ARBUSCULAR MYCORRHIZAE OF *PHORMIUM TENAX* IN A RESTORED NEW ZEALAND WETLAND ACROSS HYDROLOGICAL GRADIENTS

BY

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Abstract

New Zealand has lost over 90% of its former wetlands and many that remain are in a degraded state. Restoration projects are often impeded by the failure of native plants to establish back into non-native dominated communities. *Phormium tenax* is fast growing and acts a nurse plant in wetlands, accelerating the establishment of slower growing native woody species. The roles of below ground organisms are increasingly recognised as affecting plant community dynamics, and this study investigates the diversity of a group of pervasive organisms, the arbuscular mycorrhizal fungi (AMF), growing in symbiosis with *Phormium tenax*. Next generation sequencing was used to create two libraries to determine the sensitivity of coding and non-coding molecular markers when characterising the AMF community associated with *Phormium tenax*.

AMF communities colonising individual plants were found to be diverse, and varied across restoration stages, but uncorrelated with soil moisture. The composition of of AMF communities changed seasonally and I observed more AMF hyphae and arbuscules in winter.

Key Words

Arbuscular Mycorrhizal Fungi, wetland, restoration ecology, *Phormium tenax*, diversity, next-generation sequencing, Illumina sequencing

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Contents

Chapter 1: General overview

Wetlands are ecosystems that provide habitat for many threatened native flora and fauna, and crucial ecosystem services to humans including water filtration, flood control, and carbon sequestration (Harmsworth 2002). New Zealand has lost over 90% of former wetland areas (Cromarty and Scott 1996, Ministry for the Environment 1997) and many of those that remain are in a degraded state (Ausseil et al. 2011). Wetland restoration projects often focus on planting native vegetation; however, survival rates of trees are variable (Gillon 2014). Elsewhere, the reestablishment of trees is contingent upon positive biotic interactions (Gómez-Aparicio L. 2009), but there are gaps in the literature on the biotic interactions of native New Zealand wetland plant species commonly planted in restoration projects.

Harakeke (*Phormium tenax*) is a foundational plant species in New Zealand wetlands, often establishing early in successional sequences and persisting in mature wetland swamp forests (Wehi and Clarkson 2007). *P. tenax* is flood, drought, fire, frost and grazing tolerant, making it an ideal early colonizer in restoration projects (McGruddy 2006). *P. tenax* modifies the physical environment by stabilizing and enriching soils with its fibrous root system which grows as wide and deep as the above ground parts of the plant (>3m) (McGruddy 2006). *P. tenax* roots trap nutrient-rich sediment carried by flood waters, stabilising the substrate and forming a fertile layer of soil over time (McGruddy 2006). *P. tenax* nectar, flowers, fruits and leaves also provide food to a wide variety of fauna including invertebrates, fungi, geckos, birds and short-tailed bats (McKenzie et al. 2005). *P. tenax* is commonly believed to act as a nurse plant for later successional woody trees (Reay and Norton 1999), but the mechanisms responsible for this 'nurse effect' have not been determined. *P. tenax* is particularly good at acting as a nurse plant in ungrazed or lightly grazed pasture and grassland in which native woody species are often outcompeted by grasses (Reay and Norton 1999). Birds perching and feeding on *P. tenax* may disperse the seeds of native woody plant species into the *P. tenax* clumps, where they germinate and increase the local diversity of the vegetation. However, *P. tenax* is not in flower while the native woody species bear

fruit (Burrows 1994) so the role of *P. tenax* may primarily be as physical habitat for seed-dispersing birds (Reay and Norton 1999).

Over 80% of all plants form symbioses with arbuscular mycorrhizal fungi (AMF) (Smith and Read 1997). AMF occur in many types of wetlands (Rickerl et al. 1994, Van Hoewyk et al. 2001, Bauer et al. 2003, Gómez-Aparicio 2009) and mycorrhizal symbioses play a significant role in the growth of wetland plants (Dunham et al. 2003). Despite the ability of AMF to persist in inundated conditions, associated stressors such as anoxia and low soil pH can have a significant negative effect on mycorrhizal colonisation in wetland plants (Miller and Bever 1999, Cornwell et al. 2001, Escudero and Mendoza 2005). AMF colonisation intensity and type changes seasonally (Mayr and Godoy 1989, Mandyam and Jumpponen 2008) as does AMF community composition (Dumbrell et al. 2011). Arbuscular mycorrhizal communities change with succession in host plant communities, and there is a growing body of evidence that host plants exert control on AMF colonisation (Johnson et al. 1991, Eom et al. 2000, Sanders 2003, Limpens and Geurts 2014). In field ecological restoration projects in New Zealand, it is common practice to plant seedlings grown from locally-sourced seed to promote the native biodiversity of the area. However, the AMF associated with *P. tenax* are rarely considered during restoration and little is known of their role in in wetland restoration success. I will determine the seasonal and successional dynamics of AMF on a foundational pioneer wetland plant species (*P. tenax*). This is a base line study to assess the diversity of AMF communities across a hydrological gradient in a New Zealand wetland restoration site.

Research objectives

1. To determine the diversity of AMF communities of a native New Zealand foundational plant species *P. tenax*.

2. To examine the effect of season, hydrology and redox potential on AMF diversity and colonisation across a New Zealand wetland restoration site.

3. To compare the 18S and ITS regions of ribosomal DNA as molecular markers for arbuscular mycorrhizal fungi in a wetland plant (*P. tenax*).

Hypotheses

1. AMF diversity and community composition in *P. tenax* will differ among restoration stages at Wairio wetland. I predict that the 10-year-old restoration site will have higher diversity than the than the 1-year-old site.
2. Root colonisation of *P. tenax* by AMF will change seasonally, with greater colonisation in summer than winter but this will be insufficent to alter AMF diversity.
3. AMF colonisation of *P. tenax* will change along a moisture gradient, with relatively low rates of root colonisation where the water table and soil moisture are high.
4. AMF diversity of *P. tenax* will be inversely related to soil moisture with highest diversity in drier soils.

Thesis structure

There are three remaining chapters to this thesis, chapter two is a literature review, chapter three contains the methods and results of this study and chapter four discusses the implications of those results and the potential role of AMF in wetland restoration in New Zealand.

Research context

Wairio wetland is on the south-east coast of Lake Wairarapa, New Zealand. It has recently been a pastoral landscape but is now being restored to a functional wetland. Wairio restoration is managed by Ducks Unlimited who kindly allow students at Victoria University to conduct experimental research at the site. Previous work has included extensive hydrological mapping and modelling of the wetland (Marapara 2016) as well as survivorship and growth of restoration revegetation (Johnson 2012, Gillon 2014). Wairio provides the unique opportunity to sample plants across a hydrological gradient at a site already divided into distinct restoration stages.

Chapter 2: Literature review

Global state of wetlands

In the last few centuries, the extent of global wetlands has declined dramatically, with estimates that 50% have been destroyed (Mitsch and Gosselink 2000). The decline is mainly due to the intensification of agriculture and industry associated with huge population increases during the industrial revolution. Wetlands have not only suffered a decline in their extent but also in their state of ecological health, with many of those remaining in a state of degradation (Mitsch and Gosselink 2000). Ecological degradation is defined as the enduring loss of ecosystem function due to disturbances from which the land cannot naturally recover (Asmelash et al. 2016). There are many anthropogenic causes of wetland degradation including water pollution, hydrological alterations, sedimentation, and the introduction of invasive species (Ausseil 2011).

Wetland definition

Wetlands occur across the world in every climatic zone, from the tropics to the poles. Wetlands can be either permanent, seasonal or intermittent and either salt or freshwater. A wetland is classified as anywhere that surface water, ground water and dry land convene to support a natural ecosystem of plants and animals (Johnson and Gerbeaux 2004). Because of this broad definition, the term wetland encompasses many forms including rivers, lakes, marshes, mangroves, coral reefs, rice fields, flooded forests, peatlands, flood plains and arctic bogs.

Wetland types

Wetlands are classified based on their hydrology, soil, landscape position, climate, vegetation, and geology but there are several systems that can be used to do so. The most common classification methods are the Ramsar, hydrogeomorphology (HGM) and the Cowardin classification systems (Cowardin et al. 1979, Scott and Jones 1995).

The accepted classification of wetlands in New Zealand was created by Johnson and Gerbeaux (2004) based on adaptations from Ward and Lambie (1999) and Clarkson et al. (2003). The Johnson and Gerbeaux (2004) wetland classification system

distinguishes wetlands by the categories: hydrosystem; wetland class; wetland form; vegetation structure; and vegetation composition.

New Zealand has many types of wetland hydrosystems including: marine; estuarine; riverine; lacustrine; palustrine; geothermal; plutonic; nival and artificial (Buxton 1991, Fuller 1993). The hydrosystems are categorised at a coarse spatial scale based on the wetland's hydrology, landform, salinity, and temperature extremes (Johnson and Gerbeaux 2004).

Hydrosystems are further categorised into wetland classes based upon water regime, substrate, nutrient levels and pH. Wetland classes include bog, fen, swamp, marsh, seepage, shallow water, saltmarsh, ephemeral, Pakihi and gumland (Johnson and Gerbeaux 2004, Clarkson and Peters 2010).

Wetland forms are classified based on topography such as mire (plateau, cushion, domed, string, blanket), riparian, channel, floodplain, delta, shore, basin, swale, flat, river mouth lagoon (hapua), coastal lake and lagoon (Johnson and Gerbeaux 2004).

Vegetation structure is based on the dominant growth form and is categorised into forest, treeland, scrub, shrubland, flaxland, tussockland, fernland, reedland, rushland, sedgeland, grassland, cushionfield, herbfield, turf, mossfield, or aquatic (emergent, floating, flopating leaved, submerged, rafted). Most wetlands have diverse plant communities, nonetheless classification at a fine scale based on vegetation composition is useful for any comparison of wetlands at a regional scale.

Bogs such as peatland forests are poorly drained and are characterised by thick beds of peat (Johnson and Gerbeaux 2004). Bogs are acidic environments (pH <4.8) due to the growth of peat mosses such as *Sphagnum spp*. which bind nutrients and release hydrogen ions lowering the pH of bog soils and water (Clymo 1963). Bogs have slow decomposition rates due to their anaerobic, acidic soil which results in thick layers of peat; a process known as paludification (Johnson and Gerbeaux 2004). Bogs are stagnant, with constant water tables that are just above or near the ground surface and get all their water from rain (Johnson and Gerbeaux 2004). Bogs do not have a groundwater supply and so receive no nutrients from adjacent soils, consequently they are nutrient poor environments (Johnson and Gerbeaux 2004).

Bogs occur on flatlands or gentle slopes and can become domed due to the accumulation of peat (Johnson and Gerbeaux 2004). Bogs support a unique community of cushion plants, bryophytes, lichens and rushes and are widespread across Waikato, Southland, Westland, and the Chatham Islands (Johnson and Gerbeaux 2004).

Fens are similar to bogs but have a shallower peat substrate that is more decomposed as they receive nutrients through groundwater (Johnson and Gerbeaux 2004). Fen soils are less acidic (pH 4–6) and more fertile than bogs and are found on similar topologies as bogs (Johnson and Gerbeaux 2004). Typical vegetation found in New Zealand fens include Manuka, tussock grasses, sedges and some tangle terns (Johnson and Gerbeaux 2004). Fens may be an early successional stage of wetland and as peat builds up over geologic time, fens can develop into bogs (Johnson and Gerbeaux 2004).

Unlike bogs and fens, swamps are high in nutrients and minerals and fed from both runoff and groundwater from surrounding land (Johnson and Gerbeaux 2004). Because of this higher nutrient availability, swamps support greater plant productivity and a wide variety of flora and fauna. Common New Zealand swamp plants include harakeke (*Phormium tenax*), manuka (*Leptospernum scorparium*), sedges (*Cyperaceae*), ti kouka (*Cordyline australis*), maire tawake (*Syzygium maire*), pukatea (*Laurelia novae-zealandiae*) and kahikatea (*Dacycarpus dacrydiodes*).

Wetland formation

Wetlands can be unstable and transient environments, which are often associated with lakes that form due to landslides, tectonic movement, volcanic activity, or changing river courses (Scott 1996). Many of New Zealand's wetlands formed at the end of the last glacial period (18,000 years BP) although transient processes such as landslides and river course changes are continually creating new wetlands (McGlone 2009).

At the beginning of the Holocene (11 500 years BP) many wetlands shifted from herbaceous fens to shrub or bog–fen systems (McGlone 2009). This shift in wetland

type is due to peat aggradation as fens became isolated from groundwater inflow due to a warming climate (McGlone 2009). The climatic warming through the late-glacial and early Holocene periods caused wooded wetlands to increase and spread across New Zealand (McGlone 2009).

Succession

Succession is the process in which plant community composition changes over time due to changes in the biotic and abiotic environment. The goal of restoration projects is often to manipulate and speed up the rate of natural succession. However, predicting the trajectory of natural or manipulated succession is tricky due to the many contributing factors influencing plant community composition. Autogenic succession is driven by the biotic elements of an ecosystem, for example if organic matter accumulates from vegetation debris it can change the soil pH or nutrients. This change in soil nutrients would then would make it either more or less favourable for future seedlings to grow. On the other hand, allogenic succession is driven by abiotic factors such as wind, rain, flooding and volcanic activity.

Clements (1916) was first to describe the theory of plant succession and theorised it was a predictable and directional process driven by autogenic processes that would ultimately form a ‘climatic climax community’. The opposite school of thought is Gleason (1917) who suggested succession is driven by allogenic (abiotic) influences on the biotic community and stochastic events. Tansley (1935) proposed 'polyclimax thoery' whereby vegetation climaxes are controlled by soil moisture, nutrients, topography and fauna. Whittaker (1953) developed 'climax pattern theory' in which the type of climax community varies as the environment changes due to both autogenic forces (e.g. weed species outcompeting native species) and allogenic (e.g. natural water table fluctuation). Lewontin (1969) introduced the 'alternative stable state theory' in which there is no single 'climax community' but many, which shift over time.

Plant–plant facilitation is an ecological process in which the presence of one plant enhances the growth, survival or reproduction of a neighbouring plant. Plant

facilitation is species-specific, as nurse species tend to encourage the establishment of specific facilitated species more strongly than others (Valiente-Banuet and Verdú, 2007, 2008; Castillo et al., 2010). Plant–plant facilitation was traditionally thought to be driven by the amelioration of abiotic stresses such as wind shelter or moisture availability (Callaway 2007) however this does not explain the specificity that exists in plant-plant facilitation. It appears that the amelioration of abiotic stressors via facilitation is necessary in tree establishment regardless of abiotic factors, suggesting that biotic factors drive this facilitation (Gómez-Aparicio 2009). Another potential mechanism of species specific plant–plant facilitation may be due to the presence of beneficial arbuscular mycorrhizal fungal (AMF) (Van der Heijden and Horton 2009; Van der Putten 2009). AMF can promote plant–plant facilitation via mycorrhizal networks (MNs) which allow the transfer of nutrients between plants of the same or different species (Simard et al. 2012).

