CONIFER ESTS

In this thesis work, a Galaxy-based analytical pipeline (Baldwin et al., 2012) identified a larger number of putative SNPs (single nucleotide polymorphisms) for testing. Galaxy provided a user friendly online interface for designing HRM specific primers from high through put (e.g. Illumina) sequencing data that contained (SNPs). SNP calls were stored in a variant call file (VCF) where each line of the file represented a SNP and the information pertaining to it (Figure 36). This included: the position of SNP in the reference genome; allele in the reference genome and other alleles found; the filters not passed by the SNP; the genotypes found with its abundances; SNV (Single Nucleotide Variation) annotation, which is the frequency at which a particular change is observed at a given loci; VCF comparison; and statistics calculation for SNP calls. The SNP call data was based on the alignment of read data to contigs of interest (in this study these were for nuclear totara sequence that matched other conifer EST sequences). A range of bioinformatics scripts embedded in Galaxy was then used to design primers appropriate for HRM based on considerations for GC content, amplicon length, 3' and 5' annealing parameters. The resulting text file output from Galaxy included suggested primer pairs in tabular format

The successful implementation of Galaxy depends on parameters used to filter SNP calls. It is a very fast and reproducible method that can generate large sets of primers for screening. Its use removes subjective and human error, but relies heavily on the quality of data received. From the SNP calling parameters (Samtools, bcftools) we filtered data so that the most promising SNPs were identified. This method proved to be an effective method for designing DNA markers for this project.

Ten HRM markers were designed and eventually chosen using the Galaxy pipeline as being ones most useful for screening and distinguishing native *Podocarpus* species. However, of the 390 SNPs calls from the VCF input file, Galaxy was able to identify 336 primer pairs with a 93% amplification rate. Thus, the Galaxy pipeline proved to be a very successful tool in this study for marker development.

4.3.2 HIGH RESOLUTION MELTING (HRM) SCREENING AND ANALYSIS

4.3.2.1 SCREENING MARKERS - *PHASE 1-2*

Phase 1 screening was carried out on 132 primer pairs of which 123 showed successful amplification (93%). The 7% failure for PCR was attributed to poor PCR kinetics and in particular primers not annealing efficiently to template. This was most likely due to mismatch between the primers and template DNA. This might have resulted from sequencing error in the primer sequence due to uneven Illumina read coverage across contigs. In such instances, the consensus sequence inferred from the read data can sometimes be incorrect, and if so result in the misidentification of bases, and incorrect sequence determination for primers. These errors are common to all sequencing technologies but are greatly reduced in next-generation sequencing platforms (Metzker, 2010; Voelkerding, Dames, & Durtschi, 2009).

A significant proportion of samples from phase 1 displayed informative profiles. Fifty five primer pairs gave profiles that distinguished the four *Podocarpus* species, and 45 were carried through to phase 2 screening. In phase 2, 11 of these markers displayed differences that were too complex to interpret (Chapter 3 Results Figure 25). Primers (markers) were considered “successful” and good candidates for further screening if

there was a large relative fluorescence difference for the HRM melting curves between at least one or more *Podocarpus* species or if there were different melting peak curve shapes for these species. The 45 markers were located within a wide range of genes (Table 13, Results Chapter) which included genes of unidentified function (16/46), cysteine-rich receptor-like protein kinases (10/46), and Polymerase superfamily proteins (8/46).

In contrast to the PCR primer pairs designed using Galaxy, the primers transferred from radiata pine were not considered good candidates for subsequent phase screening as the HRM curves obtained using these primers were complex (Chapter 3 Results, Figure 25), suggesting high levels of intraspecific variation. This might be expected, since the pine primers were originally designed to identify differences in wood traits within *Pinus radiata* and thus they might also be detecting intra specific variation within the totara HRM data. This possibility would need to be further evaluated. Preliminary observations suggest that of the pine primers ATUB and UAE in particular might be most useful for studying intra-specific variation within *Podocarpus* species. Alternatively, one alternative explanation for the complex HRM profiles within the pine primers is that these are not locus specific in *Podocarpus* and that amplification of multiple sites has occurred, resulting in mixed allele signals.

Forty five markers met the criteria for phase 2 screening. These had a minimum relative signal difference of 3.0, between at least 2 groups of *Podocarpus* species. Analysis of melting curve differences was preferred over use of melting peak profiles as it was difficult to distinguish species for most markers using melting peak. However, if markers displayed a relative signal difference below three, but where there were distinct melting peak shapes then these HRM profiles were considered informative and their primers were carried through to next phase (Figure).

The 45 markers that were successful in phase 1 were screened on 16 new samples with phase 1 samples used as a positive control. Interspecific differences became less distinct when tested on more samples and as a result 45 markers were reduced to 15. The decrease in markers was due to signal differences being less pronounced. This is

possibly due to DNA quality between samples interfering with detection of SNP variation using specific primers (Figure 24). PCR reactions are sensitive to template quality and this is likely to affect robustness of HRM analyses.

A further consideration concerns the eight samples used as reference species. We assume that these 8 accessions (2 of each *Podocarpus* species: *P. totara*, *P. hallii*, *P. nivalis* and *P. acutifolius*) are true representatives of each *Podocarpus* species and this assumption is based on morphotyping. It remains possible that introgression and or incomplete lineage sorting (Joly et al., 2009; Yu et al., 2011) cause some alleles to be more similar to those of other species than to their expected species designations. More likely it was that the two accessions from each species were insufficient to eliminate intraspecific variation which would account for higher complexities observed when increasing sample size.

Screening has been used in many other studies as an efficient design strategy (Gady et al., 2009; Ganopoulos, Argiriou, & Tsaftaris, 2011; Jeong, Jo, Park, & Kang, 2010). In the present study, phase screening helped identify the most useful markers as it filtered out indistinctive HRM results. Phase 2 screening revealed that 15 markers were appropriate for screening a larger data set, of which five markers could differentiate all four species (T-H-A-N).

4.3.2.1 VALIDATION OF MARKERS - *PHASE 3-4*

The aim of the study was to develop DNA markers that could distinguish *P. totara* and *P. hallii*. To do this we needed to develop markers also capable of distinguishing other New Zealand *Podocarpus* species, including *P. acutifolius* and *P. nivalis*. Markers that passed screening phases 1 and 2 were put through validation phases 3 and 4 in an effort to test the efficiency of the markers on a wider set of samples. Firstly, the 16 successful markers from phase 2 were screened on a greater number of samples to detect whether or not the marker exhibited intraspecific variation. Phase 3 screening identified 13 of the 15 markers that identified clear interspecific differences.

The 13 successful markers from phase 3 were validated by screening transect samples from four locations. The four locations included Mount Taranaki, Mount Pureora, Mount Makatiti, and Mamaku Ranges. These sites were chosen because they are known to have natural totara stands. A transect of samples starting from *P. totara* at low altitude and ascending into *P. hallii* at higher altitudes was collected at all sites except for Makatiti (as no natural *P. totara* could be identified). The markers were tested to see if they could firstly, distinguish *P. totara* from *P. hallii*, and then secondly to see if there was any evidence of hybridisation among the transect samples. In addition, three synthetic hybrid samples (mixed DNA of *P. totara* and *P. hallii*) as well as representatives of *P. aurea* and *P. lawrencii* were tested.

Phase 4 validation screening found that New Zealand *Podocarpus* species could be discriminated into two or more groupings for ten markers (Table 7 Appendix). 94% (168/179) of the samples showed genotype assignments that matched phenotype assignments. When these markers were screened against synthetic hybrids (mixed DNA from *P. totara* and *P. hallii*), two markers (GA113, GA99) showed melting curves intermediate between curves of different species or species groups. While DNA from both species was expected to be amplified in these samples (synthetic hybrids), some markers preferentially amplified the gene regions from *P. totara.* This result might be explained by greater similarity of the PCR primers for the *P.totara* allele over the *P. hallii* allele and such phenomenon has been demonstrated in other studies (Kamiri et al., 2011; Maughan et al., 2011).

In screening transect samples, no hybrid melting curves were observed. However, at Mamaku the Neighbor-Joining tree and Neighbor-Net split graph indicate that some *P. totara* morphotypes had genotypes expected for *P. hallii*. This is an observation of interest given the bias observed with synthetic hybrids in preferentially detecting *P. totara*. This suggests that the *P. totara* alleles might have been lost from these accessions a result that might be explained by either incomplete lineage sorting or introgression.

GA90 was the only marker that consistently discriminated all four species in all phases of screening (1-4). GA90 distinguished synthetic hybrids from other samples. It also distinguished Mamaku *P. totara* accessions as being different from other *P. totara* samples. All other markers split species into 2-3 groupings with GA15 and GA58 the only markers that could not discriminate *P. totara* from *P. hallii*.

Conflicting results were obtained for some of the Mamaku samples (117-123; 127-130) meaning that some species designations differ from phenotypic assignments (highlighted red in Table 6 of Appendix). This finding is most evident from the Neighbor-Joining and splits graph for the Mamaku samples (Figure 38, Results Chapter) which show tight clustering of *P. totara* and *P. hallii* accession, where sequence genotypes are most similar to the members of each other's species. *P. totara* samples that group closely with *P. hallii* in these graphs occurred at high altitude (>500m). However, there were also *P. totara* morphological types (121-123) also found beside the road (~300m), which had *P. hallii* like genotypes.

Analyses of some of the HRM markers revealed interpopulation differences. For example: GA90 separated *P. totara* samples from Mamaku as distinct from other *P. totara* populations. GA84 separated *P. hallii* samples from Taranaki as distinct from other *P. hallii* accessions (Table 6 in Appendix). Thus, both markers provide a means to identify the source populations for these accessions.

The Australian endemic *P. lawrencii* exhibited unique HRM profiles for the ten markers. *P. lawrencii* (sample 22, 23) was represented by a distinct curve with three of the markers (GA90, GA113, GA54) and as distinct from the totara species, however it grouped with either *P. totara aurea* or the synthetic hybrids with three markers (GA15, GA84, GA78).

Most of the HRM markers screened in this study are dominant. We have assumed that if there are any hybrids present they would have been *P. totara* and *P. hallii* hybrids. HRM melting profiles clearly showed heterozygous melting curves in some instances, suggesting the presence of hybrids. However, the HRM method is limited because it does not provide information on alleic composition. The ten markers developed in this

study that collectively distinguished *P. totara* samples from *P. hallii* (Table 12) suggested various patterns of relationships between the New Zealand *Podocarpus* species. The strength and extent of conflict in respect of these relationships is indicated by the split graphs, since the degree of 'boxiness' of the graphs gives an indication of support for and against patterns of relationship suggested by the HRM profiles Only three markers demonstrated unique melting curves for the synthetic hybrid samples. The reason for synthetic hybrids not exhibiting an intermediate melting curve difference for all markers may be due to certain alleles being preferentially amplified. This has been demonstrated in various studies and is possibly due to higher similarity of PCR primers to specific alleles (Kamiri et al., 2011; Riginos, Sukhdeo, & Cunningham, 2002; Wehling, Hackauf, & Wricke, 1994).

The Neighbor Joining and Neighbour Net graphs obtained for the Mamaku site produced results for *P. totara* and *P. hallii* that are consistent with hybridization between these species at this site. However, incomplete lineage sorting of alleles is also a possible explanation for the graphs obtained. In general, distinguishing hybridisation and lineage sorting is known to be difficult. Sequencing of the HRM alleles and application of the statistical tests of Joly et al. (2009) and Gerard et al. (2011) would provide a means of obtaining definitive results. However, such work was beyond the scope of the present study.

4.3.3 EVALUATION OF MARKER DEVELOPMENT STRATEGY

Based on our screening results, an effective strategy for design primers involved identifying all SNPs in nuclear contigs and then choosing only SNPs that a) had coverage depth of at least ten reads b) had transversion base changes and c) were also of high mapping quality (read coverage across the length of the contig). For this study SNP call data was only generated for nuclear data that hit EST databases (*Pinus taeda, Picea glauca, Picea sitchensis, Cryptomeria japonica*). A limitation of this approach was that the non-coding regions, including regulatory regions of genes are excluded from the discovery of polymorphisms. Nuclear regions either too diverged or not represented in the EST libraries will also not be used.

In the present study a minimum read depth of seven was used and this meant some SNPs that looked to have potential were not examined. However, by using a greater read depth, there was greater confidence in the putative SNPs that were identified. Greater read depth with the read coverage score was expected to minimise primers mis-annealing to template and produce more successful PCR amplifications.

Transfer of markers from *Pinus* was not useful in this study. Such transfers have been shown to be successful in other studies, however these markers might still be useful in a study where intraspecific variation is being examined.

The marker development strategy employed in this study was not optimal for multi-species assay. The data were biased in terms of finding markers distinguishing only two of the four *Podocarpus* species (*P. totara* and *P. hallii*) as next-generation sequence data was derived from mixed accessions of the two species. This is reflected in the results where *P. totara* and *P. acutifolius* demonstrated same melting profiles for markers. The generation of sequence data for all four species would be most useful.

Certain details relating to the ten markers developed in this project are unknown. The gene origin is known for six of the ten markers, and only the gene family is known. The exact location in the genome is only unknown, as there is currently no reference *Podocarpus* genome sequence and *P. totara* is a non-model organism. The type of marker is not definite; HRM provides evidence for sequence difference but not what the exact difference is for example: SNP, insertion, deletion. Sequencing of the HRM loci would provide this information.

4.3.4 ANNOTATION OF NUCLEAR MOLECULAR MARKERS

The ten markers derived from this study are from a range of genes (Table 15). These genes include two cystein-rich receptor-like kinase 8, three DNA/RNA polymerase superfamily proteins, one polynucleotidyl transferase superfamily protein, and four of unknown origin. The exact gene origins are unknown as *P. totara* is a non-model organism and are from a range of genes of different functions.

Table 15: Description of gene origins for ten nuclear markers.

Marker Name	Gene Description
GA15	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8
GA48	CRK8 | cysteine-rich RLK (RECEPTOR-like protein kinase) 8
GA54	unknown
GA58	ORF158 | DNA/RNA polymerases superfamily protein
GA78	unknown
GA84	unknown
GA90	ORF158 | DNA/RNA polymerases superfamily protein
GA92	unknown
GA99	ORF120 | Polynucleotidyl transferase, ribonuclease H-like superfamily protein
GA113	ORF158 | DNA/RNA polymerases superfamily protein

4.4 THE CHLOROPLAST GENOME

4.4.1 ASSEMBLY AND ANNOTATION OF THE TOTARA CHLOROPLAST GENOME

Eight super contigs were constructed and aligned to the chloroplast genome of *Halocarpus kirkii* (unpublished Lockhart lab).The extent of mismatch was such that the complete circular molecule could not be closed; hence PCR methods were employed to close the gaps. The initial alignment of totara and *Halocarpus* chloroplast genomes allowed construction of 8 supercontigs (Figure 42) and by PCR and Sanger sequencing the orientation and order was deduced and gaps closed.