Traditionally, competition was thought to be the primary mechanism structuring plant communities in all ecosystems, especially productive environments such as wetlands (Gómez-Aparicio 2009). For this reason, succession by inhibition was thought to control the structure of plant communities (Connell and Slatyer 1977). Because of this, wetland restoration projects often focus on the removal of undesirable plant species and weed control before planting target species. However, examples of facilitative succession being more important in community structuring than competition are becoming increasingly prevalent; for example, a meta-analysis by Gómez-Aparicio (2009) found that plant-plant facilitation was necessary for the survival of woody species in all ecosystems, including relatively benign and resource-rich environments such as wetlands. The stress gradient hypothesis suggests that positive interactions (facilitation) should be most common in harsh, ‘stressful’ environments, while competition dominates in more productive, mesic and stable habitats (Bertness and Callaway 1994). Because wetlands are not considered stressful environments (Bertness and Callaway 1994), succession of wetland species via AMF has not been studied in New Zealand.

Wetland hydrology

The hydrology of a wetland is dependent on the amount and type of water, topography of the land, soil type and frequency of floods (Campbell and Jackson 2004), and as a result shows considerable temporal variation. The hydrology of a wetland including the water flow and quantity regulates the inputs and outputs of nutrients and sediments to a wetland. This flow of water and nutrients alters or maintains the soil chemistry, which determines the local flora and fauna and therefore the productivity of the wetland (Campbell 2010, Van der Valk 2006).

Wetlands are dynamic environments and the hydrology of a wetland may change daily, seasonally or annually (Van der Valk 2006). These changes in hydrology affect biodiversity and can alter ecosystem productivity and plant composition (Mitsch and Gosselink 2000, Rokosch et al. 2009).

One of the most important aspects of hydrology in a wetland is surface flooding which can kill or damage vegetation by prolonged saturation of the root zone, eroding soils, or by removing organic matter and nutrients (Bendix and Hupp 2000). In wetlands, the duration and frequency of inundation varies with elevation creating topographical patterns of soil properties and vegetation composition (Dugan 2005, Van der Valk 2006). These gradients are known as hydrological zones and they are classified into the dryland zone (upland soils on slopes or free-draining sites that are usually dry), mesic zone (upland soils that may be waterlogged for short periods of time but are normally dry), moist zone (soils are seasonally saturated and dry in summer), saturated zone (soils are waterlogged most of the year with the water table at or near the ground surface), emergent zones (permanent shallow water with plants partially submerged) and aquatic zones (permanent water with plants submerged) (Buxton 1991, Clarkson and Peters 2010). In this study, I sample *P. tenax* from the saturated and moist zones.

Characteristics of Hydric soils and sediments

Pristine wetlands have natural hydrological fluctuations that control the physical and biotic organic composition of the soil. The hydric soils that form in wetlands are often anaerobic due to periods of inundation (Mitsch and Gosselink 2000).

These hydric soils often have low concentrations of available nutrients due to a slow decomposition rate of organic materials (Bohrer et al. 2004). Hydric soils tend to contain minerals in their reduced form, which may be less available for plants and microbes. However, some minerals may be mobilised in such environments, depending on pH and availability of oxygen. Therefore, wetland flora and fauna must be adapted to deal with stressors such as anoxia, low nutrients, sedimentation and water level fluctuations. Vegetation growth in inundated soils is limited by the redox potential (Eh) of the minerals in the soil (Laanbroek 1990) and the plant's tolerance to inundation.

Reduction and Oxidation (Redox)

A reduction reaction causes a compound to gain an electron, while the opposite process oxidation occurs when a compound loses an electron. In wetlands, inundation lowers the dissolved oxygen available in soil and leads to the reduction of minerals such as sulphide, iron and manganese ions (Vepraskas and Faulkner 2001). Minerals in this reduced state can be toxic to plants and inhibit their growth (Mitch and Gosselink 2000, Vepraskas and Faulkner 2001). The oxidation-reduction (redox) potential of soil is an accurate measure of how much oxygen is available in the root zone of the soil; the more anaerobic the soil is the lower the redox potential (millivolts) and the more reduced minerals in the soil will be (Vepraskas and Faulkner 2001). Dry soil Eh is above +380 to +400 whereas saturated soil Eh is below 350 to 250Mv (Husson 2013). The Eh and pH of soils are negatively correlated (Bohrerova et al. 2004, Van Breemen 1987).

Soil Moisture

The amount of water in soils can be measured either volumetrically or gravimetrically. Volumetric soil moisture (VSM) measures the volume of water in soil pores and can be measured in situ with time-domain reflectometer (TDR) probes. Gravimetric soil moisture (GSM) measures the weight of soil moisture per sample of dry soil. Soil moisture is an important measurement of hydric soils as it indicates the amount of free air available to fungal hyphae and plant roots.
The depth of wetlands water tables varies spatially and temporally, which creates many habitats with differing nutrient levels. The patchiness of soil nutrient levels and the depth of water tables determines plant community structure, as plants are best adapted to specific areas of the wetland (Weiher and Keddy 1995, Bedford et al. 1999).

Present New Zealand values around wetlands/Ecosystem services

Wetlands are of intrinsic value regardless of their functional roles (Fuller 1993). There is increasing awareness of the ecological importance of wetlands leading to increasing interest in wetland restoration. Wetlands provide a range of ecosystem services to humans including water filtration, sediment transport, groundwater recharge, nutrient cycling, flood control, shoreline protection and erosion protection (Buxton 1991, Barbier et al. 2011, Smith et al. 1995, Novitski et al. 1996). Due to these various ecosystem services to society, there are many values surrounding New Zealand's wetlands today including social, cultural, economic, utility, productivity, economic, intrinsic and conservation values.

Provisioning services

Wetlands are highly productive and once provided various food sources for Māori including birds, fish and edible roots. The vegetation of wetlands are also sources of fibre and fuel that lead to a booming flax trade in the 1860s-1930s (Wehi et al. 2009). Wetlands provide other materials that are of value to society such as the raw materials for biochemical extraction of medicines and genetic materials.

Regulating services

Wetlands naturally regulate many systems including carbon sequestration, hydrological flows, and groundwater recharge/discharge. Wetlands can improve

water quality by removing or transforming excess nutrients such as nitrogen and phosphorous as well as metals, toxic organics and sediments. Wetlands can filter water in this way as these substances are removed through uptake of growing plants or by microbes in wetland soil (Buxton 1991). Wetlands act as buffers by slowing down the movement of water through a landscape therefore regulating floods, and protecting against erosion by retaining soils and sediments. Wetlands also provide habitat for pollinators therefore influence pollination services.

Supporting Services

Wetlands support soil formation, sediment retention and the accumulation of organic matter, nutrient cycling storage, recycling, processing, and fisheries maintenance.

Cultural Services

Wetlands are of social value to people as a place of recreation, sport or aesthetic pleasure including waterfowl hunters, fishers, boaters, and bird watchers.

Wetlands are of great historical and current cultural value to Māori and their Kaitiakitanga (guardianship) belongs to the iwi of the area. Māori believe genetic resources are connected to the identities of tangata whenua i.e. they are not public goods and therefore permission needs to be granted before non-iwi access these.

Wetland utility and productivity values include water regime control for flood control, purification systems for improving water quality, erosion control and coastal protection, biomass and protein production in food chains, as well as recharging underground aquifers (Culver and Lemly 2013).
Wetlands are of conservation value due to their genetic, species and habitat diversity, but also as Science and research opportunities (Fuller 1993).

Wetlands are of economic value to people who earn income from tourism, shooting, fishing, harvesting plant species (e.g. Sphagnum and *P. tenax*), as an energy source (peat), as well as a spawning and nursery site for commercial and recreational fish species (Myers et al. 2013).

Wetlands as animal habitats

Wetlands provide an important source of food and habitat for fauna, including plankton, invertebrates and vertebrates (Culver and Lemly 2013). Wetlands are unique habitats for a variety of aquatic and terrestrial animals in New Zealand including birds, fish, reptiles, amphibians and invertebrates. Most of New Zealand's wetland animals are endemic, including mātātā, paradise shelducks, black teal, and giant kokopu (Marlborough District Council, 2004). Wetlands are home to 30% of New Zealand's native freshwater fish species (for example common bully, Northland mudfish, black mudfish, Canterbury mudfish, inanga, giant kokopu, the short finned eel, and banded kokopu) and 22% of our total diversity of bird species (Marlborough District Council, 2004).

Wetland vegetation and community dynamics

Plant community composition is affected by many factors such as soil type, wind exposure, temperature, fire, seasonality. One of biggest influences on plant community composition is the hydrology of the site. Hydrology affects plants as their tolerable range of water stress (droughts, floods, and root saturation) will influence where they can successfully establish. Wetlands are constantly changing and shifting, over time community composition also varies as biotic interactions and physical factors (competition, shade tolerance) lead to new communities (Fuller 1993).

Plants that are dominant in early succession are hardy, fast-growing species that can withstand harsh conditions (wind, drought, frost) and may be ephemeral species. Plants that establish better in late successional stages include longer lived, shade tolerant species that may be more susceptible to frost or wind when establishing and are crucial to restoring a functional wetland.

Kahikatea swamp forests at an early seral stage are often dominated by harakeke although gradually with decreasing wetness as wetlands fill in, kahikatea become more dominant and form large forest stands at the late climax stage (Wardle 1974).

Wetland vegetation affects water and soil chemistry, operating both as a nutrient source (at the base of the food chain), and as a nutrient sink (by removing compounds from the water column and by stabilising shorelines), which improves water quality (Culver and Lemly 2013). The anoxic and wet soils typical of wetlands are stressful and harsh environments for many plants. Despite this, there are a variety of plants that are specially adapted to thrive in these conditions, with the highest plant diversity in mesic or moist zones (Buxton 1991).

In New Zealand, a disproportionately large number of threatened plants are found in wetlands; a result of habitat loss and ongoing degradation (Clarkson and Peters 2010). An example is the endemic giant jointed rush (*Sporadanthus traversii*) which can only be found in four wetlands in New Zealand.

Because wetland plant species have adapted to fluctuating water conditions, the Department of Conservation (DoC) introduced a 'Standard Operating Procedure for Translocation of New Zealand's Indigenous Flora and Fauna', 2002 to advise those involved in restoration plantings the plant species best suited to each hydrological zone.

Plants can be classified by the hydrological zone habitats they typically occupy (dryland, mesic, moist, saturated, emergent and aquatic) (Buxton 1991, Clarkson and Peters 2010). Wetlands are often dominated by monocots namely sedges, grasses, reeds, rushes and flax (Mitsch and Gosselink 2000). Although these plants may occur across entire wetlands, the community composition of plants changes across the hydrologic zones.

Native New Zealand plants that commonly grow in Dryland zones include totara (*Podocarpus totara*), manuka (*Leptospermum scorparium*) and bog pine (*Halocarpus bidwillii*) (Clarkson and Peters 2010).

Common plants in mesic zones are kowhai (*Sophora microphylla*), mahoe (*Melicytus ramiflorus*), pukatea (*Laurelia novae-zelandiae*), and kahikatea (*Dacrycarpus dacrydioides*) (Clarkson and Peters 2010). In moist zones kahikatea, toetoe (*Cortaderia toetoe*), cabbage tree (*Cordyline australis*) and harakeke (*Phorium tenax*) dominate (Clarkson and Peters 2010). Vegetation most common in saturated zones include raupo (*Typha orientalis*), swamp maire (*Syzygium maire*), and

baumea (*Baumea arthrophylla*) (Clarkson and Peters 2010). One physiological adaptation that assist plants living in wetlands are buttressed roots of which help stabilise plants in wet soil. Kahikatea and pukatea are examples of plants with this morphology that allows them to establish on ground too wet for many other tree species, leading to the formation of kahikatea swamp forests (Wardle 1974).

Survival strategies of wetland plants

Wetlands are especially vulnerable to climatic variation and extreme events as they are a frontier type ecosystem with frequent disturbance regimes. Flooding, erosion, storms and salt water encroachment (in coastal areas) are common disturbances to wetlands and many wetland plants have evolved to regenerate quickly after these disturbances (McGlone 2009). Many wetland plants reproduce asexually and have rhizomes (underground stems) that promote with rapid regrowth after inundation or drought-induced mortality which are common in wetlands (Greater Wellington Regional Council 2005). Another advantage of asexual reproduction is that it enables the colonisation of plants in waterlogged soils where seeds cannot germinate and successfully establish (Greater Wellington Regional Council 2005). Raupo is an example of a wetland plant with this vigorous adaptation for asexual reproduction; it dies back over the winter but quickly recolonizes in the spring from underground rhizomes (Williams and Imber 1970). Many wetland plants, including Carex, set seed when water levels are low in summer. These seeds can often stay viable for years in the soil allowing germination when the conditions are right (Greater Wellington Regional Council 2005). Aerenchyma cells in the roots of many monocotyledons conduct oxygen from above ground which can diffuse into the surrounding rhizosphere, aerating the anoxic soil and buffering young roots from toxic mineral damage (Marschner 1992).

Land use history of wetlands in New Zealand

Wetlands have always been an important part of the New Zealand environment as such there is an extensive history of modification and use of wetlands by both Māori and Europeans (Cromarty and Scott 1995, Harmsworth 2013).

Pre-history: It is estimated that wetlands once covered 24,000km^2 of New Zealand, nearly 10% of the country (Hansford 2014). Natural processes such tectonic movement and succession can modify land type, creating, draining or filling-in wetlands.

Māori settlement: Māori first settled around wetlands and inland waterways including coastal estuaries, rivers, lakes, lagoons and springs. Māori used wetlands for sourcing food (eels, fish and birds), for building and weaving materials (harakeke, raupo, and dried moss), clothing and adornment materials (feathers, *P. tenax*), and medicinal plants (*P. tenax*) and for transportation (e.g. waka were most commonly made from tōtara, kahikatea, or kauri (Harris et al. 2005). Māori understood the connection between water and health and used separate sources and purities of water for different purposes depending on its mauri (life force) (Durie 2005). These water resources are taonga (treasures) which are spiritually significant and connected to the identities of the Tangata Whenua through whakapapa. Māori legend tells us water is the blood of Papatūānuku, the Earth Mother, and the tears of Ranginui, the Sky Father. Waterways are also sacred due to taniwha (spiritual beings) who reside in them and protect tangata whenua. Iwi and hapū are responsible for the kaitiaki of the wetlands within their boundaries and failure to do so results in diminished mana for the iwi.