Reads were mapped to the totara chloroplast (cp) genome using Bowtie. SNP regions were visualised using Tablet *v.* 1.6.0_20. The alignment summary statistics indicated that of the 4,251,552 raw reads that were in the Illumina read file, only 2% aligned unambiguously to the cp genome. This low statistic value is most likely due to the large amount of nuclear and mitochondria DNA present in the sample prep. It is an indication of quantity and not necessarily quality of sequence data.

The *P. totara* genome has similar gene content (81 genes in total present and no inverted repeat) to *P. halocarpus* but differences in organisation (Figure 7 and Figure 43). Part of the genome is inverted and changed position including the genes such as ycf2, rps12 and rrn16. The chloroplast genome has an inverted repeat missing, a characteristic feature common to many conifers (Wu et al., 2011).

4.4.2 IDENTIFYING POLYMORPHIC "HOTSPOT" REGIONS IN THE CHLOROPLAST GENOME

As in Ahmed et al. (2013) the distribution of oligonucleotide repeats was calculated and displayed on the circular genome. The distribution of repeats appeared more randomly distributed and less concentrated than observed in *Colocasia esculenta* (and also in *Corynocarpus,* Lockhart per comm.), which makes it more difficult to *a priori* identify regions to target for future marker development. However, of interest is the observation that a number of gene regions containing repeats were also previously identified as hot spots for nucleotide variation in Ahmed et al. These include the ycf1 gene, and intergenic gene regions between ycf1-tRNA asp and tRNA try-psbB.

4.5 RECOMMENDATIONS FOR THE APPLICATION OF HRM MARKERS FOR TOTARA SPECIES IDENTIFICATION

This study has revealed that ten markers, which passed criteria for all 4 phases of screening, could differentiate all four totara species (*P. totara, P. hallii, P. nivalis, and P. acutifolius*). *P. totara* can be distinguished from *P. hallii* using the 8 markers: GA48, GA54, GA78, GA84, GA90, GA92, GA99, and GA113. *P. totara* can be distinguished from *P. acutifolius* with the markers: GA58 and GA90. *P. totara* can be distinguished from *P. nivalis* with all ten markers: GA15, GA48, GA54, GA58, GA78, GA84, GA90, GA92, GA99, and GA113 (Table 16).

These markers are expected to be of use to many groups including nursery growers, plant conservationists, iwi, and commercial native timber companies. The markers can be implemented for DNA diagnostics to help identify source populations of logged timber (such as might be investigated by Scion, Slipstream Automation Ltd, GenomeNZ or DNATURE Ltd, Gisborne, New Zealand) to mass assay juveniles of totara which have unknown species designation (seeds may indicate *P. totara* but species designation could be uncertain if collected from higher altitudes). The assay could be used by commercial companies to ensure juveniles grown for timber production are of *P. totara* origin and hence carry those specific timber characteristics. The use of this assay kit would also be of value for totara revegetation projects.

Similarly, nurseries growing such plants for either commercial or eco-restoration projects could use this assay to ensure confidence in the verification of species identification of seedlings for sale. It is of great interest to use a seedling of known provenance for eco-restoration projects. This assay will assist in projects for locations that have not been sampled in this study, and also ensure that genetically diverse populations of *P. totara* (example: Pureora forest seedlings) is used to replant forest across New Zealand.

An assay based on the markers developed also has the potential to identify whether a juvenile are of hybrid origin. For this purpose further validation (as discussed above) is first required using sequence data from the HRM markers. However, in principle

analyses to detect hybrids could be conducted in conjunction with altitude and seed morphology information. It is unknown as to whether *P. totara* and *P. hallii* hybrids have optimal timber characteristics but if this was to be studied in the future, this assay could be used in conjunction to find trees of optimal characteristics.

Table 16: Summary of what marker to use when differentiating *P. totara* from other species

		P. totara & *P. hallii*	*P. totara* &*P. acutifolius*	*P. totara* & *P. nivalis*	Hybrids of *P. totara* & *P. hallii*
GA15	TAH-N			√	
GA48	TA-H-N	√		√	
GA54	TA-H-N	√		√	
GA58	TH-AN		√	√	
GA78	TA-HN	√		√	
GA84	TA-H-N	√		√	
GA90	T-H-A-N	√	√	√	
GA92	TA-HN	√		√	
GA99	TA-H-N	√		√	√
GA113	TA-H-N	√		√	√

CHAPTER 5 CONCLUSION

This thesis project has identified ten HRM markers that can be used to screen New Zealand *Podocarpus* seed lots and when collectively screened can discriminate the four species *P. totara*, *P. hallii*, *P. nivalis* and *P. acutifolius*. Phylogenetic analyses using Neighbor Joining and Split Network indicated relationships suggesting *P. acutifolius* accessions were genetically most similar to *P. totara*. While *P. nivalis* was the most genetically distinct of all species. *P. hallii* and *P. totara* were genetically distinct and could be readily distinguished using the markers. However at the Mamaku site some accessions of these species (*P. totara* and *P. hallii*) clustered closely together. Since this finding occurred for numerous independent nuclear markers we suggest that hybridization between *P. totara* and *P. hallii* is the most likely explanation for this finding. Similar evidence for hybridisation was not observed at the other field sites.

The sequence of the *P. totara* chloroplast has been completed and annotated. Potential SNP variable regions have been identified via mapping, and repeat regions have been identified and illustrated in the form of a circos plot. These genome regions provide a further potential resource for obtaining molecular markers that could differentiate source populations of New Zealand *Podocarpus* species.

Consultation with iwi contributed to the success of the current project providing valuable information for contextualisation of the research as well as sampling and access through private land.

5.1 FUTURE WORK

Future work could involve sequencing of the HRM markers which would then allow for statistical analyses of putative hybrids using the tests of Joly et al. (2009) and Gerard et al. (2011). This would provide insight into the nature of the mismatch between phenotypic and genotypic assignments at the Mamaku site.

Of much interest would be implementation of the ten nuclear markers discovered in this project as an "Application kit" (methods, primer sequence, HRM conditions, and PCR conditions) that could be used for seedlings and tissue of commercial, historical and/or cultural interest. Development of this kit might include the development of additional chloroplast markers, using polymorphic regions identified in this study, and then screening on a larger set of accessions. Blocked probes could be utilised as an alternative method for SNP genotyping. The sequencing of homologous regions across all four New Zealand *Podocarpus* species (*P. totara*, *P. hallii*, *P. nivalis*, and *P. acutifolius*) would be ideal, and then inter-species SNPs could be detected and then screened.

To increase robustness of marker application kit accessions of New Zealand *Podocarpus* species might be further sampled. This could be from transects, as done in the present study, which consider patterns of morphological variation that potentially suggest hybrid zones. Other *Podocarpus* species could additionally be screened such as *P. totara* var. Waihoensis and the Australian *Podocarpus* species *P. gnidioides*, *P. nubigenus* and *P. smithii*.

Other work could involve optimising the DNA extraction to increase the throughput of a marker application kit. For this study DNA was extracted in 96 sample lots. Alternatively, mass screening of samples using a robotic unit, such as a Biomek® FXP Laboratory automation workstation, could be employed. An in-house protocol developed by colleagues at slipsteam Lab, Plant and Food Research, Palmerston North, New Zealand might be used to cheaply screen seeds and larger numbers of accessions, including those from nursery lots.

REFERENCES

Ahmed, I., Biggs, P. J., Matthews, P. J., Collins, L. J., Hendy, M. D., & Lockhart, P. J. (2013). Mutational Dynamics of Aroid Chloroplast Genomes. *Genome Biology and Evolution*, *4*(12), 1316–1323.

Allan, H. H. (1961). *Flora of New Zealand: Vol. I. Indigenous tracheophyta: psilopsida, lycopsida, filicopsida, gymnospermae, dicotyledons* (p. 1085). Wellington: Government Printer.

Arif, I., Bakir, M., Khan, H., Al Farhan, A. H., Al Homaidan, A., Bahkali, A. H., Sadoon, M. Al, et al. (2010). A brief review of molecular techniques to assess plant diversity. *International Journal of Molecular Sciences*, *11*(5), 2079–2096. doi:10.3390/ijms11052079

Baldwin, S., Roopashree Revanna, Thomson, S., Pither-Joyce, M., Wright, K., Crowhurst, R., Fiers, M., Chen, L., et al. (2012). Toolkit For Bulk PCR-Based Marker Design From Next-Generation Sequence Data: Application For Development Of A Framework Linkage Map In Bulb Onion (Allium cepa L.). *BMC Genomics*, *13*(1), 637.

Bennett, C. R., & Cambie, R. C. (1967). Chemistry of the podocarpaceae-XIII. Constituents of the heartwoods of Podocarpus nivalis hook and Podocarpus acutifolius kirk. *Phytochemistry*, *6*(6), 883–887.

Bergin, D. (2003). *Totara: Establishment, Growth, and Management*. Rotorua: N.Z. Forest Research.

Bergin, D., & Ecroyd, C. (1987). *Totara provenance trial - seed collection and measurement* (p. 19). Forest Research Institute Project Record No. 1751. (Unpubl.).

Bergin, D., & Kimberley, M. O. (1992). Provenance variation in Podocarpus totara. *New Zealand Journal of Ecology*, *16*(1), 5–13.

Best, E. (2005a). *Forest Lore of the Maori*. Wellington: Te Papa Press.

Best, E. (2005b). *Maori Storehouses and Kindred Structures*. Wellington: Te Papa Press.

Beveridge, A. E. (1964). Dispersal and destruction of seed in central North Island podocarp forests. *Proceedings of the New Zealand Ecological Society* (Vol. 11, pp. 48–55).

Biffin, E., Conran, J. G., & Lowe, A. J. (2011). Podocarp Evolution: A Molecular Phylogenetic Perspective. *Smithsonian Contributions to Botany*, (95), 1–20. doi:10.5479/si.0081024X.95.1

Biffin, E., Hill, R. S., & Lowe, A. J. (2010). Did Kauri (Agathis: Araucariaceae) really survive the Oligocene drowning of New Zealand? *Systematic Biology*, *59*(5), 594–602. doi:10.1093/sysbio/syq030

Billington, H. L. (2005). Effect of population size on genetic variation in a dioecious conifer. *Conservation Biology*, *5*(1), 115–119.

Bräutigam, a, & Gowik, U. (2010). What can next generation sequencing do for you? Next generation sequencing as a valuable tool in plant research. *Plant Biology*, *12*(6), 831–841.

Brown, G. R., Kadel, E. E., Bassoni, D. L., Kiehne, K. L., Temesgen, B., Van Buijtenen, J. P., Sewell, M. M., et al. (2001). Anchored reference loci in loblolly pine (Pinus taeda L.) for integrating pine genomics. *Genetics*, *159*(2), 799–809.

Cato, S. A., & Richardson, T. E. (1996). Inter-and intraspecific polymorphism at chloroplast SSR loci and the inheritance of plastids in Pinus radiata D. Don. *Theoretical and Applied Genetics*, *93*(4), 587–592.

Chagné, D., Chaumeil, P., Ramboer, A., Collada, C., Guevara, A., Cervera, M. T., Vendramin, G. G., et al. (2004). Cross-species transferability and mapping of genomic and cDNA SSRs in pines. *Theoretical and Applied Genetics*, *109*(6), 1204–1214.

Clifton, N. C. (1990). *New Zealand Timbers: The complete guide to exotic and indigenous woods* (p. 170). Wellington: GP Publications Ltd.

Collins. (1979). *New Zealand trees: conifers*. Auckland: New Zealand.

Conesa, A., Götz, S., García-Gómez, J. M., Terol, J., Talón, M., & Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics*, *21*(18), 3674–3676. doi:10.1093/bioinformatics/bti610

Cox, M. P., Peterson, D. A., & Biggs, P. J. (2010). SolexaQA□: At-a-glance quality assessment of Illumina second-generation sequencing data. *BMC Bioinformatics*, *11*(1), 485.

Cruzan, M. B. (1998). Genetic markers in plant evolutionary ecology. *Ecology*, *79*(2), 400–412.

Dang, X.-D., Kelleher, C. T., Howard-Williams, E., & Meade, C. V. (2012). Rapid identification of chloroplast haplotypes using High Resolution Melting analysis. *Molecular Ecology Resources*, *12*(5), 894–908. doi:10.1111/j.1755-0998.2012.03164.x

Davies, B. J., Brien, I. E. W. O., & Murray, B. G. (1997). Systematics and Evolution Karyotypes , chromosome bands and genome size variation in New Zealand endemic gymnosperms. *Plant Systematics and Evolution*, *208*, 169–185.

Drummond, A., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Duran, C., Field, M., et al. (2011). Geneious v5.5. Retrieved from www.geneious.com

Eagle, A. (2006). Eagle's Complete Trees and Shrubs of New Zealand. Wellington: Te Papa Press.

Eckert, A. J., Pande, B., Ersoz, E. S., Wright, M. H., Rashbrook, V. K., Nicolet, C. M., & Neale, D. B. (2008). High-throughput genotyping and mapping of single nucleotide polymorphisms in loblolly pine (Pinus taeda L.). *Tree Genetics & Genomes*, *5*(1), 225–234. doi:10.1007/s11295-008-0183-8

Erlbeck, H. H., & Waipara, M. O. te R. (1998). *Footsteps of the gods*. Auckland: Reed Publishing Ltd.

Everett, M. V., Grau, E. D., & Seeb, J. E. (2011). Short reads and nonmodel species: exploring the complexities of next-generation sequence assembly and SNP discovery in the absence of a reference genome. *Molecular Ecology Resources*, *11*, 93–108. doi:10.1111/j.1755-0998.2010.02969.x

Fleming, C. A. (1975). The geological history of New Zealand and its biota. (G. Kushel, Ed.)*Biogeography and Ecology in New Zealand*, 1–86.

Francis, L. (1956). *Maori musical instruments / [compiled by Lindo Francis and typing and drawings by G.W.M. Baker]*. 1956.

Gady, A. L., Hermans, F. W., Van de Wal, M. H., Van Loo, E. N., Visser, R. G., & Bachem, C. W. (2009). Implementation of two high through-put techniques in a novel application: detecting point mutations in large EMS mutated plant populations. *Plant Methods*, *5*, 13. doi:10.1186/1746-4811-5-13

Ganopoulos, I., Argiriou, A., & Tsaftaris, A. (2011). Microsatellite high resolution melting (SSR-HRM) analysis for authenticity testing of protected designation of origin (PDO) sweet cherry products. *Food Control*, *22*(3-4), 532–541. doi:10.1016/j.foodcont.2010.09.040

Gerard, D., Gibbs, H. L., & Kubatko, L. (2011). Estimating hybridization in the presence of coalescence using phylogenetic intraspecific sampling. *BMC evolutionary biology*, *11*(1), 291. doi:10.1186/1471-2148-11-291

Government, N. Z. (2012). Te Ara: The encyclopedia of New Zealand. *Te Ara*. Retrieved from http://www.teara.govt.nz/en/maori-creation-traditions

Grace, P., Grace, W., & Potton, G. (2006). *Earth, Sea, Sky: Images and Maōri Proverbs from the Natural World of Aotearoa New Zealand*. Huia Pub.

Hamlin, B. (1962). *Native Trees* (p. 64). Auckland: Reed.

Hamza, N. B. (2010). Cytoplasmic and nuclear DNA markers as powerful tools in populations ' studies and in setting conservation strategies. *Journal of Biotechnology*, *9*(29), 4510–4515.

Hawkins, B. J. (1988). Investigations of the Physiology and Genetics of the New Zealand Conifers: Rimu, Kahikatea and Totara: a Thesis Submitted in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in the University of Canterbury. University of Canterbury.

Hawkins, B. J., Sweet, G. B., Greer, D. H., & Bergin, D. O. (1991). Genetic variation in the frost hardiness of Podocarpus totara. *New Zealand Journal of Botany*, *29*(4), 455–458.

Hinds, H. V., & Reid, J. S. (1957). Forest trees and timbers of New Zealand. *New Zealand Forest Bulletin 12*.

Hohenlohe, P. a., Amish, S. J., Catchen, J. M., Allendorf, F. W., & Luikart, G. (2011). Next-generation RAD sequencing identifies thousands of SNPs for assessing hybridization between rainbow and westslope cutthroat trout. *Molecular Ecology Resources*, *11*, 117–122. doi:10.1111/j.1755-0998.2010.02967.x

Huson, D. H., & Bryant, D. (2006). Application of Phylogenetic Networks in Evolutionary Studies. *Molecular Biology in Evolution*, *2*, 256–267.

Imelfort, M., Duran, C., Batley, J., & Edwards, D. (2009, May). Discovering genetic polymorphisms in next-generation sequencing data. *Plant Biotechnology Journal*. doi:10.1111/j.1467-7652.2009.00406.x

Jaiswal, R., Beuria, T. K., Mohan, R., Mahajan, S. K., & Panda, D. (2007). Totarol Inhibits Bacterial Cytokinesis by Perturbing the Assembly Dynamics of. *Biochemistry*, *46*(14), 4211–4220.

Jeong, H.-J., Jo, Y. D., Park, S.-W., & Kang, B.-C. (2010). Identification of Capsicum species using SNP markers based on high resolution melting analysis. *Genome*, *53*(12), 1029–1040.

Joly, S., McLenachan, P. A., & Lockhart, P. J. (2009). A statistical approach for distinguishing hybridization and incomplete lineage sorting. *The American Naturalist*, *174*(2), E54–E70.

Kamiri, M., Stift, M., Srairi, I., Costantino, G., El Moussadik, A., Hmyene, A., Bakry, F., et al. (2011). Evidence for non-disomic inheritance in a Citrus interspecific tetraploid somatic hybrid between C. reticulata and C. limon using SSR markers and cytogenetic analysis. *Plant Cell Reports*, *30*(8), 1415–1425. doi:10.1007/s00299-011-1050-x

Khoshoo, T. N. (1960). *Chromosome Numbers in Gymnosperms* (pp. 1–9).

Kim, S., Lee, Y.-P., Lim, H., Ahn, Y., & Sung, S.-K. (2009). Identification of highly variable chloroplast sequences and development of cpDNA-based molecular markers that distinguish four cytoplasm types in radish (Raphanus sativus L.). *TAG. Theoretical and Applied Genetics.*, *119*(1), 189–98. doi:10.1007/s00122-009-1028-z

Kubo, I., Muroi, H., & Himejima, M. (1992). Antibacterial activity of totarol and its potentiation. *Journal of Natural Products*, *55*(10), 1436–40. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1453180

Kurtz, S., Choudhuri, J. V, Ohlebusch, E., Schleiermacher, C., Stoye, J., & Giegerich, R. (2001). REPuter: the manifold applications of repeat analysis on a genomic scale. *Nucleic Acids Research*, *29*(22), 4633–4642. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=92531&tool=pmcentrez&rendertype=abstract

Langmead, B., Trapnell, C., Pop, M., & Salzberg, S. L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology*, *10*(3).

Leslie, A. B., Beaulieu, J. M., Rai, H. S., Crane, P. R., Donoghue, M. J., & Mathews, S. (2012). Hemisphere-scale differences in conifer evolutionary dynamics. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(40), 16217–16221. doi:10.1073/pnas.1213621109

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., et al. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics (Oxford, England)*, *25*(16), 2078–2079. doi:10.1093/bioinformatics/btp352

Liao, P.-Y., & Lee, K. H. (2010). From SNPs to functional polymorphism: The insight into biotechnology applications. *Biochemical Engineering Journal*, *49*(2), 149–158. doi:10.1016/j.bej.2009.12.021

Lockhart, P. J., McLenachan, P. A., Havell, D., Glenny, D., Huson, D., & Jensen, U. (2001). Phylogeny, radiation, and transoceanic dispersal of New Zealand alpine buttercups: molecular evidence under split decomposition. *Annals of the Missouri Botanical Garden*, 458–477.

Matsui, T., Wilson, J. B., Zealand, N., West, C. J., & Conservancy, S. (2004). Can Podocarpus totara and P . hallii be distinguished by bark thickness?: A study on the southern coast of Southland / Otago , New Zealand. *New Zealand Journal of Botany*, *42*, 313–320.

Maughan, P., Smith, S., Fairbanks, D., & Jellen, E. (2011). Development, Characterization, and Linkage Mapping of Single Nucleotide Polymorphisms in the Grain Amaranths (Amaranthus sp.). *The Plant Genome Journal*, *4*(1), 92. doi:10.3835/plantgenome2010.12.0027

McConchie, D. (1999). Quality native timber from future managed stands. *Proceedings of Native Tree Forum*. Hamilton: Forest Research.

McCouch, S. R., Zhao, K., Wright, M., Tung, C.-W., Ebana, K., Thomson, M., Reynolds, A., et al. (2010). Development of genome-wide SNP assays for rice. *Breeding Science*, *60*(5), 524–535. doi:10.1270/jsbbs.60.524

McGlone, M. (1988). New Zealand. In H. B & W. T (Eds.), *Vegetation History* (11th ed., pp. 557–600). Klywer Academic Publishers.

McLenachan, P. A., Stöckler, K., Winkworth, R. C., McBreen, K., Zauner, S., & Lockhart, P. J. (2000). Markers derived from amplified fragment length polymorphism gels for plant ecology and evolution studies. *Molecular Ecology*, *9*(11), 1899–903. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11091325

Mead, H. M. (1995). *The art of Maori carving = Te Toi whakairo*. Auckland: Reed.

Mende Biotech Ltd. (2006). Totarol. Retrieved from http://www.totarol.com/

Metcalf, L. (2002). *Trees of New Zealand* (p. 128). Auckland: New Holland Publishers Ltd.

Metzker, M. L. (2010). Sequencing technologies - the next generation. *Nature Reviews. Genetics*, *11*(1), 31–46. doi:10.1038/nrg2626

Meudt, H. M., & Clarke, A. C. (2007). Almost forgotten or latest practice? AFLP applications, analyses and advances. *Trends in Plant Science*, *12*(3), 106–117. doi:10.1016/j.tplants.2007.02.001

Mildenhall, D. C. C., Hollis, C. J. J., & Naish, T. R. R. (2004). Orbitally-influenced vegetation record of the Mid-Pleistocene Climate Transition, offshore eastern New Zealand. *Marine Geology*, *205*(1-4), 87–111. doi:10.1016/S0025-3227(04)00019-2

Milne, I., Bayer, M., Cardle, L., Shaw, P., Stephen, G., Wright, F., & Marshall, D. (2010). Tablet--next generation sequence assembly visualization. *Bioinformatics*, *26*(3), 401–402. doi:10.1093/bioinformatics/btp666

Mohi, C. (1993). *Matauranga Maori - A national resource: A paper prepared for the Ministry of Resesearch, Science and Technology* (pp. 13–26). Wellington: NZ.

Naresh, V., Yamini, K. N., Rajendrakumar, P., & Dinesh Kumar, V. (2009). EST-SSR marker-based assay for the genetic purity assessment of safflower hybrids. *Euphytica*, *170*(3), 347–353. doi:10.1007/s10681-009-9995-3

New Zealand Government. (1965). Te Ao Hou. *Te Ao Hou: The Maori Magazine*. Retrieved from http://teaohou.natlib.govt.nz/journals/teaohou/issue/Mao52TeA/c5.html

Nybom, H. (2004). Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. *Molecular Ecology*, *13*(5), 1143–55. doi:10.1111/j.1365-294X.2004.02141.x

NZ Plant Conservation network. (2012). New Zealand Plant Conservation Network. Retrieved from http://www.nzpcn.org.nz/flora_search.aspx?scfSubmit=1&scfLatin_Common_Name=totara

NZ Wood. (2010). Totara. NZ Wood. Retrieved from http://www.nzwood.co.nz/species/totara

Ogden, J., Stewart, G. H., & Allen, R. B. (1996). Ecology of New Zealand Nothofagus forests. *The ecology and biogeography of Nothofagus forests. Yale University Press, New Haven and London*, 25–82.

Patel, R. N. (1967). Wood anatomy of Podocarpaceae indigenous to New Zealand. *New Zealand Journal of Botany*, *5*, 307–321.

Phillips, W. J. (1997). Maori carving illustrated. Auckland: Reed Books.

Phillips, W. J., & Wadmore, J. (1950). *The great carved house, Mataatua of Whakatane*. Wellington: Polynesian Society.

Red Seal Natural Health. (2010). Taradent Tootpaste. Retrieved from http://www.redseal.co.nz/

Riginos, C., Sukhdeo, K., & Cunningham, C. W. (2002). Letter to the Editor Evidence for Selection at Multiple Allozyme Loci Across a Mussel Hybrid Zone. *Molecular Biology and Evolution*, *19*(3), 347–351.

Riley, M., & Enting, B. (1994). *Māori healing and herbal: New Zealand ethnobotanical sourcebook / Murdoch Riley ; photographs by Brian Enting* (p. 528). Paraparaumu, N.Z.: Viking Sevenseas.

Rouleau, E., Lefol, C., Bourdon, V., Coulet, F., Noguchi, T., Soubrier, F., Bièche, I., et al. (2009). Quantitative PCR high-resolution melting (qPCR-HRM) curve analysis, a new approach to simultaneously screen point mutations and large rearrangements: application to MLH1 germline mutations in Lynch syndrome. *Human Mutation*, *30*(6), 867–75. doi:10.1002/humu.20947

Rozen, S., & Skaletsky, H. (1999). Primer3 on the WWW for General Users and for Biologist Programmers. *Bioinformatics Methods and Protocols* (Vol. 132, pp. 365–389). Human Press.

Salmon, J. T. (1998). *Native Trees of New Zealand*. Auckland: Reeds.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences*, *74*(12), 5463–5467.

Seeb, J. E., Carvalho, G., Hauser, L., Naish, K., Roberts, S., & Seeb, L. W. (2011). Single-nucleotide polymorphism (SNP) discovery and applications of SNP genotyping in nonmodel organisms. *Molecular Ecology Resources*, *11*, 1–8. doi:10.1111/j.1755-0998.2010.02979.x

Shaw, J., Lickey, E. B., Schilling, E. E., & Small, R. L. (2007). Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. *American Journal of Botany*, *94*(3), 275–288.

Sudheer Pamidiamarri, D. V. N., Pandya, N., Reddy, M. P., & Radhakrishnan, T. (2009). Comparative study of interspecific genetic divergence and phylogenic analysis of genus Jatropha by RAPD and AFLP: genetic divergence and phylogenic analysis of genus Jatropha. *Molecular Biology Reports*, *36*(5), 901–7. doi:10.1007/s11033-008-9261-0

Tanes Tree Trust. (2010). Native trees for the future. Retrieved from http://www.tanestrees.org.nz/indes.html

Taylor, C. F. (2009). Mutation scanning using high-resolution melting. *Biochemical Society Transactions*, *37*(Pt 2), 433–7. doi:10.1042/BST0370433

Te Rito, J. (2007). Whakapapa and whenua: An insider ’s view. *MAI Review*, *3*, 1–8.

Telfer, E., Graham, N., Stanbra, L., Manley, T., & Wilcox, P. (2013). Extraction of Pine Genomic DNA for use in a High-Throughput Genotyping Platform. *New Zealand Journal of Forestry Science*.

Townrow, J. A. (1967). On Rissikia and Mataia podocarpaceous conifers from the Lower Mesozoic of southern landstle. *Proceedings of Royal Society of Tasmania*, *101*, 103–136.

Voelkerding, K. V, Dames, S. a, & Durtschi, J. D. (2009). Next-generation sequencing: from basic research to diagnostics. *Clinical Chemistry*, *55*(4), 641–58. doi:10.1373/clinchem.2008.112789

Vossen, R. H. a M., Aten, E., Roos, A., & Den Dunnen, J. T. (2009). High-resolution melting analysis (HRMA): more than just sequence variant screening. *Human Mutation*, *30*(6), 860–866. doi:10.1002/humu.21019

Walker, R. (2008). *Tohunga whakairoa: Paki Harrison: The story of master carver*. North Shore: Penguin.

Wardle, P. (1963). Growth habits of New Zealand subalpine shrubs and trees. *New Zealand Journal of Botany*, *1*, 18–47.

Wardle, P. (1972). Podocarpus totara var. waihoensis var. nov.: the Result of Introgressive Hybridisation between P. totara and P. acutifolius. *New Zealand Journal of Botany*, *10*, 195–201.

Webby, R. F., Markham, K. R., & Molloy, B. P. J. (1987). The characterisation of New Zealand Podocarpus hybrids using flavonoid markers. *New Zealand Journal of Botany*, *25*, 355–366.

Wehling, P., Hackauf, B., & Wricke, G. (1994). Identification of S-locus linked PCR fragments in rye (Secale cereale L.) by denaturing gradient gel electrophoresis. *The Plant Journal: For Cell and Molecular Biology*, *5*(6), 891–893. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8054994

Williams, P. M. E. (1996). *Te Rongoa Maori: Maori Medicine*. Auckland: Reed.

Wilson, V. R., & Owens, J. N. (1999a). The reproductive biology of Totara (Podocarpus totara) (Podocarpaceae). *Annals of Botany*, *83*, 401–411.

Wilson, V. R., & Owens, J. N. (1999b). Cytoplasmic Inheritance in Podocarpus totara (Podocarpaceae). *IV International Conifer Conference 615* (pp. 171–172).

Winitana, C., & Reisinger, A. (2010). *Legends of Aotearoa*. Auckland, N.Z.: HarperCollins.