When Māori first arrived in New Zealand, they burnt heavily wooded areas of wetlands, shifting the dominant wetland type from stable forested fens and bogs to more dynamic swamps and lagoons (McGlone 2009). As oligotrophic-forested wetlands were burnt, this created wetter, more herbaceous systems (McGlone 2009). Because of the increased productivity of these wetlands, birds, tuna and freshwater fish thrived (McGlone 2009).

Māori have a different attitude and view of the land then Westerners who traditionally have an anthropocentric view of natural resources. Māori do not consider themselves separate from nature, they believe everything in the world is related and the relationships between things can be traced through whakapapa. Whakapapa is told through kōrero (stories) that explain the relationships between

all things and help Māori to place themselves within the world as tangata whenua (people of the land).

European Settlement: At the time of European settlement, New Zealand had roughly 6700 km^2 of freshwater wetlands, by the early 1900s this had been reduced 1000 km^2 (Buxton 1991, Hansford 2014). European settlers were largely Christian and their attitude towards the environment can be summed up in the bible by 1:26-28 "And God said unto them… have dominion over the fish of the sea, and over the fowl of the air, and over every living thing that moveth upon the earth". This anthropocentric view of natural resources resulted in the decline of wetlands by land drainage and clearance, gold mining, flood control, agriculture, flax milling and kauri gum digging. Now less than 2 percent New Zealand's total land area is wetland, which equates to a 90 percent loss in wetlands former size (Buxton 1991). The loss of wetlands is uneven across the country, with the North Island losing 95 percent and the South Island 84 percent (Hansford 2014). The loss of New Zealand's wetlands had direct consequences such as the decline in aquatic birds and native freshwater fish (McDowall 1984). People started to realise the importance of wetlands for flood control (Campbell and Jackson, 2004) and in the 1980S the New Zealand government and several voluntary programs began to manage the conservation of wetlands.

History of Wairio

Wairio wetland on the South-East shore of Lake Wairarapa is part of the largest wetland complex in the lower North Island which supports flora and fauna of national and international importance (Fuller, 1993). It is culturally important to Ngati kahungunu ki wairarapa and Rangitane o wairarapa as one of the oldest pre-European Maori settlement sites (Forest and Bird 2014). Māori first settled here around 800 years ago and hunted the plentiful waterfowl and freshwater eel (Forest and Bird 2014).

In the 1840s Europeans began to settle the area and clear vegetation for farming leading to disagreement between Māori who wanted to protect the flood prone

wetlands and farmers who wanted dry pasture for agriculture (Airey et al. 2000). The Crown took ownership of the area in 1896 and over the subsequent 91 years Lake Wairarapa was partially drained, the land was further cleared of forest and converted to pasture (Airey et al. 2000).

During the 1960s-1970s willow (*salix*) planted in the Wairarapa Valley for erosion control as part of the Lower Wairarapa Valley Development Scheme (LWVDS) invaded the wetland and were later bulldozed into parallel wind- rows running across the Wetland (Airey et al., 2000). In 1987 the Department of Conservation (DOC) was given stewardship of the area for conservation and in 2005 a land management agreement to restore Wairio wetlands was signed between DOC and Ducks Unlimited (Ducks Unlimited, 2012).

Currently Wairio wetland is being restored as a part of the Wairarapa Moana Wetland's Project (WMWP) with a goal to "restore a pristine wetland to the site" by focussing on Water Supply and Retention, Re-vegetation, Earth Works and Predator Control (Ducks Unlimited 2012). The vision for this restoration project is in 100 years "Wairio will be a fully functional wetland supporting abundant native flora and fauna, with natural hydrological regimes linked to the wider Wairarapa-Moana complex, where people can visit for recreation and to appreciate a natural ecosystem restored to pristine condition" (Ducks Unlimited 2016).

Vegetation of Wairio

At present Wairio wetland is a pastoral landscape with remnant native trees including kahikatea (*Dacrycarpus dacrydioides*), totara (*Podocarpus totara*), and cabbage trees (*Cordyline australis*). Wairio wetland has many non-native invasive plants including tall fescue (*Schedonorus arundinaceus*), beggarticks (*Bidens frondosa*) and browntop (*Agrostis capillaris*). Four blocks have been fenced off within the wetland and restored to varying degrees within the last ten years (stages 1-4). Stage 1 had modest earth works and replanting in 2005-2006 and in some areas topsoil scraped and then further replanting in 2012. Stage 2 had an earth dam constructed with areas of soil scraped and built up into islands, then was planted in

2007. Stage 3 had significant earthworks to create a dam and was replanted in 2012 (Ducks Unlimited, 2012). Stage 4 is the most recent area of restoration and was replanted in 2014.

Restoration ecology

Restoration ecology is the deliberate manipulation of succession to promote movement towards a specific ecosystem state that promotes greater ecosystem services or benefits. However, what a successful restoration project encompasses differs with people and their values, making it important to acknowledge these values when planning a restoration project.

Water fowl hunters: The main wetland restoration goal of water fowl hunters is to increase the number of game birds. This may be achieved through predator control, and increasing available habitat, nesting sites, vegetation changes.
Māori cultural purposes: Māori environmental concepts focus on keeping specific parts of the natural environment pure, unpolluted, and connected to retain mauri (Durie 2005). Māori view wetlands as taonga.

Fish habitat: The restoration goals of recreational fishers, Māori and commercial fisheries do somewhat overlap, although all differ slightly. Commercial fisheries want wetlands to provide nursery and spawning sites for commercially valuable fish species such as eel and whitebait. Recreational fishers want fish habitat to support a wide variety of desirable fish, and Māori are concerned with restoring traditional fishing areas and increasing populations of traditionally important fish including eel and whitebait.

C-sequestration: Wetlands soils facilitate carbon sequestration (C-sequestration) as the saturated anaerobic soils and the slow decomposition rate act as carbon sinks (Whiting and Chanton 2001). If increased C-sequestration is a goal in for wetland restoration this can be achieved through changing the hydrology of the wetland to increase the surface area of saturated soils.

Fungal communities

Fungi are common in wetlands from obligate aquatic, semi aquatic and terrestrial species all of which play a large role in decomposing organic litter (Van der Valk 2006). Most fungi are aerobes and this is partly the reason why decomposition rates are slow in waterlogged anaerobic soils which leads to the build-up of organic matter in wetland soils (Van der Valk 2006). A group of soil fungi called mycorrhizae are plant symbionts that typically increase growth of wetland plants (Dunham et al. 2003). Mycorrhizae comes from the Greek words μύκης / myc- meaning fungus and ῥίζα /rhiza meaning root and refers to the symbiotic relationship between the two (Bonfante and Anca 2009). Arbuscular mycorrhizal fungi (AMF) are ancient root symbionts that are thought to have been instrumental in the colonisation of plants on land (Hardie and Leyton, 1981, Allen 1991, Smith et al. 2003, Simon et al. 1993, Helgason and Fitter 2005, Smith and Read, 2008). This long history of this symbiosis has resulted in coevolution in an estimated 90% of vascular plants and more than 80% of all living land plant families (Wang and Qui 2006, Parniske 2008) although many plant species have yet to be examined for the presence of AMF (Brundrett 2002).

In AMF, both the physical and genetic extents of an individual is hard to determine as no formal operational species concept exists (Taylor et al. 2000, Rosendahl 2008). Some estimates have put the biomass of living and dead AMF hyphae at over 50% of total soil microbial biomass (Read and Perez-Moreno 2003) and the length of AMF mycelium on the order of metres per cubic centimetre of soil (Olsson et al. 2002).

AMF increase plant nutrition and the availability of limited resources, predominantly phosphorus (P) (Allen 1991, Schweiger and Jakobsen 1999) and micronutrients such as copper, cobalt, magnesium, manganese and zinc (Cooper and Tinker 1978, Clark and Zeto 2000, Leake 2004). AMF can also benefit the host plant by improving water balance (Augé 2001, Augé et al. 2014), reducing the host's uptake of phytotoxic heavy metals (Göhre and Paszkowski 2006) and reducing root invasion by microbial soil-borne plant pathogens (Newsham et al. 1995). AMF can

also increase soil particle aggregation which benefits the plant by increasing stability and resistance to flooding (Rillig and Mummey 2006). AMF hyphae extend nutrient access to plants as they can reach into smaller soil pore spaces than fine roots can penetrate. In return, AMF receive carbohydrates from the host plant with carbon at 10–20% of the plants net primary productivity (Leake 2004, Hogberg and Read 2006).

AMF and Plant Community Dynamics

AMF affect plant diversity and community composition through carbon and nutrient transfer via their mycelial networks (Read, 1991; Francis and Read, 1994; Hartnett and Wilson, 1999; van der Heijden and Sanders, 2002).

It is possible for the mycelium of a single AMF to be connected to several plants (Newman 1988) and in natural systems this mycelial network can connect different plants over large areas (Giovannetti et al. 2004). Mycelial networks may influence the outcome of plant-plant competition, biodiversity and ecosystem productivity through carbon transfer (van der Heijden and Sanders 2002, Hart and Reader 2002, Mummey and Rillig 2006, Toljander et al. 2007, Van der Heijden et al. 2008, Smith and Smith 2011).

AMF have high functional diversity as different mixtures of host plants and AMF have diverse effects on the various aspects of symbiosis. Therefore, the different functions of AMF in an ecosystem and how this is related to their genetic classification is increasingly studied (Lee et al. 2013)

AMF diversity is likely underrepresented when classified based on morphology, and currently only approximately 240 species have been described this way within a fungal phylum, Glomeromycota (Krüger et al. 212, Schüβler A, Walker 2010). Molecular studies have proposed that diversity of these fungi is far greater (Fitter 2005). High genetic variation of AMF has been described within species, even within a single AMF spore (Clapp et al. 2001, Vandenkoornhuyse and Leyval 1998).

Plant Host Specificity

Many AMF have low host specificity (Smith and Read 2008) and reciprocal rewards between symbionts stabilise the AM symbiosis (Kiers et al 2011). It is possible to form associations of almost any mycorrhizal plant with any AMF in the lab (Smith and Read 2008). However, in nature, closely related plants often host dissimilar AMF communities (Veresoglou and Rillig 2014). These AMF communities may vary significantly among members of a plant community (Oepik et al. 2008), and the AMF communities in plants are non-random assemblages (Davidon et al. 2011). While many AMF are broad host generalists, there are some that appear to be specialists, as they occur exclusively on a single host (Lang et al. 2011).

Environmental proclivity of AMF

Although some AMF persist in inundated conditions, associated stressors such as anoxia and reductions in soil pH can have a significant negative effect on the mycorrhizal colonisation in wetland plants (Miller and Bever 1999, Cornwell et al. 2001, Escudero and Mendoza 2005). Field studies show that inundation usually has either no significant or a negative effect on AMF colonisation of plants (Bauer et al. 2003; Bohrer et al. 2004) although this is dependent on the degree of colonisation before inundation (Dolinar and Gaberščik 2010). Previous research has found that once AMF colonisation is established (late in the growing season), subsequent inundation does not affect colonisation levels (Miller and Sharitz 2000, Dolinar and Gaberščik 2010). Arbuscular mycorrhizal communities have been shown to change over time with succession of host plant communities and there is a growing body of evidence that suggests host plants control which AMF colonize their roots (Johnson et al. 1991, Eom et al. 2000, Sanders 2003, Limpens and Geurts 2014).

Seasonality effect

The richness and colonisation intensity of AMF have been shown to fluctuate seasonally in wetlands (Bohrer et al. 2004). Colonisation intensity of AMF is defined as the percent root length colonised by AMF arbuscules, vesicles, spores, and hyphae (Bohrer et al. 2004). Some studies link this seasonal fluctuation in AMF richness and colonisation intensity to abiotic factors such as water table levels

and/or soil moisture levels (Anderson et al. 1984, Miller and Bever 1999). Results of other studies suggest that plant phenological events including flowering, root and vegetative growth are the primary drivers of seasonality in AMF communities (Miller 2000, Carvalho et al. 2001, Bohrer et al. 2004). It has been suggested that high levels of AMF late in the growing season may be caused by the higher demands of P in plants due to flowering (Mandyam and Jumpponen 2008). It is probably a combination of plant phenological events and abiotic factors that determine wetland colonisation intensity depending on the season and time of year.

Life cycle and morphology of AMF

There is no evidence that the AMF reproduce sexually. Under ideal conditions AMF spores germinate, form appressoria on host plant roots and establish a new mycorrhizal symbiosis. Spores may be formed on the hyphae either within (endospores) or outside the root. There are some species of AMF that can colonise new host plants directly from hyphal fragments in the soil (Smith and Read, 1997). Because of these differences in strategies of colonisation, it is difficult to elucidate the effect of AMF colonisation type on the host plant.

Wetland plants and AMF

Early studies postulated that AMF would not be important in wetland ecosystems as they are obligatory aerobic therefore would not survive frequent inundation (Shah 2014, Sondergaard and Laegaard 1977, Farmer 1985, Newman and Reddell 1987, Rickerl et al. 1994, Miller et al. 1999, Turner et al. 2000, Cornwell et al. 2001). However, subsequent studies have found some AMF tolerate anoxic conditions possibly by their host plant ameliorating these effects (Van der Valk 2006, Weishampel and Bedford 2006).

It has been suggested that some AMF species may have lower oxygen requirements than previously thought, and it is thought these AMF obtain their oxygen directly from the host root or as indirectly as oxygen diffuses out of the root and into the rhizosphere (Brown and Bledsoe 1996, Miller and Bever 1999, Cornwell et al. 2001).

It is now known that AMF occur in many types of wetlands (Rickerl et al. 1994, Van Hoewyk et al. 2001, Bauer et al. 2003, Gómez-Aparicio 2009) and mycorrhizal symbioses play a significant role in the growth of wetland plants (Dunham et al. 2003).

Methods of detection and quantification of AMF

Techniques used to detect and quantify AMF in soil and roots are becoming increasingly sophisticated from traditional microscopy and staining methods first used in the 20th century, to biochemical methods based on serology (Koide and Mosse 2004), isozyme variation revealed by gel electrophoresis (Hepper 1986), lipids (Bentivenga and Morton 1994) and sterols (ergosterol) to molecular methods based on genotyping and DNA sequencing (e.g. Sanger, next generation amplicon sequencing) (Redecker 2000).

Microscopy methods:

AMF have traditionally been classified based on asexual spore morphology, although this is error prone as many AMF have similar looking spores (Redecker et al. 2003). In absence of spores, AMF colonisation can be classified and quantified by staining and counting the percentage of root length colonized by fungal structures such as hyphae and arbuscules (Koide and Mosse 2004, Kaminskyj et al. 2008, Vierheilig et al. 2005, Wright 2005, Wang et al. 2010).

Biochemical methods:

Ergosterol is a fungal specific sterol that can also be used to estimate biomass of mycorrhizal fungi in roots and soil (Niemenmaa et al. 2008). Recently AMF quantification has been developed using fatty acid (phospholipid and neutral lipids) analysis. The fatty acid (16:1ω5c) is used as an indicator of AMF abundance in soils and roots as AMF contain it in greater concentrations than other fungi and it does not occur in plant cells (Olsson 1999, Olsson and Johansen 2000). Phospholipid fatty acid analysis (PLFA) is an accurate measure of live AMF biomass (hyphae, arbuscules, coils and vesicles) and neutral lipid fatty acid analysis (NLFA) is a good measure of AMF storage structures such as vesicles and spores (Olsson 1999).

Molecular methods:

Molecular techniques allow the identification of the fungal community in soil or roots which is useful for classifying the active community colonising host plants as well as other species present in the soil (Redecker 2002, Redecker et al. 2003). Several molecular methods have been developed to identify AMF colonizing a host plant or to identify AMF in soil, including: sequencing (Kuhn et al. 2001); restriction fragment length polymorphism (RAPD) (Lanfranco et al. 1995, Powell et al. 1996); amplified fragment length polymorphism (AFLP) (Powell et al. 1996, Rosendahl and Taylor 1997); Metabarcoding (Stockinger et al. 2010, Bianciotto et al. 2011, Schmidt et al. 2013); Single Stranded Conformation Polymorphism (SSCP) (Kjøller and Rosendahl 2000); and, the amplification of different regions of the ribosomal DNA (Fuchs and Haselwandter 2008, Kjøller and Rosendahl 2000). One drawback of molecular analysis is the need for specific primers for AMF (Krüger et al. 2009, Kohout et al. 2014). Ribosomal RNA (rRNA) regions differ in their ability to differentiate closely related AMF species (species resolution power) as well as the degree to which well-determined sequences are represented in public sequence databases (Kohout et al. 2014). This makes the selection of SSU (small subunit of rRNA), LSU (large subunit of ribosomal RNA) or internal transcribed spacers of ribosomal DNA (ITS) extremely important for accurate fungal community representation (Kohout et al. 2014). ITS is a noncoding region of rRNA which is often used in AMF studies as it is universal and the fungal barcode. One drawback of ITS is that there are no fungal specific primers for the region and so when amplifying AMF from plant roots, you may get a lot of non-target amplification such as plant or bacteria. The 18 SSU has AMF specific primers (e.g WANDA-AM1) however, as a coding region many areas are highly conserved and therefore are not variable enough to identify sequences at high resolution.

To detect and quantify AMF in this study I will be using a combination of microscopy and molecular sequencing methods. Microscopy will be used on stained root samples to determine colonization levels of AMF in *P. tenax*. A Next generation sequencing approach will be used to characterize AMF communities of *P. tenax.* Next generation RNA sequencing has clear advantages over other transcriptomic

approaches: it has high sample throughput; is sensitive enough for very low levels of RNA; it has low background noise; it can detect transcripts without a known genomic sequence and is relatively low cost for large samples (Salvioli and Bonfante 2013). Due to the different specificity of ITS and 18 SSU primers, I will characterise the AMF communities of *P. tenax* using two sets of primers to target both regions of the gene. This will generate two fungal libraries and I can compare the two molecular markers ability to detect AMF community diversity. The SSU (small subunit of rRNA) is the most appropriate region to amplify for this study as it provides the highest AMF species resolution power which will provide accurate fungal community representation (Kohout et al. 2014).

Chapter 3: Methods and Results

Study site

Wairio wetland is on the southwest shores of Lake Wairarapa in the Lower North Island of New Zealand and is a part of a larger wetland complex known as the Wairarapa Moana. Wairio is 132.3ha and is divided into 4 sites with different ages of restoration phases. The first site, Stage 1, is an 8-hectare area that underwent earthworks and restoration planting in 2002. It is the oldest stage of restoration, and was 10-years old at the time of sampling. Stage 2, is an 8.3ha area that was established in 2004, it was 8-years old at the time of sampling. Stage 4 was planted in 2013, and was less than 1-year old at the time of sampling. It is the largest restoration stage at 30.6ha (Ducks Unlimited, 2016). The land between the stages is a mixture of paddocks (intermittently grazed by cattle), bodies of permanent water and ephemeral bogs. The species used for the restoration plantings were *P. tenax, Dacrycarpus dacrydioides, Cordyline australis, Olearia virgate, Podocarpus totara, Coprosma robusta, Coprosma propinqua, Pittosporum tenuifolium,* and *Leptospermum scoparium.* There are also several remnant *Dacrycarpus dacrydioides* and *Cordyline australis* scattered across the wetland that are likely to be 50- 100 years old. Wairio wetland sits in a depression between the Rimutaka/ Tararua ranges to the North-West and the Ruamahanga/ Aorangi ranges to the South-East and, like most of Wairarapa valley, it has alluvial soils. The climate is generally dry and mild with a mean annual temperature is 12 ^{0}C (Beadel et al. 2000). The area surrounding Wairio receives 800-1300 mm of precipitation annually, with the highest rainfall occurring in spring (September to November) (Beadel et al. 2000). The wetland is primarily fed through shallow ground water, but rain, and surface runoff from adjacent pastures and sub-surface flow from both Lake Wairarapa and the pastures all contribute to the water body (Mahapara 2016).

Map of Wairio and surrounding areas. Yellow shading denotes the restoration sites in Wairio. From left to right: 10-year old restoration site, 8-year old restoration site, 1-year old restoration site. Map from google earth.

Sample collection was carried out at Wairio wetland in early December 2014 (summer) and mid July 2015 (winter). Within each restoration stage, three sites were sampled. At each site, a water regime was determined (from inundated areas to dry) and plots constructed. Plots were either low elevation (with a high-water table) or high elevation (with a low-water table). Within each plot, 3 *P. tenax* were randomly selected and 2 soil/root cores taken using a 15x5cm bulb planter. In order to sample a variety of roots from each plant, one core was collected from near the root crown and a second was collected near the 'drip line' of the plant. These two cores were pooled to a single sample for downstream analysis. A total of 96

samples were collected, 48 in summer and 48 in winter. Samples were kept on ice in a chilly bin until back in the lab where they were refrigerated at 4°C. The following data were collected from soil samples: pH; electrical conductivity (EC); percent moisture by weight (MCw); and percent soil moisture by volume (MCv). Soil properties were measured within 48 hours of collection.

Laboratory analysis of soil properties

Soil Moisture

Volumetric soil moisture content is the ratio of water volume relative to the soil volume. Volumetric soil moisture was calculated in situ using a Time Domain Reflectometry (Trime, HD2) soil moisture probe to give percent soil moisture by volume (MCv).

Soil moisture was also assessed gravimetrically. Gravimetric soil moisture content is the ratio of water weight relative to dry soil weight. Samples were scooped into pre-weighed aluminium pie dishes and weighed to determine the wet soil weight. These pie dishes were then placed in a contherm oven at 100°C until there was no further weight/water loss (72 hours). The samples were then reweighed to measure the dry soil weight and the percent moisture by weight (MCw) was calculated.

Oxidation reduction potential

Oxidation reduction potential was calculated in the lab using an EcoSense PH100 and the Hanna HI98121 ORP/pH/Temperature Tester. Samples were placed in a beaker and an equal volume of distilled water was added to make a slurry. Three measurements of redox potential (mV) were taken per sample and one of pH and temperature.

Root sampling

P. tenax roots were removed from soil cores by hand by gentle shaking and rinsing in tap water. Isolated roots were then washed in distilled water and cleaned of soil particles by sonication using a Kudos SK3300BT. Clean roots were stored at -18°C.

Quantification of fungal colonisation

Subsamples of roots from each soil core were taken to quantify the percent of root length colonised by AMF. Subsamples of 5 fine, clean, turgid and entire 1 cm root segments were cleared and stained using the acid fuchsin microwave irradiation method (Dalpé and Séguin 2013). Briefly, roots were cleared in KOH 2.5 % to empty cortical cells of cytoplasm and nuclei and to facilitate stain penetration into root tissues. For 5 roots, 50-mL beakers were filled with 15‑20 mL of KOH 2.5 %, deposited in circle around the middle of the turning plate inside the microwave oven with a maximum of five beakers per clearing assay. The tests were performed in a domestic microwave oven (Kenmore, model #89780, 2450 MHz, 900 W). For the root-clearing step, samples were microwaved on high power in 15-30s increments until roots were soft and depigmented. After staining roots were stored in 50% glycerol solution at 4°C.

Cleared and stained root tips were randomly dispersed in a petri dish with 1cm grid lines drawn on. Mycorrhizal colonisation was assessed using both a dissecting microscope and a compound microscope at 400x power by counting the number of root intercepts and number of mycorrhizas separately (Giovannetti and Mosse 1980).

Characterisation of the fungal community

Root DNA extraction

Several different DNA extraction techniques were trialled to find the best possible protocol for extracting AMF from *P. tenax* roots (Appendices table 5).
DNA extraction techniques trialled included the powersoil MOBIO kit, the Genejet plant DNA kit, and combinations of the kits using different root crushing techniques including liquid nitrogen, garnet beads, and carbide beads with variations of the mass of beads used.

The DNA extraction technique that produced the best results was using a CTAB-chloroform extraction on -18°C frozen roots, ground with a micro pestle sand

manually. The CTAB protocol was modified from Doyle and Doyle (1990). The detailed protocol I developped appears in Apendix 10.

PCR conditions and equipment

A 537bp region of the AMF 18S rRNA (SSU) gene was selectively amplified using the AMF specific primer pair WANDA and AM1 (Dumbrell et al. 2011).
Polymerase chain reactions (PCR) were set up in 25 µL volumes comprising:
12.5 µL Thermo Master Mix, 0.5µM forward primer, 0.5µM reverse primer, 1µl template DNA. The thermal conditions that were applied were as follows: initial denaturation step of 95°C for 2 minutes, followed by 30-35 cycles of 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 50 seconds with a final extension step of 5 minutes.

In some cases, where samples failed to amplify using the above protocol, samples were first amplified with NS1-NS4 (12 samples), this amplicon was diluted 1:100 and then used as the template for a nested PCR using the WANDA–AM1 primer pair. The thermal conditions for NS1-NS4 were as follows: initial denaturation step of 94°C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds, 40°C for 60 seconds, 72°C for 60 seconds with a final extension step of 10 minutes.

The second Internal Transcribed Spacer (ITS2) region of the AMF ribosomal RNA gene was selectively amplified using the eukaryotic primer pair ITS3–ITS4 designed by White et al. (1990). Polymerase chain reactions (PCR) were set up in 25 µL volumes comprising: 12.5 µL Thermo MM, 0.5µM forward primer, 0.5µM reverse primer, 0.5µM Magnesium chloride, 1M betaine and 0.4mg/ml BSA, 30 µg PVP.
The thermal conditions that were applied were as follows: initial denaturing step of 95°C for 2 minutes, followed by 35 cycles of 95°C for 30 seconds, 60°C for 37 seconds, 72°C for 50 seconds with a final extension step of 5 minutes.

PCR products were run on 1.5% agarose gels at 70 volts for 40 minutes then stained with ethidium bromide and visualised by UV transillumination. PCR products of the correct size were purified using a zygmo clean up kit.

For 19/48 of samples the ITS2 amplicon consisted of two products of similar size, which appeared as a double band on the agarose gels. It was not possible to resolve this by PCR optimisation. For these samples the PCR products were run on 1% agarose TAE gels and the 500bp band was excised carefully under UV light. The selected amplicons were then cleaned using a Zymo spin filter kit (kit name/number).

Sequencing

Two metagenomic libraries were generated, one produced with the molecular marker 18s and the other with ITS. Next-generation sequencing with the Illumina MiSeq sequencing platform number was used to sequence DNA. Sequencing and bioinformatics was carried out by the Australian Genome Research Facility (AGRF). Paired-ends reads were assembled by aligning the forward and reverse reads using PEAR1 (version 0.9.5). Primers were identified and trimmed. Trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8) 4 USEARCH 2,3 (version 8.0.1623) and UPARSE software. Using usearch tools sequences were quality filtered, full length duplicate sequences were removed and sorted by abundance. Singletons or unique reads in the data set were discarded. Chimera checking was performed, and chimeric sequences were discarded. Remaining sequences were clustered in Operational Taxonomic Units using appling a 97% sequence identity threshhold Using Qiime, taxonomy was assigned using the UNITE database 7 (Kõljalg et al. 2005).

Statistical Analysis

I used rarefaction to determine that I sampled the AMF community in *P. tenax* roots sufficiently to determine the response of AMF communities to the experimental treatments (depth of water table, season of sampling, restoration stage) (Appendix. 6).

AMF species richness was counted as the number of OTUs in a sample. I calculated the proportional abundance of OTUs by dividing the number of each OTU per sample by the total number of OTUs per sample.
I constructed root sample X AMF OTU matrices for
1) the presence and absence of OTUs
2) the absolute abundance of OTUs
3) the proportional abundance of OTUs

I calculated Bray-Curtis dissimilarity matrices for each matrix. I used non-metric multidimensional scaling (NMDS) ordination to visualize plant root samples in OTU-space (i.e. "species-space") and selected the method that maximized AMF community dissimilarity by treatment.
I used Dufrene-Legendre Indicator Species Analysis to test for siginifcant indicator OTUs for season and restoration site both 18S and ITS datasets based on MONTE CARLO tests. I calculated AMF and total fungal species richness using Shannon-Wiener Index (Shannon 1948) across season and restoration site age for the 18S and ITS datasets.
I calculated species eveness using Pielou's evenness measure (Pielou 1966) across season and restoration site age for both the 18S and ITS dataset.
I used Kruskall-Wallis chi-squared test to determine if AMF colonisation (arbuscules, hyphae, spores) on *P. tenax* roots changed significantly in to the experimental treatments (depth of water table, season of sampling, restoration stage).