Wu, C.-S., Lin, C.-P., Hsu, C.-Y., Wang, R.-J., & Chaw, S.-M. (2011). Comparative chloroplast genomes of pinaceae: insights into the mechanism of diversified genomic organizations. *Genome biology and evolution*, *3*, 309–319. doi:10.1093/gbe/evr026

Wu, C.-S., Wang, Y.-N., Hsu, C.-Y., Lin, C.-P., & Chaw, S.-M. (2011). Loss of different inverted repeat copies from the chloroplast genomes of Pinaceae and cupressophytes and influence of heterotachy on the evaluation of gymnosperm phylogeny. *Genome Biology and Evolution*, *3*, 1284–1295. doi:10.1093/gbe/evr095

Wyman, S. K., Jansen, R. K., & Boore, J. L. (2004). Automatic annotation of organellar genomes with DOGMA. *Bioinformatics*, *20*(17), 3252–3255.

Yu, Y., Than, C., Degnan, J. H., & Nakhleh, L. (2011). Coalescent histories on phylogenetic networks and detection of hybridization despite incomplete lineage sorting. *Systematic Biology*, *60*(2), 138-149.

Zeng, Y.-F., Liao, W.-J., Petit, R. J., & Zhang, D.-Y. (2010). Exploring species limits in two closely related Chinese oaks. *PloS one*, *5*(11), e15529. doi:10.1371/journal.pone.0015529

Zerbino, D. R., & Birney, E. (2008). Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Research*, *18*(5), 821–9. doi:10.1101/gr.074492.107

APPENDIX

TABLE 1 NUCLEAR PRIMERS

Primer name	Forward sequence	Reverse sequence
T101	GTAACCCTGAAACCCACCA	CATGGACGAAAAAGGATACGA
T102	TCGGTCAAGCTCGTATCCTT	GGGCATATTTGGGTGGATTT
T103	CTGCGAAAACCACAAAATCC	GGCGAGCAAAAATTGGTATG
T104	AGAAGCCAATGCCAAGGTC	CCAGGAATTTGTGTCCTCCA
T105	CGTTTATCGGTGGCTTCTTT	GCAGATAGCTTAGGTGCAGGA
T106	CTTGGATGTGGTAGCCGTTT	GACGGGTGACGGAGAATTAG
T107	AAGAAGCCACCGATAAACGA	TTCGCCATGCAATAGTTGTG
T108	GCAAAAAGGTGCAAACATCA	CCCAATTAAGCAGGCTAGGG
T109	CATCAGGGTGGATGTTAGGG	GACAGACCGAGCCCATTTT
T110	GGGAGGCAAATCGTGTTACT	GGATGAAGGAGCAAGGTCAA
T111	TCGTTTATCGGTGGCTTCTT	TGATCGCCATGAGCAGTTAG
T112	GCAGCCCATTGTCTTGATTT	CATGCTTTGCCTTCTCAACA
T213	CTGCGAAAACCACAAAATCC	GGCGAGCAAAAATTGGTATG
T214	GTTCGAGGAAACCCCAAAAC	ATACGAGCTTGACCGAGTGG
T215	GGCTCATCCCAATTTTTGTC	TTCATGCTTCGTTCAAGTGG
T216	CCTGGTTGTCCCATTTTTGT	CCTGGTTGTCCCATTTTTGT
T217	AGCAAGCACAAATGGATCTTC	TTCGCAACCACTTCCCATAC
T218	AGCTATGACGCCACTCCAAC	CAACACCAACTCGACCAACA
T219	GTTTCCGTTGTTGGTCGAGT	CATTGGATCTTCCTGCTTGG
T220	TTGATGGAGCAGACAGAGAGA	ATCGACACACAATGCACAGG
T220	GGCATTGTGAAGAACGGAGT	GTTTGGTATTTGCCCTCCAA
T221	ACTCGACCAACAACGGAAAC	AGCTATGACGCCACTCCAAC
T223	CTTGGAAAGGGCTAGAAGCA	TTACCTGCCCACATCTCCTC
T224	ATGGTGGGGAATTTTGCAGT	CATGCTTCTAGCCCTTTCCA
T225	ATTGGAATGTTGGCATTGTG	TGCCTCTTGATTACTTGTGCTT
T226	ACTCGACCAACAACGGAAAC	AGCTATGACGCCACTCCAAC
T227	TGGGAGAGATTGATTGAAGTTG	TAGCCTGATGGAGCGTGTGA
T228	TCGTTTATCGGTGGCTTCTT	TGATCGCCATGAGCAGTTAG
T229	GACACAAATTCCTGGCCTTC	TCTCCCTGCATCAAATCAAA
T230	TCAGCCGATAGACCTTGCTT	CTTAGCCATAGCAGCCCAGT
T231	GCCTTCCACATTTCTTTTGG	CAAGGATTGCATTGGGGTAA
T232	GCCCATTGTCTTGATTTGCT	CACATGCTTTGCCTTCTCAA
T233	TGTTTGCCTCAAGAATAAGGTC	AACAGGGAGAGGTGTGGAAA
T234	AAATTCTCCTCCCAGGTCCA	GGTGCATTGTGTTGTGGTTT
T235	TTTTCCCCCTTGTTTGTTTG	CACTTATTGGGCATCCTGGT
T236	GTGGGGCCTAGACTTTGTTG	AATAATCTGTGGCCGTTTGG

TABLE 2 PINE PRIMERS

Scion	Sequence
1-AGP	not available
2-DHN	not available
3-SAM	not available
4-Ces4	not available
5-Btub	not available
6-C4H	not available
7-Laccase	not available
8-Atub	not available
9-Ces7	not available
10-Ces8	not available
11-CAD	not available
12-UAE	not available

TABLE 3 GALAXY PRIMERS

Primer Name	Forward sequence	Reverse Sequence
GA1	GTGTTAGATTTGGGATCAAAAGG	CGGTTCTTCCTTTCAGCAAC
GA2	TGGACTATGGCCTATGGTATCC	TACACATCCTGCCCAATCTG
GA3	TCTTGAAAAAGGGCATGGTC	TGGAGAGGTACCGCTTGATT
GA4	TGAATCCCACTTTGTATAAAAGCAT	GCATACATTATATCTGGGCGTGT
GA5	GGAATGCTAAAAATAGTTTCTGTGAA	TTGACCCAGTCTTAGATTTCATTTC
GA6	CTGCCACATATTCAGCTTCAGT	TGGATCAGGTTTGATATCATGG
GA7	TGTTCGAACTATCCTCAGATTCA	TCAAAAGGAAGAACAGTAAAAGAAA
GA8	GGATCCTCTCCCAACTGAAA	GGCAAAATTAAGGAAGAAGTCTACA
GA9	TCCATTTGATATACTTTGAAATTTTTG	GCTCCAGTAGCAAGACTAGAAGC
GA10	TGTATCAAGCCTGGAGTACCAT	TGCAAACTGAAGAAGGCTCT
GA11	CCCAAAGGAAGTACATTGGAGA	TCCATTGGAGTGGAAAGAGG
GA12	CAACCTTTGTGGTAAGGAGTTT	CCACATCTTTCTTTGGAAGC
GA13	TTTGTTGACTGGGATGATTCAA	TATCATCACCACCAGGCTCA
GA14	GCTTTCAATCTACATTCATGTTCAA	AACAAGTGATAAAAGTGACAAACAATG
GA15	TTTCATCATTTGTTTCATCCACA	ATTATCCACCGCAGAAGCAG
GA16	CCTGAAGACTCCTCAAGGTTTT	CGAGGTCGATCCTAAGAAAGTC
GA17	GACAGTCCAAATGGCATCCT	TTCAAGACTGCTTTTACCACCA
GA18	CCACAAGTCTTGCCTTGTACC	GGGCACTTACTAATAAATGGGTGT
GA19	TGATGCACGATGTAGTTGAGG	AAACATCCCAATGTTGCTCTC
GA20	TACTCCCCCTGAGATTGTGC	ACGAAAGAAATTGGGAGTGGT
GA21	ACACCTTTGCAAGGAGGATG	GAGAAACATAAGACTACCAACAATGC

GA22	TTTCCCAAAGCACATCCTTC	CATCTCAAGCAATGGTATGTGG
GA23	TCTTTGAATCTTGTTTCAATAGGTG	TCACTCATGTGTCCAAGCCTA
GA24	TCCAATCATTGACCTGTACTGAG	CTATGACGACAGGCTGCAAA
GA25	TGCTTCTTCAATAGCAGACACCT	AGATCATGTCATCGTGAAAAAGG
GA26	CATGCTTTCCCATGATCAAA	CCCAAAATCTGTTTGTGAAGC
GA27	CAAGGCAAGATCCAGGCTAT	CAAGAGCTCGTTAAACGGATG
GA28	CATGGTTCACTCTTTGCATCC	CAGAGTTTTTGGGTGCTTGG
GA29	AGAAGAATCGGGTCTGCTTG	CCAAGTCTCTTATGCCATAGCC
GA30	CAAGGCAAGATCCAGGCTAT	CAAGAGCTCGTTAAACGGATG
GA31	ATTCGAGGCCACATTCTCAG	GCATGAATTTGAATTAGGAGCAG
GA32	ACTGGAAATCCTCCTCGAATTA	TCCGGATCAAATTTTTATTGGT
GA33	TTCAAAGAGAAAAGCATACAGGTG	CATCAACACGAACATCAGCA
GA34	TCACATTTTTGTTGGCTGGT	GCAATGGAGGAAGAGCTCAC
GA35	CAACAACTTCTCCCTATGCAA	GGAAGAACATGTTGTACATCTTAGAC
GA36	CTTCGGCAGTTGGGAGTTTA	CCACTGCTTGTGCTATATCAGG
GA37	TAGGCAATCCTCCCACAAAA	TGGGTTTGTATCAGCCACTTC
GA38	TTATGTGCCTTGGGATTCG	GGAGTAGCAGGGGCTGTATATC
GA39	AGCGGTACCTCTCCACTCCT	CGCCGAAAGGTAGAGCTG
GA40	TACAGGAGGAGGTTCGAGCA	GTGCATCTGCTGCTGCTTTA
GA41	TTGTTGATATTAGATGACTCATGGTG	GCTCAAAATAGGCTCGGTGA
GA42	ACTCCTGAACTTCTCTTCCAACT	TTTTTGTTGGTTGACTCTGCT
GA43	ACATTTGCTTTGCCAGGTTT	TGGAAGAATTGTGAGGTGGTT
GA44	TGGAGCATGTATTTTCTCCAAA	TGGATGTCCTGATCCTTAATTCT
GA45	TTTGGCTGACTCTTCAAATTTTAGT	AAGGAGAACATGATGGAGCTG
GA46	CGCAATGGAGAGAACCATTC	GGCATTGACTATGAGGAGACCT
GA47	TGCATTATTGCAACACTGAGG	GCTTTACAAATTGACGTGTAGCA
GA48	GGGGAAGGATTTCACACTCAT	CGCCACTAGTGCTTTTCCTG
GA49	CAAAGCCTTTAAAAATCAAGTAGAAA	TGAACTCACCTCCTCTGTCTG
GA50	CACCAGTGGAGGAGCATTTT	TGATAAGGATACTGAGTCTTGCTTTTT
GA51	GGCACAGTAAAAAGCAAGACTC	CTGTGTGCAGCAAGAAGTTG
GA52	CATGGGAATTCTTGTCATGTG	TTGGAGAACTGGTCAATAAAAGC
GA53	TTTTAAAAATAGCTAGAGCAAGGAA	TGACTTTGAAGAAACCTTTGC
GA54	TGCTGCTGAAAACTGTCTCC	AGAGGAGGTGAATTCATTTCAGA
GA55	CGTGCATCATGTCATGGAAA	GCCTTTTGGCCTTAAGAACG
GA56	GACATCTCATCAAATGGTACATGG	AAGCACACCCTCCACACACT
GA57	GAGCAAGAATGAGCCCACA	TCAAATCATCAACATAAAGAGAAACA
GA58	TGGAAACCCACTGAGGGTAG	GATCAAGGCCGAGATTCAGA
GA59	TTGGTGAACAACAGAATGATCC	TGGATTGTATGCCAACTGGA
GA60	CCCATGGTCGTGTAGGAACT	TGCAGTGTAAGCAAACCCAAT
GA61	TTGATGGATGTCCATACCTTCTT	CTTTGCAACCTTTACCAGCA
GA62	TTGGAACATTCCAGAAAAATCA	TGACGAGGTAAACAAGAAGTTCC
GA63	ATGCGGTATGAATGCTGGTA	GGCTCTGGAGACTTCTAAGCA
GA64	TTTAAATCTTTCGCTTCTTCTATGG	GGTATTACGCTCACTTCCTCAAA
GA65	GCTTTAAACCTTGTAATACCTCCAA	GCCGTTCAACACAAAACAAA