I then used permutational multivariate analysis of variance (perMANOVA) to determine if the AMF community on *P. tenax* roots changed significantly in response to the experimental treatments (depth of water table, season of sampling, restoration stage). The statistical significance of differences in the AMF communities among treatments was assessed using the analysis of Multiple Response Permutation Procedure (MRPP), where delta values indicate the overall weighted measure mean distances among groups and p-values indicate the statistical signifcance of difference among these means. Statistical analyses were performed using R (R Core Team 2013) and PC-ORD (McCune and Mefford 2011).

Results

OTU richness

Across all 48 samples, I identified 51 operational taxonomic units (OTUs) for the 18 SSU sequences and 691 OTUs for the ITS sequences, at the 97% sequence similarity threshold. Of these, 48 18 SSU and 563 ITS OTUs putatatively belonged to the phylum Fungi. Figure 1 shows the overall composition of the fungal libraries at the Phylum and Family taxonomic ranks.

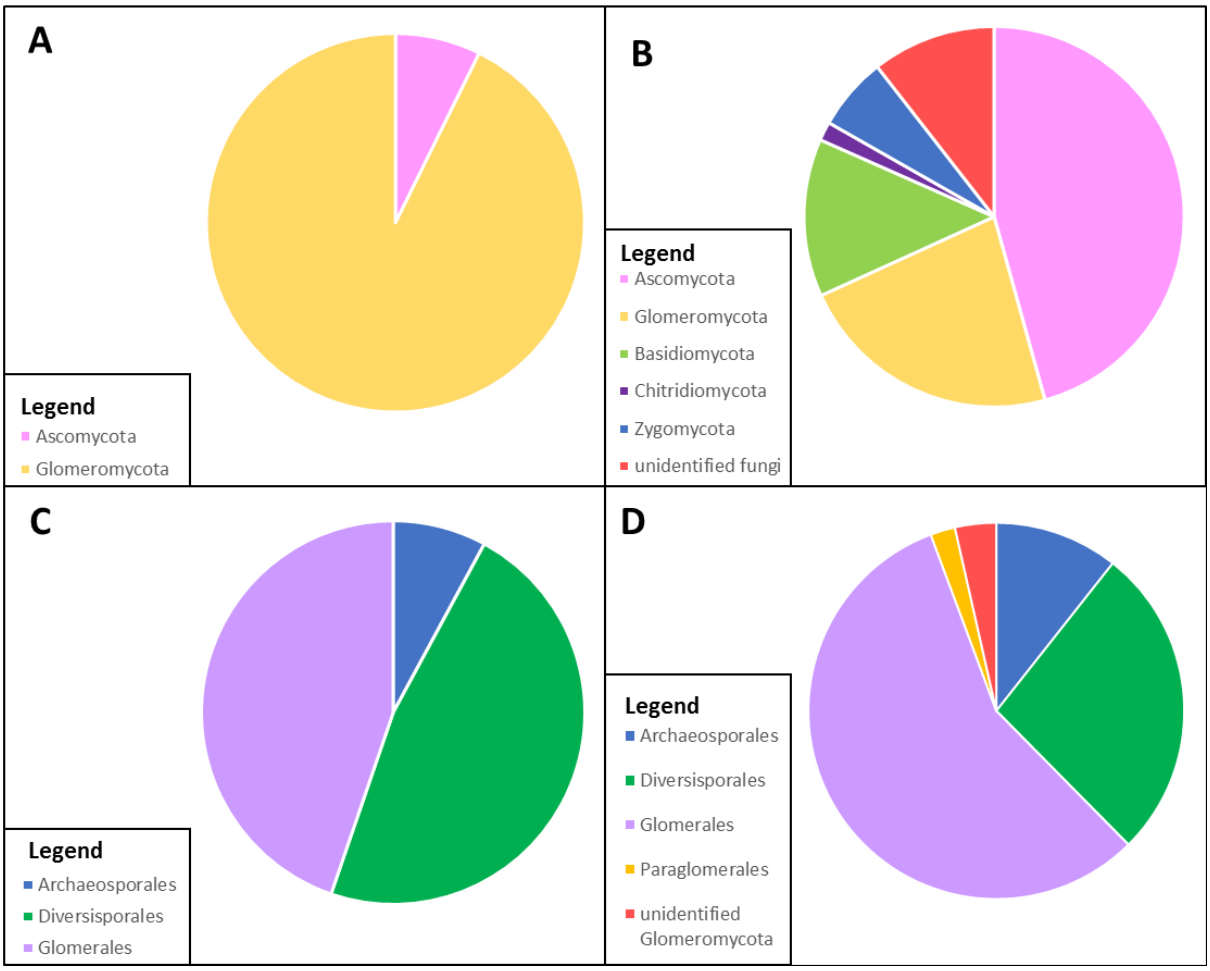


Figure 1: Proportional distribution of fungal taxa identified from *P. tenax* soil cores. (A) 18s sequence assignment of fungi by phylum. (B) 18s sequence assignment of Glomeromycota by order. (C) ITS sequence assignment of fungi by phylum. (D) ITS sequence assignment of Glomeromycota by order.

18S:

18s sequence assignment produced 51 OTUs, 10 of which could not be assigned to any taxanomic rank. Among the 41 fungi (Fig. 1A) 3 belonged to the phylum Ascomycota and 38 belonged to the phylum Glomeromycota.

Among the Glomeromycota (Fig 1B), 18/38 were Diversisporales (47%), followed by 17/38 Glomerales (45%), and 3/38 Archaeosporales (8%). Only 8/18 Diversisporales could be assigned to three families. Acaulosporaceae 3/8 (37.5%) and Gigasporaceae 3/8 (37.5%) were more commonly found then Diversisporaceae 2/8 (25%).

ITS:

ITS sequence assignment produced 693 OTUs, 12 of which could not be assigned to any taxanomic rank. Of the 681 OTUs, 626 (91.9%) were assigned to Kingdom Fungi. Of the remaining non-fungal OTUs 54 (7.9%) represented Kingdom Plantae and 1 Kingdom Protista (0.2%). Among the 626 fungi (Figure 1C) 286 (45.8%) belonged to Ascomycota, 141 (22.5%) belonged to Glomeromycota, 84 (13.4%) belonged to Basidiomycota, 10 (1.6%) belonged to Chitridiomycota, 39 (6.2%) belonged to Zygomycota and 66 (10.5%) were unidentified fungi.

Among the 141 Glomeromycota (Figure 1D) 15/141 (10.6%), were Archaeosporales 38/141 (27%) were Diversisporales, 80/141 (56.7%) were Glomerales, 3/141 (2.1%) were Paraglomerales and 5/141 (3.5%) were unidentified beyond Glomeromycota. ITS Identified more OTUs in Glomeromycota, and to higher taxonomic resolution than 18S. The OTUs in Glomeromycota were from four orders; the Glomerales, Diversisporales, Archaeosporales and the Chaetothyriales. The most dominant genera were in the order Diversisporales, followed by Glomerales, representing approximately 68%, and 30% of the total OTUs, respectively (Fig. 1A). Of the 51 OTUs identified, 2% could not be identified beyond phylum.

Of the 563 ITS OTUs detected, 147 (26.1%) were putatatively identified as belonging to Ascomycota, 45 (7.9%) were Basidiomycota, 128 (22.7%) were Glomeromycota, seven (0.1%) were Chytridiomycota, and 236 (41.9%) were unidentifiable at the phylum level. From the 48 sequenced root samples, we obtained 46 18s OTUs,

excluding possible chimeras, non-fungal sequences. Of the 46 18s OTUs detected, 1 (2.2%) was Ascomycota, 39 (84.8%) were Glomeromycota, and 6 (13.0%) were unidentifiable at the phylum level. The highest OTU richness found on an individual P. tenax plant was 110 OTUs with the ITS dataset and 29 OTUs with the 18s data.

OTU richness across restoration sites

The pattern of OTU richness by restoration site was different for ITS and 18S (Fig. 2A and 2B). This suggests that the two markers discriminate against different taxonomic groups and that the sites have different proportions of these taxa.

For the 18S library, in summer the 1-year old restoration site was dominated by Glomeromycetes; Diversporales and Glomerales. The 8-year site was also dominated by Glomeromycetes; Diversisporales, Glomerales; uncultured Glomus. The 10-year old site Glomeromycetes; Diversisporales; Acaulosporaceae, Gigasporaceae; Gigaspora, Glomerales; Glomeraceae, uncultured Glomus.

OTU richness varied within and among restoration site age with the highest richness occurring in the one-year old restoration site for the ITS dataset (Fig. 2A and 2B). Both molecular markers showed differences in AMF diversity across restoration sites.

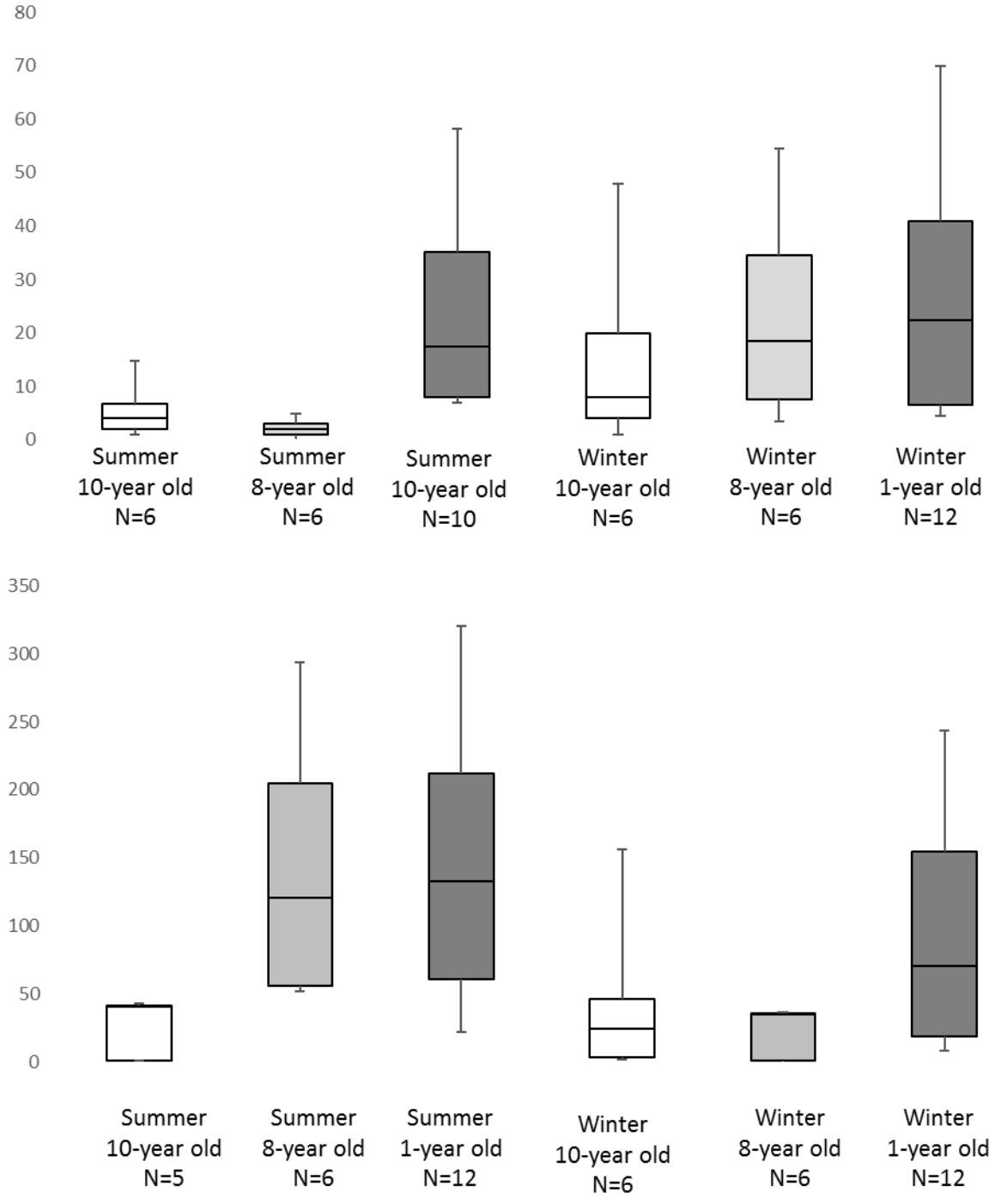


Figure 2. OTU richness across restoration stages in Wairio wetland using the molecular marker (A)18S and (B) ITS.

Table 2: Results of Non-parametric MANOVA analysis of the Bray-Curtis dissimilarities for 18S based fungal OTU community structure in *P. tenax* roots in relation to soil Eh (redox), pH, TDR (soil moisture using time domain reflectometry), season, restoration age, gravimetric soil moisture, and their interaction. Df = degrees of freedom; SS = sum of squares; MS = mean sum of squares; Pseudo-F = F value by permutation. Bold face indicates statistical significance (P < 0.05); P-values are based on 9999 permutations (i.e. the lowest possible P-value is 0.0001)

Fator	d.f.	F	P
Soil Eh	37	0.81895	0.924
pH	38	1.0368	0.394
TDR	39	2.4654	0.001***
Season	1	2.4654	0.001***
Gravimetric	36	0.98251	0.522
Restoration age	2	**3.4919**	**0.001 *** **

Table 1: Effects of treatments (season, restoration stage) and environmental factors (soil Eh (redox), pH, TDR (soil moisture using time domain reflectometry), gravimetric soil moisture, and their interactions, on ITS fungal community structure in *P. tenax* roots as determined by Multiple Response Permutation Procedure (MRPP) of the Bray-Curtis dissimilarities among fungal ITS amplicons. d.f = degrees of freedom; SS = sum of squares; MS = mean sum of squares; Pseudo-F = F value by permutation. Bold face indicates statistical significance (P < 0.05); P-values are based on 9999 permutations (i.e. the lowest possible P-value is 0.0001).

Factor	d.f.	F	P
Soil Eh	38	0.99956	0.463
pH	40	0.76551	0.954
TDR	41	0.86305	0.786
Season	1	1.7437	0.038*
Restoration age	2	2.3533	0.001**
Gravimetric soil moisture	36	0.98295	0.524

Season and restoration age influence on OTU communities

Non-metric multidimensional scaling revealed that AMF communities from different restoration sites and seasons were grouped separately (Fig. 3A, based on the relative abundance of OTUs per sample for the ITS library; Fig. 3B, based on the relative abundance of OTUs per sample for the 18S library). This pattern was verified by the MRPP results (Table 3).