GA66	TTGGCCTTAAGAATGCAGGT	TCATGCATCATATCATGGAAAAC
GA67	ATATTCCACCACCGCTCATC	CCACCCTGATGAAGATAACGA
GA68	TGATGCTCTCATCAAGAATGG	ATCCAATCGGTTTGGTTCC
GA69	TCATGCTTCTTGCCATTTCA	ACCTCTCCCTACACACCACAA
GA70	GGATGCCAAAATCCAGTGTT	GCTCCTTCCGAGATCCATTT
GA71	CAGGTCAATGATTGGAAGTTTG	TCGAGCAACCATACATACTGCT
GA72	TCTCAGTCGCACTCTGGTTG	GCAAAGACCACTGCGAGAC
GA73	TCCCCCTTCATCAAAAATTG	AAGCTTACAAGTTCTATAATCCCAAGA
GA74	TGGAGTACCATGCTCGAGGT	AGAGGATCCTAATCTGGTATGCAA
GA75	TGATTCTTGATCATCTTTATCAGTTTT	TGTGGTGGTGTTGGTCATTT
GA76	GGCATACACTTCTACTTTTTGATGC	TTATCACGTGCCTCAAGGTG
GA77	CCAATAACAGTACAAAAGCCAAAA	CTTCTGCTCCATCCGATTGT
GA78	GAATTGGCAGCCATAGTTCA	ACAATAGGTCGACCCAGGAG
GA79	TCTCTGTCATCATAGTTCTAGCCATC	CAAAATGGGGTTGTGGAAAG
GA80	TGAGAGGCAAACCATGAACA	TATGGCACCTTCGATTTGGT
GA81	GGGGCATATCATATCCGAAG	AGGAACAGACCAATTCTGCAT
GA82	TTGTCGAATGAGCACAAGAGA	TGGTTCTTCGAAATGTGCTCT
GA83	GGCACAAAATCCAGTCCATC	CTTCTCCACCATGTCTCTTATGAA
GA84	GCCACTGCTTTGTTGAGGAA	ATCCATCCCTGGCATATCCT
GA85	AAAGGAATGCAATGGAGCAT	TGTCATTTTGTTTTTCTTCATTCAG
GA86	CACCAGTGGAGGAGCATTTT	TGATAAGGATACTGAGTCTTGCTTTTT
GA87	AAGTTTCCAAGAGGGGAAGC	GTTTGCATAGGCCCACAAAG
GA88	GTGCATCGCAATGCTAGTTT	TTTGCACAAATAGCAAATCTCAA
GA89	GCAATGGAGGAAGAGCTCAC	CATTTTTGTTGTCTGGTCTAGGAA
GA90	CCTGAAGACTCCTCAAGGTTTT	CGAGGTCGATCCTAAGAAAGTC
GA91	TCTCTGTCATCATAGTTCTAGCCATC	CAAAATGGGGTTGTGGAAAG
GA92	CAGTATGGATGAAATGCATGG	TCCTTTTCTGAGGGTTTTGC
GA93	AACCTGTGAGCACCCCTATG	TTGGTGCTTTATCATCCTTGC
GA94	AGCGGTACCTCTCCACTCCT	GTCAGTCGCCGAAAGGTAGA
GA95	GATATACAGCCCCTGCTACTCC	CGCATTTCTTGCTTTAGAATCAT
GA96	CCACAAGTCTTGCCTTGTACC	GGGCACTTACTAATAAATGGGTGT
GA97	CTGCCCTCATCCACTAGACC	CCAAGCCTGACAGCATAACC
GA98	GGAAGCAAAACTCCAAACACA	GCATGTATGCTTCGATTGTCTC
GA99	GGTGTAAATTGCAGTACTCACAGC	TGGCTAGATGTATGTTGGGAAA
GA100	GCTATTACCGATATGCCTCCTC	GACGGATAGCCTGGATCTTG
GA101	GCAGCTAAAGGTTCCAAATTCA	TGACAAAATTAGTTTCTTCAGGATCA
GA102	CCAATTGTTCAAGAAATAAATTTTCA	CCAGTTGTTAAGATGACCTCCA
GA103	CTCCCTCTTTGTCTTCTTCCTCT	GCTTTTGCATCCAAAGAAGG
GA104	GAGAGAATCCTAATCTGGTATGCAA	TGGAGTACCATGCTCGAGGT
GA105	AACACTCAGGTTCACCACTATCA	AAGGACCAGAAAACCTCCTG
GA106	ATCTTCGCAAGAAGCTAGGC	CTCCCCCTGAGAATGTGAAG
GA107	AGGAAAATCAGCTAATTGTTGTTG	CTCATTCTTGCTTCCTCCAAA
GA108	CTTTTGATGCCATTTTGTGTG	GAGGTGGAGAGTATGCTGCTG
GA109	AAATGAGGATTTGGAGGAACAA	AGCCCAGTTATTTCCTCTTCAA

GA110	CCAATTGTTCAAGAAATAAATTTTCA	CCAGTTGTTAAGATGACCTCCA
GA111	CAACAACTTCTCCCTATGCAA	GGAAGAACATGTTGTACATCTTAGAC
GA112	TTGGTCAGAAAATTGTTTGCAT	GCTTGTCATTGTTGTCTATGTAGATG
GA113	AGGTGTCATGTTAGCCCAAAA	TCCACGAGTGTCCGACTAAG
GA114	TGATGCACGATGTAGTTGAGG	AAACATCCCAATGTTGCTCTC
GA115	CAAAATAGATGTATGATGAGACCACA	GCCTCACTGTAGCTTTTCTTCC
GA116	CGTGTTGATGATGAGGATGC	GAAAGGCTATTTTAAAGAGTTTCCAA
GA117	AGAGATTACTATCCACCATACCTTTTT	CCCCGAGCATGGTACTCTA
GA118	TGGCAGCTGAAAGTTGTCTT	CAGAGGAGGAGAGTTCATTTCTG
GA119	GCTTTCAATCTACATTCATGTTCAA	AACAAGTGATAAAAGTGACAAACAATG
GA120	GGAAATTGGAGAGGAGCAAA	GGGTTTTGTGCCAACTTGAT

TABLE 4 SAMPLES USED FOR SCREENING

Phase 1	Sample #	Species	Sample Origin	Tally
	189	*P. totara*	Kaikoura	1
	203	*P. totara*	Opotiki	2
	55	*P. hallii*	Pureora	3
	160	*P. hallii*	Makatiti	4
	12	*P. acutifolius*	DBG	5
	20	*P. acutifolius*	DBG	6
	4	*P. nivalis*	DBG	7
	10	*P. nivalis*	DBG	8
			Total	**8**
Phase 2	**Sample #**	**Species**	**Sample Origin**	**Tally**
	183	*P. totara*	New Plymouth	1
	213	*P. totara*	Kauaeranga	2
	211	*P. totara*	Pohangia	3
	187	*P. totara*	Banks Peninsula	4
	56	*P. hallii*	Pureora	5
	90	*P. hallii*	Mamaku	6
	92	*P. hallii*	Mamaku	7
	163	*P. hallii*	Makatiti	8
	11	*P. acutifolius*	Rotorua	9
	13	*P. acutifolius*	Rotorua	10
	15	*P. acutifolius*	DBG	11
	16	*P. acutifolius*	DBG	12
	1	*P. nivalis*	Rotorua	13
	25	*P. nivalis*	Rotorua	14

	17	*P. nivalis*	DBG	15
	26	*P. nivalis*	DBG	16
	Phase1	2x each species (positive control)	Various	8
			Total	**24**
Phase 3	**Sample #**	**Species**	**Sample origin**	**Tally**
	51	*P. hallii*	Pureora	1
	52	*P. hallii*	Pureora	2
	53	*P. hallii*	Pureora	3
	54	*P. hallii*	Pureora	4
	87	*P. hallii*	Mamaku	5
	88	*P. hallii*	Mamaku	6
	89	*P. hallii*	Mamaku	7
	90	*P. hallii*	Mamaku	8
	173	*P. hallii*	Makatiti	9
	174	*P. hallii*	Makatiti	10
	175	*P. hallii*	Makatiti	11
	176	*P. hallii*	Makatiti	12
	8	*P. hallii*	Rotorua	13
	19	*P. hallii*	Rotorua	14
	14	*P. hallii*	Rotorua	15
	18	*P. hallii*	Rotorua	16
	TH1	*P. hallii*	Taranaki	17
	TH2	*P. hallii*	Taranaki	18
	TH3	*P. hallii*	Taranaki	19
	131	*P. totara*	Kaikohe	20
	134	*P. totara*	Urewera	21
	137	*P. totara*	Raglan	22
	140	*P. totara*	Herbert	23
	142	*P. totara*	Catlins	24
	178	*P. totara*	Featherston	25
	180	*P. totara*	Kaikoura good	26
	182	*P. totara*	Harihari	27
	184	*P. totara*	New Plymouth	28
	186	*P. totara*	Whangamomona	29
	188	*P. totara*	Banks peninsula	30
	190	*P. totara*	Kaikoura poor form	31
	192	*P. totara*	Peel Forest	32
	194	*P. totara*	Pelorus River	33
	196	*P. totara*	Otaki	34
	198	*P. totara*	Gisborne	35
	200	*P. totara*	Masterton	36
	202	*P. totara*	Dean Forest	37
	204	*P. totara*	Opotiki	38
	206	*P. totara*	Nelson Wai-iti	39

	208	*P. totara*	Taumarunui	40
	210	*P. totara*	Kaikohe	41
	212	*P. totara*	Pohangia	42
	214	*P. totara*	Kauaeranga	43
	216	*P. totara*	Mamaku	44
	218	*P. totara*	Hawkes Bay	45
	220	*P. totara*	Hunterville	46
	222	*P. totara*	Hurukia	47
	119	*P. totara*	Mamaku	48
	1	*P. nivalis*	DBG	49
	2	*P. nivalis*	DBG	50
	3	*P. nivalis*	DBG	51
	4	*P. nivalis*	DBG	52
	5	*P. nivalis*	DBG	53
	17	*P. nivalis*	DBG	54
	7	*P. nivalis*	DBG	55
	9	*P. nivalis*	DBG	56
	10	*P. nivalis*	DBG	57
	11	*P. acutifolius*	DBG	58
	13	*P. acutifolius*	DBG	59
	15	*P. acutifolius*	DBG	60
	16	*P. acutifolius*	DBG	61
	189/55	Synthetic hybrid *P. totara* & *P. hallii*	Kaikoura/Pureora	62
	203/160	Synthetic hybrid *P. totara* & *P. hallii*	Opotiki/Makatiti	63
	211/163	Synthetic hybrid *P. totara* & *P. hallii*	Pohangia/Makatiti	64
	Phase1	2x each species (positive control)	Various	8
			Total	**72**
Phase 4	**Sample #**	**Species**	**Sample origin**	**Tally**
	27-56	*P. hallii*	Pureora	30
	57-86	*P. totara*	Pureora	30
	87-116	*P. hallii*	Mamaku	30
	117-130	*P. totara*	Mamaku	14
	147-176	*P. hallii*	Makatiti	29
	T1-T11	*P. totara*	Taranaki	11
	TH1-TH20	*P. hallii*	Taranaki	20
	21	*P. aurea* 'golden totara'	DBG	1
	22	*P. lawrencii* 'Purple king'	DBG	1
	23	*P. lawrencii* 'Purple king'	DBG	1
	24	*P. nivalis* 'Bronze'	DBG	1
	185/54	Synthetic hybrid *P. totara* &*P. hallii*	Whangamoana/Pureora	1
	217/170	Synthetic hybrid *P. totara* & *P. hallii*	Hawkes Bay/Makatiti	1
	181/TH2	Synthetic hybrid *P. totara* & *P. hallii*	Harihari/Taranaki	1
	Phase1	2x each species (positive control)	Various	8
			Total	**179**

TABLE 5 SAMPLE DETAILS

Location	Sample No.	Species	Gender	DBH (cm)	Height (m)	Latitude	Longitude	Altitude (m)
Scion	1	N	n/a	20	2	5771280 N	1886025 E	280
Scion	2	A	n/a	30	5	5771280 N	1886025 E	280
Scion	3	H_1	n/a	25	3	5771280 N	1886025 E	280
Scion	4	A	n/a	15	2	5771280 N	1886025 E	280
Scion	5	H_1	n/a	40	10	5771280 N	1886025 E	280
Scion	6	H	n/a	30	8	5771280 N	1886025 E	280
Scion	7	T	n/a	28	8	5771280 N	1886025 E	280
Scion	8	H_1	n/a	18	2	5771280 N	1886025 E	280
Scion	9	H_1	n/a	23	3	5771280 N	1886025 E	280
Scion	10	H	n/a	42	10	5771280 N	1886025 E	280
Scion	11	H_1	n/a	21	3	5771280 N	1886025 E	280
Scion	12	H_1	n/a	25	7	5771280 N	1886025 E	280
Scion	13	H_1	n/a	28	5	5771280 N	1886025 E	280
Scion	14	H_1	n/a	28	5	5771280 N	1886025 E	280
Scion	15	H_1	n/a	21	4	5771280 N	1886025 E	280
Scion	16	H_1	n/a	20	3	5771280 N	1886025 E	280
Scion	17	H_1	n/a	19	3	5771280 N	1886025 E	280
Scion	18	H_1	n/a	17	3	5771280 N	1886025 E	280
Scion	19	H_1	n/a	16	3	5771280 N	1886025 E	280

Scion	20	H	n/a	24	7	5771280 N	1886025 E	280
Scion	21	H	n/a	24	7	5771280 N	1886025 E	280
Scion	22	H	n/a	30	10	5771280 N	1886025 E	280
Scion	23	T	n/a	40	12	5771280 N	1886025 E	280
Scion	24	T	n/a	12	2	5771280 N	1886025 E	280
Scion	25	N	n/a	10	2	5771280 N	1886025 E	280
Scion	26	A	n/a	12	3	5771280 N	1886025 E	280
Pureora	27	H	n/a	33.	10.5	5731356 N	1830559 E	788
Pureora	28	H	n/a	5	4.8	n/a	n/a	n/a
Pureora	29	H	n/a	12.7	7.5	n/a	n/a	n/a
Pureora	30	H	n/a	4.4	6.6	n/a	n/a	n/a
Pureora	31	H	n/a	11.2	11.3	5731017 N	1830235 E	804
Pureora	32	H	n/a	6.9	7.4	5731018 N	1830127 E	856
Pureora	33	H	M	9.9	6.9	5731012 N	1830084 E	832
Pureora	34	H	M	60	18	n/a	n/a	n/a
Pureora	35	H	F	84	20	5731011 N	1829986 E	807
Pureora	36	H	n/a	42.4	16	n/a	n/a	n/a
Pureora	37	H	n/a	46.1	14	n/a	n/a	n/a
Pureora	38	H	n/a	43	11.5	n/a	n/a	n/a
Pureora	39	H	F	76	1	5731044 N	1829793 E	782
Pureora	40	H	F	48.2	15.5	n/a	n/a	n/a
Pureora	41	H	n/a	54	18	n/a	n/a	n/a
Pureora	42	H	F	51.9	19	n/a	n/a	n/a
Pureora	43	H	F	68	20	n/a	n/a	n/a
Pureora	44	H	n/a	72.5	16.5	n/a	n/a	n/a

Pureora	45	H	F	60.	15.5	5730648 N	1829436 E	769
Pureora	46	H	n/a	58.5	16.5	n/a	n/a	n/a
Pureora	47	H	F	66	12	5730617 N	1829 44.4 E	746
Pureora	48	H	F	26.3	10	n/a	n/a	n/a
Pureora	49	H	n/a	65	15	n/a	n/a	n/a
Pureora	50	H	F	32	12.5	5730413 N	1829408 E	759
Pureora	51	H	n/a	23.1	8	n/a	n/a	n/a
Pureora	52	H	M	35	10.75	n/a	n/a	n/a
Pureora	53	H	M	27	8	5730307 N	1829357E	1019
Pureora	54	H	n/a	72	10.6	5730303 N	1829331 E	1046
Pureora	55	H	F	11.2	4.9	n/a	n/a	n/a
Pureora	56	T	M	37.4	7.3	5730170 N	1829297 E	1022
Pureora	57	T	n/a	10.8	4	5734098 N	1822958 E	559
Pureora	58	T	M	39.6	10	5734083 N	1822936 E	554
Pureora	59	T	F	60	10	5734081 N	1822929 E	556
Pureora	60	T	n/a	48.8	8	5734159 N	1823184 E	559
Pureora	61	T	n/a	120	10	5734192 N	1823211 E	556
Pureora	62	T	n/a	54	8	n/a	n/a	n/a
Pureora	63	T	n/a	50	8	5734231 N	1823269 E	559
Pureora	64	T	n/a	70	12	5734247 N	1823305 E	556
Pureora	65	T	n/a	62	8	5734289 N	18 23 36.0 E	555
Pureora	66	T	M	49.8	8	5734311 N	1823476 E	561
Pureora	67	T	n/a	71	11	5734374 N	1823429 E	568
Pureora	68	T	F	29.5	6	5734418 N	1823411 E	567
Pureora	69	T	n/a	150	12	5734373 N	1823383 E	560

Pureora	70	T	n/a	200	25	5734165 N	1823300 E	588
Pureora	71	T	F	200	30	5734163 N	1823421 E	579
Pureora	72	T	F	150	35	5734145 N	1823513 E	577
Pureora	73	T	n/a	100	18	5734102 N	1823653 E	576
Pureora	74	T	n/a	120	30	5734048 N	1823780 E	578
Pureora	75	T	M	120	30	5733920 N	1824043 E	584
Pureora	76	T	n/a	150	35	5733895 N	1824208 E	575
Pureora	77	T	n/a	110	30	5733948 N	1824069 E	577
Pureora	78	T	n/a	20	6	5733829 N	1824424 E	593
Pureora	79	T	n/a	100	25	5733819 N	1824523 E	587
Pureora	80	T	n/a	100	30	5733621 N	1825218 E	595
Pureora	81	T	n/a	100	35	5733462 N	1825308	596
Pureora	82	T	n/a	100	30	5733323 N	1825414E	614
Pureora	83	T	n/a	200	30	5733290 N	1825437 E	575
Pureora	84	T	n/a	50	8	5733172 N	1825602 E	597
Pureora	85	T	n/a	30	7	5732958 N	1825621 E	617
Pureora	86	T	n/a	200	32	5732926 N	1825650 E	622
Mamaku	87	H	n/a	50	8	5775242 N	1869859 E	617
Mamaku	88	H	n/a	1	5	578120 N	1866831 E	557
Mamaku	89	H	n/a	20	5	5780961 N	1866676 E	554
Mamaku	90	H	n/a	n/a	7	5781020 N	1866648 E	553
Mamaku	91	H	n/a	n/a	7	n/a	n/a	n/a
Mamaku	92	H	n/a	30	6	5781114 N	1866568 E	550
Mamaku	93	H	n/a	30	6	n/a	n/a	n/a
Mamaku	94	H	n/a	30	5	N5781314 N	1866336 E	546

Mamaku	95	H	n/a	50	8	N5781520 N	1866262 E	548
Mamaku	96	H	n/a	50	8	n/a	n/a	n/a
Mamaku	97	H	n/a	50	8	n/a	n/a	n/a
Mamaku	98	H	n/a	n/a	7	5786446 N	1862756 E	529
Mamaku	99	H	n/a	n/a	6	5786452 N	1862520 E	521
Mamaku	100	H	n/a	n/a	10	5787248 N	1860110 E	473
Mamaku	101	H	n/a	n/a	n/a	n/a	n/a	n/a
Mamaku	102	H	n/a	n/a	n/a	5787365N	1859962 E	472
Mamaku	103	H	n/a	n/a	n/a	n/a	n/a	472
Mamaku	104	H	n/a	n/a	n/a	n/a	n/a	472
Mamaku	105	H	n/a	n/a	n/a	n/a	n/a	472
Mamaku	106	H	n/a	n/a	n/a	n/a	n/a	472
Mamaku	107	H	n/a	n/a	n/a	5787645 N	1859540 E	466
Mamaku	108	H	n/a	n/a	n/a	n/a	n/a	466
Mamaku	109	H	n/a	n/a	n/a	n/a	n/a	466
Mamaku	110	H	n/a	n/a	n/a	n/a	n/a	466
Mamaku	111	H	n/a	n/a	n/a	5787835 N	1859432 E	461
Mamaku	112	H	n/a	n/a	n/a	n/a	n/a	461
Mamaku	113	H	n/a	n/a	n/a	n/a	n/a	461
Mamaku	114	H	n/a	n/a	n/a	n/a	n/a	461m
Mamaku	115	H	n/a			5788073 N	1859205 E	441m
Mamaku	116	H	n/a	n/a	n/a	n/a	n/a	
Mamaku	117	T	n/a	n/a	n/a	5786453 N	1862321 E	521m
Mamaku	118	T	n/a	n/a	n/a	5786467 N	1862271 E	506m
Mamaku	119	T	n/a	n/a	n/a	5787365 N	1859962 E	472m

Mamaku	120	T	n/a	n/a	n/a	5788073 N	1859205 E	441m
Mamaku	121	T	n/a	n/a	n/a	5790320 N	1854419 E	283m
Mamaku	122	T	n/a	n/a	n/a	5790320 N	1854419E	283m
Mamaku	123	T	n/a	n/a	n/a	5790320 N	1854419 E	283m
Mamaku	124	T	n/a	n/a	n/a	n/a	n/a	283m
Mamaku	125	T	n/a	n/a	n/a	n/a	n/a	283m
Mamaku	126	T	n/a	n/a	n/a	n/a	n/a	283m
Mamaku	127	T	n/a	n/a	n/a	5785389 N	1868115E	283m
Mamaku	128	T	n/a	n/a	n/a	5785389 N	1868115E	283m
Mamaku	129	T	n/a	n/a	n/a	5785389 N	1868115E	283m
Mamaku	130	T	n/a	n/a	n/a	5785389 N	1868115E	283m
Kaikohe	131	T	n/a	n/a	n/a	n/a	n/a	n/a
Kaikohe	132	T	n/a	n/a	n/a	n/a	n/a	n/a
Kaikohe	133	T	n/a	n/a	n/a	n/a	n/a	n/a
Urewera	134	T	n/a	n/a	n/a	n/a	n/a	n/a
Urewera	135	T	n/a	n/a	n/a	n/a	n/a	n/a
Urewera	136	T	n/a	n/a	n/a	n/a	n/a	n/a
Raglan	137	T	n/a	n/a	n/a	n/a	n/a	n/a
Raglan	138	T	n/a	n/a	n/a	n/a	n/a	n/a
Raglan	139	T	n/a	n/a	n/a	n/a	n/a	n/a
Herbert	140	T	n/a	n/a	n/a	n/a	n/a	n/a
Herbert	141	T	n/a	n/a	n/a	n/a	n/a	n/a
Catlins	142	T	n/a	n/a	n/a	n/a	n/a	n/a
Catlins	143	T	n/a	n/a	n/a	n/a	n/a	n/a
Catlins	144	T	n/a	n/a	n/a	n/a	n/a	n/a

Makatiti	147	H	n/a	20	3.5	5774266 N	1904952 E	846
Makatiti	148	H	n/a	10	4	n/a	n/a	n/a
Makatiti	149	H	n/a	11	3.5	n/a	n/a	n/a
Makatiti	150	H	n/a	12	4	n/a	n/a	n/a
Makatiti	151	H	n/a	10	3.5	n/a	n/a	n/a
Makatiti	152	H	n/a	8	2	n/a	n/a	n/a
Makatiti	153	H	n/a	7	1.8	n/a	n/a	n/a
Makatiti	154	H	n/a	n/a	n/a	n/a	n/a	n/a
Makatiti	155	H	n/a	n/a	n/a	n/a	n/a	n/a
Makatiti	156	H	n/a	n/a	n/a	n/a	n/a	n/a
Makatiti	157	H	n/a	n/a	n/a	n/a	n/a	n/a
Makatiti	158	H	n/a	n/a	n/a	5774192 N	1904974 E	850
Makatiti	159	H	n/a	n/a	n/a	n/a	n/a	n/a
Makatiti	160	H	n/a	n/a	n/a	n/a	n/a	n/a
Makatiti	161	H	n/a	n/a	n/a	n/a	n/a	n/a
Makatiti	162	H	n/a	n/a	n/a	n/a	n/a	n/a
Makatiti	163	H	n/a	n/a	n/a	n/a	n/a	n/a
Makatiti	164	H	n/a	n/a	n/a	n/a	n/a	n/a
Makatiti	165	H	n/a	n/a	n/a	5774195 N	1904861 E	855
Makatiti	166	H	n/a	n/a	n/a	n/a	n/a	n/a
Makatiti	167	H	n/a	n/a	n/a	n/a	n/a	n/a
Makatiti	168	H	n/a	n/a	n/a	5774124 N	1904765E	861
Makatiti	169	H	n/a	n/a	n/a	n/a	n/a	n/a
Makatiti	170	H	n/a	n/a	n/a	n/a	n/a	n/a
Makatiti	171	H	n/a	n/a	n/a	n/a	n/a	n/a

Makatiti	172	H	n/a	n/a	n/a	n/a	n/a	n/a
Makatiti	173	H	n/a	n/a	n/a	n/a	n/a	n/a
Makatiti	174	H	n/a	n/a	n/a	n/a	n/a	n/a
Makatiti	175	H	n/a	n/a	n/a	n/a	n/a	n/a
Makatiti	176	H	n/a	n/a	n/a	N5774038	E1903996	896m
Feather.	177	T	n/a	19-22	10-11	n/a	n/a	n/a
Feather.	178	T	n/a	19-22	10-11	n/a	n/a	n/a
Kaikoura	179	T	n/a	19-22	10-11	n/a	n/a	n/a
Kaikoura	180	T	n/a	19-22	10-11	n/a	n/a	n/a
Harihari	181	T	n/a	19-22	10-11	n/a	n/a	n/a
Harihari	182	T	n/a	19-22	10-11	n/a	n/a	n/a
New Ply.	183	T	n/a	19-22	10-11	n/a	n/a	n/a
New Ply.	184	T	n/a	19-22	10-11	n/a	n/a	n/a
Whanga.	185	T	n/a	19-22	10-11	n/a	n/a	n/a
Whanga.	186	T	n/a	19-22	10-11	n/a	n/a	n/a
Banks p.	187	T	n/a	19-22	10-11	n/a	n/a	n/a
Banks p.	188	T	n/a	19-22	10-11	n/a	n/a	n/a
Kaikoura	189	T	n/a	19-22	10-11	n/a	n/a	n/a
Kaikoura	190	T	n/a	19-22	10-11	n/a	n/a	n/a
Peel Forest	191	T	n/a	19-22	10-11	n/a	n/a	n/a
Peel Forest	192	T	n/a	19-22	10-11	n/a	n/a	n/a
Pelorus R.	193	T	n/a	19-22	10-11	n/a	n/a	n/a
Pelorus R.	194	T	n/a	19-22	10-11	n/a	n/a	n/a
Otaki	195	T	n/a	19-22	10-11	n/a	n/a	n/a
Otaki	196	T	n/a	19-22	10-11	n/a	n/a	n/a

Gisborne	197	T	n/a	19-22	10-11	n/a	n/a	n/a
Gisborne	198	T	n/a	19-22	10-11	n/a	n/a	n/a
Masterton	199	T	n/a	19-22	10-11	n/a	n/a	n/a
Masterton	200	T	n/a	19-22	10-11	n/a	n/a	n/a
Dean F.	201	T	n/a	19-22	10-11	n/a	n/a	n/a
Dean F.	202	T	n/a	19-22	10-11	n/a	n/a	n/a
Opotiki	203	T	n/a	19-22	10-11	n/a	n/a	n/a
Opotiki	204	T	n/a	19-22	10-11	n/a	n/a	n/a
Nelson Wai	205	T	n/a	19-22	10-11	n/a	n/a	n/a
Nelson Wai	206	T	n/a	19-22	10-11	n/a	n/a	n/a
Tauma.	207	T	n/a	19-22	10-11	n/a	n/a	n/a
Tauma.	208	T	n/a	19-22	10-11	n/a	n/a	n/a
Kaikohe	209	T	n/a	19-22	10-11	n/a	n/a	n/a
Kaikohe	210	T	n/a	19-22	10-11	n/a	n/a	n/a
Pohangina	211	T	n/a	19-22	10-11	n/a	n/a	n/a
Pohangina	212	T	n/a	19-22	10-11	n/a	n/a	n/a
Kaua.	213	T	n/a	19-22	10-11	n/a	n/a	n/a
Kaua.	214	T	n/a	19-22	10-11	n/a	n/a	n/a
Mamaku	215	T	n/a	19-22	10-11	n/a	n/a	n/a
Mamaku	216	T	n/a	19-22	10-11	n/a	n/a	n/a
Hawkes B.	217	T	n/a	19-22	10-11	n/a	n/a	n/a
Hawkes B.	218	T	n/a	19-22	10-11	n/a	n/a	n/a
Hunterville	219	T	n/a	19-22	10-11	n/a	n/a	n/a
Hunterville	220	T	n/a	19-22	10-11	n/a	n/a	n/a
Hurakia	221	T	n/a	19-22	10-11	n/a	n/a	n/a

Hurakia	222	T	n/a	19-22	10-11	n/a	n/a	n/a
DBG	1	N	n/a	n/a	n/a	n/a	n/a	n/a
DBG	2	N	n/a	n/a	n/a	n/a	n/a	n/a
DBG	3	N	n/a	n/a	n/a	n/a	n/a	n/a
DBG	4	N	n/a	n/a	n/a	n/a	n/a	n/a
DBG	5	N	n/a	n/a	n/a	n/a	n/a	n/a
DBG	6	H	n/a	n/a	n/a	n/a	n/a	n/a
DBG	7	N	n/a	n/a	n/a	n/a	n/a	n/a
DBG	8	H	n/a	n/a	n/a	n/a	n/a	n/a
DBG	9	N	n/a	n/a	n/a	n/a	n/a	n/a
DBG	10	N	n/a	n/a	n/a	n/a	n/a	n/a
DBG	11	A	n/a	n/a	n/a	n/a	n/a	n/a
DBG	12	A	n/a	n/a	n/a	n/a	n/a	n/a
DBG	13	A	n/a	n/a	n/a	n/a	n/a	n/a
DBG	14	H	n/a	n/a	n/a	n/a	n/a	n/a
DBG	15	A	n/a	n/a	n/a	n/a	n/a	n/a
DBG	16	A	n/a	n/a	n/a	n/a	n/a	n/a
DBG	17	N	n/a	n/a	n/a	n/a	n/a	n/a
DBG	18	H	n/a	n/a	n/a	n/a	n/a	n/a
DBG	19	H	n/a	n/a	n/a	n/a	n/a	n/a
DBG	20	A	n/a	n/a	n/a		n/a	n/a
DBG	21	Au	n/a	n/a	n/a	n/a	n/a	n/a
DBG	22	L	n/a	n/a	n/a	n/a	n/a	n/a
DBG	23	L	n/a	n/a	n/a	n/a	n/a	n/a