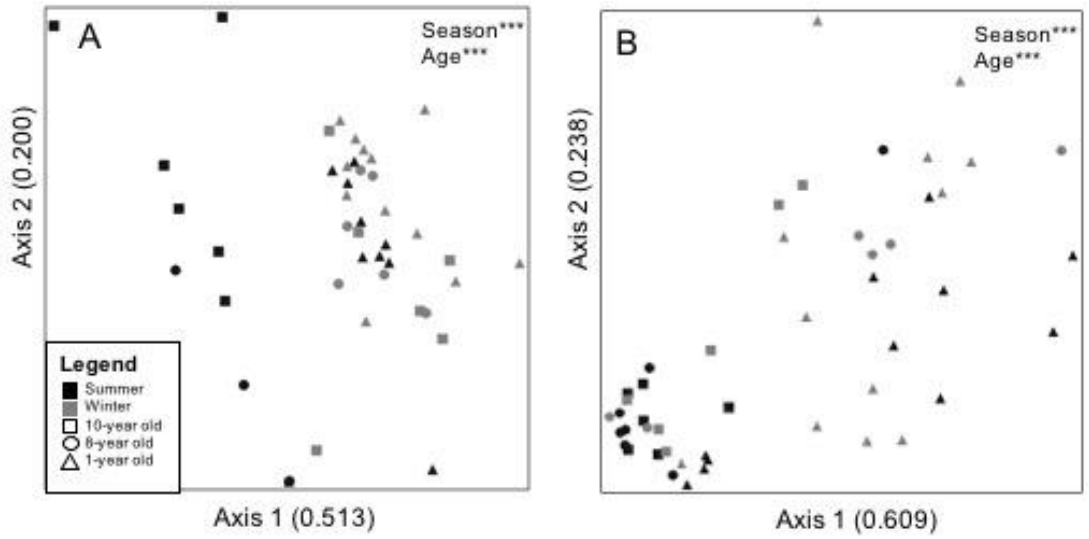


Figure 3. Nonmetric multidimensional scaling (NMS) plots demonstrating the effects of restoration site age and season on AMF communities along a wetland restoration site in New Zealand using the data of taxonomic composition (Bray–Curtis) on (A) 18S libraries and (B) ITS. Each point represents the centroid of the AMF community of each plant per restoration site and season.

MRPP

Table 3. MRPP Summary statistics for the effect of Stage and season on rhizosphere fungal community composition as characterised by Sorensen (Bray-Curtis) distances among A) 18S OTUs and B) ITS OTUs.

	Observed delta	Expected delta	Variance of delta	Skewness of delta	Test statistic: T	A	P
18S	0.543237	0.697125	0.20982763E-03	-0.632199	-10.623669	0.220747	0.00000000
ITS	0.650566	0.720812	0.16383179E-03	-0.833446	-5.488126	0.097454	0.00011780

Axis 1 of the the NMS ordination for samples in 18S-OTUs space was uncorrelated to any of the measured soil environmental variables, suggesting that moisture, pH and redox were not the primary drivers of AMF community composition.

Axis 1 of the the NMS ordination for samples in ITS-OTUs space was significantly negatively correlated with volumetric soil moisture (Pearson and Kendall r=-0.013, p=-0.043), indicating that the total soil fungal community was strongly structured by soil moisture and that older sites tended to be wetter than the younger one.

18S NMS (Fig. 3A) shows a trend towards a different AMF communities between summer and winter.

For 18S there is a trend towards a different AMF community structure in the summer and winter which accounts for over 50% of the total variation in the community (Fig. 4A). There are less species spaces/diversity of OTU composition in the 1-year old samples regardless of time of year.

Table 4. Dufrene-Legendre Indicator Species Analysis of 18S OTUs. Siginifcant indicator OTUs are based on MONTE CARLO tests with 4999 permutations.

Column	Putatative identification	Season and site	Indicator value	Mean	S.Dev	p *
OTU_1	Unknown member of Acaulosporaceae	Summer 10-year old	28.9	16.9	5.88	0.0702
OTU_16	Unknown member of Acaulosporaceae	Summer 10-year old	41.7	11.4	7.68	0.0108
OTU_6	uncultured Glomus sp.	Winter 8-year old	28.2	19.5	3.55	0.0008
OTU_8	uncultured Glomus sp.	Winter 8-year old	34.7	18.8	4.57	0.0002
OTU_47	Unindentified member of the Diversisporales	Summer 1-year old	32.7	14.5	6.74	0.0206
OTU_48	Unindentified member of the Diversisporales	Summer 1-year old	32.5	17.1	5.78	0.0088

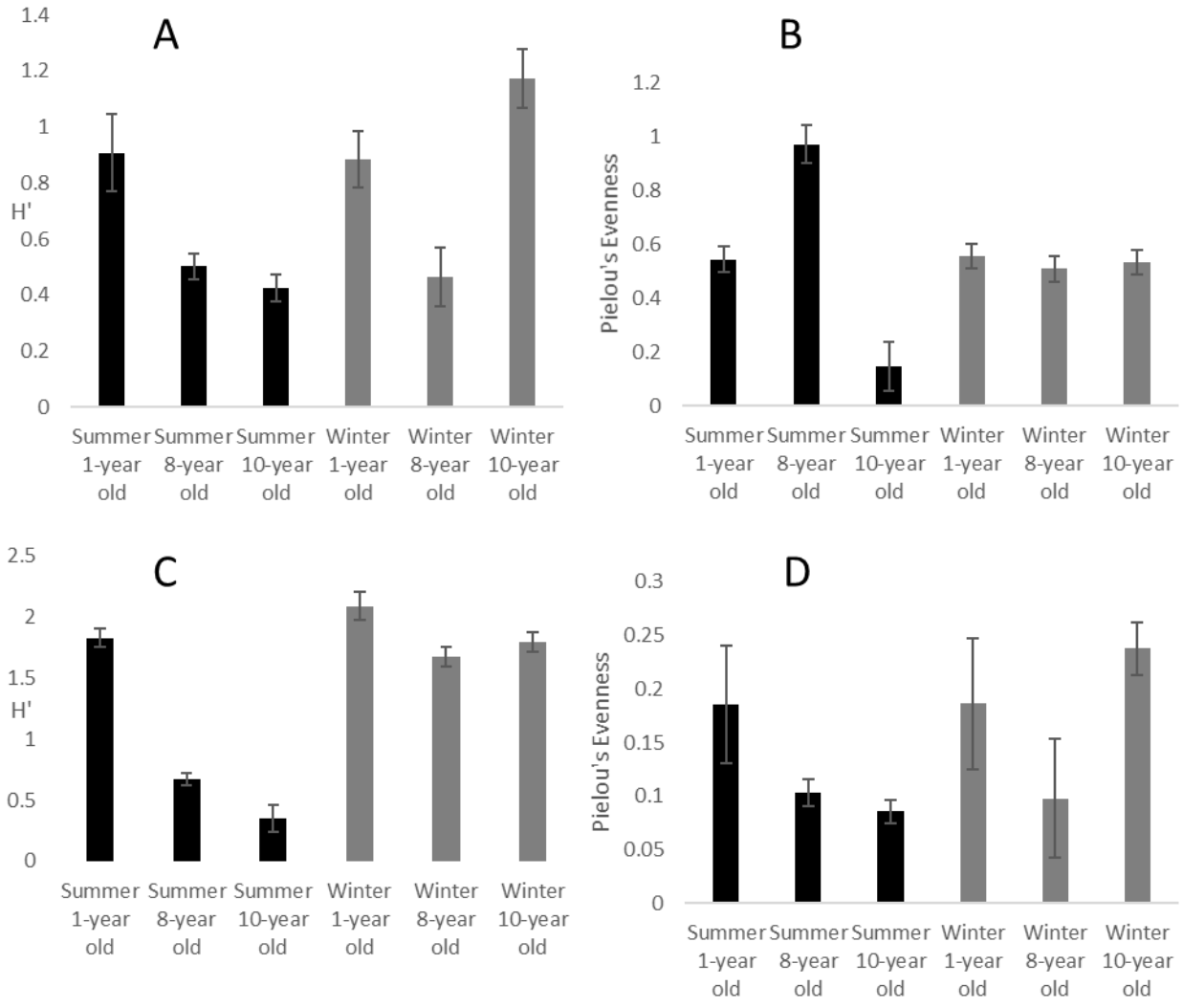


Figure 4. Shannon's diversity Index of arbuscular mycorrhizal fungi colonizing *P. tenax* roots in different restoration stages using the molecular marker (A) 18S and (C) ITS. Pielou's evenness of arbuscular mycorrhizal fungi colonizing *P. tenax* roots in different restoration stages using the molecular marker (B) 18S and (D) ITS. A score of 0 means there is no evenness and the presence of a dominant species, a score of 1 means somplete evenness between AMF communities. Means and standard error are shown.

18s:

In winter, there is relatively high diversity and moderate evenness of AMF in samples from all restoration site ages (Fig. 4A). In summer one-year old samples

follow a smiliar pattern to the winter samples with a reasonably high diversity of AMF but a moderate evenness score. However, the summer 8-year old have a relatively low diversity and high evenness, meaning the composition of the AMF community are quite even. In contrast, the summer 10-year old have low AMF diversity and low evenness, meaning the community is likely dominated by one or more species.

ITS:

In summer, there is relatively high fungal diversity in the 1-year old site, and moderately low diversity in the 8-year old and 10-year old. The community evenness of these summer samples follows the same trend with moderately high evenness for the 1-year old and low evenness for the 8 and 10-year old. This indicates a shift from an even community of many fungi to a community dominated by certain fungi that is lower in diversity as a restoration site ages.

AMF Colonisation and environmental variables

Table 3: Effect of treatments and environmental variables on the number of AMF structures visible in *Phormium tenax* roots, as determined by a Kruskall-Wallis chi-squared

Test variables	d.f.	Kruskal-Wallis chi-squared	P
Hyphae * restoration age	2	0.04668	0.9769
Hyphae * season	**1**	**13.694**	**0.0002151*** **
Hyphae * water table height	1	0.19723	0.657
Hyphae * gravimetric soil moisture	2	1.6417	0.4401
Arbuscules * season	**1**	**4.5323**	**0.03326 ***
Arbuscules * restoration age	2	0.89753	0.6384
Arbuscules * gravimetric soil moisture	2	0.14075	0.932
Vesicles * season	1	3.3855	0.06577τ
Vesicles * restoration age	2	2.8611	0.2392
Vesicles * gravimetric soil moisture	2	1.6458	0.4391
Spores in roots * season	1	1.5517	0.2129
Spores in roots * restoration age	2	0.34483	0.8416
Spores in roots * gravimetric soil moisture	2	0.6498	0.86207

Environmental factors

There were significant correlations between soil redox, soil pH and soil moisture measurements (both TDR and gravimetric) (Appendix 5). There were also seasonal differences between soil pH and gravimetric soil moisture (Appendix 5 and 7). I found no correlation between season and TDR soil moisture (Appendix 5 and 7). There is a seasonal difference in AMF community in *P. tenax* (fig. 4A and 4B). There is significantly higher AMF diversity in summer than winter ITS. This trend was highly significant for both ITS data (PerMANOVA ITS $P<0.05$) and 18S ($P=0.001$). The soil Eh across all sites were between -100 to +100Mv and are classified as moderately reduced.

Chapter 4: Discussion and Conclusions

OTU richness by molecular marker choice

AMF diversity was assessed with 2 molecular markers which vary in their ability to characterise the fungal community present in *P. tenax* roots. ITS markers identified more OTUs in Glomeromycota, and to higher taxonomic resolution than 18S. It seems in some ways that ITS is the better choice of marker for assessing AMF diversity on *P. tenax*. However, the amplification of AMF by ITS was variable. Some samples in which 18S detected AMF showed no AMF when using ITS. The reason for this is ITS is a noncoding region and so is hypervariable. ITS is universal so it can amplify not only fungi but also plants and bacteria, meaning a lot less specificity and more non-target 'junk' may be amplified than 18S. However, 18S is a coding region which is highly conserved so ITS is more of a 'coarse filter' capturing a lot more fungal diversity overall but also missing some AMF whereas 18S is more of a 'fine filter' capturing only AMF.

AMF Community structure

There is a difference in AMF community composition in *P. tenax* between site restoration ages at Wairio wetland. The youngest restoration site had the most diversity overall for the 18S dataset, but this was highly variable between individual plants. The 10-year old restoration site had the lowest AMF diversity, in the summer for both 18S and ITS. However, there was one *P. tenax* in the 10-year old restoration site that in winter had the second highest overall number of OTUs detected on one plant using 18S (28). That same P*. tenax* only had 8 OTUs during summer, which shows the high seasonal variability of AMF communities.

The overall pattern detected by 18S is that young stands of *P. tenax* have reasonably diverse and species rich AMF communities which are structured relatively evenly. In the summer, the most abundant AMF in the 1-year old stands are members from Glomales and Diversisporales including Gigaspora. The 1-year old *P. tenax* maintain similar levels of AMF species richness and evenness in the winter although Glomales is the most common AMF. Diversisporales were found to

be indicator species of *P. tenax* in summer at the 1-year old restoration site age. As the *P. tenax* ages, the diverse AMF community is replaced by a few dominant species. In the 8-year old *P. tenax* just 2 species from Diversisporales were found in the summer with the 18S dataset. This caused the Pielou's evenness scores to be very high, because many *P. tenax* had no OTU's at all and those that did only had 1 or 2 OTU's total. For the 8-year old site in the winter, Glomus were indicator species for the 18S dataset. As the *P. tenax* grew even more, the species richness decreased further and the AMF community is dominated by members of Acaulosporaceae.
The properties of AMF that make them abundant in differently aged vegetation is a combination of their life strategy (including the rate of colonisation formation, duration of dormancy and persistence) and their effect on the host plant (Bever et al. 2001, Hart et al. 2001). What the overall pattern shows is that when *P. tenax* is first planted there is a diverse and species rich AMF community colonising the roots with a dominance of Glomus species. Glomus are extensive and fast root colonisers (Maherali and Klironomos 2007) and their presence are thought to reduce root infection by soil pathogens (Newsham et al. 1995). Eventually as *P. tenax* ages, Glomus becomes outcompeted by Acaulosporaceae which is an indicator species for the 10-year old restoration site in the summer. Acaulosporaceae are slower growing than Glomus and do not colonise the soil or roots very extensively (Maherali and Klironomos 2007). It seems that Acaulosporaceae are the preferred AMF of *P. tenax* and when *P. tenax* have a large biomass they feed carbon to the Acaulosporaceae (preferentially over other AMF species). This has a reinforcing effect, allowing the Acaulosporaceae to dominate the AMF community.
ITS shows the same fungal community structure pattern as 18S although not as strong. ITS data was more variable than 18S in terms of diversity and evenness. Young stands of *P. tenax* have high fungal diversity and evenness in both summer and winter. As *P. tenax* stands mature, their fungal diversity and evenness decreases, except for 10-year old winter samples which maintain high diversity and evenness. The fungi that were dominant in mature winter stands and drove this pattern were all Ascomycetes, including members of the *Helotiales* order which are usually saprobes, root pathogens such as *Ilyonectria radicicola* and species that can have wide ranging symbiotic associations from parasitic to mutualistic such as

Leptodontidium orchidicola. Another highly abundant fungi at this site in winter was the genus Cladophialophora which are black yeast-like fungi often found in soils but can also be human pathogens (Badali et al. 2008). The ITS dataset showed a high dominance of unidentified fungi followed by Ascomycota across all other restoration sites. Ascomycota is common in wetlands where many decompose leaf litter and woody debris (Wang et al. 2005). Nikolcheva, Cockshutt and Barlocher (2003) found that Ascomycetes made up 75% of the fungal biomass on decaying leaves in a stream. Ascomycota are suited to these anaerobic conditions (Wang et al. 2005) and it is likely they play an important role in decomposition in Wairio wetland. It makes sense that Ascomycota would dominate the fungal community in winter at the 10-year old restoration site, mature *P. tenax* have a larger biomass than the juveniles and therefore the most decomposing leaf litter around them. The large amount of decomposing leaf litter would cause a larger diversity of fungi who colonise plant debris to be most active at this time when conditions for rapid decomposition are favourable.