DBG	24	N	n/a	n/a	n/a	n/a	n/a	n/a
DBG	25	N	n/a	n/a	n/a	n/a	n/a	n/a
DBG	26	N	n/a	n/a	n/a	n/a	n/a	n/a
DBG	27	N	n/a	n/a	n/a	n/a	n/a	n/a
Taranaki	TH1	H	n/a	10	2	39 18.419 S	174 05.992 E	749
Taranaki	TH2	H	n/a	5	2	39 16.188 S	174 05.804 E	922
Taranaki	TH3	H	n/a	40	10	39 16.186 S	174 05.816 E	923
Taranaki	TH4	H	n/a	30	8	39 16.189 S	174 05.824 E	921
Taranaki	TH5	H	n/a	10	2	39 16.191 S	174 05.827 E	921
Taranaki	TH6	H	n/a	30	7	39 16.206 S	174 05.835 E	920
Taranaki	TH7	H	n/a	3	1.5	39 16.246 S	174 05.861 E	900
Taranaki	TH8	H	n/a	20	5	39 16.249 S	174 05.868 E	904
Taranaki	TH9	H	n/a	10	2.5	39 16.262 S	174 05.573 E	906
Taranaki	TH10	H	n/a	10	3.5	39 16.263 S	174 05.873 E	905
Taranaki	TH11	H	n/a	8	3	39 16.274 S	174 05.869 E	907
Taranaki	TH12	H	n/a	8	3	39 16.273 S	174 05.876 E	908
Taranaki	TH13	H	n/a	7	2	39 16.258 S	174 05.912 E	901
Taranaki	TH14	H	n/a	5	1.5	39 16.269 S	174 05.895 E	906
Taranaki	TH15	H	n/a	3	.75	39 16.273 S	174 05.887 E	908
Taranaki	TH16	H	n/a	20	5	39 18.945 S	174 09.072 E	654
Taranaki	TH17	H	n/a	8	3	39 18.945 S	174 08.963 E	663
Taranaki	TH18	H	n/a	20	5	39 18.943 S	174 08.904 E	669
Taranaki	TH19	H	n/a	4	2	39 18.950 S	174 08.894 E	670
Taranaki	TH20	H	n/a	10	4	39 18.945 S	174 08.860 E	675
Taranaki	T1	T	n/a	30	7	39 06.155 S	174 07.261 E	66

Taranaki	T2	T	n/a	40	6	39 06.163 S	174 07.252 E	68
Taranaki	T3	T	n/a	100	15	39 06.167 S	174 07.238 E	70
Taranaki	T4	T	n/a	20	6	39 06.167 S	174 07.229 E	69
Taranaki	T5	T	n/a	45	5	39 06.205 S	174 07.229 E	69
Taranaki	T6	T	n/a	60	18	39 06.209 S	174 07.191 E	70
Taranaki	T7	T	n/a	120	16	39 06.208 S	174 07.170 E	70
Taranaki	T8	T	n/a	20	7	39 06.215 S	174 07.119 E	70
Taranaki	T9	T	n/a	70	10	39 06.245 S	174 07.116 E	71
Taranaki	T10	T	n/a	60	8	39 06.246 S	174 07.115 E	70
Taranaki	T11	T	n/a	10	5	39 06.224 S	174 07.072 E	72

Key

T	*P. totara*
H	*P. hallii*
A	*P. acutifolius*
N	*P. nivalis*
H_1	Hybrid of *P. acutifolius/P. totara*, or P. *hallii/ P. totara*
Au	*P. aurea*
L	*P. lawrencii*
Whanga.	Whangamoana
New Ply.	New Plymouth
Feather.	Featherston
Banks p.	Banks Peninsula
Dean F.	Dean Forest
Pelorus R.	Pelorus River
Tauma.	Taumaranui
Kaua.	Kauaeranga
Hawkes B.	Hawkes Bay
n/a	Not available
$Species_2$	- Species based on phenotypic assignment
DBG	Dunedin Botanical Garden

TABLE 6 HRM SCREENING RESULTS – PHASE 4

							Grouping	TAH-N	TA-H-N	TA-H-N	TH-AN	TA-HN	TA-H-N	T-H-A-N	TA-HN	TA-H-N	TA-H-N
Sample ID	**Sample Site**	**Site Descrip.**	**Geographic origin**	**Alt. (m)**	**Phenotypic Assignment**	**Species based on markers**	**Pos (384well)**	**GA15**	**GA48**	**GA54**	**GA58**	**GA78**	**GA84**	**GA90**	**GA92**	**GA99**	**GA113**
27	Pureora	NF	Same	788	*P. hallii*	H	A1/12	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	H	Neg
28	Pureora	NF	Same	n/a	*P. hallii*	H	B1/13	THA	H	H	TH	HN	H2	H	HN	H	H
29	Pureora	NF	Same	n/a	*P. hallii*	H	C1/13	THA	H	H	TH	HN	H2	H	HN	H	H
30	Pureora	NF	Same	n/a	*P. hallii*	H	D1/13	THA	H	H	TH	HN	H2	H	HN	H	H
31	Pureora	NF	Same	804	*P. hallii*	H	E1/13	THA	H	H	TH	HN	H2	H	HN	H	H
32	Pureora	NF	Same	856	*P. hallii*	H	F1/13	THA	H	H	TH	HN	H2	H	HN	H	H
33	Pureora	NF	Same	832	*P. hallii*	H	G1/13	THA	H	H	TH	HN	H2	H	HN	H	H
34	Pureora	NF	Same	n/a	*P. hallii*	H	H1/13	THA	H	H	TH	HN	H2	H	HN	H	H
35	Pureora	NF	Same	807	*P. hallii*	H	I1/13	THA	H	H	TH	HN	H2	H	HN	H	H
36	Pureora	NF	Same	n/a	*P. hallii*	H	J1/13	THA	H	H	TH	HN	H2	H	HN	H	H
37	Pureora	NF	Same	n/a	*P. hallii*	H	K1/13	THA	H	H	TH	HN	H2	H	HN	H	H
38	Pureora	NF	Same	n/a	*P. hallii*	H	L1/13	THA	H	H	TH	HN	H2	H	HN	H	H
39	Pureora	NF	Same	782	*P. hallii*	H	M1/13	THA	H	H	TH	HN	H2	H	HN	H	H
40	Pureora	NF	Same	n/a	*P. hallii*	H	N1/13	THA	H	H	TH	HN	H2	H	HN	H	H
41	Pureora	NF	Same	n/a	*P. hallii*	H	O1/13	THA	H	H	TH	HN	H2	H	HN	H	H

42	Pureora	NF	Same	n/a	*P. hallii*	H	P1/13	THA	H	H	TH	HN	H2	H	HN	H	H
43	Pureora	NF	Same	n/a	*P. hallii*	H	A2/14	Neg	H	H	TH	HN	H2	H	HN	H	H
44	Pureora	NF	Same	n/a	*P. hallii*	H	B2/14	THA	H	H	TH	HN	H2	H	HN	H	H
45	Pureora	NF	Same	769	*P. hallii*	H	C2/14	THA	H	H	TH	HN	H2	H	HN	H	H
46	Pureora	NF	Same	n/a	*P. hallii*	H	D2/14	THA	H	H	TH	HN	H2	H	HN	H	H
47	Pureora	NF	Same	746	*P. hallii*	H	E2/14	THA	H	H	TH	HN	H2	H	HN	H	H
48	Pureora	NF	Same	n/a	*P. hallii*	H	F2/14	THA	H	H	TH	HN	H2	H	HN	H	H
49	Pureora	NF	Same	n/a	*P. hallii*	H	G2/14	THA	H	H	TH	HN	H2	H	HN	H	H
50	Pureora	NF	Same	n/a	*P. hallii*	H	H2/14	THA	H	H	TH	HN	H2	H	HN	H	H
51	Pureora	NF	Same	n/a	*P. hallii*	H	I2/14	THA	H	H	TH	HN	H2	H	HN	H	H
52	Pureora	NF	Same	n/a	*P. hallii*	H	J2/14	THA	H	H	TH	HN	H2	H	HN	H	H
53	Pureora	NF	Same	1019	*P. hallii*	H	K2/14	THA	H	H	TH	HN	H2	H	HN	H	H
54	Pureora	NF	Same	1046	*P. hallii*	H	L2/14	THA	H	H	TH	HN	H2	H	HN	H	H
55	Pureora	NF	Same	n/a	*P. hallii*	H	M2/14	THA	H	H	TH	HN	H2	H	HN	H	H
57	Pureora	NF	Same	559	*P. totara*	T	N2/14	THA	TA	TA	TH	T	T2	T	TA	TA	TA
56	Pureora	NF	Same	1022	*P. hallii*	H	O2/14	THA	H	H	TH	HN	H2	H	HN	H	H
58	Pureora	NF	Same	554	*P. totara*	T	P2/14	THA	TA	TA	TH	T	T2	T	TA	TA	TA
59	Pureora	NF	Same	556	*P. totara*	T	A3/15	THA	TA	TA	TH	T	T2	T	TA	TA	TA
60	Pureora	NF	Same	559	*P. totara*	T	B3/15	THA	TA	TA	TH	T	T2	T	TA	TA	TA
61	Pureora	NF	Same	556	*P. totara*	T	C3/15	THA	TA	TA	TH	T	T2	T	TA	TA	TA
62	Pureora	NF	Same	n/a	*P. totara*	T	D3/15	THA	TA	TA	TH	T	T2	T	TA	TA	TA
63	Pureora	NF	Same	559	*P. totara*	T	E3/15	THA	TA	TA	TH	T	T2	T	TA	TA	TA
64	Pureora	NF	Same	556	*P. totara*	T	F3/15	THA	TA	TA	TH	T	T2	T	TA	TA	TA
65	Pureora	NF	Same	555	*P. totara*	T	G3/15	THA	TA	TA	TH	T	T2	T	TA	TA	TA

66	Pureora	NF	Same	561	*P. totara*	T	H3/15	THA	TA	TA	TH	T	T2	T	TA	TA	TA
67	Pureora	NF	Same	568	*P. totara*	T	I3/15	THA	TA	TA	TH	T	T2	T	TA	TA	TA
68	Pureora	NF	Same	567	*P. totara*	T	J3/15	THA	TA	TA	TH	T	T2	T	TA	TA	TA
69	Pureora	NF	Same	560	*P. totara*	T	K3/15	THA	TA	TA	TH	T	T2	T	TA	TA	TA
70	Pureora	NF	Same	588	*P. totara*	T	L3/15	THA	TA	TA	TH	T	T2	T	TA	TA	TA
71	Pureora	NF	Same	579	*P. totara*	T	M3/15	THA	TA	TA	TH	T	T3	T	TA	TA	TA
72	Pureora	NF	Same	577	*P. totara*	T	N3/15	THA	TA	TA	TH	T	T2	T	TA	TA	TA
73	Pureora	NF	Same	576	*P. totara*	T	O3/15	THA	TA	TA	TH	T	T2	T	TA	TA	TA
74	Pureora	NF	Same	578	*P. totara*	T	P3/15	THA	TA	TA	TH	T	T2	T	TA	TA	TA
75	Pureora	NF	Same	584	*P. totara*	T	A4/16	THA	TA	TA	TH	T	T2	T	TA	TA	TA
76	Pureora	NF	Same	575	*P. totara*	T	B4/16	THA	TA	TA	TH	T	T2	T	TA	TA	TA
77	Pureora	NF	Same	577	*P. totara*	T	C4/16	THA	TA	TA	TH	T	T2	T	TA	TA	TA
78	Pureora	NF	Same	593	*P. totara*	T	D4/16	THA	TA	TA	TH	T	T2	T	TA	TA	TA
79	Pureora	NF	Same	587	*P. totara*	T	E4/16	THA	TA	TA	TH	T	T2	T	TA	TA	TA
80	Pureora	NF	Same	595	*P. totara*	T	F4/16	Neg	TA	TA	TH	T	T2	T	TA	TA	TA
81	Pureora	NF	Same	596	*P. totara*	T	G4/16	THA	TA	TA	TH	T	T2	T	TA	TA	TA
82	Pureora	NF	Same	614	*P. totara*	T	H4/16	THA	TA	TA	TH	T	T2	T	TA	TA	TA
83	Pureora	NF	Same	575	*P. totara*	T	I4/16	THA	TA	TA	TH	T	T2	T	TA	TA	TA
84	Pureora	NF	Same	597	*P. totara*	T	J4/16	THA	TA	TA	TH	T	T2	T	TA	TA	TA
85	Pureora	NF	Same	617	*P. totara*	T	K4/16	THA	TA	TA	TH	T	T2	T	TA	TA	TA
86	Pureora	NF	Same	622	*P. totara*	T	L4/16	THA	TA	TA	TH	T	T2	T	TA	TA	TA
87	Mama.	NF	Same	617	*P. hallii*	H	M4/16	THA	H	TA	TH	HN	H2	H	HN	H	H
88	Mama.	NF	Same	557	*P. hallii*	H	N4/16	THA	H	H	TH	HN	H2	H	HN	H	H
89	Mama.	NF	Same	554	*P. hallii*	H	O4/16	THA	H	H	TH	HN	T2	H	HN	H	H

90	Mama.	NF	Same	553	*P. hallii*	H	P4/16	THA	H	H	TH	HN	H2	H	HN	H	H
91	Mama.	NF	Same	n/a	*P. hallii*	H	A5/17	THA	H	H	TH	HN	H2	H	HN	H	H
92	Mama.	NF	Same	550	*P. hallii*	H	B5/17	THA	H	H	TH	HN	H2	H	HN	H	H
93	Mama.	NF	Same	n/a	*P. hallii*	H	C5/17	THA	H	H	TH	HN	H2	H	HN	H	H
94	Mama.	NF	Same	546	*P. hallii*	H	D5/17	THA	H	H	TH	HN	H2	H	HN	H	H
95	Mama.	NF	Same	548	*P. hallii*	H	E5/17	THA	H	H	TH	HN	H2	H	HN	H	H
96	Mama.	NF	Same	n/a	*P. hallii*	H	F5/17	THA	H	H	TH	HN	H2	H	HN	H	H
97	Mama.	NF	Same	n/a	*P. hallii*	H	G5/17	THA	H	H	TH	HN	H2	H	HN	H	H
98	Mama.	NF	Same	529	*P. hallii*	H	H5/17	THA	H	H	TH	HN	H2	H	HN	H	H
99	Mama.	NF	Same	521	*P. hallii*	H	I5/17	THA	H	H	TH	HN	H2	H	HN	H	H
100	Mama.	NF	Same	473	*P. hallii*	H	J5/17	THA	H	H	TH	HN	H2	H	HN	H	H
101	Mama.	NF	Same	n/a	*P. hallii*	H	K5/17	THA	H	H	TH	HN	H2	H	HN	H	H
102	Mama.	NF	Same	472	*P. hallii*	H	L5/17	THA	H	H	TH	HN	H2	H	HN	H	H
103	Mama.	NF	Same	n/a	*P. hallii*	H	M5/17	THA	H	H	TH	HN	H2	H	HN	H	H
104	Mama.	NF	Same	n/a	*P. hallii*	H	N5/17	THA	H	H	TH	HN	H2	H	HN	H	H
105	Mama.	NF	Same	n/a	*P. hallii*	H	O5/17	new	H	H	TH	HN	H2	new	U	H	H
106	Mama.	NF	Same	n/a	*P. hallii*	H	P5/17	new	H	H	TH	HN	H2	new	HN	H	H
107	Mama.	NF	Same	466	*P. hallii*	H	A6/18	THA	H	H	TH	HN	H2	H	HN	H	H
108	Mama.	NF	Same	n/a	*P. hallii*	H	B6/18	THA	H	H	TH	HN	H2	H	HN	H	H
109	Mama.	NF	Same	n/a	*P. hallii*	H	C6/18	THA	H	H	TH	HN	H2	H	HN	H	H
110	Mama.	NF	Same	n/a	*P. hallii*	H	D6/18	THA	H	H	TH	HN	H2	H	HN	H	H
111	Mama.	NF	Same	461	*P. hallii*	H	E6/18	THA	H	H	TH	HN	H2	H	HN	H	H
112	Mama.	NF	Same	n/a	*P. hallii*	H	F6/18	THA	H	H	TH	HN	H2	new	HN	H	H
113	Mama.	NF	Same	n/a	*P. hallii*	H	G6/18	THA	H	H	TH	HN	H2	new	HN	H	H