Nineteen OTUs that occurred at the youngest restoration site were absent from the 10-year old restoration site. Six of these OTUs were Glomerales, four Diversisporales, one Archaeosporales and one Hypocreales. Three of these OTUs displayed a gradient spatial distribution across the stages with highest occurrence in the 1-year old site, medium occurrence in the 8-year old site and no occurrence at the 10-year old site. There were no OTUs that were found exclusively at the 8-year old restoration site, or most commonly at the 8-year old site. There were five OTUs found at the 10-year old site that did not occur at the 1-year old site; one unidentified, one Glomerales, one Archaeosporales, and two Diversisporales (of which one was a Gigaspora). It seems likely that these patterns are caused by treatment effects however, I cannot account for variation in AMF community structure caused by stochastic spatial patterns. To disentangle exactly which fungal groups are acquired during the restoration process and which are lost, sampling the same plants over several years is needed to confirm the pattern found in this study.

Indicator species analysis showed there were six significant indicator species across the restoration sites at different seasons. In the 1-year old restoration site in summer the two-indicator species were from Glomerales and Diversisporales. In the 10-year old restoration site in summer there were two significant OTU indicator species which were both Acaulosporaceae. In winter, there was only significant indicator species for the 10-year old restoration site both of which were uncultured Glomus. This pattern seems to support evidence of phylogenetic clustering as more closely related AMF are more likely to occur in the same age restoration site each season. Phylogenetic clustering in AMF can occur due to habitat filtering (taxa that have similar traits respond similarly to environmental factors); plant–AMF interactions (the host plant selects phylogenetically clustered AMF assemblages) or interactions with the biotic soil community (that support assemblages of conserved traits) (Horn et al. 2014).

Colonisation

There is a seasonal difference in AMF colonisation in *P. tenax* (Fig.4). There were significantly more arbuscules and hyphae colonising *P. tenax* in winter than summer. A seasonal difference in the colonisation intensity of AMF has been well documented (Anderson et al. 1984, Bohrer et al. 2004, Turner and Friese 1998, Miller 2000). There was no significant effect of soil moisture, pH or soil redox on the level of total colonisation, the number of hyphae, arbuscules or endospores (table 3). It has been reported the number of spores in wetland soil are reduced by flooding (Aziz et al. 1995), and affected by soil moisture and redox potential (Anderson et al. 1984, Khan 1993a, 1993b). The seasonal variation of AMF colonisation has been linked to the seasonal variation in water table levels and/or soil moisture levels in wetlands. It has been suggested that the seasonal variation of water regimes in wetlands allows the soil to occasionally aerate, providing AMF with periods of increased oxygenation for survival (Anderson et al. 1984, 1986; Cooke et al. 1993; Miller and Bever 1999). Results from this study show AMF colonisation levels are not affected by water table height or soil moisture. This

indicates that the seasonal variation of AMF colonisation was controlled by some other seasonal dynamic.

One possible driver of seasonal dynamics in AMF community composition could have been plant phenology. AMF seasonal variability have been linked to both plant phenology and phosphorus availability in terrestrial ecosystems (Rabatin 1979, Brundrett 1991)

If plant phenology was controlling AMF colonisation, it would make sense for the highest rates of colonisation to be in spring during flowering when phosphorous demands are highest. Perhaps the high level of colonisation in winter is because soon the *P. tenax* have a higher demand for resources during spring growth. This study did not measure AMF colonisation in the spring and further research is needed to assess this. *P. tenax* flower on average every three years, however some individuals flower reliably every year (Harris et al. 2010). Once the *P. tenax* has finished flowering during summer there is less demand for limited resources and so the AMF colonisation decreases. Some *P. tenax* were flowering at the time of sampling although I did not record which specific plants were. If AMF colonisation levels were compared between flowering and non-flowering years, it would give a better insight into the controlling factors of AMF colonisation.

In this study, it is possible that seasonal shifts in AMF colonisation were due to a multitude of different environmental factors that triggered a plant response, in turn affecting AMF colonisation. There was a strongly significant difference in soil redox values between winter and summer ($p < 0.001$, appendix figure 6 and 7) suggesting that the rhizosphere was more anaerobic in the winter. Flooded soils are more anaerobic as the rate of oxygen diffusion through the soil is drastically reduced when soil pore spaces are filled with water (Mitsch and Gosselink 2000). Oxygen diffuses through solution such as inundated soil around 10,000 times slower than through porous drained soil (Greenwood 1961, Gambrell and Patrick 1978).

Another possibility is that the seasonal differences in AMF colonisation are simply due to the seasonal community composition shift in AMF. We know that Different

taxonomic groups differ in their hyphal structure and have been shown to colonise roots at different rates (Hart and Reader 2002). Members of the Glomeraceae tend to contact roots quickly and produce vast mycelium in roots, whereas Gigasporaceae usually are slower to contact roots and establish a large mycelium in the soil rather than in roots (Hart and Reader 2002). Acaulosporaceae are also slow to contact roots but they have a much less extensive mycelium than members of the other two families, in either roots or soil (Hart and Reader 2002). The samples with the highest arbuscules and hyphae in winter were from 1 and 8-year old restoration sites. The most common fungi at those sites during winter was Glomus and Gigaspora. Glomus is known to have a high rate of root colonisation and Gigaspora is known to have extensive hyphae so it is likely the abundance of these taxonomic groups was the cause of the different seasonal colonisation rates.

There is no difference in AMF colonisation of *P. tenax* along a moisture gradient, with equal rates of root colonisation where the water table is high or low. Wetzel and van der Valk (1996) and Turner and Friese (1998) found that AMF seasonal trends are mainly influenced by flooding and/or soil moisture levels in freshwater wetlands. AMF colonisation in wetlands has been linked to seasonal variation in water table levels and/or soil moisture levels. One possibility why there was no relationship between AMF colonisation and soil moisture is that my sampling design did not encompass a sufficient range of moisture levels to detect this. It may also be due to the sample roots experiencing the same redox potential consistently due to the natural fluctuations in the water table. The soil moisture levels in Wairio seemed to vary more spatially than seasonally so samples that were inundated at the time of sampling may just be due to a prior rainfall rather than being consistently exposed to this.

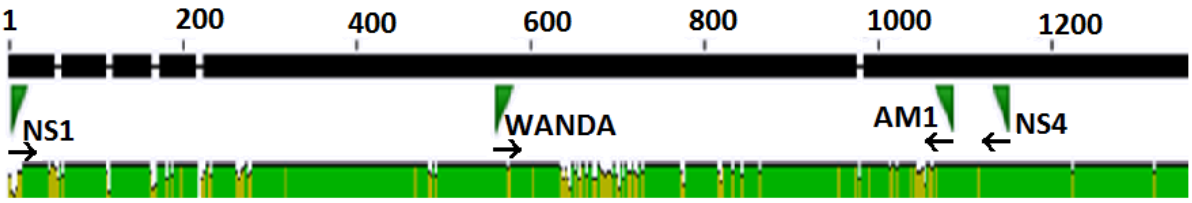
Conclusion

The purpose of this thesis was to characterise the AMF community of a foundational New Zealand wetland plant. Because we know that plants have an effect on the AMF in the soil, *P. tenax* affects the success of wetland restoration. My work shows that there are trade-offs when targeting 18S or ITS to characterise the AMF community. We would expect that AMF community to shift significantly over time as the restoration progresses and with season. AMF of *P. tenax* are dynamic and change seasonally, possibly in response to plant phenology. AMF also change over the lifetime of a *P. tenax* plant, the communities becoming less diverse due to the reinforcing effect of the host plant on it's preffered fungus. *P. tenax* feeds Acaulosporaceae carbon until it dominates the AMF community in the older restoration sites. It would be interesting to know if this pattern can be generalised to the AMF community structure of other native wetland plants. Perhaps a site dominated by Acaulosporaceae rather than Glomus is an indicator of a maturing site in wetland restoration.

Appendices

Appendix 1. Primer sequences for PCR amplification of fungal DNA, used in chapters 2 and 3.

Primer name	Sequence	Melting Temperature (ºC)	References
AML1	5'- ATC AAC TTT CGA TGG TAG GAT AGA -3'	53	Lee et al. (2008)
WANDA	5'- CAG CCG CGG TAA TTC CAG CT -3'	59	Dumbrell et al. (2011)
NS31	5'- TTG GAG GGC AAG TCT GGT GCC -3'	61	Simon et al. (1992)
AML2	5'- GAA CCC AAA CAC TTT GGT TTC C -3'	55	Lee et al. (2008)
AM1	5'- GTT TCC CGT AAG GCG CCG AA -3'	60	Helgason et al. (1998)



Appendix 2: Hypervariable region of SSU based on sequences of New Zealand plants from Genbank and areas of attachment of primers. Note the use of the AMF specific primer pair WANDA-AM1 anneal outside of the hypervariable regions and capture the diversity within.

Appendix 3. DNA extraction techniques trialled

Root sample treatment	Temperature at crushing	Crushing treatment	Beating step	DNA extraction
Fresh/frozen	Liquid nitrogen	Carbide beads	Bead beater	Genejet kit
				Powersoil kit
				Ctab
Fresh/frozen	Liquid nitrogen	sand	manual	Genejet kit
				Powersoil kit
				Ctab
Fresh/frozen	Liquid nitrogen	Garnet beads	Bead beater	Genejet kit
				Powersoil kit
				Ctab
Fresh/frozen	Room temperature	Carbide beads	Bead beater	Genejet kit
				Powersoil kit
				Ctab
Fresh/frozen	Room temperature	sand	manual	Genejet kit
				Powersoil kit
				Ctab
lyophilised	Liquid nitrogen	Garnet beads	Bead beater	Genejet kit
				Powersoil kit
				Ctab
lyophilised	Liquid nitrogen	sand	manual	Genejet kit
				Powersoil kit
				Ctab

lyophilised	Liquid nitrogen	Carbide beads	Bead beater	Genejet kit
				Powersoil kit
				Ctab
lyophilised	Room temperature			Genejet kit
				Powersoil kit
				Ctab

Appendix 4.

1. 350 µL of CTAB lysis buffer was added to 50mg of frozen root tips, 1g sterile sand and ground into solution using a micropestle in a 1.5mL microcentrifuge tube. Crush with pestle making sure the root tissue is in solution and not in a clump at the bottom of the tube.
2. Incubate at 55-60°C in heatblock/waterbath for 40-60mins, inverting every 10 minutes. Samples can stay in the water bath for a few hours if necessary.
3. Remove samples from waterbath and **IN FUME HOOD**, add 500 uL of chloroform and mix by gently shaking tubes. Shake vigorously then leave to stand for 2 mins.
4. Spin at 10000 rpm for 2 mins, if not separated spin for longer until separated.
5. Extract upper aqueous phase and transfer to a new labelled Eppendorf (can use a p1000 tip with the end cut larger to ensure the DNA is undamaged). Be careful to avoid transferring any chloroform.
6. Add 500 uL isopropanol, mix well and leave on ice for 10 min. If cloud not visible leave in freezer for a few hours (can leave overnight).
7. Remove DNA (little cloud) <u>if visible</u> with cut pipette tip and transfer to a fresh Eppendorf tube <u>or</u> remove isopropanol if it is a pellet at the bottom. Discard isopropanol into chemical waste jar. Be careful not to dislodge pellet.
8. If you can't see DNA, pulse samples in centrifuge to 6000rpm for 30 seconds, you should see a cloud or pellet.
9. Add 600 µl of 80% Ethanol and GENTLY mix (invert 5-10 times)
10. Spin at 4000 rpm for 60 seconds
11. Discard supernatant (ethanol) be careful not to dislodge pellet
12. Add 600 µl of 80% Ethanol and GENTLY mix (invert 5-10 times)
13. Spin at 4000 rpm for 60 secs
14. Extract ethanol

15. Invert tubes on a clean kimwipe and allow to dry for 10-15 minutes upside down, or until pellet looks dry. If the pellet dried too long upside down, it will fall out. Continue to dry upright but covered by a kimwipe for 30-45 minutes.
16. Hydrate pellets with 40 uL TE. Allow to resuspend then store the DNA in the refrigerator/freezer if not using the next day.

10 mL CTAB extraction buffer:

0.2g CTAB

0.1g PVP 40

2.8 mL 5 M NaCl

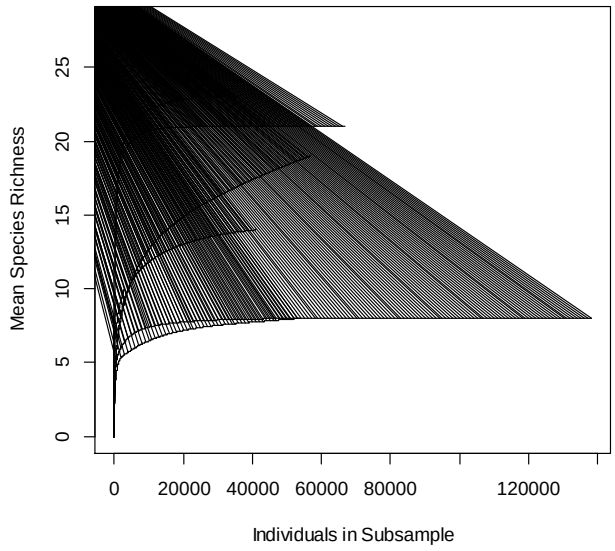
2.0 mL 100mM EDTA

1.0 mL 1 M Tris/HCl

4.0 mL ddH_2O

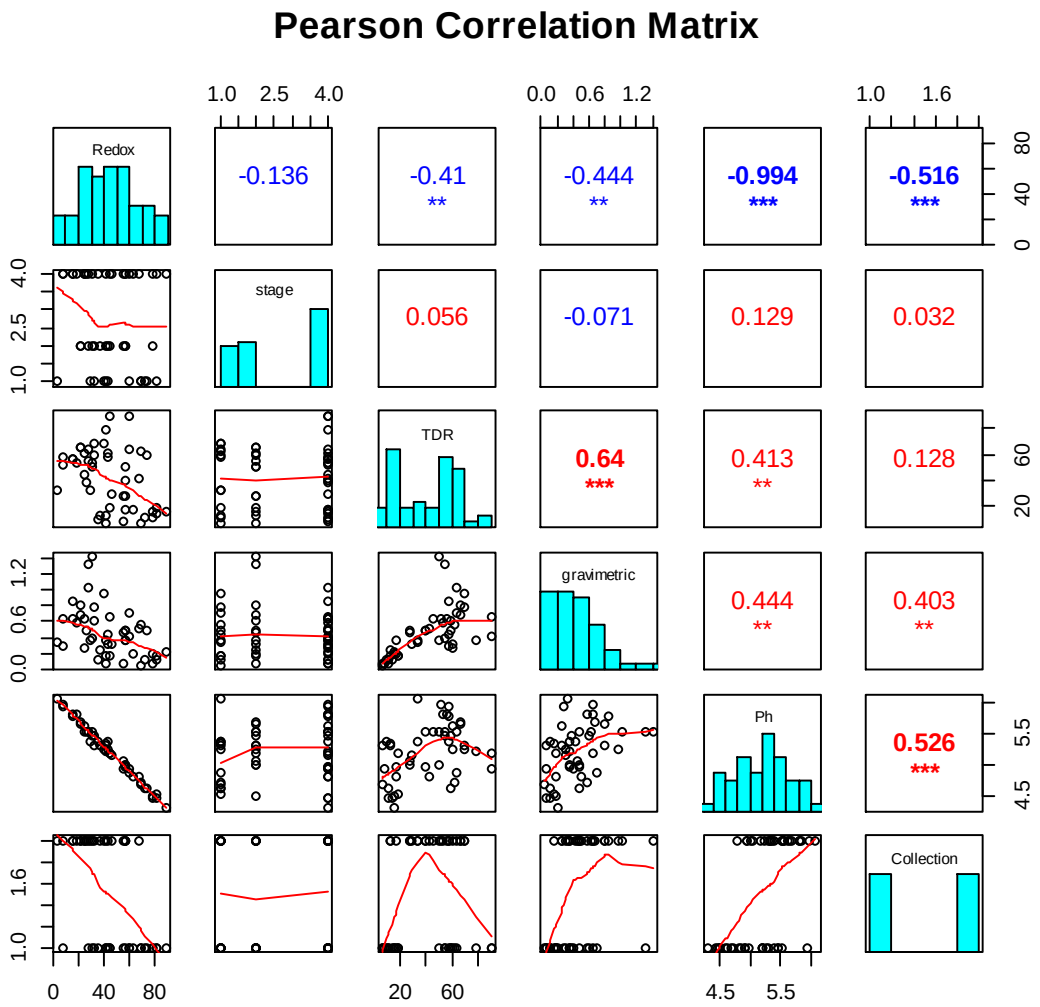
Appendix 5. Environmental variables

	Test statistic	p-value
Soil Eh & pH	-0.99	0.001***
Soil Eh & TDR	-0.41	0.01**
Soil Eh & season	-0.52	0.001***
Soil Eh & gravimetric	-0.44	0.01**
pH & TDR	0.41	0.01**
pH & season	0.53	0.001***
pH & gravimetric	0.44	0.01**
TDR & season	0.13	1
TDR & gravimetric	0.64	0.001***
Season & gravimetric	0.40	0.01**

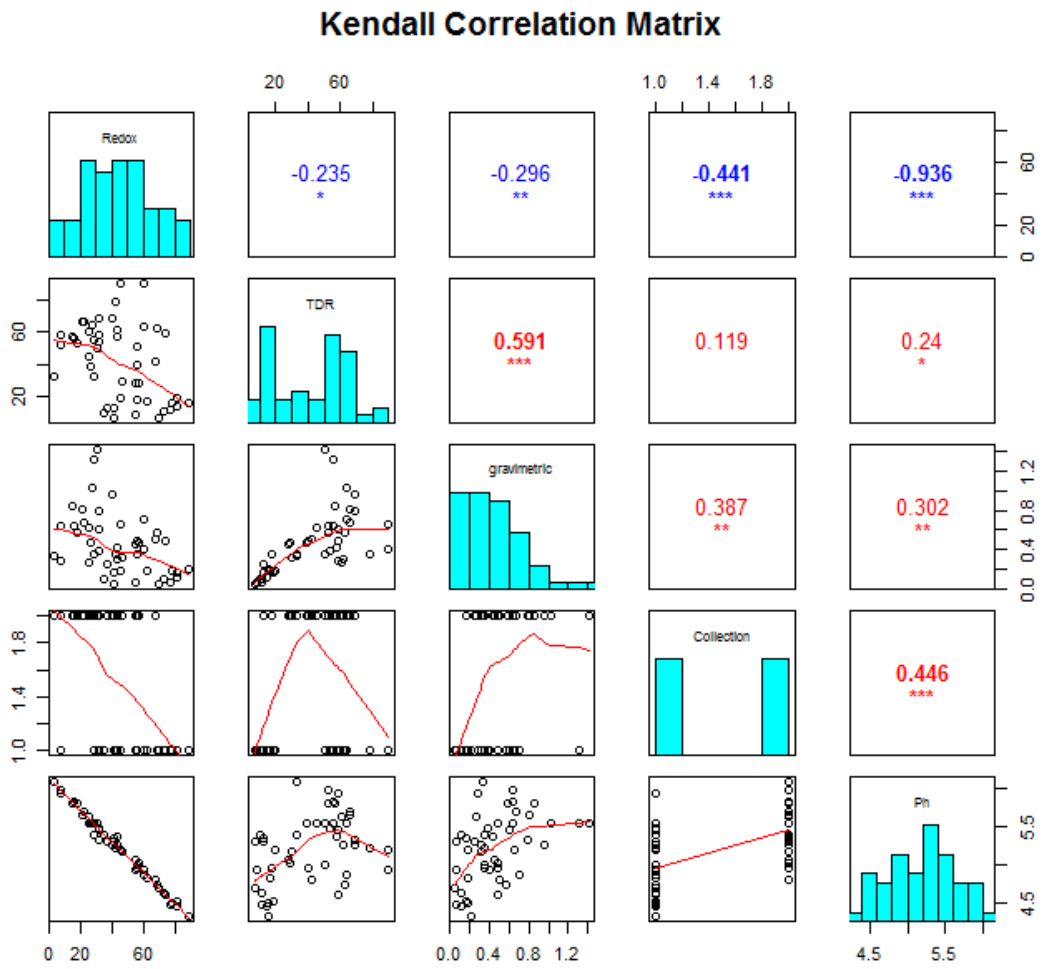


Appendix 6. Rarefaction of summer samples

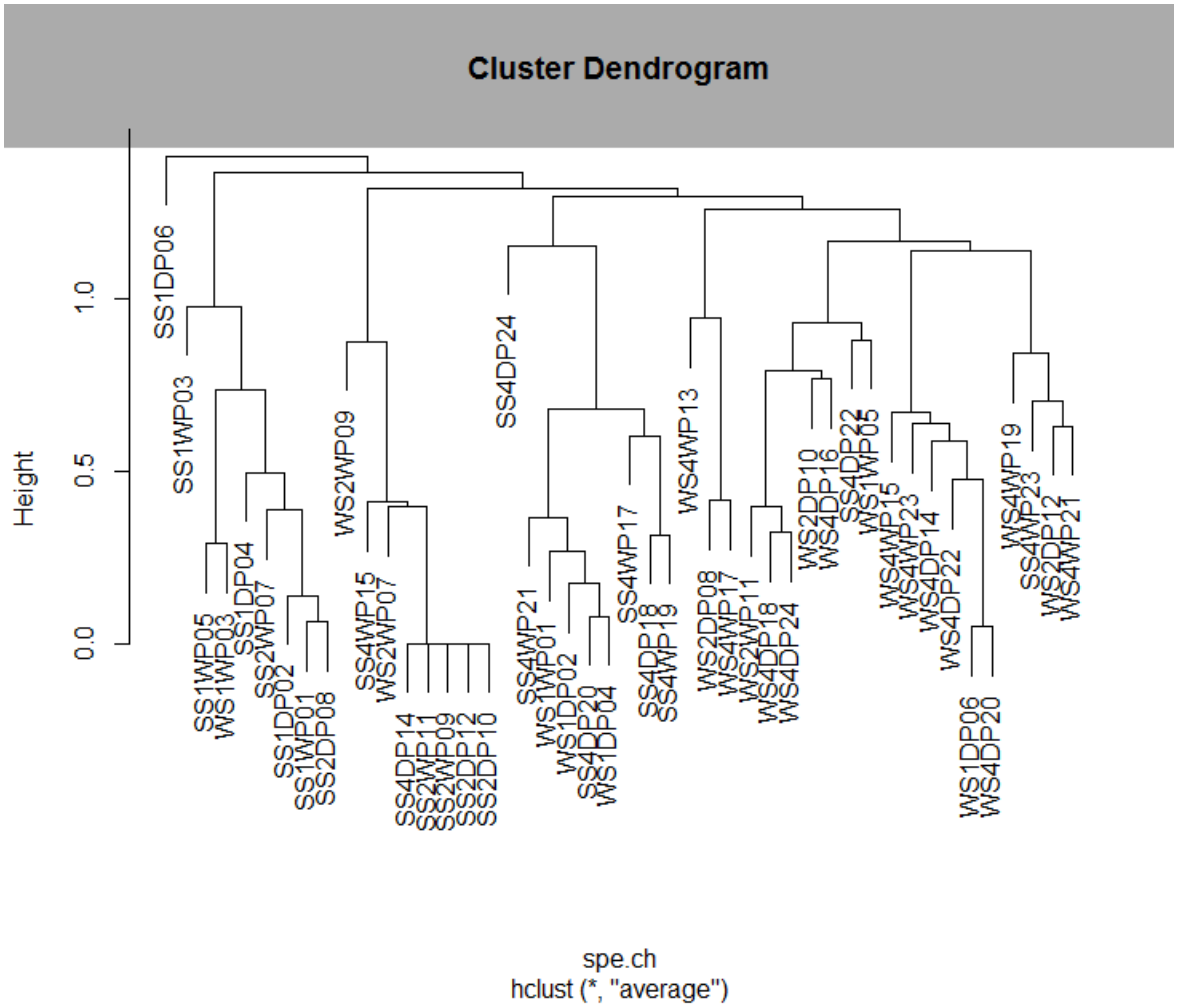
Rarefaction generally showed sampling was sufficient, the exception being one site that does not asymptote.



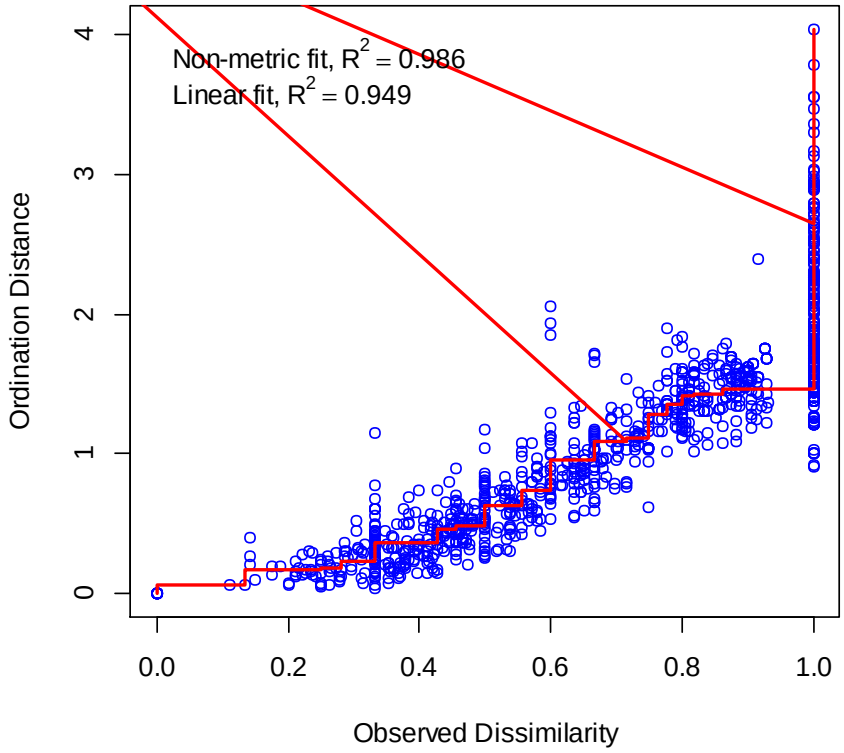
Appendix 7. Pearson correlation matrix between abiotic variables at Wairio. Redox: soil Eh, Stage: restoration site, TDR: soil moisture measurement, gravimetric: soil moisture, pH: of soil, collection: season when samples collected. Significance P<0.05 *, P<0.01 **, P<0.001 ***



Appendix 8. Kendall correlation matrix between abiotic variables at Wairio. Redox: soil Eh, Stage: restoration site, TDR: soil moisture measurement, gravimetric: soil moisture, pH: of soil, collection: season when samples collected. Significance P<0.05 *, P<0.01 **, P<0.001 ***



Appendix 9. UPGMA clustering of a matrix of chord distance among sites (species data). Cophenetic correlation = 0.9053354



Appendix 10: Shepard stress plot of 46 OTU's from the 18s data shows scatter around the regression between the interpoint distances in the final configuration (i.e., the distances between each pair of communities) against their original dissimilarities. Final stress=0.1192964.

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