114	Mama.	NF	Same	n/a	*P. hallii*	H	H6/18	THA	H	H	TH	HN	H2	new	HN	H	H
115	Mama.	NF	Same	441	*P. hallii*	H	I6/18	THA	H	H	TH	HN	H2	new	HN	H	H
116	Mama.	NF	Same	n/a	*P. hallii*	H	J6/18	new	H	H	TH	HN	H2	new	HN	H	H
117	Mama.	NF	Same	521	***P. totara***	**H**	K6/18	THA	H	H	TH	HN	H2	new	HN	H	H
118	Mama.	NF	Same	506	***P. totara***	**H**	L6/18	new	H	H	TH	HN	H2	new	HN	H	H
119	Mama.	NF	Same	472	***P. totara***	**H**	M6/18	THA	H	H	TH	HN	H2	new	HN	H	H
120	Mama.	NF	Same	441	***P. totara***	**H**	N6/18	new	H	H	TH	HN	H2	Neg	HN	H	H
121	Mama.	SOR	Same	283	***P. totara***	**H**	O6/18	THA	H	H	TH	HN	H2	new	HN	H	Neg
122	Mama.	SOR	Same	280	***P. totara***	**H**	P6/18	THA	H	H	TH	HN	H2	new	HN	H	H
123	Mama.	SOR	Same	280	***P. totara***	**H**	A7/19	THA	H	H	TH	HN	H2	H	HN	H	H
124	Mama.	SOR	Same	n/a	*P. totara*	T	B7/19	THA	TA	TA	TH	T	T2	T	TA	TA	TA
125	Mama.	SOR	Same	n/a	*P. totara*	T	C7/19	THA	TA	TA	TH	T	T2	T	TA	TA	TA
126	Mama.	SOR	Same	n/a	*P. totara*	T	D7/19	THA	TA	TA	TH	T	T2	T	TA	TA	TA
127	Mama.	NF	Same	560	***P. totara***	**H**	E7/19	THA	H	H	TH	HN	H2	H	HN	H	H
128	Mama.	NF	Same	560	***P. totara***	**H**	F7/19	THA	H	H	TH	HN	H2	H	HN	H	H
129	Mama.	NF	Same	560	***P. totara***	**H**	G7/19	THA	H	H	TH	HN	H2	H	HN	H	H
130	Mama.	NF	Same	560	***P. totara***	**H**	H7/19	THA	H	H	TH	HN	H2	H	HN	H	H
147	Makatiti	NF	Same	846	***P. hallii***	**H**	I7/19	THA	H	H	TH	HN	H2	H	HN	H	H
148	Makatiti	NF	Same	n/a	*P. hallii*	H	J7/19	THA	H	H	TH	HN	H2	H	HN	H	H
150	Makatiti	NF	Same	n/a	*P. hallii*	H	K7/19	THA	H	H	TH	HN	H2	H	HN	H	H
151	Makatiti	NF	Same	n/a	*P. hallii*	H	L7/19	THA	H	H	TH	HN	H2	H	HN	H	H
152	Makatiti	NF	Same	n/a	*P. hallii*	H	M7/19	THA	H	H	TH	HN	H2	H	HN	H	H
153	Makatiti	NF	Same	n/a	*P. hallii*	H	N7/19	THA	H	H	TH	HN	H2	H	HN	H	H
154	Makatiti	NF	Same	n/a	*P. hallii*	H	O7/19	THA	H	H	TH	HN	H2	H	HN	H	H

155	Makatiti	NF	Same	n/a	*P. hallii*	H	P7/19	THA	Unkn.	H	TH	HN	H2	new	HN	H	H
156	Makatiti	NF	Same	n/a	*P. hallii*	H	A8/20	THA	H	H	TH	HN	H2	H	HN	H	H
157	Makatiti	NF	Same	n/a	*P. hallii*	H	B8/20	THA	H	H	TH	HN	H2	H	HN	H	H
158	Makatiti	NF	Same	850	*P. hallii*	H	C8/20	THA	H	H	TH	HN	H2	H	HN	H	H
159	Makatiti	NF	Same	n/a	*P. hallii*	H	D8/20	THA	Unkn.	H	TH	HN	H2	H	HN	H	H
160	Makatiti	NF	Same	n/a	*P. hallii*	H	E8/20	THA	H	H	TH	HN	H2	H	HN	H	H
161	Makatiti	NF	Same	n/a	*P. hallii*	H	F8/20	THA	H	H	TH	HN	H2	H	HN	H	H
162	Makatiti	NF	Same	n/a	*P. hallii*	H	G8/20	THA	H	H	TH	HN	H2	H	HN	H	H
163	Makatiti	NF	Same	n/a	*P. hallii*	H	H8/20	THA	H	H	TH	HN	H2	H	HN	H	H
164	Makatiti	NF	Same	n/a	*P. hallii*	H	I8/20	THA	H	H	TH	HN	H2	H	HN	H	H
165	Makatiti	NF	Same	n/a	*P. hallii*	H	J8/20	THA	H	H	TH	HN	H2	H	HN	H	H
166	Makatiti	NF	Same	n/a	*P. hallii*	H	K8/20	THA	H	H	TH	HN	H2	H	HN	H	H
167	Makatiti	NF	Same	n/a	*P. hallii*	H	L8/20	THA	H	H	TH	HN	H2	H	HN	H	H
168	Makatiti	NF	Same	861	*P. hallii*	H	M8/20	THA	H	H	TH	HN	H2	H	HN	H	H
169	Makatiti	NF	Same	n/a	*P. hallii*	H	N8/20	THA	H	H	TH	HN	H2	H	HN	H	H
170	Makatiti	NF	Same	n/a	*P. hallii*	H	O8/20	THA	H	H	TH	HN	H2	new	HN	H	H
171	Makatiti	NF	Same	n/a	*P. hallii*	H	P8/20	THA	H	H	TH	HN	H2	new	HN	H	H
172	Makatiti	NF	Same	n/a	*P. hallii*	H	A9/21	THA	H	H	TH	HN	H2	H	HN	H	H
173	Makatiti	NF	Same	n/a	*P. hallii*	H	B9/21	THA	H	H	TH	HN	H2	H	HN	H	H
174	Makatiti	NF	Same	n/a	*P. hallii*	H	C9/21	THA	H	H	TH	HN	H2	H	HN	H	H
175	Makatiti	NF	Same	n/a	*P. hallii*	H	D9/21	THA	H	H	TH	HN	H2	H	HN	H	H
176	Makatiti	NF	Same	896	*P. hallii*	H	E9/21	THA	H	H	TH	HN	H2	H	HN	H	H
T1	Taranaki	NF	Same	66	*P. totara*	T	F9/21	THA	TA	TA	TH	T	T2	T	TA	TA	Neg
T2	Taranaki	NF	Same	68	*P. totara*	T	G9/21	THA	TA	TA	TH	T	T2	T	TA	TA	TA

T3	Taranaki	NF	Same	70	*P. totara*	T	H9/21	THA	TA	TA	TH	T	T2	T	TA	TA	TA
T4	Taranaki	NF	Same	69	*P. totara*	T	I9/21	THA	TA	TA	TH	T	T2	T	TA	TA	TA
T5	Taranaki	NF	Same	69	*P. totara*	T	J9/21	THA	TA	TA	TH	T	T2	T	TA	TA	TA
T6	Taranaki	NF	Same	70	*P. totara*	T	K9/21	THA	TA	TA	TH	T	T2	T	TA	TA	TA
T7	Taranaki	NF	Same	70	*P. totara*	T	L9/21	THA	TA	TA	TH	T	T2	T	TA	TA	TA
T8	Taranaki	NF	Same	70	*P. totara*	T	M9/21	THA	TA	TA	TH	T	T2	T	TA	TA	TA
T9	Taranaki	NF	Same	71	*P. totara*	T	N9/21	THA	TA	TA	TH	T	T2	T	TA	TA	TA
T10	Taranaki	NF	Same	70	*P. totara*	T	O9/21	THA	TA	TA	TH	T	T2	T	TA	TA	TA
T11	Taranaki	NF	Same	72	*P. totara*	T	P9/21	THA	TA	TA	TH	T	T2	T	TA	TA	TA
TH1	Taranaki	NF	Same	749	*P. hallii*	H	A10/22	THA	H	H	TH	HN	H	H	HN	H	H
TH2	Taranaki	NF	Same	922	*P. hallii*	H	B10/22	THA	H	H	TH	HN	H	H	HN	H	H
TH3	Taranaki	NF	Same	923	*P. hallii*	H	C10/22	THA	H	H	TH	HN	H	H	HN	H	H
TH4	Taranaki	NF	Same	921	*P. hallii*	H	D10/22	THA	H	H	TH	HN	H	H	HN	H	H
TH5	Taranaki	NF	Same	921	*P. hallii*	H	E10/22	THA	H	H	TH	HN	H	H	HN	H	H
TH6	Taranaki	NF	Same	920	*P. hallii*	H	F10/22	THA	H	H	TH	HN	H	H	HN	H	H
TH7	Taranaki	NF	Same	900	*P. hallii*	H	G10/22	THA	H	H	TH	HN	H	H	HN	H	H
TH8	Taranaki	NF	Same	904	*P. hallii*	H	H10/22	THA	H	H	TH	HN	H	H	HN	H	H
TH9	Taranaki	NF	Same	906	*P. hallii*	H	I10/22	THA	H	H	TH	HN	H	H	HN	H	H
TH10	Taranaki	NF	Same	905	*P. hallii*	H	J10/22	THA	H	H	TH	HN	H	new	HN	H	H
TH11	Taranaki	NF	Same	907	*P. hallii*	H	K10/22	THA	H	H	TH	HN	H	H	HN	H	H
TH12	Taranaki	NF	Same	908	*P. hallii*	H	L10/22	THA	H	H	TH	HN	H	H	HN	H	H
TH13	Taranaki	NF	Same	901	*P. hallii*	H	M10/22	THA	H	H	TH	HN	H	H	HN	H	H
TH14	Taranaki	NF	Same	906	*P. hallii*	H	N10/22	THA	H	H	TH	HN	H	new	HN	H	H
TH15	Taranaki	NF	Same	908	*P. hallii*	H	O10/22	THA	H	H	TH	HN	H	H	HN	H	H

TH16	Taranaki	NF	Same	654	*P. hallii*	H	P10/22	THA	H	H	TH	HN	H	H	HN	H	H
TH17	Taranaki	NF	Same	663	*P. hallii*	H	A11/23	THA	H	H	TH	Unkn.	H	H	HN	H	H
TH18	Taranaki	NF	Same	669	*P. hallii*	H	B11/23	THA	H	H	TH	HN	H	H	HN	H	H
TH19	Taranaki	NF	Same	670	*P. hallii*	H	C11/23	THA	H	H	TH	HN	H	H	HN	H	H
TH20	Taranaki	NF	Same	675	*P. hallii*	H	D11/23	THA	H	H	TH	HN	H	H	HN	H	H
21	DBG	Pl	n/a	n/a	*P. au*	Au	E11/23	AuL	Au	H	SH	AuL	AuL	H	HN	SH	H
22	DBG	Pl	n/a	n/a	*P. l*	L	F11/23	AuL	N	L	AN	AuL	AuL	L	TA	TA	L
23	DBG	Pl	n/a	n/a	*P. l*	L	G11/23	AuL	N	L	AN	AuL	AuL	L	TA	TA	L
24	DBG	Pl	n/a	n/a	*P. NB*	N	H11/23	N	N	N	AN	HN	N	N	HN	N	N
189	TTS	Pl	Kaikoura	n/a	*P. totara*	T	I11/23	THA	TA	TA	TH	neg	T	T	TA	TA	TA
203	TTS	Pl	Opotiki	n/a	*P. totara*	T	J11/23	THA	TA	TA	TH	T	T	T	TA	TA	TA
55	TTS	Pl	Pureora	n/a	*P. hallii*	H	K11/23	THA	H	H	TH	HN	H	H	HN	H	H
160	TTS	Pl	Makatiti	n/a	*P. hallii*	H	L11/23	THA	H	H	TH	HN	H	H	HN	H	H
12	DBG	Pl	n/a	n/a	*P. au*	A	M11/23	THA	TA	TA	AN	A	A	A	TA	TA	TA
20	DBG	Pl	n/a	n/a	*P. au*	A	N11/23	THA	TA	TA	AN	A	A	A	TA	TA	TA
4	DBG	Pl	n/a	n/a	*P. nivalis*	N	O11/23	N	N	N	AN	HN	N	N	HN	N	N
10	DBG	Pl	n/a	n/a	*P. nivalis*	N	P11/23	N	N	N	AN	HN	N	N	HN	N	N
H1	Pureora /TTS	NF/Pl	Pureora/ whangamata	1046-n/a	*P. totara / P. hallii*	HY	A12/24	THA	TA	TA	TH	T	T	T	TA	SH	SH
H2	Makatiti /TTS	NF/Pl	Makatiti/ Hawkes Bay	870-n/a	*P.totara/ P. hallii*	HY	B12/24	THA	TA	TA	TH	T	T	T	TA	SH	SH
H3	Taranaki /TTS	NF/Pl	Taranaki-Harihari	927-n/a	P.totara/ *P. hallii*	HY	C12/24	THA	TA	TA	SH	T	T	T	TA	TA	SH

Key

T	– *P. totara*
H	– *P. hallii*
A	– *P. acutifolius*
N	– *P. nivalis*
Au/P. au	–*P. aurea*
L/P.l	–*P. lawrencii*
P.NB	–*P. nivalis* Bronze
TTS	– Tapapakanga Trial Site
DBG	–Dunedin Botanical Garden
New	–Group of melting curves different to four totara species (based on controls I11/23 -P11/23
New2	–Second different type of melting curve
Mama.	–Mamaku Ranges
NF	–Natural forest
PL	–Planted
SH	– Synthetic Hybrid where DNA is mixed from a *P. totara* and *P. hallii* sample.
SOR	–Side of road
Unkn.	–Unknown genotype
Neg	–Negative no amplification
n/a	– Information not available